Pharmacological study of nanoparticular selenium compounds – new opportunities for selenium supplementation

by Éva Ungvári

Supervisor: Ilona Benkő, MD, PhD

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF PHARMACEUTICAL SCIENCES

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Supervisor: Ilona Benkő, MD, PhD

Doctoral School of Pharmaceutical Sciences (Programme of Pharmacology)
University of Debrecen

Head of the Examination Committee: Árpád Tósaki, DP, PhD, DSc
Members of the Examination Committee: Emese Kiss, MD, PhD, DSc
Attila Kiss, MD, PhD

The Examination takes place at the Library of Department of Pharmacology, Faculty of Pharmacy, University of Debrecen; 7th May 2015. at 11:00

Head of the Defense Committee: Árpád Tósaki, DP, PhD, DSc
Reviewers: István Szegedi, MD, PhD
Attila Kónya, PhD
Members of the Defense Committee: Emese Kiss, MD, PhD, DSc
Attila Kiss, MD, PhD

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen; 7th May 2015. at 14:00
1. INTRODUCTION

In our modern era one of the most threatening jeopardy for human organism is the increased oxidative stress caused by chemicals and environmental pollution. Fumes of vehicles, industry and household heating, cigarette smoke, UV and ionizing radiation (γ- and X-rays) lead to formation of large amount of free radicals resulting increased oxidative stress. Excessive amount of reactive oxygen species (ROS) may damage DNA, proteins and membrane lipids, moreover, they can weaken the immune system. As a consequence they correlate with development of many diseases. ROS play a critical role in diseases leading mortality lists worldwide – like cardiovascular and malignant diseases – as well as in early aging with increased risk for degenerative disorders.

Endogenous antioxidant defense mechanisms limit and counterbalance the effects of oxidative stress by neutralizing ROS derived both from endogenous and exogenous sources. These endogenous mechanisms may be supported by exogenous sources of antioxidant materials. Healthy diet contains proper amount of such components but our diet has been moved away from the desirable one in recent decades. Consequently currently there can be an imbalance between protective and damaging mechanisms.

The first line of endogenous defense against free radicals consists of some selenium containing enzymes. Selenium is essential micronutrient mainly because of the antioxidant effects of these selenoproteins. Being a cofactor of antioxidant glutathione-peroxidase (GPx) enzymes selenium takes part in scavenging free radicals, thus protects cells, membranes and cell organelles from lipid peroxidation, enzymes and nucleic acids from harmful effects of ROS. Selenium as dietary supplement has relative narrow optimal dose range, the recommended dietary allowance (RDA) and tolerable upper intake level are close to each other, so it is easy to overdose. Selenium supplement via food is doubtful, because its selenium content depends on Se concentration of soil, and Hungarian soils are mostly selenium deficient.

The latest trend in selenium supplementation is the application of selenium nanoparticles (SeNP). According to many studies SeNPs are more effective than organic and inorganic Se compounds. Their therapeutic application is currently studied in oncology. The results on their toxicity are contradictory, therefore more research are needed.

The ways of production and use of SeNPs are untapped field for researchers.
2. PURPOSES

My aim was to produce a functional food and dietary supplement, which provide an opportunity to safer selenium supplementation.

I. Production of functional food

It is known that lamb meat contains high levels of micronutrients including selenium. Increasing the Se-content of lamb meat by per os NanoSe administration would result a functional food, which would be an effective way to sufficient amount of Se intake with no risk of overdose.

I searched answers for the following questions:
1. Is it possible to increase selenium level of lamb meat by per os NanoSe administration of lambs?
2. Are there any changes in selenium level after drying of meat?
3. Is there any effect of lamb meat and selenium-enriched lamb meat on antioxidant defense system of animals?
4. It is found that white blood cells and especially their dividing progenitors are sensitive to xenobiotics. Now I would like investigate whether DMBA cause cell apoptosis, and which is the most vulnerable cell population.
5. It is well-known, that DMBA treatment has myelotoxic effect through oxidative damages. The most vulnerable population in bone marrow are progenitors of granulocytes and macrophages (CFU-GM). Damage of these progenitors halts the renewal of phagocytes resulting neutropenia. I would like to know if DMBA have an effect on hematopoiesis especially on granulopoiesis.
6. Are lamb meat and Se-enriched lamb meat able to reduce DMBA-induced myelotoxicity?

