

PROTECTIVE EFFECTS OF SELENIUM ON HEPATOTOXICITY CAUSED BY SUBACUTE EXPERIMENTAL COMBINED EXPOSURE TO CADMIUM AND LEAD IN RATS

BOGDAN GABRIEL ŞLENCU^{1*}, CONSTANTIN CIOBANU¹, RODICA CUCIUREANU¹, ALINA ANTON², STELIAN CIOBANU², GHEORGHE SOLCAN², CARMEN SOLCAN²

¹Faculty of Pharmacy, "Grigore T. Popa" University of Medicine and Pharmacy Iasi, 16 Universităţii Street, 700115, Iaşi, Romania

²Faculty of Veterinary Medicine, "Ion Ionescu de la Brad" University of Agricultural Sciences and Veterinary Medicine Iasi, 3 Mihail Sadoveanu Alley, 700490, Iaşi, Romania

*corresponding author: bogdan.slencu@umfiasi.ro

Manuscript received: January 2018

Abstract

Cadmium (Cd) and lead (Pb) toxicity is mediated by multiple mechanisms. The purpose of this study was to assess the potential protective effects of selenium (Se) in the case of experimental Cd, Pb or combined Cd and Pb exposure through drinking water in rats. Male Wistar rats were used (8 experimental groups, n = 6). The experiment lasted for 56 days. The groups were: Control (distilled water), Se (Se - 0.2 mg/L), Cd (Cd - 150 mg/L), Pb (Pb - 300 mg/L), Cd+Pb (Cd - 150 mg/L; Pb - 300 mg/L), Cd+Se (Cd - 150 mg/L; Se - 0.2 mg/L), Pb+Se (Pb - 300 mg/L; Se - 0.2 mg/L), Cd+Pb+Se (Cd - 150 mg/L; Pb - 300 mg/L; Se - 0.2 mg/L). Cd exposure resulted in an increase in transaminases activities and a modification in the serum protein fractions ratios, which was alleviated by Se. Cd or/and Pb exposure altered the normal hepatic histology, which was improved by Se. Se exhibited potential protective action by improving some biochemical parameters and the hepatic histological architecture and reducing the intensity of p53 and caspase-3 immunostaining.

Rezumat

Toxicitatea cadmiului (Cd) şi plumbului (Pb) este mediată de mecanisme multiple. Scopul acestui studiu a fost de a evalua efectele potenţial protectoare ale seleniului (Se) în cazul expunerii experimentale la Cd şi Pb separat şi simultan, prin intermediul apei de băut, la şobolani. Au fost utilizaţi şobolani Wistar masculi (8 loturi, n = 6). Perioada experimentală a fost de 56 de zile. Loturile au fost: Control (apă distilată), Se (Se - 0,2 mg/L), Cd (Cd - 150 mg/L), Pb (Pb - 300 mg/L), Cd+Pb (Cd - 150 mg/L; Pb - 300 mg/L), Cd+Se (Cd - 150 mg/L; Se - 0,2 mg/L), Pb+Se (Pb - 300 mg/L; Se - 0,2 mg/L), Cd+Pb+Se (Cd - 150 mg/L; Pb - 300 mg/L; Se - 0,2 mg/L). Expunerea la Cd a determinat creşterea activităţii transaminazelor şi o modificare a fracţiilor proteinelor serice, modificările fiind atenuate de administrarea de Se. Expunerea la Cd sau/şi Pb a determinat modificarea histologiei hepatice normale, proces ce a fost atenuat de către Se. Se a manifestat acţiune potenţial protectoare prin îmbunătăţirea unor parametri biochimici şi a histoarhitectonicii hepatice şi prin reducerea intensităţii imunocolorării pentru p-53 şi caspaza-3.

Keywords: selenium, cadmium, lead, hepatotoxicity

Introduction

Cadmium (Cd) and lead (Pb) are ubiquitous environmental pollutants, with toxic effects in humans, animals and plants. The general human population is exposed to cadmium through food and water intake, cigarette smoking and inhalation of ambient air [63]. Diet is the main source of Cd exposure in non-smokers. Occupational exposure to Cd occurs mainly through inhalation, in the course of industrial processes in which Cd-containing materials are heated, such as smelting and electroplating [2]. People are exposed to lead by eating food or drinking water that contains lead and by cigarette smoking [1, 52]. Occupational exposure to lead occurs especially through inhalation, in case of the lead-emitting industries, such as lead smelters or

lead acid battery recycling plants [52]. International and national institutions have established guidelines regarding the maximum allowable cadmium or lead levels in food products, medicinal plants, drinking water and environment [1, 2, 33, 63].

Cadmium has several toxic effects in animals and humans, including nephrotoxicity, hepatotoxicity, carcinogenicity, teratogenicity, endocrine disruption, reproductive toxicity and undesired effects on bones [43, 48]. The distribution of Cd between various tissues depends on many endogenous and exogenous factors and acute exposure results in a different distribution pattern throughout the body than the chronic exposure does. Cadmium accumulates mainly in the kidneys and in the liver [58]. It generally has a very low excretion rate, thus it has a long biological half-life. In mice and rats the half-life

can range between 200 and 700 days, depending on the level of exposure and other factors [43]. Cadmium is not known to undergo direct metabolic conversions [2]. The interaction between Cd and metallothionein plays a critical role in its toxicokinetics and toxicodynamics [2, 27, 43]. It is retained in the kidneys and in the liver mainly bound to metallothionein [2, 27]. Generation of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and hydroxyl radicals is the main mechanism of Cd toxicity in the case of acute poisoning, but in the case of chronic poisoning acquired Cd tolerance occurs, with aberrant gene expression [32]. Cadmium may replace some essential metals such as copper, iron, manganese, zinc in their biological systems [40].

Lead exposure produces various harmful effects at hematopoietic, renal, nervous, bone and gastrointestinal tract level [46, 52]. After exposure, Pb accumulates in blood, soft tissues and bones. In blood, approximately 99% of Pb is found in erythrocytes while in soft tissues it accumulates mainly in the liver and in the kidneys [1, 46, 52]. While the half-life for Pb from blood generally does not exceed one month, Pb from bones can have a half-life of decades in humans [1, 52]. Pb has the ability to substitute polyvalent cations of essential metals (especially calcium - Ca^{+2} and zinc - Zn^{+2}) in their biological systems and subsequently perturb significant processes, including metal transport, energy metabolism, apoptosis, inter- and intracellular signalling, cell adhesion and enzymatic processes [21]. The main mechanism of Pb toxicity, leading to the reduction of mental activity in children, is considered to be related to the alteration of calcium channels functioning and of glutamate and cholinergic systems in the developing brain [1, 21]. Oxidative stress has been reported as another mechanism of Pb toxicity, with two simultaneous pathways: a) generation of ROS such as hydroperoxides, singlet oxygen and hydrogen peroxide and b) depletion of antioxidant reserves, such as the inactivation of glutathione [19, 52].

In the majority of experimental studies with the objective of assessing Cd or Pb toxicity a single metal is usually used, in high doses. Combined Cd and Pb toxicity has been investigated much less. Co-exposure to Cd and Pb generally results in additive toxic effects [20, 34, 67]. It is possible that Cd and Pb could also exert synergistic toxic effects on particular systems that have not been investigated within experimental studies.

