

*Research article***EFFECTS OF SELENIUM AND THYROID HORMONE DEFICIENCY ON PERITONEAL MACROPHAGES ADHESION AND OCCURRENCE OF NATURAL IgM ANTIBODIES IN JUVENILE RATS**

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Both selenium, as an effector and regulator of antioxidative enzymes activity, and thyroid hormones are potent immunomodulators. Besides, selenium incorporated into iodothyronine deiodinases is involved in the thyroid function and thus indirectly regulates the immune response. Studies of the mutual influence of selenium and thyroid hormones on the immune response are scarce, hence we analyzed the effects of an iodothyronine deiodinases blocker, propylthiouracil (PTU), and selenium deficiency on the function of peritoneal macrophages, and titer of naturally occurring anti-sheep red blood cells (SRBC) IgM antibodies in juvenile rats.

The experiment was carried out on 64 Wistar male rats allotted to 4 groups: control-selenium adequate PTU- group; selenium adequate, PTU+ group; selenium deficient, PTU- group; and selenium deficient, PTU+. The selenium adequate and selenium deficient groups were fed a diet containing 0.334 and 0.031 mg Se/kg, respectively. PTU+ groups received PTU (150 mg/L) in drinking water. After 3 weeks, thyroxine (T₄), triiodothyronine (T₃), and thyroid stimulating hormone (TSH) were determined. The animals having “intermediate” concentrations of T₃ (1.56–1.69 nmol/L) and T₄ (41–50 nmol/L) were excluded from further analysis. Thus, PTU+ groups included hypothyroid animals (T₃ ≤ 1.55 nmol/L; T₄ ≤ 40 nmol/L), while PTU- groups included euthyroid rats (T₃ ≥ 1.70 nmol/L; T₄ ≥ 50 nmol/L). Both groups of selenium deficient rats had, when compared to the control group, a significantly lower activity of glutathione peroxidase GPx1 and GPx3. Neither selenium deficiency nor PTU influenced the adherence of peritoneal macrophages. Selenium deficiency significantly decreased the peroxide synthesis in macrophages and significantly increased the titer of anti-SRBC IgM. Hypothyroidism alone or in combination with selenium deficiency had no influence on these parameters.

Key words: glutathione peroxidase, IgM, macrophage adherence, macrophage peroxide production, rats, selenium, thyroid hormones

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INTRODUCTION

Selenium is an essential microelement which achieves its biological effects in the form of selenocysteine incorporated into selenoproteins. Selenium is an important member of the antioxidative systems and improves the parameters of oxidative and general stress. At the same time it is crucial for optimal functioning of the endocrine and immune systems [1,2].

Selenium status is of great significance for the function of the immune system. Data showed that selenium deficiency is accompanied with an inefficient immune response to various antigens (viruses, tumors, allergens) [3,4], and development of autoimmune diseases [5,6]. Most of its immunomodulatory effects have been explained by its role in antioxidant protection, i.e. in controlling the activities of reactive oxygen species (ROS)-mediated glutathione peroxidases (Gpx) and thioredoxin reductase. Selenium deficiency results in an inadequate activity of reactive oxygen species (ROS), controlling selenoproteins, and oxidative stress mediated cells damage or, in the case of selenium excess, reduced microbicidal effect of neutrophils, monocytes/macrophages and NK cells [2,7-10]. Selenium influences cells of adaptive immunity and its deficiency is accompanied by a reduced number of T lymphocytes [11] and their decreased proliferation and differentiation [12-14]. Selenium deficiency also reduces the number of B lymphocytes but its effect on antibody synthesis is antigen dependent [10].

Thyroid hormones are unambiguously confirmed as powerful regulators of the immune response, able to modulate the activity of both innate and adaptive immune systems [15]. After binding to specific receptors expressed on immune cells of hematopoietic origin and/or on non-hematopoietic immunoregulatory cells, thyroid hormones modulate cell signaling, which results in the modulated expression of various membrane antigens, secretion of cytokines and other pro- and anti-inflammatory mediators [15]. Hypothyroidism is accompanied with an increased proinflammatory activity (increased phagocytosis and increased ROS generation) of macrophages and neutrophils, depressed activity of NK cells, increased maturation of antigen presenting dendritic cells, and reduced number and lower effector function of T lymphocytes [15-18]. Data on the effect of hypothyroidism on humoral immunity are often contradictory and depend on the implemented experimental protocols and analyzed antigens [15].

