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Original Research Article

THE PROOXIDANT-ANTIOXIDANT SYSTEM IS THE PRIMARY STAGE OF THE ANSWER OF ORGANISM ON UNBALANCED DIET

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Abstract
The influence of the by animal proteins and vitamins unbalanced diet (PVU diet) on some characteristics of prooxidant and antioxidant systems activity in rats obtaining on such diet during 2 months was investigated. This diet was not influence the somatometric characteristics of animals but the products of free radical reactions increased in liver microsomes and mitochondria and also in the blood serum. Such changes of prooxidant activity were shown simultaniusely with decreased activity of glutathione peroxidase and other antioxidant enzymes. The changes in the balance of pro – and antioxidants not depended on age of experimental animals. Addition of *Aronia melanocarpa* fruits to the diet of animals did not affect the activity of antioxidant enzymes, but decreased the content of peroxidation products to the control level. This effect is due to the presence of antioxidant non enzymatic components in fruits of *Aronia melanocarpa*. The peroxidation in animals on the PVU can be achieved by the inclusion of sodium selenite in such diet. PVU-induced changes in the prooxidant system of the animals can be removed by different strategies.

Keywords: Unbalanced diet, *Aronia melanocarpa*, antioxidant enzymes.

Introduction

Modern food technology is using a variety of methods of deep processing, canning and food modifications. These necessary procedures inevitably lead to a deep change of food components, first of all to change and loss of components essential to the organism: vitamins, amino acids, unsaturated fatty acids and trace chemical elements. Modifying the composition of food is concerned not only with deep processing of food, but also with changes in the system of agricultural cultivation. Using these products, we are faced with the problem of unbalanced diets, and the problem affects all the segments of the human population.

As it is well known, the obtaining of unbalanced in amino acid, vitamin and trace

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element composition diet may be accompanied by the formation of a variety of pathologies [1-3], impairs the life quality [4] and its duration [5]. The obesity is the epidemic, swept many countries, it is also connected with the unbalanced diets [6, 7]. This problem is particularly acute at early stages of ontogenesis, when are forming food preferences and epigenotype [8, 9]. Many experts see the solution to this problem in the development of the concept of functional foods [10, 11], food additives [12] and enrichment of food products by the components essential for organism.

Despite the intensive study of the problem of unbalanced diets, the mechanisms of pathologies, temporary stages of formation of metabolic changes and their contribution to certain pathological manifestations till now remain unclear. On the background of an unbalanced diet relations between various metabolic changes and the role of these changes in the development of pathology are poorly understood.

The unbalanced diet is of great interest in the study of the mechanisms of aging and control of lifespan. It is well known that the calorie restricted, but a balanced diet induce increase of lifespan of experimental animals [13 - 15]. The consumption of unbalanced diet is accompanied by symptoms of disadaptation, a decrease in non-specific resistance to environmental stress [16].

The crucial issue of the problem unbalanced nutrition is determination of those metabolism links that first respond to the lack of food nutrients. One can assume that active prooxidant- antioxidant system can be such a primary link of metabolism that able to "respond" to the diet.

The definition of "primary" link of response to the unbalanced diet will give an opportunity to developt of a simple test-system for dietology; to identify the critical periods in the formation of persistent metabolic changes, i.e. formation of imprinting; and to investigate the mechanisms of influence of different diets on the aging process.

In this regard, we investigated the characteristics of prooxidant system (lipid hydroperoxide content, Schiff bases, OH radicals) and activity of antioxidant enzymes (glutathione peroxidase, glutathione-S-transferase, glutathione reductase) in the mitochondrial and microsomal fractions of liver cells in experiment animals after 2 months of obtaining the diet unbalanced in proteins and vitamins.

Materials and Methods

Materials

Investigations were done on 3-months old (young) and 18-months old male Wistar rats. Over 2 months control group of animals (10 rats) received standard diet and the test group (7 animals) received a diet with reduced protein content by 35 % compared with control and proteins consisted only of vegetable proteins. Vitamin E content was reduced by 37 % compared to controls. When the animal proteins were replaced by vegetable proteins, as a consequence, essential amino acids content decreased by 2-3 times.

After 2 months rats were decapitated with the rules of the European Convention for the Protection of Vertebrate Animals used for experimental and scientific purposes.

