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Cent. Eur. J. Chem. • 10(5) • 2012 • 1442-1451
DOI: 10.2478/s11532-012-0064-8

Central European Journal of **Chemistry**

Effect of selenium in organic and inorganic form on liver, kidney, brain and muscle of *Wistar* rats

Invited Paper

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Received 4 January 2012; Accepted 18 April 2012

Abstract: Selenium is a micronutrient, localized in the active sites of enzymes such as glutathione peroxidase and thioredoxin reductase, and participating together with these enzymes in an antioxidant defence system of organisms against free radicals. Administration of selenium is necessary for maintaining oxidative homeostasis. The present experiment is aimed at investigation of selenium impact on basal metabolic processes and selected antioxidants in a Wistar rat model, fed selenium in organic and inorganic forms. Liver, kidney, brain and muscle were sampled during a month-long feeding with four different doses of selenium (0.075 mg or 1.5 mg of inorganic and/or organic selenium per kg of feed). We found a significant reduction in glutathione level in liver tissue regardless of the form of the administered selenium. On the other hand, selenium caused a decreased glutathione reductase level in the liver and metallothionein level in the liver, kidney and muscle.

Keywords: *Selenium • Oxidative Stress • Glutathione • Glutathione Reductase • Metallothionein*

1. Introduction

Selenium is a nonmetal chemical element and micronutrient. The essentiality of selenium for animals was recognized in 1950s [1]. Additionally, selenocysteine has been found in numerous proteins as a coded amino acid [2]. Selenium simply undergoes redox changes as it alters its valence. Biological activity of selenium in enzymes is based solely on these redox changes. For this reason, selenocysteine residues are present in the active sites of enzymes such as glutathione peroxidase and thioredoxin reductase, participating in protection from oxidative stress [3]. Deficiency of selenium in the diet can lead to development of some carcinogenesis processes, cardiomyopathy – such as Keshan disease – and dysregulation of inflammation [4-6].

From the point of view of diet, selenium can be taken up in two basic forms: organic (selenocysteine, selenocystine, selenomethonine) and inorganic (selenites, selenides). All of these sources are suitable for mammals and can be digested [7]. In the body, these compounds are transformed into selenide and then into selenophosphate upon consumption of ATP and catalysis by selenophosphate synthetase [8]. In spite of the essential nature of this element, selenium overdose can cause toxic symptoms [9]. The primary aim of the

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Table 1. Composition of feed expressed in %, where one percent means 0.075 mg of feed per kg of the experimental animal.

Group	1 (controls)	2 (selenite)	3 (Sel-Plex)	4 (selenite)	5 (Sel-Plex)
Composition\% of Se	0%	50%	50%	1000%	1000%
Wheat	60.54	60.54	60.54	60.54	60.54
Pollards from soya (47%)	12	12	12	12	12
Sunflower oil	2	2	2	2	2
Lysine 78%	0.46	0.46	0.46	0.46	0.46
PremixPO MAR 01	5	5	5	5	5
Maizena	20	19	19	0	0
Selenite (diluted)	0	1	0	20	0
Sel-Plex (diluted)	0	0	1	0	20

here-reported study is investigation of the effects of both inorganic and organic selenium on oxidative stress homeostasis. The experiment can answer the question of whether selenium causes only improvement of antioxidant barriers or can also contribute to detrimental consequences resulting in oxidative insult or other related pathologies.

2. Experimental procedure

2.1. Laboratory animal experiment

In total, 80 male, specific, pathogen-free *Wistar* rats (Biotest, Konarovice, Czech Republic) were used in the experiment. At the beginning of experiment, the animals were 28 days old and differences in body weight were in a range ±2.5 g. The animals were kept in an air conditioned room with stable temperature 23±1°C, light period 12 hours and humidity 60%. Food and water were provided *ad libitum* for the whole experiment.

The experiment started after 8 days quarantine. The animals were divided into five groups, each having 16 specimens. One group served as a control and these rats were not fed a selenium-supplemented diet. The remaining four groups' diets were supplemented with selenium according to the following scheme: 1) the group called "inorganic 50%" was fed with 50% of recommended selenium intake in form of sodium selenite, *i.e.*, 0.075 mg of selenium per kg of feed; 2) the group called "organic 50%" was fed with 50% of recommended selenium intake in form of selenomethonine as Sel-Plex (Alltech, Czech Republic), *i.e.*, 0.075 mg of selenium per kg of feed; 3) the group called "inorganic 1000%" was fed with 1000% of recommended selenium intake in form of sodium selenite, *i.e.*, 1.5 mg of selenium per kg of feed; 4) the group called "organic 1000%" was fed with 1000% of recommended selenium intake in form of selenomethonine as Sel-Plex. The composition of the feed is shown in Table 1. Two animals were put to death at specific time intervals (1st, 4th, 7th, 10th, 13th, 16th, 19th and 22nd day of the experiment) and femoral muscle, brain, liver and kidney were collected.

