UDC 57.013:577.353 doi: https://doi.org/10.15407/ubj93.04.093

BIOCHEMICAL AND TENSOMETRIC ANALYSIS OF C_{60} FULLERENES PROTECTIVE EFFECT ON THE DEVELOPMENT OF SKELETAL MUSCLE FATIGUE

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Received: 19 May 2021; **Accepted:** 07 July 2021

The protective effect of water-soluble C_{60} fullerenes on the development of slow and rapid fatigue of rat skeletal muscles was analyzed. It was found that the reduction of muscle contraction force (muscle soleus) by 50% of the initial values is almost twice as slow as stimulation with a frequency of 1 Hz (slow muscle fatigue) than with 2 Hz (rapid muscle fatigue) stimulation after intramuscular injection of C_{60} fullerenes (dose 0.5 mg/kg). There is a clear tendency to decrease the values of biochemical parameters of the blood of animals with the therapeutic effect of water-soluble C_{60} fullerenes by approximately 45-60% and 35-40% with the development of slow and rapid muscle fatigue, respectively. Thus, C_{60} fullerenes, as powerful antioxidants, are able to efficiently affect the prooxidant-antioxidant homeostasis of muscle tissue and thus help maintain its normal physiological state.

K e y w o r d s: skeletal muscle, fatigue, biochemical and tensometric analysis, C_{60} fullerene.

Skeletal muscles are able to perform physical work only for a certain period of time, the duration of which is inversely proportional to the amount of load, after which there is a gradual decrease in the maximum level of strength that can be generated and maintained by skeletal muscle. This phenomenon is called muscle fatigue [1, 2].

Muscle fatigue is a protective mechanism of the body against overload and further development of pain sensitivity of muscle [1]. Its nature and optimal degree are key factors for the formation of adaptation and increase the level of functional and physical capabilities of the organism. There are two main reasons for losing function by muscle due to prolonged irritation. The first is accumulation of metabolic products (lactic acid, free radicals, etc.) during muscle contraction. Some of these products, as well as potassium ions, diffuse from the fibers to the outside and inhibit the ability of the stimulated membrane of myocytes to generate action potentials. Another cause of muscle fatigue is the gradual depletion of energy reserves. During long-term muscle functioning, there is a sharp decrease in glycogen reserve, which disrupts the processes of resynthesis of ATP and creatine phosphate required for muscle contraction [2].

Under natural conditions, fatigue of the musculoskeletal system during prolonged work develops more complex and depends on many factors. Firstly, due to the fact that body's muscle is continuously supplied with blood and receives a certain amount of nutrients (glucose and amino acids) and is released from metabolic products that disrupt the normal functioning of muscle fibers. Secondly, in body fatigue depends not only on the dynamic processes in the muscle, but also on the processes that develop in the nervous system and are involved in the management of motor activity [3]. For example, fatigue is accompanied by incoordination and dysfunction of many muscles that do not participate

in the work. Other muscles are involved into the work, first synergists, which compensate for the decrease in the strength of the main muscles, and then, as the discoordination increases, other muscles, in particular antagonists [4]. The movements become less precise, their pace slows down [5]. Thus, the level and quality of the body's protective response against overload depends on the rate of development of pathological processes in an actively functioning muscle. One of the important unresolved issues is still the physiological difference between the formation of rapid muscle fatigue and its slow onset. This is primarily due to the adaptive correction of muscle fatigue processes, which do not take into account the fast and slow ways of its development.

The greatest influence on the development of muscle fatigue is caused by free radicals, in particular superoxide anion, hydrogen peroxide and hydroxyl radicals [6]. This was initially confirmed in rabbit muscles, where free radicals were registered before and after strenuous exercise [7]. Later, the effect of free radicals on muscle fatigue of human limbs was demonstrated in [8]. Exercise has been shown to increase markers of lipid peroxidation, protein carbonylation, and DNA oxidation [9, 10]. The development of muscle fatigue disrupts the activity of antioxidant enzymes, induces the oxidation of glutathione, which belongs to the non-enzymatic part of the antioxidant system of protection of the cell from free radicals, which leads to a general decrease in its concentration [11].