To determine the effect of fruits of melanocarpa *Aronia* animals (10 rats), kept on the animal proteins and vitamins unbalanced diet (PVU diet) additionally received 0.6 grams of dried fruits melanocarpa per 100 g body weight. A separate group (7 animals) received sodium selenite in dose 1.2 μg per 100 g body mass of animal on the background of the PVL.

For 12-14 hours before slaughter the animals were starved.

Methods

Preparation of blood serum.

Blood was collected in plastic tubes, serum was collected after erythrocytes

sedimentation and centrifugation at 1000 g for 10 min.

Isolation of hepatocytes

Decapitation of rats was performed under ether anesthesia, liver perfusion was performed with medium containing 250 mM sucrose; 5 mM KCl; 0,4 mM Na2HPO4; 1 mM MgSO4; 5,5 mM EDTA, pH 7,4. The perfusion rate was 10 ml/min , temperature 37°C, 10 min. Hepatocytes isolated as described by [17].

Viability of the obtained hepatocytes was assessed using the vital dye - trypan blue [18], the viability was 85-90 %.

Preparation of mitochondria, postmitochondrial fraction (microsomes + cytosol) and microsomes.

Mitochondria and postmitochondrial microsome fraction was isolated by differential centrifugation [19]. The isolated mitochondria are suspended in the medium: 0.3 M sucrose - 10 mM Tris -HCl buffer, pH 7.4 and their concentration was adjusted to 60-80 mg/ml total protein.

The isolated microsomes were suspended in 125 mM KCl, 10 mM Tris -HCl buffer, pH 7.4.

Analytical methods

Determination of the rate of generation of superoxide radical in microsomes

Generation of O_2 in the microsomes suspension at NADPH oxidation microsomes was measured by the formation of adrenochrome with adrenaline in a medium: 0.15 M potassium phosphate buffer, pH 7.8, 10^{-4}M EDTA, 10^{-4}M NADPH, 5.10^{-4}M adrenaline microsomes at 250 mcg protein per ml. The rate of O_2 generation was determined by molar extinction coefficient of adrenochrome - 4.02 10^{-3} m $^{-1}$cm $^{-1}$ [20], taking into account for oxidation of 1M of adrenaline 1.4 M of superoxide radicals is needed [21].

Determination of the rate of generation of reactive oxygen species in isolated hepatocytes

The rate of generation of OH^- and O_2 was determined in the medium: 250 mM sucrose, 5 mM KCl; 0.4 mM KH_2PO_4; 0.5 mM Na_2HPO_4; 1 mM $MgSO_4$; 1.5 mM $CaCl_2$ and 2.5 10^6 hepatocytes in 1 ml.

In the case of determining the generation of OH radicals in the medium deoxyribose was added 4.0 mM additionally. Determination was performed as described [22].

In the case of determination of O_2 generation 50 mM tetrazolium blue was added instead deoxyribose. Generation rate was expressed as nmol of formazan/min per 10^6 cells.

Determination of lipid hydroperoxide.

Hydroperoxides content in hepatocytes and subcellular fractions was determined by the method of Ohkawa et al. [23] and in the serum by the method of Asakawa et al. [24]. The content of lipid hydroperoxides was expressed in equivalent quantities of Malone dialdehyde (MDA) per 1 mg of plasma or serum.

Shiff bases content was measured fluorimetrically after lipid extraction by chloroform: methanol (1 : 2, v/v) mixture by the method [25].

Determination of antioxidant and antiradical activity in system in vitro

The antioxidant activity of chockberry *Aronia* extract was determined on a model of yolk lipoproteins as described earlier [26]. Antiradical activity was determined by the rate of OH-radicals trapping using deoxyribose as described earlier [22].

Determination of antioxidant enzymes activity

Catalase activity (EC 1.11.1.6) was determined as described earlier [27].

Glutathione peroxidase activity (EC 1.11.1.9) in samples was determined as described [28], and glutathione-S-transferase activity (EC 2.5.1.18) was determined spectrophotometrically [29]. The glutathione reductase activity (EC 1.6.4.2) was determined by the method [30].

The protein content was determined by Lowery method modified by [31].

