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C_{60} FULLERENE EFFECT ON THE FUNCTIONAL ACTIVITY OF RAT *GASTROCNEMIUS MUSCLE* DURING ITS REGENERATION AFTER THE OPEN INJURY

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Open injuries are one of the most common skeletal muscle traumas. The study aimed to estimate the effect of the oral administration of C_{60} fullerene aqueous solution (C_{60}FAS) daily at a dose of 1 mg/kg on the restoration of rat skeletal muscle functional activity on the 5th, 10th and 15th day after the open trauma. Male Wistar rats were randomly divided into three groups of 12 animals in each: control, with muscle injury and with muscle injury+C_{60}FAS. The isolated gastrocnemius muscle was subjected to open injury by transverse dissection with a depth of 1 mm. Stimulation of muscle efferents was carried out by electrical impulses generated using a strain gauge generator. The content of C-reactive protein, creatinine, lactate, reduced glutathione and the activity of catalase and superoxide dismutase in the rat blood were determined. According to the data obtained, application of C_{60}FAS promotes the restoration of the functional activity of injured muscle, which was confirmed by a significant increase in gastrocnemius muscle force impulse, attenuation of the inflammatory and development of fatigue and normalization of pro- and antioxidant balance in the process of regeneration.

K e y w o r d s: C_{60} fullerene, gastrocnemius muscle, open injury, muscle force impulse, protein C, lactate, pro-antioxidant balance.

Open injuries are one of the most common skeletal muscle traumas, which are often the leading cause of death and disability among civilians and military personnel [1, 2]. It is related to the fact that there is not only a high heterogeneity in the severity of the injuries sustained, but they occur in different muscle groups, which makes it very difficult to conduct clinical trials.

Post-traumatic skeletal muscle repair is a series of complex processes involving coordinated stages of degeneration, inflammation, regeneration and fibrosis. The sequence of events includes initial damage, which is primarily mechanical, and secondary, metabolic or biochemical damage, which peaks 1-5 days after the traumatic events. Recovery from injury is usually completed within 30 days [3]. However, with incomplete or poor-quality repair, mechanical loads on the injured skeletal muscle usually exceed its biomechanical capabilities, causing additional acute injury. In addition, the superficial location of many muscles makes them even more vulnerable to reinjury due to the occurrence of tears [4]. Also, preclinical studies [5] have revealed that muscle mass remaining after injury or incomplete repair functions suboptimally and the percentage of strength deficit is significantly higher than can be explained by the initial mass loss of the contractile apparatus.

Thus, the long healing time combined with a high frequency of repeated injuries of the musculoskeletal system make them the main cause of work capacity loss and subsequent disability. Given this, there is a clinically justified need to accelerate the kinetics of tissue healing after open injuries [6]. The phase of muscle regeneration is complicated by a large amount of free radicals produced during the developed inflammatory process. Since inflammation is the main factor determining the level of adverse pathologies, a promising direction is the search for

drugs that reduce its development in muscle injuries [7, 8]. Antioxidant therapy is effectively used for this purpose [9]. In particular, biocompatible C_{60} fullerene [10] is known as a powerful antioxidant capable of simultaneously attaching up to 34 free methyl radicals [11]. In our previous works, it was shown that the application of C_{60} fullerene aqueous solution (C_{60}FAS) leads to significant therapeutic effects in muscle pathologies of different nature [12-14]. Based on the above data, the aim of the present study was to investigate the effect of C_{60}FAS (daily oral administration at a dose of 1 mg/kg throughout the experiment) on the process of restoration of functional activity of rat skeletal muscle (gastrocnemius muscle) on the 5th, 10th and 15th day after the initiation of open trauma.

Materials and Methods

To obtain C_{60}FAS, we used a method based on the transfer of C_{60} molecules from toluene to water, followed by sonication [15]. The resulting C_{60}FAS at a maximum concentration of 0.15 mg/ml remains stable for 12-18 months at 4-25°C. It is a typical nanocolloid containing both single C_{60} molecules as well as their nanoaggregates [16].

