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THE EFFECT OF C_{60} FULLERENES ON THE RECOVERY OF *MUSCLE SOLEUS* CONTRACTION DYNAMICS IN RATS AFTER CHRONIC ALCOHOLIZATION

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Background. It has been shown that the available therapeutic agents do not eliminate the consequences of miotic pathologies in chronic alcoholism, the most significant of which are disturbances in the dynamics of muscle contraction. A positive effect of biocompatible water-soluble C_{60} fullerenes on the contraction parameters of damaged muscles has been established. In addition, administration of C_{60} fullerene aqueous solution during chronic alcoholization of rats protects muscle tissue from damage caused by oxidative stress.

Materials and Methods. Biomechanical parameters such as the values of the minimum and maximum contraction force and the muscle force impulse were evaluated using tensometry. The blood levels of creatine phosphokinase and lactate dehydrogenase, creatinine and lactate as well as the level of oxidative processes in muscle tissue of experimental animals (content of hydrogen peroxide, activity of catalase, glutathione peroxidase and superoxide dismutase) as markers of muscle damage were determined using methods of biochemical analysis.

Results. The C_{60} fullerene aqueous solution effect on the skeletal muscle contraction dynamics in rats after chronic alcoholization for 9 months and cessation of alcohol consumption for 1 month was investigated. It was established that water-soluble

C_{60} fullerenes (daily dose of 1 mg/kg) reduce the effects of chronic alcoholization by 35–40±2 % on the studied biomechanical parameters and by 20±1 % on the studied biochemical parameters compared to the group of alcoholized animals, thus increasing the energy capabilities of the muscular system.

Conclusions. The obtained data indicate a pronounced protective effect of C_{60} fullerenes on the *muscle soleus* contraction dynamics during the development of alcoholic myopathy, which opens up the potential possibility of their use for the prevention and correction of miotic damage.

Keywords: *muscle soleus*, alcoholization, C_{60} fullerene, biomechanical parameters of skeletal muscle contraction, biochemical indicators

INTRODUCTION

In the case of excessive alcohol consumption, along with other serious pathological consequences, such as cirrhosis of the liver, metabolic, physiological and structural changes in muscle tissue occur (Preedy *et al*. 2001). Alcoholic myopathy is one of the most frequent manifestations of alcoholic disease and occurs in patients with chronic ethanol intoxication in 40–60 % of cases (Rehm *et al.*, 2009; World Health Organization's Global status report on alcohol and health, 2014). With alcoholic myopathy, people can experience chronic fatigue and muscle discomfort, tremor dysfunction of the limbs, walking problems, etc. The destructive effect of ethanol on skeletal muscles is also manifested through the increase in peroxidation processes initiated by the excessive formation of free radicals and the development of oxidative stress, which destroys the membrane structures of myocytes (Adachi *et al.*, 2003) and disrupts the functions of their enzyme systems: a decrease in the activity of Na^+/K^+-ATPase and an increase in the activity of Ca^{2+}-ATPase (Tseyslyer *et al.*, 2014). The direct effect of alcohol on the myocyte membrane components is a violation of the electromechanical excitation-contraction coupling due to the inhibition of the release of Ca^{2+} from the sarcoplasmic reticulum into myofibrils of striated skeletal muscles (Hong-Brown *et al.*, 2001; Cofan *et al.*, 2000). The consequences of the development of this pathology are the progressive loss of control over the performance of precise goal-directed limb movements and the development of chronic muscle weakness (Nozdrenko *et al.*, 2005). Experiments on rats show that long-term alcohol consumption reduces the content of muscle proteins and muscle fibers protein synthesis, which affects the development of muscle strength and increases the duration of muscle fatigue. Biopsy almost always showed a significant decrease in the muscle fibers diameter against the background of alcoholic myopathy (Hong-Brown *et al.*, 2001; Cofan *et al.*, 2000).

Thus, alcoholic myopathy leads to muscle fiber ultrastructural disintegration, myodystrophy, changes in pro-oxidant-antioxidant balance and, as a result, to a violation of electrolyte homeostasis, energy and structural metabolism. These pathological manifestations are present for long periods of time after the cessation of alcohol consumption.