2.2. Tissues processing

The tissue samples were spread in grinding mortars after being frozen in liquid nitrogen. The crude homogenates were dissolved in 50 mM phosphate buffer pH 7.0 in a ratio 1:10, sonicated (Sonoplus, Bandeline Electronic, Germany) for five minutes, and mixed by Vortex-2 Genie (Scientific Industries, USA) for 15 minutes. Using Eppendorf 5402 device, the suspensions were spun at 16 400 RPM at 4°C for 30 minutes. Finally, the freshly received supernatant was separated and processed as a sample.

2.3. Assay of metallothionein

In total, 200 µL of sample were analysed using differential pulse voltammetry (DPV) based on the Brdicka reaction. The assay was performed in compliance with a previously described protocol [10]. 746 VA Trace Analyzer and 695 Autosampler (Metrohm, Zofingen, Switzerland), standard reaction cell with three electrodes and cooled sample holder (4°C) were used for assay purposes. A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 served as a working electrode. A silver/silver chloride electrode was the reference and a platinum electrode was used as the auxiliary electrode. The sample was injected into the reaction cell containing 1,800 µL of Brdicka supporting electrolyte (1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer consisting of $NH_3(aq)$ + NH_4Cl, pH = 9.6). The DPV assay parameters were as follows: initial potential of −0.7 V, end potential of −1.75 V, modulation time 0.057 s, time interval 0.2 s, step potential 2 mV, modulation amplitude −250 mV. All experiments were carried out at 4°C (Julabo F12 cooler).

2.4. Assay of reduced and oxidized glutathione

Reduced (GSH) and oxidized (GSSG) glutathione were assayed using high performance liquid chromatography with electrochemical detection (HPLC-ED) under experimental conditions described by Pohanka *et al*. [11] and Diopan *et al*. [12]. Two solvent delivery pumps (Model 582 ESA Inc., Chelmsford, MA, USA) operating in a range of 0.001–9.999 mL min^{-1}, colony Zorbax eclipse AAA C18 (150×4.6; 3.5 μm particle size; Agilent Technologies, Santa Clara, CA, USA) and a CoulArray electrochemical detector (Model 5600A, ESA) with three cells including carbon porous working and auxiliary electrodes and Pd/H_2 reference electrode were used for assay purposes. The assayed solution was injected using an autosampler (Model 542 HPLC, ESA) with the carousel temperature adjusted to 8°C. The column was heated to 32°C. Mobile phase consisted of 80 mM trifluoroacetic acid (A) and methanol (B). The compounds of interest were separated by the following linear gradient: 0 → 1 min (3% B), 1 → 2 min (10% B), 2 → 5 min (30% B), 5 → 6 min (98% B). Mobile phase flow rate of 1 mL min^{-1} and working electrode potential of 900 mV were used. Time of analysis was 20 min.

2.5. Determination of total protein content

Determination of total proteins was done using Bradford method. A 190-μL volume of Reagent Coomassie Brilliant blue G-250 (0.01% Coomassie Brilliant Blue G-250, 4.7% ethanol, 8.5% H_3PO_4, *v/v*) was injected into one cuvette and administration of the sample (10 μL) followed. The freshly prepared mixture was incubated at 37°C for 10 min. After incubation, absorbance was measured at 595 nm against the reagent (blank) using an automated spectrophotometer BS-400 (Mindray, China). The spectrophotometer was composed of a cuvette space tempered at 37±1°C and reagent space with a carousel for reagents and sample tempered at 4±1°C. Bovine serum albumin (Sigma-Aldrich, Saint Louis, Missouri, USA) was used for calibration purposes in the assay.

2.6. Determination of aspartate aminotransferase (AST)

A 150-μL volume of solution *AST R1* (80 mM Tris buffer pH 7.8, 240 mM L-aspartate, 1200 U L^{-1} malate dehydrogenase) was pipetted into a plastic cuvette with subsequent addition of 15 μL of the sample. The solution was incubated for 270 s. Subsequently, 30 μL of solution *AST R2* (15 mM 2-oxoglutarate, 0.18 mM NADH) were added and the solution was incubated for 90 s, after which measurement of absorbance at 340 nm started and lasted for 180 s. The same device and conditions were used for AST as in the case of the total protein assay. AST activity was determined kinetically and the assay was based on the rate of NADH consumption during the reaction.

2.7. Determination of alanine aminotransferase (ALT)

In total, 150 μL of solution *ALT R1* (100 mM Tris buffer pH 7.5, 500 mM L-alanine, 1200 U L^{-1} lactate dehydrogenase) was combined with 15 μL of the sample and incubated for 270 s. Subsequently, 30 μL of solution *ALT R2* (15 mM 2-oxoglutarate, 0.18 mM NADH) were added and the solution was incubated for 90 s. Absorbance at 340 nm was subsequently measured for 180 s, and activity of ALT was calculated from the absorbance decrease. The same device and conditions were used for ALT as in the case of total protein assay.

2.8. Determination of alkaline phosphatase (ALP)

Firstly, a 150-μL volume of solution *ALP R1* (0.9 M 2-Amino-2-methyl-1-propanol, pH 10.4, 1.6 mM $MgSO_4$, 0.4 mM $ZnSO_4$, 2 mM N-(2-hydroxyethyl) ethylenediarninetriacetic acid) were combined with 3 μL of the tissue sample in a disposable plastic cuvette. The solution was incubated for 270 s. Subsequently, 30 μl of solution *ALP R2* (16 mM p-nitrophenylphosphate) were added and the solution was incubated for 90 s. After the incubation, absorbance was measured at 405 nm for 180 s. The same device and conditions were used for alkaline phosphatase (ALP) as in the case of total protein assay. The mean increase of absorbance per minute was calculated and used for ALP assay.