One of the most powerful antioxidants that can be used to correct skeletal muscle fatigue is the biocompatible water-soluble C_{60} fullerene nanoparticles [12-14]. The C_{60} molecule has a high reproducibility - it can attach up to six electrons. Due to this property, C_{60} fullerenes act in biological systems as effective scavengers of free radicals, in particular ROS (reactive oxygen species), the hyperproduction of which leads to many pathologies, including ischemic injuries [15], traumatic genesis [16]. It is confirmed that C_{60} fullerenes normalize cellular metabolism and nervous processes, increasing resistance to stress, enhance the activity of enzymes and tissue regenerative capacity, show pronounced antiviral, anti-inflammatory and anti-allergenic effects [17, 18]. It has been experimentally confirmed that C_{60} fullerenes and their derivatives can be adjuvants in complex therapy due to their ability to intensify the protective functions of the immune and antioxidant systems of the human body [19, 20].

No toxic effects or fatalities were observed in studies of C_{60} fullerenes after oral administration to rats at a total dosage of 2 g/kg for 14 days [21].

The above data indicate the high prospects for the use of water-soluble C_{60} fullerenes for therapeutic purposes, the antioxidant effect of which far exceeds that of known natural antioxidants - vitamins C, E and carotenoids [22], in particular as potential nanoagents to improve the functioning of human skeletal muscle by modification of ROS-dependent mechanisms that play an important role in the development of muscle fatigue. Therefore, the aim of this study was to evaluate the protective effect of water-soluble C_{60} fullerenes on the dynamics of muscle contraction and biochemical composition of rat blood in the formation of rapid and slow skeletal muscle fatigue due to its prolonged activation.

Materials and Methods

To obtain water-soluble C_{60} fullerenes, a method was used that is based on the transfer of C_{60} molecules from toluene to water with subsequent sonication [23]. C_{60} fullerene aqueous solution (C_{60}FAS) is a typical polydisperse nanocolloid [24], stable for 12-18 months at a storage temperature of 4°C.

The atomic force microscopy (AFM) was performed to determine the size of C_{60} fullerene particles (their aggregates) in aqueous solution. Measurements were done with the "Solver Pro M" system (NT-MDT, Russia). A drop of investigated solution was transferred on the atomic-smooth substrate to deposit layers. Measurements were carried out after complete evaporation of the solvent. For AFM studies, a freshly broken surface of mica (SPI supplies, V-1 grade) was used as a substrate. Measurements were carried out in a semicontact (tapping) mode with AFM probes of the RTPESPA150 (Bruker, 6 N/m, 150 kHz) type.

Experiments on rats were performed in accordance with international guidelines for medical and biological studies with the use of animals and European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986). Experiments on animals were carried out in accordance with the rules of treatment of experimental animals, which was approved by the Academic senate of Taras Shevchenko National University of Kyiv and in accordance with 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 in accordance with the Law of Ukraine N3446 - IV 21.02.2006, Kyiv, on the Protection of Animals from Cruelty during medical and biological research.

The experiments were performed on white nonlinear rats weighing 135-140 g. The animals selected for the experiment were divided into five groups (n = 30): intact animals (n = 6); animals with skeletal muscle stimulation by electrical impulses with a frequency of 1 Hz (slow muscle fatigue; n = 6); animals with skeletal muscle stimulation by electrical impulses with a frequency of 2 Hz (rapid muscle fatigue; n = 6); animals with skeletal muscle stimulation by electrical impulses with a frequency of 1 Hz (slow muscle fatigue; n = 6) along with intramuscular injection of C_{60}FAS at a dose of 0.5 mg/kg 1 h before the experiment; animals with skeletal muscle stimulation by electrical impulses with a frequency of 2 Hz (rapid muscle fatigue; n = 6) along with intramuscular injection of C_{60}FAS at a dose of 0.5 mg/kg 1 h before the experiment.

The selected dose of C_{60}FAS is based on experimentally established data that indicate a high protective effect of water-soluble C_{60} fullerenes in the model of ischemia-reperfusion [25]. Also, it should be noted that the used dose was significantly lower than the LD_{50} value, which after oral administration to rats was 600 mg/kg body weight [26], and after intraperitoneal administration to mice – 721 mg/kg [27].