It has been shown that the available therapeutic agents do not eliminate the consequences of miotic pathologies in chronic alcoholism (Estruch *et al.*, 1993), the most significant of which are disturbances in the dynamics of muscle contraction (Nozdrenko *&* Bogutska, 2005). Currently, exogenous antioxidants are used in the treatment of muscle pathologies of various nature. Thus, it was established (Mach *et al.*, 2010) that the use of pycnogenol is accompanied by an increase in muscle endurance. It has been proven

that β-alanine (Harris & Sale, 2012) accelerates muscle recovery in the event of muscle fatigue. A positive therapeutic effect of biocompatible water-soluble C_{60} fullerenes on the contraction parameters of damaged muscles has been found (Nozdrenko *et al.*, 2018; Nozdrenko *et al.*, 2020). In addition, administration of C_{60} fullerene aqueous solution (C_{60}FAS) during chronic alcoholization of rats protects muscle tissue from damage caused by oxidative stress (Motuziuk *et al.*, 2023).

Therefore, the purpose of this study was to analyze the effect of C_{60}FAS on reducing the consequences of muscle system dysfunctions, which are manifested after nine months of chronic alcoholization in experimental animals and one month after the cessation of alcohol consumption.

MATERIALS AND METHODS

Preparation of C_{60}FAS. To obtain C_{60}FAS, a method based on the transfer of C_{60} molecules from toluene into water, followed by sonication, was used (Prylutska *et al.*, 2007). The obtained C_{60}FAS at a maximum concentration of 0.15 mg/mL is a typical colloid containing both single C_{60} molecules and their nanoparticles (Prilutski *et al.*, 1999) that remains highly stable for 18 months at a temperature of +4 °C. C_{60} fullerene is a hydrophobic molecule and is able to be embedded into biological membranes and thus to penetrate into the cell (Foley *et al.*, 2002; Grebinyk *et al.*, 2019; Prylutska *et al.*, 2019).

According to our previous data (Prylutska *et al.*, 2009; Tolkachov *et al.*, 2016), water-soluble C_{60} fullerenes at concentrations up to 14.4 as well as 24 μg/mL did not manifest any toxic effects in rat erythrocytes and thymocytes as well as in human mesenchymal stem cells, respectively. It was shown that intraperitoneal administration of C_{60} fullerene suspension at a dose of 2.5 g/kg does not lead to mice death or to violations of their behaviour within 8 weeks (Moussa *et al.*, 1996). It was established that the radiolabeled C_{60} fullerenes after intravenous administration to mice accumulate mainly in the liver, spleen, stomach, and blood and are excreted from the body within 72 h mainly with urine (Ji *et al.*, 2006; Nikolic *et al.*, 2009). Our recent results (Prylutskyy *et al.*, 2023) indicate the prolonged kinetics of water-soluble C_{60} fullerenes elimination from the body of rats, which contributes to their long-term (at least 48 h) compensatory activation of the endogenous antioxidant system in response to muscle stimulation.

***In vivo* experiments.** All experiments were carried out on laboratory animals in compliance with the international principles of 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 generally accepted norms of bioethics and biological safety. The protocols of the experiments were approved by the Bioethics Commission of the ESC “Institute of Biology and Medicine” of Taras Shevchenko National University of Kyiv (protocol No. 2 dated September 2, 2022).

Experiments were performed on 30 male Wistar rats aged from 1 month to 10 months (at the end of the experiment).

The control group of animals (n = 10) received 100 % drinking water.

Alcoholization model: each animal of the “alcoholization” group (n = 10) was placed in a separate cage to obtain 40 % of ethanol in drinking water. The ethanol consumption was calculated as 0.5 % of the animal’s body weight. Recalculation of the ethanol dose was carried out every day during the entire experiment (D’Souza El-Guindy *et al.*, 2010). The duration of alcoholization was 9 months.

Experimental animals of "alcoholization+C_{60}" group (n = 10) received alcohol and were administered C_{60}FAS orally at a daily dose of 1 mg/kg rat body weight.

It is important to note that the selected dose of C_{60}FAS (1 mg/kg) in our experiments, as the most effective one, was chosen on the basis of previously conducted research (Motuziuk *et al.*, 2023). Moreover, this dose does not present any toxicity: it is significantly lower than the LD_{50} value, which was 600 mg/kg body weight when administered orally to rats (Gharbi *et al.*, 2005) and 721 mg/kg when administered intraperitoneally to mice (Prylutska *et al.*, 2019).