Selenium (Se) is an essential microelement for animals and humans. It enters in the structure of some proteins and enzymes with multiple physiological roles, of which the most important are the glutathione-peroxidases, the deiodinases of the thyroid hormones and the thioredoxin reductases. In high amounts, selenium can exert toxic effects [49].

Some studies have found that Se protects animals against toxicity caused by exposure to and/or intake of metals such as cadmium, lead, mercury, arsenic, vanadium, silver [42]. There are multiple mechanisms involved in Se protective effects. Selenium compounds are known for their ability to scavenge ROS [11]. The glutathione-peroxidase catalytic cycle in which glutathione acts as the reducing substrate involves the formation of active selenol, which reduces peroxide [11, 49]. Some Se compounds have been shown to prevent metal-induced ROS generation by binding metals, such as copper or iron, *in vivo* and *in vitro* [10, 11, 65].

The primary aim of this study was to assess the effects of selenium exposure in case of experimental exposure to cadmium, lead or a combination of the two metals through drinking water on histopathological changes of the liver and on some blood biochemical parameters.

Materials and Methods

Reagents

All reagents were of analytical grade. Distilled water was used as drinking water for the animals. Standard dry chow for rats was purchased from "Cantacuzino" National Institute for Research and Development in Microbiology and Immunology. Cadmium chloride hemipentahydrate ($\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$), lead acetate trihydrate ($\text{Pb}(\text{CH}_3\text{COO})_2 \cdot 3\text{H}_2\text{O}$) and sodium selenite pentahydrate ($\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$) were used as the sources of Cd, Pb and Se. Ketamine 10%, injectable solution for veterinary use (Kepro VB, Holland) was used as anaesthetic. A Hydragel $\beta 1$ - $\beta 2$ (Sebia, France) kit was used for the determination of serum proteins; the other blood biochemical parameters were determined using specific assay kits from Cormay (Poland). Novocastra[®] lyophilized mouse monoclonal antibody CPP32 (Caspase-3) (Leica Biosystems, Germany), p53 protein (DO-7) mouse monoclonal antibody (Vector Laboratories, USA) and Vectastain Elite ABC kit (Vector Laboratories, USA) were used for immunohistochemistry.

Instruments

Serum was separated on a Hettich Rotina 38R (Andreas Hettich GmbH, Germany) centrifuge. An Accent 200 (Cormay, Poland) automatic biochemical analyser was used to determine biochemical parameters from blood. The gels for serum proteins were generated using a Sebia system, scanned using a Sebia Gelscan system, analysed and quantified with Sebia's Phoresis software (Sebia, France). The stained liver sections were analysed using an Olympus CX41 microscope with an integrated DP21 camera.

Animals and procedures

The animal protocol used in this work was evaluated and approved by the Ethics Committee for Research of "Grigore T. Popa" University of Medicine and Pharmacy, Iași, Romania. All procedures were

conducted in accordance with the 86/609/EEC directive, with the internal regulations of the university and with other applicable regulations.

A number of 48 male Wistar rats, purchased from "Cantacuzino" National Institute for Research and Development in Microbiology and Immunology – Băneasa, Romania, animal facility, having the initial weight: 250 - 400 g and the age approximately 8 months were randomly divided into 8 experimental groups ($n = 6$) and subsequently marked. The rats were maintained throughout the experiment at 18 - 25°C, with a light/dark cycle of 12/12 h and were housed in collective cages (each experimental group in one collective cage). The animals had free access to food (dry chow) and water (containing the elements of interest), *ad libitum*. Special water dispensers with stoppers were used and the water consumption for every group was measured on a daily base. The animals were weighed every 7 days. The substances of interest were dissolved in the drinking water.

The experimental groups were: Control group (only distilled water); Se group (Se - 0.2 mg/L); Cd group (Cd - 150 mg/L); Pb group (Pb - 300 mg/L); Cd+Pb group (Cd - 150 mg/L and Pb - 300 mg/L); Cd+Se group (Cd - 150 mg/L and Se - 0.2 mg/L); Pb+Se group (Pb - 300 mg/L and Se - 0.2 mg/L); Cd+Pb+Se group (Cd - 150 mg/L, Pb - 300 mg/L and Se - 0.2 mg/L).

The experiment lasted for 56 days and the animals were subsequently anaesthetised. Ketamine (100 mg/kg body weight (bw)) was injected intraperitoneally to induce anaesthesia. After the onset of the ketamine effect, the rats were immobilised and the thorax and

the abdomen were opened. Blood was collected through cardiac puncture in special vacutainers. The livers were removed, examined and weighed.

Body weight, liver weight, water consumption and element intake

The evolution of the body weight was calculated as percent of increase/decrease in comparison to the initial body weight, for every rat, at every weighing moment. The mean percent was then calculated for every experimental group.

The relative liver weight was expressed for every rat as g of liver/100 g bw at the end of the experiment. Means were then calculated for every experimental group.

Daily approximate water consumption for one rat from a group was estimated by dividing the volume of water consumed by that experimental group during 24 hours to 6 (the number of rats in a group). Means for 7 days intervals were then calculated.

Daily approximate water consumption reported to kg bw for a group was estimated by dividing the volume of water consumed by that experimental group during 24 hours to the total weight of the rats from that group (the previous weighing). Means for 7 days intervals were then obtained.

Based on the daily approximate water consumption reported to kg bw for each group and on the concentrations of Cd, Pb and Se in the drinking water, the daily approximate intake reported to kg bw for each element was obtained. The means for the entire experimental period of 56 days were then calculated. The estimated intake of each element for each experimental group is presented in Table I [53, 54].

Table I

Estimated intake of cadmium, lead and selenium through the drinking water for the experimental groups

Group	Cd (mg/kg bw)	Pb (mg/kg bw)	Se (µg/kg bw)
Control	-	-	-
Se	-	-	15.48 ± 0.42
Cd	10.48 ± 0.41	-	-
Pb	-	25.34 ± 0.68	-
Cd+Pb	8.80 ± 0.35	17.60 ± 0.70	-
Cd+Se	7.36 ± 0.23	-	9.81 ± 0.30
Pb+Se	-	23.84 ± 0.64	15.90 ± 0.43
Cd+Pb+Se	7.28 ± 0.24	14.56 ± 0.48	9.71 ± 0.32

Note: Results are expressed as mean ± SEM ($n = 6$).

Blood parameters

After clothing, the blood was centrifuged at 3500 rounds *per minute* for five minutes and serum was separated.

Total proteins, total cholesterol, serum triglycerides (TG), total bilirubin, direct bilirubin, serum alanine aminotransferase activity (ALT) and serum aspartate aminotransferase activity (AST) were determined on the automated biochemical analyser.

Gel electrophoresis was employed to separate and quantify serum protein fractions. Serum albumin

(ALB), serum alpha 1 globulins (α 1-GLO), serum alpha 2 globulins (α 2-GLO), serum beta 1 globulins (β 1-GLO), serum beta 2 globulins (β 2-GLO) and gamma globulins (γ -GLO) were expressed as percentage from the total amount of proteins. Albumin/globulin ratio (ALB/GLO) was automatically calculated by the software. Each kit was employed according to the manufacturer's instructions for use.

