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BIOCHEMICAL PARAMETERS OF BLOOD AND TISSUE OF THE GASTROCNEMIUS MUSCLE IN CHRONICALLY ALCOHOLIZED RATS UNDER ORAL ADMINISTRATION OF C_{60} FULLERENE AQUEOUS SOLUTION

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Biochemical indices of blood and tissue of the gastrocnemius muscle chronically alcoholized (for 3, 6 and 9 months) rats were studied. C_{60} fullerene aqueous solution (C_{60}FAS) was administered orally as a pharmacological agent at a dose of 1 mg/kg daily throughout the experiment in a three routes: 1 h before alcohol intake (preventive regimen), together with alcohol (therapeutic regimen I) and 1 h after alcohol intake (therapeutic regimen II). Creatine phosphokinase (CPK), lactate dehydrogenase (LDH), catalase, superoxide dismutase, glutathione peroxidase (GPx) activity and the level of creatinine, lactate, hydrogen peroxide, reduced glutathione were estimated with clinical diagnostic kits. A pronounced upward trend in creatinine and lactate content, CPK and LDH activity with increasing degree of alcoholic myopathy during experiment was detected. Administration of C_{60}FAS was shown to reduce the biochemical indices of muscle injury and to reduce oxidative processes by maintaining the balance between pro-oxidant and antioxidant systems. The maximum positive effect was observed when C_{60}FAS was administered together with alcohol (therapeutic regimen I). The results indicate on C_{60} fullerene ability to correct the pathological condition of the muscular system arising from alcohol intoxication.

K e y w o r d s: C_{60} fullerene, alcohol intoxication, gastrocnemius muscle, creatine phosphokinase, lactate dehydrogenase, antioxidant system.

Ethanol is a toxic substance, it causes direct and indirect effects on the muscular system, which leads to alcoholic myopathy. The metabolism of alcohol is closely related to enzymes involved in oxidative stress and the generation of reactive oxygen species (ROS), such as superoxid anion ($O^{2\bullet-}$) and hydrogen peroxide (H_2O_2), which cause damage to cells and tissues [1]. Oxidative stress can be considered a consequence of an imbalance in the formation of ROS and the body's antioxidant defense system. Alcohol is mainly metabolized to acetaldehyde by alcohol dehydrogenase and cytochrome p450 2E1 (CYP2E1) in the liver [2]. In rats that constantly consumed alcohol, a decrease in the levels of several antioxidant enzymes was observed, including total and free glutathione, as well as the activity of glutathione reductase, glutathione peroxidase and superoxide dismutase-2 [3]. In addition, skeletal muscle shows increased protein carbonylation [4], as well as the increased levels of hydrogen peroxide and malondialdehyde [5], which are markers of oxidative damage. Increased oxidative stress promotes protein degradation, including increased expression of the UPP system (ubiquitin-proteasome pathway) in muscle fibers.

Oxidative damage can occur due to an increase in the number of free radicals or a decrease in the activity of the antioxidant system. In alcoholics, excessive production of ROS by the microsomal system and mitochondria has been observed [6]. Mitochondrial damage disrupts fatty acid oxidation and increases lipid peroxidation (LPO). A clinical study found mitochondrial muscle damage in 57 alcoholics (28%) [7].

Chronic alcohol intake leads to glycolytic disorders in rat skeletal muscle [8]. For example, the

activity of the glycolytic enzymes aldolase, pyruvate kinase, and lactate dehydrogenase significantly decreased after prolonged ethanol administration [9], which was consistent with the results in humans [10]. At the same time, the opposite effects were observed in the white gastrocnemius muscles of male rats, indicating a potential effect of ethanol on fibre type on glycolytic enzyme activity [11, 12].

Alcohol-induced changes in glucose metabolism are associated with decreased muscle performance in athletes due to impaired gluconeogenesis and glucose release, decreased lactate and glycerol levels [13, 14], as well as decreased glucose uptake and glycogen storage [15]. Previous studies have shown that muscle damage caused by chronic alcohol exposure was not associated with mitochondrial metabolic imbalances in rats (oxygen uptake and ATPase activity) and humans (oxygen uptake, cytochrome content, respiratory chain complexes) [16, 17]. At the same time, there is evidence of a direct link between skeletal muscle damage and dysregulation of mitochondrial metabolism due to alcohol consumption. In particular, it has been shown that alcohol alters the basic energy metabolism by reducing the quantitative ratio of NAD^+/NADH, causing the accumulation of tricarboxylic acid cycle intermediates and malfunctioning of the respiratory chain [18].

