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Applying C_{60} Fullerenes Improve the Physiological State of Rats with Ischemia–Reperfusion Injury of Skeletal Muscle

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The effect of water-soluble pristine C_{60} fullerenes as powerful antioxidants on the biochemical parameters of blood of rats under the ischemia–reperfusion injury of the skeletal muscle depending on its active and inactive states, as well as duration of this pathology, is studied. Levels of enzymes (creatine phosphokinase and lactate dehydrogenase) and their metabolic products (creatinine and lactic acid) in blood are measured for the evaluation of general physiological state of experimental rats. Moreover, levels of some components of antioxidant system, namely catalase, reduced glutathione, thiobarbituric-acid reactive substances, and hydrogen peroxide as indicators of lipid peroxidation and oxidative stress, are also measured. The pronounced tendency to decrease of these biochemical parameters of the blood at average by 20–25% in the experimental groups (1 mg/kg intramuscular introduction of water-soluble pristine C_{60} fullerene immediately after muscle reperfusion) compared to animals without the C_{60}-fullerene introduction is shown regardless of the muscle-ischemia duration of 1, 2 or 3 h.

Досліджено вплив водорозчинних немодифікованих C_{60}-фуллеренів як потужніх антиоксидантів на біохемічні показники крові щурів за ішемічно-реперфузійного пошкодження скелетного м'яза залежно від його активного та неактивного станів, а також тривалости цієї патології. Для оцінки загального фізіологічного стану піддослідних щурів визначали рівні ферментів (креатинфосфокінази та лактатдегідрогенази) й їхніх метаболічних продуктів (креатиніну та молочної кислоти) у крові. Крім того, були визначені рівні деяких компонентів антиоксидантної системи, зокрема каталази, відновленого глутатіону, реактивних речовин тіобарбітурової кислоти та пероксиду водню як індикаторів перекисного окиснення ліпідів та окиснювального стресу. Показано, що у дослідних групах тварин (1 мг/кг-внутрішньом'язове введення водорозчинного немодифікованого C_{60}-фуллерену одразу після реперфузії м'яза) спостерігається виражена тенденція до зменшення цих біохемічних показників крові в середньому на

20–25% порівняно з контролем (тварини без введення C_{60}-фуллерену) незалежно від тривалости ішемізації м'яза в 1, 2 чи 3 години.

Исследовано влияние водорастворимых немодифицированных C_{60}-фуллеренов как мощных антиоксидантов на биохимические показатели крови крыс при ишемически-реперфузионном повреждении скелетной мышцы в зависимости от её активного и неактивного состояний, а также продолжительности этой патологии. Для оценки общего физиологического состояния подопытных крыс измеряли уровни ферментов (креатинфосфокиназы и лактатдегидрогеназы) и их метаболических продуктов (креатинина и молочной кислоты) в крови. Кроме того, были измерены уровни некоторых компонентов антиоксидантной системы, в частности каталазы, восстановленного глутатиона, реактивных веществ тиобарбитуровой кислоты и пероксида водорода в качестве индикаторов перекисного окисления липидов и окислительного стресса. Показано, что в опытных группах животных (1 мг/кг-внутримышечное введение водорастворимого немодифицированного C_{60}-фуллерена сразу после реперфузии мышцы) наблюдается выраженная тенденция к уменьшению этих биохимических показателей крови в среднем на 20–25% по сравнению с контролем (животные без введения C_{60}-фуллерена) независимо от продолжительности ишемизации мышцы в 1, 2 или 3 часа.

Key words: C_{60} fullerene, skeletal muscle, ischemia–reperfusion injury, biochemical blood parameters.

Ключові слова: C_{60}-фуллерен, скелетний м'яз, ішемічно-реперфузійна травма, біохемічні показники крові.

Ключевые слова: C_{60}-фуллерен, скелетная мышца, ишемически-реперфузионная травма, биохимические показатели крови.

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1. INTRODUCTION

Ischemia–reperfusion injury of skeletal muscle could be explained *via* the cascade of cellular events, which were triggered by the following reperfusion [1]. During ischemia, the constant decline of the cellular energy store takes place. Despite the role of creatine phosphate that is present in muscles, the concentration of ATP falls. Also by producing a little energy and lactate, the glycogen store becomes depleted [2]. Under the reperfusion, reactive hyperaemia develops that leads to the increase of blood circulation in a muscle. However, this flow of blood washes out the substances, which are needed as precursors of adenine nucleotide resynthesize.

Reactive oxygen species (ROS) are formed in a process of membrane lipid peroxidation (LPO) and reoxygenation under the effect of impaired phosphorylation in mitochondria. Sequestration of white blood

cells and molecules of leucocyte adhesion into the muscle provokes the enlargement of injury during reperfusion. This leads to the consequential damage of organs, which are located far from a place of injury, namely, lungs, heart and kidneys [3]. Therapy of ischemia injuries depends on its level that has a connection with the time of ischemia induction. Early-stage treatment of the light forms of ischemia gives near 100% positive results. In contrast, curing of severe forms of ischemia almost always is ineffective. Time wasted for incorrect therapy of full damaged muscle often leads to the complications related to organism intoxication or even death. The future of therapeutic approaches for minimization of ischemia trauma belongs to those, which are focused on modulation of ischemia–reperfusion effect for effective diagnostic of severity of ischemia-induced damage. In this context, an antioxidant therapy may play an important role [3, 4].

