The Study of Biochemical Parameters of Liver Mitochondria as Markers of Hypoxia in Burn Regeneration after Experimental Thermal Injury

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The aim of this work was to study pro- and antioxidant systems and energy-generating functions of mitochondria in case of combined thermal injury.

Materials and Methods. The experiment was carried out on male rats of Wistar line. Two groups were formed: control group 1 (n=10) of intact healthy animals; experimental group 2 (n=10) of animals with combined thermal injury (20% contact burns and thermal inhalation impact of hot air and combustion products). Animals were taken out of the experiment on days 1, 7, and 14 post-injury by decapitation under anesthesia (Zoletil 100 + XylaVET).

Mitochondria were obtained by differential centrifugation. For the mitochondria identification, an electron microscopic study was conducted. In the liver mitochondria, the intensity of free radical oxidation, the activity of catalase, superoxide dismutase, succinate dehydrogenase, and cytochrome c oxidase were evaluated. The research results were processed using Statistica 6.0 (StatSoft Inc., USA).

Results. An increase of intensity of free radical oxidation in the liver mitochondria on days 7 and 14 post-injury was registered. Meanwhile, total antioxidant activity of blood plasma and catalase activity in erythrocytes in case of thermal injury were decreasing on all test days after the burn as compared to the control group.

Towards days 7 and 14, superoxide dismutase activity significantly decreased in comparison with the healthy animals. The study of succinate dehydrogenase and cytochrome c oxidase showed a decrease in specific activity of enzymes in the liver mitochondria on days 1, 7, and 14 after combined thermal injury. The most pronounced decrease in the activity of succinate dehydrogenase and cytochrome c oxidase was observed on day 14 after the burn.

Conclusion. A presence of oxidative stress during combined thermal injury was revealed, as well as a comprehensive mechanism of its formation, implicating both activation of free radical oxidation and decrease in antioxidative capacity imbalance in the functioning of prooxidant and antioxidant systems of the body. Inhibition of energy supply of cells, reduction in the cell aerobic and increased anaerobic oxidation were revealed.

Key words: mitochondria; combined thermal trauma; free radical oxidation; antioxidant system; succinate dehydrogenase; cytochrome c oxidase.

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Introduction

The study of structural and functional features of mitochondria enhances understanding about the origins and development of many pathological processes at the cellular level and in the organism. Damages associated with characteristic abnormalities of the biochemical processes of the cell form the basis of specific clinical manifestations of human diseases. Burns are one of the most important problems of modern medicine as well as of surgical practices. Pathological changes developing in the liver in burn disease lead to the hypoxia and the disruption of many organism functions. A comprehensive study of the processes occurring in the conditions of

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oxygen lack is vital in the burn disease. In this situation, the metabolic disorder is one of the most characteristic hypoxia manifestations [1].

Increase of free radical oxidation (FRO) and the disruption of the antioxidant defense system are the most pathological changes induced in organs and tissues during hypoxia and lead to oxidative stress [2]. Oxidative stress causes an increase in intracellular generation of reactive oxygen species (ROS) and oxidative damage of molecular structures in cells [3].

Mitochondria accumulate a lot of the oxidative metabolic pathways and contain numerous redox vectors and sites potentially capable to one-electron reduction of oxygen into superoxide anion ($O_2^{\bullet-}$) — the precursor of other ROS [4]. Generated $O_2^{\bullet-}$ may be involved in the regulation of cell metabolism or eliminated by mitochondrial superoxide dismutase [5]. The energy and oxidative metabolism of hepatocytes are determined by the state of the respiratory chain of mitochondria, which depends on the functioning of the liver.

The level of production of ROS in the mitochondria of hepatocytes significantly increases in pathologies of the liver [6]. However, the mechanism of increased production of $O_2^{\bullet-}$ in the respiratory chain of mitochondria in thermal injury is practically not studied. In addition, a study of the metabolic state of rat liver mitochondria could help in the development of adequate tactics of burn disease treatment.

Evaluation of the activity of succinate dehydrogenase (SDH) can serve as one of the criteria of hypoxia. SDH largely determines the rate of consumption of oxygen and formation of adenosine triphosphate (ATP) in the respiratory chain of mitochondria [7].