Anesthesia of animals was performed by intraperitoneal administration of nembutal (40 mg/kg). Soleus muscle of rat was released from the surrounding tissues and its tendon was cut across in distal part. The ventral roots were cut in places of their exit from the spinal cord for the modulated stimulation of efferents. Changes in muscle contraction were recorded using strain gauges to which tendons of the test muscle were attached. Programmable signal generators of special shape were used to generate stimulus signals. Distributed stimulation allowed to obtain a monotonous and uniform muscle contraction with low-frequency (1 and 2 Hz) stimulation of individual filaments. Stimulation was performed with electrical pulses lasting 2 ms for 60 min through platinum electrodes. The external load on the muscle was controlled by a system of mechanical stimulators. The perturbation of the load was carried out by a linear electromagnetic motor.

The development of muscle fatigue was assessed by calculating the time of reduction of its contraction force by 50% from the initial value during stimulation.

The levels of creatinine, creatine phosphokinase (CPK), lactate (LA), lactate dehydrogenase (LDH), thiobarbituric acid reactive substances (TBARS), hydrogen peroxide (H_2O_2), reduced glutathione (GSH) and catalase (CAT) activity as markers of muscle injury [28], were determined in blood plasma of experimental animals using clinical diagnostic equipment - a haemoanalyzer [25].

Statistical processing of results was performed by methods of variation statistics using software Original 8.0. Biochemical data are expressed as the means ± SEM for each group. The differences among experimental groups were detected by one-way analysis of variance followed by Bonferroni multiple comparison test. Values of $P < 0.05$ were considered significant.

Results and Discussion

The monitoring of C_{60} fullerene particle' size in aqueous solution is important for controlling the degree of C_{60} fullerene aggregation which may influence its toxicity [29, 30]. The prepared C_{60}FAS (concentration 0.15 mg/ml) was characterized by AFM technique.

The study of C_{60} fullerene films deposited from an aqueous solution revealed a high degree of molecules dispersion in solution. It turned out that prepared C_{60}FAS contains both single C_{60} fullerene (~0.7 nm) and its labile nanoaggregates with size of 1.4-20 nm (Fig. 1). The majority of C_{60} molecules were located chaotically and separately along the surface, or in the form of bulk clusters consisting of several tens C_{60} molecules [24]. Such arrangement of C_{60} molecules formed because of electrostatic repulsion between them: the zeta potential value was -25.3 mV at room temperature [27], indicating a high solute stabilization.

To ensure long-term development of muscle fatigue, rat *muscle soleus* stimulation was performed with electrical pulses of 1 Hz (slow muscle fatigue) and 2 Hz (rapid muscle fatigue) without a period of relaxation. Thus, with stimulation at a frequency of 1 Hz, the time to reach the force of muscle contraction 50% of the initial values was 3080 s (Fig. 2).

At the same time, the analysis of muscle mechanograms after water-soluble C_{60} fullerenes administration revealed that the force of muscle contraction decreased to 50% of the initial values for the time exceeding the duration of stimulation (3600 s), - 4300 s.

