

Effect of C₆₀ Fullerene on Muscle Injury-Induced Rhabdomyolysis and Associated Acute Renal Failure

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Introduction: Rhabdomyolysis, as an acute stage of myopathy, causes kidney damage. It is known that this pathology is caused by the accumulation of muscle breakdown products and is associated with oxidative stress. Therefore, the present study evaluated the effect of intraperitoneal administration (dose 1 mg/kg) of water-soluble C₆₀ fullerenes, as powerful antioxidants, on the development of rat kidney damage due to rhabdomyolysis caused by mechanical trauma of the *muscle soleus* of different severity (crush syndrome lasting 1 min under a pressure of 2.5, 3.5, and 4.5 kg/cm², respectively).

Methods: Using tensometry, biochemical and histopathological analyses, the biomechanical parameters of *muscle soleus* contraction (contraction force and integrated muscle power), biochemical indicators of rat blood (concentrations of creatinine, creatine phosphokinase, urea and hydrogen peroxide, catalase and superoxide dismutase activity), glomerular filtration rate and fractional sodium excretion value, as well as pathohistological and morphometric features of muscle and kidney damages in rats on days 1, 3, 6 and 9 after the initiation of the injury were studied.

Results: Positive changes in biomechanical and biochemical parameters were found during the experiment by about 27–30 ± 2%, as well as a decrease in pathohistological and morphometric features of muscle and kidney damages in rats treated with water-soluble C₆₀ fullerenes.

Conclusion: These findings indicate the potential application of water-soluble C₆₀ fullerenes in the treatment of pathological conditions of the muscular system caused by rhabdomyolysis and the associated oxidative stress.

Keywords: *muscle soleus*, C₆₀ fullerene, rhabdomyolysis, biomechanical parameters of muscle contraction, blood biochemical indicators, histopathological analysis

Introduction

Acute renal failure (ARF) is a condition in which there is a rapid decline in kidney function, in particular, there is a decrease or even complete absence of urine production, which leads to the accumulation of nitrogen metabolism products in the blood. Recently, a significant number of ARF cases have been associated with rhabdomyolysis of skeletal muscle. Rhabdomyolysis is a clinical syndrome of muscle disorder caused by the destruction of skeletal muscle fibers, large artery occlusion, epileptic status, infections, etc. It is characterized by the release of skeletal muscle cell contents into the bloodstream, including myoglobin, labile iron ions, and various toxic metabolites,¹ followed by a potentially lethal accumulation of toxins, including potassium, lactic acid, and myoglobin, leading to multiple organ failure, primarily acute kidney injury.² This pathology affects up to 46% of hospitalized patients and 80% of patients requiring intensive care for rhabdomyolysis.³ Even with effective medical care, the mortality rate exceeds 25%.⁴ Over the past

decade, the incidence of rhabdomyolysis has increased 10-fold,⁵ particularly for wounded military personnel due to ballistic trauma to skeletal muscle,^{6,7} who are 5 times more likely to develop rhabdomyolysis than civilians.⁸ Global mortality from rhabdomyolysis is estimated at 10% to 46% depending on the severity, cause, early treatment, and the presence of comorbidities and complications.⁹

Currently, there is no specific treatment for rhabdomyolysis. Dialysis remains the main treatment for patients with ARF caused by rhabdomyolysis. Dialysis is believed to remove myoglobin released from damaged muscles and metabolic toxins from the bloodstream. Standard medical interventions are usually massive hydration, urine alkalization, and forced diuresis. It is reported that 28% to 65% of patients who develop rhabdomyolysis require mandatory dialysis therapy.¹⁰

Note that traumas of various natures can cause acute kidney injury (AKI). It is the early diagnosis of AKI, in particular, thanks to the use of nanoparticles¹¹ and recently discovered biomarkers,¹² ensures its effective therapy in the future. The burst of reactive oxygen species (ROS) plays a key role in the pathological progression of AKI. Therefore, mitochondrial-targeted antioxidant therapy is very promising because mitochondria are the main source of ROS in AKI. It was shown that antioxidant nanodrugs, in particular ultra-small Tungsten-based nanodots, with actively targeted mitochondria have achieved encouraging success in many oxidative stress-induced diseases.¹³ Many new antioxidant nanodrugs with intrinsic kidney targeting through the control of size, shape, and surface properties have opened exciting prospects for the treatment of AKI.^{14,15}