Histology and immunohistochemistry

Liver fragments from each rat were preserved in 10% neutral buffered formalin (approximately 4%

formaldehyde, pH ~ 7.4), processed according to the standard technique and embedded in paraffin. Immunostaining was conducted according to the manufacturer's suggested techniques. Sections of 5 μ m were obtained from the paraffin blocks and dewaxed. The epitopes were revealed by heating at 95°C in 10 mmol of citrate acid buffer (pH = 6) for 10 minutes in a microwave oven, after which were left at room temperature for 20 minutes. The sections were washed twice in phosphate buffer solution (PBS) with a pH of 7.5 for 5 minutes, after which were incubated with primary anti-p53 and anti-caspase-3 antibodies and diluted 1:100 at room temperature for 1 h, in a humid chamber. After washing with PBS, the sections were incubated with the secondary antibody (horseradish peroxidase goat anti-mouse IgG) for 1 h, in a humid chamber, at 4°C, then washed with PBS and incubated with 3,3' diaminobenzidine substrate for 7 minutes, counter-stained with Harris haematoxylin, clarified with xylene and mounted. The slides were examined under the microscope and relevant photographs were taken.

Statistical analysis

Results of biochemical blood parameters, water consumption, estimations of elements intake, body weight evolution and liver weight were expressed as mean $\pm$ SEM. The normal distribution of the values of biochemical blood parameters was assessed by using the Shapiro-Wilk test, then the values were subjected to one-way ANOVA, followed by Tukey's posthoc test in order to estimate data variance between experimental groups. The minimum level of significance was set at 0.05. IBM SPSS Statistics 20 and MS Excel 2007 were used for the statistical analysis and graphical representation. GIMP 2.8 was used for editing photographs.

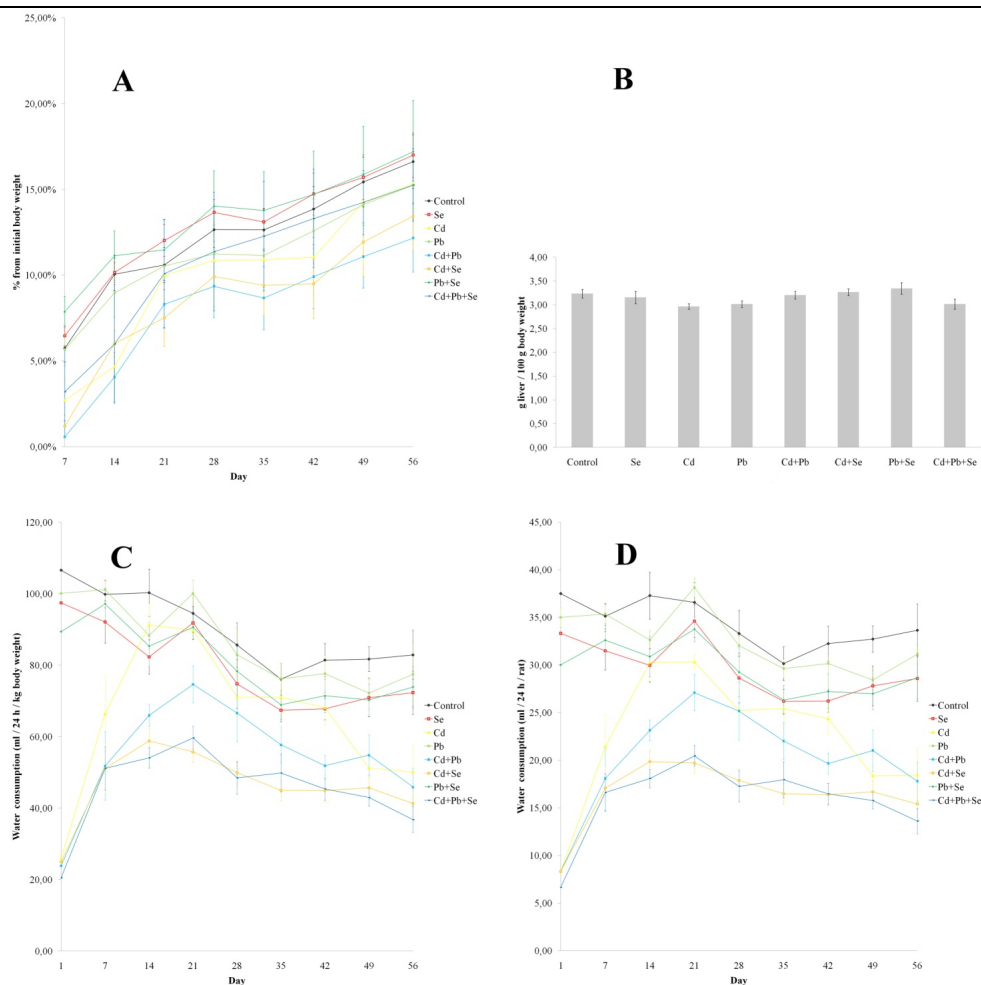
Results and Discussion

Figure 1A shows that the rate of body weight gain was lower in case of Cd, Pb and Cd+Pb groups when compared to Control; the lowest was observed in case of Cd+Pb group. The rate was slightly higher in case of Se group in comparison to Control. Co-exposure to selenium alleviated the decrease in body weight gain in case of lead and cadmium + lead exposure (Pb+Se > Pb; Cd+Pb+Se > Cd+Pb), while it apparently had no effect on cadmium exposure (Cd+Se < Cd, overall). Cadmium intoxication is known to cause reduction or stagnation in weight gain, depending on dose, exposure time, route of exposure and other factors [2, 7, 67]. The explanation could be that weight gain is influenced by the availability and absorption of nutrients and cadmium decreases the absorption and digestion of some nutrients [18].

Lead is also known to cause reduction or stagnation in weight gain [1, 4, 24] through several mechanisms, among which it is the impairment of zinc status in zinc-dependent enzymes, which are necessary for a multitude of metabolic processes, the induced disruptions leading to a metabolic imbalance [24]. Combined exposure to cadmium and lead results in a more reduced weight gain ratio than the exposure to individual elements [13]. Co-exposure to selenium partially alleviates weight gain in case of cadmium [9, 26] or lead intoxication [4]. The results regarding body weight gain ratios in our study are in accordance to the results reported by other authors, with the exception of the rats exposed to cadmium and selenium (Cd+Se group), for which the weight gain ratio was lower when compared to those exposed only to cadmium (Cd group). Still, this exception could be due to the fact that only 6 animals were attributed to each experimental group, slightly reducing the statistical power of the experiment and increasing the probability of errors.

There was no significant difference among groups regarding relative liver weight (Figure 1B).

Figure 1C and Figure 1D show that the estimated water consumption was overall lower in case of Cd, Pb and Cd+Pb groups, in comparison to Control; the lowest was observed in the case of Cd+Pb group. Water consumption was lower for the Se group than for the Control group. The water consumption was lower in the case of co-exposure to selenium (Cd+Se < Cd; Pb+Se < Pb; Cd+Pb+Se < Cd+Pb). The lowest water consumption was observed in the case of the Cd+Pb+Se group. Some studies have also reported decreased fluid intake in case of cadmium or lead exposure [8, 39], while other studies did not [41, 59]. One cause for the decrease in fluid intake, when the drinking water contains cadmium and/or lead can be the alteration of taste. It has been demonstrated that cadmium chloride in solutions is aversive to rats and the decrease in drinking water intake occurs rapidly, so the physiological impairment could be excluded as a cause [14]. Another study concluded that *Meriones shawi* rodents maintained a homeostasis state and presented an adaptation to regulate volume during cadmium exposure by decreasing diuresis and increasing urine osmolality [35]. It is generally known that fluid intake decreases with the increase in the osmolality of the drinking water, so another less plausible cause for the difference in water consumption among groups in this study could be the increase in the osmolality of the drinking water. Still, the concentrations of the salts were not high enough in order to lead to hypertonic solutions.