II. Production of dietary supplement

In my experiments I wanted to study a nanoparticular selenium species produced by a new technology. My aims were to compare the new LactoMicroSel® (LMS) product to current organic, inorganic and NanoSe compounds on aspects of effect on antioxidant system and bone marrow function under physiological and hyperoxidative circumstances.

1. What are the toxicological characteristics of LMS? Does LMS have effect on survival, white blood cells and GM-CFU progenitors?
2. Are there any histopathological changes in liver after selenium administration at 50 ppm?
3. Are selenium species able to increase the GPx activity under physiological and hyperoxidative circumstances?
4. Are they able to moderate DMBA-induced myelotoxicity?
5. Which selenium species has the best toxicological characteristics?

3. MATERIALS AND METHODS

3.1. General aspects

Experiments of the present research were divided into two arms. First effects of lamb meat and Se-enriched lamb meat on antioxidant defense system were studied, then LMS was compared to current inorganic, organic and nano-size selenium compounds.

3.1.1. Animals

BDF1 mice (male, 20 weeks-old) were purchased from National Oncology Institute (Budapest, Hungary). They were housed in an animal room with 12-hour light and dark periods a day, temperature of 23±2 °C, relative humidity of 60±10% with 5 animals per pen. The animals can eat food and tap water ad libitum. The present experiments conform to the European Community's guiding principles for the care and use of laboratory animals. The experimental protocol has been approved by the Ethics Committee for Animal Research, University of Debrecen (4/2011 DE MAB).

3.1.2. Chemicals

DMBA. 7,12-dimethylbenz(a)anthracene) was purchased from Sigma Aldrich Ltd. (Budapest, Hungary) in powder form and it was dissolved in vegetable oil.

FRAP-reagent:

- acetate buffer: 300 mM, pH 3,6
- 2,4,6-tripyridyl-s-triazine: 10 mM (Sigma Aldrich Ltd., Budapest, Hungary)
- FeCl₂: 20mM dissolved in distilled water (Sigma Aldrich Ltd., Budapest, Hungary)

Ransel kit purchased from Randox Laboratories Ltd. (Crumlin, United Kingdom).
3.1.3. Production of nano-sized elemental selenium

NanoSe of 100 to 500 nm diameter was produced by József Prokisch et al. Briefly, the Se source and the inoculum (probiotic yogurt bacteria) were added to 1 L of MRS medium. As Se source, sodium hydroxidene (NaHSeO3) stock solution (10 g/L) was used. Then, 20 ml of selenite stock solution was added to 980 ml of MRS medium, for a final concentration of 0.2 g/L selenite. A 10-ml mixture of strains containing *Lactobacillus acidophilus*, *Streptococcus thermophilus*, and *Lactobacillus casei* served as an inoculum. After these additions to the medium, fermentation was carried out for 48 h in a rotary shaker at 37°C.

3.1.4. Sampling

Blood was collected from the retroorbital plexus of mice into tubes containing 2 µl aprotinin (Gordox, Richter Gedeon Rt., Budapest, Hungary) and 5 µl EDTA (Sigma-Aldrich, Hungary). One part of the blood was used for counting blood cells (CellDyn 3700, Abbott, Santa Clara, CA, U.S.A) and the other to determine total antioxidant capacity and GPx activity.

After the mice were exterminated, femurs were removed aseptically, then bone marrow was washed out with 1 ml McCoy’s 5A modified medium (GIBCO GRAND Island, NY, USA) by the help of a sterile needle.