Thus, the study of regulation of mitochondrial functions is one of the most important problems, the solution to which will contribute to the normalization of energy processes playing a critical role in the metabolism of cells in hypoxia after burn disease.

The aim of this work was to study prooxidant and antioxidant systems, energy-generating functions of mitochondria in case of combined thermal injury.

Materials and Methods

The experiment was carried out on 20 white male rats of Wistar line weighing among 200–250 g received from the Stolbovaja farm (Moscow, Russia). All animals were kept in standard vivarium conditions in cages with free access to food and water. Research work was carried out according to the rules of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 2006). After the quarantine (14 days) two groups of animals were formed: control group 1 (n=10) of intact healthy animals; experimental group 2 (n=10) of animals with combined thermal injury (CTI).

Combined thermal injury including contact burns on 20% of the body surface and thermo-inhalation exposure to hot air and combustion products for 20–30 s in the inhalation chamber was performed under anesthesia (Zoletil 100, VIRBAC, France (60 mg/kg) + XylaVET, Pharmamagist Ltd., Hungary (6 mg/kg)) [8]. Animals were taken out of the experiment on days 1, 7, and 14 by decapitation with preliminary carotid artery cut performed under anesthesia (Zoletil (60 mg/kg) + XylaVET (6 mg/kg)).

Mitochondria were obtained by differential centrifugation. For the mitochondria identification, the electron microscopic study was conducted. For electron microscopic study, the material was preliminarily fixed according to the standard procedure in 2.5% glutaraldehyde in phosphate buffer followed by additional fixation with tetroxide of osmium. After washing with buffer solution, transaction in alcohols of the increasing strength, and impregnation, the material was enclosed in epoxy resin. Preparation of ultrathin sections of 100 nm from the polymerized Epon-Araldite blocks was carried out on the PC Power Tome ultramicrotome (RMC Products, USA). For the contrast enhancement, we used a double staining of ultrathin sections with uranyl acetate and lead citrate. Electron microscopic study of the mitochondria fraction was performed on the transmission electron microscope HT7700 (Hitachi, Japan).

Prooxidant and antioxidant status in the liver mitochondria was assessed. The activity of FRO was studied using the method of induced biochemiluminescence on the BHL-06 device (Medozons, Russia). The following parameters of biochemiluminescence were evaluated: tg 2α, characterizes the speed of reduction of FRO processes in the plasma and indicates total antioxidant activity; S, the light sum of chemiluminescence in 30 s, reflects the potential ability of a biological object to FRO. The concentration of malonic dialdehyde was evaluated by Mihara, Uchiyama and Fukuzawa [9]. For the evaluation of catalase activity, we used the spectrophotometric method based on the determination of the rate of decomposition of hydrogen peroxide by catalase from the sample [10]. The activity of superoxide dismutase (SOD) was determined by inhibition of formation of adrenaline oxidation product [11]. The activity of SDH was determined by a method based on the use of artificial electron acceptors [12]. The catalytic activity of cytochrome c oxidase was recorded according to the rate of oxidation of cytochrome c by the method Schwitzguebel and Siegenthaler [13]. Protein concentration was calculated by the modified method of Lowry [14].

Statistical data processing was performed by the software (Statistica 6.0 (StatSoft Inc., USA). The significance of differences between groups was assessed using Student's t-test. The differences were considered statistically significant at $p<0.05$.

Results

In the first phase of the study, the quality of the mitochondrial fractions obtained with the chosen method was assessed and evaluated. The obtained samples

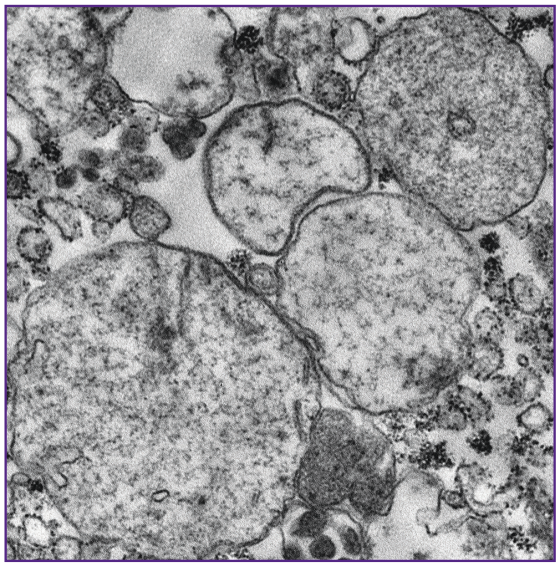

Figure 1. Electron microscopic image of the mitochondria fraction from rat liver, transmission electron microscope HT7700 Hitachi (38,000×)