The ability of the biocompatible C_{60} fullerenes and their derivatives to inactivate the ROS was first demonstrated in Ref. [5]. C_{60} fullerene is more powerful antioxidant than natural antioxidant Vitamin E in preventing the integrity of membranes from damage and, thus, contributes to the maintenance of transmembrane potential [6]. It has been established that pristine C_{60} fullerenes have a dose-dependent protective effect against oxidative-mediated muscle trauma [7, 8].

The majority of experimental data show that pristine C_{60} fullerenes are not toxic nanostructures of *in vitro* and *in vivo* systems [9, 10]. So, C_{60} fullerene aqueous colloid solutions show no *in vivo* toxicity in doses lower 25 mg/kg [11]. Water-soluble C_{60} fullerene derivatives do not show acute *in vivo* toxicity for 200–500 mg/kg [12]. It was established that intraperitoneal introduction of C_{60} fullerene suspension in a dose of 2.5 g/kg does not lead to mice death or to violations of their behaviour within 8 weeks [13].

Moreover, C_{60} fullerene aqueous colloid solutions protect rats' liver for the ROS [14, 15]. Herewith, the concentration of accumulated C_{60} fullerenes in hepatocytes decreased in time that indicates their ability to excrete from rats' liver.

Water-soluble pristine C_{60} fullerenes in low concentrations are not toxic for normal cells [16]. Recently, the impact of water-soluble pristine C_{60} fullerene on *Drosophila melanogaster* at DNA, tissue and organism levels was tested [17]. It was found that C_{60} fullerene at the concentration of 40 μg/ml does not affect the reproductive system and embryogenesis. At the same time, C_{60} fullerene can induce DNA damage, but activation of DNA-repair decreases this negative effect at the organismal level. It contributes for further medical and biological applications of C_{60} fullerene.

Given the accumulated data about the powerful antioxidant properties of C_{60} fullerenes [18, 19], the purpose of this work was to study their effect on the some biochemical parameters of blood in rats under

ischemia–reperfusion injury of the skeletal muscle depending on its active and inactive states as well as duration of this pathology.

2. EXPERIMENTAL

2.1. Nanomaterials

A highly stable reproducible C_{60}-fullerene aqueous colloid solution (C_{60}FAS) was prepared according to protocol [20, 21]. Briefly, for the preparation of C_{60}FAS, we used a saturated solution of pure C_{60} fullerene (purity > 99.99%) in toluene with a C_{60} molecule concentration corresponding to maximum solubility near 2.9 mg/ml, and the same amount of distilled water in an open beaker. The two phases formed were treated in ultrasonic bath. The procedure was continued until the toluene had completely evaporated and the water phase became yellow coloured. Filtration of the aqueous solution allowed separating the product from undissolved C_{60} fullerenes. The purity of prepared C_{60}FAS samples was determined by HPLC and GC/MS techniques using standard programs. The concentration of C_{60} fullerene in the prepared

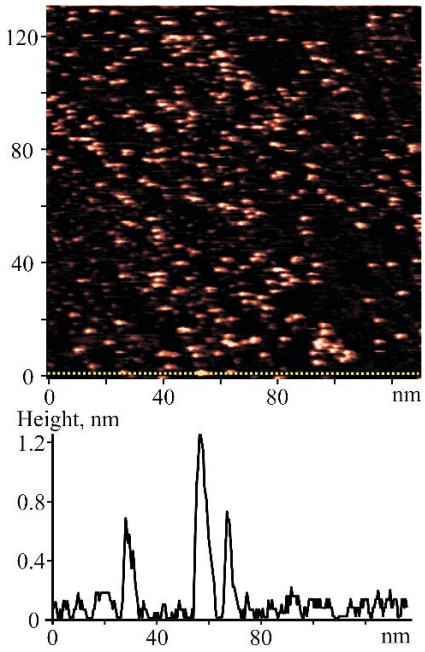

Fig. 1. The scanning tunnelling microscopy image of C_{60} fullerenes deposited from C_{60}FAS (0.15 mg/ml) on Au(111) surface (SPI Supplies, USA). Scanning parameters: I_t = 93 pA, U_t = 713 mV. The height of nanoparticles was estimated along the cross-section line (Z-profile) marked by yellow.

C_{60}FAS sample was determined as the concentration of total organic carbon in aqueous solution (Analytik Jena TOC Analyser multi N/C 3100). In our experiments, the C_{60}FAS sample with 0.15 mg/ml concentration of C_{60} fullerene was used.