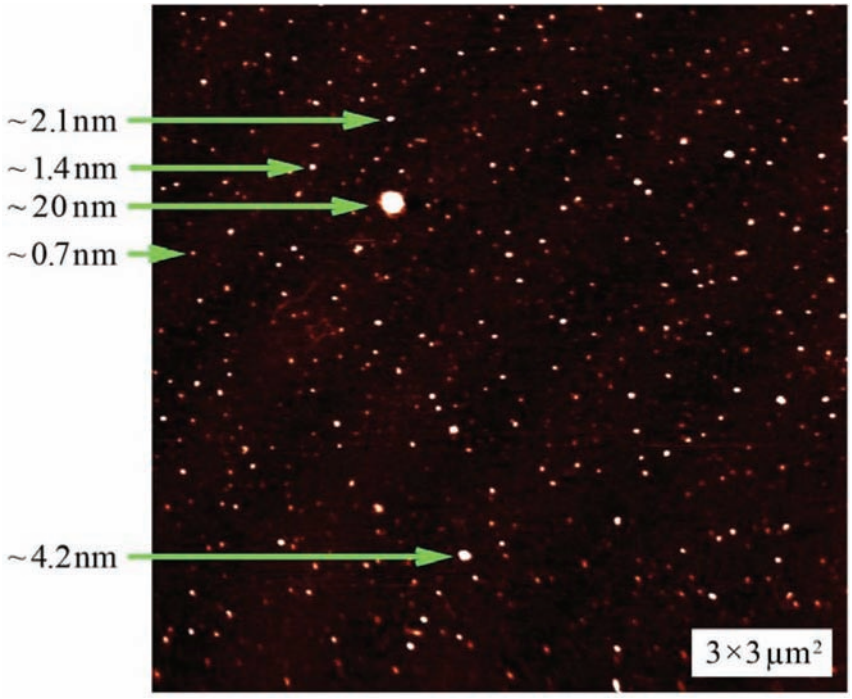

Fig. 1. AFM image of the C_{60} fullerene layer. Numbers with arrows show the height of nanoobjects

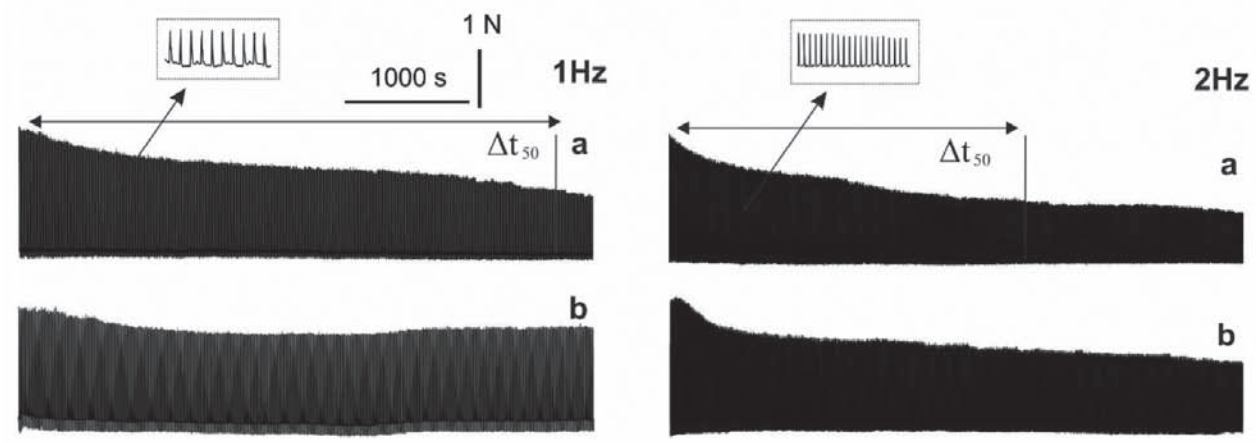

*Fig. 2. Curves of contraction force generation of rats muscle soleus caused by electrical stimulation with frequency 1 and 2 Hz and duration 3600 s: mechanograms of muscles in control (**a**); muscle mechanograms with prophylactic administration of water-soluble C_{60} fullerenes (**b**); Δt_{50} – time of reduction of the maximum force to 50% of the initial level*

During stimulation with frequency of 2 Hz the time to reach the force of muscle contraction 50% of the initial level decreased significantly and amounted to 1890 s (Fig. 2). After injections of water-soluble C_{60} fullerene to animals, this time increased to 2340 s. It should also be noted that after the prophylactic use of water-soluble C_{60} fullerenes, the force of isometric muscle contraction, after some fall, reached a steady level. This confirms the fact that without the addition of the drug, there is a constant decrease in the level of muscle contraction throughout the period of stimulation, meanwhile after administration of C_{60}FAS, this decrease is significantly slowed down.

