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The Change in the Biochemical Parameters of the Rat Blood after Skeletal Muscle Injury with C_{60} Fullerene Injection

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The biochemical markers as creatinine, creatine phosphokinase, lactic acid and lactate dehydrogenase, thiobarbituric acid reactive substances, hydrogen peroxide, reduced glutathione, and catalase activity are analysed in rat blood after skeletal muscle (*soleus muscle*) injury and with aqueous colloidal solution of C_{60}-fullerenes' application. As shown, 1 mg per kg of animal weight is the most optimal therapeutic dose of C_{60}-fullerene intramuscular injection to restore muscle functioning after injury. The studied rats' blood biochemical parameters positive changing compared to the control confirms the water-soluble C_{60}-fullerenes effectiveness, as powerful antioxidants, to correct muscular system pathological conditions from trauma.

Проаналізовано рівні таких біохемічних показників крові щурів як креатинін, креатинфосфокіназа, молочна кислота та лактатдегідрогеназа, реактивні речовини тіобарбітурової кислоти, пероксид водню, відновлений глутатіон та активність каталази після травматичного пошкодження скелетного м'яза (*soleus muscle*) за дії водного колоїдного розчину C_{60}-фуллеренів. Встановлено, що 1 мг на кг маси тварини є найоптимальнішою терапевтичною дозою внутрішньом'язового введення C_{60}-фуллерену для відновлення активного функціонування м'яза після травми. Позитивна зміна досліджуваних біохемічних показників крові піддослідних щу-

рів порівняно з контролем підтверджує ефективність водорозчинних C_{60}-фуллеренів, як потужних антиоксидантів, кориґувати патологічні стани м'язової системи, що виникають за травм.
Key words: C_{60} fullerene, muscle trauma, dynamics of muscle contraction, biochemical analysis of blood, rats.

Ключові слова: C_{60}-фуллерен, м'язова травма, динаміка м'язового скорочення, біохемічна аналіза крові, щури.

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1. INTRODUCTION

Understanding the mechanisms of muscle injury development at the molecular, cellular and tissue levels underlies their specific therapy, in particular, is of great importance for the development and application of new drugs with effective anti-inflammatory activity. Once the muscle injury has been initiated, the inflammatory process begins with damage to the integrity of muscle tissue and sarcolemma. This leads to extracellular calcium entry into damaged cells, calcium-dependent proteases and phospholipases activation, which in turn activate calcium-dependent necrosis [1]. As a result, the muscle fibres are in an overstressed state. Proteins that get into blood from damaged tissue, in particular creatine kinase, present in the cytosol of muscle cells and usually appear only in blood samples after mechanical stress or muscle degenerative diseases [2]. As a result, muscle tissue loses its ability to generate and retain the contraction force.

Usually, mechanical injuries cause damage to the connective tissue, which results in necrosis, hematoma and inflammatory processes [3]. Effective regeneration ensures optimal muscle regeneration over time so that it can resume its optimal functional activity. At the same time, exacerbation of inflammatory reaction worsens muscle recovery and leads to pathological displacement of homeostasis [4].

It is known that, because of muscle injury, products of incomplete oxygen oxidation (reactive oxygen species (ROS)) are formed: free radicals, oxygen ions, etc. [5]. It has been shown that lipid peroxidation (LPO) reduces the content of unsaturated fatty acids and forms various fatty acid derivatives, in particular such metabolites as malondialdehyde and hydroperoxide [6]. Excessive accumulation of ROS (oxidative stress) leads to serious functional disorders, because different components of cells are damaged [6]. An example is LPO of biological membranes, contributing to the disturbance of their structure and increasing permeability [7]. Cell protection against such damages is provided by the antioxidant system. Recently the influence of exogenous antioxidants on various manifestations of ROS in muscle tissues has

been intensively studied [8–10]. Therefore, Mach *et al.* [8] used a pycnogenol as an antioxidant. It has been found that its use is accompanied by an increase in the level of both oxidized and reduced NAD^+ in the blood serum, as well as increased muscle endurance.

The ability of C_{60} fullerenes and their derivatives to inactivate ROS was first demonstrated by Krustic *et al.* [11]. It has been shown that C_{60} fullerene is more effective than a natural antioxidant—vitamin E in preventing LPO, preventing damage to membrane integrity, and thus contributing to the maintenance of membrane potential. Water-soluble C_{60} fullerenes show dose-dependent protective effect against skeletal muscle ischemic pathologies [12–14].