Selenium is an integral component of all three iodothyronine deiodinases. Iodothyronine deiodinases are enzymes involved in the process of activation and inactivation of thyroid hormones [4,19,20]. By regulating the activity of thyroid hormones selenium indirectly regulates the immune response. The discovery of this function of selenium has pointed to the need for re-evaluation of previous data and re-interpretation of the mechanisms of immune regulation mediated by selenium and thyroid hormones. We performed this study in order to explore the mechanisms of interaction between these two immune regulatory systems and to define their individual and cross effects on the components of the immune system involved in the recognition and

elimination of previously encountered antigens: macrophages and naturally occurring IgM antibodies. Macrophages are potent and long-lived phagocytes which within the immune system function as sentinel cells and antimicrobial effector cells, providing the second line of innate immune defense. Besides, they regulate adaptive immunity by acting as antigen presenting cells and secreting numerous pro- and anti-inflammatory mediators [21-23]. Naturally occurring IgM antibodies are mainly secreted by self-renewing B-1 lymphocytes, without requirement for exogenous antigenic stimulation. Thanks to their low specificity they can recognize a wide range of evolutionary conserved polysaccharides and lipids of microbial antigens and some autoantigens, and provide the first line of defense against invading pathogens. Naturally occurring IgM antibodies directly neutralize or inhibit pathogens, aid the synthesis of immune IgM and IgG antibodies, and together with them protect the organism from bacterial and viral infections. Moreover, naturally occurring IgM suppress inflammatory responses initiated by mechanisms of innate and adaptive immunity (both T and B cell dependent) and protect from uncontrolled inflammation and development of inflammatory and autoimmune diseases [24-26]. However, when precise control of immunological reactivity is lost, as in selenium deficiency and hypothyroidism, both macrophages and naturally occurring IgM can be factors contributing to the development of long-term uncontrolled inflammation and autoimmune diseases [21,24-26]. Therefore, we performed this study, using a juvenile rat model, with the aim to analyze if individual and combined effects of nutritional selenium deficiency and chemically PTU- induced hypothyroidism modulate the function of peritoneal macrophages (adherence and peroxide production) and synthesis of naturally occurring IgM antibodies reactive to membrane antigens of sheep red blood cells (SRBC).

MATERIAL AND METHODS

Animals and experimental design

The experiment was designed as a group-control system on 64 male Wistar rats, 21 days old, weighing 48.6 ± 7.8 g, Charles-River origin (Hungary). The use of animals was approved by the Ethical Committee of the Faculty of Veterinary Medicine, University of Belgrade in accordance with the National Regulation on Animal Welfare.

Rats were supplemented either with selenium or propylthiouracil (PTU). Selenium adequate groups were fed a specially formulated feed containing 0.334 mg Se/kg (C-1000, Altromin, Germany), and selenium deficient groups were fed a diet containing 0.031 mg Se/kg (C-1045, Altromin, Germany). PTU+ groups received PTU (Sigma) in drinking water at a concentration of 150mg/L, while PTU- groups were given pure drinking water. Four different groups were formed: (1) control group - selenium adequate, PTU- group (Se_{adeq} PTU-); (2) selenium adequate, PTU+ group (Se_{adeq} PTU+); (3) selenium deficient, PTU- group (Se_{def} PTU-); (4) selenium deficient, PTU+ group (Se_{def} PTU+).

After a period of three weeks the rats were anesthetized with ether. Approximately 5 mL of blood was sampled by cardiac puncture into glass tubes containing heparin (15 IU/mL). Selenium concentration and activity of glutathione peroxidase (GPx1) were determined in the whole blood samples. The blood plasma was obtained by centrifugation for 20 min at 1000 x g. Plasma samples were stored at -20°C for the determination of concentration of triiodothyronine (T₃), thyroxin (T₄), and thyroid - stimulating hormone (TSH), glutathione peroxidase 3 activity (GPx3), and the concentration of anti SRBC IgG and IgM antibodies.

Concentration of T₃, T₄, and TSH

Concentrations of free and bound T₃ and T₄ were measured using commercial RIA kits (INEP, Zemun, Serbia). The concentration of TSH was measured using RIA kits produced by MP Biomedicals, Belgium. The radioactivity of the sediment was measured with a gamma scintillation counter CompuGamma LKB (Belgium).

Concentration of selenium, GPx1 and GPx3

Whole blood selenium concentration was measured using the hydride technique, on the atomic absorption spectrophotometer (Thermo electron Solar AA, Series 4) with a hydride module and electrical heating of quartz cuvettes in an EC 90 furnace. Quality control was carried out with the aid of the referent material BCR 185 (IRMM, Belgium). The obtained values in replicate were within the range of the certified values.

