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Research Article

The Residual Effect of C_{60} Fullerene on Biomechanical and Biochemical Markers of the Muscle Soleus Fatigue Development in Rats

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Muscle fatigue as a defense body mechanism against overload is a result of the products of incomplete oxygen oxidation such as reactive oxygen species. Hence, C_{60} fullerene as a powerful nanoantioxidant can be used to speed up the muscle recovery process after fatigue. Here, the residual effect of C_{60} fullerene on the biomechanical and biochemical markers of the development of muscle soleus fatigue in rats for 2 days after 5 days of its application was studied. The known antioxidant *N*-acetylcysteine (NAC) was used as a comparison drug. The atomic force microscopy to determine the size distribution of C_{60} fullerenes in an aqueous solution, the tensiometry of skeletal muscles, and the biochemical analysis of their tissues and rat blood were used in this study. It was found that after the cessation of NAC injections, the value of the integrated muscle power is already slightly different from the control (5%–7%) on the first day, and on the second day, it does not significantly differ from the control. At the same time, after the cessation of C_{60} fullerene injections, its residual effect was 45%–50% on the first day, and 17%–23% of the control on the second one. A significant difference (more than 25%) between the pro- and antioxidant balance in the studied muscles and blood of rats after the application of C_{60} fullerene and NAC plays a key role in the long-term residual effect of C_{60} fullerene. This indicates prolonged kinetics of C_{60} fullerenes elimination from the body, which contributes to their long-term (at least 2 days) compensatory activation of the endogenous antioxidant system in response to muscle stimulation, which should be considered when developing new therapeutic agents based on these nanoparticles.

1. Introduction

Muscle fatigue is usually a short-term and reversible process manifested as a lack of energy [1]. The main causes of fatigue are related to overexertion, insufficient relaxation time, or physical trauma [2, 3]. However, muscle fatigue can be persistent and more severe when associated with pathological conditions, or chronic exposure to certain drugs and toxic compounds [4–6]. Although the origin of fatigue is multifactorial [7], muscle fatigue in pathological states is inextricably linked to the occurrence of muscle-mass loss and difficulty in performing precise goal-directed movements [8].

Fatigue can be caused by many mechanisms, ranging from metabolite accumulation in muscle fibers [9] to inadequate motor command generation [10]. Therefore, there is no single mechanism responsible for muscle fatigue; the mechanisms that cause fatigue are specific to the particular

task performed [11]. Quantitatively, the development of muscle fatigue is defined as a decrease in maximum muscle strength or power, meaning that maximum contractions cannot be maintained after the onset of muscle fatigue [12].

Any stress response of an organism is accompanied by an increase in reactive oxygen species (ROS). Today it is believed that free-radical processes may occupy one of the key positions in the regulatory mechanisms that determine the possibility of cell survival, its death, or transformation in stressful situations [13]. The condition in which the generation of free-radical processes increases more than the capacity of the antioxidant system due to the action of any factors is defined by researchers as oxidative stress [14]. Literature evidence suggests that free radicals are the main pathogenic factor in the process of muscle fatigue [15, 16]. They include the initiation of lipid peroxidation (LPO), direct inhibition of mitochondrial respiratory chain enzymes and ATPase (adenosine 5′-triphosphatase) activity, inactivation of glyceraldehyde-3-phosphate dehydrogenase, and membrane sodium channels [17, 18]. When stress is of sufficient strength, oxidative stress acts as a serious danger to the functioning of the organism, so there is a complex multilevel protection system against the excessive formation of free-radical transformation products in all cellular components [19]. The link of antioxidant reactions in the mechanism of protective processes is leading and most powerful, because it not only prevents the development of free-radical reactions, and the accumulation of superoxide anions, and peroxide, but also maintains the high activity of redox processes, provides elimination of final oxygen metabolites, promotes the activity of synthetic processes, including antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GP_x), which form, respectively, the first and second lines of protection [20].

At the moment, the search and development of new drugs are being actively conducted to reduce the manifestation of fatigue processes in skeletal muscles, changes in the biochemical parameters of the oxidative status, and energy metabolism under their influence. The main biochemical markers of the therapeutic effect of the drugs are the content of lactic acid, creatine, creatine kinase, SOD, and GP_x [20, 21]. However, the concentration of these markers in the blood and fatigued muscles can differ significantly and, thus, the interpretation of the kinetics of the therapeutic effect of the studied drug is much more difficult [21]. Biocompatible carbon nanostructures, C_{60} fullerenes, can be considered potential antioxidants [22]. They easily attach up to six electrons and thus can act as effective free radical scavengers [23]. We previously tested the powerful antioxidant properties of C_{60} fullerenes in experiments on ischemia, fatigue, and skeletal muscle injury [24–26]. However, the development of fatigue involves processes at all levels of the motor pathway between the brain and muscles [3]. Central fatigue is the inability of the nervous system to maximize muscle control [7]. It is defined as a progressive decrease in arbitrary activation or nerve impulse to a muscle caused by exercises [8]. On this basis, it cannot be excluded that the components of the applied drug on the nerve conduction of afferents may contribute to the resulting therapeutic effects [4, 5, 10, 25, 26] and thus contribute to improving the kinetics of neuronal mediator recovery. Since the question of the duration of the therapeutic effect after the agent application remains important within the framework of an adequate analysis of the dynamics of the muscle antifatigue therapy, we studied the residual effect of C_{60} fullerene on biomechanical and biochemical markers of fatigue development in rat muscle soleus during 2 days after its 5-day application. The known antioxidant *N*-acetylcysteine (NAC) [27, 28] was used as a comparison drug.