Experimental data show that water-soluble C_{60} fullerenes at low concentrations do not have toxic effects *in vitro* and *in vivo* systems [15, 16]. Thus, toxic effects of aqueous colloidal solutions of C_{60} fullerenes *in vivo* experiments are not detected at total doses up to 25 mg/kg [17]. It has been established that intra-abdominal injection of C_{60} fullerenes (2.5 g/kg) during 8 weeks into mice does not lead to death of animals or disturbance in their behaviour [18].

So, water-soluble C_{60} fullerenes do not cause acute and chronic toxicity in normal cells, at least at low concentrations, which contributes to the potential possibility of their biomedical application. Considering the accumulated data on the powerful antioxidant properties of C_{60} fullerenes [19, 20], it is important to study their influence on the course of free-radical oxidation processes in damaged skeletal muscles.

2. EXPERIMENTAL

2.1. Animals

For experimental studies, 30 male rats of the Wistar inbred line at the age of 150 days were used. The research protocol was approved by the Commission on Bioethics of ESC "Institute of Biology and Medicine", according to the rules of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" and the norms of biomedical ethics, according to the Law of Ukraine No.: 3446-IV 21.02.2006, Kyiv, "On protection of animals from cruelty" with medical and biological research.

To register the skeletal muscle contraction force, an original strain gauge device created at the Department of Biophysics and Medical Informatics of the ESC "Institute of Biology and Medicine" was used. The device is a complex consisting of the following components: force and length sensors, synchronous pulse generator, thermal control system, oscilloscopes, ACC–CAC complex [21]. The muscle was stimulated by electric pulses of rectangular form with the duration of 2 ms, which were formed by pulse generator controlled by ACC through platinum

electrodes. The duration of the stimulation signal was 6 s [22].

Anaesthesia of animals was performed by intra-abdominal administration of nembutal (40 mg/kg). The muscle injury was caused mechanically by clutching the muscle for 1 min at an applied pressure of 3.5 kg/cm^2 [23]. The technique of Crush Syndrome was applied, which led to the systemic manifestation of pathological changes in the skeletal muscle, in particular, the release of muscle cell components (creatine kinase, lactic acid, myoglobin, *etc.*) in the extracellular environment, which served as a marker of muscle injury.

2.2. Nanomaterials

An original method based on the transfer of C_{60} molecules from toluene into water with subsequent ultrasound treatment was used to produce a C_{60} fullerene aqueous solution (C_{60}FAS) [24, 25]. The obtained C_{60}FAS is a typical colloidal solution containing both single C_{60} molecules (~ 0.72 nm) and their nanoaggregates 1.2–100 nm in size. In addition, C_{60}FAS was stable for 12 months at +4°C.

C_{60}FAS was administered once intramuscularly in a dose of 0.5, 1 or 2 mg per kg animal weight.

2.3. Biochemical Analysis

The level of enzymes content in the blood of experimental animals, as markers of muscle injury, was determined using clinical diagnostic equipment—a haemoanalyser [26].

2.4. Statistical Analysis

The statistical processing of the results was carried out using the methods of variation statistics using the Origin 9.4 software. 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 tests. Values of $p < 0.05$ were considered significant.

3. RESULTS AND DISCUSSION

Figure 1 shows mechanograms of the injured *musculus soleus* contraction by electrostimulatory irritation lasting 6 s. It can be concluded that *muscle soleus* dysfunction after the initiation of its injury leads to the complete inability of the muscle to maintain a constant value of force during tetanic contraction. After the therapeutic application of

C_{60}FAS, the dynamics of muscle contraction tended to increase the maximum power responses of the muscle. As can be seen, the injection of C_{60}FAS in a dose of 0.5 mg/kg has almost no effect on the power response of the damaged muscle; with increasing the dose of C_{60}FAS to 1 mg/kg, the muscle response rate increases by almost 17%. Finally, as the dose of C_{60}FAS increases up to 2 mg/kg the muscle contraction force continues to increase, but no more than 6%. Therefore, an increase in the effective dose of C_{60}FAS from 1 to 2 mg/kg (for 100%) leads to a negligible therapeutic effect.

Changes of animal blood biochemical indicators in the development of inflammatory process after the initiation of muscle injury allow us to assess the therapeutic effect of the applied drug on the studied pathology.