Glutathione peroxidases activities were measured in whole blood and plasma samples using a coupled test as described by Gunzler *et al.* [27], modified by Sankari [28]. All chemicals were obtained from Sigma Aldrich. Blood samples were hemolysed using Drabkin's reagent (1.6 mM KCN, 1.2 mM K₂Fe(CN)₆ and 0.023 M NaHCO₃). The final concentrations of the used reagents were: 100 mM phosphate buffer (pH 7.4), 4 mM ethylenediaminetetraacetic acid (EDTA), 6 mM glutathione (GSH), 0.375 IU/mL glutathione reductase (GR), 0.3 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH) and 1.575 mM tertiary butyl hydroperoxide (TBH). The low concentration of TBH ensures that only the activity of selenium-dependent GPx enzymes is registred. The decrease in NADPH concentration was measured for 3 min at 366 nm using a Cecil Ce2021 spectrophotometer with a Peltier thermostat unit. The activity of GPx1 and GPx3 was expressed in $\mu\text{kat/L}$.

Assays for peritoneal macrophages adherence and peroxide production

Peritoneal macrophages were induced by intraperitoneal application of 8 mL of filtered 2.4% thioglycollate medium seven days prior to sacrifice. After sacrifice the abdominal cavity was lavaged with 15 mL sterile PBS and then centrifuged at 500 x g for 10 min. The cell pellet was diluted in 2 mL PBS. Cell concentration was determined using manual hemocytometer and then diluted with PBS at a concentration of $2.5 \times 10^6/\text{mL}$.

Adhesion of peritoneal macrophages to plastic (polystyrene) was assessed with an *in vitro* spectrophotometric assay performed in 96 flat bottom microtiter plates [29]. The assay was carried out in such a way that 50 μ L of buffer was added per well, and then 50 μ L of the macrophages suspension was placed in each well. The final concentration of macrophages was 1.25×10^6 /L. The cells were incubated at 37 °C for 10, 30, and 60 minutes, rinsed with warm (37 °C) PBS, fixed with 100 μ L methanol for 6 minutes, and stained by adding 100 μ L 0.1% filtered methylene blue. After 10 minutes the plates were rinsed with water until discolored and dried overnight at 37 °C. After drying the dye was dissolved with 0.1 M HCl and the plates were incubated at 37 °C in a humid chamber for 4h. The intensity of the developed color was measured on a microplate reader (GDV, Microplate Reader DV 990 BV 4/6, Italy) at 620 nm.

The capacity of induced peritoneal macrophages to generate peroxide production was tested *in vitro* where the production of H_2O_2 was stimulated with phorbol miristate acetate (PMA). The method is based on the peroxidase-dependent transformation of phenol-red in a compound with an enhanced absorbance at 600-610 nm [30]. In brief: 100 μ L of the prepared peritoneal macrophages suspension was added to each well. After 2h incubation at 37°C the non-adherent cells were removed by double washing with Minimum Essential Medium. The peroxide production was stimulated with 100 μ L 25 nM PMA in phenol-red. Immediately after plating the cells, H_2O_2 production was stopped by adding 10 μ L NaOH. The plates were incubated for 1 h at 37 °C and the reaction was stopped with 10 μ L NaOH. Optical density (OD) was measured at 620 and 690 nm. The standard curve for the determination of the production of H_2O_2 was constructed according to standard H_2O_2 solutions in the concentration range of 1-40 μ M.

Determination of titer of anti-sheep red blood cells (SRBC) IgM and IgG antibodies in rat plasma

Titers of anti-SRBC IgM and IgG antibodies in rat plasma were determined with commercial ELISA kits: Rat Anti-SRBC IgM ELISA Kit (Life Diagnostics, Inc. West Chester, PA, USA) and Rat Anti-SRBC IgG Kit (Life Diagnostics, Inc. West Chester, PA, USA). ELISA plates were coated with SRBC membranes (antigens). ELISA was performed according to the manufacturer's instructions. In brief: diluted rat plasma (1:50) and serially diluted standard rat sera containing anti-SRBC IgM and IgG antibodies (3-50 U/mL 50 anti-SRBC IgM and 6-50 U/mL 50 anti-SRBC IgG) were incubated with coated antigen. After 45 min incubation and removal of unbound proteins the plates were incubated with secondary anti-rat IgM or anti-rat IgM conjugated with horseradish peroxidase. The reaction of SRBC antigens and specific antibodies was visualized using a peroxidase substrate 3,3',5,5'-tetramethylbenzidine (TMB). The reaction products were measured on the multi-plate reader at OD 450 nm and the results were given as U/mL of specific anti-SRBC IgM and anti-SRBC IgG. The results were calculated based on the standard curve constructed by plotting a series of IgM and IgG standard sera dilutions.

