

## PECULIARITIES OF THE FREE RADICAL PROCESSES IN RAT LIVER MITOCHONDRIA UNDER TOXIC HEPATITIS ON THE BACKGROUND OF ALIMENTARY PROTEIN DEFICIENCY

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*The rate of superoxide anion radical, hydroxyl radical and hydrogen peroxide generation, the level of oxidative modification of mitochondrial proteins in the liver of rats with toxic hepatitis was investigated on the background of alimentary protein deficiency. We did not find significant increases of the intensity of free radical processes in liver mitochondria of rats maintained on the protein-deficient ration. The most significant intensification of free radical processes in liver mitochondria is observed under the conditions of toxic hepatitis, induced on the background of alimentary protein deprivation. Under these conditions the aggravation of all studied forms of reactive oxygen species generation was observed in liver mitochondria. The generation rates were increased as follows:  $O_2^-$  – by 1.7 times,  $H_2O_2$  – by 1.5 times,  $\cdot OH$  – practically double on the background of accumulation of oxidized mitochondria-derived proteins. The established changes in thiol groups' redox status of respiratory chain proteins insoluble in 0.05 M sodium-phosphate buffer (pH 11.5), and changes of their carbonyl derivatives content may be considered as one of the regulatory factors of mitochondrial energy-generating function.*

*Key words: alimentary protein deficiency, acetaminophen, induced liver injury, reactive oxygen species, oxidative protein modification, mitochondrial proteins.*

Active oxygen metabolites, such as superoxide anion radical, hydrogen peroxide, and hydroxyl radical [1], are known to participate in intracellular signaling, regulation of cellular functions, non-specific immune response etc. [2, 3]. Nevertheless, due to their high oxidative potential, the reactive oxygen species (ROS) are capable of damaging cell structure and inducing a number of chain reactions leading to uncoupling of integrative functions in the organism [4].

The mitochondrial respiratory chain is the primary producer of ROS-generating electrons [5, 6]. Certain cellular mechanisms protect against overproduction of  $O_2^-$  in mitochondria and products of its following transformations [7]. The disruptions in these mechanisms may be considered the starting point of mitochondrial degradation, marked by opening of mitochondrial permeability transition pores in the inner mitochondrial membrane, decrease in membrane potential with simultaneous cytochrome *c* release, and  $Ca^{2+}$ -dependent activation

of hydrolytic enzymes [8-10]. The sensitive target of ROS is mtDNA and the mitochondrial proteins [11].

The problem of damage to functioning of energy biotransformation system under alimentary deprivation of microelements remains insufficiently studied as yet [12, 13].

The deficiency of mitochondrial respiratory chain substrates, primarily NADH, is crucial for disruptions to functioning of energy production systems. As we had demonstrated earlier [14], the protein-deficient animals with hepatitis display increased ratio of mitochondrial  $NAD^+/NADH$ , indicating lacking in NADH concentration. Nevertheless, the animals with acetaminophen-induced hepatitis fed standard ration do not develop NADH deficiency, despite damage to functional activity of mitochondrial respiratory chain enzyme complexes in liver.

On the other hand, the defining factor in disruptions of energy production may be the changes in activity of energy-producing systems resulting from

damaging effects of ROS on structural organization of nucleus-encoded or mitochondrion-encoded enzymatic complexes of mitochondrial respiratory chain may be [15].

Therefore, it is important to study the limiting factors of mitochondrial energy-producing systems under toxic liver lesions in animals maintained on rations with varying protein content.

The aim of the present study was to investigate the rate of ROS generation and oxidative modifications of mitochondrial proteins in liver of rats with toxic hepatitis on the background of alimentary protein deficiency.

### Materials and Methods

The experiments were conducted on white rats of 90-100 g body mass aged 2-2.5 months. The experimental work was conducted in accordance with rules set by the 'European convention for the protection of vertebrate animals used for experimental and other scientific purposes' (Strasbourg, 1986). The animals were separated into solitary plastic cages with sand bedding, with *ad libitum* access to water. The daily rations were regulated according to principles of pair feeding.