The scanning tunnelling microscopy (STM; NT-MDT, Russia) data (Fig. 1) clearly indicates the presence in C_{60}FAS of both the individual C_{60} fullerenes (0.72 nm in diameter) and their nanoparticles with a height more than 1.2 nm.

2.2. Animals

Animals (male rats of *Wistar* line) were kept under standard conditions in the vivarium of the ESC 'Institute of Biology and Medicine', Taras Shevchenko National University of Kyiv. Animals had free access to food and water. All experiments were conducted in accordance with the international principles of the European Convention for protection of vertebrate animals under a control of the Bio-Ethics Committee of the above-mentioned institution.

All studied animals (weight 150–170 g) were divided into following groups: intact group ($n = 10$), control group (animals after ischemia with saline 1 mg/kg intramuscular injection immediately after reperfusion; $n = 10$), and experimental group (animals after ischemia with C_{60}FAS 1 mg/kg intramuscular injection immediately after reperfusion; $n = 10$).

Under deep anaesthesia (ketamine; 100 mg/kg 'Pfizer', USA), after tracheotomy, an artificial lung ventilation has been started. In a region of popliteal fossa, a *musculus soleus* was separated, cut proximally and mounted to the force transducers. The animal was immobilized in the stereotaxic frame with strong fixation of head, pelvis and limbs. Nerve that innervates a *musculus soleus* was mounted on the bipolar platinum electrode, which was used for stimulation. During the surgery and the whole experiment, the animal heart rate was constantly monitored. To develop ischemia injury, the branch of femoral artery, which supplies blood flow to muscle, was ligated for 1, 2 or 3 h.

The control of muscle contraction force was performed using strain gauges [22]. Electrical impulses lasted 2 ms with frequency 2 Hz were applied during 30 min for stimulation of afferents in L7-S1. External load on the muscle was controlled by the system of mechanical stimulators [23].

2.3. Biochemical Analysis

Levels of enzymes (creatine phosphokinase (CPK) and lactate dehydrogenase (LDH)) and their metabolic products (creatinine and lactic acid

(LA)), as well as components of antioxidant system (catalase (CAT), reduced glutathione (GSH), thiobarbituric acid reactive substances (TBARS) and hydrogen peroxide (H_2O_2)) in blood of rats, which characterize their muscle activity, were examined by clinic-diagnostic methods and equipment (Selectra Pro XL EliTechGroup, France).

Biochemical data are expressed as the means ± SEM for each group. The differences among experimental groups were detected by one-way ANOVA followed by Bonferroni's multiple comparison test. Values of $p < 0.05$ were considered as significant.

3. RESULTS AND DISCUSSION

Such oxygen active compounds as superoxide and hydroxyl radicals could mediate microvascular and parenchyma damage induced by ischemia–reperfusion injury of the skeletal muscle [3, 4]. Death of the majority of muscular cells occurs to chemical substances, which are formed during and after ischemia and can last several days even after normalization of blood supply [3]. That is why biochemical changes in blood under ischemia are important parameters during the prophylactic treatment and, especially, for making fast decisions.

Until now, it is known that, after 2 h of the skeletal muscle ischemia with following reperfusion, an ATP concentration decreases dramatically. At the same time, the level of lactate significantly increases. After 3 h of ischemia, the pool of ATP in a muscle is only 5% from initial value, and glycogen stored falls up to 88% [2]. This indicates the significant amount of energy used for homeostasis maintenance, especially, during the first hour of ischemia [24]. As a result, the metabolic disturbance leads to a marked increase of muscular fatigue.

However, the ROS production is the main pathological cause in a process of ischemia–reperfusion damage of muscular tissue. Free radical processes include triggering of LPO, direct inhibition of mitochondrial respiratory chain enzymes, inactivation of glyceraldehyde-3-phosphate-dehydrogenase, suppression of APTase activity, inactivation of membrane sodium channels, and some other pathological events [3, 25].

All facts listed above make ischemia–reperfusion trauma to be considered as the complex pathological process that affects many metabolic aspects directly in site of injury as well as in the whole organism. Because biochemical changes in blood were found to reflect those in a muscle during pathological process, evaluation of blood biochemical content give direct data about biochemical changes in an active muscle. Blood parameters selected for this investigation display a strong tendency to enlarge both with the development of pathology and after intensive physical activity [2, 3, 24, 25].

For the evaluation of the general physiological state of the ischemic

muscle, we chose those markers, which could be measured using standard biochemical methods and are specific for ischemia-damaged muscles. Such markers include CPK, LDH, creatinine and LA.