**Figure 1.**

Evolution of the body weight, relative liver weight and water consumption. **(A)** Evolution of the body weight, expressed as percent of increase/decrease in comparison to the body weight at the beginning of the experiment; **(B)** Relative liver weight of the rats from all the experimental groups, expressed as grams of liver *per* kg bw; **(C)** Estimated water consumption during 24 hours *per* kg bw; **(D)** Estimated water consumption during 24 hours *per* rat. Error bars are SEM

Table II

Effects of cadmium and/or lead and/or selenium exposure on some biochemical blood parameters

Group	Total cholesterol (mg/dL)	TG (mg/dL)	Total bilirubin (mmol/L)	Direct bilirubin (mmol/L)	ALT (IU/L)	AST (IU/L)
Control	70.33 ± 6.11	92.81 ± 22.39	0.088 ± 0.009	0.058 ± 0.009	85.26 ± 2.55 ^c	213.53 ± 13.21 ^c
Se	86.00 ± 4.76 ^e	90.91 ± 15.16	0.071 ± 0.013	0.030 ± 0.008	87.40 ± 6.69 ^c	235.06 ± 25.16 ^c
Cd	79.16 ± 5.32	105.66 ± 13.83	0.068 ± 0.003	0.041 ± 0.001	128.21 ± 8.61 ^{ab}	351.98 ± 37.40 ^{ab}
Pb	84.33 ± 4.25	104.58 ± 0.81	0.080 ± 0.004	0.050 ± 0.005	91.61 ± 8.53	291.40 ± 21.74
Cd+Pb	66.00 ± 3.86 ^b	105.88 ± 9.17	0.131 ± 0.066	0.046 ± 0.006	113.58 ± 11.50	296.40 ± 32.09
Cd+Se	56.83 ± 3.56 ^{bcd}	104.43 ± 22.73	0.110 ± 0.012	0.071 ± 0.006 ^{bc}	100.36 ± 4.45	273.25 ± 13.78
Pb+Se	56.16 ± 1.30 ^{bcd}	86.93 ± 8.93	0.093 ± 0.005	0.041 ± 0.004	92.05 ± 8.29	245.05 ± 10.00 ^c
Cd+Pb+Se	56.83 ± 4.19 ^{bcd}	102.20 ± 8.78	0.086 ± 0.002	0.061 ± 0.006 ^b	114.76 ± 12.57	241.15 ± 11.14

Results are expressed as mean ± SEM (n = 6); statistical analysis consisted in one-way ANOVA followed by Tukey's test; a value of $p < 0.05$ was considered significant; ^a – significant difference when compared to Control; ^b – significant difference when compared to Se; ^c – significant difference when compared to Cd; ^d – significant difference when compared to Pb; ^e – significant difference when compared to Cd+Pb

Table II shows the values determined for total cholesterol, TG, total bilirubin, direct bilirubin ALT and AST.

Selenium co-exposure reduced total cholesterol levels (Cd > Cd+Se; Pb > Pb+Se). As in the case of

this study, an attenuation caused by selenium co-administration was also observed by other research groups [44].

Apparently cadmium or lead resulted in a small decrease, statistically insignificant, in total bilirubin

and direct bilirubin levels, when compared to Control (Control > Cd; Control > Pb). Apart from this change, there was no consistent and coherent influence of exposure to a combination of any of these elements on total bilirubin and direct bilirubin levels. Total bilirubin level is a marker of liver damage [55]. Bilirubin is a product of haemoglobin degradation and is a marker of hepatobiliary damage, especially cholestasis and biliary effects [45]. Some researchers reported elevated total bilirubin levels in the case of cadmium [29] or lead [5, 24] exposure. Another study reported no marked difference between groups of control rats, rats exposed to cadmium and rats exposed to cadmium and selenium [3].

Exposure to cadmium determined an increase in ALT and AST activity, in comparison to Control (Cd > Control) while co-exposure to selenium had a decreasing effect (Cd > Cd+Se). An increase in serum trans-aminases usually accompanies damage to the liver through a necrotic mechanism [55]. Serum ALT activity is usually a more specific biomarker of liver function than AST activity, because AST is released from damaged myocytes as well as hepatocytes [45]. Injured hepatocytes release enzymes into the blood stream, resulting in ALT and AST increase. The high ALT and AST activities are accompanied by high liver microsomal membrane fluidity, alteration in the liver tissue histogram and ROS generation [24]. Although the kidney is generally the main target for cadmium toxicity because it accumulates preferably in high quantities in this organ, cadmium also accumulates in the liver and exerts hepatotoxic effects [2, 30, 63]. Lead is also accumulated in the liver and manifests its toxicity in this organ [36, 61]. Our results are in agreement with those observed within other studies, which showed an increase in ALT and AST in case of cadmium [16, 29] intoxication. In this study, combined exposure to cadmium and lead did not result in a significant increase in transaminases when compared to individual exposure, which is in accordance with the results obtained by

other researchers on mice, during a 60 day exposure time through the drinking water [13]. This can be due to the fact that water and the subsequent elements intake were lower for the combined exposure groups than for the individual exposure groups. Other studies have reported a decrease in AST and ALT in the case of selenium co-exposure or co-administration with cadmium [15, 28] or lead [4, 44].

Table III shows the values determined for total serum proteins and for serum protein fractions. No significant differences in total serum proteins were observed between groups, with the exception of the Cd+Pb group, for which the level was significantly reduced when compared to Control (Cd+Pb < Control). Exposure to cadmium and lead resulted in a significant decrease in ALB and ALB/GLO (Control > Cd+Pb), while the co-exposure to selenium apparently had no significant or marked effect when compared with the Cd+Pb group. Exposure to cadmium or lead increased β 2-GLO. Albumin, the most abundant individual serum protein is responsible for maintaining osmotic pressure and serves as a transport protein [23]. Among the globulins, α 1-GLO and α 2-GLO include many clinically important acute-phase proteins [68]. α 1-GLO include, among other proteins, α 1-antitrypsin, α 1-antichymotrypsin and α 1-lipoprotein while α 2-GLO include α 2-globulins, haptoglobin, α 2-macroglobulin, ceruloplasmin and α 2-antiplasmin [60]. Beta globulins include other acute-phase proteins in addition to complement and various proteins important in coagulation [60, 68]. The γ -GLO include the immunoglobulins (IgA, IgM, IgE and IgG) but some immunoglobulins can migrate in the β area [68]. Other studies observed that cadmium or lead caused a decrease in albumin levels while selenium partially alleviated the cadmium-induced changes [15, 16, 51]. The reduction in total proteins and ALB may be caused by an inhibition of protein bio-synthesis through the specific enzymes involved in cell processes and a low excretion of some hormones involved in protein biosynthesis [24].

