Pharmacological Reports 70 (2018) 1073–1078

Contents lists available at ScienceDirect

Pharmacological Reports

journal homepage: www.elsevier.com/locate/pharep

Original article

Effects of Selol 5% supplementation on tissue antioxidant enzyme levels and peroxidation marker in healthy mice

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ARTICLE INFO

Article history:
Received 8 January 2018
Received in revised form 27 April 2018
Accepted 14 June 2018
Available online 18 June 2018

Keywords:
Selenium
Selol
Glutathione peroxidase
Thioredoxin reductase
Malondialdehyde

ABSTRACT

Background: Selenium (Se) is an essential micronutrient for animals and humans used in the prevention or treatment of cancer. Selol is a mixture of selenitetriglycerides, containing Se(IV). It does not exhibit mutagenic activity and is less toxic than inorganic sodium selenite containing Se(IV). The antioxidant properties of the Selol were demonstrated using the blood of healthy animals. The aim of the study was to evaluate Selol as a Se supplement by determining the effect of its administration on the Se level and the antioxidant status in the tissues.
Methods: We examined the effect of long-term (28-day) Selol 5% supplementation on the activity of antioxidant enzymes, including the main selenoenzymes in healthy mice organs, such as liver, brain, lungs, and testis. Enzyme activities of the tissue homogenates and the concentration of malondialdehyde (MDA) as a biomarker of oxidative stress were measured using spectrophotometric methods. The selenium concentrations in the tissues were determined by inductively coupled plasma mass spectrometer (ICP-MS) as well.
Results: A significant increase in glutathione peroxidase, thioredoxin reductase, and glutathione S-transferase activity as well as the MDA concentration was observed in most of the studied tissues during the Selol 5% supplementation.
Conclusions: Long-term supplementation with the new Se(IV) compound - Selol 5% significantly affects the activity of antioxidant enzymes and the redox state in healthy mice organs. In the healthy population Selol 5% seems to be a promising new antioxidant compound.

Introduction

Epidemiological studies have revealed that the modern busy lifestyle and unbalanced diet have a considerable effect on the prevalence of metabolic disorders, which lead to chronic diseases such as diabetes, hypertension, neurological disorders and various types of cancer. Thus, effective and safe compounds with preventive, anticancer activity and limited side effects are sought. Recently, considerable attention has been paid to the relationship between the content of selenium (Se) in the organism's diet and the incidence of certain cancers and neurological diseases as well [1,2].

Se is known for its antioxidant effect on cells owing to its being a component of the active centers of some antioxidant enzymes, such as glutathione peroxidase (GPx) and thioredoxin reductase (TrxR). These enzymes are crucial for intracellular redox homeostasis and human health, and their lower activity was observed in Se-deficient subjects [3,4]. Decreased activity of Se-dependent is associated with oxidative stress (OS) and is important factor of neurological disorders like Alzheimer's and Parkinson's disease [2]. In contrast to low Se(IV) doses, a high Se supplementation can induce OS, which is responsible for the cytotoxic effect of Se as well as its anti-cancer properties. The Se pro-oxidative effect is mediated by reactive oxygen species (ROS). An increase in their generation enhances the inactivation of many important enzymes such as antioxidant enzymes. Consequently, the increased ratio of oxidized thiols to reduced thiols is observed. This imbalanced redox state in cells results in the inactivation of crucial enzymes and, consequently, in apoptosis [5].

Previous research has shown that Se deficiency due to its insufficient amount in soil or water may result in several health problems. However, Se at higher doses is a toxic micronutrient. As

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https://doi.org/10.1016/j.pharep.2018.06.003
1734-1140/

