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Original research article

Effects of Selol 5% supplementation on the activity or concentration of antioxidants and malondialdehyde level in the blood of healthy mice

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ABSTRACT

Background: Selol is a novel organoselenium Se(IV) compound. It reveals lower potential of toxicity than sodium selenite and does not exhibit mutagenic activity. Its antioxidant and anticancer properties including overcoming cancer cell resistance to standard therapy of the drug were proven. This is the first publication describing the influence of Selol 5% on the activity of blood antioxidant status *in vivo*.
Materials and methods: We investigated the influence of Selol 5% short-term (24 h) and long-term (28 days) administration on the activity of antioxidant enzymes, including the main selenoenzymes, in healthy mice plasma and erythrocytes. Plasma oxygen radical absorbance capacity value (ORAC) and the concentration of malonyldialdehyde (MDA) in plasma as a biomarker of oxidative stress as well as the value of selenium (Se) concentration in erythrocytes were shown.
Results: A significant increase of the selenium dependent glutathione peroxidase (Se-GSHPx) activity in plasma and erythrocytes, plasma selenoprotein P concentration, ORAC values, and Se concentration were observed during long-term supplementation as well as after Selol 5% single-dose administration, with two distinct increases of activity a few hours after the beginning of the experiment and before its end. We found a decreased thioredoxin reductase (THRR) activity and an increased MDA level during Selol 5% long-term supplementation. Glutathione S-transferase activity (GST) remained unchanged.
Conclusion: Selol 5% supplementation *in vivo* affects the selenoenzymes activities as well as the antioxidant status of plasma and erythrocytes. Selol 5% is an inhibitor of thioredoxin reductase activity, which can be important in anticancer therapy.
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Introduction

Several epidemiological and clinical studies showed that selenium (Se) is a trace element essential to human health, playing an important role as an antioxidant and anticancer agent [6,36]. As a component of selenocysteine, selenium is present in approximately 30 proteins including selenoenzymes, where it is present in their active centers [21], *e.g.* thioredoxin reductase or glutathione peroxidase [7,13]. Apart from its role as an antioxidant and cancer prevention agent, selenium also exhibits anticancer properties. Its supplementation together with drugs and radiation can increase the effectiveness of anticancer treatment, where Se acts as a pro-oxidant rather than an antioxidant inducing apoptosis through oxidative stress pathway [9]. The Se compounds with the highest activity as free radical scavengers and the greatest anticancer potency contain selenium at the +4 oxidation state [25].

Selol, the structure of which is presented, is a mixture of selenitetriglycerides synthesized from sunflower oil and is a novel organic compound of Se(IV). As an organic drug, it reveals lower potential for toxicity than sodium selenite, which is an inorganic Se(IV) compound. It was found that in single-dose toxicity studies performed on rats, after oral administration of Selol, LD_{50} was 100 mg Se kg^{-1} body mass [18]. Moreover, Selol did not exhibit any cumulative toxicity or mutagenicity, which was proved in investigations with *Salmonella* strains [29].

Selenium antioxidant properties may result from its incorporation in the structure of selenoenzymes. However, the mechanism

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may depend on the pharmaceutical dosage form and the drug metabolism. Although *in vitro* studies revealed that Selol 5% is a promising anticancer drug that can overcome the resistance of cancer cells [34], there are no *in vivo* data concerning the influence of Selol 5% single-dose administration on the activity of some enzymes related to oxidative stress [33]. The evidence describing the biochemical effects of long-term Selol supplementation in healthy animals is inconsistent with other studies on selenium compounds [40]. Therefore, the aim of the present study was to describe (1) the activity of selenoenzymes: selenium dependent glutathione peroxidase (Se-GSHPx) and thioredoxin reductase (THRR); (2) the concentration of selenoprotein P; (3) other than selenoenzymes antioxidant enzymes activities: total glutathione peroxidase (GSHPx), glutathione S-transferase (GST) in mice plasma and/or red blood cells after Selol 5% long-term supplementation and single-dose administration. In addition, we investigated the concentration of malonyldialdehyde (MDA) as biomarker of oxidative stress, the value of oxygen radical absorbance capacity (ORAC) in plasma and selenium (Se) concentration in red blood cells whose concentration is greater than in plasma.

Materials and methods

Chemicals

The synthesis of Selol was carried out in the Department of Drugs Analysis at Warsaw Medical University (Polish Patent 1999). In the present experiment Selol 5% at a dose of 4 mg Se kg^{-1} body mass and 17 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, 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), fluorescein stock solution, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX), hemoglobin standard were purchased from Sigma–Aldrich. Drabkin's solution was obtained from Human. Mouse selenoprotein P ELISA kit and mouse MDA ELISA kit were purchased from Wuhan EIAab Science. For preparation of solutions double distilled deionized water was used.

Biological material

The experiment was performed on healthy male mice, Swiss race, weighing 20–22 g. The animals were kept under conventional conditions (room temperature 22.5–23.0 °C, relative humidity 50–70%).

Long-term supplementation

Mice were divided into five groups: one control group ($n = 5$ animals per group) and four study groups ($n = 5$ animals per group). Each of the study groups was supplemented daily *per os* for 3, 5, 10, and 28 days with a single dose of Selol 5% diluted with vegetable oil equivalent to 4 mg Se kg^{-1} body mass. The control groups were fed the standard diet with the same rate of pure vegetable oil as the study groups. Blood samples from mice were collected into heparinized test tubes before morning feeding.

Short-term supplementation

Mice were randomly allocated to the study group ($n = 5$ animals per each subgroup) and control group ($n = 5$ animals per group, which were sacrificed over the time the experiment was performed). The control group was fed the standard diet with the same rate of pure vegetable oil as the study group. In the study group the experiment was performed for 24 h. Blood samples from mice were collected into heparinized test tubes before morning feeding after 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 20, and 24 h of Selol 5% oral administration at a single dose of 17 mg Se kg^{-1} body mass.

