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Immunomodulatory effect of selenosemicarbazides and selenium inorganic compounds, distribution in organs after selenium supplementation

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Abstract

Antioxidant properties of selenium producing a protective barrier against free radicals play an important role in numerous metabolic and immunologic processes associated with oxidation- reduction reactions which take place during intracellular digestion of phagocyted bacteria. The aim of our study was to examine the properties of an organic compound of selenium, 4-(*o*-tolilo)- selenosemicarbazide of p-chlorobenzoic acid in terms of its retention in organs, effect on erythropoesis and phagocytic abilities of neutrophiles as well as antioxidant properties in neutrophiles tested with NBT test. This compound as well as inorganic sodium selenate was given to Swiss mice at the dose of 10^{-3} g Se/kg for the period of 10 days. The concentrations of selenium in livers of mice treated with sodium selenate and selenosemicarbazide were found to be higher than in controls (18,7 μg lg^{-1} and 23.2 μg lg^{-1} vs. 12 μg lg^{-1}, respectively). Analysis of blood cells count has shown a significant decrease in neutrophile levels in both groups treated with selenium. The influence of selenium compounds on phagocytosis and especially NBT test has been determined (3.8% of positive cells in the controls vs. 2.2% and 0.9% in the groups treated with sodium selenate and selenosemicarbazide, respectively). Our preliminary investigations suggest that selenosemicarbazides are biologically active compounds and can modify neutrophile functions.

Introduction

The antioxidant properties of selenium producing a protective barrier against free radicals play an important role in numerous metabolic and immunological processes associated with the oxidation-reduction reactions (Chan *et al.* 1998; Winnefeld 1997) which are involved in intracellular digestion of phagocyted bacteria. The biological activity of the selenium compounds is related to the selenium presence in the selenoprotein and enzyme molecules in which selenium constitutes the activity centre. The following selenodependant enzymes are important in the human metabolic processes: glutathione peroxidase, formate dehydrogenaze, xanthene dehydrogenase, glycine reductase, thioredoxin reductase (Hori *et al.* 1997) which are involved in various reduction processes. The selenium compounds protect membrane lipids against oxidation, result in peroxide destruction, i.e., the reduction of hydrogen peroxide and organic peroxides, reduction of the oxidized glutathione form. Selenium is found in all organs and its deficiency is associated with various metabolic disorders while even its border excess is highly toxic for metabolic processes and immune mediators. The subtoxic selenium intakes which increase the inducibility of interleukin-2 receptor, high-dose vitamin E and possibly chromium may counteract the down-regulatory effect of cAMP on interleukin-2 activity (McCarty 1997). Selenium is assimilated in the alimentary canal, its organic

compounds show higher capacity of assimilation and the most favourable therapeutic effects are observed in combination with vitamin A, C and E (McCarty 1997). The main trends of the selenium studies concern their most suitable chemical form, toxicity limits, organ cumulation and their effects on the immunological mechanisms. The selenium configuration which is similar to sulphur results in the fact that the properties of its organic derivatives are comparable with the ones of model sulphur compounds. New form of selenium the 4-(*o*-tolilo)-selenosemicarbazide of p-chlorobenzoic acid obtained by us in the addition reaction of o-tolilo isoselenate with p-chlorobenzoic acid is comparable with the suitable thiosemicarbazide (Bielak & Biliński 1996). To determine the biological properties of this compound the analysis of selenium cumulation in organs was performed, its effects on blood formation, phagocytosis and antioxidative properties in mouse neutrophiles after the supplementation with 4-(*o*-tolilo)-selenosemicarbazide of p-chlorobenzoic acid were examined and compared with the effects induced by the inorganic selenium compounds.