Data obtained in tensometric experiments (Fig. 2) showed that the decrease in muscle contraction force after administration of water-soluble

C_{60} fullerenes is almost two times slower than in the control after stimulation with frequency 1 Hz, and 45-55% slower than in control after stimulation with frequency 2 Hz. Significant reduction in the maximum level of strength developed by the muscle during the entire period of stimulation after application of the drug was 23 and 26% for slow and rapid muscle fatigue, respectively, while in the control (without drug administration) this figure reached 56 and 58%, respectively. This confirms our previously obtained data on the protective effect of water-soluble C_{60} fullerenes on the functions of the immune and antioxidant systems of the body in inflammatory processes and fatigue [16, 31]. However, a comparison of the development of rapid and slow skeletal muscle fatigue shows that the therapeutic effect of injections of C_{60}FAS significantly affects the development of slow fatigue and almost in two times differs from the effect after rapid fatigue.

Analysis of the biochemical composition of the blood of animals makes it possible to assess the functional changes that occur in the muscle due to its contraction, as well as the effectiveness of the therapeutic effect of the drug on fatigue. The biochemical parameters selected in the study, in particular creatinine, CPK, LA and LDH, are markers of physiological disorders in muscle tissue due to the development of fatigue.

The change in the level of creatinine, a product formed in muscle fibers during their destruction, allowed us to assess the level of myocyte damage during the prolonged contractions. This parameter increased from 59 ± 2 μM in the group of intact animals to 121 ± 4 and 147 ± 2 μM after stimulation with frequency 1 and 2 Hz, respectively (Fig. 3). Preliminary administration of C_{60} fullerenes reduced these values to 97 ± 5 and 132 ± 3 μM, respectively (Fig. 3).

The level of changes in LDH, an enzyme that catalyzes the oxidation of lactic acid, made it possible to assess the general state of muscle performance. The increase from 210 ± 5 Units/l in the group of intact animals to 381 ± 12 and 582 ± 12 Units/l after stimulation with frequency 1 and 2 Hz, respectively, is evidence of the development of significant muscle dysfunction associated with the development of fatigue. The increase in the LDH fraction in the blood is the result of both physiological destruction of myocyte walls and an increase in lactate content with prolonged muscle activation. Administration of C_{60}FAS reduced LDH levels to 303 ± 11 and 521 ± 19 Units/l, respectively (Fig. 3), which may be the evidence of reduction in mechanical damage to muscle fibers and in lactate concentrations in the muscular system in general.

In active muscle, most metabolic processes occur under anaerobic conditions, as a result muscle uses a significant amount of mitochondrial enzymes and, thus, accumulates a large amount of LA, which does not have time to oxidize with prolonged muscle stimulation. An increase in the level of lactic acid in the active muscle will indicate that its level of uptake into cells exceeds the level of its oxidation and excretion. Studies of individual muscle fibers have shown that acidification affects isometric strength and rate of contraction. At the same time, it has been found that muscle can recover faster than the pH returns to normal [32]. The effect of acidification on the development of muscle fatigue is not constant: a strong correlation was found between the level of acidosis and the development of muscle fatigue at a temperature of ≤15°C [33]. Experiments on individual muscle fibers [34] and whole mice muscle [33] have shown that at a temperature of 30°C, acidification has a minimal effect on the contractile activity of the muscle. Thus, under physiological temperatures, muscle contraction is weakly dependent on acidification. However, a possible alternative way for acidosis to alter muscle strength is energy metabolism. It is known that the key enzymes in glycogenolysis and glycolysis - phosphorylase and phosphofructokinase - are inhibited at acidic pH. There is also a theory that acidification significantly affects the release of calcium ions from the sarcoplasmic reticulum during muscle contraction. Acidification is thought to affect ryanodine-sensitive Ca^{2+}-channels and interfere with intracellular calcium release [35].

In the group of intact animals, the level of LA was 10.6 ± 2 M. After stimulation with a frequency 1 and 2 Hz, its value increased to 15.3 ± 1.0 and 23.4 ± 3.0 M, respectively. Injections of C_{60}FAS reduced LA levels to 11.3 ± 1.0 and 21.4 ± 1.0 M, respectively (Fig. 3). Thus, C_{60} fullerene therapy resulted in an increase in LA oxidation to almost control values after 1 Hz stimulation (slow muscle fatigue).