Determination of thyroxin and triiodthyronine in blood serum was determined by radioimmunoassay using the «Total T4 RIA» and «Total T3 RIA» kit, Immunotech (Czech Republic).

Statistic analysis of the results

All the experiments were repeated at least 5 times, statistic analysis of the results was based on a software program "Statistica V6" using the Student *t*-test.

Characteristics of used reagents

We used reagents: NADPH, GSH, GSSG, TRIS (hydroxymethyl) aminomethane, adrenaline, human serum albumin, mannitol – "Reanal" production (Hungary), triton X-100 «Ferak» production (Germany), cumene hydroperoxide, KH_2PO_4, glutathione reductase, $CaCl_2$, Na_2HPO_4; $NaHCO_3$ – "Sigma" (USA), α-tocoferol ICN (USA).

Results

Somatometric indexes in animals obtained for two months the animal proteins and vitamins unbalanced diet (PVU diet)

The transfer of 3 -month animals on proteins and vitamins unbalanced (PVU) diet was not accompanied by significant change in body weight, liver and heart weight (Table 1).

It should be noted that the increase of PVU diet period more than 3 months was accompanied by body mass retardation as compared to control, at the same time the mass coefficients of the heart and liver increased.

When the old (20-months) animals were transferred on the PVU, their somatometric indexes didn't change.

Table 1: Some somatometric indexes of young rats maintained on PVU during two months

Index	**Housing conditions**	
	Standard diet	**PVU-diet**
Body weight, g	200.0 ± 7.3	180.0 ± 7.7
Liver weight, g	5.56 ± 0.28	5.76 ± 0.49
Heart weight, g	0.65 ± 0.02	0.68 ± 0.03
Liver mass coefficient, g/g	2.87 ± 0.011	3.18 ± 0.18
Heart mass coefficient, g/g	0.327 ± 0.016	0.378± 0.012

Characteristics of prooxidant- antioxidant system of hepatocytes

Two-month maintenance of young (3 months) rats on the PVU diet was accompanied by significant increase in lipid hydroperoxide by 30%, Schiff bases - by 33% , and OH radicals by 56 % and O_2 at 23 % compared to the control level (Fig. 1).

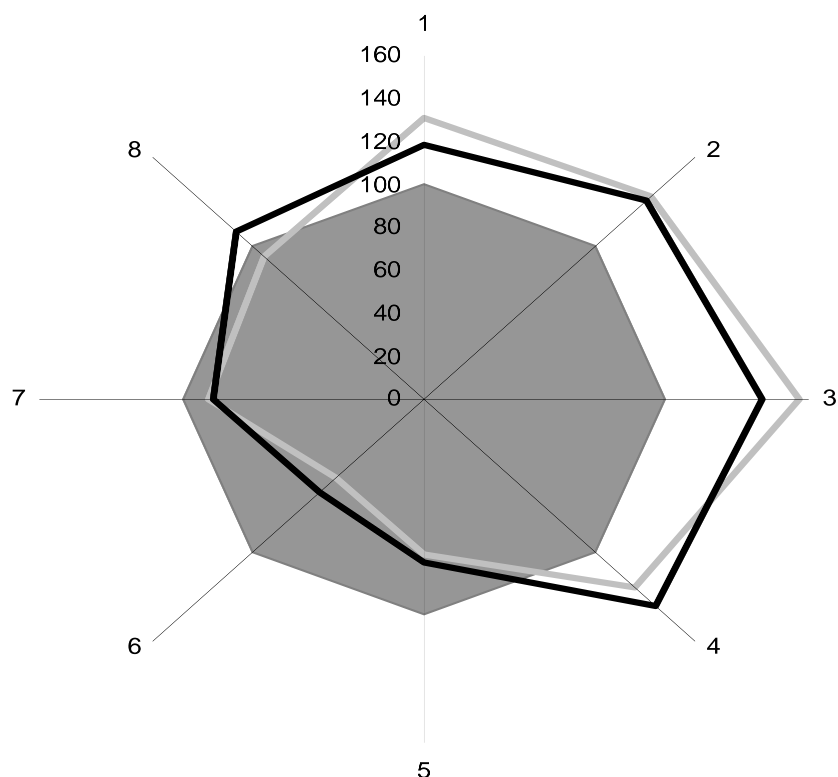

Figure 1. Relative change characteristics of prooxidant and antioxidant systems of hepatocytes (in percent) in the control animals, the values of which are taken as 100 % (■) , in the 3-month animals obtaining the PVU 2 months (▬) and at 20 months animals obtaining the PVU 2 months **(▬)** Axes: 1 - lipid hydroperoxides; 2 - Schiff bases; 3 - OH radical content; 4 - O2 content; 5 – activity of total glutathione peroxidase; 6 – activity of selen-dependent glutathione peroxidase; 7 – activity of glutathione reductase; 8 – activity of selen-independent glutathione peroxidase.