3.1.5. Colony assay

Changes in bone marrow function were characterized by measuring total cellularity, frequency and total amount of granulocyte-macrophage colony forming units (CFU-GM). Granulocyte-macrophage colony forming units (CFU-GM), the common progenitors of granulocytes and macrophages are the main target of cytostatic drugs. Their damage prevents renewal of phagocytes with development of neutropenia in blood. CFU-GMs are not identifiable by morphological methods so I used a functional test, a special colony assay for their detection. Briefly 2x10^5 bone marrow cells were plated into petri dishes containing methylcellulose (Methocel, FLUKA, Buchs, Switzerland) in 1.2 % final concentration as support matrix for semisolid culture. Cultures were grown in triplicates for 7 days in a CO2 incubator. Following this the colonies were counted under a dissecting microscope (Olympus, Hamburg, Germany).
3.2. Effect of Se-enriched lamb meat on antioxidant defense system and bone marrow function in mice

3.2.1. Lamb meat

István Monori and colleagues of Research Institute of Karcag of the Center for Agricultural and Applied Economic Sciences of University of Debrecen produced lamb meat for animal model experiments. The selenium nanoparticles were mixed up with the lambs’ forage, and were transported into their muscles after digesting. Mice were fed in a mono diet system with lamb meat from either lambs on standard feeding or on nano-sized selenium-rich diet. It was created a dehydrating box in which the effect of sunshine can be simulated. Temperature was maintained at 38 °C inside the box by two thermostats to avoid damage structure of meat proteins. The process of the drying took 48 hours. Meat slices were turned four times at the 4th, 8th, 15th and 23th hour. Sizes of the meat slices were 4-5 x 20-30 x 100-400 millimeters. Content of moisture of the end-product was less than 7%. Each mouse was fed with this meat for 4 weeks practically in the same quality.

3.2.2. Measurement of selenium content of lamb meat

To estimate the total selenium content of the lamb meat Hydride Generation Atomic Fluorescence Spectrometry (HG-AFS) was used by József Prokisch et al. Wet digestion was used for samples, briefly it was added 5 ml of 65% HNO₃ to 1 g of sample, digested it at 60°C for 60 minutes, then at 120°C for 240 minutes, after adding 3 ml of 30% H₂O₂. The digested samples were diluted to 15 ml with 3M HCl and then filtered. The use of HCl for dilution was necessary for the hydride generation reaction.

3.2.3. Determination of total antioxidant capacity (TAC)

Ferric Reducing Ability of Plasma (FRAP-assay), as a measure of antioxidant power, was applied to estimate TAC (total antioxidant capacity). This assay uses antioxidants in a redox-linked colorimetric method and represents total antioxidant capacity of the blood. The collected blood was centrifuged at 10000 rpm for 2 minutes, then 300 µl FRAP reagent (ferric chloride and tripyridyl-5-triazine) was added to the 10 µl serum. Change in the color was read at 593 nm on a spectrophotometer (Perkin Elmer, Germany). As a standard ascorbic acid (100-1000 µM) was used and processed in the same way.

3.2.4. Study design

Sixty mice were randomly divided into 3 groups. The first 20 mice were fed with standard laboratory chow, the second group got dried lamb meat from animals on standard
feeding and the third group was fed with dried lamb meat from animals on selenium enriched diet. At the end of the feeding period, on the 28th day all the groups were assigned into further two subgroups. Oxidative stress was induced in 3x10 animals by a single intraperitoneal injection of DMBA in a dose of 200 mg/kg body weight, the remaining 3x10 mice in the other subgroups received only vehicle (vegetable oil). Following further feeding according to the previous pattern, blood and bone marrow samples were obtained 48 hours after the DMBA injection to determine changes in antioxidant capacity of the blood, bone marrow function, number and morphology of blood cells.

3.3. Comparative study of new nanoparticular selenium compound

3.3.1. Production of LactoMicroSel®

LactoMicroSel® was produced by József Prokisch et al. Solid sodium nitrite and for inoculation a mixture of the three yogurt strains *L. acidophilus*, *S. thermophilus*, and *L. casei* were used. Fermentation took place in a rotary shaker at 37°C for 48 h. At the end of the fermentation process, Se-rich pink- or red-colored yogurt was obtained. The yogurt was centrifuged for 5 min at 2,000 g to get rid of most of the water. After decantation, the solid phase was placed into a 50 to 60°C dryer for 16 h. Grinding was followed by mixing lactoselenium into the feed.