2. Materials and Methods

2.1. Preparation and Characterization of C_{60}FAS. A highly stable reproducible C_{60} fullerene aqueous colloid solution (C_{60}FAS) was prepared according to the protocol [29, 30]. 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^{-1}, and the same amount of distilled water in an open beaker. The two phases formed were treated in an ultrasonic bath. The procedure was continued until the toluene had completely evaporated and the water phase became yellow-colored. Filtration of the aqueous solution allowed to separate the product from undissolved C_{60} fullerenes. The concentration of C_{60} fullerene in the prepared C_{60}FAS sample was determined as the concentration of total organic carbon in an aqueous solution (Analytik Jena TOC Analyser multi N/C 3100). In our experiments, the C_{60}FAS sample with 0.15 mg mL^{-1} concentration of C_{60} fullerene was used. The prepared C_{60}FAS is stable within 12–18 months at temperature +4°C.

The atomic force microscopy (AFM) was performed to determine the size of C_{60} fullerene particles (their aggregates) in an aqueous solution [31]. Measurements were done with the "Solver Pro M" system (NT-MDT, Russia). A drop of investigated solution was transferred to 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 RTPE-SPA150 (Bruker, 6 N/m, 150 kHz) type.

2.2. Animals. Male Wistar rats (170 ± 12 g, 2 months old) were bred and housed in standard temperature conditions (21–23°C), lighting (12/12 hr light–dark cycle), and humidity (30%–35%). All animals had unlimited access to chow and tap water. The study was carried out in strict accordance with the European convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986) and was approved by the Bioethical Committee of the ESC "Institute of Biology and Medicine" of the Taras Shevchenko National University of Kyiv, Ukraine (study protocol no. 10 dated October 20, 2021).

The following groups of animals were tested: two experimental groups after 5 days of C_{60}FAS ($n = 7$) and NAC ($n = 7$) administration on the 1st and 2nd day after the cessation of the respective drug injection; the control ("fatigue,"

saline administration) group ($n = 7$) and the intact ("norm," no fatigue) group ($n = 7$).

Based on previously obtained data [28], the research protocol involved intraperitoneal injection of C_{60}FAS and NAC at a daily dose of 1 and 150 mg kg^{-1}, respectively, 1 hr before the experiment for 5 days.

It is important to note that the selected dose of C_{60}FAS in our experiments is significantly lower than the LD_{50} (lethal dose) value, which was 600 mg kg^{-1} body weight when administered orally to rats [32] and 721 mg kg^{-1} when administered intraperitoneally to mice [33].

2.3. Biomechanical Analysis. The object of the study was the rat muscle soleus. In preliminary preparation for the experiment, anesthesia was performed by intra-abdominal injection of Nembutal (40 mg kg^{-1}). Standard preparation included cannulation (a. carotis communis sinistra) for pressure measurement and laminectomy at the lumbar spinal cord level. Muscle soleus was released from surrounding tissues, and their tendon parts were connected to force measurement sensors in the distal part. To prepare for modulated efferent stimulation, the ventral roots in the respective segments were transected directly at their exit points from the spinal cord.

The dynamic properties of muscle contraction were studied under conditions of muscle activation using the method of modulated efferent stimulation [34]. Fatigue was induced by successive stimulation impulses with a frequency of 50 Hz and a duration of 5 s each, without a relaxation period between them. The sum of such stimulation signals was 500 s, followed by 5 min of relaxation. The number of stimulation pools was three. The external load on the muscle was controlled by using a system of mechanostimulators. Changes in contraction force were measured by strain gauges. During the analysis of the results, we used quantitative parameters-integrated muscle power (calculated area under the strength curve), which is an indicator of its general performance under the applied stimulation pools, and levels of maximum and minimum strength generation of contraction, which are indicators of the general dysfunction of the muscular system in the development of fatigue [4, 5, 24, 26].

2.4. Biochemical Analysis. LPO was measured from the formation of thiobarbituric acid-reactive substances (TBARS) using the method of Buege and Aust [35]. TBARS were isolated by boiling tissue homogenates for 15 min at 100°C with thiobarbituric acid reagent (0.5% 2-thiobarbituric acid/10% trichloroacetic acid/0.63 mM (millimolar) hydrochloric acid) and measuring the absorbance at 532 nm. The results were expressed as nM (nanomolar) mg^{-1} of protein using $\varepsilon = 1.56 \times 10^5$ mM^{-1} cm^{-1}.

The hydrogen peroxide (H_2O_2) concentration in the tissue homogenates was measured using the method, which is based on the peroxide-mediated oxidation of Fe^{2+}, followed by the reaction of Fe^{3+} with xylenol orange (*o*-cresolsulphonephthalein 3′,3″-bis(methylimino) diacetic acid, sodium salt). This method is extremely sensitive and is used to measure low levels of water-soluble hydroperoxide present in the aqueous phase. To determine the H_2O_2 concentration, 500 μL of the incubation medium was added to 500 μL of assay reagent (500 μM ammonium ferrous sulfate, 50 mM H_2SO_4, 200 μM xylenol orange, and 200 mM sorbitol). The absorbance of the Fe^{3+}-xylenol orange complex (A560) was detected after 45 min. Standard curves of H_2O_2 were obtained for each independent experiment by adding variable amounts of H_2O_2 to 500 μL of basal medium mixed with 500 μL of an assay reagent. Data were normalized and expressed as μM H_2O_2 per mg protein [36].

Total SOD activity was measured by the method [37], which is based on the inhibition of autooxidation of adrenaline to adrenochrome by SOD contained in the examined samples. The results were expressed as specific activity of the enzyme in units per mg protein. One unit of SOD activity is defined as the amount of protein, causing 50% inhibition of the conversion rate of adrenaline to adrenochrome, under specified conditions.

CAT activity was measured by the decomposition of hydrogen peroxide, determined by a decrease in the absorbance at 240 nm [38].