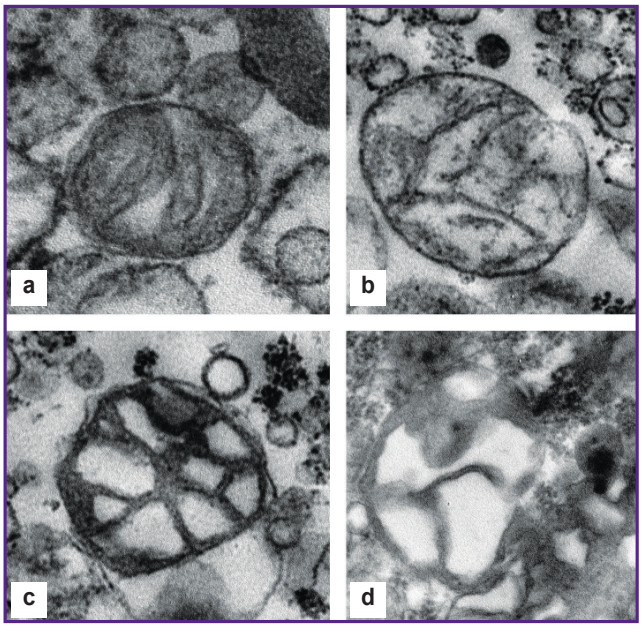

Figure 2. Heterogeneity of ultrastructure of the mitochondrial fraction from rat liver, transmission electron microscope HT7700 Hitachi (22,000×):
(a) a dense matrix and clear-cut cristae of the mitochondria; (b)–(d) a change of the electron density of the mitochondrial matrix, its focal lysis, clarification as well as shortening, fragmentation, and reduction of cristae

are informative and suitable for high-quality electron microscopic photographs (Figure 1).

The study revealed the polymorphism of mitochondrial fractions: small and large mitochondria with intact membranes were present in the examined samples. A part of the mitochondria had a dense matrix and clear-cut cristae (Figure 2 (a)).

In addition to mitochondria with preserved structure, swollen mitochondria with violation of intra-mitochondrial architectonics were found. In some mitochondria a change of the electron density of the mitochondrial matrix, its focal lysis, clarification as well as shortening, fragmentation, and reduction of cristae (Figure 2 (b)–(d)) were observed. A part of the mitochondria, in contrast, had a dense granular matrix, masking the cristae.

Also, electron-dense rosettes of glycogen granules, inclusions of cisterns of the endoplasmic reticulum and multiple ribosomes were present in the studied samples, including in the form of clusters on the mitochondrial membranes (Figure 3).

All of the aforementioned ultrastructural changes in the form of the degeneration of the elements in the mitochondrial fraction may represent a consequence of the peculiarities of sample preparation. The quality of the material appears to be satisfactory and sufficient for further evaluation of metabolic changes with the selected methods.

Mitochondria are the indicators of the functional state of cells involved in metabolism via the Krebs cycle and transport of electrons in the respiratory chain. The energy produced by mitochondria is converted and stored within the molecules of ATP as the result of oxygen-dependent free radical reactions related to the oxidation in the respiratory chain of mitochondria [6].