After both procedures blood was centrifuged (1000 × *g*, for 15 min at 4 °C) to obtain plasma. Next red blood cells were washed twice with 0.9% NaCl and with plasma refrigerated and stored at −80 °C before beginning the analysis. On the day of analysis, the samples were thawed at room temperature. The blood for enzymatic measurements was hemolyzed using an equal volume of 3 mM phosphate buffer pH = 7.4 with 1 mM EDTA. Then the hemolysates were centrifuged (1000 × *g*, for 20 min at 4 °C) and the supernatants were used for further analysis.

Analytical methods

Measurements of enzyme activities

Plasma selenium dependent glutathione peroxidase activity (plSe-GSHPx), plasma total glutathione peroxidase activity (plGSHPx), red blood cells activities of selenium dependent glutathione peroxidase (Se-GSHPx) and total glutathione peroxidase activity (GSHPx) were measured spectrophotometrically at the wavelength of 340 nm, using the method developed by Paglia and Valentine modified by Wendel [28,38]. Before assay the hemolysates were mixed with an equal volume of Drabkin's solution to eliminate the activities of other peroxidases in the sample. In the final reaction mixture in the volume of 220 μl the concentration of glutathione (GSH) was 1.0 mM, of NADPH was 65 μM, and of sodium azide was 0.17 mM. The reaction was carried out at 25 °C in the 50 mM sodium phosphate buffer with 0.40 mM EDTA, pH = 7.0. The tertbutyl hydroperoxide substrate was used at a concentration of 0.02 mM which ensured that the Se-GSHPx was the major determined enzymatic activity. For determination the activity of GSHPx, cumene hydroperoxide at a concentration of 1.05 mM was used.

Glutathione S-transferase (GST) activity was measured spectrophotometrically at the wavelength of 340 nm, using Habig method [14]. The final reaction mixture contained 2 mM glutathione (GSH) and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. The reaction was carried out at 25 °C in the 50 mM sodium phosphate buffer with 0.50 mM EDTA, pH = 7.5.

Thioredoxin reductase (THRR) activity was measured spectrophotometrically at the wavelength of 412 nm, using Hill et al. amendments, with 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) as a substrate at a concentration of 4 mM in the final reaction mixture. The specific inhibitor of the studied enzyme ATM (1 mM) was used. The reaction was carried out at 37 °C with 50 mM sodium phosphate buffer with 1 mM EDTA, pH = 7.0 with NADPH as the cofactor of the enzymatic reaction at the final concentration of 2 μM [12,15,24].

The enzymatic activity and protein absorbance were measured with a Synergy Mx Spectrophotometer microplate reader (Biotek). The presented results of the enzymatic activity were the mean of three independent experimental data and were expressed as units per gram of hemoglobin for hemolysates of red blood cells and units per ml for plasma.

Hemoglobin concentration was assayed spectrophotometrically at the wavelength of 546 nm using standard Human assay kit.

Selenoprotein P and MDA concentration were measured spectrophotometrically using standard ELISA kits.

ORAC measurement

The ORAC-FL (oxygen radical absorbance capacity-fluorescein) assay was performed according to Ou et al. [27]. The ORAC value

was determined fluorometrically with a Fluorescence Spectrophotometer plate reader F-7000 (Hitachi) using black 96-well plates (Grainer) with excitation wavelength of 485 nm and an emission wavelength of 520 nm. All used solutions (fluorescein, AAPH) were prepared daily in PBS buffer, pH 7.4. The final reaction mixture contained: 13 mM AAPH and fluorescein at a concentration of 40 nM. AAPH is widely used as a free radical generator. A fraction of free radicals generated by AAPH is inactivated by plasma antioxidants while the remaining part oxidizes fluorescein, causing the decay of fluorescence. Fluorescence of the remaining fluorescein was measured every minute for 90 min after AAPH addition. The ORAC value in Trolox equivalents (TE) was determined using the standard curve for Trolox, a water soluble vitamin E derivative.

Determination of selenium (Se) concentration

To determine the total Se in red blood cells, the ICPMS technique was used with a VG PlasmaQuad 3 inductively coupled plasma mass spectrometer [26]. Erythrocytes (0.1 g) were collected from 5 mice in each group. After the addition of 3 ml of 65% HNO_3, the samples were mineralized in Teflon crucibles with a microwave mineralizer MDS-2100.

Statistical methods

The data are presented as the mean ± standard error (SE). Statistical analyses were carried out using the Student's *t*-test. A *p*-value less than 0.05 was considered significant with respect to control. The strength of the relationship between variables was measured by the Spearman's rank correlation coefficient. Statistical tests were performed using Statistica 10.

Results

Determination of Se-GSHPx and GSHPx activity

Long-term supplementation

Selol 5% supplementation caused significant changes in plasma and red blood cells of Se-GSHPx and GSHPx activities. A significant increase of plSe-GSHPx (Fig. 1A) and plGSHPx (Fig. 1B) activities, in comparison with the control group (0), were observed starting from the 3rd day of the experiment (for both enzymes, respectively: $p = 0.0015$; $p = 0.0130$ for the 3rd day, $p = 0.0360$; $p = 0.0447$ for the 5th day, $p = 0.0318$; $p = 0.0464$ for the 10th day, $p = 0.0058$; $p = 0.0027$ for the 28th day). In red blood cells a significant increase of Se-GSHPx (Fig. 1C) activity was observed only from the 10th to the 28th day ($p = 0.0061$; $p < 0.0001$, respectively), whereas an increase in the GSHPx (Fig. 1D) activity was observed on the 3rd day ($p = 0.0027$), on the 10th day ($p = 0.0416$) and on the 28th day ($p = 0.0002$) of supplementation There were no significant differences in red blood cells of Se-GSHPx and GSHPx activity on the 5th day of Selol 5% administration (Fig. 1C and D).