the range between a therapeutic and a toxic dose of Se is quite narrow, effective treatment with this microelement is still a challenge. The additional problem is the difference in the bioavailability of Se, which is a result of not only interpersonal differences but also the form of supplementation [6]. Organic and inorganic forms of Se have different toxic levels and effects on an organism. Organic forms are less toxic and more effective and efficient than inorganic forms [7]. One of the organic Se compounds with potential anticancer activity is Selol. Selol is a mixture of selenitetriglicerides obtained by the chemical modification of sunflower oil, which contains Se in the +4 oxidation state. This formulation is believed to have significant benefits. As an organic drug, it is significantly less toxic than sodium selenite, which is an inorganic form of the Se(IV) compound frequently used in medicine. In single-dose toxicity studies performed on rats, researchers observed that after an oral administration of Selol, LD_{50} (lethal concentration, 50%) was as high as 100 mg Se kg^{-1} body mass [8]. Moreover, Selol did not exhibit chronic toxicity, accumulate, or show mutagenic activity [9]. Thus far, no Se content drug with both pro-oxidant characteristics and low toxicity has been developed. Thus, Selol because of its safety and high Se(IV) content seems to be a promising supplement in anticancer therapy. However, further studies need to be conducted prior to its potential medical application. For instance, no data are currently available on the tissue distribution of the drug and the effects of long-term Selol supplementation. As penetration into organs where the tumor is developing is crucial to target the tumor, drug distribution in the organism is an important medical issue.

Therefore, the aim of the present study was to determine the Se distribution in tissues as well as the activity of the antioxidant enzymes and the concentration of the lipid peroxidation marker after Selol 5% daily supplementation in the healthy animal organs.

Materials and methods

Chemicals

Selol was synthesized at Department of Bioanalysis and Drugs Analysis, Warsaw Medical University [(Patent, Pol.PL 176530 (Cl. A61K31/095)]. In the experiments, Selol 5% at a dose of 4 mg Se kg^{-1} body mass was used. Glutathione (GSH), the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), sodium azide, glutathione reductase, 1-chloro-2,4-dinitrobenzene (CDNB), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), sodium aurothiomalate (ATM), cumene peroxide, and tert-butyl peroxide were purchased from Sigma. A mouse MDA ELISA kit was purchased from Wuhan EIAab Science. For the preparation of solutions, Mili-Q water was used.

Biological material

All procedures were in compliance with Animal Care Committee guidelines (nr 33/2009).

The studies were performed on healthy male Swiss mice, weighing 20–22 g, fed with the standard laboratory diet based on commercial LSM pellets, and given water *ad libitum*. The mice were kept under conventional standardized conditions (room temperature 22.5–23.0 °C; relative humidity 50%–70%; 12-h day/night cycle). Animals were divided randomly into five groups. Each group consisted of five mice. The control group, which was sacrificed during the experiment, received sunflower oil in a volume equivalent to that administered to the study groups. The study groups were supplemented daily *per os* for 3, 5, 10, and 28 days with a single dose of Selol 5% (5 mg of Se(IV) in 1 mL) diluted with sunflower oil (4 mg Se(IV) kg^{-1} body mass) in a volume of approximately 10 ml kg^{-1}. Then, before the morning feed, mice were anaesthetized with ether. After that, the animals were decapitated, and tissues were collected and placed in liquid nitrogen for a few minutes. The tissues–livers, brains, lungs, and testis–were stored at −80 °C and processed within 2 months.

Prior to the measurements, the tissues were homogenized using a manual glass homogenizer. Samples for the enzyme activity measurements were homogenized in a cold medium containing 5 mM phosphate buffer, 0.25 mM sucrose, and 0.5 mM EDTA (pH = 7.2), while for MDA the cold medium used contained 5 mM pyrophosphate buffer (pH = 7.4). The cytosolic fraction was separated by centrifugation of the homogenates at 10,000 × g for 20 min at 4 °C. In the supernatants of the tissue homogenates, the following parameters were determined: 1) activity of Se-GPx and 2) of total GPx, 3) activity of TrxR, 4) activity of glutathione S-transferase (GST), 5) the concentration of malondialdehyde (MDA, lipid peroxidation marker), and 6) Se concentration.

Analytical methods

Determination of Se-dependent (Se-GPx) and total glutathione peroxidase (GPx) activity

Se-GPx and GPx activities were measured spectrophotometrically at the wavelength of 340 nm, using the method developed by Paglia and Valentine, and modified by Wendel as described previously [10–12]. The reaction was performed at 25 °C in the 50 mM sodium phosphate buffer with 0.40 mM EDTA (pH = 7.0). The supernatant (10 μl) was used to analyze enzymes activities. In the final reaction mixture, in a volume of 220 μl, the concentration of reduced glutathione (GSH) was 1.0 mM, that of NADPH was 65 μM, and that of sodium azide was 0.17 mM. A tertbutyl hydroperoxide substrate was used at a concentration of 0.02 mM, which ensured that the Se-GPx activity was determined. For the determination of the GPx activity, cumene hydroperoxide at a concentration of 1.05 mM was used. The decrease in absorbance (proportional to Se-GPx and GPx activity) was measured every 10 min at 340 nm. The results were expressed as U/mg protein.