Biomechanical analysis. A 12-bit analog-to-digital and digital-to-analog converter (ADC-DAC) was used to record electrophysiological signals. DAC output pulses were triggered by isolated stimulators (DS2A, Digitimer), which performed nerve stimulation. Efforts were measured using semiconductor strain gauges that were glued to rigid steel beams and mounted on moving parts of the linear motor. Stimulation of efferents was carried out with electric pulses lasting 2 ms, generated by the pulse generator. Control of the external load on the muscle was carried out using a system of mechanostimulators. When analyzing the miotic response of the studied muscle (*muscle soleus*), such biomechanical parameters as the values of the minimum and maximum contraction force were analyzed, which serve as markers of certain link dysfunctions in the "excitation-response" chain. The muscle force impulse, as a calculated area under the force curve using the Origin 9.4 software, is an indicator of the muscle general working capacity under the applied stimulation pools (Motuziuk *et al.*, 2023).

It should be noted that in this research we studied the process of recovery of the dynamics of muscle contraction after long-term alcohol intoxication 1 month after the cessation of alcohol consumption. The choice of this particular term is related to data (Jung *et al.*, 2011; de la Monte *et al.*, 2014), which show that with complete abstinence from alcohol, the muscular and nervous systems recover for at least 30 days.

Biochemical analysis. The activity of enzymes (creatine phosphokinase (CPK) and lactate dehydrogenase (LDH)), creatinine and lactate in the blood plasma of experimental animals, as well as the level of oxidative processes in muscle tissue (content of hydrogen peroxide, activity of catalase, selenium-dependent glutathione peroxidase (GP_x) and superoxide dismutase (SOD)), as markers of muscle damage, were determined using clinical diagnostic equipment – biochemical analyzers RNL-200 (Netherlands), ABX Micros ESV60 (France) and automatic analyzer Pentra C400 (France).

Statistical analysis. Statistical processing of the measurement results was carried out by methods of variation statistics using the Origin 9.4 software. Each of the experimental kinetic curves is the result of averaging 10 similar measurements. No less than five repeats were performed for each biochemical measurements. The differences among experimental groups were detected by ANOVA followed by Bonferroni's multiple comparison test. Values of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Low-frequency stimulation provides an opportunity to effectively analyze the development of muscle fatigue. One month after the alcoholization cessation and the application of the specified frequency and a durable non-relaxation stimulation, a significant decrease in the force response of the muscle was recorded (**Fig. 1**).