Short-term supplementation

After Selol 5% single-dose administration we observed a significant increase in plSe-GSHPx and plGSHPx activities. Plasma Se-GSHPx (Fig. 2A) activity increased significantly during the 2nd and 3rd hour of the experiment ($p = 0.0010$, $p = 0.0324$, respectively) and from the 14th to the 18th hour ($p = 0.0014$ for the 14th hour; $p < 0.0001$ for the 16th hour; $p = 0.0014$ for the 18th hour, respectively) after Selol 5% single-dose administration. Similarly, we observed a significant increase in GSHPx (Fig. 2B) activity during the 2nd, 3rd and 4th hour of the experiment ($p < 0.0001$; $p = 0.0328$; $p = 0.0419$, respectively) and from the 10th to the 18th hour ($p = 0.0132$ for the 10th hour; $p = 0.0166$ for the 12th hour; $p = 0.0105$ for the 14th hour; $p = 0.0006$ for the 16th hour and $p = 0.0107$ for the 18th hour). As presented in Fig. 2C, a significant increase of red blood cells Se-GSHPx activity was observed during the 3rd, 4th and 12th hour of supplementation ($p = 0.0024$; $p = 0.0049$; $p = 0.0201$ respectively) whereas for GSHPx (Fig. 2D) activity there was a significant increase only during the 4th hour ($p = 0.0264$) and the 12th hour ($p = 0.0165$) after Selol 5% administration.

Determination of THRR and GST activity

Long-term supplementation

No significant differences in plasma GST (Fig. 1E) activity were observed on the 3rd, 5th, 10th and 28th day of Selol supplementation. Moreover, after 5 days of Selol 5% daily administration there was also a significant decrease in RBC GST activity (Fig. 1F). On 3rd, 10th and 28th day of supplementation there was a significant decrease in plasma and red blood cells THRR activity in comparison with the control group (0) (Fig. 1G and H).

Short-term supplementation

Selol 5% administration caused no significant changes in plasma GST (Fig. 2E) activity during the experiment, the only exception was the 1st hour ($p = 0.0277$) after Selol 5% administration. In red blood cells the increase of GST (Fig. 2F) activity was observed after the 2nd and the 3rd hour ($p = 0.0007$; $p = 0.0104$) and from the 16th to the 20th hour ($p = 0.0026$ for the 16th hour; $p = 0.0035$ for the 18th hour; $p = 0.0016$ for the 20th hour, respectively) of single-dose oral administration.

Moreover, we observed a significant increase in plasma THRR (Fig. 2G) activity during the 1st hour ($p = 0.0035$) after Selol 5% administration and from the 10th to the 16th hour ($p = 0.0351$ for the 10th hour; $p = 0.0234$ for the 12th hour; $p = 0.0282$ for the 14th hour, and $p = 0.0028$ for the 16th hour, respectively) of Selol 5% distribution. Similarly Selol 5% administration caused a significant increase of red blood cells THRR (Fig. 2H) activity from the 2nd to the 4th hour and from the 12th to the 20th hour of the experiment (for all study groups $p < 0.0001$).

Determination of selenoprotein P and MDA concentration

Long-term supplementation

Selenoprotein P (Fig. 1I) concentration in plasma was significantly higher on the 3rd day ($p = 0.0063$) and on the 28th day ($p = 0.0041$), whereas MDA level was significantly higher on the 10th day ($p = 0.0122$) and on the 28th day of administration in comparison with the control group (0) (Fig. 1J).

Short-term supplementation

Selol 5% single-dose administration caused a significant increase ($p < 0.0001$) in selenoprotein P concentration in plasma from the 4th to the 24th hour of the experiment (Fig. 2I).

From the 1st to the 8th hour of the experiment, Selol 5% distribution caused no significant changes in MDA (Fig. 2J) concentration, with the exception of the 8th hour when a significant increase occurred ($p = 0.0334$). From the 10th hour to the end of the experiment we observed a statistically significant decrease (except the increase during the 20th hour $p < 0.0001$) of MDA content in plasma ($p = 0.0026$ for the 10th hour; $p = 0.0293$ for the 12th hour; $p = 0.0200$ for the 14th hour; $p = 0.0006$ for the 14th hour; $p < 0.0001$ for 24 h of the experiment, respectively).

Determination of ORAC level

Long-term supplementation

The ORAC values in plasma of the study groups were higher than those in the control group ($p < 0.0001$ for each comparison) (Fig. 1K).