In rats, chronic alcohol-induced oxidative stress and decreased antioxidant potential are manifested in changes in glutathione and oxidized glutathione concentrations, increased LPO, and increased total protein oxidation [19]. Overall, these findings demonstrate that oxidative stress and mitochondrial dysfunction can delay muscle regeneration and recovery and contribute to alcohol-related dysfunctions. This cascade of processes can lead to an imbalance in the time to establish smooth tetanus, a decrease in muscle strength response, and disruption of muscle group interaction [20].

C_{60} fullerene, as the third allotropic form of carbon materials, has a stable spherical-like hollow structure with a diameter of 0.72 nm, which is close to that of the polypeptides' α-helix and steroid molecules. Thus, the steric compatibility of C_{60} fullerene with biological structures such as receptor recognizing sites or enzyme active centers may be suggested [21, 22]. The surface of C_{60} molecule consists of 60 carbon atoms, which are connected by sp^2 single and double bonds. One of the main physicochemical properties of C_{60} fullerene responsible of its various biomedical effects [21-24], in particular to protect biological systems against cell damage and tissue abnormalities, is the ability to scavenge a large number of free radicals [25, 26]. Thirty carbon double bonds in the C_{60} fullerene cage give it an aromatic character and resulting in C_{60} fullerene acting as a highly efficient "free radical sponge". C_{60} molecule can be reversibly reduced up to six electrons. So, Wang et al. [27] reported that C_{60} fullerene and some its derivatives efficiently can prevent peroxidation and membrane breakdown triggered by free radical species as well as were more effective in inhibiting lipid peroxidation than vitamin E, which is a natural antioxidant. They could weaken certain inflammatory processes (e.g. arthritis and acute inflammation in rats) and possibly facilitate the recovery of damaged tissue [28, 29].

C_{60} fullerene is hydrophobic molecule and is able to be embedded into biological membranes and thus to penetrate into the cell [30, 31], It was shown that water-soluble C_{60} fullerene derivatives can enter mitochondria, where free radicals are generated [32].

The pristine C_{60} fullerene has very low solubility in water. However, it can form aggregates in aqueous solutions and make stable colloid solutions which contain both individual C_{60} fullerene and its nanoclusters [33].

As known, C_{60} fullerene is active only in a soluble form when its carbon double bounds are freely accessible [25]. Recently we demonstrated, that pristine C_{60} fullerene aqueous colloid solution (C_{60}FAS) prevents the restraint stress-induced oxidative disorders in rat' brain and heart tissues [34] as well as CCl_4-induced acute liver injury [35], diminishes the muscle fatigue in rats comparable to the known exogenous antioxidant N-acetylcysteine [36, 37], has a positive therapeutic effect following the initiation of atrophy [38] and injury [39, 40] in skeletal muscles.

Thus, the aim of the present study was to evaluate the effects of C_{60}FAS on changes in biochemical parameters of blood and muscle tissue of chronically alcoholic rats in a dose- and application-dependent manner.

Materials and Methods

In vivo models. The experiments were performed on male Wistar rats aged 1 to 10 months (at the end of the experiment). The study protocol was approved by the Bioethics Committee of the ESC "Institute of Biology and Medicine", Taras

Shevchenko National University of Kyiv (No. 1 dated 18.01.2022) 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 №3447 - IV 21.02.2006, Kyiv, The Protection of Animals from Cruelty during biomedical research.

The animals were euthanized by the cervical dislocation of the cervical vertebrae.

A control group of n = 10 animals received 100% drinking water.

Experimental rats in a number of n =10 (experimental group "alcoholization") were randomly selected and each of them was placed in a separate cage to receive 40% of ethanol in drinking water, which meant that the rats had no access to 100% of water until the full consumption of dosed ethanol [41]. Ethanol intake was calculated as 0.5% of the animal's body weight. The ethanol dose was recalculated every 24 h throughout the experiment [42]. The duration of alcoholization was 3, 6 and 9 months. The target value of 40% ethanol in drinking water was chosen due to the fact that it repeats the blood alcohol concentration reported in chronic alcoholics [43].