3.3.2. Determination of Se concentration of NanoSe and LactoMicroSel®

Se concentration was measured by József Prokisch et al. Flame emission atomic absorption spectrometer (Thermo ICE 3000) and atomic fluorescence spectrometer (PSA Thermo, Excalibur) was used to determine the final selenium concentration of ready nanoselenium samples. In the purified selenium samples 200-500 mg/L selenium concentration was measured, while in the liofilised LMS samples Se concentrations was 1000-3000 mg/kg.

3.3.3. Other selenium species

NanoSe and LMS was compared to current inorganic and organic selenium compounds that were the follows: inorganic selenite (Na₂SeO₄), inorganic selenite (Na₂SeO₃), organic Sel-Plex® containing selenomethionine.

3.3.4. Study design

Selenium species were administered by adding them at 5 or 50 ppm to the diet of BDF1 male mice weighing an average of 27 g. At the end of the feeding period, on the 14th day
oxidative stress was induced by a single intraperitoneal injection of DMBA in a dose of 200 mg/kg body weight in a half of the animals fed with Se-enriched food at 5 ppm.

3.3.4.1. Toxicological studies

Survival was studied after Se compounds administration at 50 ppm. In further animals blood and bone marrow samples was collected as described earlier. Liver and spleen were excised from dissected mice and rinsed in ice-cold saline. The weight of organs was determined. Liver tissue samples were stored at -30°C before sectioning.

3.3.4.1.1. Histological sections

Histological samples were made by Gábor Nagy et al. Liver samples were obtained from the left outer lobe of the organ. Protocols involved fixation, dehydration, embedding, slicing into 15-µm-thick sections, hematoxylin staining of sectioned preparations, and viewing under a microscope. Fixation of liver tissue samples took place under standard condition in 10% paraformaldehyde solution for 12 h. Fixation was followed by dehydration at increasing concentrations of alcohol from 50% ethanol solution through 60, 70, 80, 90, 96%, and absolute ethanol. Each dehydration step lasted for 30 min. After the final step in 100% ethanol, xylene was used to remove alcohol, and to prepare the samples for embedding. Following dehydration the samples were embedded in paraffin. This process lasted for 1 d and consisted of three separate phases: (1) xylene–paraffin mixture (1:1) for 6 h; (2) a first paraffin embedding for 6 h; and (3) a second embedding in paraffin for 12 h. The embedding took place in a 56°C thermostat. Final embedding in paraffin lasted for 1 d prior to sectioning. A sliding microtome (Reichert, Diversified Equipment) was used to cut tissue slices of 15 µm corresponding to the thickness of approximately three cell layers.

3.3.4.1.2. Hematoxylin–eosin staining

Paraffin was removed from tissue slices in two consecutive steps by keeping samples in xylene for 10 min each. For rehydration, the opposite gradient of ethanol concentrations was used (96, 90, 80, 70, 60, 50%) for 10 min and finally distilled water for 5 min. After rehydration, the samples were placed in a staining solution that contained alum–hematoxylin and methanol. The nuclear staining lasted for 5 min. The samples were treated with acidic-alcohol (1 ml HCl dissolved in 99 ml 70% ethanol) for 2 s. The samples were washed in tap water for 5 min in two consecutive steps, and then in running tap water for 45 min. After washing, samples were dehydrated in 50% ethanol, followed by 60 and 70% ethanol for 5 min each. Samples were background-stained with eosin-staining solution (containing 70%
ethanol). Final dehydration was in 80, 90, 96%, and absolute ethanol, each for 3 min. The alcohol was removed with xylene in two steps for 5 min each. Slides containing tissue samples were embedded in one drop of Entellan, covered with coverslips, and used for microscopy.

3.3.4.2. Effect of Se species at 5 ppm concentration

3.3.4.2.1. Determination of white blood cell count
White blood cell count was determined by Kornél Miszti-Blasius et al.

3.3.4.2.2. Effects on antioxidant system
Se increases the activity some antioxidant enzymes containing Se in their active centre. In this experiment activity of one of the best diagnostic marker for Se status, glutathione-peroxidase (GPx) was measured. Ransel kit (Randox Laboratories Ltd., Crumlin, United Kingdom) was used for enzyme activity measurement.

3.3.4.2.3. Determination of Se concentration of plasma
Plasma Se concentration was measured by Edina Baranyai et al. Hydride generation flame emission atomic absorption spectrometer (Agilent Technologies 240 FS AA) was used for determination of Se-concentration of plasma.