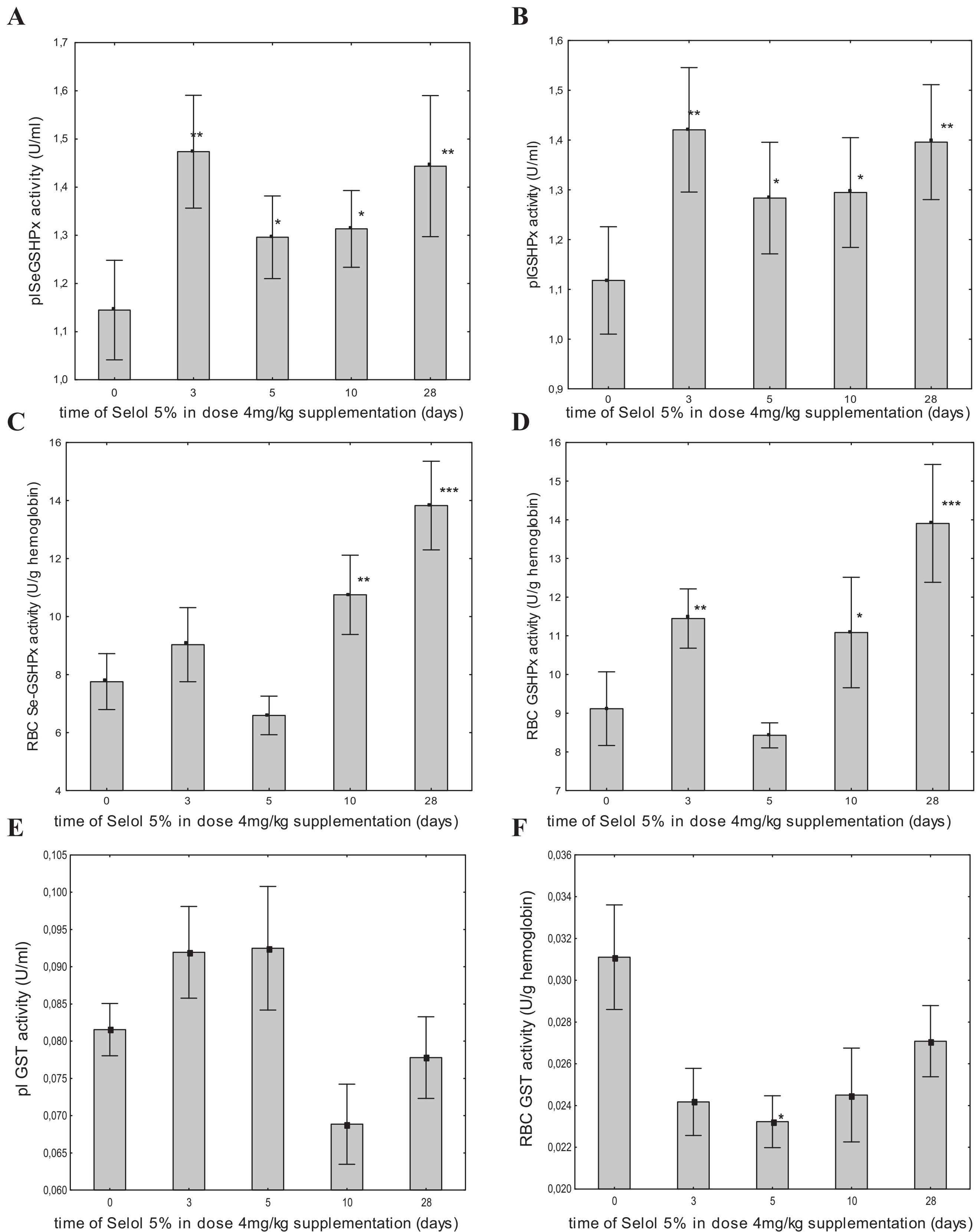


Fig. 1. Effect of the Selol 5% in dose 4 mg Se/kg daily (long-term supplementation) on the activity of (A) plasma selenium dependent glutathione peroxidase (plSe – GSHPx), (B) plasma total glutathione peroxidase (plGSHPx), (C) red blood cells selenium dependent glutathione peroxidase (RBC SeGSHPx), (D) red blood cells total glutathione peroxidase (RBC GSHPx), (E) plasma glutathione S-transferase (plGST), (F) red blood cells glutathione S-transferase (RBC GST), (G) plasma thioredoxin reductase (pl THRR), (H) red blood cells thioredoxin reductase (RBC THRR), (I) concentration of selenoprotein P, (J) MDA concentration and (K) ORAC value in plasma, (L) red blood cells Se concentration. (E) concentration of selenoprotein P, (F) MDA concentration and (G) ORAC value in plasma and on the (H) Se concentration in red blood cells. The p values indicate significant difference between control and study groups and are signed as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

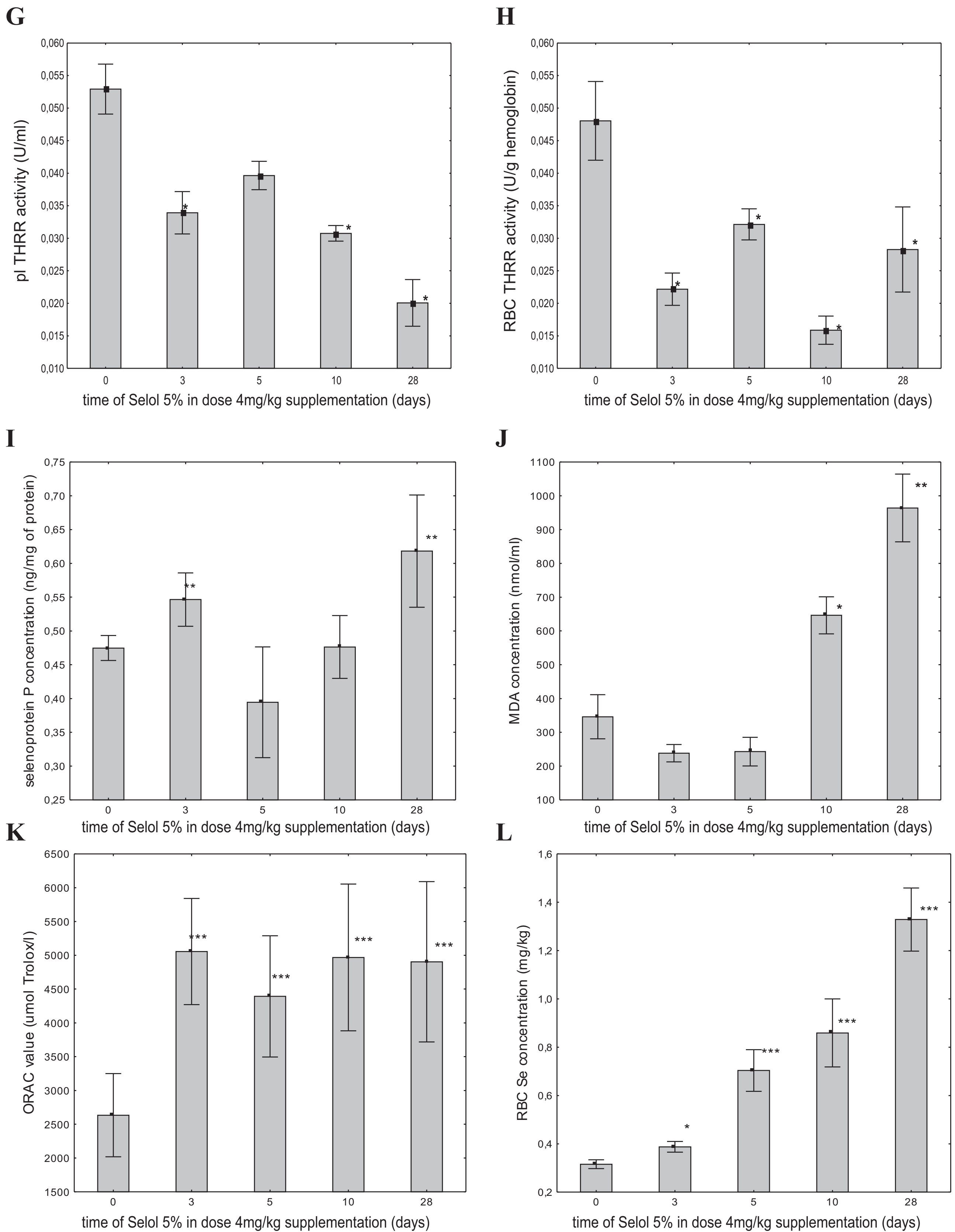


Fig. 1. (*Continued*).