CPK is an enzyme from the energy supply system of musculoskeletal fibers that catalyzes the transfer of the phosphate group from ATP to the creatine molecule to form the high-energy compound creatine phosphate, which is used by the body as an energy substance after increasing physical ac-

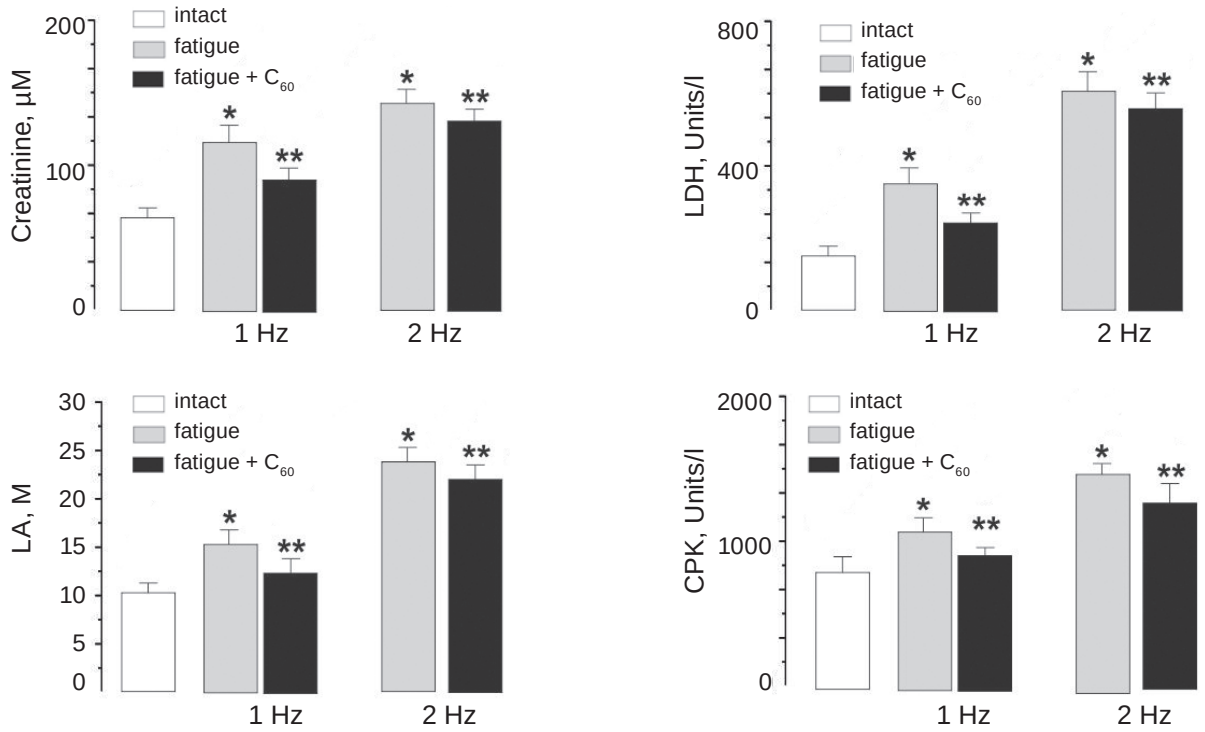

*Fig. 3. Biochemical parameters of muscle fatigue: levels of creatinine, LDH, LA and CPK in the blood of rats after stimulation with frequency 1 and 2 Hz of muscle soleus for 3600 s (*P < 0.05 relative to the intact group; **P < 0.05 relative to the corresponding control fatigue group)*

tivity. Changes in its concentration are one of the key markers of pathological processes in the muscle, which characterizes the depletion of energy reserves of the cell. Prolonged contraction results in mechanical damage to the muscles and the release of this enzyme from the cells and, consequently, an increase in its activity in the blood. The increase in CPK fraction from 780 ± 18 Units/l in the group of intact animals to 1010 ± 27 and 1202 ± 20 Units/l with muscle stimulation of frequency 1 and 2 Hz, respectively, is the result of physiological damage to the myocyte membrane, which increases with active long non-relaxation contraction. After administration of C_{60}FAS, the CPK level was 920 ± 24 and 1112 ± 28 Units/l, respectively (Fig. 3). Thus, therapeutic injections of C_{60} fullerene significantly increase the energy capacity of a functioning muscle, almost equally affecting the development of slow and rapid fatigue.