The measures of prooxidant system of old (20 months) rats increased significantly, but to a less extent, with the exception of O_2 (Fig. 1).

The increase of free radical products content may be due to a significant increase in their production or decreased activity of the antioxidant system. To elucidate this question, activity of glutathione peroxidase and glutathione reductase in hepatocytes was determined. It was found that glutathione remained unchanged in both young and aged animals maintained during 2 months on the PVU (Fig. 1). The total glutathione peroxidase activity in hepatocytes was 25-30% lower compared to the control in young and old animals (Fig. 1).

Glutathione peroxidase (GP) is known to be consisted of selenium-dependent and selenium-independent GP. Determination of selenium-independent GP showed that its activity remained unchanged, as compared to controls (Fig. 1). At the same time, the activity of selenium-dependent GP was less than the control level both in young and in old rats by 46 and 40 % respectively.

Therefore two-month maintenance of animal on PVU was accompanied by an

increase of free radical products that may be connected with inhibition of activity of selenium-dependent GP and, possibly, of other antioxidant enzymatic and non-enzimatic systems, and also with vitamin deficiency.

Characteristics of prooxidant-antioxidant system in subcellular fractions of hepatocytes

Since mitochondria and microsomes are the basic components of pro/antioxidant system, the effects of PVU may be more pronounced on the hepatocytes level.

The content of lipid hydroperoxide in mitochondria was increased by 27 %, and in microsomes by 37% in young animals maintained on the PVU during 2 months (Fig. 2). Equally, the hydroperoxides content in the plasma of these animals was increased (Fig. 2).

Such stable changes in the content of hydroperoxides in plasma, hepatocytes, liver microsomes and mitochondria allows to use their content in the plasma when evaluating the effect of diet on the pro/antioxidant system.

If the increase of free radical reactions products is due to lack of vitamins or minerals, presumably the addition of these components in the diet can reverse the identified effects. With purpose to check this hypothesis 0.6 g of dry powder of *Aronia melanocarpa* fruits per 100 g of animal body weight were added in the PVU diet. It was found that in this case the content of lipid hydroperoxide in mitochondria; microsomes and plasma differ from the control level (Fig. 2).

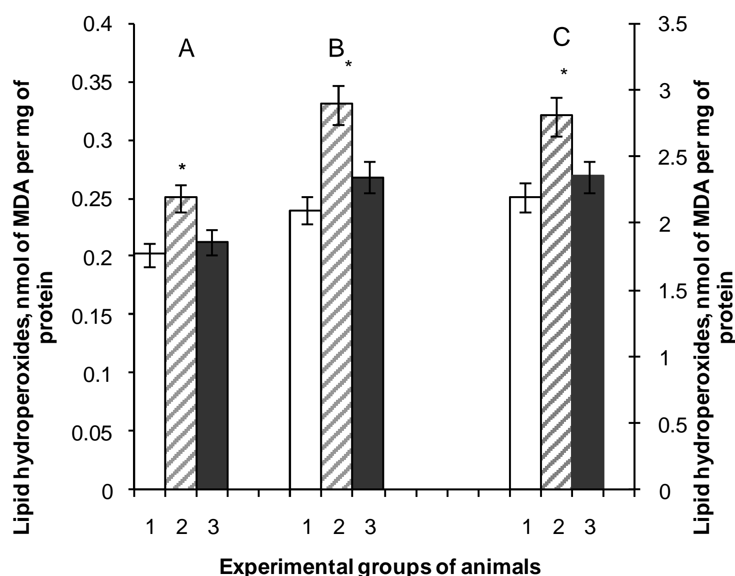

Fig. 2. Contents of lipid hydroperoxides in mitochondria (A), liver microsomes (B) and plasma (B) in the control group of animals (1), the animal obtaining the PVU (2) and animals obtaining and additionally treated with dry fruits of Aronia in dose 0.12 µg per 100 g body mass (3). Average data from 8 experiments. * - difference P <0.05 compared to the control

Revealed effect of decrease of the lipid hydroperoxide content after the addition of *Aronia melanocarpa* can be explained by: 1 - antioxidant properties of melanocarpa; 2 - activation of cellular antioxidant components; 3 - decreased production of free radicals; 4 - induction simultaneously several security systems.