CPK is an enzyme that catalyses transfer of phosphate group from ATP to creatine with formation of creatine phosphate, which is the energy substrate. When muscle is damaged, this enzyme is released from cells into the blood. We suggest that increase of a CPK fraction with the increase of ischemia time (Fig. 2) from 789 units/l in a control up to 1178, 1346 and 167 units/l after 1, 2 and 3 h of ischemia (without muscle stimulation), respectively, is the result of pathological destruction of myocytes' cell membrane that is followed by intracellular-enzymes' release into the tissue environment. This is also the reason for the increase in CPK level after activation of ischemia-damaged muscle from 854 units/l in a control (intact rats) to 1334, 1664 and 2208 units/l after 1, 2 and 3 h of ischemia, respectively. It is important to note that, in active state of a muscle, a CPK level after 1 h of ischemia is almost equal to that after 2 h of ischemia but for an inactive muscle. After 2 h of ischemia in active muscle, a CPK output is the same as in resting muscle after 3 h of ischemia.

LDH catalyses oxidation of lactate to pyruvate with generation of

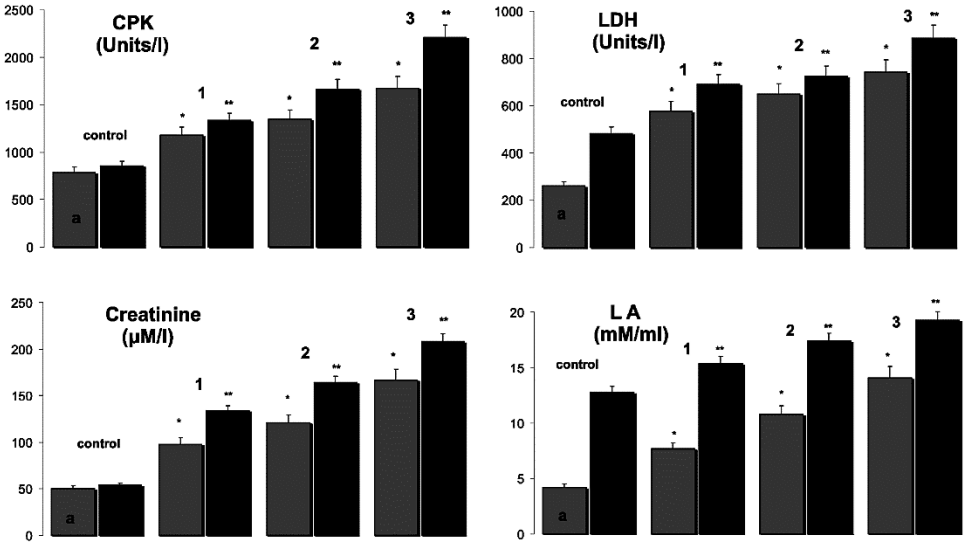

Fig. 2. Levels of enzymes (CPK and LDH) and their metabolic products (creatinine and LA) in blood of rats without muscle stimulation (*a*) and after 30 min of stimulation with 2 Hz-frequency of a current (*b*): control—intact rats; 1, 2 and 3—experimental rats under ischemia–reperfusion trauma of muscle for 1, 2 and 3 h, respectively. Values are $M \pm SD$, $n = 10$; $^{*}p < 0.05$ in comparison with control (experiment without muscle stimulation); $^{**}p < 0.05$ in comparison with control (experiment with muscle stimulation).

NADP in almost all cells in the body. In diseases, which are followed with tissue damage and cell destruction, the LDH blood level increases. The LDH concentration had elevated after even 1 h of ischemia of *musculus soleus* (without stimulation): from 261 units/l in a control (intact rats) to 578 units/l (Fig. 2). Moreover, it was increasing with the enlargement of ischemia period: up to 649 and 742 units/l for 2 and 3 h of ischemia, respectively. These data are consistent with those from other studies [2, 3, 22] in the aspect of LDH concentration increases in response to enlargement of ischemia duration. Activation of ischemic muscle results in significant increase of LDH level, namely, up to 690, 724 and 887 units/l for 1, 2 and 3 h of ischemia, accordingly. It is worth mentioning that mostly LDH concentration was higher in active ischemic muscle than its level in inactive muscle during increase of ischemia time.

Creatinine is formed in muscles from creatine, which provides the body with energy for muscle contraction. In case of ischemia–reperfusion trauma, significant increase of creatinine release from impaired tissue occurs. This creatinine elevation is observed after even 1 h of muscle ischemia (Fig. 2) and reaches from 50 to 98 μM/l comparatively to the control group (intact rats). At ischemia time of 2 or 3 h, a creatinine level rises up to 161 or 167 μM/l, respectively. This is the evidence of serious mechanic damage of muscular fibres in experimental limb. At 30 min of muscle activation, a creatinine level increases a little (from 50 to 54 μM/l) in a control (intact rats) and illustrates long relaxation activity of experimental muscle. The described elevation of a creatinine level is much higher under ischemia: 98 μM/l without activation and 134 μmol/l during muscle activation at 1 h of ischemia, 121 and 164 μM/l—at 2 h of ischemia, as well as 167 and 208 μM/l after 3 h of ischemia. Finally, as well as in case of a CPK, in active state of a muscle, a creatinine level after 1 and 2 h of ischemia is almost equal to that after 2 and 3 h of ischemia, respectively, but for an inactive muscle.