Statistical analysis

Statistical analysis was carried out using MS Excel 2007 and Graph Pad Prism 5 statistical software packages. The data were tested for homogeneity and normality. As average values, arithmetic means were determined for homogeneous data and medians for heterogeneous values in a group. Therefore, we have presented both arithmetic means \pm SD and medians. The significance of the differences between average values/medians was evaluated by using Student's t-test for homogenous and Mann Whitney U test for heterogeneous data. Results were deemed as statistically significant when the p value was <0.05 . Correlations between selected parameters were tested by linear regression analysis.

RESULTS

As presented in the Material and Method section, hypothyreosis was induced with PTU. Animals not treated with PTU were considered as euthyroid. Nevertheless, all the animals having "intermediate" concentrations of T_3 (range 1.56 – 1.69 nmol/l) and T_4 (the range 41-50 nmol/L) were excluded from further analysis, after blood analyses on thyroid status. Thus, PTU+ groups included only hypothyroid animals ($T_3 \leq 1.55$ nmol/L and $T_4 \leq 40$ nmol/L), while PTU- groups included euthyroid rats ($T_3 \geq 1.70$ nmol/L and $T_4 \geq 50$ nmol/L). The data showed, as expected, that concentrations of T_4 and T_3 were significantly lower in groups treated with PTU. Also, in these groups, the level of TSH was significantly higher compared to PTU non treated groups (Table 1).

Table 1. Influence of selenium deficiency and PTU on blood selenium concentration and activity of erythrocyte cytosolic glutathione peroxidase (GPx1) and blood plasma glutathione peroxidase (GPx3)

| | Selenium adequate | | Selenium deficient | |
|----------------------------|-------------------------|--------------------------------------|--------------------------------------|--------------------------------------|
| | Euthyroid (n=14) | Hypothyroid (n=10) | Euthyroid (n=15) | Hypothyroid (n=7) |
| Se ($\mu\text{g/L}$) | 360 \pm 113 460 | 369 \pm 129 291 | 61 \pm 11 ^c 63 | 71 \pm 20 ^c 71 |
| GPx1 ($\mu\text{kat/L}$) | 166 \pm 43 170 | 159 \pm 43 177 | 59 \pm 19 ^c 58 | 109 \pm 36 ^{a, #} 103 |
| GPx3 ($\mu\text{kat/L}$) | 55.3 \pm 17.8 63.3 | 83.2 \pm 25.0 ^a 83.2 | 37.4 \pm 19.8 ^b 30.7 | 46.1 \pm 11.3 ^a 41.1 |

The summary data are presented as the mean \pm SD and *median*

(^a) – $p < 0.05$; (^b) – $p < 0.01$; (^c) – $p < 0.001$ compared to control selenium adequate, euthyroid group; ([#]) – $p < 0.01$; compared to selenium deficient, euthyroid group.

For the assessment of selenium status of experimental rats, whole blood selenium concentration and erythrocyte glutathione peroxidase (GPx1), as well as plasma glutathione peroxidase (GPx3) activities were measured (Table 2). There were no statistically significant differences in whole blood selenium concentrations between selenium adequate groups. Selenium deficient rats had significantly lower selenium

concentrations than selenium adequate rats. There was no significant difference in Se concentration between PTU- and PTU+ rats. Likewise, GPx1 and GPx3 activities were significantly higher in selenium adequate than Se deficient rats. Although both groups of selenium deficient rats had, comparing to the control group, a lower activity of GPx1, the activity was higher in PTU treated, selenium deficient rats. Besides, GPx3 activity in selenium adequate, PTU+ rats was higher than in the control group.

Table 2. Influence of selenium deficiency and PTU on the concentration of thyroxin (T4), triiodothyronine (T3), and thyroid stimulating hormone (TSH).

| | Selenium adequate | | Selenium deficient | |
|-------------|---------------------|----------------------------------|---------------------|----------------------------------|
| | Euthyroid (n=14) | Hypothyroid (n=10) | Euthyroid (n=15) | Hypothyroid (n=8) |
| T4 (nmol/L) | 90.7 ± 22.0 88.7 | 22.7 ± 11.3 ^c 25.4 | 90.4 ± 14.1 91.9 | 20.0 ± 12.1 ^c 14.9 |
| T3 (nmol/L) | 2.27 ± 0.26 2.27 | 0.82 ± 0.44 ^c 0.70 | 2.14±0.26 2.09 | 0.86± 0.42 ^c 0.88 |
| TSH (ng/L) | 4.1±2.7 4.2 | 82.8±10.3 ^c 84.0 | 5.6±2.9 5.1 | 86.3±9.4 ^c 90.0 |

The summary data are presented as the mean ± SD and *median*. (c) – p < 0.001 compared to control selenium adequate, euthyroid group.