The activity of selenium-dependent GP_x was determined according to the method [39]. Briefly, the reaction mixtures consisted of 50 mM KPO_4 (pH 7.0) 1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM NaN_3, 0.2 mM NADPH (nicotinamide adenine dinucleotide phosphate), 1 mM GSH (reduced glutathione), 0.25 mM H_2O_2, 226 U mL^{-1} glutathione reductase, and rates of NADPH oxidation followed at 340 nm.

The GSH was determined as described [40]. The tissue sample was mixed with sulphosalicylic acid (4%) and incubated at 4°C for 30 min. Thereafter, it was centrifuged at 1,200 × g for 15 min at 4°C and 0.1 mL of this supernatant was added to phosphate buffer (0.1 M, pH 7.4) containing DTNB (5,5′-dithio-bis-(2-nitrobenzoic acid) in abs. ethanol. The yellow color developed was read immediately at 412 nm. The GSH content was calculated as mM GSH/mg protein ($\varepsilon_{412} = 13.6 \times 10^3$ M^{-1} cm^{-1}).

The levels of TBARS, hydrogen peroxide, GSH, GP_x, SOD, and CAT activity as markers of total antioxidant status [41], were determined in the blood plasma of experimental animals using clinical diagnostic equipment—a haemoanalyzer (Erba, Czech Republic) [24].

2.5. Statistical Analysis. Statistical processing of the measurement results was performed by methods of variational statistics using the software Origin 9.4. Each of the experimental force curves obtained in the work is the result of averaging 10 similar experiments. At least three repetitions were performed for each biochemical measurement. Data are expressed as means ± SEM (standard error of the mean) for each group. Differences from experimental groups were indicated by one-way ANOVA described Bonferroni's multiple comparison test. Values of $p < 0.05$ were considered significant.

3. Results and Discussion

3.1. Characterization of C_{60}FAS. When studying the layers deposited from C_{60}FAS, 3D images of AFM revealed a monodisperse film (~1.1 nm thick) in the form of a solid mesh

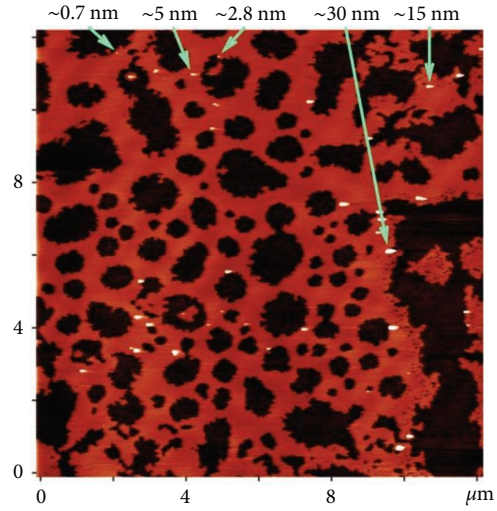

FIGURE 1: AFM image of the C_{60} fullerene layer deposited from C_{60}FAS (0.15 mg mL^{-1}) on the mica surface. Arrows indicate the heights of nanoscale objects.

superstructure and individual islands (Figure 1). As one can see, in addition to individual C_{60} molecules (a height of ~0.7 nm), its composition includes nanoaggregates of C_{60} fullerenes with a height of 2.8–30 nm. It is important to note that the size distribution of C_{60} fullerene nanoparticles in an aqueous solution did not change for 6 months, which indicates the absence of additional aggregation of nanoparticles and confirms their suitability for in vivo studies.

3.2. Biomechanical Analysis. The phenomenological approach to the analysis of pathological processes affecting the mechanical properties of the muscle makes it possible to establish important relationships between the macroscopic parameters of the muscle state, such as strength, speed, elasticity, and the level of afferent activity. In many cases, this is sufficient to analyze the processes of regulation of motor activity and the degree of the pathological condition and assess the impact of therapeutic measures.

Figure 2 shows the change in contractile strength of rat muscle soleus when applying 50 Hz stimulation for 5 s in three successive pools for 500 s each with 5 min of relaxation between them after C_{60}FAS and NAC administration for 5 days. The three-component muscle stimulation was chosen based on our earlier data [4, 5, 12, 25, 26, 28] on the maximum level of fatigue processes development exactly at this type of fatigue stimulation, which exhausts the possibilities of adaptive restructuring of the muscle system to great physical loads and implies the presence of the full range of muscle fatigue dysfunctions.

Analysis of the value of integrated power allows us to evaluate the kinetics of muscle fatigue formation in the system of equilibrium "force of contraction-external load," which is a physiological analog of the performance of the muscular system as a whole (Figure 3(a)). It turned out that already after the first pool of stimulation, integrated power decreased significantly by $58 \pm 4\%$. After the relaxation period, it decreased progressively at the 2nd and 3rd stimulation pools and was $39 \pm 2\%$ and $24 \pm 3\%$ of control values, respectively.

Application of NAC during 5 days increased the value of this index: its effect was $32 \pm 3\%$, $28 \pm 2\%$, and $25 \pm 2\%$ at the 1st, 2nd, and 3rd stimulation pools, respectively. On the second day after cessation of NAC therapy, its residual effect was no more than 5% of control values at the 1st, 2nd, and 3rd stimulation pools and remained virtually unchanged thereafter.

Injection of C_{60}FAS increased the level of this parameter. On day 5 of therapy, its effect was $41 \pm 3\%$, $69 \pm 6\%$, and $75 \pm 3\%$ at the 1st, 2nd, and 3rd stimulation pools, respectively. On the second day after cessation of C_{60}FAS injection, its residual effect was 39%, 30%, and 25% of the control values at the 1st, 2nd, and 3rd stimulation pools, respectively, and did not change significantly thereafter.