In the next set of experiments the activity of antioxidant enzymes such as glutathione peroxidase, glutathione-S-transferase, glutathione reductase in mitochondria postmitochondrial fraction and the liver microsome fraction was determined.

It was found that the activity of glutathione peroxidase in liver mitochondria of

rats maintained on PVU during two months was decreased by 2 times compared with the control (Table 2). Addition of *Aronia melanocarpa* in the PVU has no effect on glutathione peroxidase activity in mitochondria and microsomes and in postmitochondrial fraction it has even decreased (Table 2).

Table 2. Activity (nmol NaDPH / min mg protein) of the total glutathione in mitochondria and postmitochondrial fraction of rat liver microsomal fractions on a standard diet, obtaining the PVU and the PVU with the addition of *Arónia melanocárpa*

Experimental group	Subcellular fraction		
	mitochondria	post mitochondrial	microsomal
Standard diet	185.5 ± 20.0	397.9 ± 24.3	12.7 ± 0,84
PVU-diet	85.4 ± 8.8*	316.4 ± 18.3*	9.8 ± 0.55*
PVU with *Arónia melanocárpa*	82.5 ± 6.8*	264.2 ± 18.8*	9.3 ± 0.67*

The selenium-dependent glutathione peroxidase activity in postmitochondrial fraction of liver in animals maintained on PVU was decreased compared with the control by 2.7 times (Fig. 3). Addition of Aronia melanocarpa to the diet had no effect on the activity of this enzyme, and it remained 2 times below the control (Fig. 3).

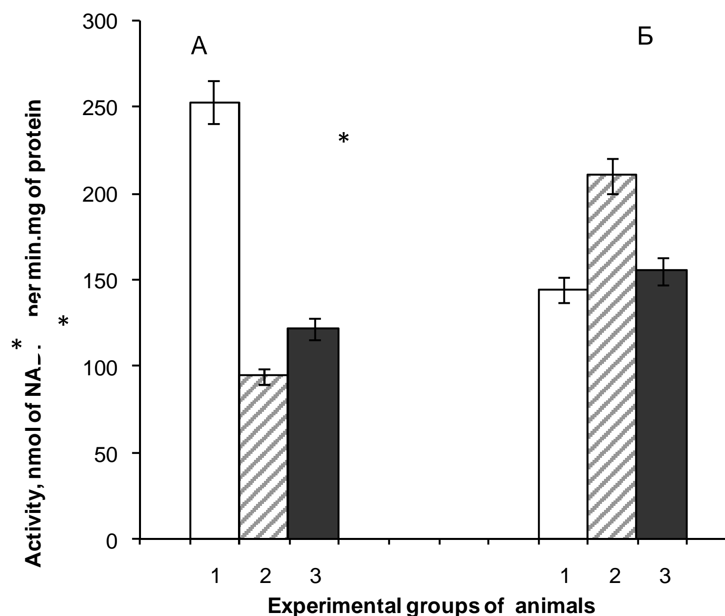

Fig. 3 Activity of selen dependent (A) and selen independent (B) glutathione peroxidase in postmitochondrial fraction of liver of control 3-month animals (1), the animal obtaining 2 months the PVU (2) and animals obtaining the PVU 2 months and fruits of Aronia melanocarpa (3)

At the same time, the activity of selenium-dependent glutathione peroxidase has even been increased by 45% in animals on PVU and was completely restored to control level on the diet with the addition of *Aronia melanocarpa* (Fig. 3).

Consequently, the selenium-dependent glutathione peroxidase activiry decreases during two months of PVU apply makes the main contribution to the total decrease of glutathione peroxidase activity.