Selected blood parameters have a pronounced upward tendency in the inflammatory process development in damaged muscle tissue. One of the well-known markers of pathological processes in skeletal muscle is changes in concentration of creatine phosphokinase—an enzyme from the skeletal muscle energy supply system, which catalyses the transfer of phosphate group from ATP to creatine molecule with the formation of high-energy product—creatine phosphate, which is used by the body as an energy substance with increased physical activity. Creatine phosphokinase is an enzyme that is present in high concentrations in skeletal muscles. In case of mechanical muscle damage, the release of the enzyme from the cells is observed and, consequently, the increased activity of creatine kinase in the blood. The increase of creatine phosphokinase fraction by induction of muscle injury (Fig. 2) from 500 (norm) to 2700 Units/l is the result of nonspecific physiological destruction of myocytes with partial yield of enzymes in the extracellular space [27]. However, with intramuscular injection of C_{60}FAS at the doses of 0.5, 1 and 2 mg/kg (Fig. 2) the level of creatine phosphokinase decreases to 2480, 2120 and 20180 Units/l, respectively. It

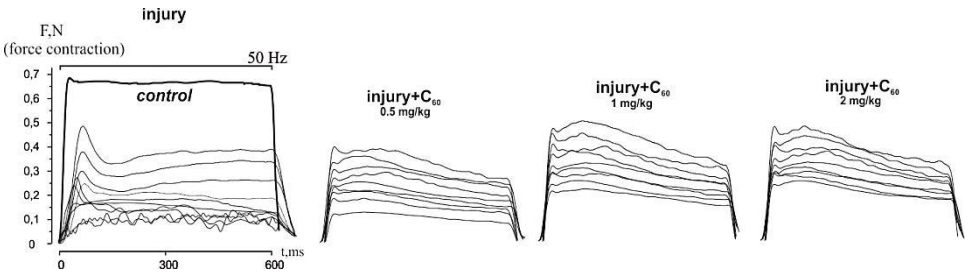

Fig. 1. *Musculus soleus* force contraction curves, induced by 50 Hz stimulation and 6 s duration, 1 h after initiation of muscle injury (control) and with C_{60}FAS injections at 0.5, 1 and 2 mg/kg, respectively: injury+C_{60} 0.5 mg/kg, injury+C_{60} 1 mg/kg and injury+C_{60} 2 mg/kg.