2.9. Determination of glutathione reductase (GR)

A Glutathione Reductase Assay Kit (Sigma Aldrich) was used for GR activity determination. Reagents *GR R1* and *GR R2* were prepared by dissolving in an assay buffer (100 mM potassium phosphate buffer, pH 7.5, with 1 mM EDTA). A 260-μL volume of reagent *GR R1* (1.15 mM oxidized glutathione in the assay buffer) was combined with 10 μL of sample and 30 μL volume of reagent *GR R2* (1 mM NADPH in GR assay buffer) in a plastic cuvette. The decrease in absorbance was measured at 340 nm using a kinetic program for 126 s. The same device and conditions were used for GR as in the case of total protein assay.

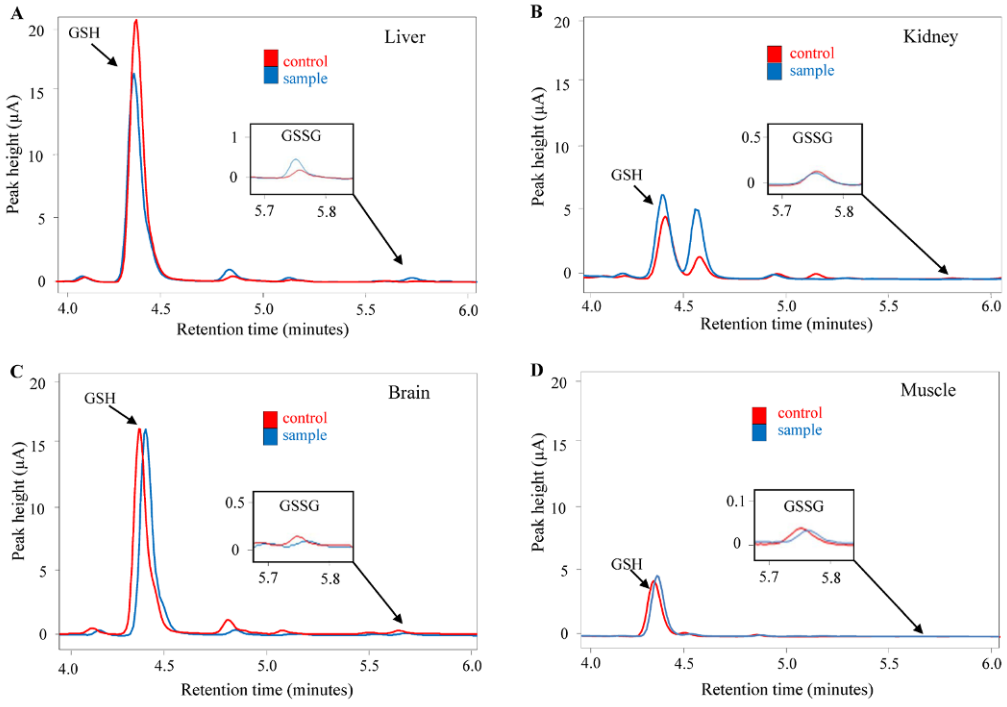

Figure 1. Overlay of chromatograms of control and real sample from variant 1000% inorganic after 22 days for (**A**) liver, (**B**) kidney, (**C**) brain and (**D**) muscle. For other experimental details of chromatographic measurements, see Chapter 2.4.

3. Results and discussion

Alimentarily administered selenium is commonly considered as a factor enhancing antioxidant barriers in organisms. It was proven as an effective substance in diet given to diabetic mice [13]. The mice exhibited amelioration of the adverse effects caused by hyperglycaemia, including oxidative stress. The anticancer effect of selenium has been discussed by many authors and beneficial effects of selenium were expected [14]. However, clinical trials have not confirmed these expectations [15]. The indistinct effect of selenium on the cancer patients' prognosis is not surprising when considered with the similar finding for melatonin and its effect on neurodegenerative disorders and similar detrimental processes [16]. One may suggest that there could be a connection between metabolism of selenium and reactive oxygen species. Therefore, we aimed to assay some of the biochemical markers connected with scavenging of reactive oxygen species, such as reduced (GSH) and oxidized (GSSG) glutathione, metallothionein, low-molecular-mass cysteine-rich protein [17-20], total protein content, and activity of the following enzymes: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and glutathione reductase (GR) in rats treated with organic and inorganic selenium. The results of the experimental groups were related to the control group. Results for the control group were set as one hundred percent and experimental group results were recalculated by an equation X% = (100/K)*E, where X is the result in percent, K is the marker value for the control group and E is the marker value for the experimental group.