The spontaneous (non-stimulated) adherence of peritoneal macrophages in the control group was equal at all estimated times. The recorded OD₆₂₀ values were 0.043±0.004, 0.047±0.006, and 0.051±0.012, for 10, 30 and 60 min. incubation. There were no statistically significant differences in the adherence of peritoneal macrophages between different groups after 10, 30 (*data not shown*) and 60 min incubation (Table 3). Macrophages peroxide production was significantly higher in selenium adequate rats, but there was no significant difference between PTU- and PTU+ animals (Table 3). A significant correlation between the macrophages adherence and selenium concentration was not found (R=0.194; p>0.10; n=27) (Figure 1A), but the level of peroxide synthesis in peritoneal macrophages significantly correlated with selenium concentration (R=0.44; p<0.02; n=29) (Figure 1B).

Table 3. Influence of selenium deficiency and PTU on 60 min peritoneal macrophage adherence and peroxide production *in vitro*.

| | Selenium adequate | | Selenium deficient | |
|-------------------|------------------------|------------------------|-----------------------------------|-------------------------------|
| | Euthyroid (n=14) | Hypothyroid (n=10) | Euthyroid (n=15) | Hypothyroid (n=8) |
| Adherence (OD620) | 0.051 ± 0.010 0.046 | 0.051 ± 0.003 0.052 | 0.070±0.030 [‡] 0.046 | 0.060±0.015 0.052 |
| Peroxide (µM) | 4.1 ± 2.9 3.3 | 4.7 ± 4.1 3.4 | 1.2 ± 0.8 1.0 ^b | 1.2 ± 0.9 0.6 ^b |

The summary data are presented as the mean ± SD and *median*. (b) – p < 0.01 compared to control selenium adequate, euthyroid group; (‡) – Difference between euthyroid, selenium adequate and selenium deficient groups close to statistically significant, p = 0.08.

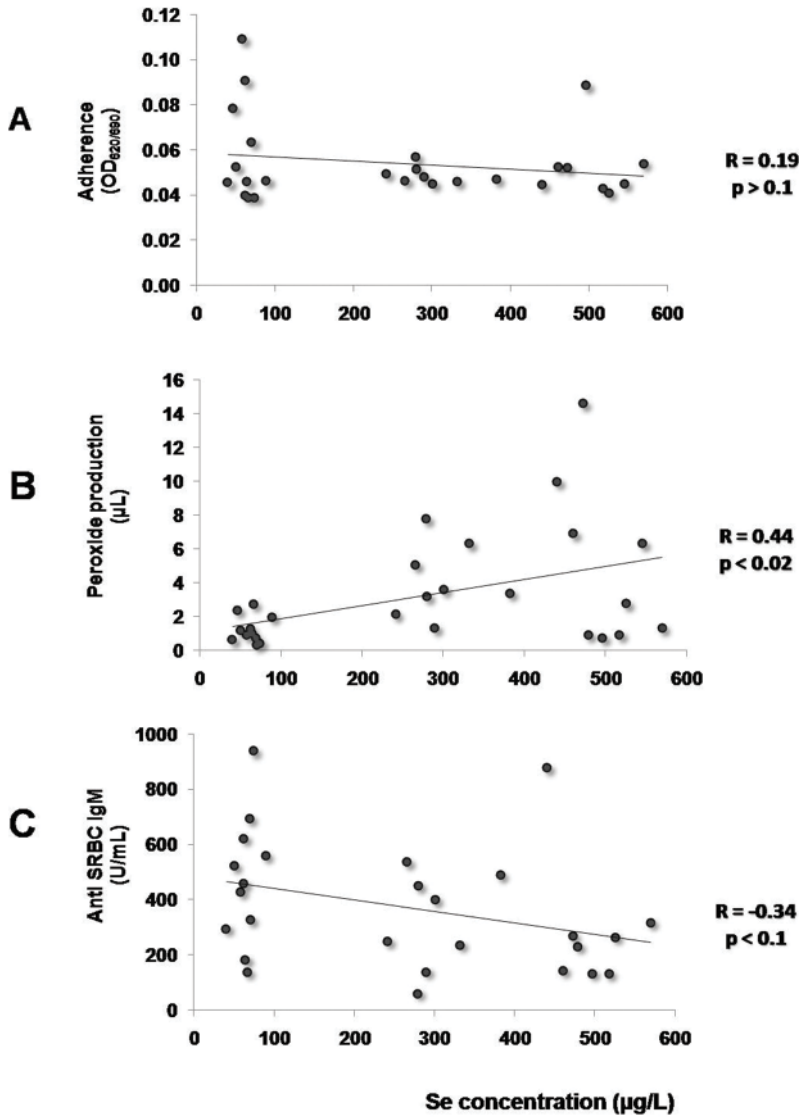


Figure 1. Correlation between blood selenium concentration and macrophages adhesion (A) macrophages peroxide production (B) and titer of anti-sheep red blood cells (SRBC) IgM antibodies (C). (●) – Experimental data; (—) – Linear regression analysis.