We investigated the activity of other antioxidant enzymes of liver postmitochondrial fraction. It was shown that the catalase activity of animals on PVU decreased insignificantly as compared with control, and addition to the PVU diet of fruits of *Aronia melanocarpa* didn't influences the catalase activity decrease (Table 3).

The activity of glutathione-S-transferase and glutathione reductase was the same in animals on PVU and animals additionally applying *Aronia* (Table 3).

Consequently, the effect of decrease of hydroperoxides in animals maintained during 2 months on PVU and applying the *Aronia melanocarpa* can be connected not with increase of antioxidant activity of enzymes but probably with antioxidant properties of *Aronia* components. We checked the antoxidant activity of the *Aronia melanocarpa* extracts in model experiments.

Table 3. Enzymatic activity in the postmitochondrial fraction of liver in control animals on a standard diet, and animals on different diets

Experimental group	Activity of enzymes, μmol H_2O_2/min mg of protein)		
	Catalase	Glutathione-S-transferase	Glutathione reductase
Standard diet	213.3 ± 13.2	1362.0 ± 108.6	73.2 ± 6.2
PVU-diet	155.0 ± 16.1	1266.7 ± 124.9	69.5 ± 3.1
PVU with *Arónia melanocárpa*	147.8 ± 8.7	1295.6 ± 92.4	73.3 ± 2.7

It has been found that the antioxidant activity of the Aronia melanocarpa was less than the activity of α-tocopherol. The antioxidant activity of Aronia increased almost linearly in dependence on concentration of its components in the diet (Fig. 4). So, to obtain the same effect as that of α-tocopherol, it was needed to add 4 times more biomass of fruits of Aronia melanocarpa. At the same time the antiradical activity of Aronia fruits estimated on the ability to intercept the OH radical in the model system was shown to be higher than the activity of mannite (Fig. 4).

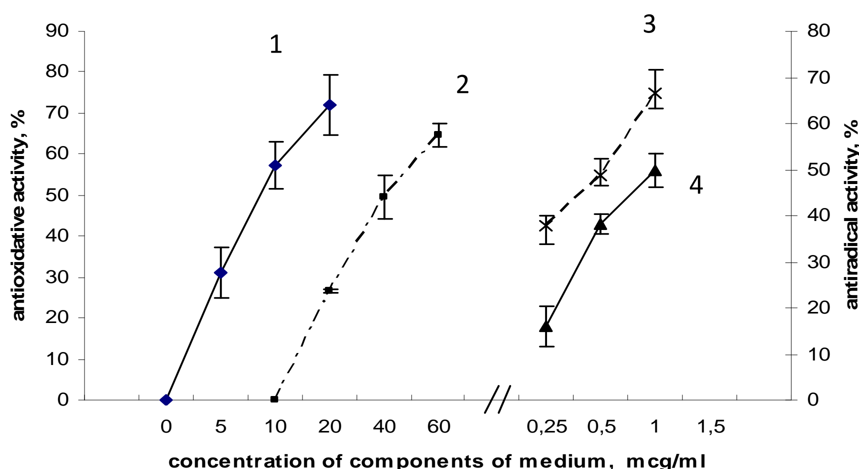

Fig. 4. Antioxidative activity in model systems of α-tocopherol (1), fruit extract of *Arónia melanocárpa* (2) and antiradical activity of mannitol (3) and fruits of *Arónia melanocárpa* (4) with addition of different amounts of the tested components

Consequently, the *Aronia* fruit has the pronounced antioxidant properties that cancel the prooxidant effect induced by two-month PVU.

However it is still unclear why the selenium-dependent glutathione peroxidase is decreased mostly at PVU and if selenium-dependent glutathione peroxidase is the main cause in antioxidant system of the cell.

To solve this problem the additive experimental series on the sodium selenite influence on the selenium dependent glutathione peroxidase and lipid hydroperoxide content in mitochondria were conducted.

It was found that the lipid hydroperoxides content in liver mitochondria of rats maintained 2 months on PVU was increased more than by 40% and glutathione peroxides activity was decreased by 3 times (Fig. 5). In that case if these animals apply microelements and selenium additively the glutathione peroxidase activity didn't differ from control level (Fig. 5). Maintenance of glutathione peroxidase activity in animals on PVU applying selenium additively eliminated entirely the increased level of hydroperoxides in liver mitochondria of these animals (Fig. 5).