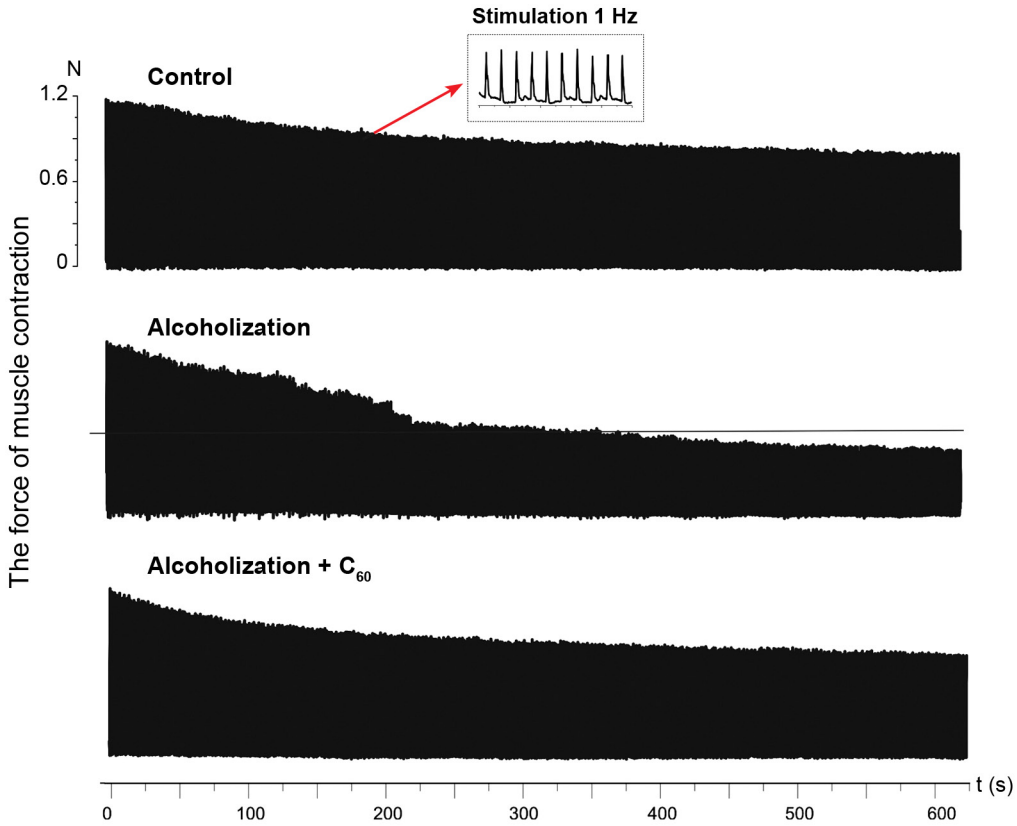

Fig. 1. The *muscle soleus* contraction strength of rats applying under 1 Hz stimulation lasting 600 s after 9 months of chronic alcoholization and 1 month after cessation of alcohol consumption: "alcoholization" and "alcoholization + C_{60}" – rats receiving alcohol and a mixture of alcohol and C_{60}FAS (daily dose of 1 mg/kg), respectively, throughout the entire period of alcoholization

In the group of alcoholized rats the muscle strength impulse was 63±3 % (in the control – 96±2 %), and in the group of animals that received a mixture of alcohol and C_{60}FAS it was 84±2 % (**Fig. 2**).

A change in the level of minimum contraction force is one of the most sensitive markers of muscle dysfunction. In the control group, this indicator was at the level of 0.87±0.10 N at the last contraction, in the group of alcoholized rats – 0.63±0.10 N, and in the group of animals that received a mixture of alcohol and C_{60}FAS – 0.84±0.10 N (**Fig. 2**).

A change in the level of the maximum contraction force is an indicator of the muscular system general dysfunction, which indicates a decrease in the maximum possible force response during the development of pathology. The value of this marker in the control group was 0.85±0.10 N, in the group of alcoholized rats – 0.56±0.10 N, and in the group of animals that received a mixture of alcohol and C_{60}FAS – 0.78±0.10 N (**Fig. 2**).

It is important to note that in the "alcoholization + C_{60}" group of animals there is almost no diversity among the difference between the maximum and minimum muscle contraction forces (the value of this difference is responsible for the quality and efficiency of precision movements) compared to the control.

Thus, the effects of pathological changes in the skeletal muscle contraction dynamics caused by a 9-month alcoholization of rats and 1 month after the cessation of alcohol use amounted to 27±1 % for the muscle force impulse index, 46±2 % and 34±2 % for the minimum and maximum force, respectively, regarding control. In the case of consumption of alcohol and C_{60}FAS mixture by experimental animals, the processes of muscle fatigue development differed from the control values by 10–12±1 %.