3.1. Reduced and oxidized glutathione

Primarily, we aimed our attention at determination of the both GSH and GSSG, which was expressed as GSH/GSSG (Figs. 1 and 2). Typical chromatograms of liver, kidney, brain and muscle are shown in Figs. 1A, 1B, 1C and 1D, respectively. Glutathione is found almost exclusively in its reduced form, since the enzyme which reverts it from its oxidized form (GSSG) – glutathione reductase – is constitutively active and inducible upon oxidative stress. In fact, the ratio of reduced to oxidized glutathione within cells is often used as a marker of cytotoxicity and also as a marker of considerable oxidative stress [21-24]. GSH/GSSG was detected in all sampled tissues. The GSH/GSSG ratio was increased in the liver once selenium was administered in organic or inorganic form and its level doubled by the end of experiment when compared to the beginning (Fig. 2). No specific differences were observed between the individual groups. The brain, kidney and muscle had an increased ratio of GSH/GSSG in the first week after administration (Fig. 2). However, the value decreased

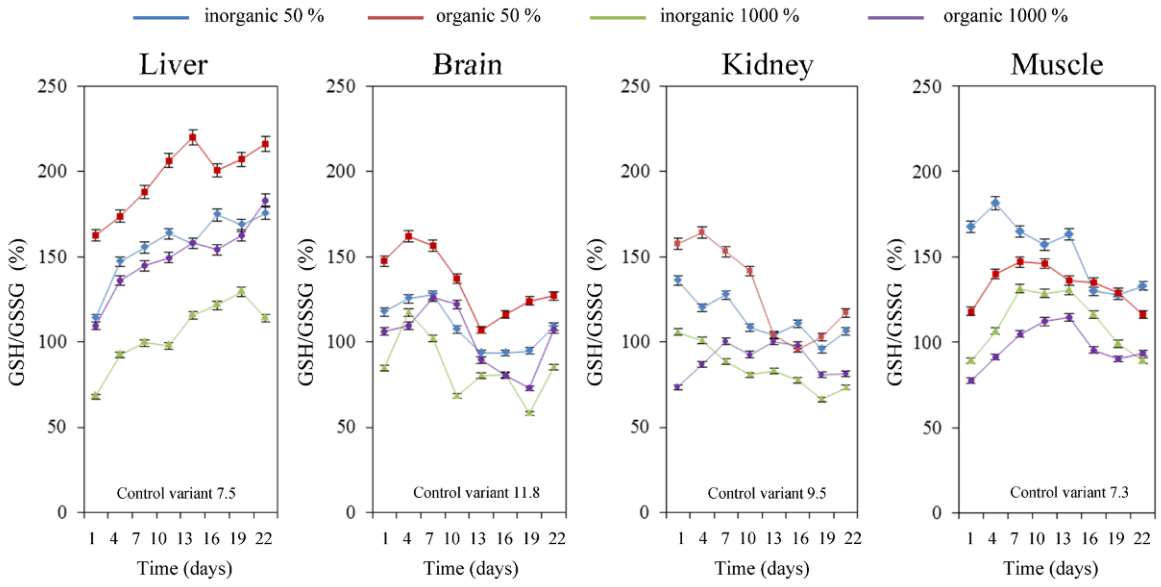

Figure 2. GSH/GSSG levels in the examined organs. The level was related to the controls. Error bars indicate standard error of mean for three specimens. Both reduced and oxidized glutathione were quantified using high performance liquid chromatography with electrochemical detection. The GSH/GSSG results were related to a control group and recalculated as a percentage of control. Control group was determined as a one hundred percent from individual values as follows: liver 7.5, brain 11.8, kidney 9.5 and muscle 7.3.

back to the initial level at the end of the second week. The increase of GSH/GSSG ratio was influenced by improvement of GSH level in the exposed animals. The findings are in a good agreement with experiments reported by other authors [13,25,26]. The effect of selenium on GSH/GSSG is probably not influenced by whether selenium is provided in organic or inorganic form. It could be explained by a fast engaging of the selenium in catabolism of selenium in enzymes interacting with GSH. It is not surprising when considered with the conclusions of the other investigators [27]. The effect of selenium on GSH level, particular on GSH/GSSG ratio, is not clear at molecular level. It may be dependent on enhancement of antioxidant barriers, although glutathione peroxidase, an enzyme with selenium in its active site, uses GSH as a substrate [28]. For this reason, one would expect depletion of GSH rather than its enhancement. On the other hand the depletion of GSH in the presence of glutathione peroxidase is possible when the hydrogen peroxide concentration in cell is enhanced, which may come about due to the presence of free metal ions, particularly transition metal ions, in the cytosol [29]. Moreover, the enhancing trend of GSH/GSSG ratio was observed in rats both when the selenium was administered with and without magnesium [30]. Influence of selenium on aluminium liver toxicity was found to be negligible but the overall intracellular redox status determined via GSH/GSSG ration was ameliorated as well [31]. A second important preferred mechanism for removing the hydrogen peroxide using GSSG as an acceptor of two hydrogen protons is the glutathione-ascorbate cycle whose three included enzymes are not dependent to selenium ions [23]. Thus we can assume that in the case of selenium only the enormous occurrence of metal ions can rapidly decrease the GSH/GSSG ratio and this was partially confirmed. In the tissues which are better equipped with Reactive oxygen species (ROS) detoxication mechanisms (as the liver seems to be), the decrease in the ratio could be minimal or even none. The explanation might also be linked to the higher amount of mitochondria in liver cells, which can generate the hydrogen peroxide physiologically [32].