LA level significantly increased compared to the control value 4.2 mM/ml for 1, 2 and 3 h of muscle ischemia and was 7.7, 10.8 and 14.1 mM/ml without muscle activation, accordingly (Fig. 2). After stimuli, these rates increased to 15.4, 17.4 and 19.3 mM/ml, respectively, with a significant increase in the level of LA in control (intact rats)—12.8 mM/ml.

Membrane lipids become more sensitive for peroxidation after cell death, which occurs in severe forms of ischemia. This should be taken into account in evaluation of the general state of ischemic muscle. There are well-known mechanisms for elimination of the harmful hydroperoxides of fatty acids [2, 26]. Nevertheless, when they are overloaded, it is obvious that damaged cell is going to die. This actually happens under severe ischemia. Collagen and hyaluronic acids are

those extracellular components, which are mostly affected by free radicals. Thus, an activation of free-radical formation under ischemia triggers the complex process that complicates determination of the level of ischemic trauma based on biochemical parameters [27].

Obtained data give the evidence that ischemic cell damage is a two-stage process with the primary effect during ischemia itself and secondary during reperfusion [28]. Primary effects of ischemia are well characterized; energy deficit in the cell that causes homeostasis and ion gradients shifting accompanied with enzymatic insufficiency lead to the cell death. Injury due to reperfusion is developed because of free radicals, endothelial factors and neutrophils [28]. Under ischemia injury during the reperfusion time, several types of free radicals are formed, among which the most reactive is hydroxyl due to its ability to trigger LPO. Endothelium is the most sensitive in this case. Its damage changes the intracellular environment, can provoke muscular fibre fragmentation and neutrophils chemotaxis. Ischemia–reperfusion impairs the balance that maintains homeostasis in microcirculation. It results in involvement, activation, adhesion and migration of neutrophils that leads to local tissue destruction by released proteases and free radicals [3, 4].

At the same time under prolonged stimulation of a muscle, the changes in its metabolism rise that is the main factor in the development of muscular fatigue. Special tests have revealed accumulation of LPO derivatives and changes in antioxidants quantity in muscular tissue that becomes fatigued [29, 30]. Obtained data demonstrate the elevated level of such indicators of LPO and oxidative stress as CAT, TBARS, GSH and H_2O_2 after activity of muscle.

Experiments on ischemic inactive muscles show that these biochemical markers were significantly higher in comparison with intact muscles and, for 1, 2 and 3 h of ischemia, they were as follow: 2.9%, 145.1% and 224.3%—for CAT; 62.5%, 109.2% and 161.7%—for TBARS; 71.6%, 138.1% and 245.6%—for GSH, as well as 262%, 329% and 414%—for H_2O_2 (Fig. 3).

After 30 min of muscle stimulation, levels of these substances substantially rise in comparison with inactive muscle with the same rate of ischemic trauma. Increase of H_2O_2 under physical activity of ischemic muscle leads to the elevation of CAT activity. CAT develops protective antioxidant function facilitating transformation of hydrogen peroxide into water and oxygen [30]. In experiments, a CAT level significantly raises from 2.21 to 2.83 μM/min/ml (28%) in a muscle under 1 h of ischemia, from 3.31 to 4.12 μM/min/ml (24%) in 2 h of ischemia, and from 4.48 to 5.41 μM/min/ml (22%) in case of 3 h of ischemia (Fig. 3).

During an activation of ischemic muscle, the elevation of a TBARS level was found from 4.21 to 5.25 nM/ml (24%), from 5.42 to 6.26 nM/ml (15%), and from 6.78 to 7.32 nM/ml (7.9%) for 1, 2, and 3 h of