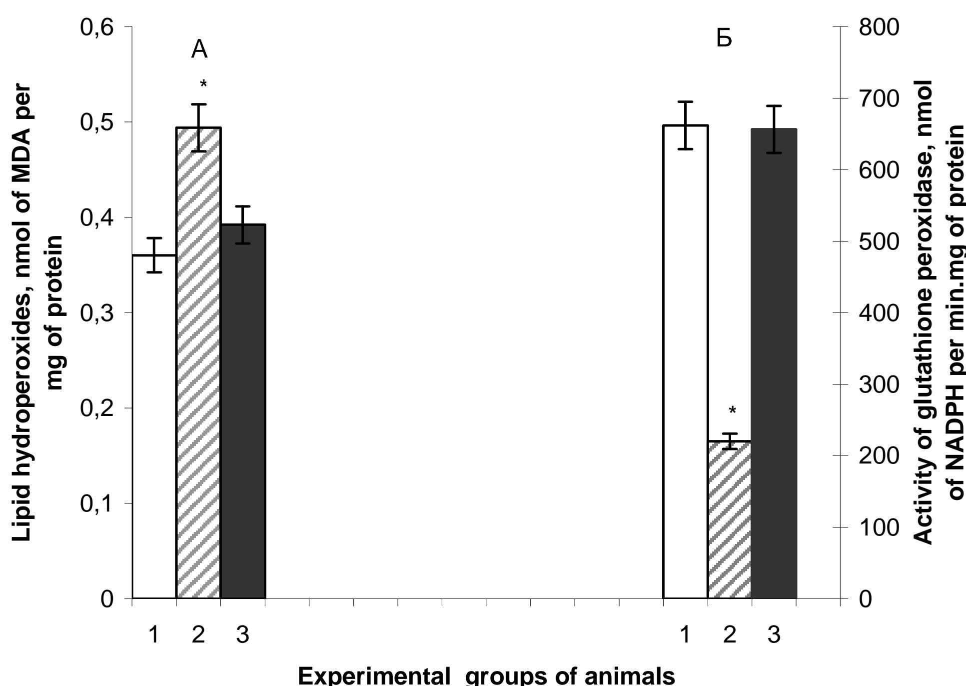

Fig. 5. Contents of lipid hydroperoxides (A) and glutathione peroxidase activity (B) in the mitochondria of young animals on a standard diet (1) and the PVU (2) and animals obtaining the PVU with the addition to the diet of sodium selenite in dose 1.2 µg per 100 g of body mass (3)

Consequently, selenium-dependent glutathione peroxidase really can be regarded as one of the key enzymes of glutathione antioxidant system and restoration of its activity eliminates increase of hydroperoxides induced by PVU.

The decrease of selenium-dependent glutathione peroxidase activity in animals on PVU diet can be explained by the set of factors: inhibition of enzyme synthesis due to the lack of essential amino acids, decrease of enzyme activity due to the lack of selenium and also by change of regulation due to change of hormonal status of such animals. The content of thyroxin and triiodothyronine in blood serum in animals

kept 2 months on PVU diet was increased more than twofold and by 30% accordingly (Table 4).

These results are indicative of multivariate system of regulation of glutathione peroxidase activity and system response to 2-month maintenance on PVU diet.

Table 4. Content of thyroid hormones in blood serum of three-month-old rats of the control group kept on a standard diet, and animals kept 2 months on PVU-diet

Experimental group	Gormones of content (nmol/L)	
	Triiodothyronine	Thyroxine
Standard diet	0.70 ± 0.09	76.4 ± 4.6
PVU-diet	1.9 ± 0.20*	98.7 ± 9.1*

Discussion

Maintenance of animals on the diet limited in proteins and vitamins over 2 months didn't influence the studied somatometric indicators (body weight, liver weight), the appearance and behavior. From this it follows that such duration of diet is not critical for both young and old animals. At the same time the prooxidant and antioxidant system underwent significant changes, which were reflected in significant (30-40%) increase in peroxidation products and the loss of activity of prooxidant enzymes and first of all of the selenium-dependent glutathione peroxidase. It can be assumed that the manifestation of the negative effects due to an unbalanced diet is implemented in several stages: 1. Compensatory reorganization of metabolism. It is difficult to distinguish these changes, since the metabolic variability is quite high and is the result of a combination of a complex set of global and local factors [32]. Further "fate" of metabolic status will depend on the nature of the change or constancy of diet.