Determination of glutathione S-transferase (GST) activity

The GST activity was measured spectrophotometrically at the wavelength of 340 nm, using the Habig method [13]. The reaction was performed at 25 °C in the 50 mM sodium phosphate buffer with 0.50 mM EDTA (pH = 7.5). The supernatant (10 μl) was used to analyze enzyme activity. The final reaction mixture, in a volume of 200 μl, contained 2 mM GSH and 1 mM CDNB as a substrate. The enzyme activity was measured in the sample for 10 min at 25 °C. GST activity was expressed as U/mg protein.

Determination of thioredoxin reductase (TrxR) activity

The TrxR activity was measured spectrophotometrically at the wavelength of 412 nm, using the Hill et al. amendments. The reaction was performed at 37 °C in the 50 mM sodium phosphate buffer with 1 mM EDTA (pH = 7.0). The supernatant (10 μl) was used to analyze enzyme activity. Final concentrations of reagents in the measurement mixture, in a volume of 200 u l were: 4 mM for DTNB as a substrate, 2 μM for NADPH as the cofactor of the enzymatic reaction and 1 mM for ATM as a specific inhibitor of the studied enzyme. The activity of the thioredoxin reductase enzyme is defined as the difference in the enzymatic activity of the samples measured without inhibitor and with its addition. The measurements were carried out at 37 °C for 10 min [14–16]. TrxR activity was expressed as U/mg protein.

Determination of protein concentration

Protein concentration in supernatants was measured by spectrophotometric assay using Bradford reagent. The absorbance of protein-bound dye (Coomassie Brilliant Blue G-250) was

measured at 595 nm. Protein concentration was measured using a standard curve, where bovine albumin (BSA) was the standard.

Determination of MDA concentration

The MDA concentration as an indicator of lipid peroxidation was measured by ELISA spectrophotometric assay kit (Wuhan EIAAB Science Co., LTD.) as recommended by the manufacturer. MDA concentration was expressed as μmol/mg of protein.

The enzymatic activity and protein absorbance were measured with a Synergy Mx Spectrophotometer microplate reader (Biotek).

Determination of selenium concentration

To determine the total Se concentration in the tissues, an inductively coupled plasma mass spectrometer (ICP-MS) (Plasma-Quad, Thermofisher Scientific) was used [17]. The tissue samples were mineralized by adding 3 ml of 65% HNO_3 in Teflon PTFE crucibles, using a microwave energy. Optimal measuring range of the ICP-MS method for this element was 0.1–2.5 μg/l. Se concentration was expressed as μg/g of tissue.

Statistical analysis

The data are presented as the mean ± standard deviation (SD). Statistical analyses were performed using one-way ANOVA followed by the Dunnett *post hoc* test. A *p*-value of less than 0.05 was considered significant. The *p* values indicate differences between control (time 0) and study groups and were signed as $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$. Statistical tests were performed using the Statistica 10 software (StatSoft).

Results

As selenocysteine is a structural component of the active centers of Se-dependent antioxidant enzymes, the activity of these enzymes was examined in this study.

Determination of Se-GPx and GPx

Selol 5% supplementation caused significant changes in the Se-GPx and GPx activities in the animal tissues. In the control group, the highest activity of the enzymes (approx. 10 times higher than in the other tissues) were measured in the liver (0.430 ± 0.059 U/mg of protein), followed by in the testis (0.027 ± 0.002 U/mg of protein), brain (0.012 ± 0.003 U/mg of protein), and lungs (0.013 ± 0.003 U/mg of protein). The highest increase in Se-GPx activity after 28 days of supplementation was observed in the lungs (5-fold) and then, in the brain tissues (2-fold). In the testicular tissue, no statistically significant changes in the activity of the Se-GPx and GPx as compared to the control group were observed. The organs that responded the fastest to Selol were liver and lungs, where after 3 days of supplementation, the activity of both Se-GPx and GPx increased significantly (Fig. 1A, B).