3.2. Metallothionein

Metallothioneins (MT) are low molecular mass (from 2 to 16 kDa) proteins with a unique abundance of cysteine residues (more than 30% from all amino acids). The main functions of MT in organisms are metal ion transport, maintenance of the oxidative-reducing conditions and regulation of gene expression [17]. DP voltammograms of tissue extracts from liver, kidney, brain and muscle are shown in Figs. 3A, 3B, 3C and 3D, respectively. The cat2 peak height was used for quantification of this protein [33-35]. Metallothionein level tended to decrease in the liver, kidney and muscle (Fig. 4). The metallothionein level decreased by about 20% at the end of experiment when compared to the experiment beginning. Compared with the liver, kidney and muscle, the brain metallothionein decreased

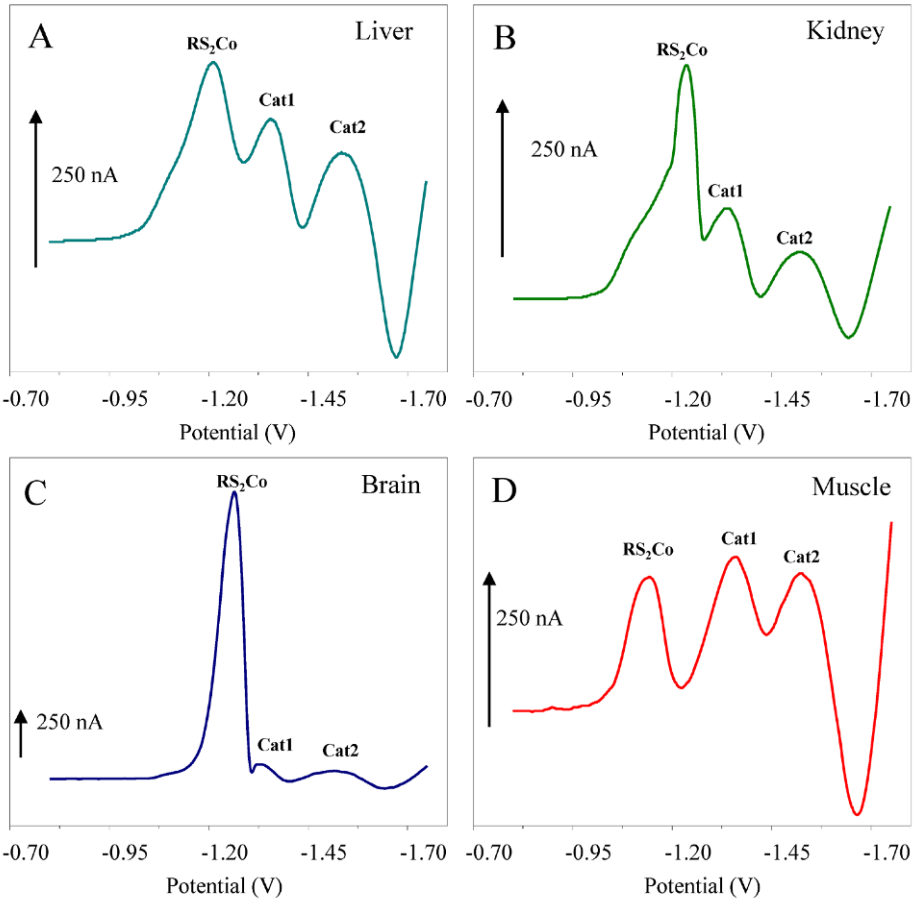

Figure 3. Overlaid voltammetric records of metalothionein in the liver (A), kidney (B), brain (C), muscle (D).