Experimental rats (experimental groups "alcoholization+C_{60}") before alcoholization (n = 10) and during alcoholization (two groups of n = 10 animals in each) received C_{60}FAS at a dose of 1 mg/kg animal weight. This dose, as the most optimal one, was chosen on the basis of our earlier studies [23-26]. The dose was recalculated every 24 h throughout the experiment. Three regimens of C_{60}FAS administration were used: 1 h before alcohol intake (preventive regimen), together with alcohol (therapeutic regimen I) and 1 h after alcohol intake (therapeutic regimen II).

Preparation of C_{60}FAS. C_{60} fullerene (Sigma Cat. No. 379646) aqueous colloid solution with purity of more than 99.96% has been prepared according to the protocol [44, 45]. The method is based on transferring C_{60} molecules from organic solution into the aqueous phase by ultrasonic treatment. The maximal concentration of C_{60} fullerenes in water obtained by this method was 0.15 mg/ml. The prepared C_{60}FAS is stable within 18 months at temperature +4°C.

Biochemical analysis. The levels of enzymes (creatine phosphokinase (CPK) and lactate dehydrogenase (LDH)) as well as creatinine and lactate (LA) in the blood plasma of the test animals and assessment of the level of oxidative processes in muscle tissues (content of hydrogen peroxide and reduced glutathione (GSH) as well as catalase (CAT), selenium-dependent GP_x and SOD activities), as markers of muscle damage [46], were determined using clinical diagnostic equipment (hemoanalyzers RNL-200 (Netherlands), ABX Micros ESV60 (France) and automatic analyzer Pentra C400 (France)).

Blood alcohol concentration was determined at the end of the experiment by cardiac puncture using an AM1 alcohol analyser (Analox Instruments Limited, UK).

Statistical analysis. Statistical processing of the measurement results was carried out by methods of variation statistics using the Origin 9.4 software. No less than five repeats were performed for each biochemical measurement. Data are expressed as the means ± SEM for each group. The differences among experimental groups were detected by one-way ANOVA followed by Bonferroni's multiple comparison test. Values of $P < 0.05$ were considered significant.

Results and Discussion

At the end of the experiment, the blood alcohol concentration value in rats after chronic ethanol consumption ranged from 140 mg/dl (3 months of alcohol consumption) to 252 mg/dl (9 months of alcohol consumption). These data agree well with the results of a study [41].

The use of C_{60}FAS in different regimens showed no significant changes in these parameters throughout the experiment.

The analysis of biochemical blood markers, particularly creatinine, CPK, LA and LDH levels [42], made it possible to assess physiological changes in skeletal muscle function caused by prolonged alcoholization.

Studies have shown that all these markers show a pronounced upward trend with increasing degree of pathology (Fig. 1).

The change in the level of creatinine, a product formed in muscles during the destruction of intramuscular structures, allowed the level of myocyte damage to be assessed. When C_{60}FAS was used in therapeutic regimen I, creatinine levels decreased by 16 ± 1%, 18 ± 2% and 37 ± 2% at 3, 6 and 9 months of alcoholization, respectively (Fig. 1). When C_{60}FAS was used in the preventive regimen and therapeutic regimen II, the previously observed effect decreased by an average of 8 ± 1% and 11 ± 3%, respectively.

One important marker of pathological processes in muscle is a change in the concentration of CPK, an enzyme from the energy supply system of skeletal muscle cells. When a muscle is mechanically damaged during active muscle function, there is a release of this enzyme from the cells and a corresponding increase in CPK levels in the blood. Administration of C_{60}FAS in therapeutic regimen I resulted in a 13 ± 1%, 18 ± 2% and 28 ± 2% decrease in CPK levels with alcoholization lasting 3, 6 and 9 months, respectively (Fig. 1). When C_{60}FAS was used in the preventive regimen and therapeutic regimen II, the previously observed effect decreased by an average of 10 ± 1% and 16 ± 2%, respectively.

During the cascade of pathological reactions after the development of muscular myopathies, there is a significant depletion of cellular energetic substances, leading to a disturbance of homeostasis and a loss of ionic gradient due to cell membranes. As a consequence, there is an accumulation of LA and H^+ ions and, consequently, an acidification of the pH in the intra- and extracellular media. C_{60}FAS administration in therapeutic regimen I decreased LA levels by 19 ± 1%, 27 ± 2% and 38 ± 2% with alcoholization lasting 3, 6 and 9 months, respectively (Fig. 1). When C_{60}FAS was used in the preventive regimen and the therapeutic regimen II, the previously observed effect was reduced by an average of (9-10) ± 3%.