Material and methods

The selenosemicarbazide synthesis

The compound was synthetized using the addition reaction of suitable substrates and the final product was chemically analyzed. 0.1 mol of o-tolilo isoselenocyanate, 0.1 mol of p-chlorobenzoic acid hydrazide in 30 ml of methanol were heated for 30 min. The sediment formed after cooling was crystallized 3 times from 20 ml of 95% ethanol. The obtained compound, o-CH_3-C_6H_4-NH-CSe-NH-NH-CO-C_6H_4-p–Cl was identified examining the percentage of C, H and N. Calculated: 49.12% of C, 3.84% of H, 11.46% of N. Obtained: 48.80% of C, 3.68% of H, 11.17% of N. The UV, IR, H NHR spectrometry was performed. IR spectrum: CO group band was determined (amide I): 1662 cm^{-1}. UV spectrum: λ_{max}[nm]/ϵ band: 234/23205; 271/13200. H-NMR: DMSO-d 6: 10.67 (s. 1H. NH); 10.10(s. 1H. NH); 9.98 (s. 1H. NH) 7.12–7.99 (m. 8H. ar); 2.18 (s. 3H. CH_3-ar). The product was kept in its crystalline form, undissolved in water.

The experiment description

Selenosemikarbazid was suspended in the emulsion composed of olive oil, arabic gum and water in the following proportion 2:1:1.5. The compound suspended in the emulsion was administered to SWISS mice by stomach tube in the dose 10^{-3} g/kg body weight daily for 10 days. The study was carried out in the group of 10 females. The comparable group consisted of 10 females which were administered sodium selenite (Na_2SeO_3) also in the doses of 10^{-3} mg Se g^{-1} of body weight once a day for 10 days. The dose was subtoxic to determine the selenium cumulation in the form of selenosemicarbazide in comparison with commonly examined sodium selenite and to detect the immunological effects of the down-regulatory reaction. Used doses by other authors which ranged from 0.1–0.3 mg Se kg^{-1} (Wilson *et al.* 1992) to 0.1–1 mg Se kg^{-1} (Iton & Suzuki 1997) and 0.5–8 mg Se kg^{-1} of body weight (Gu *et al.* 1998). The control group included another 10 females without selenium supplementation. The age and weight of the animals kept in identical conditions were comparable. Ten days later the blood samples were collected from the heart in partial anaesthesia and the organs were taken to examine the selenium contents.

Methods

At the beginning of the experiment the animal's body weight ranged from 23 to 25 g. After the experiment the body weight was 24.6 g $\pm$ 1.7 g - 24.1 g $\pm$ 1.2 g in the control group. In the second group it was 23.9 g $\pm$ 1.6 g and in the third group: 24.2 g $\pm$ 1.3 g. No statistically significant differences were observed. They were fed with standard LSM food stuffs and watered ad libitum. The blood samples were collected to heparinized test tubes and the red blood and white blood count, haematocrit and haemoglobin concentration were studied. In the smears stained with May–Grunwald method the percentage of white morphotic elements of blood was determined which was used to calculate the absolute cell number in mm^3 of blood.

To evaluate the neutrophil phagocytic capacity, the phagocytic reaction with Bacto-Latex (Difco, USA) was used.

The neutrophil oxidation-reduction potential was examined using NBT test in which the positive cells were those whose oxidated form of a yellow water-soluble dye was converted into a dark-blue water-insoluble diformazon upon reduction. The total NBT

reduction to diformazon form requires 4 electrons. The reaction with superoxide anion-radical proceeds in 4 unielectron stages.

In both tests 100 cells were calculated. In the phagocytic test the cells with at least 3 latex granules were considered the positive ones. In the NBT test the positive cells were those in which big formazon granules were observed.

The number of positive cells in 100 analysed cells is determined by the test indicators.