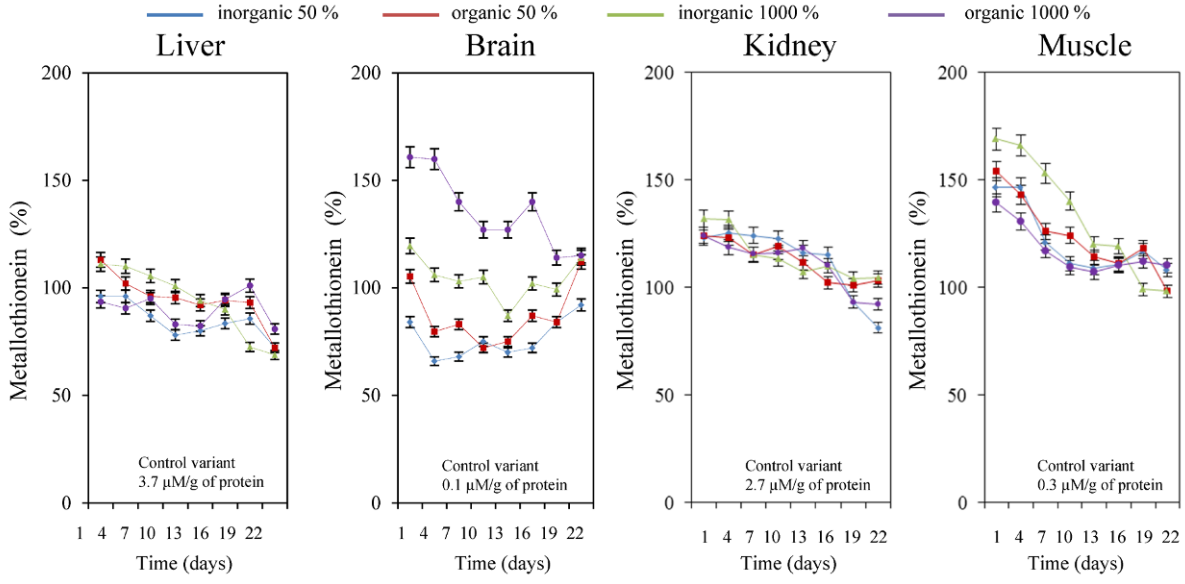

Figure 4. Metallothionein (MT) level in the examined organs. The level was related to the controls. Error bars indicate standard error of mean for three specimens. Metallothionein was determined using differential pulse voltammetry Brdicka reaction. The MT results were related to a control group and recalculated as a percentage of control. Control group was determined as a one hundred percent from individual values as follows: liver 3.7 μM, brain 0.1 μM, kidney 2.7 μM and muscle 0.3 μM.

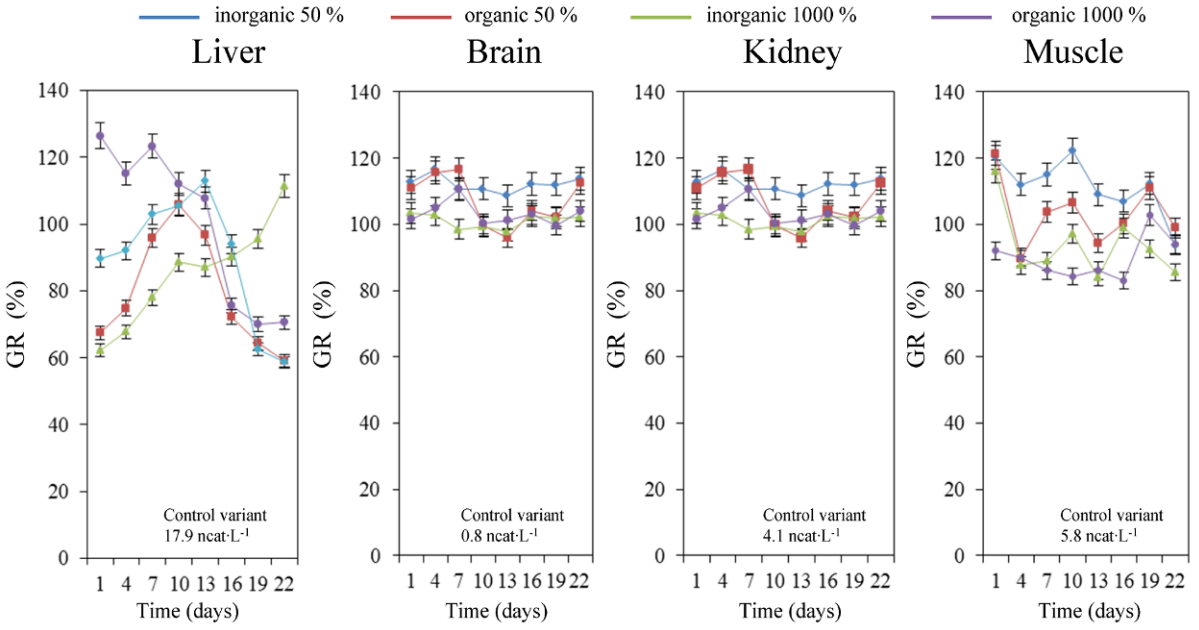

Figure 5. GR activity in the examined organs. The level was related to the controls. Error bars indicate standard error of mean for three specimens. GR was determined by spectrometric assay. The GR activity results were related to a control group and recalculated as a percentage of control. Control group was determined as a one hundred percent from individual values as follows: 17.9 ncat L^{-1}, brain 0.8 ncat L^{-1}, kidney 4.1 ncat L^{-1} and muscle 5.8 ncat L^{-1}.