Selenium deficient rats had a significantly higher titer of blood plasma anti-SRBC IgM antibodies comparing to selenium adequate rats (Table 4). The difference in plasma anti-SRBC IgM antibodies titer between PTU- and PTU+ rats was not found. The negative correlation between the titer of anti-SRBC IgM and the blood concentration of selenium was recorded ($R=-0.34$; $p= 0.08$; $n=27$) (Figure 1C).

Table 4. Influence of selenium deficiency and PTU on the titer of naturally occurring anti-SRBC antibodies.

| | Selenium adequate | | Selenium deficient | |
|------------|-------------------------|-------------------------|--------------------------------------|-------------------------------------|
| | Euthyroid (n=12) | Hypothyroid (n=10) | Euthyroid (n=11) | Hypothyroid (n=8) |
| IgM (U/mL) | 254 ± 240 <i>182</i> | 268 ± 144 <i>258</i> | 480 ± 250 ^a <i>475</i> | 444 ± 84 ^a <i>465</i> |
| IgG (U/mL) | – | – | – | – |

The summary data are presented as the mean ± SD and *median*. (–) – Below the detection level; ^(a) – p < 0.05 compared to control selenium adequate, euthyroid group.

Titers of anti-SRBC antibodies of IgG class were below the detection level (Table 4).

DISCUSSION

In this study selenium status was determined on the basis of blood selenium concentration and activity of plasma GPx3 and erythrocyte GPx1. Activities of these enzymes correlate to a high degree with dietary selenium and represent excellent biomarkers for the estimation of selenium status [31]. Based on the measured values of these biomarkers it was estimated that the minimal selenium requirements are 0.06 mg Se/kg feed (for plasma GPx3) and 0.08 mg/kg feed (erythrocyte GPx1) [32]. In accordance with this it can be concluded that the diet containing 0.33mg Se/kg feed fed to the rats in our experiment ensured the maximal activity of GPx3 and GPx1 in selenium adequate animals. In contrast to this, selenium deficient rats, fed a diet containing ten times less selenium, had approximately six times lower blood selenium concentration (p<0.001). This result unambiguously confirmed that in our experiment feeding the low selenium diet resulted in its real deficiency. The real selenium deficiency was also confirmed by the results showing that both GPx1 and GPx3 activities were lower in selenium deficient animals. The activity in selenium deficient rats was by 38% lower compared to the selenium adequate groups. Hill et al. [33] reported that in selenium deficient animals GPx1 declines more rapidly compared to GPx3 which is in agreement to our results where we measured a 65% decline in GPx1 activity compared to GPx3.

After the three week experimental period concentrations of T₄, T₃ and TSH in the blood of the euthyroid selenium adequate animals were within the physiological range [34]. It has been proven that PTU induces hypothyroidism in different animal species [35,36] including rats [37]. PTU doses recommended for the induction of hypothyreosis in rats are in the range from 0.01% to 0.10% [37]. Thus the PTU dose of 150 mg/L drinking water (0.015%) used in our experiment is sufficient to induce hypothyroidism, which was confirmed by the measured T₄, T₃ and TSH concentrations in the blood of both selenium adequate and selenium deficient rats. Two possible mechanisms of achieving these effects have been proposed. The first implies the

inhibition of thyrocytes' peroxidase which oxidases iodine (I⁻¹→I⁰) and incorporates it into the thyroglobulin tyrosine residues [34]. The second possible mechanism implies the inhibition of iodothyronine deiodinases type-1 which is responsible for the conversion of T4 to T3 [38].

Macrophages as components of innate immunity provide the second line of innate immune defense. During an inflammatory response ROS and cytokine synthesis by tissue resident macrophages activate endothelial cells. Adhesion of circulating monocytes to inflammatory activated endothelial cells and recruitment to an inflamed tissue is the first step in their activation, and it is followed by ROS generation [21-23]. In this study we analyzed the functional characteristics of peritoneal macrophages which were induced i.e. whose number was increased by intraperitoneal injection of sterile thioglycollate solution leading to blood monocytes recruitment [39,40]. These obtained "inflammatory macrophages" differ functionally from resident cells and are characterized by a higher plasma membrane turnover, increased capacity for phagocytosis and enhanced respiratory burst. Modulatory effects of thyroid hormones and selenium status on functional properties of the macrophages were studied using two *in vitro* assays: adherence to plastic (polystyrene) and peroxide production.