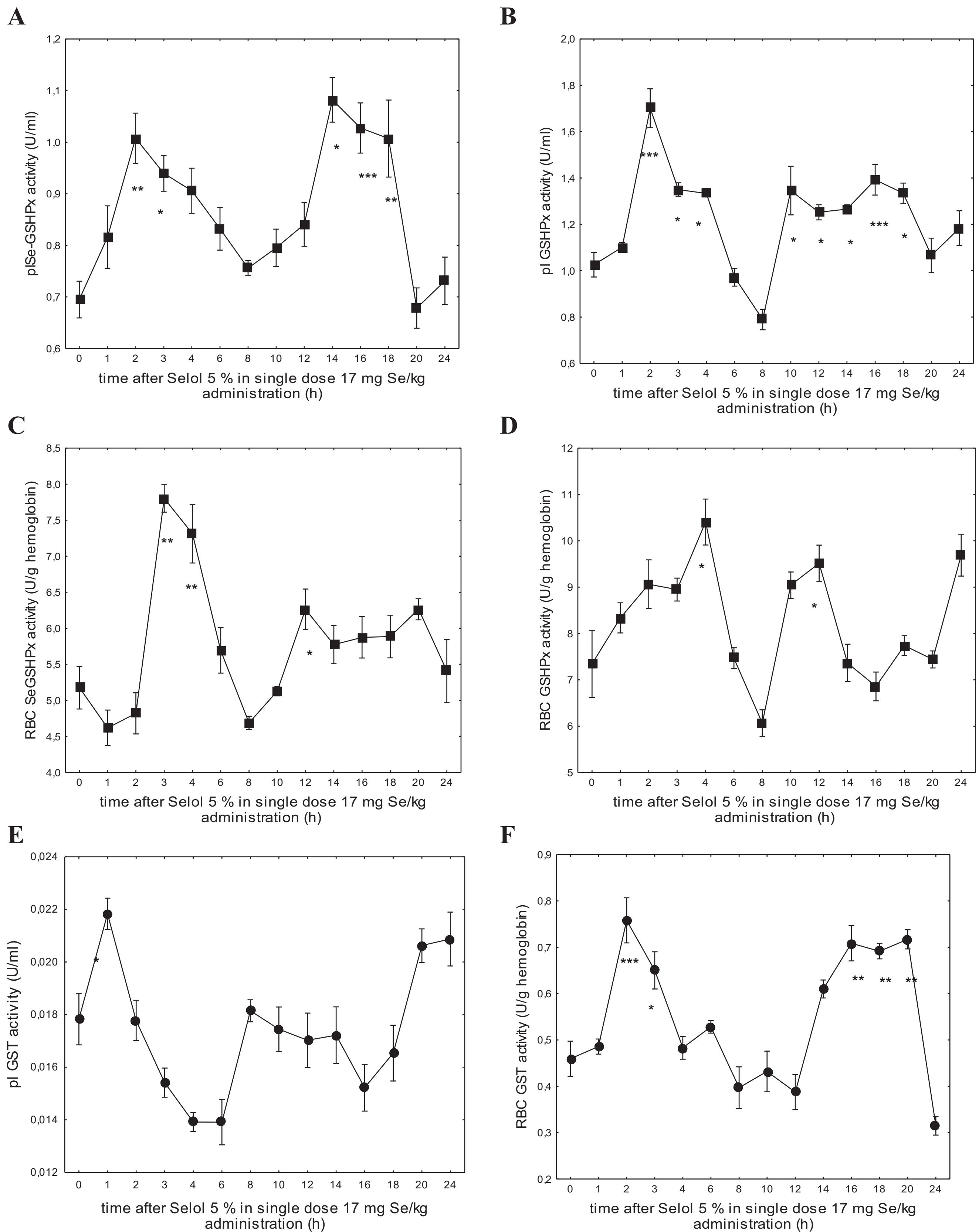
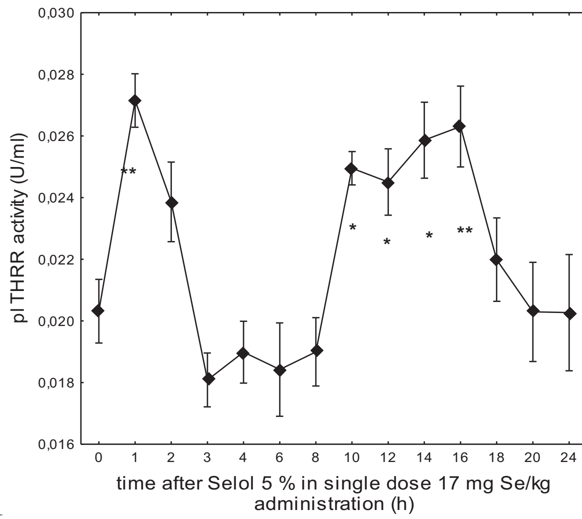


Fig. 2. Effect of the Selol 5% in dose 17 mg Se/kg single dose oral administration (short-term administration) on the activity of (A) plasma total glutathione peroxidase (plGSHPx), (B) plasma selenium dependent glutathione peroxidase (pl Se-GSHPx), (C) red blood cells total glutathione peroxidase (RBC GSHPx) (D) red blood cells selenium dependent glutathione peroxidase (RBC SeGSHPx), (E) plasma glutathione S-transferase (plGST), (F) red blood cells glutathione S-transferase (RBC GST), (G) plasma thioredoxin reductase (pl THRR), (H) red blood cells thioredoxin reductase (RBC THRR), (I) concentration of selenoprotein P, (J) MDA concentration and (K) ORAC value in plasma, (L) red blood cells Se concentration. The *p* values indicate significant difference between control and study groups and are signed as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