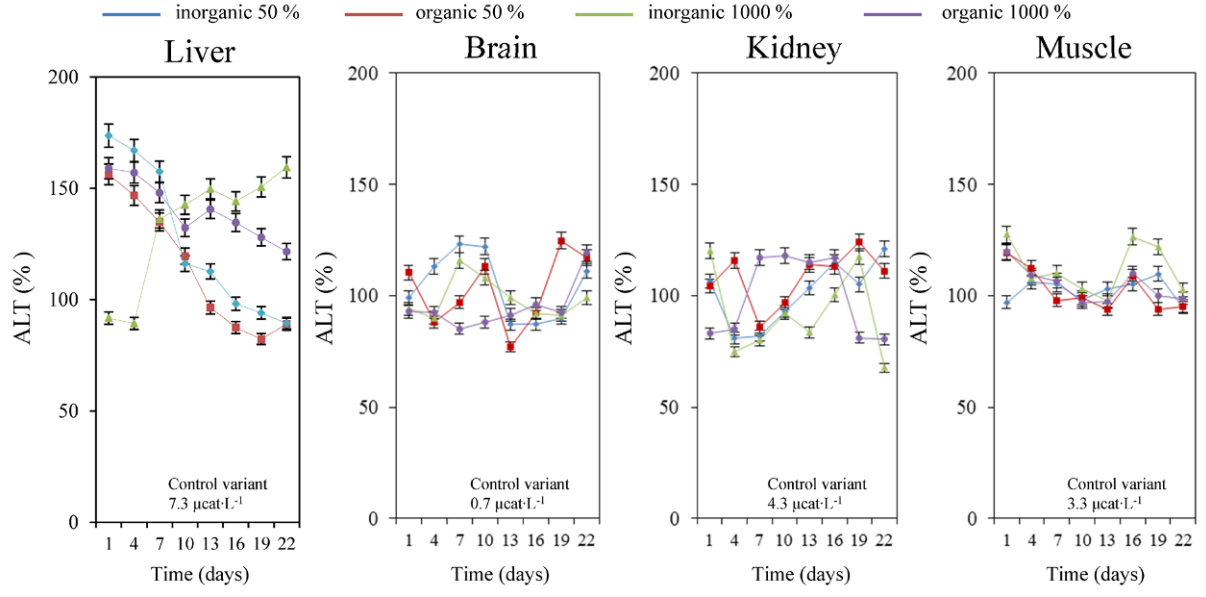

Figure 6. ALT activity in the examined organs. The level was related to the controls. Error bars indicate standard error of mean for three specimens. ALT was determined by spectrometric assay. . The ALT activity results were related to a control group and recalculated as a percentage of control. Control group was determined as a one hundred percent from individual values as follows: liver 7.3 μcat L^{-1}, brain 0.7 μcat L^{-1}, kidney 4.3 μcat L^{-1} and muscle 3.3 μcat L^{-1}.

only in the first half of the experimental period. The animals receiving selenium at 50% of recommended dose in any form and the animals receiving 1000% of recommended doses in inorganic form were able to recover to the original metallothionein level in the end of the experiment. The organic form of selenium in the upper dose was able to deplete approximately 30% of metallothionein in the brain in the end of experiment. On the contrary, a protein with antioxidant properties, metallothionein, was depleted in the liver, kidney and muscle tissue in the course of selenium diet (Fig. 4). The finding well correlates with the experiment reported

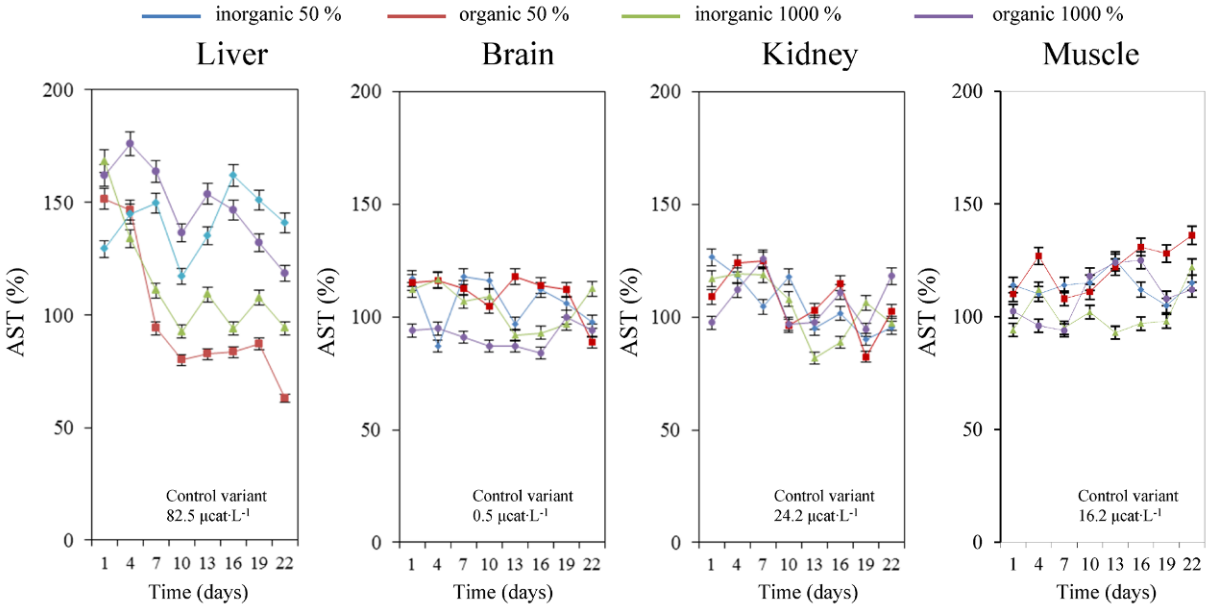

Figure 7. AST activity in the examined organs. The level was related to the controls. Error bars indicate standard error of mean for three specimens. AST was determined by spectrometric assay. The AST activity results were related to a control group and recalculated as a percentage of control. Control group was determined as a one hundred percent from individual values as follows: liver 82.5 μcat L^{-1}, brain 0.5 μcat L^{-1}, kidney 24.2 μcat L^{-1} and muscle 16.2 μcat L^{-1}.