Changes in the levels of generation of maximum and minimum contraction strength can be associated both with the development of fatigue processes in the neural component and with myotic components of the pathology under study. When performing sufficiently simple single-joint movements, these markers are the main indicators of muscle dysfunction, the phenomenological analysis of which makes it possible to establish the presence of causal relationships between the level of decrease in the biomechanical activity of the muscles, the main mechanical parameters of movements, and the level of development of the pathological process.

The analysis of the obtained mechanograms (Figure 3(b)) showed changes in the maximum strength of contractions in the control measurements, which were 0.7 ± 0.1 N, 0.42 ± 0.03 N, and 0.39 ± 0.03 N at 1st, 2nd, and 3rd stimulation pools, respectively.

The use of NAC did not significantly change the maximum contraction strength at any of the three studied stimulation pools during the 5 days of its application. On the first day after discontinuation of NAC, its residual effect was 8%, 6%, and 5% on 1st, 2nd, and 3rd pools, respectively, and on the second day, no significant differences in mechanokinetics of muscle contraction were recorded

The use of C_{60}FAS for 5 days increased the described values. Its residual effect was 39%, 26%, and 21% at 1st, 2nd, and 3rd pools, respectively, on day 1 after discontinuation of the agent and 16%, 9%, and 6% on day 2.

The minimum force response of the studied muscle in the control (Figure 3(b)) was 0.50 ± 0.04 N, 0.21 ± 0.02 N, and 0.10 ± 0.01 N at the 1st, 2nd, and 3rd pools, respectively.

Application of NAC injections during 5 days increased minimum strength values. On the first day after cessation of NAC use, its residual effect was 8% and 5% at 1st and 2nd pools, respectively, and at 3rd pool, as well as on day 2 after cessation of NAC use, no significant differences in mechanokinetics of contraction were recorded.

Injections of C_{60}FAS during 5 days have significantly changed the mechanokinetics of the contractile process: its effect was 44%, 80%, and 470% at the 1st, 2nd and 3rd pools, respectively. As can be seen, the maximum effect is observed on the 3rd pool of contraction, on which, in turn, the most severe disturbances of contraction biomechanics are observed

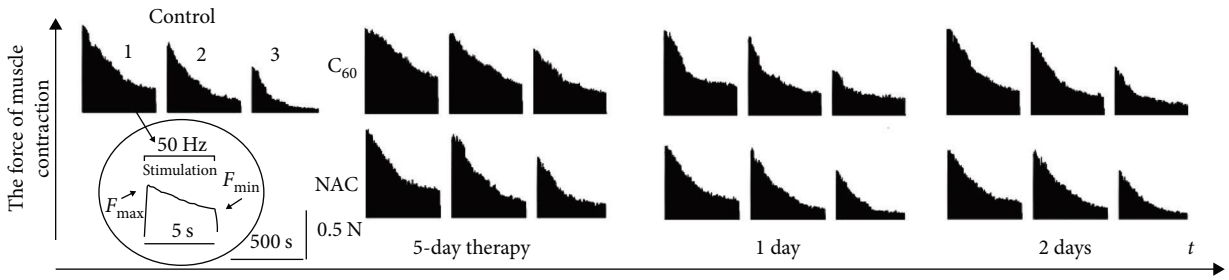

Figure 2: Recording the contractile force of rat muscle soleus when applying 50 Hz stimulation for 5 s in three consecutive pools (1, 2, and 3) for 500 s each with 5 min relaxation between them: control-native muscle; 5-day therapy—mechanograms of muscle after C_{60}FAS (C_{60}) and NAC administration during 5 days; 1 day and 2 days—mechanograms of muscle on day 1 and 2, respectively, after C_{60} and NAC administration; F_{max} and F_{min}—maximum and minimum strength of a single contraction, respectively.