The level of changes in LDH, an enzyme that catalyzes lactic acid oxidation, made it possible to assess the overall performance status of the injured muscle after prolonged activation. When C_{60}FAS was used in therapeutic regimen I, LDH levels decreased by 15 ± 1%, 29 ± 2% and 41 ± 2% with alcoholization lasting 3, 6 and 9 months, respectively (Fig. 1). When C_{60}FAS was used in the preventive regimen and the therapeutic regimen II, the previously observed effect was reduced by an average of 12 ± 3%.

Thus, animals given a mixture of alcohol and C_{60}FAS (therapeutic regimen I) for the duration of the experiment (3, 6 and 9 months) showed the maximum effect: there was a (15–36) ± 3% reduction in the described biochemical blood indexes.

The explanation for the above positive effects of water-soluble C_{60} fullerenes may be their powerful antioxidant properties [25, 26]. Determination of changes in the pro- and antioxidant balance in the tissues of the muscle under study is essential to confirm this hypothesis.

Fig. 2 shows the results of tests showing the level of secondary LPO products accumulation in rat gastrocnemius muscle after prolonged alcohol intoxication.

The data obtained indicate the increased levels of peroxidation and oxidative stress after electrical stimulation of alcohol-induced muscle, induced by 6 s with non-relaxation pools of 50 Hz frequency [38-40]. Thus, H_2O_2 values were 151%, 327% and 414% when compared with control muscle for 3, 6 and 9 months of alcoholisation, respectively. Administration of C_{60}FAS in therapeutic regimen I resulted in a 28 ± 2%, 44 ± 2% and 51 ± 3% reduction of H_2O_2 in rat gastrocnemius muscle, respectively. These results confirm that water-soluble C_{60} fullerenes are able to penetrate through plasma membranes and accumulate in selected tissues, including muscle, without evidence of damage [30-32].

Antioxidant defense enzymes such as SOD, CAT and GP_x play a key role in the regulation mechanisms of free-radical and peroxide processes. The most powerful natural antioxidant and the enzyme of the first link of antioxidant protection is SOD, which carries out the reaction of superoxide anion-radical dismutation and converts them into less reactive hydrogen peroxide molecules [47]. Studies [48] showed that SOD activity and accumulation in rat skeletal muscle increased during exercise or development of pathological processes. At the same time, SOD activity increased predominantly in oxidative muscles with a high content of type I and IIa fibres [49]. In our experiment with electrical stimulation of muscles against the background of C_{60}FAS in therapeutic regimen I, a gradual decrease of SOD activity in rat gastrocnemius muscle by 21 ± 1%, 29 ± 1% and 44 ± 2% was found after 3, 6 and 9 months of alcoholization, respectively.

The changes in CAT activity in rat muscle gastrocnemius when C_{60}FAS was administered in therapeutic regimen I were 12 ± 1%, 34 ± 2% and 48 ± 2%, compared to the “alcoholization” group after 3, 6 and 9 months of alcohol intake, respectively.

Cellular mechanisms of antioxidant protection are also associated with the functioning of a powerful glutathione link [47]. The glutathione system is one of the active components of the body’s antioxidant defense and plays a significant role in attenuating the pathological process in the development of muscle myopathy, as it not only prevents the course of free-radical reactions, but also provides effective elimination of the final metabolites of LPO [50]. In our study, after administration of C_{60}FAS in therapeutic regimen I, GP_x activity in rat gastrocne-