Table III

Effects of cadmium and/or lead and/or selenium exposure on total serum proteins and on serum protein fractions

Group	Total proteins (g/dL)	ALB (%)	α 1-GLO (%)	α 2-GLO (%)	β 1-GLO (%)	β 2-GLO (%)	γ -GLO (%)	ALB/GLO
Control	6.03 $\pm$ 0.12 ^c	61.18 $\pm$ 0.78 ^c	19.78 $\pm$ 0.68	4.91 $\pm$ 0.24	2.83 $\pm$ 0.45	1.38 $\pm$ 0.27 ^{cd}	8.39 $\pm$ 1.39 ^c	1.50 $\pm$ 0.09 ^c
Pb	5.61 $\pm$ 0.21 ^c	60.16 $\pm$ 1.19 ^c	18.55 $\pm$ 1.11	3.93 $\pm$ 0.70	4.08 $\pm$ 0.29	1.93 $\pm$ 0.60 ^d	11.33 $\pm$ 0.29	1.52 $\pm$ 0.07 ^c
Cd	6.04 $\pm$ 0.14 ^c	56.88 $\pm$ 0.48	15.20 $\pm$ 0.62	4.96 $\pm$ 0.48	5.41 $\pm$ 0.27	4.43 $\pm$ 0.47 ^a	13.10 $\pm$ 0.27	1.32 $\pm$ 0.02
Pb	5.81 $\pm$ 0.13 ^c	54.45 $\pm$ 1.10	17.71 $\pm$ 0.82	5.16 $\pm$ 0.47	5.50 $\pm$ 0.64	4.68 $\pm$ 0.18 ^{ab}	12.48 $\pm$ 0.50	1.20 $\pm$ 0.05
Cd+Pb	4.74 $\pm$ 0.22 ^{abcd}	51.80 $\pm$ 0.76 ^{ab}	18.25 $\pm$ 0.55	4.28 $\pm$ 0.51	6.58 $\pm$ 0.91	3.68 $\pm$ 0.61	15.16 $\pm$ 0.55 ^a	1.08 $\pm$ 0.03 ^{ab}
Cd+Se	6.28 $\pm$ 0.14 ^c	57.61 $\pm$ 1.25	13.88 $\pm$ 0.35 ^a	5.68 $\pm$ 0.51	5.70 $\pm$ 0.38	3.96 $\pm$ 0.27	13.15 $\pm$ 0.54	1.36 $\pm$ 0.07
Pb+Se	5.98 $\pm$ 0.18 ^c	53.38 $\pm$ 3.54 ^a	16.41 $\pm$ 2.59	7.35 $\pm$ 0.26 ^{bc}	5.50 $\pm$ 0.38	3.75 $\pm$ 0.31	13.60 $\pm$ 1.09	1.19 $\pm$ 0.14
Cd+Pb+Se	6.30 $\pm$ 0.13 ^c	52.75 $\pm$ 2.18 ^a	14.93 $\pm$ 1.02	8.23 $\pm$ 0.96 ^{abcde}	6.48 $\pm$ 1.92	7.38 $\pm$ 1.28 ^{abce}	10.21 $\pm$ 0.50	1.14 $\pm$ 0.10 ^b

Results are expressed as mean $\pm$ SEM (n = 6); statistical analysis consisted in one-way ANOVA followed by Tukey's test; a value of p < 0.05 was considered significant; ^a – significant difference when compared to Control; ^b – significant difference when compared to Se; ^c – significant difference when compared to Cd; ^d – significant difference when compared to Pb; ^e – significant difference when compared to Cd+Pb

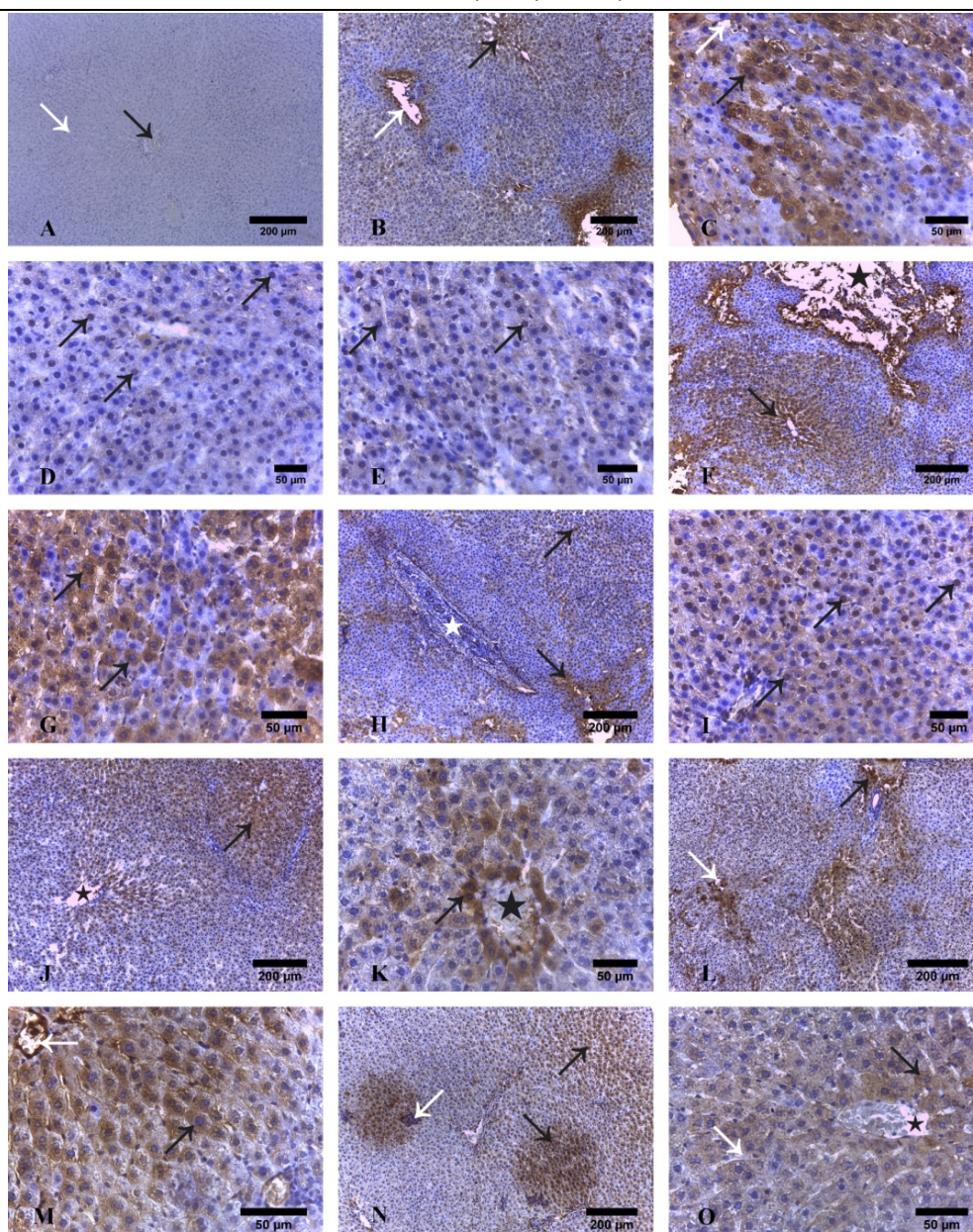


Figure 2.