G



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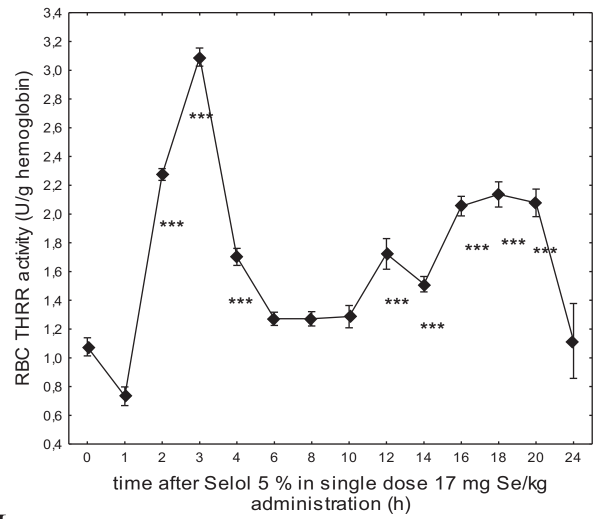

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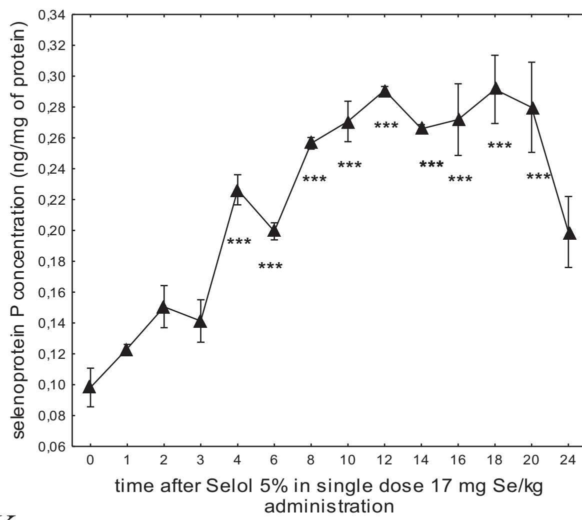

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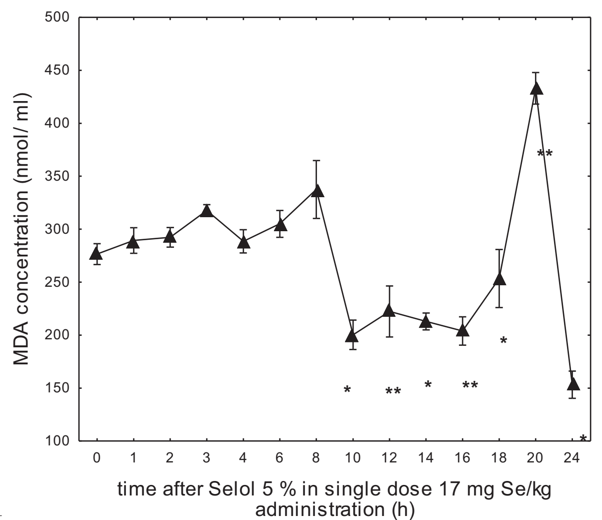

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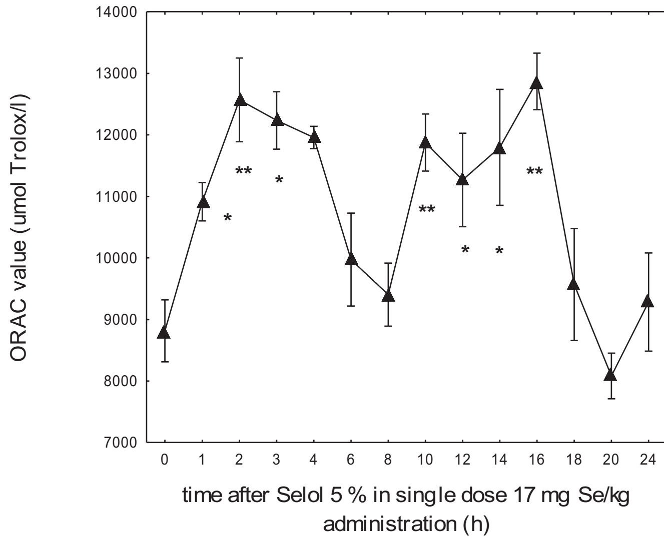

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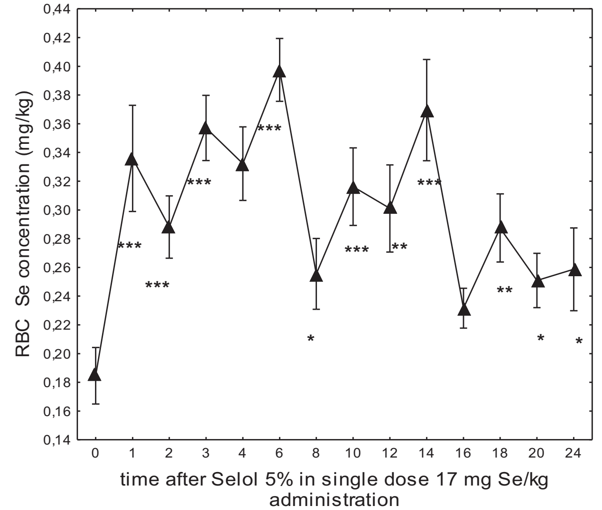

Fig. 2. (*Continued*).

Short-term supplementation

During the experiment there was a statistically significant increase in the ORAC value in plasma from the 2nd to the 3rd hour ($p = 0.0012$; $p = 0.0100$, respectively) and from the 10th to the 16th hour of experiment ($p = 0.0053$ for the 10th hour; $p = 0.00389$ for the 12th hour; $p = 0.0258$ for the 14th hour; $p = 0.0010$ for the 16th hour, respectively) (Fig. 2K).

Determination of selenium (Se) content

Long-term supplementation

The concentrations of Se in red blood cells were significantly higher as compared with the control group (0) after 3 days ($p = 0.0376$), 5 days ($p < 0.0001$), 10 days ($p < 0.0001$) and 28 days ($p < 0.0001$) of Selol 5% supplementation (Fig. 1L).