Taking into account that development of muscle fatigue is associated with an increase in the concentration of ROS, cellular antioxidant systems can be considered as components of the body's defense system against the effects of muscle fatigue.

Thus, the change in the level of TBARS was following: 2.63 ± 0.3 and 3.18 ± 0.4 μM (2.02 ± 0.20 μM in the group of intact animals) after development of fatigue caused by 1 and 2 Hz muscle stimulation, respectively, and 2.09 ± 0.20 and 2.93 ± 0.40 μM after development of fatigue caused by 1 and 2 Hz muscle stimulation and administration of C_{60}FAS, respectively (Fig. 4).

The level of H_2O_2 was 2.49 ± 0.40 and 2.91 ± 0.50 mM (1.43 ± 0.1 mM in the group of intact animals) with the development of fatigue after muscle stimulation with frequency 1 and 2 Hz, respectively, and 2.19 ± 0.6 and 2.82 ± 0.6 mM with the development of fatigue after muscle stimulation with frequency 1 and 2 Hz and administration of C_{60}FAS, respectively (Fig. 4).

CAT activity increased from 0.68 ± 0.1 mM/min in the group of intact animals to 0.81 ± 0.3 and 0.93 ± 0.1 mM/min after muscle stimulation with frequency 1 and 2 Hz, respectively, and decreased to 0.62 ± 0.1 and 0.76 ± 0.1 mM/min under the action of C_{60}FAS (Fig. 4).

The content of reduced GSH was 2.43 ± 0.5 and 2.92 ± 0.3 M (1.32 ± 0.4 M in the group of intact