Selenium contents in organ tissues

The selenium content was studied in liver, spleen and kidney. The collected organs were weighed and frozen until mineralization was performed according to Shimoishi method. 500 mg of tissue in 10 ml of concentrated nitric acid were placed in Kjeldahla flask and slowly heated to 150 °C for about 1 h in silicon bath. After cooling, 3 ml of 1 mol carbamide solution were added and heated for 10 min to decompose the nitrogen oxides. After cooling the solution was diluted with 10 ml of distilled water and 1 ml of 0.6% DANB solution (1,2-diamino-4-nitrobenzene). The solution was left for 2 h in order to form 5-nitropiazselenol and extracted with 5 ml of toluene. The toluene layer was initially treated with 20 ml of 1 mol NaOH and then washed with 10 ml of 7.5 mol hydrochloric acid solution. The selenium content was analysed spectrophotometrically (specord H-40, Carl Zeiss Jena) determining the toluene solution absorbance at the wavelenght of 350 nm. The concentration was calculated against the standard curve plotted from DANB and standard solution of 100 μg Se ml^{-1} in 0.1 mol hydrochloric acid solution (Bem 1979).

Results

The studies of kidneys liver and spleen after the supplementation with sodium selenite and selenosemicarbazide revealed the decreased weight of kidneys and spleen, and the differences in comparison with the control group were statistically significant in the case of selenosemicarbazide while with sodium selenite only the spleen's weight decreased significantly (Table 1). The highest cumulation of selenium was observed in the liver; after the selenosemicarbazide administration almost a double increase of concentration was statistically significant, 12.04 $\pm$ 4.0 μg g^{-1} in the control group and 23.27 $\pm$ 6.8 μg g^{-1} of tissue. After the sodium selenite administration the concentration increased to 18.72 $\pm$ 7.7 μg g^{-1} of tissue. The selenium content in kidneys following the sodium selenite administration increased to 27.13 $\pm$ 6.7μg g^{-1} while the organic selenium resulted in the increase to 31.82 $\pm$ 6.5μg g^{-1}. Those values were not statistically significant. The surprising results were observed in spleens as after the selenium supplementation Se concentration was markedly lower than in the control group. The initial selenium concentration in spleen was comparable to the kidney's content being 26.28 μg g^{-1} of tissue. The studies of blood parameters, erythrocyte and leukocyte count, haematocrit, haemoglobin concentration, showed the decrease of all values in both examined groups, particularly in mice treated with selenosemicarbazide. However, compared to the control groups the differences were not statistically significant (Table 2).

The studies of white cell composition in mice treated with selenium compounds revealed the decreased absolute values of the number of neutrophils and their young rod-shaped forms, the lower monocyte number and the least visible decrease of the lymphocyte number was observed only in mice treated with inorganic selenium. The most visible, statistically significant decrease was found in neutrophils, their number after the selenosemicarbazide administration dropped by half. The results are presented in Table 3.

The studies of the phagocytic functions of neutrophils after the administration of the selenium compounds showed statistically significant, almost double decrease in the group supplemented with inorganic selenium. The phagocytic functions after the selenosemicarbazide treatment almost did not changed. The analysis of the intracellular capacity of nitrotetrazolium reduction processes performed by defining the intracellular formazon showed some decrease in the group treated with inorganic selenium while in mice supplemented with selenosemicarbazide the NBT test values were significantly decreased both in comparison with the control group and the group treated with inorganic selenium (Table 4).

Discussion

The studies concerning the content and function of selenium and other elements in the living organisms provide interesting results, although their interpretations are not always univocal. The studies are mostly inspired by the observations of the disorders of the

Table 1. The selenium content in Swiss mouse organs following the supplementation with inorganic sodium selenite and organic 4-(*o*-tolilo)-selenosemicarbazide of p-chlorobenzoic acid compared to the control group