In the long-term daily Selol 5% supplementation there was a significant correlation between Se concentration and Se-GSHPx activity ($r_s = 0.49$, $p < 0.0001$) in red blood cells. We did not find any significant correlation between Se concentration and GSHPx, THRR or GST activities in red blood cells during the experiment.

Comparing selenium concentration in red blood cells and plasma enzymes activities we found a significant correlation in the activity of plSe-GSHPx ($r_s = 0.38$, $p = 0.0065$), plGSHPx ($r_s = 0.44$, $p = 0.0013$) and negative correlation for plasma THRR activity ($r_s = -0.69$, $p < 0.0001$). There was no correlation between red blood cells Se concentration and selenoprotein P concentration and plasma GST activity.

Short-term supplementation

Selol 5% single-dose administration caused a significant increase in Se concentration in red blood cells from the 1st to the 24th hour of the experiment with $p < 0.0001$ from the 1st to the 6th hour and a statistically significant increase from the 8th hour to the end of the experiment ($p = 0.03$ for the 8th hour; $p = 0.0003$ for the 10th hour; $p = 0.0018$ for the 12th hour; $p < 0.0001$ for the 14th hour; $p = 0.0062$ for the 18th hour; $p = 0.0273$ for the 20th hour; $p = 0.0381$ for the 24th hour, respectively) (Fig. 2L).

In the short-term study of Selol 5% single-dose administration, there was a significant correlation between Se concentration and Se-GSHPx activity ($r_s = 0.23$, $p = 0.0022$) in red blood cells. We did not find any significant correlation between Se concentration and GSHPx THRR, GST activities in red blood cells.

Comparing selenium concentration in red blood cells and plasma enzymes activities during the experiment we found a significant correlation only in the activity of plSe-GSHPx ($r_s = 0.23$, $p = 0.0012$). There was no correlation between red blood cells Se concentration and selenoprotein P concentration and plasma GST, GSHPx, THRR activity.

Discussion

Selenium and selenium compounds have already been shown to exhibit cancer chemopreventive effects [11]. Numerous animal studies demonstrated that dietary selenium supplementation decrease the incidence of induced tumor [8,30]. The mechanisms of cancer chemopreventive abilities and the anticancer activity of selenium compounds include (1) activation of the immune system [2], (2) an increase of oxidized glutathione (GSSG) concentration which results in inhibition of protein synthesis [31], (3) formation of cytotoxic methylated selenium metabolites like CH_3Se^- which reacts with O_2 and generates the reactive oxygen species (ROS) [16,17], (4) an increased activity of selenoproteins and related enhanced protection against oxidative stress [11]. Moreover, Se at the +4 oxidation state increases the effectiveness of anticancer therapy, it behaves as a pro-oxidant rather than an antioxidant, inducing apoptosis through oxidative stress pathway [9].

Antioxidant effects of Se action are mainly associated with its specific incorporation in selenoproteins and its +4 oxidation state. The selenocysteine (SecCys) is a part of an active site of enzymes which are able to catalyze redox reactions [32]. For the biological activity and synthesis of specific selenoproteins, selenium should be present as a selenide-like intermediate (II), which can incorporate into specific SecCys residues. The selenide can react with the reduced form of glutathione in red blood cells and form selenodiglutathione, the main compound with anticancer activity which induces apoptosis in human tumor cells [22]. Recent studies suggest that selenide is more easily formed from inorganic forms of Se than the organic ones. However, inorganic forms of selenium reveal a rather narrow therapeutic range, relatively poor absorption and consequently lower concentration of selenium circulation [23].

The studies on antioxidant enzymes activities, concentration of selenoproteins and oxidative stress biomarkers are the first steps to investigate the biochemical mechanism of action of a novel organoselenium Se(IV) compound with potential anticancer activities Selol 5%. In the present experiment the daily dose was chosen to be 4 mg of Se(IV) kg^{-1} body mass per day of Selol 5%, which refers to approximately 5% of LD_{50} (LD_{50} = 100 mg Se(IV) kg^{-1} body mass). The short-term supplementation dose was chosen to be approximately 20% of LD_{50}, 17 mg Se(IV) kg^{-1} body mass. Such high doses of selenium administration were possible owing to Selol 5% lower toxicity and slower absorption in comparison with other Se(IV) compounds, like sodium selenite. The first symptoms of harmful activity of Selol 5% in the form of lung congestion were observed after administration of 30 mg of Se(IV) kg^{-1} body mass (unpublished study).

The biological investigations into Selol carried out on healthy rats indicate that after oral, subcutaneous and intraperitoneal administration, Selol was distributed in the whole body. The obtained data showed that absorption of Selol from the rat gastrointestinal tract is high. Selol and its metabolism products are eliminated completely from the animal's body within 24 h by urinary excretion [18,19]. Therefore we decided to perform the short-term supplementation for 24 h. Selol 5% was administered orally since this is the preferred route of Selol administration in the future therapy.

Our study confirmed the hypothesis that Selol 5% supplementation affects the selenoenzymes activities in healthy animals models. The short-term study of Selol 5% single-dose administration resulted in a significant increase in plasma and red blood cells selenoenzymes activities (SeGSHPx, THRR) in two time intervals, during the 1st hours after the administration (from the 1st to the 4th hour), and a few hours before the end of the experiment (from the 10th to the 20th hour). Furthermore, starting from the 4th hour of the study we observed a significant increase of the selenoprotein P concentration, which is the antioxidant in the extracellular space. The plasma ORAC values after Selol 5% single-dose administration were shaped similarly to the activities of other antioxidant enzymes (GSHPx, Se-GSHPx, THRR, GST) with two distinct increases of activity. The pattern of changes of the antioxidant enzymes activities after Selol administration could not be easily explained yet. However, the first increase (1–4 h) may be a consequence of high dose of Se (IV) administration and related enhanced antioxidant enzymes expression *via* Nrf2/ARE pathway. Within first hours of the experiment the glutathione (GSH) level changes and redox status decrease (to be published elsewhere), whereas the second temporary increase of antioxidants (14–20 h) did not involve thiols level changes and could be a result of the influence of unknown Selol 5% metabolite on the antioxidant enzymes.