The adhesiveness of leukocytes to various matrices (natural or synthetic) has been used for *in vitro* correlation of leukocyte activation, and the adherence to polystyrene is a widely used assay since the plastic surface behaves physiologically with respect to leukocyte adhesion [29]. Tissue infiltration by blood derived macrophages implies adhesion of circulating monocytes to activated endothelial cells. L-selectin and integrin molecules on circulating monocytes are responsible for this process [22,23]. Despite the fact that a significant difference was not found, our results showed that macrophages from selenium adequate rats had a slightly lower level of adhesion. Whether this increased adhesiveness reflects an increased activity of macrophages in selenium deficiency, which can be connected with increased inflammation, is not clear at this moment. The increased adhesiveness of macrophages might be the result of a known "adaptive" mechanisms occurring in selenium deficiency (inhibition of monocyte L-selectin by shedding metalloproteinases and/or stimulation of NF- κ B transcription factors which in turn regulates the synthesis of cyclooxygenase-2, TNF- α , IL-1 β , IL-6, IL8 and adhesive molecules [41] but it cannot be proven without further investigation.

Despite the known fact that hypothyroidism modulates the expression of membrane-bound and soluble adhesion molecules which regulate lymphocyte and monocyte adherence and trans-endothelial migration [42,43] in this study we did not find that hypothyroidism modulated the adhesion of induced peritoneal macrophages. Without further studies including the analysis of expression of adhesive molecules and analysis of adhesiveness to other matrices (e.g. extracellular matrix proteins) our results cannot be fully explained.

Synthesis of ROS, catalyzed by xanthine oxidases, cyclooxygenases, lipoxygenases, nitric oxide synthase, and mitochondrial oxidases, is one of the multiple mechanisms

by which macrophages achieve their anti-microbial effects and regulate the activity of cells of both innate and adaptive immunity [44]. It is widely accepted that proinflammatory effects of selenium deficiency resulted from decreased activity of GPx, increase intracellular ROS generation, activate NF- κ B signaling and accumulate oxidative damages [45]. Therefore, we studied the effects of selenium deficiency and PTU on the synthesis of H₂O₂ in thioglycollate induced peritoneal macrophages in a response to *in vitro* PMA stimulation. H₂O₂ synthesized during cell respiratory burst plays a major role in the destruction of the phagocytized microorganisms. In this process, firstly NADPH oxidase complex releases unstable superoxide (O₂⁻) anions which, spontaneously at low pH or by the action of the enzyme superoxide dismutase (SOD) are converted into H₂O₂.

In our study selenium deficiency resulted in a decreased peroxide production within the rats' macrophages. Our result is in agreement with previously published data [2,8] showing that in *in vitro* there is a correlation between selenium concentration and peroxide production [46]. In our study such correlation has been confirmed *in vivo*, as well. The reduced oxidative burst and the subsequent decline in the production of peroxide could be explained by an inadequate metabolism of hydrogen peroxide. A decrease in hydrogen peroxide metabolism can be explained by the decreased GPx activity (detected in this study) resulting in a function loss of membrane bound NADPH oxidase [8]. The physiological significance of our result is not clear and we can only speculate that the reduced H₂O₂ synthesis in peritoneal macrophages could be an adaptive mechanism activated during selenium deficiency to protect cells and tissues from oxidative damage. Our results showed that combined selenium deficiency and hypothyroidism resulted in an increased activity of GPx1 compared to selenium deficient euthyroid rats. In order to explain these results, it would be necessary to explain if the lowering peroxide synthesis in the applied experimental conditions were the result of: 1) activation of mechanisms providing priority of preservation of the activity of selenoenzymes members of the antioxidative protection system including thioredoxin reductase; 2) activation of other enzymes of this system (SOD, catalase, hem oxygenase); 3) changed concentrations of non-enzyme components of the system (bilirubin, uric acid, ceruloplasmin, transferrin) and/or 4) inhibition of ROS generating enzymes.

In this study we showed that hypothyroidism induced with PTU treatment did not influence H₂O₂ synthesis in peritoneal macrophages after *in vitro* stimulation with PMA. Data on hypothyreosis and macrophage function are not numerous but are similar to our results. Rosa et al. [16] showed that hypothyroidism (similar as hyperthyroidism) did not influence peroxide synthesis in thioglycollate induced macrophages.