The animals were separated into the following experimental groups: I – animals receiving complete semi-synthetic ration (C); II – animals receiving low-protein ration (LPR); III – animals subjected to acetaminophen-induced liver lesions receiving complete ration (H); IV – animals subjected to acetaminophen-induced liver lesions that were previously fed semi-synthetic low-protein ration (LPR+H).

The animals of the groups I and III were fed standard fodder containing 14% protein (casein), 10% fat, and 76% carbohydrates, balanced with all the essential nutrients. The animals of the groups II and IV were fed isoenergetic fodder containing 4.7% protein, 10% fat, and 85.3% carbohydrates, calculated after recommendations of the American Institute of Nutrition [13, 14].

The animals were fed the corresponding diet for four weeks. Afterwards, the acetaminophen-induced liver lesion was modeled by per os introduction of 2% starch suspension of acetaminophen in dose of 1 g/kg body mass of animals for 2 days over 24 hrs. with corresponding probe [16].

Cervical dislocation was performed under light ether desensitization on day 31 of the experiment.

Mitochondrial fraction was separated by differential centrifugation in the following buffer

medium: 250 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl; pH 7.4 at 0-3 °C.

The rate of superoxide anion radical generation in mitochondrial fraction was measured with nitro blue tetrazolium test (NBT) [17].

Hydrogen peroxide content was measured after [18]. The working reagent was prepared by mixing reagent A (25 mM  $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$  in 2.5 M  $\text{H}_2\text{SO}_4$ ) and reagent B (100 mM sorbitol + 125  $\mu\text{M}$  xylol orange) in 1:100 ratio. In test tubes with 50 mM potassium-phosphate buffer (pH 6.0) was placed an aliquot of sample up to a total volume of 400  $\mu\text{l}$ . Control sample was 400  $\mu\text{M}$  of buffer solution. 2.0 ml of working reagent was added to each sample, mixed and incubated for 15 to 20 min. under room temperature, than optical density was measured against control at  $\lambda = 540$  nm. Hydrogen peroxide content was determined against calibration plot and calculated per 1 mg of total protein.

The rate of hydroxyl radical generation was measured after [19]. Incubation medium was as follows: 20 mmol deoxyribose, 1 mmol  $\text{H}_2\text{O}_2$ , 20 mmol sodium-phosphate buffer (pH 7.4), a sample was added containing 200  $\mu\text{g}$  of protein. The solution was incubated for 30 min. at 37 °C, than mixed with 0.5 ml of 1% solution of thiobarbituric acid in 50 mM NaOH and 0.5 ml of 2.8% solution of trichloroacetic acid. The resulting mixture was incubated for 20min. in boiling water bath, cooled, and extinction was measured at  $\lambda = 532$  nm. Rate of  $\cdot\text{OH}$ -generation was expressed as  $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  of protein.

Mitochondrial proteins were extracted by method [20] that is based on principle of insolubility of products of mitochondrial translation in 0.05 M sodium-phosphate buffer with pH 11.5. Mitochondria suspension was stored at -20 °C overnight. After thawing the suspension was centrifuged at 20 000 g for 20 min. The sediment was resuspended in 10 ml of 0.05 M sodium-phosphate buffer (pH 11.5) and centrifuged at 20 000 g for 20 min. The procedure was repeated [15].

Protein carbonylation was assayed via amount of 2,4-dinitrophenylhydrazone derivatives, that are produced in reactions of oxidized amino acid residues with 2,4-dinitrophenylhydrazine, and expressed as nmol of carbonyl protein derivatives per mg of protein.

Protein SH-group content was determined with method based on reaction of Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB)) with SH-groups. The products are disulfide and thioni-

trophenyl anion (TNPA). The amount of TNPA is directly proportional to the amount of SH-groups of proteins that reacted with DTNB. The content of free SH-groups was calculated basing on molar extinction of  $11400 \text{ M}^{-1}\cdot\text{cm}^{-1}$  and expressed as nmol/mg of protein.