Liver sections of rats from the experimental groups. (A) Control group (p53 staining); normal structure with no pathological modifications observed; (B) Cd group (caspase-3 staining); disorganization in the architecture of hepatic lobules, focal areas of necrosis near the portobiliary veins, caspase-3 positive marked cells both in the portobiliary space and around the centrolobular vein; (C) Cd group (caspase-3 staining); caspase-3 overexpression in the hepatocytes around the centrolobular vein, dilated centrolobular vein, a large number of Kupffer cells at sinusoidal capillaries level; (D) Cd group (p53 staining); positive p53 nuclei, in large numbers; (E) Pb group (p53 staining); positive p53 nuclei; (F) Pb group (caspase-3 staining); overexpression of caspase-3 in hepatocytes around the veins from the portobiliary space and around the centrolobular vein; (G) Pb group (caspase-3 staining); hepatocytes positively marked for caspase-3 and visible Kupffer cells at sinusoidal capillaries level, dilated sinusoidal capillaries; (H) Cd+Pb group (caspase-3 staining); conjunctive proliferation and large portobiliary space with newly formed biliary ducts; (I) Cd+Pb group (p53 staining); p53 positive nuclei in large number, modified architecture of the hepatic lobules; (J, K) Cd+Se group (caspase-3 staining); reduced focal areas of necrosis, reduced intensity of caspase-3 marking, typically organized hepatic lobules, sinusoidal capillaries dilated in some areas, visible Kupffer cells; (L, M) Pb+Se group (caspase-3 staining); reduced focal areas of necrosis, smaller number of cells positively marked for caspase-3, reduced intensity of caspase-3 marking, the cytoplasm has a vacuolar aspect; (N, O) Cd+Pb+Se group (caspase-3 staining); focal areas positively marked for caspase-3, reduced intensity of caspase-3 marking, reduced focal areas of necrosis.

The livers of the rats from the Control and Se groups had normal specific histological structure, without modifications. Portobiliary spaces, hepatocyte plates arranged radially from the centrolobular vein towards periphery and separated by sinusoidal capillaries can be observed in Figure 2A (Control). Areas of necrosis were observed in the livers of the rats from the Cd group, especially around the vein from the portobiliary space and in some cases around the centrolobular vein. The hepatocytes around the centrolobular vein were dense and swollen, with signs of granular and hydropic degeneration. The hepatocyte cytoplasm was positively marked for caspase-3 (Figure 2B; Figure 2C) and the nuclei were positively marked for p53 (Figure 2D). An intense degeneration in the normal architecture of the hepatic lobule and in the radially disposition of the hepatocytes around the centrolobular vein were observed in the Pb (Figure 2F; Figure 2G) and Cd+Pb groups (Figure 2H; Figure 2I). Fibroblastic proliferation in the portobiliary space, diffuse leucocytary infiltration, periductal fibrosis and focal necrosis in the portobiliary space were observed in the Cd+Pb group (Figure 2H; Figure 2I). The biliary ducts proliferated, forming new biliary ducts (Figure 2H). Positive p53 nuclei were observed in the Pb group (Figure 2E); in the case of Cd+Pb group the positive p53 nuclei were more numerous (Figure 2I). Positive caspase-3 hepatocytes were observed both in the case of Pb group (Figure 2F; Figure 2G) and in the case of Cd+Pb group (Figure 2H). The intensity of necrosis was reduced and the hepatic sinusoidal capillaries were less dilated in the Cd+Se group compared to Cd group (Figure 2J; Figure 2K), Pb+Se group compared to Pb group (Figure 2L; Figure 2M) and Cd+Pb+Se group compared to Cd+Pb group (Figure 2N; Figure 2O).

One group of researchers found that cadmium exposure resulted in various degenerative changes in the liver of rats: enlargement of cell sizes, condensed chromatin, necrosis of single cells, pycnotic nuclei, the vicinity of some sinusoids infiltrated by mononuclear cells [26]. Co-exposure to selenium resulted in the absence of nucleus fragmentation and a decrease in the necrosis of single hepatocytes and in the mononuclear cell infiltrations [26], which is in accordance to the results of our study. Another group of researchers observed that lead exposure in rats resulted in hydropic degeneration, anisokaryosis, nuclear vesiculation, binucleation, cytoplasmic inclusions, cytoplasmic swelling and necrosis [25].

Apoptosis is a complex process through which an organism kills and removes unwanted cells. Caspases are crucial in the mediation of apoptosis. Caspase-3 is a frequently activated death protease and acts in a tissue selective manner [47]. Caspase-3 is considered a marker of DNA damage [22]. Increased immunoreactivity to or expression of caspase-3 in kidneys,

prostate and other organs, in case of cadmium exposure, was previously reported [6, 22]. It is also known that lead exposure results in increased immunoreactivity to caspase-3 in rat testes [17] and leads to caspase-3 mediated cell death *in vitro* when employing N2a neuroblastoma cells [31]. In an *in vitro* experiment using human myeloid HL-60 cells, selenium, at low concentrations, activated multiple survival mechanisms, including caspase-3 expression that counteracted oxidative stress-induced cell apoptosis [62].

p53 activates caspase by releasing apoptogenic factors from the mitochondria [50]. p53 is crucial in the cellular response to DNA damage and it is primarily involved in defence mechanisms by transcriptional activation of genes responsible of growth arrest and apoptosis for the elimination of severely damaged cells [64]. It is known that cadmium interferes with the structure and function of p53, but opposite effects have been reported; some authors reported the induction of p53-mediated stress response while others reported inactivation of p53 *via* structural changes, *in vitro* [12, 37, 64]. Some researchers found that lead exposure resulted in an increase in p53 expression in mouse liver [66]. Some selenium compounds are known to modulate p53, by various mechanisms, which can differ among compounds. *In vitro*, sodium selenite causes phosphorylation of some key cysteine residues in p53, leading to its activation [56].

Conclusions

According to our results, selenium exhibited a potential protective action against hepatotoxicity induced by cadmium, lead or a combination of lead and cadmium by improving some biochemical parameters and the histological architecture of the liver and reducing the intensity of p53 and caspase-3 immunostaining. In order to extrapolate the findings of the study to humans, additional clinical studies are required, in order to establish the safety and efficacy of selenium for reducing Cd and/or Pb toxicity in humans. Selenium has very narrow safety limits that need to be carefully considered if one would intend to use it in humans for reducing Cd and/or Pb deleterious effects.

References

1. Agency for toxic substances and disease registry. *Toxicological profile for lead*. US Department of health and human Services - Agency for Toxic Substances and Disease Registry, Atlanta, USA, 2007. www.atsdr.cdc.gov.
2. Agency for toxic substances and disease registry. *Toxicological profile for cadmium*. US Department of health and human services - Agency for toxic substances and disease registry: Atlanta, USA, 2012. www.atsdr.cdc.gov.