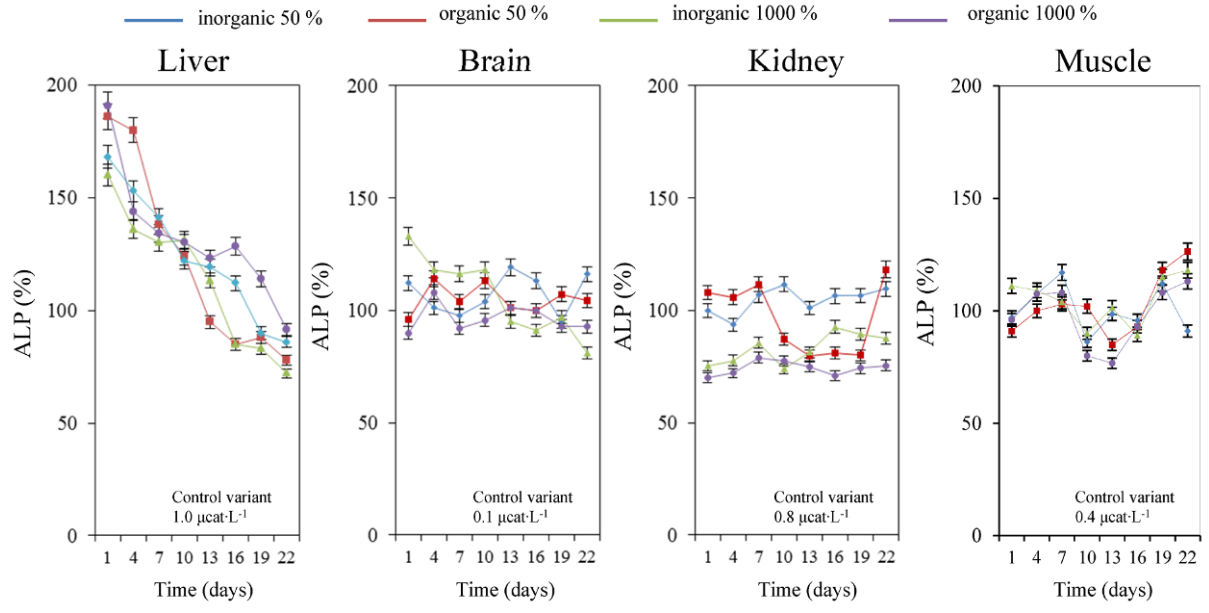

Figure 8. ALP activity in the examined organs. The level was related to the controls. Error bars indicate standard error of mean for three specimens. ALP was determined by spectrometric assay. The ALP activity results were related to a control group and recalculated as a percentage of control. Control group was determined as a one hundred percent from individual values as follows: liver 1.0 μcat L^{-1}, brain 0.1 μcat L^{-1}, kidney 0.8 μcat L^{-1} and muscle 0.4 μcat L^{-1}.

by Banni *et al.* [36,37]. They also reported a decrease in metallothionein level with contemporary reduction of oxidative stress induced by cadmium. Moreover, we also found that oxidative stress had considerable effects on MT level in rats after exposure to mycotoxins [38].

3.3. Glutathione reductase

Glutathione reductase, also known as GR, is an enzyme (EC 1.8.1.7) that reduces glutathione disulfide (GSSG) to the sulfhydryl form GSH, as discussed above. The GR activity was unchanged in the brain and kidney

(Fig. 5). Fluctuation of GR activity was seen in the muscle; however, the GR activity returned to the initial values at the end of experiment. In the liver, the GR activity decreased in all cases, with the exception of administration of the inorganic selenium at 1000% of recommended intake, in which case it increased. On the other hand, it increased to the level of the controls, indicating alteration of GR level before the experiment began. With respect to GR, our results are in opposition to those reported by Glaser *et al*. [39]. The effect may be attributed to fact that Glaser *et al*. used mice, whose GSH-utilizing enzymes have different activities from those of rats [40].

3.4. ALT, AST and ALP

The ALT, AST and ALP activities in the examined tissues are shown in Figs. 6, 7 and 8, respectively. The assayed enzymes had a stable level in the brain, kidney and muscles where only random fluctuations in their activities were found. Compared to the brain, kidney and muscles, a decrease in the enzymes' activities was found in liver in the course of selenium alimentation. At the end of the experiment, the enzymes' activities had decreased nearly 50% compared to the beginning of the experiment. ALT, AST and ALP are involved in basal metabolism and they are important markers of liver function [41]. Here, the enzymes were used as a marker of basal metabolism in the examined organs. The liver was the only impacted organ, with depression in activities of all three mentioned enzymes. The findings correspond to the papers describing selenium impact on liver tissue [42]. Due to the scale of the enzymes' alteration, we infer a significant implication of selenium in basal metabolism.

4. Conclusions

Selenium is an effective element enabling an enhancement in GSH level. The enhancement is not dependent on the form of the administered selenium. The improvement in the GSH level is balanced with a decrease in the GR activity and metallothionein level. Decrease in the liver's basal metabolism follows administration of selenium, as well. For the GR activity, the inorganic form of selenium seems to have a slightly greater effect than the organic form. The other parameters were influenced regardless of the chemical form of selenium.

Acknowledgement

Financial support from the project SIX CZ.1.05/2.1.00/03.0072 is highly acknowledged.

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