The data statistics was processed with MS Excel software, and represented as mean  $\pm$  deviation. The statistical significance was determined with standard Student's *t*-test.

## Results and Discussion

Liver mitochondria of rats with alimentary protein deprivation demonstrated no significant increase in production of superoxide anion radical (Fig. 1), hydrogen peroxide (Fig. 2) and hydroxyl radical (Fig. 3).

There were no statistically significant differences between carbonyl derivatives and free SH-groups content in liver mitochondria of this experimental group and the corresponding values of the control. We surmise that imbalance in functional activity of components of mitochondrial respiratory chain demonstrated by us [13] under alimentary protein deficiency results from interruptions in synthesis of particular subunits of the respiratory complexes, and not from a higher rate of their degradation.

We observed increased rates of generation of superoxide anion radical (1.3 times control values, Fig. 1) and hydroxyl radical (1.5 times control values, Fig. 3) in animals subjected to acetaminophen-induced liver lesions. Small quantities of ROS are normally produced in mitochondria as a result of

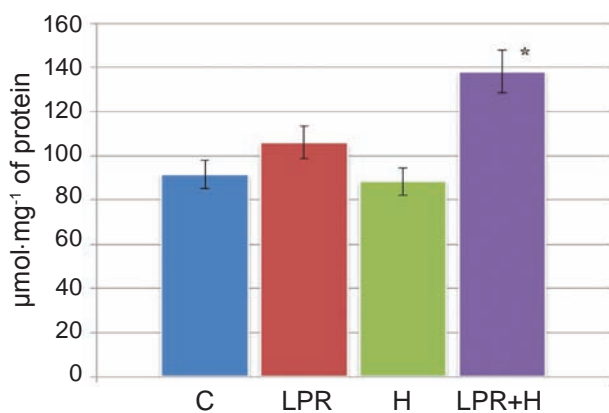


Fig. 2. Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) content in liver mitochondria under toxic hepatitis and alimentary protein deficiency

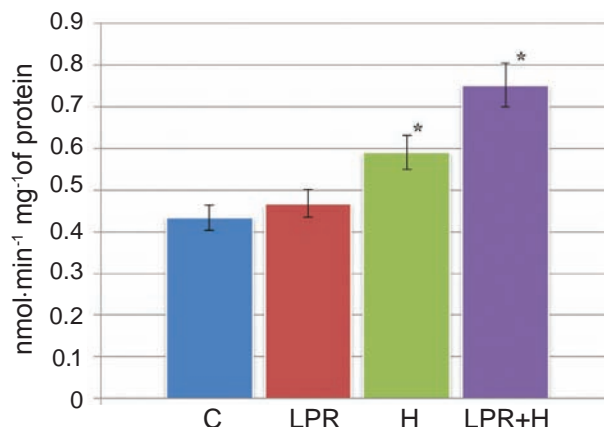


Fig. 1. Rate of superoxide anion radical ( $\text{O}_2^{\cdot-}$ ) generation in liver mitochondria under toxic hepatitis and alimentary protein deficiency. Here and in Fig. 2-5: \* difference from control significant with  $P < 0.05$ ,  $n = 9$ ; C – animals receiving complete semi-synthetic ration; LPR – animals receiving low-protein ration; H – animals subjected to acetaminophen-induced liver lesions receiving complete ration; LPR+H – animals subjected to acetaminophen-induced liver lesions that were previously fed semi-synthetic low-protein ration

electron leakage from the respiratory chain. The primary points of ROS generation in mitochondria are complexes I and III of the respiratory chain [6, 7]. Superoxide radicals from complex I dissipate into matrix and are quickly processed by mitochondrial superoxide dismutase into  $\text{H}_2\text{O}_2$ . The unstable semiquinone ( $\text{Q}^{\cdot}$ ) radical is the source of  $\text{O}_2^{\cdot-}$  in complex III. It must be noted that the superoxide