The experiments were conducted on 3-month-old male Wistar rats weighing 170 ± 5 g. The experimental animals were housed in Plexiglas cages and kept in an air-filtered, temperature-controlled (21 ± 1°C) room under 12-h light/12-h dark conditions. Rats received a standard pellet diet and water *ad libitum*. The use of the laboratory animals was approved by the Biomedical Ethics Committee of the ESC "Institute of Biology and Medicine" of Taras Shevchenko National University of Kyiv (protocol No 9 dated September 4, 2023) and performed in accordance with the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, 1986) and Article 26 of the Law of Ukraine "On the Protection of Animals from Cruelty" (No 3447-IV, 21.02.2006), as well as European Union Directive of 22 September 2010 (2010/63/EU) for the protection of animals used for scientific purposes.

The animals were randomly divided into the following experimental groups: control group (n = 12); open injury group (n = 12); open injury+C_{60} group (oral daily use of C_{60}FAS in a dose of 1 mg/kg animal body weight after initiation of injury; n = 12).

The choice of 1 mg/kg dose of C_{60}FAS is based on our previous results [12-14] demonstrating its high efficacy in the therapy of various muscle pathologies *in vivo*. Moreover, this dose is significantly lower than the LD_{50} value, which was 600 mg/kg of animal weight when administered orally to rats [17] and 721 mg/kg when administered intraperitoneally to mice [18].

Anesthesia of experimental animals was carried out by intraperitoneal injection of nembutal (40 mg/kg), which allowed putting the animals into deep surgical anesthesia for at least 2 h. If necessary, anesthesia was continued by intraperitoneal injection of ketamine/xylazine mixture (¼ of the initial dose) every 30-40 min until the end of the experiment.

Gastrocnemius muscle was isolated from surrounding tissues in the area of the hamstring fossa. All muscle branches except for those innervated, were cut. The isolated muscle was fixed on a bipolar platinum wire electrode for further electrical stimulation. The skin edges on the hind limbs of rats around the incision were sutured to the armature of the strain gauge machine, and the resulting baths with the muscle and nerve were filled with vaseline oil.

The studied *gastrocnemius muscle* was subjected to transverse dissection with a depth of 1 mm in three equidistant places [19]. After that, we sutured the wound skin opening with synthetic absorbable suture material made of copolymer (Liberti, Germany).

Analysis of mechanograms of muscle contraction and blood biochemical analysis of rats were performed 5, 10 and 15 days after the beginning of the experiment. This is due to the fact that the main processes of repairing damaged muscles in the conditions of their natural post-traumatic recovery last for 12-15 days [20].

Stimulation of *gastrocnemius muscle* efferents was carried out by electrical impulses generated using a strain gauge generator. Each series of stimulation consisted of 100 consecutive 2 ms rectangular pulses with a frequency of 1 Hz. The current intensity at which the muscle started to contract was considered as a threshold, and further stimulation was performed at an intensity of 1.3-1.4 threshold. The external load on the muscle was controlled using a mechanostimulator system. The change in the force of muscle contraction was measured using strain gauges [21].

Analyzing the obtained mechanograms, the muscle force impulse was calculated as the value of the area under the force curve using Origin 9.4

software ($S = \int f(t)dt$, where f is the force of *gastrocnemius muscle* contraction). The analysis of this parameter allows us to evaluate the activity of muscle functioning in the system of equilibrium 'force - external load', which is a physiological analog of the performance of the muscle system as a whole [12-14].

Changes in the concentrations of C-reactive protein (CRP), creatinine and lactate, as well as indicators of pro- and antioxidant balance (activity of catalase (CAT) and superoxide dismutase (SOD) and concentration of reduced glutathione (GSH)) in the blood plasma of rats were studied using clinical diagnostic equipment - biochemical analyzers RNL-200 and JN-1101-TR2 (Netherlands).