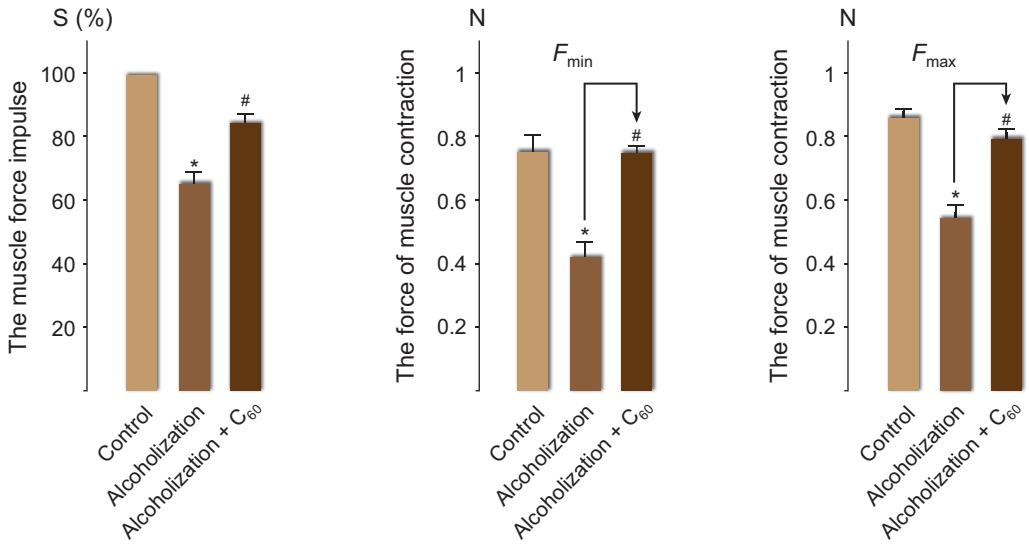

Fig. 2. Biomechanical markers of *muscle soleus* contraction in rats under 1 Hz stimulation lasting 600 s after 9 months of chronic alcoholization and 1 month after cessation of alcohol consumption: "alcoholization" and "alcoholization + C_{60}" – rats receiving alcohol and a mixture of alcohol and C_{60}FAS (daily dose of 1 mg/kg), respectively, throughout the entire period of alcoholization; *S* – muscle force impulse (relative to the control, which was taken as 100 %); F_{min} and F_{max} – the muscle minimum and maximum force response level, respectively, at the last contraction of the stimulation test. *p <0.05 – relative to the control group; #p <0.05 – relative to the alcoholization group

To confirm the obtained biomechanical results, an analysis of biochemical parameters of blood plasma, which are used to assess the development of muscle myopathies (D'Souza El-Guindy *et al.*, 2010), was carried out, in particular, changes in the levels of creatinine, CPK, lactate and LDH.

The creatinine content was 156±5 μM in the group of control animals, 209±9 μM in the group of alcoholized rats, and 16±8 μM in the group of animals that received a mixture of alcohol and C_{60}FAS (**Fig. 3**). Thus, the positive effect of C_{60}FAS on this marker was 21±1 % compared to the alcoholization group (deviation from control is 6±1 %).

The CPK content was 1240±9 Units/L in the control group, 1760±17 Units/L in the group of alcoholized rats, and 1305±19 Units/L in the group of animals receiving a mixture of alcohol and C_{60}FAS (**Fig. 3**). Thus, the positive effect of C_{60}FAS on this marker was 25±1 % compared to the alcoholization group (deviation from control is 5±1 %).

The lactate level was 13.9±0.4 M in the group of control animals, 17.1±0.4 M in the group of alcoholized rats, and 14.1±0.6 M in the group of animals receiving a mixture of alcohol and C_{60}FAS (**Fig. 3**). Thus, the positive effect of C_{60}FAS on this marker was 22±1 % compared to the alcoholization group (deviation from control is 5±1 %).

The LDH concentration was 356±3 Units/L in the group of control animals, 472±7 Units/L in the group of alcoholized rats, and 371±9 Units/L in the group of animals receiving a mixture of alcohol and C_{60}FAS (**Fig. 3**). The positive effect of C_{60}FAS on this marker was 21±1 % compared to the alcoholization group (deviation from control is 4±1 %).