Determination of TrxR and GST activity

In the control group, the highest activity of the GST enzyme was detected in the testis, which was approx. 10 times higher than in the other tissues (0.151 ± 0.063 U/mg of protein). In the other control tissues, the activity of GST was comparable: lungs (0.0298 ± 0.0111 U/mg of protein), liver (0.0276 ± 0.0050 U/mg of protein), and brain (0.0240 ± 0.0034 U/mg of protein). The highest increase in the GST activity was observed at the end of the experiment in the lung tissue (4-fold) and then, in the brain (3-fold) and the liver (2 fold). In the testis, although the GST activity was the highest, we observed a statistically significant increase in the GST activity after 28 days Selol supplementation (Fig. 1C). The

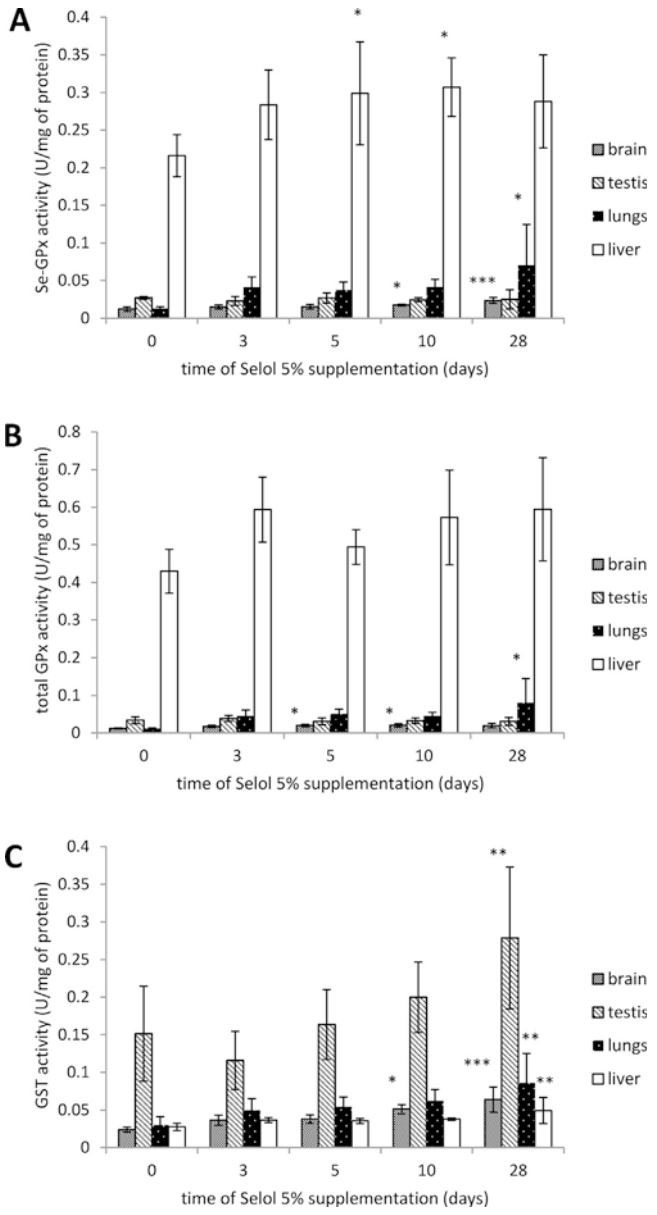

Fig. 1. Effect of Selol 5% in dose 4 mg Se/kg daily supplementation on the activity of: selenium dependent glutathione peroxidase (Se-GPx) (A), total glutathione peroxidase (GPx) (B), glutathione S-transferase (GST) (C). Data are shown as mean ± SD. The *p* values indicate differences between control (time 0) and study groups and are signed as $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

activity of TrxR in the lungs of the control group (0.832 ± 0.331 U/mg of protein) was approx. 10 times higher than in the other tissues: brain (0.0519 ± 0.0046 U/mg of protein), testis (0.0501 ± 0.0099 U/mg of protein), and liver (0.0271 ± 0.0015 U/mg of protein). The highest increase in the TrxR activity was observed in the liver on day 28 of the supplementation (2 fold) and in the lungs at days 5 and 10 of the experiment (2.5 fold) (Fig. 2A). The organs that responded the fastest to Selol were the brain and the liver, where after 3 days of supplementation, the activity of both GST and TrxR increased.