Statistical evaluation of the results was performed using analysis of variances (ANOVA) with mixed design. Two between-group factors were supposed: 1) open injury; 2) C_{60}FAS treatment (two levels – no and use of C_{60}FAS). The factor of time was supposed as within-group with three levels (5, 10 and 15 days after muscle injury initiation). The Shapiro-Wilk *W*-test was used to test for normality. Levene's test was used to assess the equality of variances across groups. Multiple pairwise comparisons between different groups and conditions were performed using Bonferroni *post-hoc* test. The differences between the groups were considered significant at $P < 0.05$. Each of the experimental force curves is the result of averaging 10 similar tests. Each biochemical measurement was carried out at least three times. The statistical evaluation was performed by software package Statistica 8.0 (Dell, USA).

Results and Discussion

Biomechanics of injured gastrocnemius muscle contraction. Fig. 1 shows the results of force changes of 100 consecutive non-relaxation contractions of muscle gastrocnemius of rats 5, 10 and 15 days after the initiation of open muscle injury. On the basis of the obtained mechanograms, the changes in the force impulse of *gastrocnemius muscle* (S, %) describing its dysfunction were calculated.

Five days after the initiation of open *gastrocnemius muscle* injury, the muscle force impulse value decreased to 23 ± 2% of control. Application of C_{60}FAS improved this parameter to 49 ± 3% relative to control. On days 10 and 15, this biomechanical parameter showed the presence of repair processes at 41 ± 3% and 51 ± 3% of the control, respectively. The application of C_{60}FAS improved this parameter to 58 ± 3% and 77 ± 4% of the control, respectively.

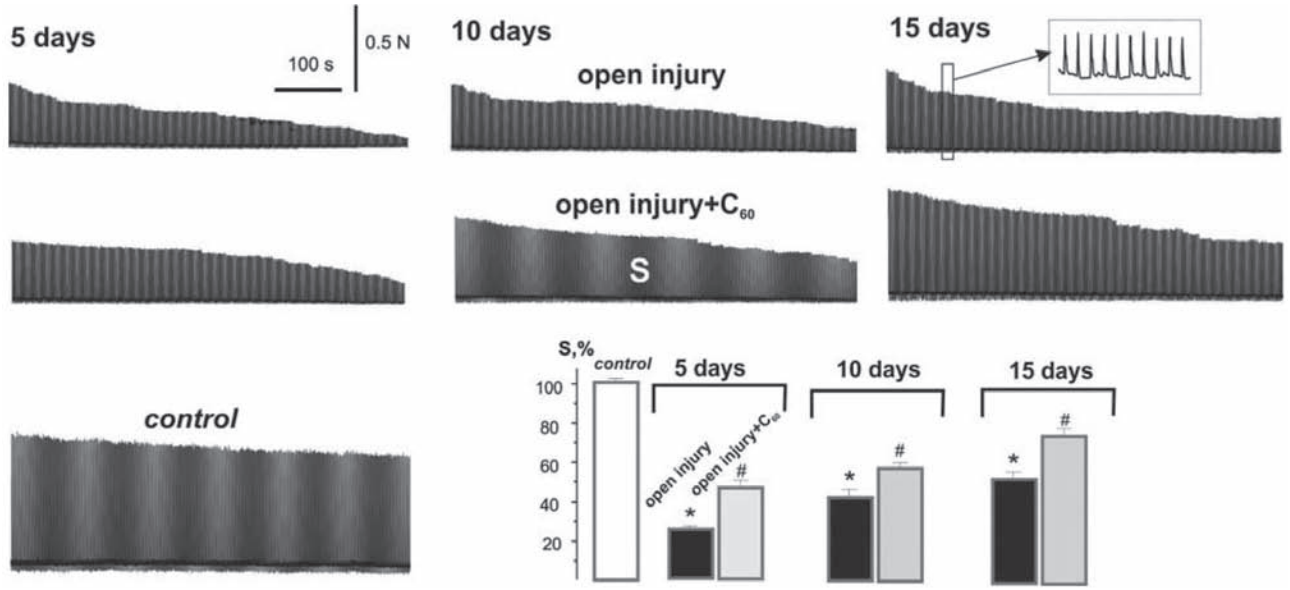

Fig. 1. The force generation curves of rat gastrocnemius muscle contraction at 100 consecutive non-relaxation contractions and application of fatigue stimulation at a frequency of 1 Hz: control, open injury and open injury+C_{60} – the control, groups of injured rats and rats receiving C_{60}FAS (daily dose 1 mg/kg) after initiation of open muscle injury, respectively; 5, 10 and 15 days – 5, 10 and 15 days after initiation of open muscle injury, respectively. S – the calculated gastrocnemius muscle force impulse (relative to the control, which was taken as 100%)