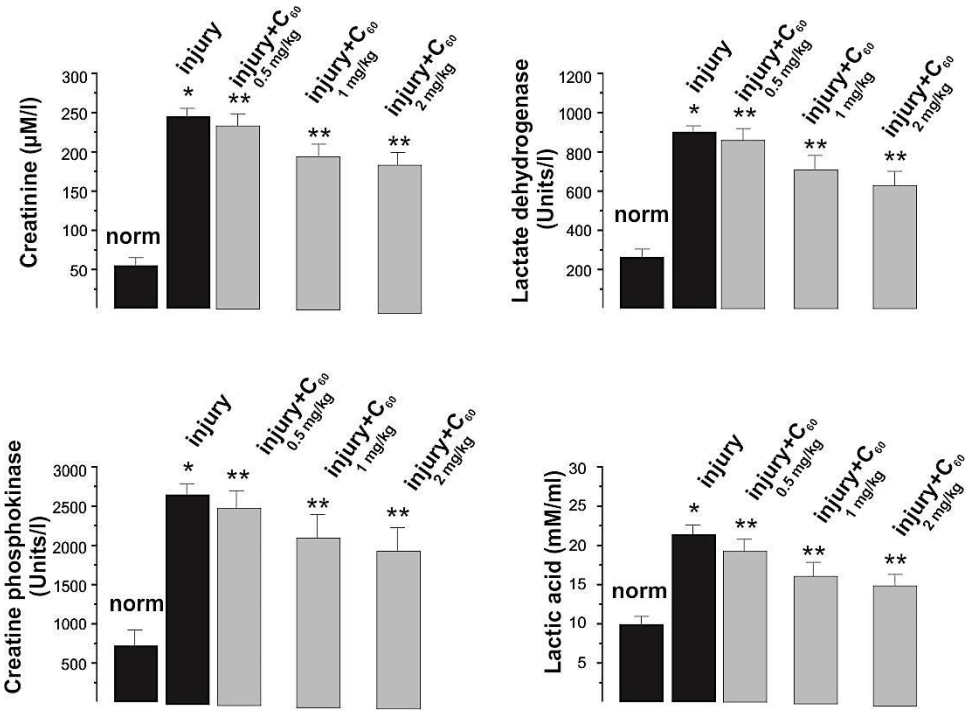

Fig. 2. Changes in creatinine, lactate dehydrogenase, creatine phosphokinase and lactic acid activity in rat blood after induced muscle injury with C_{60}FAS injections at doses of 0.5, 1 and 2 mg/kg, respectively: injury+C_{60} 0.5 mg/kg, injury+C_{60} 1 mg/kg and injury+C_{60} 2 mg/kg; $*p < 0.05$ compared with norm group; $**p < 0.05$ regarding the injury group.

is important to note that with a 2-fold increase in dose (from 1 to 2 mg/kg), the creatine phosphokinase level decreased only by 7% . This is an indication of the minor therapeutic effects of C_{60}FAS in this case.

The change of lactate dehydrogenase level (Fig. 2)—an enzyme that catalyses oxidation of lactic acid (the end product of glucose metabolism in cells during prolonged physical activity) allows to estimate the state of functional activity of injured muscle [28]. The increase in the enzyme activity from 210 (norm) to 900 Units/l (after injury) is evidence of the development of significant dysfunctions of the separate neuromuscular system associated with the development of inflammatory process. With the C_{60}FAS intramuscular administration in doses of 0.5, 1 and 2 mg/kg, a lactate dehydrogenase activity was reduced to 870, 790 and 680 Units/l, respectively. As one can see, with a 2-fold increase in the C_{60}FAS dosage (from 1 to 2 mg/kg) the activity of this enzyme changed only by 8% .

In *musculus soleus*, as in muscle with high levels of myoglobin, most

metabolic and biochemical processes occur under anaerobic conditions, regarding that, muscle accumulates a large amount of lactate, which does not have time to oxidize behind long-term muscle stimulation. An increase in lactic acid levels in the active muscle indicates that its intake to the cells exceeds oxidation and withdrawal levels. The increase of lactate dehydrogenase fraction in the blood is the result of both physiological destruction of myocytes and increase of lactate content in long-term muscle activation [29]. The decrease of this enzyme from intramuscular therapeutic administration of C_{60}FAS indicates both a decrease in mechanical damage to muscle fibres and a decrease in lactate concentration in the muscular system as a whole.

Changes in the level of creatinine, a product formed in muscles when intramuscular structures are destroyed, allow us to estimate the level of myocyte damage. From Figure 2, one can see that this indicator increases from 50 (norm) to 250 μM/l (after injury). The protective effect on these processes of C_{60} fullerene, in contrast to previous indicators, is shown at all three concentrations used. The reduction of creatinine was 230, 200 and 180 μM/l at C_{60}FAS doses of 0.5, 1 and 2 mg/kg, respectively. So, C_{60} fullerenes effectively protect the membranes of skeletal muscle cells from non-specific free-radical damage, absorbing ROS in the concentration dependence.

Biochemical tests revealed an increase in the secondary products of LPO and changes in the level of endogenous antioxidants in rat blood after muscle injury. The obtained data clearly demonstrate increased levels of peroxidation marker and oxidative stress TBARS (thiobarbituric acid reactive substances) and H_2O_2 (hydrogen peroxide) after muscle injury (Fig. 3): this increase was statistically significant and was 332% ($p < 0.05$) for TBARS and 380% ($p < 0.05$) for H_2O_2 in relation to the intact muscle (norm). At the same time, endogenous antioxidants were activated in the injured muscle: the level of GSH (reduced glutathione) tripled ($p < 0.05$) and the activity of the enzyme CAT (catalase) increased significantly (Fig. 3).

After therapeutic injections of C_{60} fullerenes, a decrease in peroxidation and oxidative stress markers was revealed (Fig. 3). Thus, the CAT activity was reduced from 4.5 (1.2 in norm) to 4.2, 4 and 3.8 μM/min/ml for the introduction of C_{60}FAS in doses of 0.5, 1 and 2 mg/kg, respectively. The change in TBARS activity occurred within the following limits: from 7.8 (2.3 in norm) to 7.4, 6.2 and 6.1 μM/ml for administration of C_{60}FAS in doses of 0.5, 1 and 2 mg/kg, respectively. The H_2O_2 concentrations were characterized by the following values: 4.8 for injury (0.9 in norm) and 4.5, 4.2 and 4.1 μM/ml for administration of C_{60}FAS in doses of 0.5, 1 and 2 mg/kg, respectively. Finally, the GSH activity was following: 6.3 for injury (1.9 in norm) and 5.8, 4.5 and 4.2 μM/ml for administration of C_{60}FAS at 0.5, 1 and 2 mg/kg doses, respectively. So, there is a clear tendency of described