3.3.4.2.4. Effects on bone marrow function
To study the bone marrow function colony assay was used as described earlier.

3.5. Statistical analysis
Statistical analysis was performed with two-way analysis of variance and Student’s t test and one-way analysis of variance followed by Bonferroni’s post-test for multiple comparisons. For statistical analysis Graphpad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA) was used. Results were expressed as mean standard error of the mean. Differences were considered statistically significant at p <0.05. (* p < 0.05, **p < 0.01, ***p < 0.001)
4. RESULTS

4.1. Effect of Se-enriched lamb meat on antioxidant defense system and bone marrow function under physiological and oxidative circumstances

4.1.1. Effect of Se supplementation in vivo on Se concentration of lamb meat (measured by J. Prokisch et al)

Selenium concentration in fresh meat of lambs fed with elemental selenium nanoparticles was about three times higher than in selenium non-enriched lamb meat (0.86±0.09 vs. 0.27±0.04 mg/kg) and this ratio remained similar after drying as it was measured 1.28±0.12 mg/kg and 0.36±0.03 mg/kg of selenium concentrations in the meat samples from selenium supplemented and the control animals, respectively.

4.1.2. Total antioxidant capacity

It was found that lamb meat had beneficial effect on antioxidant capacity of blood. A statistically significant increase in total antioxidant capacity was seen in the group fed with selenium enriched lamb meat compared to both the control mice and to the group which was fed with selenium non-enriched lamb meat.

As expected, DMBA pretreatment decreased the total antioxidant capacity of plasma probably owing to capturing of DMBA-induced free radicals. Similarly to the observations in normal mice, both selenium non-enriched and enriched lamb meat diet was protective against the total antioxidant capacity decreasing effect of DMBA pretreatment, the latter providing an even higher protection. Additionally, this protective effect prevented the decrease of total antioxidant capacity below the level observed in control mice.

4.1.3. White blood cell counts in peripheral blood

Total white blood cell (WBC) count was in normal range in groups not exposed to DMBA without significant differences but it was decreased in each group after DMBA pretreatment in a great extent. However, this decrease was the least in mice fed with selenium enriched lamb meat and the total WBC count in this group was significantly greater than in both the control and selenium non-enriched groups.

Beneficial effects of lamb meat from selenium supplemented animals on WBC are originated from the effects on granulocytes and monocytes, as there were no differences in lymphocyte counts among the groups receiving DMBA. Contrarily a great preservation of phagocytes was detected in the group which was fed with selenium enriched lamb meat.
Compared to mice fed with standard laboratory rodent diet and pre-treated with DMBA, both absolute neutrophil cell counts (ANC) and absolute monocyte cell counts were significantly higher. The protective effect was the highest for neutrophils and ANC remained in the normal range even after DMBA injections.

4.1.4. Effect of Se-enriched lamb meat diet in normal and chemical oxidative stress exposed mice on bone marrow

Similarly to the above described results, even in my preliminary experiments I observed the greatest protection with respect to circulating phagocytes which are produced by granulocyte-macrophage progenitors (CFU-GM) in bone marrow. Thus I studied the bone marrow function of mice by evaluating cellularity (i.e. the total number of nucleated cells), frequency of CFU-GM colonies and total CFU-GM content of femoral bone marrow. As all blood cells are originated and renew from bone marrow, hemopoiesis in general can be characterized by cellularity, the total cell number of the femoral bone marrow. Frequency of CFU-GM, shown by colony numbers grown from $10^5$ mononuclear bone marrow cells, indicates intensity of regeneration of granulopoiesis and the total CFU-GM content of femoral bone marrow mirrors the total regenerative capacity.

**Bone marrow cellularity.** Under normal circumstances there were no differences among any groups in the number of mononuclear cells of bone marrow. However, DMBA treatment resulted in a large decrease but the extent of this decrease was different in each diet groups. The highest decrease was observed in the control group, a lower decrease in the selenium non-enriched lamb meat group and the lowest decrease in the selenium enriched lamb meat group. According to these data, selenium supplementation through food web, using nano-selenium diet for meat-producing animals and not directly to the studied mice damaged with DMBA, could moderate the toxicity of DMBA on hemopoiesis in general.