Thus, a significant increase of *gastrocnemius muscle* force impulse was recorded when C_{60}FAS was applied to the open injury group (17-26 ± 2%) in the process of regeneration of the injured muscle. In our opinion, the effect of water-soluble C_{60} fullerenes on this biomechanical parameter can be explained by their powerful antioxidant properties, which lead to a decrease in myocyte membrane damage, the time of muscle fatigue and muscle fibrosis during the development of muscle injuries [7, 22].

Blood biochemical indicators of rats with injured *gastrocnemius muscle.* To confirm the biomechanical data obtained above, the changes in the biochemical composition of rat blood with damaged *gastrocnemius muscle* were analyzed.

CRP, a protein whose level rises in response to inflammatory processes in the body, is produced by the liver. Its concentration doubles approximately every two hours during infection or the acute phase of a reaction. It is generally accepted that CRP is a marker of systemic inflammation, but it can also be elevated due to tissue damage, including skeletal muscle damage. CRP level in the blood is determined in the clinic to monitor the inflammatory process associated with rhabdomyolysis [23, 24].

The increase of CRP concentration in blood from 0.50 ± 0.03 mg/l in control to 4.1 ± 0.3 mg/l after 5 days of experiment was revealed, which is an indicator of high-level inflammatory processes in the injured muscle (Fig. 2). The application of C_{60}FAS reduced this index by almost 40 ± 3% relative to the open injury group, which was 2.5 ± 0.1 mg/l. On the 10^{th} day of the experiment, this index was 2.3 ± 0.1 mg/l in the open injury group and 1.5 ± 0.1 mg/l in the open injury+C_{60} group. As we can see, the positive effect of C_{60}FAS in this case was about 35 ± 2% relative to the open injury group. Finally, on the 15^{th} day CRP concentration significantly decreased to 1.5 ± 0.1 mg/l, and C_{60}FAS application improved this index to 0.92 ± 0.05 mg/l, which indicates the prospective use of antioxidants in the therapy of muscle inflammation [25].

During intense prolonged work, skeletal muscles produce large amounts of lactate. Increased intramuscular pressure and fibrosis, as well as damage to the accessory muscle apparatus, shunt muscle strength. Therefore, the lactate level is a quantitative indicator of the development of fatigue processes in pathological muscle [26].

In control, the lactate concentration in blood was 5.3 ± 0.4 mM. After the initiation of open *gastrocnemius muscle* injury, its value increased to 12.5 ± 0.6, 11.2 ± 0.6 and 7.9 ± 0.3 mM on the 5^{th}, 10^{th} and 15^{th} day of the experiment, respectively. C_{60}FAS administration decreased the lactate level to 8.5 ± 0.3, 7.5 ± 0.3 and 6.6 ± 0.3 mM on the 5^{th}, 10^{th} and 15^{th} day of the experiment, respectively (Fig. 2). Thus, C_{60}FAS therapy resulted in a 16-33 ± 2% increase in lactate oxidation relative to the open injury group.

Creatinine is a product of creatine phosphate decay from muscle and protein metabolism. It is excreted by the body at a constant rate depending on muscle mass, which varies during metabolism in different physiological and pathological states of muscle [27].

Blood creatinine concentration increased from 48 ± 1 μM in the control to 179 ± 5, 149 ± 4 and 121 ± 2 μM on day 5, 10 and 15 of the experiment, respectively. The application of C_{60}FAS decreased it to 119 ± 2, 98 ± 2 and 69 ± 1 μM, respectively (Fig. 2). The significant reduction of creatinine fraction upon application of water-soluble C_{60} fullerenes by 34-43 ± 3%, in our opinion, indicates that they effectively protect skeletal muscle cell membranes from non-specific free-radical destruction by intensively absorbing free radicals.