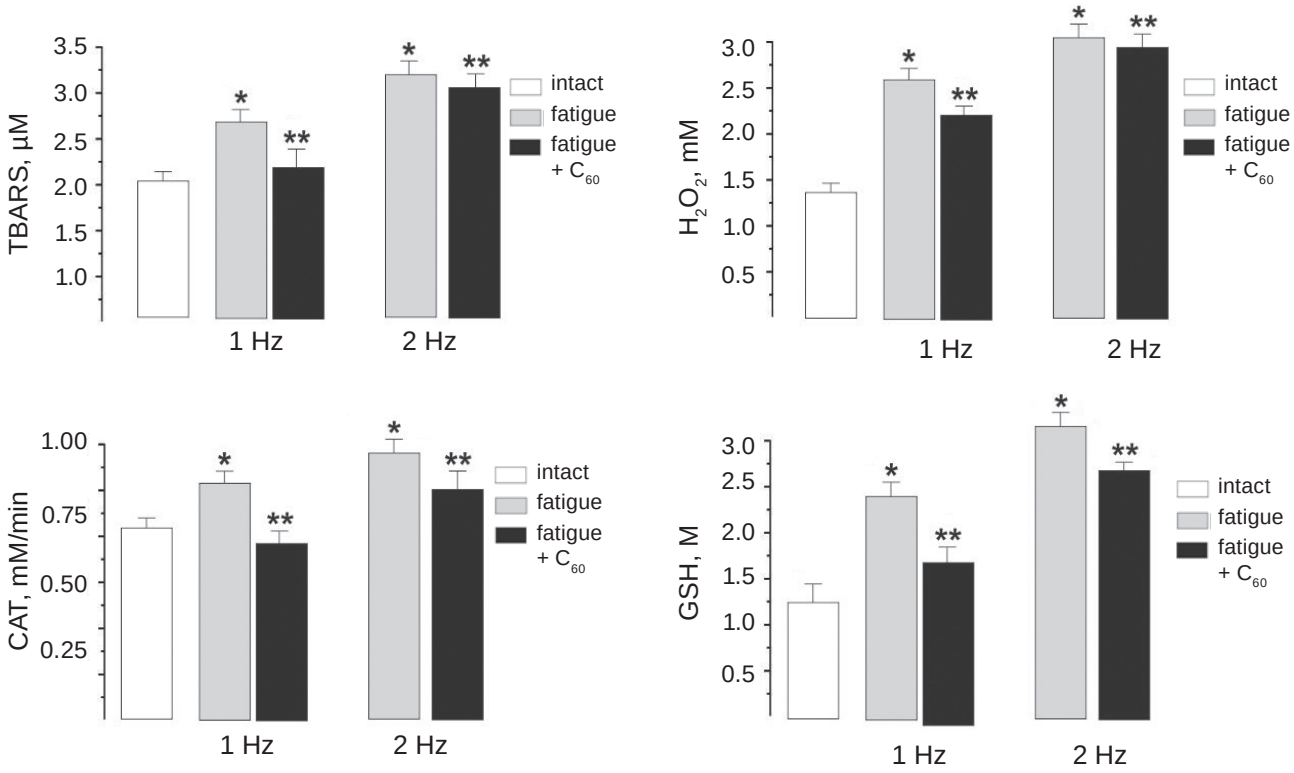

*Fig. 4. Indicators of pro- and antioxidant balance (TBARS, H_2O_2, CAT and GSH) in the blood of rats after stimulation of muscle soleus with frequency 1 and 2 Hz for 3600 s (*P < 0.05 relative to the intact group; **P < 0.05 relative to the corresponding control fatigue group)*

animals) with the development of slow and rapid fatigue, respectively, and 1.61 ± 0.2 and 2.59 ± 0.5 M with the development of slow and rapid fatigue and administration of C_{60}FAS, respectively (Fig. 4).

Thus, muscle fatigue caused the increase of the level of oxidative stress markers (TBARS and H_2O_2), compensatory activation of the anti-peroxide enzyme CAT, and increase of GSH level in blood of rats. The use of water-soluble C_{60} fullerenes reduced these values to the optimal level.

Conclusions. The analysis of the obtained data shows a clear tendency to decrease the rate of metabolic parameters in the blood of rats by approximately 45-60% with the development of slow fatigue and 35-40% with the development of rapid fatigue after administration of low doses of water-soluble C_{60} fullerenes. In addition, under conditions of long-term muscles activation C_{60} fullerene can normalize the endogenous antioxidant system, which leads to a weakening of prooxidant processes. At the same time, tensometric test data show that water-soluble C_{60} fullerenes significantly affect the development of slow fatigue (the detected effect is almost twice as different as the development of rapid fatigue). Thus, C_{60} fullerenes, as potent antioxidants, can prevent the onset of dysfunction in active muscle and thus maintain it within physiological limits throughout the contraction process. This opens the prospect of further clinical trials of C_{60}FAS as a potential therapeutic agent capable of correcting pathological conditions of the muscular system.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukr-biochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

Acknowledgments. This research was supported by the Ministry of Education and Science of Ukraine (project № 21БП018-01Р).

БІОХІМІЧНИЙ ТА ТЕНЗОМЕТРИЧНИЙ АНАЛІЗИ ПРОТЕКТОРНОЇ ДІЇ C_{60} ФУЛЕРЕНІВ НА РОЗВИТОК ВТОМИ СКЕЛЕТНИХ М'ЯЗІВ

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Проаналізовано протекторну дію водорозчинних C_{60} фулеренів на розвиток повільної та швидкої втоми скелетних м'язів щурів. Так, встановлено, що після внутрішньом'язової ін'єкції C_{60} фулеренів (доза 0,5 мг/кг) зниження сили скорочення м'яза (muscle soleus) на 50% від початкових значень відбувається майже вдвічі повільніше за стимуляції частотою 1 Гц (повільна втома м'яза), ніж за стимуляції частотою 2 Гц (швидка втома м'яза). Виявлено чітку тенденцію до зменшення величин біохімічних показників крові тварин за терапевтичної дії водорозчинних C_{60} фулеренів приблизно на 45–60% і 35–40% за розвитку повільної і швидкої втоми м'яза, відповідно. Таким чином, C_{60} фулерени, як потужні антиоксиданти, здатні ефективно впливати на про/антиоксидантний гомеостаз м'язової тканини і, таким чином, сприяти підтримці її нормального фізіологічного стану.

Ключові слова: скелетний м'яз, втома, біохімічний та тензометричний аналізи, C_{60} фулерен.

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