Determination of MDA concentration

The MDA level, marker of lipid peroxidation and OS, was significantly higher in the liver and brain than in the control group. In the control group, the highest concentration of MDA was

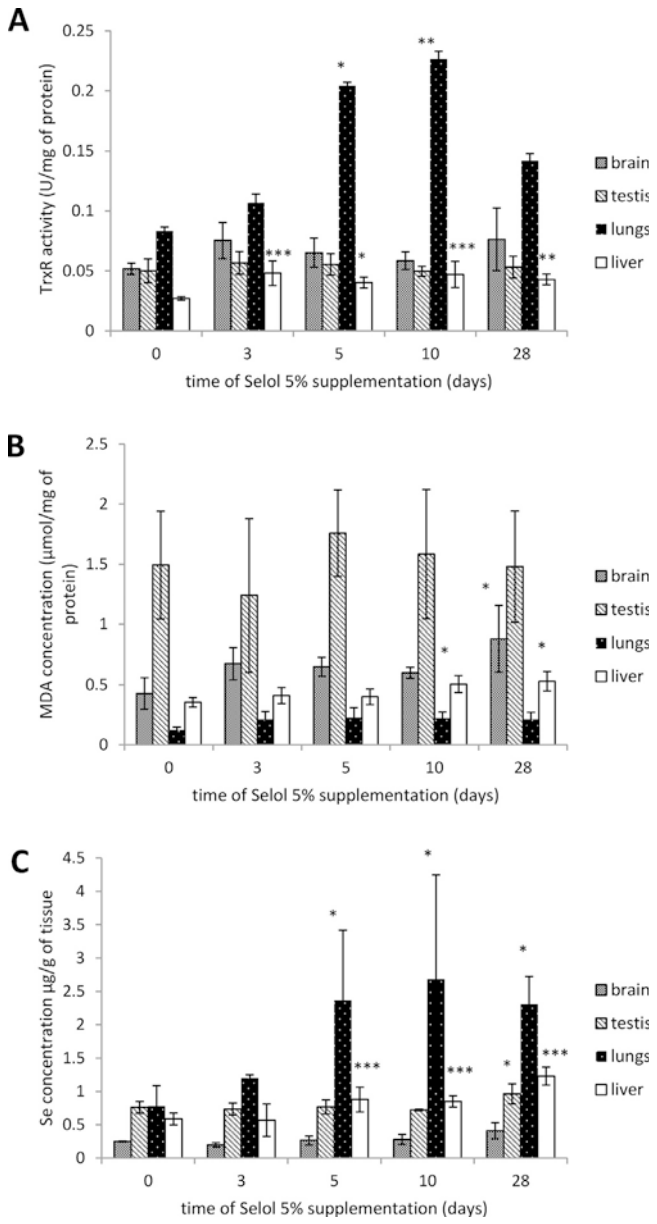

Fig. 2. Effect of Selol 5% in dose 4 mg Se/kg daily supplementation on the activity of: thioredoxin reductase (TrxR) activity (A), MDA concentration (B) and Se concentration (C). Data are shown as mean ± SD. The *p* values indicate differences between control (time 0) and study groups and are signed as $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

observed in the testicular tissues (1.5 ± 0.573 μmol/mg of protein), followed by in the brain (0.43 ± 0.13 μmol/mg of protein), liver (0.355 ± 0.040 μmol/mg of protein), and the lungs (0.124 ± 0.025 μmol/mg of protein). In the testicular and lungs tissues of the study groups, we did not observe a statistically significant change in the MDA concentration with respect to the control group. In the brain and the lungs, the MDA concentration increased the most (2-fold increase) after 28 days of Selol 5% supplementation as compared to that in the control group. The organ that responded the fastest to the Selol formulation (on day 3 of the Selol 5% supplementation) was the brain (Fig. 2B).