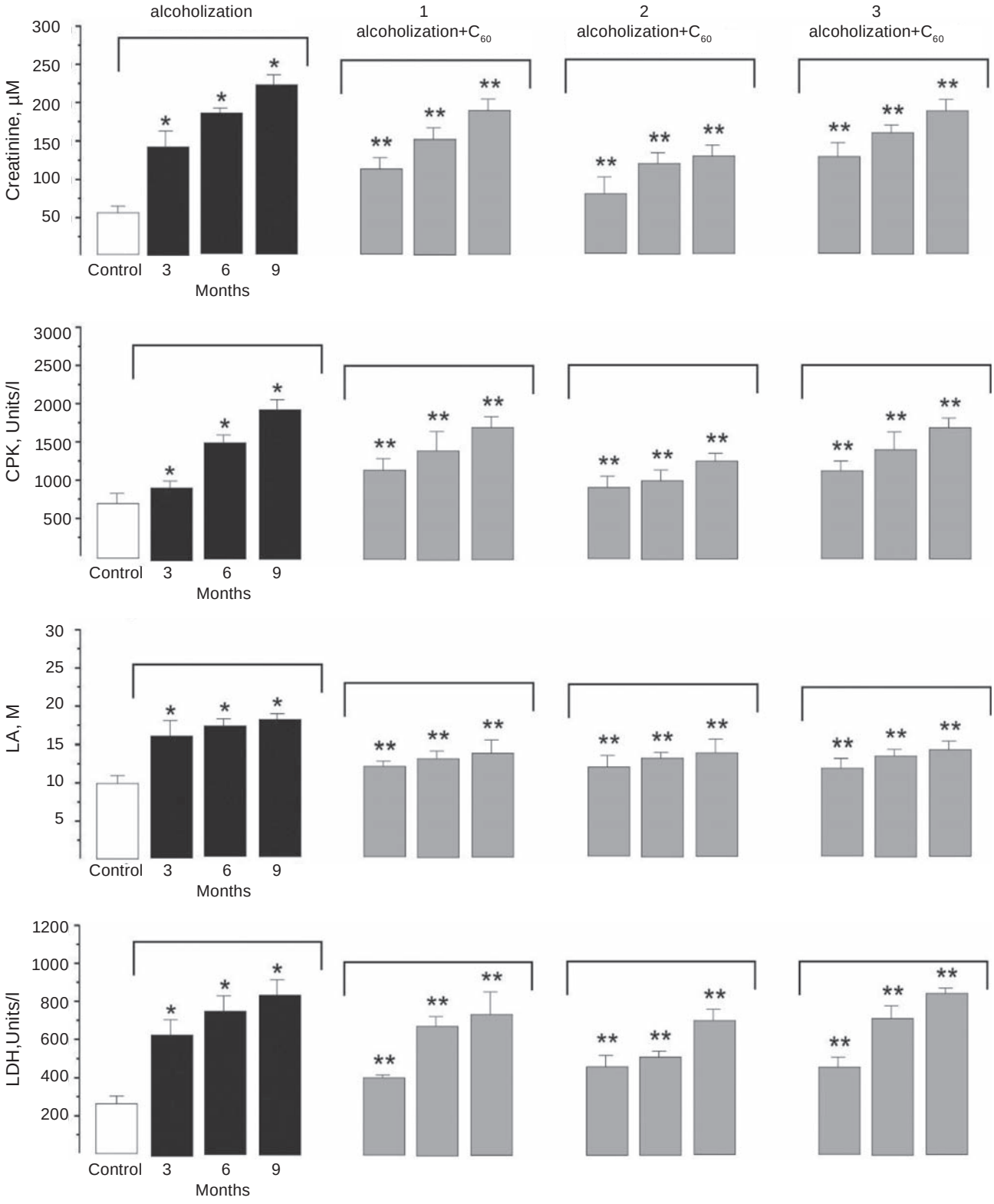

*Fig. 1. The content of creatinine, CPK, LA and LDH in blood plasma of experimental rats: control – the control group of animals; alcoholization – rats treated with alcohol; alcoholization+C_{60} – rats treated with C_{60}FAS 1 h before alcohol intake (preventive regimen) (**1**), in mixture with alcohol (therapeutic regimen I) (**2**) and 1 h after alcohol intake (therapeutic regimen II) (**3**), respectively; 3, 6 and 9 months – alcoholization lasting 3, 6 and 9 months, respectively; *P < 0.05 relative to the control group; **P < 0.05 relative to the alcoholization group*