In the case of constant diet, limited on animal proteins and vitamins, formed compensatory metabolic pattern will be "confirmed" or imprinted, and further morphofunctional changes will form the decreased ability to adapt and increased probability of development of pathologies.

If the components that will be able to support the activity of prooxidant system at a high level even "using" other elements of prooxidant system and non-enzymatic antioxidants added to the diet, trace elements as in the present study, we can provide metabolic reprogramming. It should be noted that in the first and in the second cases, although various strategies of nutritional adaptation are used, we will have different metabolic patterns which are characterized by different adaptive strategy and resistance to new extreme factors.

An important aspect of the problem of food metabolism reprogramming is to determine the period of formation and irreversible metabolic imprinting patterns in primary compensatory response.

The results of this work suggest that the characteristics of pro-antioxidant system can be considered as one of the primary elements in the metabolic response of the organism to an unbalanced diet.

Thus cell prooxidant system is activated by a wide range of toxic compounds [33]. The free radicals alter membrane function, nucleic acids, proteins, lipids, and induce an "oxidative stress" [34]. The intensity of oxidative stress is determined by the balance between the rate of formation of free radicals and the rate of their utilization. On this basis, the activity of prooxidant and antioxidant systems can serve an integral indicator of the primary metabolic response to unbalanced diet.

The characteristic of this system can be used in the formation of irreversible metabolic patterns.

As it is suggested in the present study, the 2-monthed maintenance of both young and old animals on the PVU was accompanied by

significant increase of free radical reactions products in the different compartments of the liver cells and serum, indicating the general response of the organism at the level of prooxidant system. Such a response is explained by changes in the balance of pro / antioxidant activity. Unbalanced protein diet and lack of amino acids in the organism can be accompanied by inhibition of the rate of synthesis of enzymes. Deficiency of vitamins and probably change of the contents of trace elements, in particular selenium, can explain the decline in the activity of antioxidant enzymes.

To determine the reversibility of the PVU-induced activity of pro-antioxidant system the addition of components of *Aronia melanocarpa* to this diet was used. It is well known that *Aronia* is rich with vitamines and antioxidant components [35]. Our results on its antiradical and antioxidant properties *in vitro* also suggest it.

Addition of *Aronia melanocarpa* fruits in the PVU reduce the content of the products of free radical reactions to the control level. These results support the suggestion that the change of balance pro/antioxidants in favor of the prooxidant activity on the PVU and explain the elimination of negative effects of peroxidation through various strategies.

This is supported by normalization of hydroperoxides to the control level using selenium and selenium-dependent glutathione peroxidase activation.

Glutathione peroxidases – a family of enzymes that reduce lipid peroxide in the alcohol, and hydrogen peroxide to water. On our results the selenium-dependent glutathione peroxidase showed the greatest response on the PVU. And addition of selenium to the diet increases the activity of this enzyme, which correlated with a reduction products of free radical reactions.

Consequently, the activity of glutathione peroxidases correlates with hydroperoxides content in mitochondria, microsomes and blood serum. However, if PVU decline activity of glutathione peroxidases due to a deficiency of selenium only, it would be accompanied by a decrease in the rate of conversion of thyroxine to triiodothyronine. Since the activity of deiodinase, which is selenium-dependent enzyme, the conversion of thyroxine to triiodothyronine should decrease.

In our study, on the contrary, the PVU glutathione peroxidase activity decreased and thyroxine content increased by 29 % and triiodothyronine increased more than 2 times. These results suggest that the PVU has an effect on different elements of the metabolism in different ways and, as a result, a new metabolic pattern is forming.

This metabolic pattern, which is based on oxidative stress, affects the immune system and other systems of an organism. As Beck M. has shown, food oxidative stress, which was induced by the lack of selenium and vitamin E, "allows" Coxsackie virus to mutate into virulent forms and cause heart disease [36].

Formation of various pathologies in rats obtaining PVU will depend on the duration of food oxidative stress and individual characteristics of the organism's metabolism.

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