**Frequency of CFU-GM colonies.** As mentioned previously the frequency of CFU-GM colonies provides a more detailed view about the regeneration intensity of granulopoiesis. In this aspect there were differences between the different diet groups even under normal circumstances and more intensive granulopoiesis was detected in mice fed with lamb meat but the difference was significant only in mice fed with lamb meat from selenium supplemented animals. DMBA treatment resulted in a significant decrease in CFU-GM frequency in each diet groups, showing the serious damage to granulopoiesis. However, lamb meat diet
provided some protection as the frequency of CFU-GM colonies were significantly higher in both the selenium non-enriched and selenium enriched groups than in the control group.

*Total CFU-GM content of femoral bone marrow.* The more intensive granulopoiesis resulted in the expansion of CFU-GM population even under normal circumstances in mice fed with selenium enriched lamb meat. DMBA treatment resulted in similar significant damage in the total granulopoietic regenerative capacity in any groups but the protective effect of lamb meat diet was observed with respect to this parameter too in mice fed with lamb meat, and this was again greater in mice fed with selenium enriched lamb meat.

### 4.2 Comparative study of new nanoparticular selenium compounds

#### 4.2.1. Toxicological studies (50 ppm)

*Survival.* The survival rate at high toxic (50 ppm) Se concentration shows that after 14 d of selenate consumption, two-thirds of the animals died. Selenite was less toxic and caused the death of one-third of the mice followed by nanoSe, with 17% death. All animals survived Sel-Plex and LMS treatment.

*Liver toxicity.* Liver toxicity is expressed by the percentage of liver/body weight. The relatively low, but detectable and variable reduction in the liver/body weight index can be accounted for by liver toxicity.

*Spleen toxicity.* Spleen toxicity was determined by a decreased spleen/body-weight ratio, with selenate causing a 44% decrease, selenite causing a 59% decrease, Sel-Plex causing a 57% decrease, NanoSe causing a 64% decrease, and LMS causing a 19% decrease.

*White blood cell number.* The number of white blood cells was drastically reduced at higher Se concentrations as follows: selenate 55%, selenite 32%, Sel-Plex 86%, NanoSe 77%, and LMS 70%.

*Liver histopathology (determined by Gábor Nagy).* The control liver tissue exhibited no disruptions. After mice were kept on a subacute selenate diet (50 ppm), large disruptions dominated the liver tissue stained with hematoxylin–eosin. Many small disruptions were caused by selenite. Sporadically distributed liver cell disruptions were seen after Sel-Plex treatment, NanoSe generated a few wide disruptions, and LMS induced formation of very small round holes only rarely.
Bone marrow cell number. The highest reductions (88–90%) in bone marrow cell numbers were caused by the inorganic selenate and selenite; other reductions were nearly 50% loss by Sel-Plex, 55% by NanoSe, and only 20% by LMS treatment.

Total GM-CFUs in the femur. The GM-CFUs were reduced by 20% after Sel-Plex treatment and increased by 27% after LMS administration.

Body weight. Relative to the average body weight of the murine strain (27 g), body-weight losses were as follows: selenate 8 g (30%), selenite 9 g (33%), Sel-Plex 8.5 g (31%), nanoSe 7.5 g (28%), and LMS 1.2 g (4%).

4.2.2. Effects of Se-enriched diet at tolerable dose (5 ppm)

4.2.2.1. White blood cell count (measured by K. Miszti-Blasius et al)

WBC count was in normal range in groups not exposed to DMBA but it was decreased in each group after DMBA-treatment in a great extent. The most vulnerable WBC population against DMBA-induced damages was lymphocyte’s.

4.2.2.2. Determination of Se-concentration of plasma (measured by E. Baranyai et al)

All the selenium species has good absorption characteristics, but the highest Se-concentration was observed in Sel-Plex group. In the other groups significant differences was not seen. DMBA-treatment did not affect absorption.