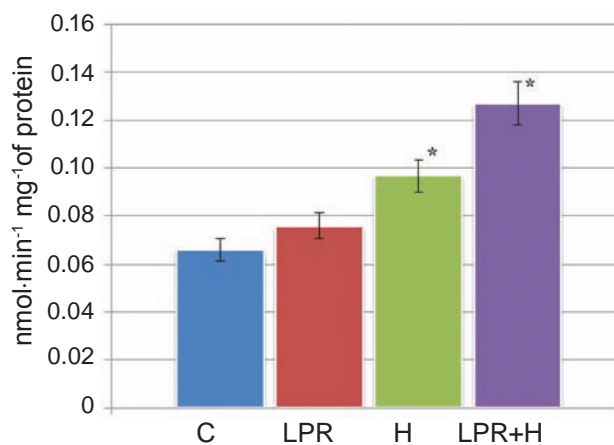


Fig. 3. Rate of hydroxyl radical ( $\cdot\text{OH}$ ) generation in liver mitochondria under toxic hepatitis and alimentary protein deficiency

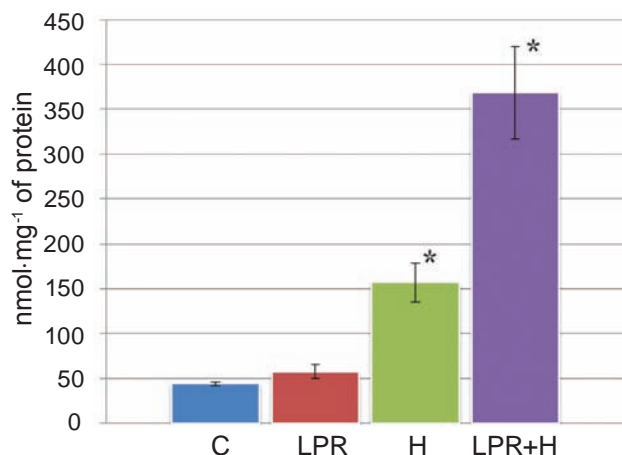


Fig. 4. Protein carbonyl derivatives content in liver mitochondria under toxic hepatitis and alimentary protein deficiency

radicals produced by complex I are vectored only in the direction of matrix, while the superoxide radicals from complex III are released both into matrix and into intermembrane space (and hence into cytosol). Other sources of ROS in mitochondria include outer membrane enzymes (cytochrome-*b*<sub>5</sub> reductase, monoamine oxidase A and B, dihydroorotate dehydrogenase, glycerol-3-phosphate dehydrogenase), inner membrane enzymes (succinate dehydrogenase), and matrix enzymes (aconitase,  $\alpha$ -ketoglutarate dehydrogenase) [21]. The ROS that are generated by complexes I and III cause their oxidative inactivation. Complex I is the most sensitive to free-radical damage, while complexes II and III are much less sensitive. Since most oxidative metabolic pathways converge on mitochondria, and they bear numerous redox transporters and centers of ROS production, the biomolecules of mitochondria are in the most vulnerable position under conditions of oxidative stress [22].

We have demonstrated that ROS production in liver mitochondria of rats with toxic liver lesions is associated with intensification of oxidative damaging to mitochondrial proteins. Content of carbonyl derivatives of proteins insoluble in 0.05 M sodium-phosphate buffer (pH 11.5) in liver of the animals from this experimental group was 3.4 times higher than the corresponding value in control (Fig. 4), while free SH-group content was 1.4 times lower (Fig. 5). The level of oxidative damage to a protein molecule depends on its amino acid composition, structure, and exposure of amino acid residues to radicals [23]. The primary targets of ROS are