To confirm the assumption about the positive effect of water-soluble C_{60} fullerenes as antioxidants, we analyzed the levels of markers of peroxidation and oxidative stress (SOD, CAT and GSH). We immediately note that the data obtained clearly demonstrate their reduction with C_{60}FAS therapy (Fig. 3).

SOD catalyzes the conversion of superoxide anions into oxygen and hydrogen peroxide, protecting cells from the toxic effects of oxygen metabolism. SOD activity in cells is induced as the first line of defense against free oxygen radicals and its value depends on the level of the inflammatory process [28].

SOD activity in blood on the 5^{th}, 10^{th} and 15^{th} day of the experiment was 5.7 ± 0.3, 4.1 ± 0.2 and 3.4 ± 0.2 Units/ml, respectively, while the control was 1.8 ± 0.1 Units/ml (Fig. 3). When C_{60}FAS was administered, this index was significantly decreased and was 4.1 ± 0.2, 2.6 ± 0.1 and 2.2 ± 0.1 Units/ml, respectively. Thus, the positive effect of C_{60}FAS was about 28-37 ± 3% relative to the open injury group.

CAT converts hydrogen peroxide formed by SOD into water and molecular oxygen. It also uses hydrogen peroxide to oxidize toxins such as phenols, formic acid, formaldehyde and alcohols. In clinical studies, directly or indirectly, CAT serves

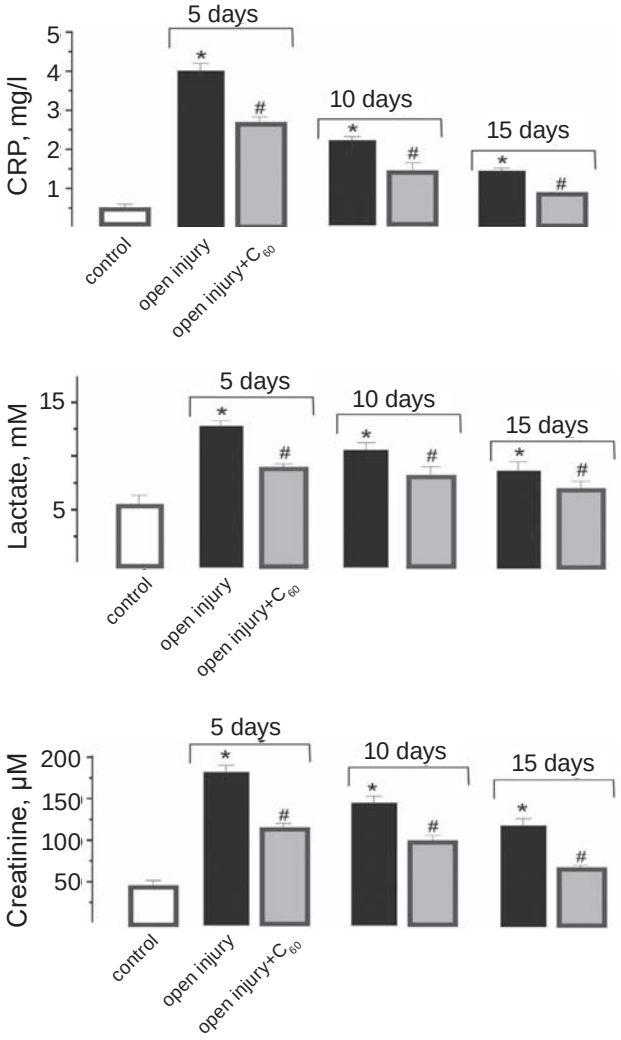

*Fig. 2. Concentrations of CRP, lactate and creatinine in rat blood plasma 5, 10 and 15 days after the initiation of open gastrocnemius muscle injury: control, open injury and open injury+C_{60} – the control, groups of injured rats and rats receiving C_{60}FAS (daily dose of 1 mg/kg) after initiation of open muscle injury, respectively; 5, 10 and 15 days – 5, 10 and 15 days after initiation of open muscle injury, respectively; *P < 0.05 relative to the control group; #P < 0.05 relative to the open injury group*

