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Effects on enzymes and the genetic apparatus of sheep after administration of samples from industrial emissions

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In the present work the influence of the administration of industrial emissions from a zinc and copper plant on aspartate aminotransferase (AST), alanine aminotransferase, gammaglutamyl transferase, creatine phosphokinase (CK), total bilirubin, serum zinc levels and the genetic apparatus was studied on seven ewes. Each animal was given a dose of 31.99 g of emissions per day. The first and the last animals died of zinc intoxication on days 42 and 58, respectively. Significantly increased zincemia could be observed from day 8 of the experiment ($P < 0.01$). In the enzymes under investigation, the most pronounced effects of the emission were seen in AST and CK activities. In comparison with the starting levels, AST values revealed significant differences on days 37 and 58 ($P < 0.05$ and $P < 0.01$, respectively), and CK on day 58 ($P < 0.01$). Significantly increased bilirubinemia ($P < 0.01$) could be observed from day 8 of the experiment. In the period prior to the first gavage of emission and day 30 of administration no significant increase of chromosome breaks per cell was observed in the experimental sheep. The genotoxic effect of the emission was also stated on the basis of recombination frequency visualized by means of the sister chromatid exchange test; on day 30, the increase of these disturbances revealed statistical significance ($P < 0.01$).

Keywords: biochemical parameters, genotoxicity, industrial emission, sheep

Introduction

Several methods have been used for the determination of the negative effects of toxic elements on animals, their choice depending on the type and chemical form of the pollutant and its dose and period of influence, as well as on the species, age and breed predispositions of the animals and, not least, on animal productivity and health (Van Der Schee *et al.* 1983, Parada *et al.* 1987, Galey & Hall 1990). Heavy metal contamination occurring in the vicinity of ore-processing plants is very dangerous for sheep. In view of their predisposition, sheep as a species seem to be most sensitive to an increased intake of copper, arsenic, cadmium and lead. The toxic effects of risk elements on sheep may become evident in health disturbances and decreased productive and reproductive indices (Riet-Correa *et al.* 1989, Smith *et al.* 1991). The functions of the liver, where necrobiotic processes take place, are most heavily affected by the metabolism of xenobiotics (Friel *et al.* 1987, Kumaratilake & Howell 1989). As during metabolic inactivation or detoxification of xenobiotics specific enzymes are activated, the latter were chosen for determination in blood serum. The animals reveal deteriorated defense abilities and become more susceptible to other diseases (Bíreš *et al.* 1990). Mutagenicity, carcinogenicity or teratogenicity could be stated for some elements (Leonard *et al.* 1986, Wong 1988). In consequence of the increased levels of risk element residues, products made of animals living in exposed regions might secondarily endanger human health. Because of the above facts, biological material from the animals is also analyzed in order to determine the toxic effects of heavy metals (Parada *et al.* 1987, Reif *et al.* 1989).

Thus, the present investigation was carried out to evaluate changes occurring during the administration of industrial emissions from a copper and zinc plant in selected biochemical indices and on the genetic apparatus of sheep.

Material and methods

Seven ewes of the Improved Wallachian breed aged 1.5–2 years and weighing 40.25 ± 3.55 kg were used. After

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morning feeding, using a laryngeal tube, each animal was given 31.99 g of the emissions. The latter were obtained by dedusting of electrolytical filters from a chimney of a copper and zinc plant in East Slovakia. The daily intake of copper, iron, zinc, molybdenum, selenium, arsenic, cadmium and lead both from the emissions and the feed is given in Table 1. The feeding ration comprised meadow hay (1.5 kg per animal per day) and grain (0.20 kg per animal per day). Access to water was unrestricted.

Administration of the emissions was carried out until the animals died of zinc intoxication. The mean age of animals in this experiment reached 46.28 ± 5.03 days, the first and the last animal dying on day 42 and 58, respectively.