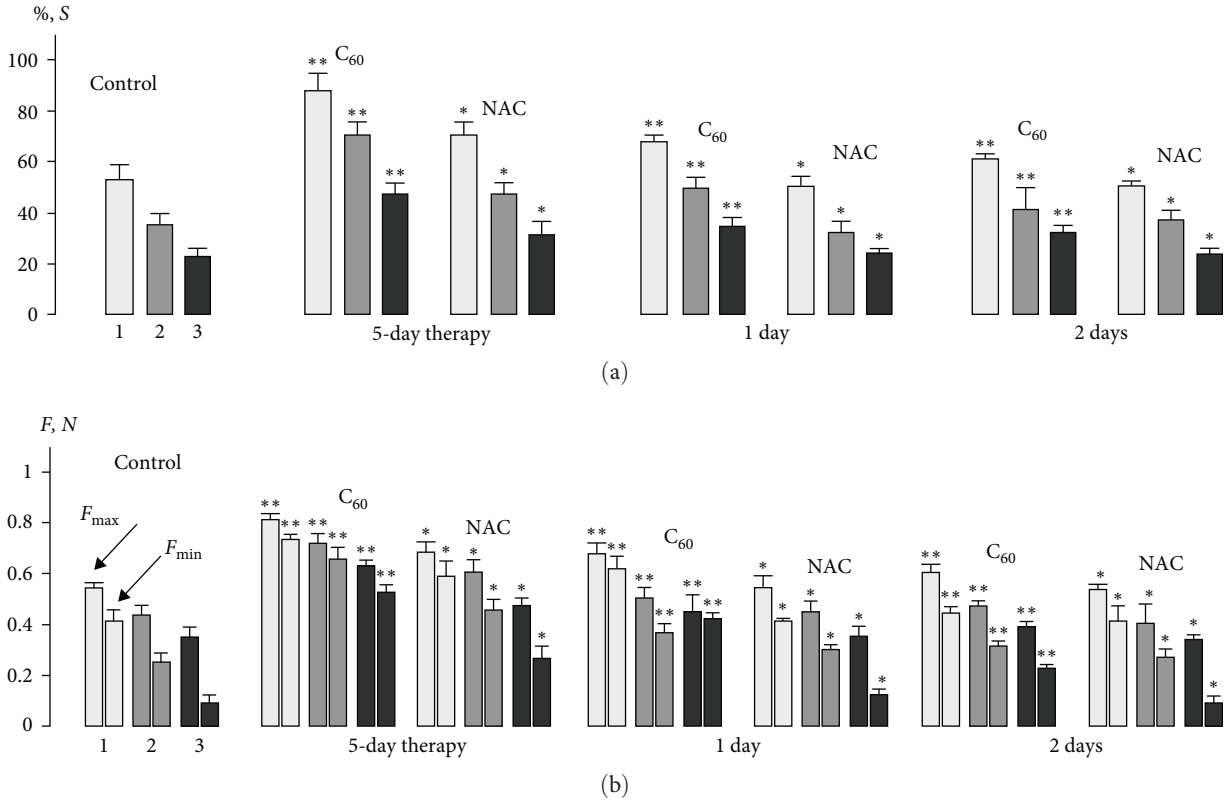

Figure 3: Integrated power (S, presented as a percentage of maximum values) (a) and peak contraction force values (F, N) (b) of rat muscle soleus when applied 50 Hz stimulation for 5 s duration in three consecutive pools (1, 2, and 3) for 500 s duration each with 5 min relaxation between them: control-native muscle; 5-day therapy—muscle mechanograms after C_{60}FAS (C_{60}) and NAC administration for 5 days; 1 day and 2 days—muscle mechanograms on day 1 and 2, respectively, after C_{60} and NAC administration; F_{max} and F_{min}—maximum and minimum single contraction force, respectively. $^{*}p<0.05$ compared with control ("fatigue" group); $^{**}p<0.05$ compared to values in the NAC group.

in the development of skeletal muscle fatigue. The residual effect of C_{60}FAS was 37%, 42%, and 227% on pools 1, 2, and 3, respectively, on the first day after discontinuation of the drug and 15%, 19%, and 158% on day 2.

Thus, the biomechanical effects of skeletal muscle described above are based on the antioxidant mechanism of C_{60} fullerene action. The increase in muscle contraction strength is probably due to the inactivation of the excess amount of secondary oxidation products in muscle fibers by C_{60} fullerenes, which leads to a slowdown in the development of the fatigue process. The use of C_{60} fullerene therapy can reduce the severity of muscle fiber microtraumas that occur during prolonged physical exertion. As is known, the main factor in membrane damage is an increase in LPO induced by an excess of free radicals. The appearance of a large number of injured myocytes leads to the occurrence of local inflammatory processes and, as a result, an increase in subfascial pressure and the occurrence of myofascial compartment syndrome. These processes increase the stiffness components of the muscles, leading to a decrease in the time of fatigue development and a significant decrease in

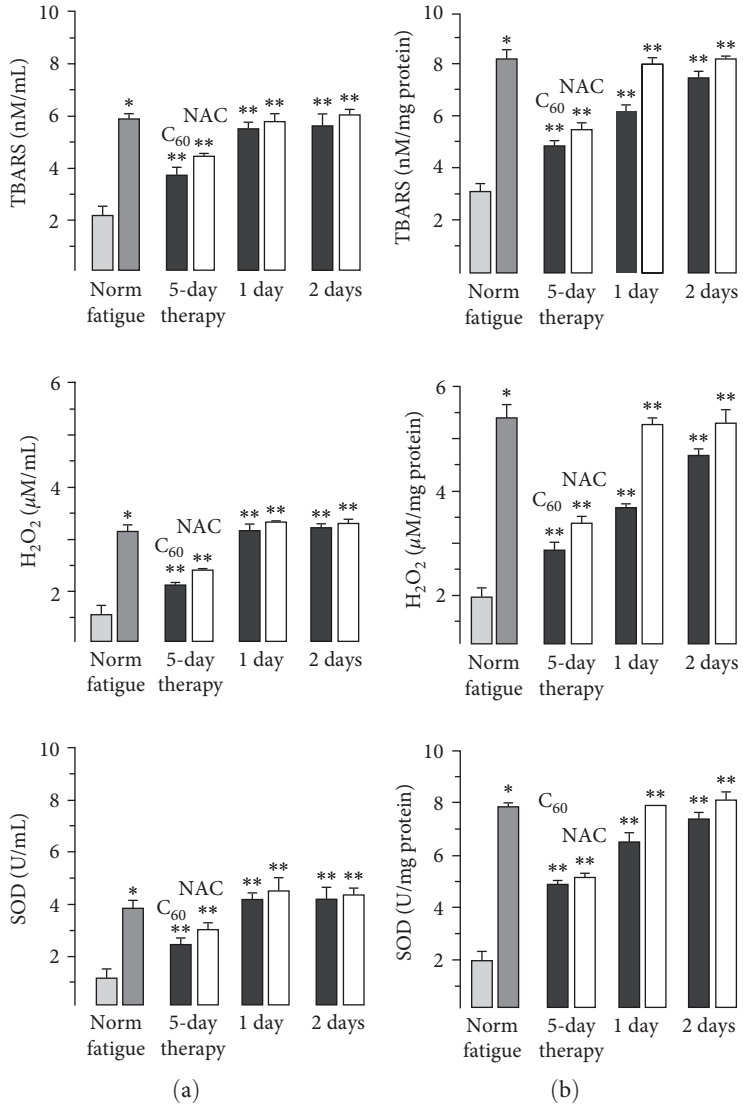

Figure 4: Indicators of pro- and antioxidant balance (TBARS, H_2O_2, and SOD) in the blood plasma (a) and muscle soleus (b) of rats after C_{60}FAS and NAC application: 5-day therapy—data after C_{60}FAS (C_{60}) and NAC administration for 5 days; 1 day and 2 days—data on the first and second day, respectively, after C_{60} and NAC application. $^{*}p<0.05$ compared with intact ("norm") group; $^{**}p<0.05$ compared with control ("fatigue" group).

muscle power. To confirm this hypothesis, it is necessary to analyze changes in the parameters of the pro- and antioxidant balance of muscle tissue and blood in rats in the described models of the development of fatigue processes.