3. Alhazza IM, Cadmium-induced hepatotoxicity and oxidative stress in rats: protection by selenium. *Res J Environ Sci.*, 2008; 2: 305-309.
4. Ali MA, Awal MA, Mostofa M, Islam MA, Ghosh A, Effects of selenium and vitamin B6 with their combination in lead acetate induced toxicities in Long Evans rats. *Bangl J Vet Med.*, 2014; 8: 63-73.
5. Annabi Berrahal A, Nehdi A, Hajjaji N, Gharbi N, El-Fazaa S, Antioxidant enzymes activities and bilirubin level in adult rat treated with lead. *C R Biologies*, 2007; 330: 581-588.
6. Arriazu R, Pozuelo JM, Henriques-Gil N, Perucho T, Martin R, Rodriguez R, Immunohistochemical study of cell proliferation, Bcl-2, p53, and Caspase-3 expression on preneoplastic changes induced by cadmium and zinc chloride in the ventral rat prostate. *J Histochem Cytochem.*, 2006; 54: 981-990.
7. Asagba SO, Adaikpoh MA, Kadiri H, Obi FO, Influence of aqueous extract of *Hibiscus sabdariffa* L. petal on cadmium toxicity in rats. *Biol. Trace Elem Res.*, 2007; 115: 47-57.
8. Asagba SO, Comparative effect of water and food-chain mediated cadmium exposure in rats. *Biometals*, 2010; 23: 961-971.
9. Banni M, Messaoudi I, Said L, El Heni J, Kerkeni A, Said K, Metallothionein gene expression in liver of rats exposed to cadmium and supplemented with zinc and selenium. *Arch Environ Contam Toxicol.*, 2010; 59: 513-519.
10. Battin EE, Perron NR, Brumaghim JL, The central role of metal coordination in selenium antioxidant activity. *Inorg Chem.*, 2006; 45: 499-501.
11. Battin EE, Brumaghim JL, Antioxidant activity of sulfur and selenium: a review of reactive oxygen species scavenging, glutathione peroxidase, and metal-binding antioxidant mechanisms. *Cell Biochem Biophys.*, 2009; 55: 1-23.
12. Chatterjee S, Kundu S, Sengupta S, Bhattacharyya A, Divergence to apoptosis from ROS induced cell cycle arrest: effect of cadmium. *Mutation Research*, 2009; 663: 22-31.
13. Cobbina SJ, Chen Y, Zhou Z, Wu X, Zhao T, Zhang Z, Feng W, Wang W, Li Q, Wu X, Yang L, Toxicity assessment due to sub-chronic exposure to individual and mixtures of four toxic heavy metals. *J Haz Mat.*, 2015; 294: 109-120.
14. Cory-Slechta DA, Weiss B, Aversiveness of cadmium in solution. *Neurotoxicology*, 1981; 2: 711-724.
15. El-Boshy ME, Risha EF, Abdel Hamid FM, Protective effects of selenium on some selective immunological and biochemical parameters in cadmium intoxicated rat. *J Invest Biochem.*, 2014; 3: 115-119.
16. El-Boshy ME, Risha EF, Abdel Hamid FM, Mubarak MS, Hadda TB, Protective effects of selenium against cadmium induced hematological disturbances, immunosuppressive, oxidative stress and hepatorenal damage in rats. *J Trace Elem Med Biol.*, 2015; 29: 104-110.
17. Elgawish RAR., Abdelrazek HMA, Effects of lead acetate on testicular function and caspase-3 expression with respect to the protective effect of cinnamon in albino rats. *Toxicol Reports*, 2014; 1: 795-801.
18. Ezedom T, Asagba SO, Effect of a controlled food-chain mediated exposure to cadmium and arsenic on oxidative enzymes in the tissues of rats. *Toxicol Rep.*, 2016; 3: 708-715.
19. Flora G, Gupta D, Tiwari A, Toxicity of lead: A review with recent updates. *Interdiscip Toxicol.*, 2012; 5: 47-58.
20. Fowler BA, Whittaker MH, Lipsky M, Wang G, Chen XQ, Oxidative stress induced by lead, cadmium and arsenic mixtures: 30-day, 90-day, and 180-day drinking water studies in rats: An overview. *Biometals*, 2004; 17: 567-568.
21. Garza A, Vega R, Soto E, Cellular mechanisms of lead neurotoxicity. *Med Sci Monit.*, 2006; 12: RA57-65.
22. Hagar H, Al Malki W, Betaine supplementation protects against renal injury induced by cadmium intoxication in rats: role of oxidative stress and caspase-3. *Environ Toxicol Pharmacol.*, 2014; 37: 803-811.
23. Hall RL, Clinical pathology of laboratory animals. In: *Animal models in Toxicology*, 2nd ed., Shayne CG, Editors. CRC (Taylor & Francis), Boca Raton, USA, 2007.
24. Ibrahim NM, Eweis EA, El-Beltagi HS, Abdel-Mobdy YE, Effect of lead acetate toxicity on experimental male albino rat. *As Pac J Trop Biomed.*, 2012; 2: 41-46.
25. Jarrar BM, Taib NT, Histological and histochemical alterations in the liver induced by lead chronic toxicity. *Saudi J Biol Sci.*, 2012; 19: 203-210.
26. Jihen EH, Imed M, Fatima H, Abdelhamid K, Protective effects of selenium (Se) and zinc (Zn) on cadmium (Cd) toxicity in the liver and kidney of the rat: Histology and Cd accumulation. *Food Chem Toxicol.*, 2008; 46: 3522-3527.
27. Klaassen CD, Liu J, Diwan BA, Metallothionein protection of cadmium toxicity. *Toxicol Appl Pharmacol.*, 2009; 238: 215-220.
28. Konar V, Kara H, Yilmaz M, Dayangac A, Karatas F, Effects of selenium and vitamin E, in addition to melatonin, against oxidative stress caused by cadmium in rats. *Biol Trace Elem Res.*, 2007; 118: 131-137.
29. Kowalczyk E, Kopff A, Fijakowski P, Kopff M, Niedworok J, Baszczyk J, Kedziora J, Effect of anthocyanins on selected biochemical parameters in rats exposed to cadmium. *Acta Biochimica Polonica*, 2003; 50: 543-548.
30. Kuester RK, Waalkes MP, Goering PL, Fishers BL, Micuskey RS, Sipes IG, Differential hepatotoxicity induced by cadmium in Fisher 344 and Sprague-Dawley rats. *Toxicol Sci.*, 2002; 65: 151-159.
31. Kumawat KL, Kaushik DK, Goswami P, Basu A, Acute exposure to lead acetate activates microglia and induces subsequent bystander neuronal death via caspase-3 activation. *Neurotoxicology*, 2014; 41: 143-153.
32. Liu J, Qu W, Kadiiska MB, Role of oxidative stress in cadmium toxicity and carcinogenesis. *Toxicol Appl Pharmacol.*, 2009; 238: 209-214.
33. Lungu C, Corciova A, Spac A, Ciobanu C, Ivănescu B, Evaluation of bioactive compounds from commercial lavender products and comparative histo-anatomical study. *Analele Științifice ale Universității „Al. I. Cuza” Iași s. II a. Biologie vegetală*, 2014; 60: 11-19.