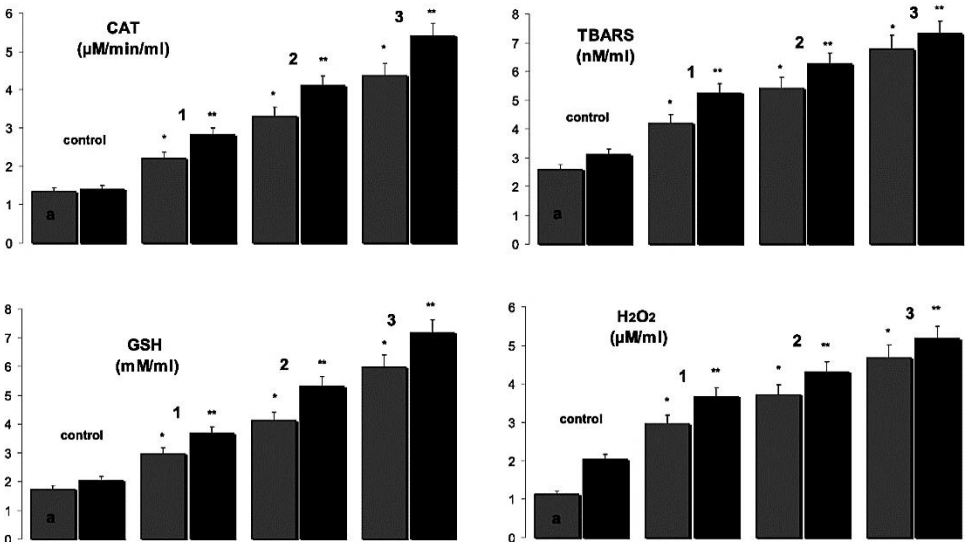

Fig. 3. Components of antioxidant system in blood of rats without muscle stimulation (*a*) and after 30 min of stimulation with 2 Hz-frequency of a current (*b*): control—intact rats; 1, 2 and 3—experimental rats under ischemia–reperfusion trauma of muscle for 1, 2 and 3 h, respectively. Values are $M \pm SD$, $n = 10$; $^{*}p < 0.05$ in comparison with control (experiment without muscle stimulation); $^{**}p < 0.05$ in comparison with control (experiment with muscle stimulation).

ischemia, respectively, compared to resting ischemic muscle (Fig. 3).

Increased quantity of GSH in stimulated ischemic muscle (in comparison with that muscle in rest) indicates a compensatory activation of endogenous antioxidant system in response to the irritant of a threshold value (Fig. 3). A significant decrease of GSH and increase of its oxidized form under intensive physical activity were described in Ref. [30]. Investigation shows the elevation of GSH level from 2.97 to 3.68 mM/ml (23%) for 1 h of ischemia, from 4.12 to 5.32 mM/ml (29%) for 2 h of ischemia, and from 5.98 to 7.18 mM/ml (20%) for 3 h of ischemia.

Based on the data obtained, it can be concluded that, during the induced muscle stimulation, the level of LPO and oxidative stress markers TBARS, CAT and GSH increased by more than 20% ($p < 0.05$) in comparison with their levels in muscle at rest.

A significant increase in the amount of H_2O_2 in the stimulated ischemic muscle in comparison with the muscle at rest was established. Quantitative indices were 3.68, 4.32 and 5.15 μM/ml, respectively, for 1, 2 and 3 h of muscle ischemia. In percentage rate to ischemic muscle without activation, the growth of H_2O_2 level was 123%, 116% and 110% (Fig. 3).

Thus, we showed that muscle stimulation led to an increase in the metabolic products (LA) and intensification of the oxidative processes, namely, a significant increase in ROS production and LPO. As a result, an increase in CAT and GSH activity relative to the intact muscle occurs. The increased LA level further reduced the pH value, which could induce various biochemical and physiological effects during muscular contractions, including glycolysis, phosphofructokinase and calcium release. Therefore, LA is an important marker for evaluating the degree of fatigue of a living organism.

With a moderate external load on the muscle, the metabolism in it occurs aerobically. In the muscle, which is actively contracting, metabolism increases significantly that leads to accumulation of products of secondary oxidation in the muscle fibres [31]. It was proved that such metabolic processes become a source of ROS and contribute to the intensification of LPO [32, 33]. The presence of such metabolic products increases the level of ischemic damage and prevents an adequate diagnosis of ischemic injury levels. In this case, an antioxidant therapy may be effective.

In the case of water-soluble C_{60} fullerene introduction, all aforementioned biochemical markers of muscle tissue damage tended to decrease regardless of muscle-ischemia duration. So, the CPK level was 978, 1123 and 1388 units/l for inactivated muscle, and 1121, 1267 and 1764 units/l for activated muscle for 1, 2 and 3 h ischemia, respectively (Fig. 4). The therapeutic effect of C_{60}FAS compared to ischemic animals without C_{60} fullerene introduction (Fig. 2) was 83%, 83% and 82% for inactivated muscle, and 84%, 76% and 79% for activated muscle for 1, 2 and 3 h of ischemia, respectively.

The LDH level was 321, 478 and 623 units/l for inactivated muscle, and 598, 678 and 776 units/l for activated muscle for 1, 2 and 3 h of ischemia, respectively (Fig. 4). The therapeutic effect of C_{60}FAS compared to ischemic animals without C_{60} fullerene introduction (Fig. 2) was 65%, 73% and 82% for inactivated muscle, and 86%, 83% and 87% for activated muscle for 1, 2 and 3 h of ischemia, respectively.

The level of creatinine was 68, 95 and 123 μM/l for inactivated muscle, and 79, 121 and 134 μM/l for activated muscle for 1, 2 and 3 h of ischemia, respectively (Fig. 4). The therapeutic effect of C_{60}FAS compared to ischemic animals without C_{60}-fullerene introduction (Fig. 2) was 69%, 73% and 79% for inactivated muscle, and 57%, 64% and 73% for activated muscle for 1, 2 and 3 h of ischemia, respectively.