3.3. Biochemical Analysis. The changes in blood chemistry during the development of fatigue processes are a reflection of biochemical shifts occurring both in the muscle complex and at the level of accessory organs and tissues (activity of liver enzymes, kidney function, etc.). We compared antioxidant enzymes isolated from the blood of the studied animals and the tissues of the muscle subjected to stimulation fatigue.

Inflammatory processes occurring immediately after the onset of fatigue in skeletal muscle are a source of ROS and contribute to the intensification of LPO processes [34, 35]. This interferes with the adequate performance of muscle work and significantly increases the recovery period. During reperfusion, oxygen entering the tissues initiates xanthine and hypoxanthine oxidation by xanthine oxidase, which leads to the formation of large amounts of superoxide anion radicals and hydrogen peroxide. As a result of biochemical tests, we have determined the number of secondary LPO products and antioxidant levels in the blood of rats after induction of fatigue. The obtained data demonstrate an increased level of markers of peroxidation and oxidative stress after the occurrence of muscle fatigue and their decrease with the applied therapeutic agents (Figures 4 and 5).

The change in TBARS level in the blood plasma on day 5 of the experiment was $5.8 \pm 0.2\,\text{nM}\,\text{mL}^{-1}$ during the development of fatigue ($2.2 \pm 0.4\,\text{nM}\,\text{mL}^{-1}$ in the intact group), 4.4 ± 0.1 and $4.1 \pm 0.4\,\text{nM}\,\text{mL}^{-1}$ after application of NAC

and C_{60}FAS, respectively. On the second day after 5 days of NAC administration, its level was 5.2 ± 0.2 nM mL^{-1} (10% residual effect). After discontinuation of C_{60}FAS, its residual effect on the first and second days was 19% and 14%, respectively (Figure 4(a)).

The change of TBARS level in rat muscle soleus on day 5 of the experiment was 8.2 ± 0.1 nM mg^{-1} protein during fatigue development (2.5 ± 0.3 nM mg^{-1} protein in the intact group), 5.8 ± 0.1 nM mg^{-1} protein and 4.8 ± 0.2 nM mg^{-1} protein after NAC and C_{60}FAS application, respectively. On day 2 after a 5-day NAC administration, its level did not differ from the control values. After discontinuation of C_{60}FAS, its residual effect on the first and second days was 6.1 ± 0.2 nM mg^{-1} protein and 7.3 ± 0.3 nM mg^{-1} protein (26% and 11% effect), respectively (Figure 4(b)).

Blood plasma H_2O_2 levels on day 5 of the experiment were $3.3 \pm 0.2\,\mu$M mL^{-1} during fatigue development ($0.8 \pm 0.1\,\mu$M mL^{-1} in the intact group), 2.4 ± 0.2 and $2.1 \pm 0.2\,\mu$M mL^{-1} after NAC and C_{60}FAS administration, respectively. On the second day after 5 days of NAC administration, its level did not differ from the control values. After discontinuation of C_{60}FAS, its residual effect on the first and second days was 2.5 ± 0.1 and $2.8 \pm 0.2\,\mu$M mL^{-1} (18% and 10% effect), respectively (Figure 4(a)).

The change of H_2O_2 level in rat muscle soleus on day 5 of the experiment was $3.3 \pm 0.2\,\mu$M mg^{-1} protein during the development of fatigue ($0.8 \pm 0.1\,\mu$M mg^{-1} protein in the intact group), $4.4 \pm 0.2\,\mu$M mg^{-1} protein and $2.7 \pm 0.1\,\mu$M mg^{-1} protein after NAC and C_{60}FAS application, respectively. On the first day after 5-day NAC administration, its residual effect was 5%, and on the second day, the H_2O_2 level did not differ from the control values. After discontinuation of C_{60}FAS, its residual effect on was $3.6 \pm 0.1\,\mu$M mg^{-1} protein and $4.7 \pm 0.2\,\mu$M mg^{-1} protein (34% and 14% effect), respectively (Figure 4(b)).

Blood plasma SOD activity on day 5 of the experiment was 3.9 ± 0.1 U mL^{-1} during fatigue development (1.3 ± 0.1 U mL^{-1} in the intact group), 3.0 ± 0.2 and 2.3 ± 0.2 U mL^{-1} after NAC and C_{60}FAS administration, respectively. On the first day after a 5-day administration of NAC and C_{60}FAS, a 9% residual effect was observed only for C_{60}FAS. On the second day, the blood SOD activity was no different from the control values for both agents (Figure 4(a)).

The SOD activity in the muscle soleus on day 5 of the experiment was 6.8 ± 0.2 U mg^{-1} protein during fatigue development (1.6 ± 0.1 U mg^{-1} protein in the intact group), 5.0 ± 0.2 U mg^{-1} protein, and 4.1 ± 0.2 U mg^{-1} protein after NAC and C_{60}FAS administration, respectively. On the first and second days after discontinuation of NAC, SOD activities were virtually indistinguishable from control values, and C_{60}FAS showed a residual effect of 5.9 ± 0.1 U mg^{-1} protein and 6.2 ± 0.1 U mg^{-1} protein (13% and 8% therapeutic effect), respectively (Figure 4(b)).