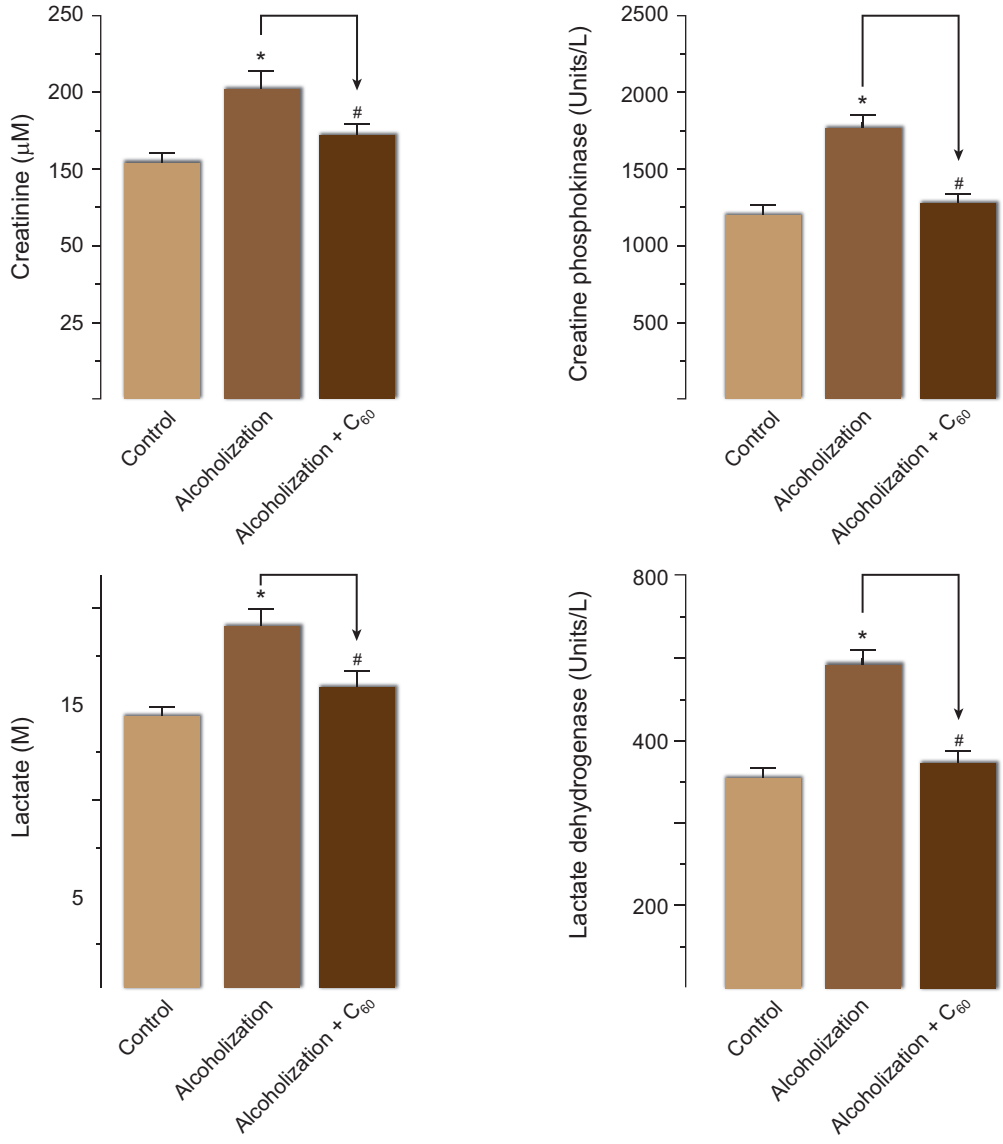

Fig. 3. The content of creatinine and lactate and activities of CPK and LDH in blood plasma of experimental rats: "alcoholization" and "alcoholization + C_{60}" – rats receiving alcohol and a mixture of alcohol and C_{60}FAS (daily dose of 1 mg/kg), respectively, throughout the entire period of alcoholization; *p <0.05 – relative to the control group; #p <0.05 relative to the alcoholization group