Mouse group	Kidney		Spleen		Liver	
	Average organ mass ± SD (g)	Average Se content in μg/g tissue ± SD	Average organ mass ± SD (g)	Average Se content in μg/g tissue ± SD	Average organ mass ± SD (g)	Average Se content in μg/g tissue ± SD
Controls without Se supplementation	0.296 ± 0.016	25.73 ± 10.2	0.163 ± 0.01	26.28 ± 11.6	1.72 ± 0.34	12.04 ± 4.0
After sodium selenite supplementation	0.271 ± 0.047	27.13 ± 6.7	0.102 ± 0.01*	4.25 ± 0.26*	1.65 ± 0.25	18.72 ± 7.7
After selenosemi-carbazide supplementation	0.250 ± 0.044*	31.82 ± 6.5	0.118 ± 0.0 1* (**)	10.55 ± 0.43* (**)	1.63 ± 0.41	23.27 ± 6.8*

SD – standard deviation.
*Statistical significance in comparison with the control group $p < 0.05$.
**Statistical significance after selenosemicarbazide supplementation in comparison with sodium selenite supplementation.

Table 2. Morphological parameters of blood in Swiss mice after the supplementation with inorganic sodium selenite and organic 4-(*o*-tolilo)-selenosemicarbazide of p-chlorobenzoic acid compared to the control group

Mouse group	Haemoglobin (g%)	Haematocrit (%)	Erythrocytes (10^6 mm^{-3})	Leucocytes (10^3 mm^{-3})
Controls without Se supplementation	15.32 ± 0.78	47.80 ± 2.16	9.39 ± 0.45	3.46 ± 1.28
After sodium selenite supplementation	14.68 ± 1.07	46.15 ± 3.43	9.08 ± 0.58	2.44 ± 0.89
After selenosemicarbazide supplementation	14.48 ± 0.89	45.10 ± 2.51	8.87 ± 0.47	2.90 ± 0.88

vital functions or severe diseases resulting from the deficiencies of trace the elements including selenium. The most severe diseases caused by the selenium deficiency observed in animals were muscular dystrophy, liver toxic dystrophy chicken oxidative diathesis and neoplastic lesions. In some regions with lower soil selenium contents the increased number of some diseases in humans was observed in association the selenium deficiency (Kossakowski & Kossakowska 1980). The selenium deficiency may affect the prevalence of neoplastic diseases, leukemias, hypertension, degenerative changes in skeletal muscles and the decreased efficiency of the immune system (Graczyk *et al.* 1994; Zawierta *et al.* 1997). Dietary selenium content 0.1–0.3 mg Se kg^{-1} is thought to be physiologically indispensable, however possible selenium protective activity against neoplasms necessitates bigger doses, i.e., 2–5 mg Se kg^{-1} (Wilson *et al.* 1992). The significantly decreased selenium concentration (Dhur *et al.* 1990) was observed in some infections, inflammations and autoimmunological diseases. The experimental and therapeutic supplementation with the selenium compounds resulted in positive changes (Gartner *et al.* 1997; Hori *et al.* 1997), which encourages to continue the studies to find less toxic and better assimilated organic selenium compounds. In order to achieve the better form of selenium the synthesis of selenosemicarbazides and their cyclic combinations was performed. Numerous diseases go hand in hand with the disturbances of the homeostasis. All healthy mammalian organisms are characterised by an equilibrium between the occurrence of high reactive oxygen species and their destruction by anti-oxidants, intermediated (Winnefeld *et al.* 1995) by glutathione peroxidase activity. The results of our studies concerning healthy mice supplemented with subtoxic inorganic selenium doses and selenosemicarbazide proved the selenium cumulation, particularly in liver and kidney, which is consistent with the literature data (Zawierta *et al.* 1997). The higher kidney selenium content following the selenosemicarbazide supplementation compared to inorganic selenium supplementation confirms the observations that the active absorption of the organic selenium compounds is easier. This particularly concerns selenomethionine which most readily builds into peptides and proteins (Zawierta *et al.* 1997).