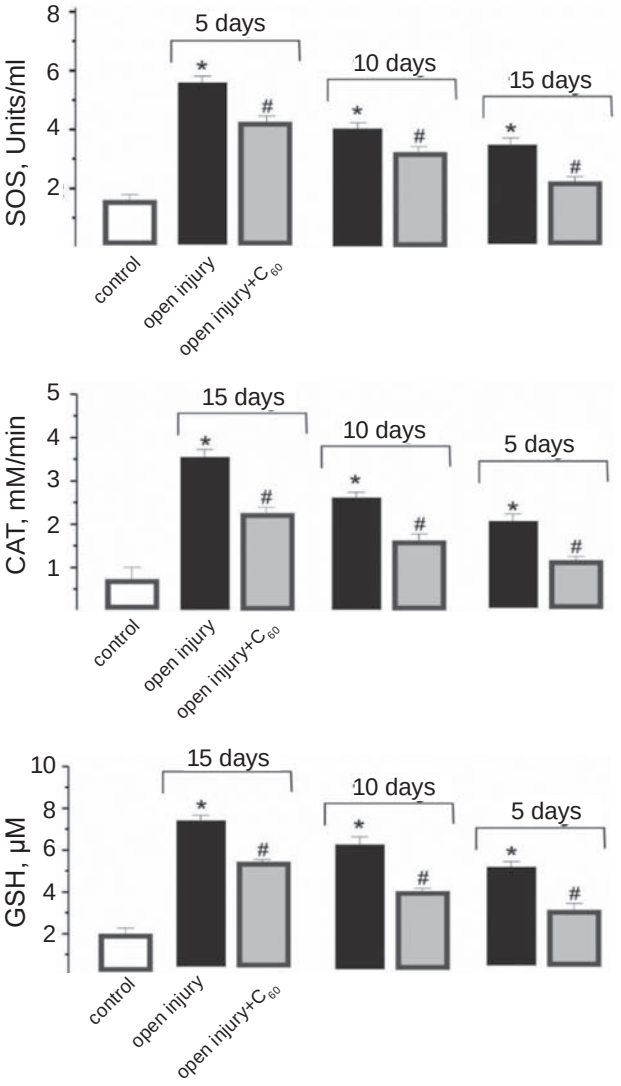

*Fig. 3. Indices of pro- and antioxidant balance (SOD, CAT and GSH) in rat blood plasma 5, 10 and 15 days after the initiation of open gastrocnemius muscle injury: control, open injury and open injury+C_{60} – the control, groups of injured rats and rats receiving C_{60}FAS (daily dose of 1 mg/kg) after the initiation of open muscle injury, respectively; 5, 10 and 15 days – 5, 10 and 15 days after the initiation of open muscle injury, respectively; *P < 0.05 relative to the control group; #P < 0.05 relative to the open injury group*

as a marker of inflammation in the development of various diseases and infections [25].

CAT activity in blood after the initiation of open trauma to the *gastrocnemius muscle* increased from 0.7 ± 0.1 mM/min in control to 3.5 ± 0.2, 2.6 ± 0.2 and 2.1 ± 0.2 mM/min on the 5th, 10th and 15th day of the experiment, respectively (Fig. 3). The application of C_{60}FAS significantly decreased this index, which was 2.2 ± 0.2, 1.5 ± 0.1 and 1.1 ± 0.1 mM/min on day 5, 10 and 15, respectively. The positive effect of C_{60}FAS was about 37-48 ± 3% relative to the open injury group.