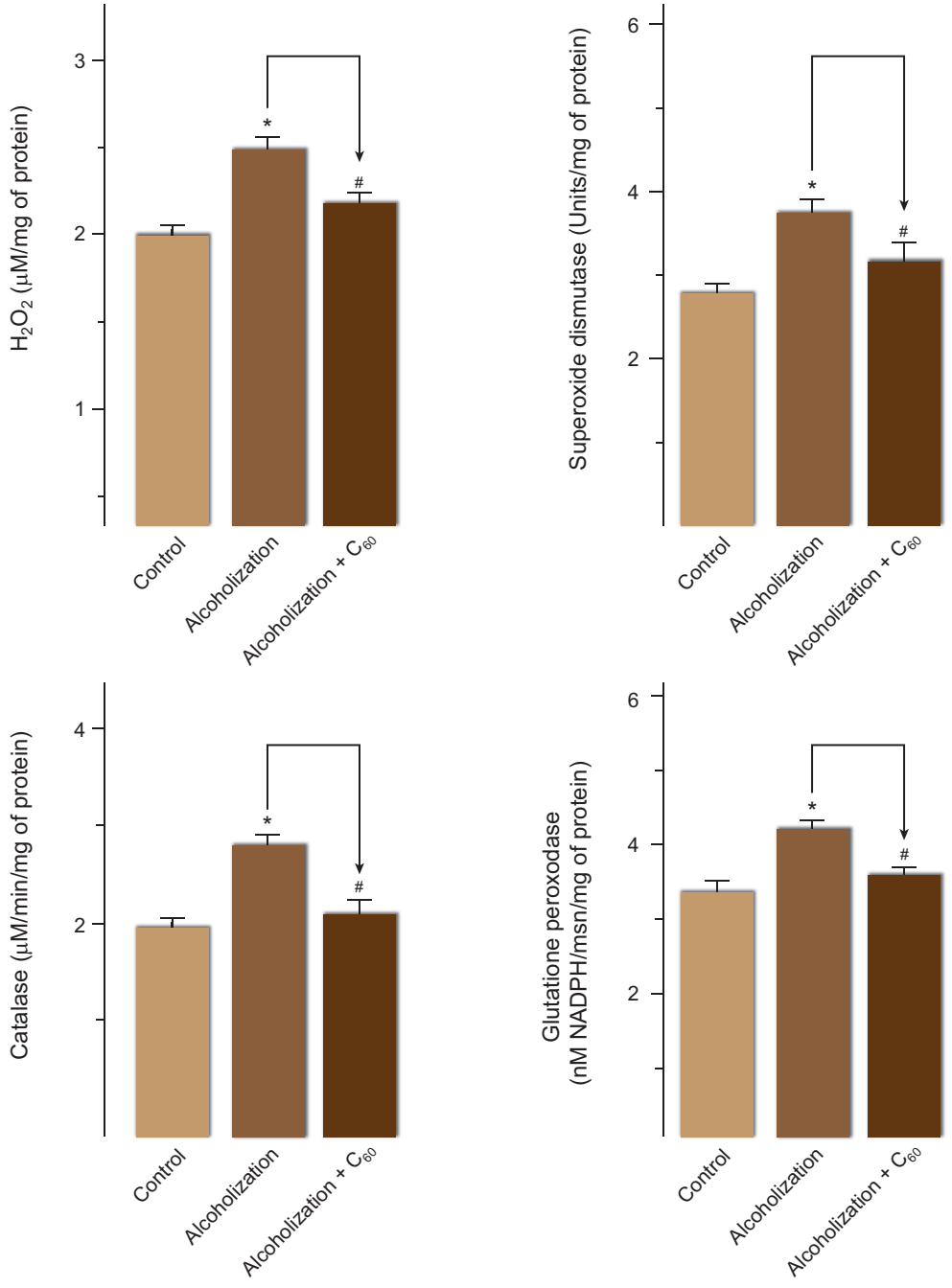

Fig. 4. The content of H_2O_2 and activities of SOD, catalase and GP_x in the *muscle soleus* homogenate of experimental rats: "alcoholization" and "alcoholization + C_{60}" – rats receiving alcohol and a mixture of alcohol and C_{60}FAS (daily dose of 1 mg/kg), respectively, throughout the entire period of alcoholism; *p <0.05 relative to the control group; #p <0.05 relative to the alcoholization group

The positive effect of water-soluble C_{60} fullerenes can be explained by their powerful antioxidant properties (Nozdrenko *et al.*, 2018, 2020). Determination of changes in the pro- and antioxidant balance levels in the studied muscle tissues is necessary to confirm this hypothesis.

Against the background of the alcoholic myopathy development the body's antioxidant protection system is able to neutralize reactive oxygen species (ROS) using SOD enzymes and catalase. On the other hand, excessive SOD activity causes excessive production of H_2O_2, which leads to the cyclic development of miotic damage (Lejay *et al.*, 2012). One of the consequences of chronic alcohol consumption is mitochondrial dysfunction, as a result of which even more free radicals are produced (Adachi *et al.*, 2001). Thus, a decrease in the activity of the antioxidant system components, namely SOD, GP_x, and catalase, plays a significant role in the pathogenesis of excessive exposure to free radicals (Brand *et al.*, 2004).

The H_2O_2 content was 1.9±0.2 μM/mg protein in the group of control animals, 2.4±0.2 μM/mg protein in the group of alcoholized rats, and 2.1±0.2 μM/mg protein in the group of animals that received a mixture of alcohol and C_{60}FAS (**Fig. 4**). Thus, the positive effect of C_{60}FAS on this marker was 13±1 % compared to the alcoholization group (deviation from control is 10±1 %).

The SOD activity was 2.9±0.2 Units/mg protein in the group of control animals, 3.8±0.3 Units/mg protein in the group of alcoholized rats, and 3.1±0.2 Units/mg protein in the group of animals that received a mixture of alcohol and C_{60}FAS (**Fig. 4**). Thus, the positive effect of C_{60}FAS on this marker was 19±1 % compared to the alcoholization group (deviation from control is 6±1 %).

The catalase activity was 2.4±0.2 μM/min/mg protein in the group of control animals, 3.1±0.2 μM/min/mg protein in the group of alcoholized rats, and 2.5±0.2 μM/min/mg protein in the group of animals that received a mixture of alcohol and C_{60}FAS (**Fig. 4**). Thus, the positive effect of C_{60}FAS on this marker was 19±1 % compared to the alcoholization group (deviation from control is 4±1 %).