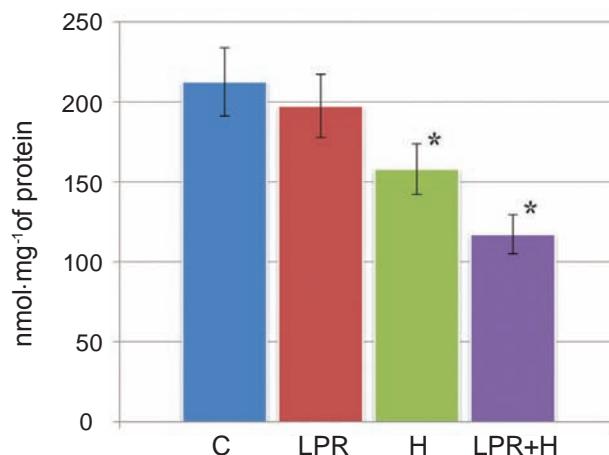


Fig. 5. Free SH-groups content in proteins of liver mitochondria under toxic hepatitis and alimentary protein deficiency

cysteine, histidine, tyrosine, phenylalanine, tryptophan, and methionine.

The intensified destruction of liver mitochondria proteins in animals with acetaminophen-induced hepatitis disrupts their functional activity. In accordance with observations from our previous experiments, the acetaminophen-induced hepatitis is associated with decreased enzymatic activity of the components of mitochondrial electron-transport chain [14, 16]. Therefore, the changes in oxidation of thiol groups in proteins of the respiratory chain insoluble in 0.05 M sodium-phosphate buffer (pH 11.5), and changes in content of their carbonyl derivatives may be perceived as one of the regulative factors of mitochondrial energy production.

Nevertheless, the most pronounced intensification of free-radical processes in liver mitochondria was observed under toxic hepatitis induced on the background of alimentary protein deprivation. Under these conditions we detected increased generation of all the investigated ROS: 1.7 times the control values for  $O_2^{\cdot-}$  (Fig. 1), 1.5 times for  $H_2O_2$  (Fig. 2), and 2 times for  $\cdot OH$  (Fig. 3). The nature of oxidative damage may vary depending on the chemical character of the particular ROS.  $\cdot OH$  chiefly causes protein aggregation, and protein fragmentation if acting concurrently with  $O_2^{\cdot-}$ . The aggregates are produced due to formation of intermolecular covalent bonds through tyrosine residues. The protein fragmentation results from cleavage of peptide bonds, and produces peptides with low molecular mass. Combined action of  $\cdot OH$  and  $O_2^{\cdot-}$  causes changes to primary, secondary and tertiary structure of the affected proteins [24].



$H_2O_2$  exhibits comparatively smaller modifying activity. Protein SH groups are the most sensitive to  $H_2O_2$ -induced oxidation. However, the damaging effect of  $H_2O_2$  on biological objects is amplified by its membrane-permeating qualities [25].

It must be noted that the most pronounced accumulation of oxidatively modified mitochondria-encoded proteins was observed under toxic hepatitis on the background of alimentary protein deficiency. The content of carbonyl derivatives of mitochondrial proteins was 6.8 times higher than the corresponding control value, and 2.3 times higher than the corresponding value for experimental animals fed standard fodder (Fig. 4). Thiol groups' content in the investigated proteins is nearly half that of control group (Fig. 5). Sulfhydryl groups (-SH) of cysteine are known to oxidize into -SOH, -SO<sub>2</sub>H, -SO<sub>3</sub>H, and disulfide derivatives [26]. Formation of intramolecular and intermolecular disulfide bonds leads to changes in protein structure or causes their dimerization, and, as a result, may affect the efficiency of respiratory chain. Changes in content of modified proteins under these experimental conditions may be viewed as a signal for rearrangement of mitochondrial energy generation.

Thus, the under toxic liver lesions on the background of alimentary protein deficiency we observed the most pronounced intensification of free radical-induced processes in liver mitochondria, associated with increased production of superoxide anion radical, hydroxyl radical, and hydrogen peroxide, as well as oxidative modifications of mitochondrial proteins.

These results lead to the conclusion that alimentary protein deficiency under toxic liver lesion conditions is a critical factor leading to increased oxidative damage to mitochondrial proteins.