Blood samples were taken from the vena jugularis prior to the first administration of the emission, and on days 8, 15, 23, 37, 44 and 58. In the blood serum, photometrical determination was carried out of aspartate aminotransferase (AST; EC 2.6.1.1.), alanine aminotransferase (ALT; EC 2.6.1.2.), gammaglutamyl transferase (GGT; EC 1 4.1.3.) and creatine phosphokinase (CK; EC 2.7.3.2) activities using the Biola test (Lachema, Brno, Slovakia). Total bilirubin levels in the blood serum were stated photometrically according to Jendrassik & Grof (1938). Serum zinc levels (zincemia) were analyzed by atomic absorption spectrophotometry on a Perkin-Elmer 1100 device.

The potential genotoxic effects of the emissions on the sheep were evaluated in a two-stage procedure by chromosome analysis and the method of sister chromatid exchange (SCE); the latter is based on the visualization of DNA recombination frequency. Both tests were carried out using cells of the bone marrow that was obtained by sternal puncture of segment 3–4 after shearing, disinfection and degreasing of the site of puncture.

Biological material for chromosome analysis was taken from five experimental sheep prior to the first administration of the emission and on day 30 of the experiment when most of the animals displayed symptoms of chronic zinc intoxication. The material (0.25 ml) was cultured in two cultivation dishes with 10 ml RPMI 1640 (ÚSOL, Praha, Slovakia) medium adjusted for cell cultures. The material was further processed according to the method of Lojda & Mráz (1982).

Evaluation of the test was based on the re-calculation of structural changes of chromosomes per one cell examined, i.e. chromatid and chromosome breaks and chromosome exchanges. The increase of the break per cell ratio between the starting value and that on day 30 was taken as the basic criterion. In each animal under observation, 50 metaphases were evaluated, i.e. a total of 250 metaphases both at the beginning and on day 30 of the experiment.

In the same animals, the SCE test was carried out according to Perry & Wolf (1974) by a method modified by Quin & Huang (1984). Visualization was achieved by staining in a bisbenzimid solution (Hoechst 332-58; Merck, location) for 30 min. The stain concentration presented 10^{-3} mg Hoechst 332-58 per 1 ml of 0.15 M phosphate buffer, pH 7.0. This was followed by photoactivation of the wet preparations with the same stain for 30 min under a UV lamp. Then incubation of the preparations was carried out in a 2 × SSC (sodium saline citrate; 0.3 M sodium chloride and 0.03 M trisodium citrate) solution for 60 min. After profound rinsing with 0.15 M phosphate buffer, pH 8.0, the preparations were stained in 5% Giemsa with the same buffer for 5 min.

The SCE frequency was evaluated in 41 metaphases prior to starting the experiment and in 50 metaphases on day 30 of investigation.

The results of the analysis are given as mean ± SD. Student's *t*-test was used to compare the values of the indices observed in the experimental and control groups.

Measurements of copper, iron, zinc, molybdenum, selenium, arsenic, cadmium and lead in the feed and emissions were carried out by atomic absorption spectrophotometry using the Perkin-Elmer 1100 device and the HGA 500 graphit tube. See Table 1.

Results

Zinc levels in the blood serum of sheep were found to be on the lower border of the reference value (11.78 ± 2.38 μmol l^{-1}) (Figure 1). A significant increase of zincemia was seen as early as day 8 of investigation ($P < 0.01$). Later, serum zinc levels also revealed an increasing trend, reaching their maximum at the end of the experiment (299.7 ± 128.8 μmol l^{-1}). Throughout the experiment, serum zinc levels revealed significant differences between the starting values and the values determined in the single periods ($P < 0.01$).

Until day 23 of the experiment, serum AST activity did not reveal significant changes in comparison with the starting values and remained within the physiological range (Figure 2). Significantly increased AST levels were observed on days 37 and 58 of the experiment ($P < 0.05$ and $P < 0.01$, respectively). AST activity reached its

Table 1. Daily intake of copper, iron, zinc, molybdenum, selenium, arsenic, cadmium and lead from emission and feed (mg per ewe)

Source	CU	Fe	Zn	Mo	Se	As	Cd	Pb
Emission	402.02	95.97	6158.07	1.436	2.975	15.38	0.598	22.14
Meadow hay	12.66	275.1	79.35	0.601	1.035	2.934	0.005	0.08
BAK feed mixture	18.06	17.4	12.84	0.1	0.45	0.18	0.046	0.367
Portable water	2.43	0.954	1.22	0.14	2.41	trace	trace	trace
Total	435.35	389.45	6251.48	2.28	6.87	18.49	0.649	22.59