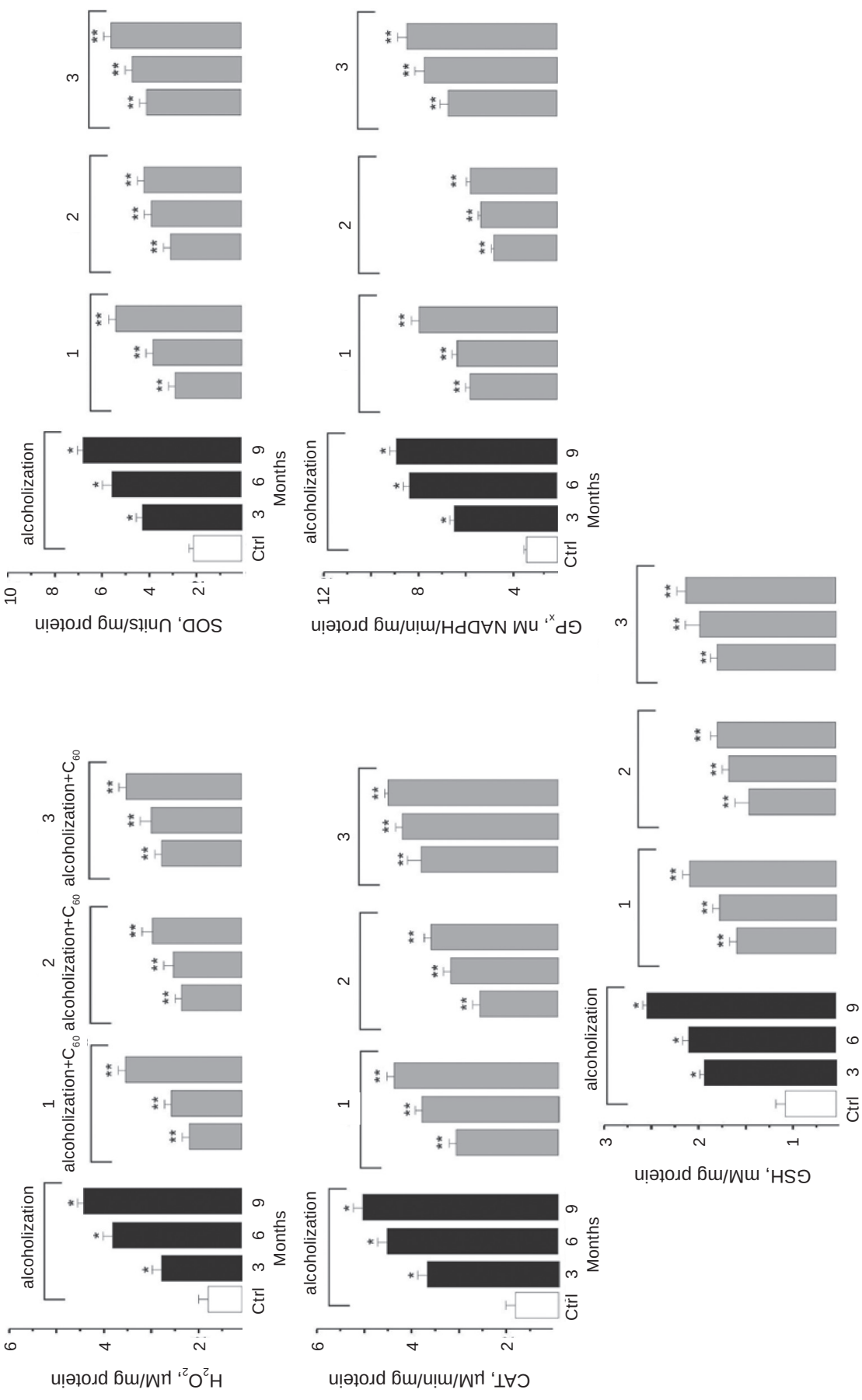

*Fig. 2. Indices of pro- and antioxidant balance (H_2O_2, SOD, CAT, GP_x and GSH) in rat gastrocnemius muscle: control (Ctrl) – the control group of animals; alcoholization – rats treated with alcohol; alcoholization+C_{60} – rats receiving C_{60}FAS 1 h before alcohol intake (preventive regimen) (**1**), in mixture with alcohol (therapeutic regimen I) (**2**) and 1 h after alcohol intake (therapeutic regimen II) (**3**), respectively; 3, 6 and 9 months – alcoholization lasting 3, 6 and 9 months, respectively; *$P < 0.05$ relative to the control group; **$P < 0.05$ relative to the alcoholization group*

mius muscle was decreased by 17 ± 1%, 23 ± 1% and 39 ± 2% compared to the "alcoholization" group after 3, 6 and 9 months of alcohol intake, respectively. Finally, application of C_{60}FAS in therapeutic regimen I resulted in a 13 ± 1%, 27 ± 1% and 42 ± 2% change in GSH content in muscle gastrocnemius of rats compared to the "alcoholization" group after 3, 6 and 9 months of alcohol intake, respectively. Consequently, administration of C_{60}FAS led to an increase in the efficiency of the antioxidant defence system by increasing GSH content in muscle, thereby increasing muscle tolerance to physical exertion. This result agrees well with the data of [34], where it was shown that water-soluble C_{60} fullerenes can influence the synthesis and metabolism of glutathione in various tissues, including muscle, under various pathological conditions by modulating the Nrf2/ARE-antioxidant pathway.