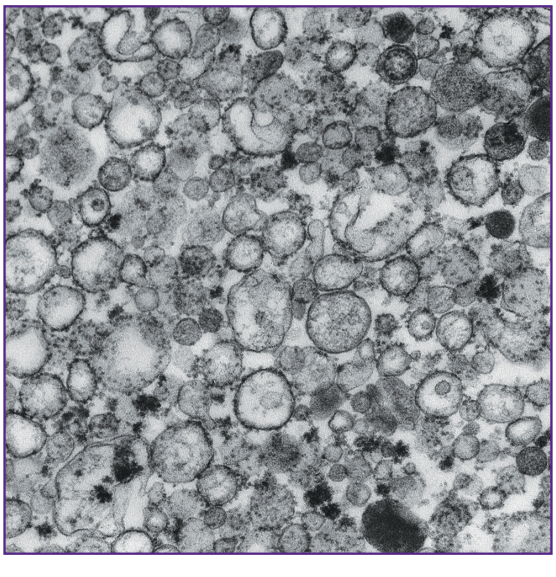

Figure 3. Electron microscopic image of the mitochondria fraction from rat liver, transmission electron microscope HT7700 Hitachi (10,000×)

According to biochemiluminescence (S) and content of malonic dialdehyde in liver mitochondria no statistically significant differences between control and experimental groups (day 1 after the trauma) were revealed. This was probably caused by an increase of consumption of fatty acids, mainly polyunsaturated [15]. An increase in the

intensity of FRO was observed in the liver mitochondria on day 7 (by 1.2 times, p=0.017) and day 14 (by 1.4 times, p=0.023) after injury (see the Table). Similar results were obtained in the study of the concentration of the intermediate FRO product. The malonic dialdehyde concentration in the liver mitochondria increased on day 7 after CTI by 1.1 times (p=0.035), on day 14 — by 1.4 times (p=0.041) in comparison with that of the intact rats. The growth of FRO during the burn was not accompanied by an increase in the total antioxidant protection. While there was a decrease in total antioxidant activity on all tested days after thermal injury: on day 1 after CTI — by 1.7 times (p=0.018), on day 7 — by 1.2 times (p=0.009), on day 14 — by 1.7 times (p=0.011) in comparison with the control.

A compensatory increase of the specific activity of a key enzyme of the antioxidant system SOD by 1.1 times (p=0.037) was observed on day 1 after CTI in comparison to healthy animals. On days 7 and 14 SOD activity decreased by 1.8 times (p=0.036 and p=0.041, respectively) in comparison to healthy animals (Figure 4).

The intensity of free radical oxidation in liver mitochondria of rats in the experiment with combined thermal injury

The conditions of the experiment	S (RU)	tg 2α (RU)	Malonic dialdehyde (μmol/L)
Control	9.66±0.12	0.929±0.011	4.579±0.045
Burn, day 1	9.64±0.23	0.534±0.015*	4.494±0.051
Burn, day 7	11.93±0.17*	0.755±0.022*	4.984±0.028*
Burn, day 14	13.66±0.34*	0.534±0.030*	6.476±0.018*

* Differences were statistically significant compared with the control (p<0.05).

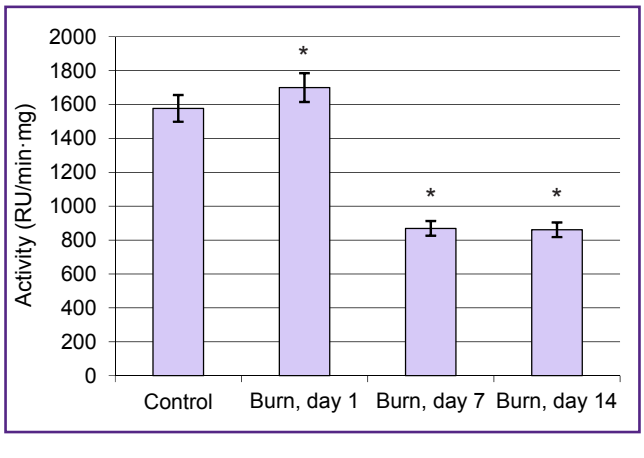

Figure 4. Superoxide dismutase activity in the liver mitochondria of rats in the experiment with combined thermal injury

* Differences were statistically significant compared with the control (p<0.05)