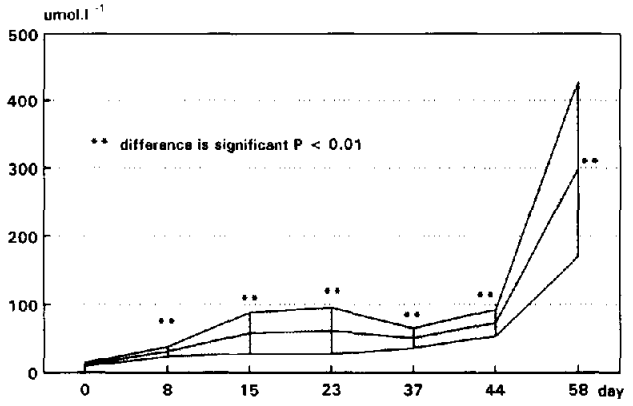

Figure 1. The concentrations of zinc in blood serum of sheep. Number of animals, 7; —, average; □, ± SD.

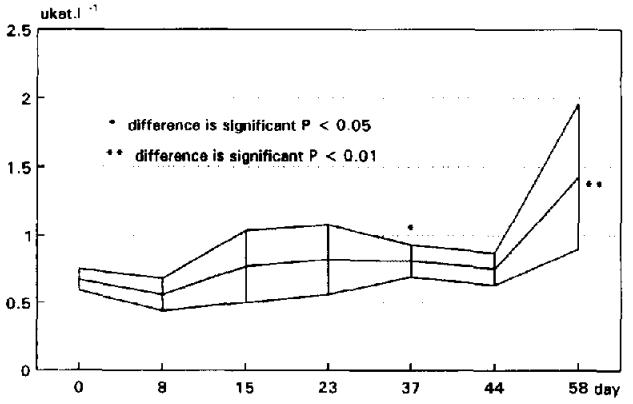

Figure 2. The activity of AST in blood serum of sheep. Number of animals, 7; —, average; □, ± SD.

maximum at the end of the experiment (1.43 ± 0.53 μkat l^{-1}).

In comparison with the starting values, the dynamics of ALT activity revealed a declining tendency between days 15 and 44 (Figure 3). The difference between ALT levels observed in this period and prior to the first gavage of emissions was at the level of $P < 0.01$. The highest ALT activity—significantly ($P < 0.01$) increased compared with the starting value—was recorded at the end of the investigation.

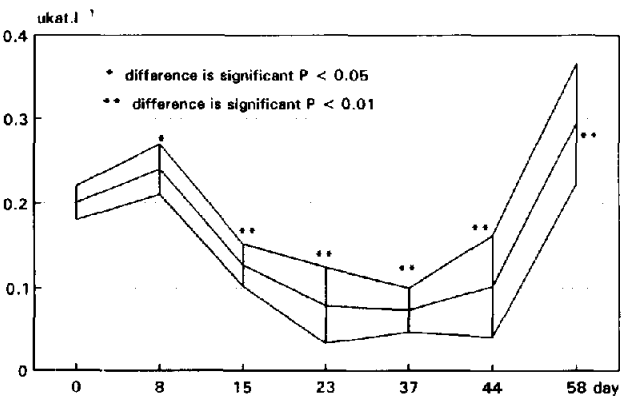

Figure 3. The activity of ALT in blood serum of sheep. Number of animals, 7; —, average; □, ± SD.

Elevated GGT values (1.86 ± 0.53 μkat l^{-1}) were observed at the start of the experiment (Figure 4) while decreased GGT activity occurred between days 15 and 44 ($P < 0.01$). On day 58 GGT levels reached 1.60 ± 0.40 μkat l^{-1} and still surpassed the upper physiological margin.

From day 37, CK dynamics revealed an increasing tendency (Figure 5). In comparison with the starting levels, CK activity was significantly elevated at the end of the experiment ($P < 0.01$). From day 37 of gavage, CK activity surpassed the upper reference margin.