The CAT activity in the blood plasma on day 5 of the experiment increased from $0.9 \pm 0.1\,\mu$M min^{-1} mL^{-1} in the intact group to $4.3 \pm 0.1\,\mu$M min^{-1} mL^{-1} after the development of muscle fatigue, decreasing to 3.4 ± 0.1 and 2.8 ± 0.1 M min^{-1} mL^{-1} with NAC and C_{60}FAS injections, respectively. On the first day after a 5-day application of NAC and C_{60}FAS, a residual effect of 7% was observed only for C_{60}FAS. On day 2, the activity of CAT in the blood no longer differed from the control values for both drugs (Figure 5(a)).

The CAT activity in the muscle soleus on day 5 of the experiment was $1.8 \pm 0.1\,\mu$M min^{-1} mg^{-1} protein in the intact group, $4.6 \pm 0.2\,\mu$M min^{-1} mg^{-1} protein after fatigue induction, $4.1 \pm 0.1\,\mu$M min^{-1} mg^{-1} protein and $3.4 \pm 0.3\,\mu$M min^{-1} mg^{-1} protein when applying NAC and C_{60}FAS, respectively. A residual effect (13%) was recorded only for C_{60}FAS on the second day after discontinuation of its use (Figure 5(b)).

The concentration of GSH in the blood plasma on day 5 of the experiment was 2.7 ± 0.6 mM mL^{-1} during the development of fatigue (1.2 ± 0.1 mM mL^{-1} in the intact group), 2.1 ± 0.6 and 1.6 ± 0.5 mM mL^{-1} after NAC and C_{60}FAS administration, respectively. The residual effect of NAC was 9% on the first day, and for C_{60}FAS it was 17% and 11% on the first and second day, respectively, after discontinuation of its administration (Figure 5(a)).

The GSH concentration in the muscle soleus on day 5 of the experiment was 1.2 ± 0.1 mM mg^{-1} protein in the intact group and increased to 2.6 ± 0.2 mM mg^{-1} protein during the development of fatigue, decreasing to 1.7 ± 0.1 mM mg^{-1} protein and 1.5 ± 0.1 mM mg^{-1} protein after NAC and C_{60}FAS, respectively. The residual effect of NAC was 8% on the second day, and for C_{60}FAS it was 27% and 17% on the first and second days, respectively, after discontinuation of its use (Figure 5(b)).

Cellular mechanisms of antioxidant protection are also associated with the functioning of a powerful glutathione link. The protective functions of GP_x during oxidative stress are determined by the ability to catalyze the cleavage of hydrogen peroxide and fatty acid hydroperoxide with GSH. The GP_x concentration in blood plasma on day 5 of the experiment was 3.9 ± 0.4 nM NADPH min^{-1} mL^{-1} in the intact group, 7.9 nM NADPH min^{-1} mL^{-1} after fatigue initiation, 6.4 ± 0.2 nM NADPH min^{-1} mL^{-1} and 5.9 ± 0.4 nM NADPH min^{-1} mL^{-1} after NAC and C_{60}FAS, respectively. The residual effect of NAC was 8% and 4% on the first and second days, respectively, and for C_{60}FAS it was 16% and 12%, respectively, after discontinuation of its administration (Figure 5(a)).

GP_x concentration in muscle soleus on day 5 of the experiment was 4.1 ± 0.4 nM NADPH min^{-1} mg^{-1} protein in the intact group, 8.9 ± 0.6 nM NADPH min^{-1} mg^{-1} protein after fatigue initiation, 5.3 ± 0.5 nM NADPH min^{-1} mg^{-1} protein and 5.9 ± 0.4 nM NADPH min^{-1} mg^{-1} protein after NAC and C_{60}FAS administration, respectively. The residual effect of NAC was 9% and 6% on the first and second days, respectively, and for C_{60}FAS was 26% and 16%, respectively, after discontinuation of its administration (Figure 5(b)).

Our studies have shown that on day 6 and 7 of the experiment (day 1 and 2 after cessation of the administration of the corresponding drug), SOD activity remains significant in the muscles of rats against the background of decreased LPO processes. This indicates the preservation of a sufficient level of aggressive superoxide anion dismutation in the remote period of the experiment. The decrease in H_2O_2 content under these conditions is evidence of the coordinated action of the antiperoxide enzymes, CAT and GP_x, whose