The increase of MDA level was observed only at the end of the study (during the 20th hour). Between the 10th and the 24th hour of the experiment (with the exception of the 20th hour) the level of MDA was even lower than in the control group. This is also the

evidence of the antioxidant activity of Selol 5% in healthy subjects observed during the 24-h experiment.

Since the levels of GSHPx/Se-GSHPx (Fig. 2A–C), and other biomarkers (Fig. 2F–H) at 24 h were similar to those at 0 h, we conclude that short-term Selol 5% supplementation induces some temporary and reversible increases of enzyme activities.

Significant increases of the Se-GSHPx activity in plasma and red blood cells and the plasma selenoprotein P concentration were observed during long-term supplementation as well. The increase of enzyme activity was correlated with Se concentration in the blood of the study groups. The observation is consistent with many studies which showed the dependence of Se administration and selenoproteins levels in the blood of the examined animals. Therefore, we can assume that the Se from Selol is probably metabolized to selenocysteine incorporated in the structure of selenoproteins [3,5,41].

Unexpectedly we observed the decrease of other selenoenzyme-THRR in plasma and red blood cells of healthy mice during long-term Selol 5% supplementation. The results are opposite to those of short-term supplementation, where as we mentioned previously the activity of THRR increased, what can be a result of the increase of its expression, *e.g. via* Nrf2/ARE and probably the activation of the enzyme *via* direct incorporation of Se(IV) into selenocysteine (Sec) residues. However the decrease in thioredoxin reductase activity was observed with continued supplementation of relatively high dietary selenium levels what could be explained by biphasic Sec/diselenide incorporation [4]. Exposure to high doses of Se, can generate reactive Se species/metabolites like diselenide forms, which can inhibit the THRR activity over time [10]. It can be assumed that during long-term supplementation the inactivation of the THRR by unknown Selol 5% metabolites occurs, however that hypothesis is quite speculative. A similar conclusion of THRR inhibition by a novel selenium compound ethaselen was drawn *in vitro* in the experiment carried out by Wang et al., where ethaselen significantly suppressed cancer cells viability in parallel with direct inhibition of THRR activity [37]. In another *in vivo* study on tumor tissues, Wang et al. showed the decreased THRR tumor activity in mice treated with that drug [35]. However, there are no available data relative to the inhibition of the THRR activity after selenium (Se(IV)) supplementation *in vivo*, in the healthy subjects. Moreover, many articles revealed that the supply of selenium is related to increased THRR activity *in vitro* and *in vivo* healthy models [1].

Since a recent study showed that THRR protein is overexpressed in many cancers and its activity increases significantly in tumor cells, stimulating its growth and reducing apoptosis, specific inhibitors of THRR activity have been searched for. Therefore, if the obtained results of our study on healthy subjects are to be confirmed on cancer individuals, Selol 5% could be considered as THRR inhibitor as well. This indicates the possible pharmaceutical application of Selol 5% as a valuable anticancer drug, even more effective than other Se compounds without THRR inhibiting activity.

The present report also showed the increase of plasma ORAC values in all study groups after long-term Selol 5% supplementation, which can be associated with the increase of enzymes activities (Se-GSHPx, GSHPx; the increased activity of GSHPx may be due to higher activity of Se-GSHPx).

The influence of long-term Selol supplementation on selenoproteins levels was also described by Zagrodzki et al. The authors observed insignificant changes in the main selenoenzymes activities: glutathione peroxidase as well as thioredoxin reductase measured in plasma and blood of various merino sheep. The results differed from those obtained from the studies of long-term supplementation of Se compounds to herbivore animals [39]. Hence Zagrodzki et al. suggested that Se from Selol is metabolized by a different pathway than Se from classic compounds such as sodium selenite [40].On the other hand, our results showed the increase of plasma MDA (a marker of lipid peroxidation) value starting from the 10th day of Selol 5% supplementation. This might be the consequence of the increase of ORAC value and insufficient activity of antioxidant enzymes, which results in elevated oxidative stress (OS) and MDA overproduction during Selol 5% long-term supplementation. A similar observation of the pro-oxidative function was observed during *in vivo* study of sodium selenite supplementation [20].

However, further research on the changes of antioxidant enzymes, ORAC value and markers of oxidative stress level in tissues and tumors seems to be necessary to explain these phenomena. The increased plasma MDA concentration may be related not only to endogenous lipids peroxidation as a result of OS, but also to the embodiment of the Selol 5%, which is a mixture of selenitetriglycerides synthesized from sunflower oil. Therefore, in a further study the markers of oxidative stress, not related to lipid peroxidation, like nucleic acid oxidation (8-hydroxy-2′-deoxyguanosine, 8-hydroxyguanine) will be determined as well.

Surprisingly, within long term Selol 5% administration on 5th day we observed the significant decrease of Se-GSHPx level in plasma ($p = 0.0255$ *vs.* 3rd day) with no statistically significant changes in plasma GSHPx activity. Furthermore on 5th we noticed significant drop, comparing to 3rd day, of the RBC Se-GSHPx/GSHPx activity (respectively $p = 0.0054$, $p < 0.0001$), which is opposite to the overall tendency of increased enzymes activity. Similar patterns of different trends were also shown in RBC THRR activity, where the enzyme activity increased on 5th day comparing to 3rd day ($p = 0.0204$). These rebound trends could be related to drug metabolism, which was not examined so far. Therefore, other research team led by Suchocki aim at description of Selol metabolites, its pharmacokinetics in blood after short and long term supplementation. Moreover, the isolation of metabolites and *in vitro* assessment of their biological activity will be performed. We hope that obtained results confirms our hypothesis of influence of Selol metabolites on antioxidant status.

In conclusion, our results confirm the influence of Selol 5% administration on the antioxidant status of blood in healthy subjects. Moreover, Selol 5% acts as an inhibitor of thioredoxin reductase activity and induces oxidative stress elevation during its long-term supplementation, which is important in anticancer therapy. Further studies, especially those performed on tumor mice supplemented with Selol 5%, are necessary to determine the exact mechanism of its anticancer properties.

Conflict of interest

The authors disclose any actual or potential conflict of interest.

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