Bilirubinemia was observed to continuously increase from the beginning of the experiment (Figure 6), reaching significantly elevated values ($P < 0.01$) from day 8 and surpassing the physiological level from day 15. The highest total bilirubin level (18.07 ± 7.21 μmol l^{-1}) was recorded in sheep with clinical zinc intoxication.

The frequency of chromosome aberrations in sheep is given in Table 2. The level of spontaneous chromosome changes in 250 metaphases prior to the start of the experiment presented two achromatic lesions (gaps), four breaks, four hypoploidias and three polyploidias. The distribution of aberrations in 50 metaphases was as follows: sheep 1, one break, one hypoploidia, one polyploidia; sheep 2, one break, two hypoploidias; sheep 3,

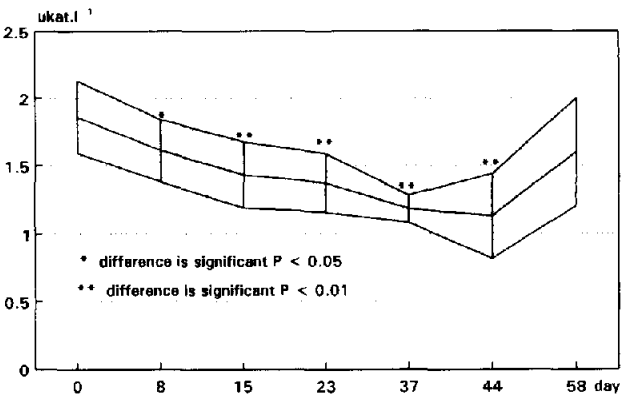

Figure 4. The activity of GGT in blood serum of sheep. Number of animals, 7; —, average; □, ± SD.

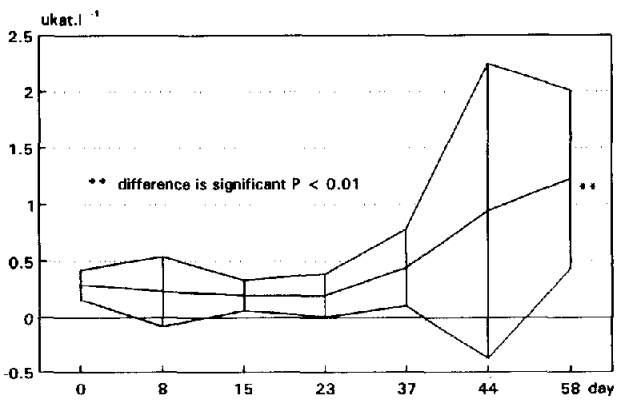

Figure 5. The activity of CK in blood serum of sheep. Number of animals, 7; —, average; □, ± SD.

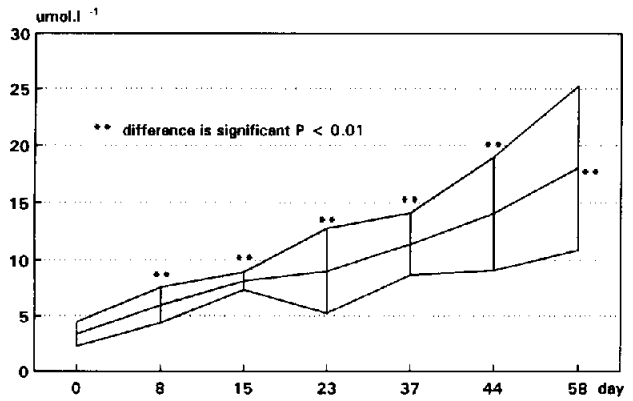

Figure 6. The concentrations of total bilirubin in blood serum of sheep. Number of animals, 7; —, average; □, ± SD.

one gap, two breaks, one hypoploidia; sheep 4, one polyploidia; sheep 5, one gap, one polyploidia.