Glutathione is an antioxidant present in almost every cell of the body and plays an important role in the detoxification of xenobiotics. In the human body, glutathione exists in two forms: reduced

(GSH) and oxidized (GSSG). GSH 'neutralizes' free-radical foci, is oxidized and becomes inactive, thus becoming GSSG. Inactive GSSG can be recycled back into the active form of GSH by the enzyme glutathione reductase. When this enzyme is overloaded and too much oxidized GSSG (compared to active GSH) accumulates, cells become susceptible to damage. Thus, GSH level is an important clinical marker of inflammatory process development [29].

After the initiation of open trauma to the *gastrocnemius muscle*, GSH concentration was 7.4 ± 0.5, 6.1 ± 0.3 and 5.1 ± 0.2 μM on the 5th, 10th and 15th day of the experiment, respectively (1.9 ± 0.1 μM in control) (Fig. 3). After the application of C_{60}FAS, this value was 5.2 ± 0.2, 4.0 ± 0.2 and 3.0 ± 0.1 μM on the 5th, 10th and 15th day of the experiment, respectively. The positive effect of C_{60}FAS was about 30-41 ± 3% relative to the open injury group.

Thus, the use of water-soluble C_{60} fullerenes significantly reduces oxidative processes in the injured muscles by maintaining the balance between prooxidants and antioxidant defense system of the organism. This indicates the presence of compensatory activation of the endogenous antioxidant system by C_{60} fullerenes in the process of pathological changes of *gastrocnemius muscle* caused by open injury.

Conclusions. Application of C_{60}FAS (daily oral dose of 1 mg/kg during the whole experiment) promotes the restoration of functional activity of injured muscle gastrocnemius of rats, which is confirmed by the data of biomechanics of muscle contraction and blood biochemistry of experimental animals. Thus, a significant increase of *gastrocnemius muscle* force impulse at the application of C_{60}FAS in the process of regeneration of the injured muscle at the level of 17-26 ± 2% relative to the open injury group was registered. In addition, there is an increase in the studied biochemical indices in the blood of rats throughout the experiment at the level of 16-48 ± 3% relative to the open injury group. In our opinion, C_{60} fullerenes influence the activity of endogenous antioxidants, suppressing the occurrence of destructions in the muscle and, thus, reduce its degradation during the repair of traumatic injuries. This opens the possibility of using water-soluble pristine C_{60} fullerenes as promising nanoagents capable of effectively correcting the state of the damaged muscle system.

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

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ВПЛИВ С60 ФУЛЕРЕНУ НА ФУНКЦІОНАЛЬНУ АКТИВНІСТЬ *GASTROCNEMIUS MUSCLE* ЩУРА УПРОДОВЖ ЙОГО РЕГЕНЕРАЦІЇ ПІСЛЯ ВІДКРИТОЇ ТРАВМИ

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Відкриті травми є одними з найпоширеніших травм скелетних м'язів. Мета дослідження – оцінити вплив щоденного перорального введення водного розчину C_{60} фулерену (C_{60}ВРФ) у дозі 1 мг/кг на відновлення функціональної активності скелетних м'язів щурів на 5, 10 і 15 добу після ініціації відкритої травми. Самців щурів Wistar випадковим чином розділили на три групи по 12 тварин у кожній: контрольну, із ушкодженням м'язів і ушкодженням м'язів+C_{60}ВРФ. На ізольованому *gastrocnemius muscle* робили поперечний розтин глибиною 1 мм. М'язові еференти стимулювали електричними імпульсами, які генерувалися за допомогою генератора тензометричної установки. У крові щурів визначали вміст С-реактивного протеїну, креатиніну, лактату, відновленого глутатіону та активність каталази та супероксиддисмутази. Згідно з отриманими даними, застосування C_{60}ВРФ сприяє відновленню функціональної активності ушкодженого м'яза, що підтверджено значним збільшенням імпульсу сили *gastrocnemius muscle*, ослабленням запалення і розвитку втоми

та нормалізацією про- та антиоксидантного балансу в процесі регенерації.

Ключові слова: C_{60} фулерен, *gastrocnemius muscle*, відкрита травма, імпульс м'язової сили, протеїн С, лактат, про-антиоксидантний баланс.

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