Determination of Se content

The concentration of Se in all of the tissues of the control group was almost the same: testis (0.760 ± 0.090 μg/g of tissue), lungs (0.777 ± 0.307 μg/g of tissue), liver (0.586 ± 0.091 μg/g of tissue). The lowest concentration was observed in the brain tissues (0.251 ± 0.007 μg/g of tissue). In the study groups, the highest Se concentration was observed in the lungs and the liver from day 5 to day 28 of the experiment. We also noticed the highest increase in the Se concentration (2–3 fold) in these tissues. In these tissues, we also noticed the highest increase in the Se concentration (2–3 fold). The earliest changes appeared on day 3 in the lungs (Fig. 2C).

Discussion

Studies on antioxidant enzymatic defense mechanisms, Se concentration, and OS biomarkers are the first steps to determining the biochemical mechanism of the action of a new selenitetriglycerides mixture containing Se(IV) called Selol. The role of selenium as an antioxidant is well documented [18,19]. Selenium as an antioxidant and the component of some antioxidant enzymes is involved in metabolic processes at the cellular level, protects cell membranes from ROS (reactive oxygen species), limits lipid peroxidation, protein oxidation, DNA and RNA damage, and reduces the risk of cancer [9]. The described chemopreventive effect was observed for inorganic selenium in a daily dose of at least 100–200 μg. Also a new organic form of selenium(IV) – Selol, has chemoprotective effect. Due to stimulation of the antioxidant systems, Selol protected against LPS-induced neuroinflammation in the brain tissues [20].

Parallel to the above theory on the antioxidant activity of selenium, recent animal studies have reported that inorganic forms of Se in the +4 oxidation state acts as a pro-oxidant rather than an antioxidant and exhibits considerably high anti-carcinogenic properties [21]. These findings have led to an increase interest in the role of Selol in cancer therapy.

The present work is a continuation of our previous studies on the effects of a Selol oral supplementation on the antioxidant activity in the blood of healthy animals [12]. This time, Selol was administrated orally, not in a single dose of 17 mg Se kg^{-1} body mass, but as a long-term supplement at 4 mg Se kg^{-1} body mass. Similarly, in the liver, lung, and brain tissues of healthy mice, supplemented daily with Selol 5%, we observed significantly higher activity of most of the investigated antioxidant enzymes [12]. The obtained data suggest that after the oral administration of Selol 5%, its absorption from the gastrointestinal tract was sufficient. A significant increase in the activity of selenoenzymes, namely Se-GPx and TrxR, suggested the specific incorporation of Se, in the form of selenocysteine at the active sites of the selenoenzymes. A similar observation was made in numerous studies, where supplementation with inorganic Se was performed and resulted in an increase in the activity of Se-GPx in the liver cells [22,23]. Long-term administration of Se compounds leads to an increase in the cytosolic Se-GPx mRNA in the liver cells, which proves the regulation of these processes at the transcription level [24]. In contrast, in the liver tissues of animals with a Se-deficient diet, TrxR exhibited low activity [15] and long-term supplementation with Se and/or its compounds led to an increase in the activity of hepatic TrxR [25]. The highest increase in the Se-dependent enzyme activity in the liver tissue, after 28 days of Selol 5% supplementation, may be attributed to the increased concentration of Se. Changes in the concentration of Se in the liver are considered to be a good indicator of the long-term storage of this element in the body and are more sensitive to changes in its concentration than the Se reservoirs in the blood plasma [17].

Furthermore, in the rat brain tissues, 20% of the overall amount of Se is present in the molecular structures of the Se-dependent peroxidase enzyme family [26]. Inorganic and organic Se supplementation increases the activity/expression of Se-GPx, glutathione reductase (GR) as well as TrxR in the brain cells

[27]. Selol also inhibits the thiols oxidation and accumulation preventing neuroinflammation [20]. Se is stored in the brain tissue in the form of selenomethionine [28]. We noticed a statistically significant increase in the Se concentration in the brain tissue until day 28 of the experiment. These results are probably related to the high lipophilicity of the Selol formulation.

Numerous studies have reported a relationship between the supplementation of Se and/or its compounds and the increased activity of antioxidant enzymes in the lung tissues [29]. An increased concentration of Se in the lung tissues was observed as early as on days 3, 5, 10, and 28 of the Selol 5% supplementation. The consequence of this is the simultaneous increase in the activity of the Se-dependent enzymes. More importantly, in the lung tissues, we observed a higher increase in the Se-dependent enzymes (Se-GPx and TrxR) than in the control group. The increased activity of TrxR in the lung tissue as a result of the normal daily Se intake has been described in the literature [30].