On day 30 of the experiment chromosome aberrations in 250 metaphases under evaluation revealed the following pattern: four gaps, five breaks, four hypoploidias and two polyploidias. In 50 metaphases the following distribution of aberrations was seen: sheep 1, three gaps, one chromatid and one chromosomal double chromatid deletion, i.e. three breaks and one hypodiploid metaphase $2n-2$ (52 chromosomes); sheep 2, one break, one hypodiploidia; sheep 3, one gap; sheep 4, one break, two hypodiploidias, one polyploidia; sheep 5, one polyploidia.

The number of aberrations calculated to the breaks/cell value reached 0.016 ± 0.0149 and 0.023 ± 0.021 at the beginning and the end of the experiment, respectively. The difference between the values revealed no significance.

Prior to starting the experiment, the SCE test for clastogenicity was carried out on the basis of 41 assessable metaphases. Seven, eight, eight, 10 and eight metaphases with a mean of 5.43, 5.38, 5.5, 5.2 and 5.0 SCE were obtained from sheep 1, 2, 3, 4 and 5, respectively. Prior to the start of the experiment the SCE test value reached 5.26 ± 1.34 (Table 2).

On day 30 of the experiment, 50 metaphases with well-identified chromatid exchanges were evaluated using SCE. From sheep 1, five, seven, 17, 11, eight and seven metaphases with a mean of 8.42, 7.29, 8.0, 7.4 and 8.28 SCE per cell were evaluated. On day 30 of emission gavage, the SCE test revealed a value of 7.76 ± 1.46. The difference of the SCE test between the start and day 30 of the experiment was observed to be significant ($P < 0.01$).

Discussion

In animals, an excessive supply of zinc salts causes anorexia, depression, diarrhea, sight disturbances and nervous symptoms (Dreosti 1982, Ogden & Edwards 1988). The liver is dystrophic; histologically, hemosiderin is observed (Pritchard *et al.* 1985). Different histological changes are also seen in the pancreas, kidneys and digestive tract (Allen & Masters 1985, Kazacos & Van Vleet 1989). In view of the composition of the emission employed, copper, arsenic and cadmium also may have participated in the toxic effects on sheep and in the development of the intoxication itself. Interactions between the single toxic metals probably played an important role in the negative impact of the emissions (Chowdhury & Chandra 1987).

For a general assessment of the effects of toxic metal-containing emissions from a copper and zinc plant on sheep, selected parameters of liver metabolism were evaluated. Transaminases are included among indicator cytoplasmic enzymes (Hořejší 1979). An increase of their activity in the serum indicates that the membranes of cells, mainly those of the hepatocytes, are damaged and hypersecretion of the enzymes into blood occurs (Waner & Nyska 1991). GGT and bilirubin activity determinations are also used to assess liver function (West *et al.* 1987, Sheabar & Yannai 1989). In the course of metal intoxication of the organism, hyperbilirubinemia and increased enzyme activity become evident even prior to the onset of clinical symptoms (Soli 1980). At chronic intoxication, increased CK levels in the blood serum of animals are connected with damage to muscle, liver, kidney, brain and erythrocyte cell membranes (Thompson & Todd 1974).

In the blood serum of emission-treated sheep the most profound changes were observed in AST and CK activities as well as total bilirubin concentrations. As compared with the starting values, AST levels were significantly increased on days 37 and 58, while those of CK and total bilirubin on day 58 and between days 8 and 58, respectively ($P < 0.01$ and $P < 0.05$). ALT and GGT activities revealed minimal dependence on the intake of emission.

Zinc levels in the blood serum significantly increased as early as day 8 ($P < 0.01$). From day 15, hyperzincemia was observed that coincided with the values recorded by Allen

Table 2. Frequencies of chromosomal aberrations and SCE test

Chromosome analysis		No. of animals	No. of examined cells	Aberrations re-calculated (breaks/cell)
	start	5	250	0.016 ± 0.0149
	day 30 of the experiment	5	250	0.023 ± 0.021
SCE-s		No. of animals	No. of examined cells	SCE/cell
	start	5	41	5.26 ± 1.34
	day 30	5	50	7.76 ± 1.46[a]

[a]The difference in the SCE test between the start and day 30 of the experiment was significant ($P < 0.01$).