4.2.2.3. Effects of Se-enriched diet on GPx activity

GPx enzymes are the main component of antioxidant defense. They are diagnostic marker for determination of actual Se status. Presence of Se is essential for their function. Se supplementation increase the activity of GPx. In my experiments all the Se compounds increased significantly the activity of GPx both in normal and oxidative stress exposed mice.

4.3.4. Effects of Se-enriched diet on bone marrow function

Bone marrow cellularity. There were no significant differences between Se-treated and control animals. Selenium did not affect number of mononuclear bone marrow cells. After DMBA injection bone marrow cellularity dramatically decreased. The decay of cells caused by ROS was at the same rate in all group. Protective effect was observed in NanoSe and LMS group.

Frequency of CFU-GM colonies. Frequency of CFU-GM colonies shows more detailed view of intensity of granulopoiesis. Under normal circumstances selenium compounds did not
affect the common progenitors of granulocytes and macrophages, numbers of CFU-GM colonies grown from $2 \times 10^5$ bone marrow cells were similar in all groups.

In oxidative stress exposed mice it was found that DMBA decreased the number of CFU-GM colonies. In the LMS group significantly higher colony numbers were detected, which can be interpreted that LMS meant more effective protection against ROS than other selenium compounds.

*Total CFU-GM content of femoral bone marrow.* Under normal circumstances there were no changes compared to control group. DMBA treatment resulted significant damage in total granulopoietic regenerative capacity in groups but protective effect of LMS-enriched diet was observed ($p<0.05$).

5. DISCUSSION

5.1. *Modeling environmental pollution-induced oxidative stress*

DMBA, a representative of PAH compounds, produces enormous amount of free radicals which consume physiological antioxidant mechanisms and result in genotoxicity with increased risk for malignant diseases. Toxicity of PAHs is typically the result of their metabolism to reactive diol epoxides, which bound to DNA-chain covalently and cause mutations, and quinone compounds, which can enter the redox cycle and generate huge amount of ROS. These metabolits qualify DMBA for modeling both malignant diseases and oxidative stress.

5.2. *Effects of Se-enriched lamb meat*

Endogenous antioxidant defense mechanisms limit and counterbalance the effects of oxidative stress by neutralizing ROS derived both from endogenous and exogenous sources. A part of the antioxidants synthesize in our organism, while the other part is needed to take in trough food like vitamin E, C, β-carotene. Healthy diet contains proper amount of such components but our diet has been moved away from the desirable one in recent decades. Selenium containing enzymes comprise a significant part of the endogenous antioxidant system and it was shown that administration of exogenous selenium may incorporate into selenium-containing antioxidant enzymes and increases their activity. Based on these data in my current experiments I studied the effect of diets containing different amount of selenium on these primary defense mechanisms and whether beneficial effects of selenium appear if we use it non-directly but through the food chain.
These effects were diminished in mice on lamb meat diet and the protective effects were significantly higher in mice fed with lamb meat from selenium supplemented animals.

Macrophages produce huge amount of free radicals and use them to kill microorganisms. In addition they often work in a high ROS environment under inflammatory circumstances. Many antioxidant mechanisms help them to become relatively resistant. DMBA reduced the number of each white blood cell. This should be, at least in part, direct toxicity, because 48 hours after DMBA exposition only a small fraction of circulating blood cells are eliminated physiologically. Monocytes were the most sensitive, their number decreased by 90%. The remarkable sensitivity of monocytes based on the lack of expression of many DNA repair proteins causing severe DNA repair defect against oxidative stress. It was observed a greater protection in the case of neutrophils than in monocytes using selenium enriched diet which may be explained by the lower expression of antioxidant systems in monocytes than in neutrophils.

CYP1B1 enzyme, also found in bone marrow, is the major contributor to metabolism of DMBA to reactive dihydrodiol epoxides and quinones. In addition, the selenoprotein containing thioredoxin reductase/thioredoxin system was described in bone marrow cells of the monocyte/macrophage pathway of differentiation as well as peripheral blood monocytes and neutrophils. It was also shown that selenium supplementation is able to enhance antioxidant capacity through thioredoxin reductase and superoxide dysmutase selenoproteins in many types of bone marrow cells. These data are compatible with the view that selenium intake could enhance not only total antioxidant capacity of plasma but it may also replenished antioxidant capacity of bone marrow cells. This might be indicated by my findings that the population of CFU-GM progenitors, responsible to renew circulating phagocyte pool, is expanded even under normal circumstances in mice fed with lamb meat in good correlation with its selenium content.