The increased activity of GST in the liver, brain, testis, and lung tissues, as compared to the control group, during the supplementation of Selol 5%, might be attributed to the activation of the second-phase enzymes responsible for the metabolism of xenobiotics such as drugs, food additives, pollutants, and insecticides. The expression of these enzymes is induced by the activation of the NRf2ARE pathway in response to the OS. A recent study reported that Selol acts as a monofunctional inducer of the phase-2 enzyme activity, where activation is mediated by the NRf2 transcription factor [31]. GST is one of the most important enzymes that neutralize the compounds involved in lipid peroxidation [32]. Lipid peroxidation may be initiated by OS; this might be attributed to the increase in the MDA concentration in the liver tissue, on days 10 and 28 of the Selol 5% supplementation. Therefore, an increase in the activity of Se-GPx, GPx, GST, and TrxR may be the body's response to the increased cellular ROS, which, generated in addition to the incorporation of Se into active selenoprotein centers, is one of the most important mechanisms of Selol activity.

The supplementation of Selol 5% led to an increased concentration of the lipid peroxidation product (MDA) in the liver tissues as well as in the brain and lung tissues on days 3, 5, 10, and 28 of the experiment. The increased MDA concentration in the brain tissues of healthy rats following the daily supplementation of the inorganic material Se, has already been described in the literature [33]. In the tissues of the mice testis, the daily supplementation of the Selol 5% did not cause a significant change in the activity of enzymes such as Se-GPx, GPx, and TrxR, and the MDA concentration. This was confirmed by the results of Kaur et al., where no change in the activity of the Se-GPx in the testis of healthy mice after the Se supplementation was observed [34]. A statistically significant increase in the Se concentration in the testis, as compared to the control, occurred as late as on day 28 of the supplementation, at which point, a significant increase in the GST activity was observed.

In conclusion, we have shown that each organ reacts differently to the daily Selol 5% supplementation. In the liver and lungs, we observed an increase in the Se-GPx, GPx, GST, and TrxR activity from day 3 of the supplementation, which correlated with an increase in the Se concentration. The highest Se concentration in the brain was observed at the end of the experiment, while the activity of the Se-dependent enzymes (Se-GPx and TrxR) and GST increased faster, *i.e.*, from day 3 of the supplementation. In the testis, a significant increase in the Se concentration on day 28 of the Selol 5% supplementation was observed, and GST was not related to the changes in the activity of the examined antioxidant enzymes. In the liver, the Se concentration correlated to the Se-GPx activity ($r_s = 0.24$, $p = 0.0342$), GST activity ($r_s = 0.56$, $p < 0.0001$), and MDA concentration ($r_s = 0.50$, $p < 0.0001$). In the brain tissues, the Se concentration correlated to the Se-GPx activity ($r_s = 0.73$, $p < 0.0001$) and the GST activity ($r_s = 0.70$, $p < 0.0001$). In the lung tissue, the Se concentration correlated to the TrxR activity ($r_s = 0.49$, $p = 0.0100$) and the MDA concentration ($r_s = 0.46$, $p = 0.0200$). Differences of the antioxidant system response after Selol 5% supplementation between organs may be an indicator of the therapy length required to achieve the therapeutic effect. The liver tissue had the fastest response to the Selol supplementation. Liver is a major organ attacked by the ROS, and liver cells are the primary cells subjected to an OS-induced injury [35]. Therefore, antioxidant enzymes such as GPx, TrxR, and GST are affected, by its induction or increase in activity, and are used as markers to evaluate the level of OS. Our study confirmed the hypothesis that long-term Selol 5% supplementation changes the activity of antioxidant enzymes and the redox state, which correlates with the results reported by Flis et al. [36]. Research on tumors of mice treated with Selol 5% is in progress.

Conflict of interest

None declared.

Acknowledgments

This work was supported by the Polish Ministry of Science and Higher Education based on contract 3606/B/P01/2010/39 as part of research project N N405 360639.

The authors would like to thank Ms. Sylwia Michorowska for the review of the paper.

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