& Masters (1985) and Thompson *et al.* (1991) in intoxicated animals. However, the levels of zinc observed in this experiment surpassed those determined in sheep that had been fed emissions from a copper or a zinc plant, as reported by Bíreš *et al.* (1991) and Reif *et al.* (1989), respectively.

In order to state the effects of the emission on the genetic apparatus of sheep, chromosome analyses and SCE tests were carried out on cells of the sternal bone marrow. As a criterion of mutagenic activity, chromosome break evaluations were also employed by Bockov *et al.* (1975), Brogger (1982) and Lojda & Mráz (1982). According to these authors, calculation of the number of structural changes per breaks presents a decisive criterion in assessing mutagenic activity. The above calculation revealed an insignificant increase in the number of breaks per cell on day 30 of treatment as compared with the starting values. In contrast to the results achieved in this work, several authors reported positive clastogenic effects of some of the metals (arsenic, cadmium, lead, copper and zinc) or their compounds found in emissions (Wong, 1988, Goldman & Dacre 1991, Magos 1991, Coogan *et al.* 1992), on the basis of testing the individual elements under *in vitro* conditions. In our tests the genotoxic influence of the emission was only expressed by the increase of SCE frequency. This fact points at a type of DNA damage that is different from chromosomal breaking. This assumption is in accordance with the potency of metal ions to bind to the DNA structure (Petrakis & Sideris, 1982 and Rossman & Molina 1986). As the results of our work suggest, in order to estimate practical environmental genotoxicity it is necessary to use different tests reflecting DNA damage at different levels.

References

Allen WM, Masters HG. 1985 Renal lesions and tissue concentrations of Zn, Cu, Fe and Mn in experimentally zinc intoxicated sheep. *Res Veter Sci* **39**, 249–251.

Bíreš J, Vrzgula L, Hojerová A. 1990 Changes in the phogocytic activity of blood leucocytes, concentrations of plasma Ig and albumin in sheep fed pollutant substrate. *Živoč Výr* **35**, 763–771.

Bíreš J, Kováč G, Vrzgula L. 1991 Mineral profile of serum in experimental copper intoxication of sheep from industrial emissions. *Vet Hum Toxicol* **33**, 431–435.

Bočkov NP, Šrám RI, Kulesov NP, Žurkov VS. 1975 Chemical substances and methods of their evaluation on mutagenicity of humans (in Russian). *Genetika* **11**, 156–170.

Brogger A. 1982 The chromatid gap-a useful parameter in genotoxicology. *Cytogen et Cell Genet* **33**, 14–19.

Chowdhury BA, Chandra RK. 1987 Biological and health implications of toxic heavy metal and essential trace element interactions. *Progr Ed Nutr Sci* **11**, 55–113.

Coogan PT, Bare RM, Waalkes MP. 1992 Cadmium-induced DNA strand damage in cultured liver cells: reduction in cadmium genotoxicity following zinc pretreatment. *Toxicol Appl Pharmacol* **113**, 227–233.

Dreosti IE. 1982 Clinical applications of recent advances in zinc metabolism. *J Food Nutr* **39**, 167–173.

Friel JK, Borgman FR, Chandra RK. 1987 Effect of chronic cadmium administration on liver and kidney concentrations of Zn, Cu, Fe, Mn and Cr. *Bull Environ Contam Toxicol* **38**, 588–593.

Galey FD, Hall JD. 1990 Field investigations of small animal toxicoses. *Vet Clin N Am: Small Animal Practice* **20**, 283–291.

Goldman M, Dacre JC. 1991 Inorganic arsenic compounds: are they carcinogenic, mutagenic, teratogenic. *Environ Geochem Health* **13**, 179–191.

Hořejší J, Matys Y, Nejedlý B, *et al.* 1979 *Základy klinické biochemie ve vnitřním lékařství*. Praha: Avicenum; 168.

Jendrassik L, Grof P. 1938 Vereinfachte photometrische Methoden zur Bestimmung des Blutbilirubins. *Biochem Zeit* **297**, 6–13.

Kazacos AE, Van Vleet JF. 1989 Sequential ultrastructural changes of the pancreas in zinc toxicosis in ducklings. *Am J Pathol* **134**, 581–589.