Further we analyzed the influence of selenium deficiency or/and hypothyroidism on the titer of naturally occurring anti-SRBC antibodies of IgM classes present in the blood plasma of non-immunized rats. Although immunization with SRBC antigens is widely applied in immune toxicology for the evaluation of T-cell dependent antibody response [47] the antigenic structure of SRBC membranes are complex and besides T

dependent antigens express phylogenetically conserved carbohydrate and phospholipid antigens which can be recognized by naturally occurring IgM antibodies secreted in the juvenile rat in absence of apparent antigenic stimulation. The absence of a detectable level of anti-SRBC IgG antibodies and absence of germinal centers in the spleen of any of the analyzed rats (data not shown) indirectly proved that anti-SRBC IgM antibodies belong to a pool of naturally occurring antibodies. In this study we found that hypothyroidism did not influence the titer of anti-SRBC naturally occurring IgM but that selenium deficiency led to an increasing titer of these antibodies. Data on the influence of selenium deficiency on the titer of naturally occurring IgM antibodies are absent in the available literature. The increasing titer of naturally occurring IgM could have a protective role based on their ability to bind and neutralize previously unrecognized microbiological antigens, to induce synthesis of acute phase proteins, to interact with cells of adaptive immune response and stimulate the synthesis of antigen specific IgG, to suppress inflammatory responses mediated by innate or adaptive immune cells, and to prevent autoimmune responses mediated by autoimmune B and T cells [24-26]. However, knowing that selenium deficiency is related to the occurrence and progression of some autoimmune diseases (Hashimoto's thyroiditis, rheumatoid arthritis) [5,48,49] and that some autoantibodies belong to naturally occurring IgM pool augment the pathological immune response [24-26,50], the recorded increased level of naturally occurring IgM antibodies does not necessarily have a protective role. To completely understand and correctly interpret these results further studies of the effect of selenium on naturally occurring antibodies in animal models of autoimmune diseases using less complex antigen systems are necessary.

In this study, using the applied experimental model of selenium deficiency and PTU induced hypothyroidism in juvenile rats, we did not find that hypothyroidism influences the synthesis of peroxide in peritoneal macrophages or the titer of naturally occurring IgM antibodies. Also we did not find combined effects of selenium deficiency and PTU treatment on the analyzed parameters. The obtained results indicate that selenium, but not thyroid hormones, is of importance for the regulation of the investigated functions of the immune system involved in the recognition and elimination of previously encountered antigens.

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Authors' contributions

BJ, IBJ and MS conceived the idea of the study, participated in its design and coordination and helped to draft the manuscript and performed part of the statistical analysis. KD and GD performed statistical data analysis. IV, KD and VO carried out

the miochemical analyses, participated in the sequence alignment and drafted the manuscript. IV and MS carried out the immunoassays. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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UTICAJ DEFICITA SELENA I TIROIDNIH HORMONA NA ATHEZIJU PERITONEALNIH MAKROFAGA I PRIRODNA IgM ANTITELA KOD JUVENILNIH PACOVA

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Selen kao efektor i regulator enzima antioksidativne zaštite, kao i hormoni tiroidne osovine su snažni imunomodulatori. Pored toga, selen, kao sastavni element enzima jodotirozin dejodinaza direktno reguliše aktivnost hormona tiroidne žlezde, a time indirektno reguliše imunski odgovor. Studije o njihovom uzajamnom dejstvu na regulaciju imunskog odgovora skoro da ne postoje. U ovom radu smo analizirali uticaj propiltiouracila (PTU), blokatora dejodinaza i deficit selen na funkciju peritonealnih makrofaga i titar prirodnih IgM antitela kod juvenilnih pacova. Studija je rađena na mužjacima Wistar pacova ($n=64$) koji su bili podeljeni u četiri grupe: kontrolna grupa, selenadekvatni, PTU-; selenadekvatni, PTU+; selendeficitni, PTU- i selendeficitni, PTU+. Selenadekvatni i selendeficitni pacovi su dobijali hranu koja je sadržala 0.334 odnosno 0,031 mg Se/kg. PTU je davan rastvoren u vodi za piće u koncentraciji 150 mg/L. Posle tri nedelje tretmana određivan je nivo tiroksina (T4), trijodtironina (T3) i tireostimulirajućeg hormona (TSH). Životinje koje su imale intermedijalne koncentracije T3 (1,56-1,69 nmol/L) i T4 (41-50 nmol/L) su isključene iz daljih analiza. Tako su PTU+ grupe činile isključivo hipotiroidne jedinke ($T3 \leq 1,55$ nmol/L; $T4 \leq 40$ nmol/L), dok su PTU- grupe činile eutiroidne jedinke ($T3 \geq 1,70$ nmol/L; $T4 \geq 50$ nmol/L). Obe grupe selendeficitnih pacova su imale nižu aktivnost glutation peroksidaza GPx1 i GPx3 u odnosu na kontrolnu grupu životinja. Ni deficit selen ni PTU nisu uticali na adherencu peritonealnih makrofaga. Deficit selen je značajno smanjio sintezu peroksida u makrofagama i doveo do povećanja titra IgM antitela na ovčje eritrocite. Sam hipotiroidizam ili u kombinaciji sa deficitom selen nije uticao na ove parametre.