5.3. LactoMicroSel®

In my second part of experiments a new selenium products were studied, syntethized in a fermentation procedure, by the help of probiotic yoghurt bacteria. While in SeNPs selenium spheres were separated from the bacteria, the new LMS product contained not only elemental selenium but also bacteria and organic microenvironment. My aim was to compare the new LMS product to current organic, inorganic and NanoSe compounds on toxicological and physiological aspects.
5.4. Toxicity of Se compounds

High Se concentrations (50 ppm, 400mg/kg body wt/d) caused multiple toxic effects in mice, manifested as moderate liver toxicity, and reduced weight of the spleen, decreased body weight, and decreased bone marrow and white blood cell numbers. The high Se dose allows us to make a clear distinction among the toxicity of Se species. Based on the organ toxicity, more organs were affected by selenite, but the lower survival rate of mice suggests that selenate is generally more toxic than selenite. In spite of these discrepancies, it was concluded that the order of toxicity of Se species is as follows: selenate > selenite > NanoSe > Sel-Plex > LMS.

Under selenosis white blood cell count drastically decreased. The most vulnerable cell type were lymphocytes and monocytes. Moderate cell death was detected in group fed with LMS.

The histopathology of livers on exposure to high selenate concentration revealed large disruptions in the texture of the liver. Selenite caused many small intercellular disruptions. As far as the histology of other Se species is concerned, the effect of nanoSe is closer to that of selenite, with many smaller discontinuities, and the effect of Sel-Plex resembling more that of selenate, with somewhat larger disruptions. The structure of liver tissue was hardly affected by LMS. The microscopy of Se toxicity confirms the toxicity order: selenate > selenite > NanoSe > Sel-Plex > LMS.

Se at high concentration correlated negatively to bone marrow function as well. Count of mononuclear bone marrow cells decrease in all groups except that fed with LMS. Due to high rate of cell death in inorganic selenium-treated animals colony assay was unsuccessful, none of CFU-GM colony were detected. Contrarily Sel-Plex and LMS did not cause damages in progenitor cells.

To summarize the toxicological results, inorganic Se compounds appeared to be the most toxic. NanoSe was more harmful than organic Sel-Plex. The most favorable compound was LMS.

5.5. Effects of Se-enriched diet at tolerable dose (5 ppm)

Increasing the activity of enzymes containing Se in their active centre Se support the antioxidant defense system. In my experiment Se increased the GPx activity both in normal and oxidative stress exposed mice. It was found that cause of increased GPx expression is oxidative stress, and condition is presence of Se.
Se did not affect bone marrow parameters like cellularity, frequency of CFU-GM colonies, total CFU-GM of femur under physiological circumstances, but in oxidative stress exposed mice LMS had significant protective effect to DMBA-induced damages. This protective effect was not observed on mature white blood cells. Lymphocytes were the most sensitive cell type. In my study NanoSe was less effective than organic selenium. According to other authors effect and toxicity of nano-sized selenium depends on size of spheres. The smaller spheres is supposed to be more bioactive and less toxic.

Although high plasma Se concentration was not measured in LMS group, LMS proved to be more effective in many parameter than the other Se compounds. My hypothesis is that absorption and distribution of LMS is fast and very good but pharmacokinetic studies are needed to verify this.

6. NEW FINDINGS

Geographical study using great number of soil samples showed that Hungarian soils are mostly Se-deficient, therefore food originated from animals and plant grown in Hungary are not a sufficient source of selenium. Dosage of selenium as dietary supplement requires careful planning, because optimal dose range of Se is really narrow, so there is a risk to overdose.

To summarize the results, it was produced a new functional food and dietary supplement, which effectively support the antioxidant defense system against free radicals, with lower risk of selenium overdose. The selenium-enriched lamb meat and LactoMicroSel® with wider optimum dose range means new opportunity for safer selenium supplementation.
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List of publications related to the dissertation

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Subacute toxicity of nano-selenium compared to other selenium species in mice.
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