Table 3. White blood count in Swiss mice after the supplementation with inorganic sodium selenic and organic 4(*o*-tolilo)-selenosemicarbazide of p-chlorobenzoic acid compared to the control group

Mouse group	Neutrophils number mm^{-3}	Bacillus number mm^{-3}	Monocytes number mm^{-3}	Lymphocytes number mm^{-3}
Controls without Se supplementation	1101±338	199±165	122±96	2014±1036
After sodium selenite supplementation	730±295*	79±77	48±38	1574±616
After selenosemicarbazide supplementation	582±234*	74±62	64±52	2173±931

*Statistical significance in comparison with the control group $p < 0.05$.

Table 4. The comparison of granulocyte phagocystic abilities and NBT in Swiss mice following the supplementation with inorganic sodium selenite and organic 4(*o*-tolilo)-selenosemicarbazide of p-chlorobenzoic acid against to the control group

Mouse group	Neutrophils number mm^3	Phagocytosis% of positive cells	NBT test% of positive cells
Controls without Se supplementation	1101±338	30.8±10.2	3.8±3.5
After sodium selenite supplementation	730±295*	16.7±7.3*	2.2±1.0
After selenosemicarbazide supplementation	582±234**	29.1±9.9**	0.9±0.5*,**

*Statistical significance in comparison with the control group $p < 0.05$.
**Statistical significance after selenosemicarbazide supplementation in comparison with sodium selenite supplementation.

Trace elements modulate immune responses through their critical role in the enzyme activity. Both deficiency and excess of trace elements have been recognised as influencing immunity (Chandra *et al.* 1986), resistance to bacterial and fungal infections (Boyne *et al.* 1986; Chan *et al.* 1998; Kovacs & Hruza 1995; Segal *et al.* 1987), virus infections. Recently, clinical applications concerning selenium show successful treatment of dermatosis (Segal *et al.* 1987) and intracellular parasites (Davis *et al.* 1998) after selenium supplementation. The dependence between selenium concentration, immunity defects and acute stage of infection has been found (Forceville *et al.* 1998). The selenium substitution significantly improves the clinical outcome of the inflammatory response (Hori *et al.* 1997). It became obvious because selenium deficiency was demonstrated to impair the ability of neutrophils to kill *C. albicans in vitro* test (Boyne & Arthur 1986). There are many reports on selenium deficiency and impaired leukocyte and lymphocyte responses in vitro although their relevance to a disease in vitro is unproven (Suttle & Jones 1989). After selenium supplementation both infection subsidence, decreased mortality (Forceville *et al.* 1998) and clinical improvement in patients with rheumatoid arthritis (Heinle *et al.* 1997) were observed. Several experimental studies suggest that severe selenium deficiency compromises T-cell dependent immune functions as blastogenic response to mitogens, decreases the antibody response, especially if associated with vitamin E deficiency (Dhur *et al.* 1990). Selenium has been linked to viral infections, enhanced T-cell functions and TNF-β induced increase in natural killer cell activity (Harbige 1996). The splenic natural killer cell activity is enhanced in selenium-supplemented healthy animals. Despite some controversies, most experimental studies on selenium-deficient animals report normal phagocytosis and altered bacterial capacity of neutrophils. The decrease in glutathione peroxidase activity of polymorphonuclear cells following selenium deficiency could explain some of these alterations (Dhur *et al.* 1990). Other reasons of different results could be the dose- dependant subtoxic doses which cause the down-regulatory activity (McCarty 1997) while modest doses of Se can improve the immune function which may increase the general re-

sistance to infections (Ilback *et al*. 1998). The subtoxic doses of Se used in our studies result in significantly decreased NBT test which is related to the antioxidative properties of Se. However, the relevant finding in favour of selenosemicarbazide concerns the results of phagocytosis since despite the comparable contents of Se given to mice supplemented with selenosemicarbazide, the phagocytosis disorders were not observed. Thus, it should be concluded that the selenosemicarbazide compound synthesised for the first time may be used analogously to the commonly applied selenium compounds.

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