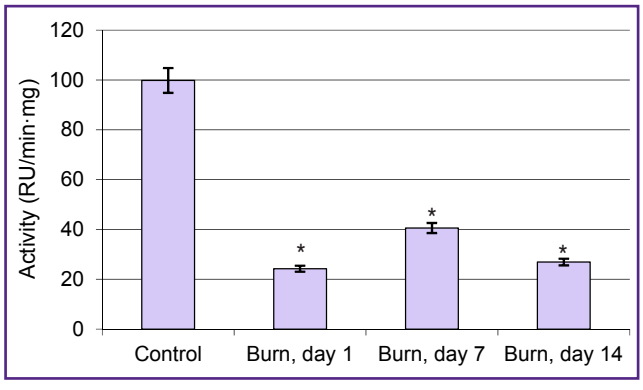

Figure 5. Catalase activity in the liver mitochondria of rats in the experiment with combined thermal injury

* Differences were statistically significant compared with the control (p<0.05)

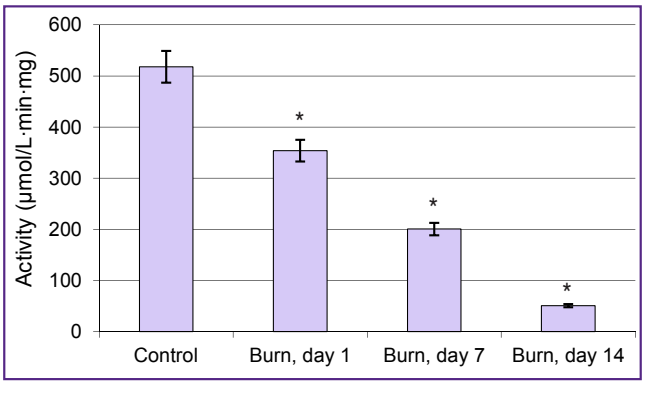

Figure 6. Succinate dehydrogenase activity in the liver mitochondria of rats in the experiment with combined thermal injury

* Differences were statistically significant compared with the control (p<0.05)

Catalase activity decreased on day 1 after CTI by 4.1 times (p=0.001), on day 7 — by 2.5 times (p=0.001), on day 14 after injury — by 3.7 times (p=0.003) in comparison to control (Figure 5).

The study of one of the key enzymes of the citric acid cycle of Krebs, SDH, involved in the reaction of dehydrogenation of succinic acid to fumaric acid showed a decrease in the specific activity of SDH (complex II) in mitochondria of the liver on days 1, 7, and 14 after CTI by 1.1 times (p=0.016), by 2.6 times (p=0.017), by 10.2 times (p=0.008), respectively in comparison with healthy rats (Figure 6).

A decrease in the activity of cytochrome c oxidase (complex IV of the respiratory chain of mitochondria) transferring electrons from cytochrome c to molecular oxygen was revealed on days 1, 7, and 14 after burn by 1.5 times (p=0.002), by 1.9 times (p=0.001), and by 2.4 times (p=0.001), respectively in comparison to healthy animals (Figure 7).

Therefore, the activity of SDH and cytochrome c

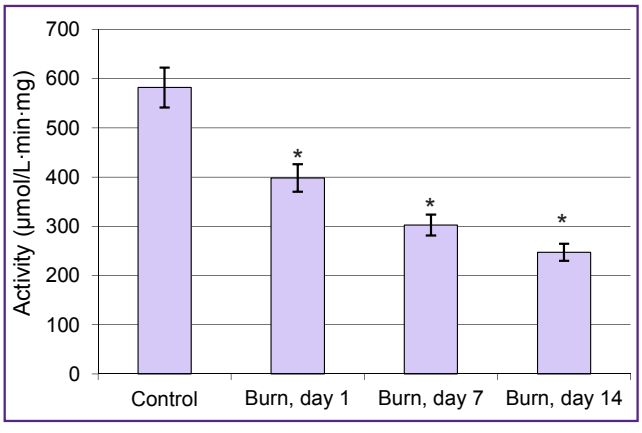

Figure 7. Cytochrome c oxidase activity in the liver mitochondria of rats in the experiment with combined thermal injury

* Differences were statistically significant compared with the control ($p<0.05$)

oxidase demonstrated the maximum decrease on day 14 after CTI.

Discussion

The reduction of catalytic properties of SOD in a burn was probably the result of the intensification of redox processes and reduction of energy metabolism in the mitochondria. The increased formation of superoxide radical during the activation of FRO leads to substrate inhibition of superoxide dismutase in liver mitochondria of rats with CTI.