Thus, in all the tests performed, a positive change in the studied biochemical parameters of about (15–30) ± 2% is observed when C_{60}FAS is administered together with alcohol (therapeutic regimen I). The other two C_{60}FAS administration regimens (preventive and therapeutic regimen II) have a (7–10) ± 1% reduction in efficacy.

It should be noted that functional muscle characteristics will be impaired after the development of alcoholic myopathy, regardless of the time elapsed since the onset of alcoholization, but these disorders increase steadily up to the development of rhabdomyolysis. Pathological changes in muscle tissue occur along with changes in tendon-muscle morphology. These abnormalities can persist for years after the rehabilitation period and disrupt non-linear processes in muscle biomechanics [20]. Therefore, in our opinion, the use of C_{60}FAS against the background of alcoholic myopathy should be of a long-term and systemic nature.

Conclusion. Based on the data obtained, the conclusion can be made that the oral administration of C_{60}FAS at an optimal dose of 1 mg/kg helps to reduce oxidative processes in muscle by maintaining the balance between pro-oxidants and the antioxidant defense system, which prevents the negative effects of ROS on cellular and subcellular structures in the development of alcoholic myopathy in rats over 3, 6 and 9 months. This indicates the presence of compensatory activation by water-soluble C_{60} fullerenes of the endogenous antioxidant system during pathological changes in muscle caused by long-term chronic alcohol consumption. In our opinion, water-soluble C_{60} fullerenes, as powerful antioxidants, inhibit the occurrence of degradation in muscle and thus reduce its degradation.

Summarizing, positive changes in the studied biochemical indices of blood and muscle gastrocnemius tissue of alcoholic rats confirm the possibility of using water-soluble C_{60} fullerenes as promising pharmacological nanoagents capable of reducing/correcting pathological conditions of the muscular system occurring in the development of alcoholic myopathy. The found significant quantitative difference between the effects within the proposed C_{60}FAS application schemes should be taken into account when developing the algorithm of rehabilitation procedures.

Conflict of interest. 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.

БІОХІМІЧНІ ПОКАЗНИКИ КРОВІ І ТКАНИНИ М'ЯЗА *GASTROCNEMIUS* ПІСЛЯ ХРОНІЧНОЇ АЛКОГОЛІЗАЦІЇ ЩУРІВ ЗА ПЕРОРАЛЬНОГО ВВЕДЕННЯ ВОДНОГО РОЗЧИНУ C_{60} ФУЛЕРЕНУ

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Досліджено біохімічні показники крові та тканини м'яза *gastrocnemius* після хронічної алкоголізації щурів упродовж 3, 6 і 9 місяців. Як фармакологічний агент використовували пероральне введення водного розчину C_{60} фулерену (C_{60}ВРФ) у дозі 1 мг/кг упродовж усього експерименту у трьох схемах застосування: за 1 год до введення алкоголю (профілактична схема), разом із алкоголем (терапевтична схема I) та через 1 год після прийому алкоголю (терапевтична схема II). Встановлено, що C_{60}ВРФ зменшував рівень патологічних станів у м'язовому апараті за тривалого розвитку алкогольної міопатії,

пригнічуючи окислювальні процеси у ньому та запобігаючи, таким чином, його деградації. У всіх проведених тестах відзначено позитивну зміну досліджуваних біохімічних показників приблизно на (15–30) ± 3% за терапевтичної схеми I введення C_{60}ВРФ. У разі застосування двох інших схем введення C_{60}ВРФ його ефективність знижувалась на (7–10) ± 3% порівняно з терапевтичною схемою I. Отримані результати свідчать про здатність фулерену C_{60} коригувати патологічний стан м'язової системи, що виникає внаслідок алкогольної інтоксикації.

Ключові слова: фулерен C_{60}, алкогольна інтоксикація, м'яз *gastrocnemius*, креатинфосфокіназа, лактатдегідрогеназа, антиоксидантна система.

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