Finally, the LA level was 5.7, 8.6 and 11.8 mM/ml for inactivated muscle, and 14.3, 16.1 and 18.7 mM/ml for activated muscle for 1, 2 and 3 h of ischemia, respectively (Fig. 4). The therapeutic effect of C_{60}FAS compared to ischemic animals without C_{60}-fullerene introduction (Fig. 2) was 74%, 77% and 83% for inactivated muscle, and 92%, 92% and 96% for activated muscle for 1, 2 and 3 h of ischemia, respec-

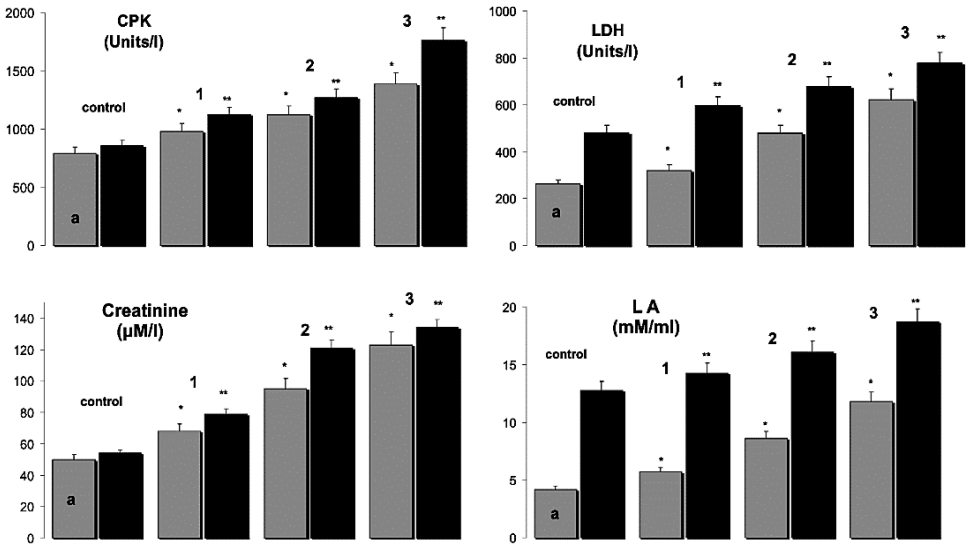

Fig. 4. Levels of enzymes (CPK and LDH) and their metabolic products (creatinine and LA) in blood of rats without muscle stimulation (*a*) and after 30 min of stimulation with 2 Hz-frequency of a current (*b*): control—intact rats after intramuscular injection of 1 mg/kg saline; 1, 2 and 3—experimental rats after intramuscular injection of 1 mg/kg C_{60}FAS under ischemia–reperfusion trauma of muscle for 1, 2 and 3 h, respectively. Values are $M \pm SD$, $n = 10$; $^{*}p < 0.05$ in comparison with control (experiment without muscle stimulation); $^{**}p < 0.05$ in comparison with control (experiment with muscle stimulation).

tively.

After water-soluble C_{60}-fullerene introduction, a decrease in the levels of LPO and oxidative stress markers was established. So, the CAT level was 2.02, 2.87 and 3.43 μM/min/ml for inactivated muscle, and 2.2, 3.41 and 4.11 μM/min/ml for activated muscle for 1, 2 and 3 h of ischemia, respectively (Fig. 5). The therapeutic effect of C_{60}FAS compared to ischemic animals without C_{60}-fullerene introduction (Fig. 3) was 91%, 86% and 78% for inactivated muscle, and 77%, 72% and 75% for activated muscle for 1, 2 and 3 h of ischemia, respectively.

The TBARS level was 3.77, 4.02 and 5.15 nM/ml for inactivated muscle and 4.05, 5.23 and 6.31 nM/ml for activated muscle for 1, 2 and 3 h of ischemia, respectively (Fig. 5). The therapeutic effect of C_{60}FAS compared to ischemic animals without C_{60}-fullerene introduction (Fig. 3) was 89%, 74% and 79% for inactivated muscle, and 77%, 82% and 79% for activated muscle for 1, 2 and 3 h of ischemia, respectively.

The GSH level was 2.21, 3.45 and 4.28 mM/ml for inactivated muscle, and 3.04, 4.56 and 5.88 mM/ml for activated muscle for 1, 2 and 3 h of ischemia, respectively (Fig. 5). The therapeutic effect of C_{60}FAS