Kumaratilake JS, Howell JMcC. 1989 Intracellular distribution of Cu in the liver of copper-loaded sheep—a subcellular fractionation study. *J Comp Pathol* **101**, 161–176.

Leonard A, Gerber GB, Leonard F. 1986 Mutagenicity, carcinogenicity and teratogenicity of zinc. *Mutat Res* **168**, 343–353.

Lojda L, Mráz I. 1982 Unstable chromosomal aberation in animals from selected coal-mining areas in north Bohemia (in Slovak). *Proc. 12.dni genetiky hospodárskych zvierat*. Nitra; 64–65.

Magos L. 1991 Epidemiological and experimental aspects of metal carcinogenesis: physicochemical properties, kinetics and the active species. *Environ Health Perspect* **95**, 157–189.

Ogden L, Edwards WC. 1988 Zinc intoxication in a dog from the ingestion of copper-clad zinc pennies. *Vet Hum Toxicol* **30**, 577–578.

Parada P, Gonzales S, Berggvist E. 1987 Industrial pollution with copper and other heavy metals in a beef cattle ranch. *Vet Hum Toxicol* **29**, 122–129.

Perry P, Wolf S. 1974 New Giemsa method for the differential staining of sister chromatids. *Nature* **251**, 156–158.

Petrakis H, Sideris EG. 1982 Evidence from mutagenesis on the possible involvement of Cu^{2+} and Mg^{2+} ions in the eucaryotic chromosome structure. *Studia Biophys* **92**, 1–9.

Pritchard GC, Lewis G, Wells GAH, Stopforth A. 1985 Zinc toxicity, copper deficiency and anaemia swilfed pigs. *Vet Rec* **117**, 545–548.

Quin S, Huang CC. 1984 A new method for sisterchromatid exchange studies *in vivo*. *Mutat Res* **141**, 189–193.

Reif JS, Ameghino E, Aaronson MJ. 1989 Chronic exposure of sheep to a zinc smelter in Peru. *Environ Res* **49**, 40–49.

Reit-Correa F, Oliveira JA, Giesta S, Schild L, Mendez MC. 1989 Intoxicaqao chronica por cobre em ovinos no Rio Grande do Sul Pesq. *Vet Bras* **9**, 51–54.

Rossman TG, Molina M. 1986 Genetic toxicology of metal compounds. Enhancement of ultraviolet light-induced mutagenesis in *Escherichia coli* WP 2. *Environ Mutagen* **8**, 263–271.

Sheabar YF, Yannai S. 1989 In vitro effects of cadmium and aresenite on glutathione peroxidase, aspartate and alanine aminotransperases, cholinesterase and glucose-6-phosphate dehydrogenase activities in blood. *Vet Hum Toxicol* **31**, 528–531.

Smith RM, Griel LC, Muller LD, Leach RM, Baker DE. 1991 Effects of dietary cadmium chloride throughout gestation on blood and tissue metabolites of primigravid and neonatal dairy cattle. *J Anim Sci* **69**, 4078–4087.

Soli NE. 1980 Chronic copper poisoning in sheep. *Nord Vet Med* **32**, 75–89.

Thompson LJ, Hall JO, Meerdink GL. 1991 Toxic effects of trace element excess. *Vet Clin N Am: Food Animal Practice* **7**, 277–306.

Thompson RH, Todd JR. 1974 Muscle damage in chronic copper poisoning of sheep. *Rec Vet Sci* **16**, 97–99.

Van Der Schee W, Van Der Assem GH, Van Der Berg R. 1983 Breed differences in sheep with respect to the interaction between zinc and accumulation of copper in the liver. *Vet Quart* **5**, 171–174.

Waner T, Nyska A. 1991 The toxicological significance of decreased activities of blood alanine and aspartate aminotransferase. *Vet Res Commun* **15**, 73–78.

West HJ, Bates A, Hynes GE. 1987 Changes in the concentrations of bile acids in the plasma of sheep with liver damage. *Res Vet Sci* **43**, 243–248.

Wong PK. 1988 Mutagenicity of heavy metals. *Bull Environ Contam Toxicol* **40**, 597–603.