The GP_x activity was 3.2±0.3 nM NADPH/min/mg protein in the group of control animals, 4.2±0.3 nM NADPH/min/mg protein in the group of alcoholized rats, and 3.1±0.2 nM NADPH/min/mg protein in the group of animals that received the mixture alcohol and C_{60}FAS (**Fig. 4**). Thus, the positive effect of C_{60}FAS on this marker was 26±1 % compared to the alcoholization group (deviation from control is 3±1 %).

CONCLUSIONS

The obtained data indicate that water-soluble C_{60} fullerenes, as powerful antioxidants, contribute to the effective restoration of the functional activity of the body's skeletal and muscular structures after nine months of chronic alcoholization and one month after the cessation of alcohol consumption. It was shown that the oral administration of C_{60}FAS at a daily dose of 1 mg/kg reduces the effects of chronic alcoholization by 35–40±2 % on the studied biomechanical parameters of skeletal muscle contraction and by 20±1 % on the studied blood's and muscle tissue's biochemical indicators compared to the alcoholized group. This indicates that the C_{60}FAS exhibits a protective effect on the *muscle soleus* contraction dynamics against the background of alcoholic myopathy, which opens up new possibilities in the therapy and prevention of miotic damage.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Human Rights: This article does not contain any studies with human subjects performed by any of the authors.

Animal Studies: All international, national and institutional guidelines for the care and use of laboratory animals were followed.

AUTHOR CONTRIBUTIONS

Conceptualization, [D.N.; O.M.; Y.P.]; methodology, [D.N.; S.P.]; investigation, [D.N.; W.N.; O.M.; S.P.; O.V.; K.I.]; data analysis, [O.D.; O.L.]; writing – original draft preparation, [D.N.; O.L.; Y.P.]; writing – review and editing, [Y.P.]; visualization, [O.M.; O.D]; supervision, [Y.P.]; project administration, [D.N.; Y.P.].

All authors have read and agreed to the published version of the manuscript.

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ВПЛИВ C_{60} ФУЛЕРЕНІВ НА ВІДНОВЛЕННЯ ДИНАМІКИ СКОРОЧЕННЯ *MUSCLE SOLEUS* ЩУРІВ ПІСЛЯ ХРОНІЧНОЇ АЛКОГОЛІЗАЦІЇ

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Обґрунтування. Наявні терапевтичні засоби не усувають наслідків патологій за хронічного алкоголізму, найсуттєвішими з яких є порушення динаміки м'язового скорочення. Раніше встановлено позитивний вплив біосумісних водорозчинних C_{60} фулеренів на параметри скорочення пошкоджених м'язів. Крім того, введення водного розчину C_{60} фулерену під час хронічної алкоголізації щурів захищало м'язову тканину від пошкодження, зумовленого окисним стресом.

Матеріали та методи. За використання тензометрії оцінювали такі біомеханічні параметри як величини мінімальної та максимальної сили скорочення, імпульс сили м'яза. Використовуючи методи біохімічного аналізу, визначали вміст у плазмі крові піддослідних тварин креатинфосфокінази та лактатдегідрогенази, креатиніну і лактату, а також рівень окисних процесів у м'язовій тканині (вміст пероксиду водню, активності каталази, глутатіонпероксидази і супероксиддисмутази) як маркерів пошкодження м'язів.

Результати. Досліджено дію водного розчину C_{60} фулерену на динаміку скорочення скелетних м'язів щурів після 9-місячної хронічної алкоголізації та через 1 місяць після припинення вживання алкоголю. Встановлено, що водорозчинні C_{60} фулерени (щоденна доза 1 мг/кг) зменшують ефекти хронічної алкоголізації на 35–40±2 % на досліджувані біомеханічні параметри та на 20±1 % на досліджувані біохімічні показники, порівняно з групою алкоголізованих тварин, підвищуючи таким чином енергетичні можливості м'язової системи.

Висновки. Одержані дані свідчать про виражений захисний ефект C_{60} фуленів на динаміку скорочення *muscle soleus* за розвитку алкогольної міопатії, що відкриває потенційну можливість їхнього застосування для профілактики і корекції міотичних пошкоджень.

Ключові слова: *muscle soleus*, алкоголізація, C_{60} фулерен, біомеханічні параметри скорочення скелетного м'яза, біохімічні показники

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