There is also evidence of an increase of FRO processes of lipids and proteins with inhibition of the antioxidant systems in patients with thermal injury in the literature [16]. Reasons for the imbalance in the system of prooxidants–antioxidants may be the impairment of lipid and protein profile of mitochondria in thermal injury [17]. On the other hand, the activation of the FRO in the mitochondria during the burn can be one of the causes of damage of the liver tissue.

One of the possible mechanisms of changes in the activity of catalase in burn disease may be a conformational modification of the enzyme molecule under conditions of oxidative stress.

Thus, the obtained results confirm information about the presence of oxidative stress manifested in the enhancement of FRO and the decrease of general and enzymatic activity of antioxidant defense system in thermal injury. This leads to the development of systemic disorders during the burn. Causes of oxidative stress under CTI may be excessive ROS production, the activation of the FRO and the negative response of reserves of the antioxidant protective system. The development of oxidative stress leads to an increase of destructive processes, which may be a pathogenetic factor of burn disease.

The decrease in the specific activity of SDH in mitochondria of the liver on days 1, 7, and 14 after CTI indicates a decrease in succinate dependent respiration in the mitochondria and reduction of activity of the citric acid cycle. SDH is associated with a chain of electron transfer and in contrast to other enzymes of the Krebs cycle is an integral protein of the internal mitochondrial membrane. Two electrons of flavin adenine dinucleotide ($FADH_2$) are directly transferred to the iron atoms of the enzyme. Specific activators of SDH are ATP and restored ubiquinone played a role as an acceptor of electrons from succinate dehydrogenase complex (where SDH is the first enzyme, and cytochrome c oxidase — the last) in the mitochondria [7].

In addition, the decrease in the activity of SDH in the conditions of O_2 deficiency, developing in the cells in thermal injury [18], is due to the activation of fumarate reductase reaction. Fumarate reductase reaction is the reverse of the reaction catalyzed by SDH in the composition of the complex II system of tissue respiration. In this process, free oxygen radicals that can directly inactivate SDH are quickly formed [19]. Therefore, the increase of their formation in conditions of lack of oxygen led to a decrease of SDH activity.

The decrease in activity of SDH and cytochrome c oxidase at CTI indicates a reduction of aerobic metabolism, enhancement of anaerobic oxidation in the cell and decrease of energy supply of the cells. These changes appear from the disruption of electron transport in the mitochondrial membrane and deficiency of ATP production.

Probably a large number of ROS generated in thermal injury are associated with the metal atoms within cytochrome c oxidase, inhibiting the enzyme.

However, according to the literature data, information about the state of the respiratory chain in burn disease is very divergent and insufficient. Some authors argue that the components of the respiratory chain in the pathology of the liver are seriously damaged therefore ATP decreases [20]. However, detailed studies of the mechanisms of these disorders have not been carried out yet. Other authors consider that the components of the respiratory chain are not damaged, but unable to change their functional activity [21]. The obtained results allow the evaluation of the energy metabolism changes during hypoxia developing in burn disease. The study obtained fundamental data on changes in aerobic and anaerobic metabolism of mitochondria of the liver, which will help to expand existing concepts on the pathogenetic mechanisms of hypoxia during pregnancy with the subsequent possibility of searching for ways of its correction. A critical decline of oxygen tension in tissue in a thermal injury leads to activation of anaerobic formation of energy in the cell.

Conclusion

The obtained data confirm, firstly, the presence of mitochondrial oxidative stress during combined thermal

injury and, secondly, a comprehensive mechanism of its formation, including activation of free radical oxidation and decrease in antioxidative potential. The inhibition of energy support of mitochondria was detected during the burn and manifested itself through decreased activity of succinate dehydrogenase and cytochrome c oxidase in mitochondria, which is a sign of reduction of aerobic oxidation and intensification of anaerobic metabolism in the cell.

In the current study, it was demonstrated that identification of the activities of antioxidant enzymes and succinate dehydrogenase as well as of cytochrome c oxidase can be used as markers of metabolic disturbances in the liver during hypoxia due to thermal injury.

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Conflict interest. The authors declare that they have no conflict interests.

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