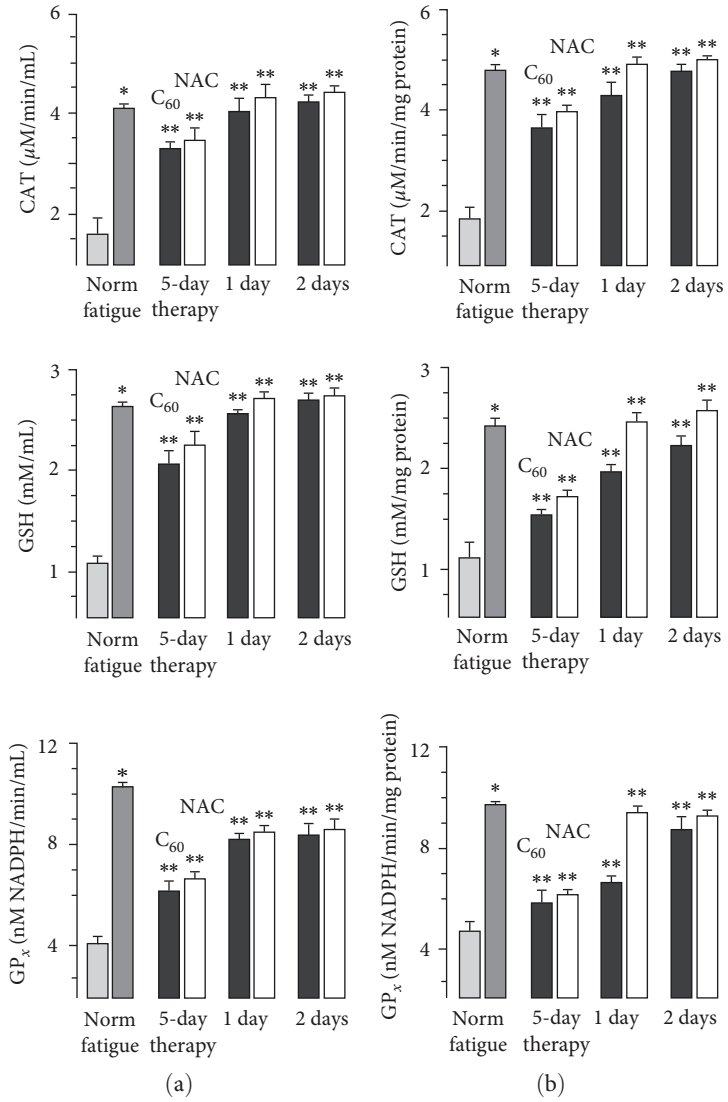

Figure 5: Indicators of antioxidant balance (CAT, GSH, and GP_x) in the blood plasma (a) and muscle soleus (b) of rats after C_{60}FAS and NAC application: 5-day therapy—data after C_{60}FAS (C_{60}) and NAC administration for 5 days; 1 day and 2 days—data on the first and second day, respectively, after C_{60} and NAC administration. $^{*}p<0.05$ compared with intact ("norm") group; $^{**}p<0.05$ compared with control ("fatigue" group).

activity also increases. This is probably due to the formation of adaptation reactions of the organism to the action of an extreme stimulus. Repeated induction of ROS during muscle stimulation results in increased cellular resistance to a stress factor and forms long-term adaptation [42]. Under these conditions ROS play the role of secondary messengers, participating in the processes of natural signal transduction in tissues. This is manifested primarily by the activation of transcription factors (hypoxia-inducible factor (HIF-1), nuclear factor kappa B (NF-κB), activator protein (AP-1)) and corresponding genes encoding antioxidant enzymes, in particular SOD, enzymes of the glutathione system, and CAT [42, 43]. Against this background, the use of C_{60} fullerenes can enhance and promote further activation of the above processes. This is proved by our preliminary studies, which confirmed the fact that the use of C_{60} fullerene under extreme conditions affects the rapid formation of adaptive reactions of the body by affecting such transcription factors as Nrf2 (NF-E2-related factor 2), NF-κB, and p53 [44]. The glutathione system plays an important role in the implementation of antiradical and antiperoxide cell protection [22]. The coordinated action of all components (GSH, glutathione-dependent, and NADPH-generating enzymes) contributes to the establishment of optimal levels of peroxide compounds and the preservation of antioxidant homeostasis [45]. Glutathione's high-redox activity with simultaneous resistance to oxygen oxidation, significant concentration in the cell, and ability to maintain its reduced state make it an important intracellular redox buffer [46]. Recent studies suggest a nonspecific nature of changes in the content of thiol compounds (primarily glutathione) for the action of extreme factors on the body, as well as their participation in the formation of adaptation processes [47]. Herewith the mechanism of GSH action can be dual. On the one hand, it neutralizes ROS by acting directly as a trap for

free radicals, or by ensuring the work of the specific peroxidases. On the other hand, GSH restores several oxidized proteins, thus restoring the functional activity of the wide range of enzymes, receptors, and transcription factors, and contributing, in this way, to the rapid formation of compensatory-adaptive responses [48]. Our results showed that the use of C_{60} fullerene enhances the synthesis of glutathione on 6th and 7th days of the experiment (−59%, $p < 0.05$). Thus, long-term use of C_{60} fullerenes promotes the faster and more efficient formation of the adaptation processes under conditions of electrical stimulation of skeletal muscle of rats.

In summary, in the process of muscle fatigue, metabolism is disturbed, and products of incomplete oxidation of oxygen, peroxides, and free radicals are formed. The use of C_{60} fullerenes, as powerful antioxidants, helps reduce oxidative processes in skeletal muscles by maintaining a balance between pro-oxidants and the antioxidant defense system.

4. Conclusion

Thus, these data indicate that after 5-day use of the studied agent C_{60} fullerene has a 50%–70% stronger effect on the resumption of muscle biomechanics after fatigue than NAC. It was found that after cessation of NAC injections, the value of integrated muscle power already on the first day did not differ significantly from the control (5%–7%), and on the second day, did not differ significantly from the control. At the same time, after cessation of C_{60} fullerene injections, its residual effect was 45%–50% on the first day, and on the second day, 17%–23% of the control.

There is a clear tendency for all the described biochemical parameters to decrease by about 15% with therapeutic administration of NAC and by 30%–40% with C_{60} fullerene after 5 days of their application. The significant difference (more than 25%) between the pro- and antioxidant balance parameters in the studied muscles and blood plasma of rats after C_{60}fullerene administration probably plays a key role in its long-term residual effect compared with NAC. This indicates prolonged kinetics of water-soluble C_{60}fullerene excretion from the body, which contributes to a long (at least 2 days) compensatory activation of the endogenous antioxidant system by C_{60} fullerene in response to muscle stimulation, which should be considered in the development of new therapeutic agents based on this powerful nanoantioxidant [49].

Data Availability

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Ethical Approval

The study protocol was approved by the Bioethics Committee of Taras Shevchenko National University of Kyiv by 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 by the Law of Ukraine No. 3447–IV 21.02.2006, Kyiv, on the Protection of Animals from Cruelty during medical and biological research.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Biomechanical analysis was performed by Dmytro Nozdrenko and Kateryna Bogutska; biochemical analysis was performed by Olga Gonchar and Svitlana Prylutska; preparation and characterization of the samples were done by Yuriy Prylutskyy, Eric Täuscher, and Uwe Ritter; coordination of the research work, analysis of the data, and preparation of the manuscript were done by Yuriy Prylutskyy, Peter Scharff, and Uwe Ritter.

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