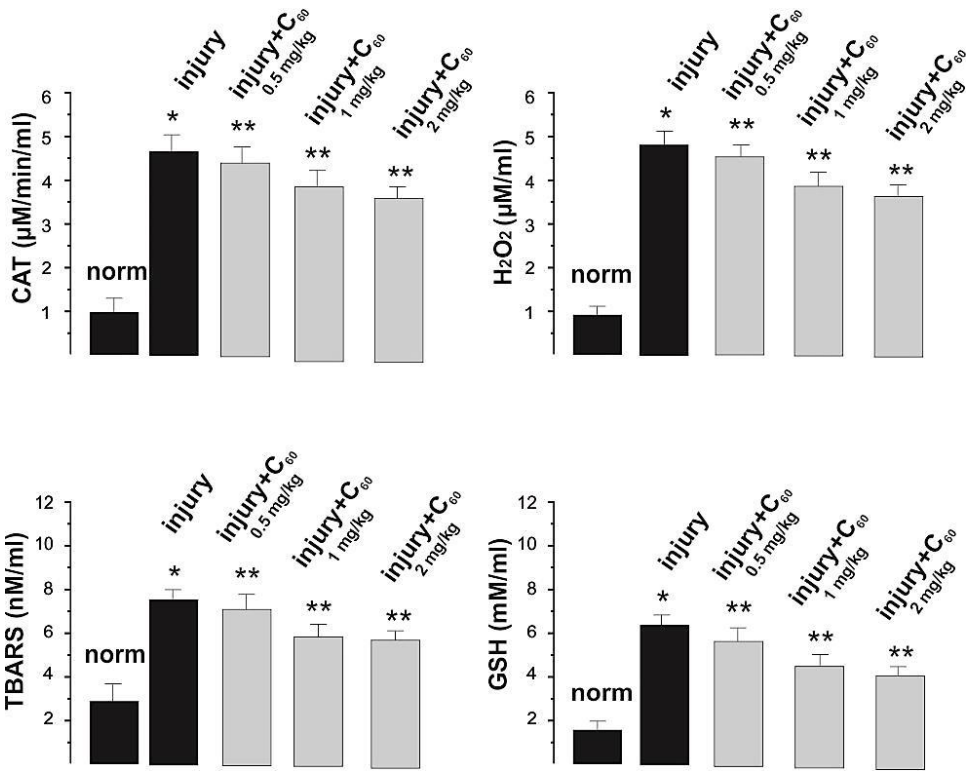

Fig. 3. Indicators of pro- and antioxidant balance in rat blood after caused muscle injury. The CAT activity and concentrations of H_2O_2, TBARS and GSH indicated with C_{60}FAS injections in doses of 0.5, 1 and 2 mg/kg, respectively: injury+C_{60} 0.5 mg/kg, injury+C_{60} 1 mg/kg and injury+C_{60} 2 mg/kg; *$p < 0.05$ compared with norm group; **$p < 0.05$ compared with injury group.

biochemical parameters to decrease by about 10–15% for the therapeutic introduction of water-soluble C_{60} fullerenes.

The results demonstrate compensatory activation of C_{60} fullerenes of the endogenous antioxidant system in response to long-term stimulation of the damaged muscle. Thus, C_{60} fullerene is able to prevent muscle dysfunction by keeping it within the physiological norm during the activation process.

Inflammatory processes occurring immediately after muscle injury are the source of ROS, which contributes to the intensification of LPO processes [30]. The presence of such metabolites usually prevents the muscles from performing their functions adequately and significantly increases their recovery time. Decrease in the content of these oxygen metabolites due to the therapeutic introduction of water-soluble C_{60} fullerenes allows improving the motor commands of the CNS in the

muscular system and helps reduce inflammation [31].

So, positive changes of investigated biomechanical parameters of muscle contraction and biochemical parameters of rat blood under the therapeutic action of C_{60}FAS in a dose of 1 mg/kg indicate a high efficiency of the use of this substance in injured skeletal muscles antioxidant therapy.

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