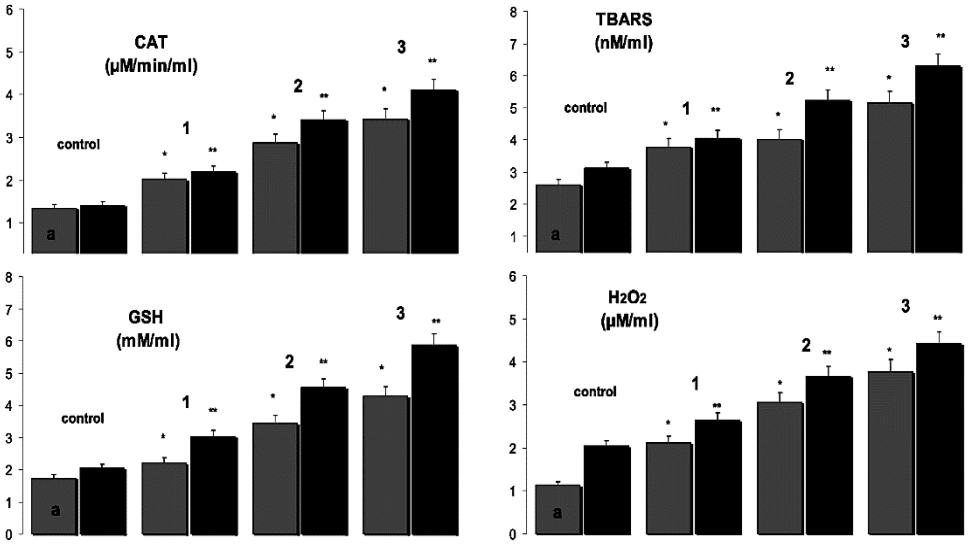

Fig. 5. Components of antioxidant system in blood of rats without muscle stimulation (*a*) and after 30 min of stimulation with 2 Hz-frequency of a current (*b*): control—intact rats after intramuscular injection of 1 mg/kg saline; 1, 2 and 3—experimental rats after intramuscular injection of 1 mg/kg C_{60}FAS under ischemia–reperfusion trauma of muscle for 1, 2 and 3 h, respectively. Values are $M \pm SD$, $n = 10$; $^{*}p < 0.05$ in comparison with control (experiment without muscle stimulation); $^{**}p < 0.05$ in comparison with control (experiment with muscle stimulation).

compared to ischemic animals without C_{60}-fullerene introduction (Fig. 3) was 74%, 83% and 72% for inactivated muscle, and 82%, 85% and 81% for activated muscle for 1, 2 and 3 h of ischemia, respectively.

Finally, the H_2O_2 level was 2.12, 3.06 and 3.78 μM/ml for inactivated muscle, and 2.65, 3.67 and 4.43 μM/ml for activated muscle for 1, 2 and 3 h of ischemia, respectively (Fig. 5). The therapeutic effect of C_{60}FAS compared to ischemic animals without C_{60}-fullerene introduction (Fig. 3) was 71%, 82% and 80% for inactivated muscle, and 72%, 84% and 85% for activated muscle for 1, 2 and 3 h of ischemia, respectively.

Thus, the treatment groups marked tendency to decrease abovementioned biochemical parameters at average by 20–25% compared to animals without C_{60}-fullerene application.

During ischemia development, there is a significant depletion of cellular energy substances, especially, ATP decomposition, which leads to a sharp disruption of homeostasis and loss of ionic gradient through cell membranes [34]. At the same time, there are NAD, lactate and H^+ accumulation, and, consequently, acidification of the intracellular and extracellular media [35]. The ATP-production decrease suppresses the

activity of Na^+–K^+-ATPase that leads to increase in intracellular Na^+ concentration and, as a consequence, intracellular Ca^{2+} [36]. The high K^+ content causes a delay in the action potential generation and, accordingly, its distribution in T-tubes [37]. It can be assumed that ionic changes impair the ability of muscle to respond to electrical impulses, because the loss of ischemic muscle ability to a contraction is associated with the depolarization of membrane systems of myocytes and violation of intracellular conductivity that prevents the development of excitation and leads to its extinction.

At the same time, the ischemic injury leads to the ROS overabundance [38]. It was shown that ROS are formed during both ischemia and reperfusion. The main radicals produced in the myocytes are superoxide anion (O_2^-) and nitric oxide (NO), which lead to the formation of several secondary reactive compounds such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($H\dot{O}$), and peroxynitrites ($ONOO^-$) [39]. In case of ischemia, a xanthine dehydrogenase, which is contained in the microvascular endothelial cells of the skeletal muscle, converted into xanthine oxidase [40], which, in turn, catalyses the conversion of hypoxanthine to xanthine by forming a superoxide anion. Other potentially important sources of free radicals' generation include NADP oxidase, NO synthase and XO [41]. It has been established that physical activity results in muscles' fatigue and H_2O_2 formation that normally do not have a significant effect on changes in myoplasmic Ca^{2+} levels during single and tetanus muscle contractions [40, 42], but, in case of ischemia, they inhibit Ca^{2+} withdrawal from a sarcoplasmic reticulum during muscle contraction.

The obtained results show the pronounced therapeutic effect of C_{60} fullerene on the ischemic pathological process development in a *musculus soleus* of rats. Such impact, in our opinion, is due to the neutralization of part of the ROS by water-soluble C_{60} fullerenes at the early stage of the development of the ischemic process.

Thus, the development of biomedical nanotechnologies using the water-soluble pristine C_{60} fullerenes, as powerful antioxidants [43], opens up new opportunities in the therapy and prevention of the skeletal-muscles' ischemic pathologies, in the basis of which is the action of free radical processes.

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