34. Masso EJ, Corredor L, Antonio MT, Oxidative damage in liver after perinatal intoxication with lead and/or cadmium. *J Trace Elem Med Biol.*, 2007; 21: 210-216.
35. Mbarek S, Saidi T, Gonzalez-Costas JM, Gonzalez-Romero E, Chekir RBC, Effects of dietary cadmium exposure on osmoregulation and urine concentration mechanisms of the semi-desert rodent *Meriones shawi* *J Environ Monit.*, 2012; 14: 2212-2218.
36. Mehana EE, Meki A, Fazili KM, Ameliorated effects of green tea extract on lead induced liver toxicity in rats. *Experim Toxicol Pathol.*, 2012; 64: 291-295.
37. Meplan C, Mann K, Hainaut P, Cadmium induces conformational modifications of wild-type p53 and suppresses p53 response to DNA damage in cultured cells. *Biol Chem.*, 1999; 274: 31663-31670.
38. Moisa C, Vicaș LG, Ganea M, Levei EA, Cadar O, Berce C, Murine studies regarding the tissue intake of different magnesium compounds. *Farmacia*, 2018; 66(1): 176-180.
39. Moreira EG, Magalhaes Rosa GJ, Moraes Barros SB, Bassilieff VS, Vassillieff I, Antioxidant defense in rat brain regions after developmental lead exposure. *Toxicology*, 2001; 169: 145-151.
40. Moulis JM, Cellular mechanisms of cadmium toxicity related to the homeostasis of essential metals. *Biometals*, 2010; 23: 877-896.
41. Nai GA, Golghetto JJ, Estrella MPS, Alves JA, Garcia LA, pH dependence of cadmium-contaminated drinking water on the development of cardiovascular injury in Wistar rats. *Biol Trace Elem Res.*, 2015; 165: 81-85.
42. Navarro-Alarcon M, Cabrera-Vique C, Selenium in food and the human body: a review. *Sci Tot Environ.*, 2008; 400: 115-141.
43. Nordberg GF, Nogawa K, Nordberg M, Cadmium. In: *Handbook on the Toxicology of metals - Volume II: Specific metals*, 4th ed., Nordberg GF, Fowler BA, Nordberg M, Editors. Academic press (Elsevier), London, UK, 2015.
44. Othman AI, El Missiry MA, Role of selenium against lead toxicity in male rats. *J Biochem Mol Toxicol.*, 1998; 12: 345-349.
45. Ozer J, Ratner M, Shaw M, Bailey W, Schomaker S, The current state of serum biomarkers of hepatotoxicity. *Toxicology*, 2008; 245: 194-205.
46. Papanikolaou NC, Hatzidaki EG, Belivanis S, Tzanakakis GN, Tsatsakis AM, Lead toxicity update. A brief review. *Med Sci Monit.*, 2005; 11: RA329-336.
47. Porter AG, Janicke RU, Emerging roles of caspase-3 in apoptosis. *Cell Death Differ.*, 1999; 6: 99-104.
48. Rani A, Kumar A, Lal A, Pant M, Cellular mechanisms of cadmium-induced toxicity: a review. *Int J Environ Health Res.*, 2014; 24: 378-399.
49. Reilly C, *Selenium in food and health*, 2nd ed. Springer, New York, USA, 2006.
50. Schuler M, Green DR, Mechanisms of p53-dependent apoptosis. *Biochem Soc Trans.*, 2001; 29: 684-688.
51. Sharma S, Raghuvanshi S, Jaswal A, Shrivastava S, Shukla S, Lead acetate-induced hepatotoxicity in Wistar rats: possible protective role of combination therapy. *J Environ Pathol Toxicol Oncol.*, 2015; 34: 23-34.
52. Skerfving S, Bergdahi IA, Lead. In: *Handbook on the Toxicology of metals - Volume II: Specific metals*, 4th ed., Nordberg GF, Fowler BA, Nordberg M, Editors. Academic press (Elsevier), London, UK, 2015.
53. Slencu BG, Ciobanu C, Solcan C, Anton A, Ciobanu S, Solcan G, Cuciureanu R, Effect of selenium supplementation on serum iron, calcium and magnesium in rats exposed to cadmium or lead. *Lucrări Științifice, Seria Medicină Veterinară*, 2013; 56: 200-206.
54. Slencu BG, Ciobanu C, Solcan C, Anton A, Ciobanu S, Solcan G, Cuciureanu R, Effect of selenium supplementation on serum amylase, lactate dehydrogenase and alkaline phosphatase activities in rats exposed to cadmium or lead. *Cercetări Agronomice în Moldova*, 2014; 47: 113-121.
55. Smith CM, Marks AD, Lieberman MA, *Marks's basic medical biochemistry: a clinical approach*, 2nd ed. Lippincott Williams & Wilkins, Philadelphia, USA, 2005.
56. Smith ML, Lancia JK, Mercer TI, Io C, Selenium compounds regulate p53 by common and distinctive mechanisms. *Anticancer Res.*, 2004; 24: 1401-1408.
57. Soliman MM, Baiomy AA, Yassin MH, Molecular and histopathological study on the ameliorative effects of curcumin against lead acetate-induced hepatotoxicity and nephrototoxicity in Wistar rats. *Biol Trace Elem Res.*, 2015; 167: 91-102.
58. Swiergosz-Kowalewska R, Cadmium distribution and toxicity in tissues of small rodents. *Microsc Res Techniq.*, 2001; 55: 208-222.
59. Tomaszewska E, Winiarska-Mieczan A, Dobrowolski P, Hematological and serum biochemical parameters of blood in adolescent rats and histomorphological changes in the jejunal epithelium and liver after chronic exposure to cadmium and lead in the case of supplementation with green tea vs black, red or white. *Exp Toxicol Pathol.*, 2015; 67: 331-339.
60. Tothova C, Nagy O, Kovac G, Serum proteins and their diagnostic utility in veterinary medicine: a review. *Veterinarni Medicina*, 2016; 61: 475-496.
61. Udipalli A, Lead hepatotoxicity & potential health effects. *Indian J Med Res.*, 2007; 126: 518-527.
62. Uguz AC, Naziroglu M, Espino J, Bejarano I, Gonzalez D, Rodriguez AB, Pariente JA, Selenium modulates oxidative stress-induced cell apoptosis in human myeloid HL-60 cells through regulation of calcium release and caspase-3 and -9 activities. *J Membrane Biol.*, 2009; 232: 15-23.
63. United Nations Environmental Programme, Final review of scientific information on cadmium - version of December 2010. United Nations Environmental Programme, Nairobi, 2010. www.unep.org.
64. Urani C, Melchiorretto P, Fabbri M, Bowe G, Maserati E, Gribaldo L, Cadmium impairs p53 activity in HepG2 cells. *ISRN Toxicology*, 2014; 1-9.
65. Valdiglesias V, Pasaro E, Mendez J, Laffon B, *In vitro* evaluation of selenium genotoxic, cytotoxic and protective effects: a review. *Arch Toxicol.*, 2010; 84: 337-351.
66. Xu J, Lian LJ, Wu C, Wang XF, Fu WY, Xu LH, Lead induces oxidative stress, DNA damage and alteration of p53, Bax and Bcl-2 expressions in mice. *Food Chem Toxicol.*, 2008; 46: 1488-1494.

67. Yuang G, Dai S, Yin Z, Lu H, Jia R, Xu J, Song X, Li L, Shu Y, Zhao X, Toxicological assessment of combined lead and cadmium: Acute and sub-chronic toxicity study in rats. *Food Chem Toxicol.*, 2014; 65: 260-268.
68. Zaias J, Mineau M, Cray C, Yoon D, Altman NH, Reference values for serum proteins of common laboratory rodent strains. *J Am Assoc Lab Anim Sci.*, 2009; 48: 387-390.