The results may be the base for understanding of mechanisms of cellular metabolic response to alimentary protein deficiency, and biochemical grounds for developments in correction of consequences of imbalance in energy exchange under toxic hepatitis on the background of dietary protein deficiency.

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## **ОСОБЛИВОСТІ ВІЛЬНОРАДИКАЛЬНИХ ПРОЦЕСІВ У МІТОХОНДРІЯХ ПЕЧІНКИ ЩУРІВ ЗА ТОКСИЧНОГО ГЕПАТИТУ НА ТЛІ АЛІМЕНТАРНОЇ ПРОТЕЇНОВОЇ НЕДОСТАТНОСТІ**

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Досліджували інтенсивність генерації супероксидного аніон-радикала, гідроксильного радикала, пероксиду водню та рівень окислювальної модифікації мітохондріальних протеїнів у печінці щурів із токсичним гепатитом на тлі аліментарної протеїнової недостатності. Встановлено, що в мітохондріях печінки тварин, яких утримували на раціоні з нестачею протеїну, вірогідного підвищення інтенсивності вільнорадикальних процесів не спостерігалось. Найвираженіша інтенсифікація вільнорадикальних процесів у мітохондріях печінки відбувалася за умов токсичного гепатиту. Показано посилення генерації всіх досліджуваних активних форм кисню:  $O_2^{\cdot-}$  – в 1,7, а  $H_2O_2$  – в 1,5 рази,  $\cdot OH$  – практично в 2 рази на тлі акумуляції окисномодифікованих протеїнів, синтез яких детермінується мітохондріальним геномом. Встановлені зміни окисно-відновного стану тіолових груп протеїнів дихального ланцюга, нерозчинних в 0,05 М Na-фосфатному буфері (pH 11,5), та зміни вмісту їхніх карбонільних похідних можна розглядати як один із факторів регуляції енергетичних функцій мітохондрій.

**Ключові слова:** аліментарна протеїнова недостатність, ацетаминофен, індуковане пошкодження печінки, активні форми кисню, окислювальна модифікація протеїнів, протеїни мітохондріального кодування.

## ОСОБЕННОСТИ СВОБОДНОРАДИКАЛЬНЫХ ПРОЦЕССОВ В МИТОХОНДРИЯХ ПЕЧЕНИ КРЫС ПРИ ТОКСИЧЕСКОМ ГЕПАТИТЕ, ИНДУЦИРОВАННОМ НА ФОНЕ АЛИМЕНТАРНОЙ НЕДОСТАТОЧНОСТИ ПРОТЕИНА

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Исследованы интенсивность генерации супероксидного анион-радикала, гидроксильного радикала, пероксида водорода и уровень окислительной модификации протеинов митохондриального кодирования в печени крыс с токсическим гепатитом, индуцированным на фоне алиментарного дефицита протеина. Установлено, что в митохондриях печени животных, содержащихся на низкопротеиновом рационе, достоверного повышения интенсивности свободнорадикальных процессов не наблюдалось. Наиболее выраженная интенсификация свободнорадикальных процессов в митохондриях печени происходит при токсическом гепатите. Показано усиление генерации всех исследуемых активных форм кислорода:  $O_2^{\cdot-}$  – в 1,7 раза,  $H_2O_2$  – в 1,5 раза,  $\cdot OH$  – практически в 2 раза на фоне аккумуляции окисленных протеинов, синтез которых детерминируется митохондриальным геномом. Установленные изменения окислительно-восстановленного состояния тиоловых групп протеинов дыхательной цепи, нерастворимых в 0,05 М Na-фосфатном буфере (pH 11,5) и изменение содержания их карбонильных производных можно рассматривать как один из факторов регуляции энергетических функций митохондрий.

**Ключевые слова:** алиментарная протеиновая недостаточность, ацетаминофен, индуцированное повреждение печени, активные формы кислорода, окислительная модификация протеинов, протеины митохондриального кодирования.

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