ARF causes a general or regional decrease in renal blood flow. Subsequent renal hypoxia and ischemia contribute to the formation of ROS, which damage biomolecules and membranes, including renal tubular cells. It has been shown that various exogenous antioxidants have a significant effect on the course of ARF induced by rhabdomyolysis. For example, ascorbate (vitamin C) reduces rhabdomyolysis kidney damage in humans.¹⁶ It has been shown that the increase in the level of lipid peroxidation products (LPO) and the activity of antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase) was reversed by the administration of D-panthenol.¹⁷ Its use improved renal morphology and reduced ARF markers through antioxidant effects and normalization of mitochondrial metabolism. It was found that allopurinol administration attenuated renal dysfunction in the ARF model associated with rhabdomyolysis by reducing oxidative stress (systemic, renal and muscle), apoptosis, and inflammation. Allopurinol also reduced oxidative stress in injured muscles, attenuated muscle damage/inflammation, and accelerated muscle recovery.¹⁸ Acetaminophen has been shown to reduce kidney damage and improve kidney function by counteracting ROS and free radical formation in a rat model of rhabdomyolysis.¹⁹ Study²⁰ found that N-acetylcysteine improves renal function. It has been shown that biocompatible and bioavailable carbon nanoparticles C₆₀ fullerenes with actively targeted mitochondria²¹ are powerful scavengers of ROS induced by muscle damage: their application in vivo resulted in significant positive therapeutic effects after the initiation of ischemic injury,²² fatigue,²³ atrophy,²⁴ and skeletal muscle injury,²⁵ as well as pesticide poisoning.²⁶ Finally, in our previous work,²⁷ we demonstrated a protective effect of water-soluble C₆₀ fullerene on the development of ARF in a rat model of glycerol-induced rhabdomyolysis.

Based on the above-mentioned data, the aim of this work was to study the effect of water-soluble C₆₀ fullerenes on the development of rhabdomyolysis caused by *muscle soleus* mechanical trauma of different degrees of severity and associated ARF by evaluating biomechanical parameters of muscle contraction (contraction force and integrated muscle power) and biochemical parameters of laboratory animals blood (concentrations of creatinine, creatine phosphokinase (CPK), urea and hydrogen peroxide, CAT and SOD activity), as well as glomerular filtration rate (GFR) and fractional excretion of sodium (FE_{Na}). Additionally, the pathohistological and morphometric features of muscle and kidney damage in rats after the initiation of the injury were evaluated.

Materials and Methods

Material Preparation

An aqueous solution of pristine C₆₀ fullerene with a purity of more than 99.95% has been prepared according to the method.²⁸ Briefly, it is based on the transfer of C₆₀ molecules from toluene to water followed by sonication. Microscopic and spectroscopic data indicate that an aqueous solution of C₆₀ fullerene at a maximum concentration of 0.15 mg/mL is

a typical colloid containing both single C₆₀ molecules (~0.7 nm in diameter) as well as their nanoaggregates (up to ~100 nm)²⁹ and remains highly stable (zeta potential value is -25.3 mV³⁰) for 18 months at a temperature +4 °C.

It is important to note that due to its nanosized almost spherical shape, high chemical stability, and unique physical properties, C₆₀ fullerene is of the greatest interest for biomedical research.³¹ In particular, C₆₀ fullerene exhibits a strong reducing ability, readily attaching up to six electrons simultaneously. Due to this, it acts in *in vitro* and *in vivo* systems as a powerful scavenger of free radicals,^{32,33} the overproduction of which leads to many pathologies.

Animal Model

All experiments were performed on rats in accordance with the international principles of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986), Article 26 of the Law of Ukraine “On the Protection of Animals from Cruelty” (No. 3447-IV, February 21, 2006) as well as the European Union Directive (2010/63/EU) “On the protection of animals used for scientific purposes”. The research protocols were approved by the Bioethics Committee of the ESC ‘Institute of Biology and Medicine’ of the Taras Shevchenko National University of Kyiv (Protocol No. 2 of September 2, 2022).

Experiments were performed on male Wistar rats weighing 150–180 g, randomly divided into the following groups:

- control group (n = 7);
- model pathology groups of 1 (n=7), 2 (n=7), and 3 (n=7) severity degrees;
- model pathology groups of 1 (n=7), 2 (n=7), and 3 (n=7) severity degrees after administration of water-soluble C₆₀ fullerenes.

Muscle trauma was induced by compressing the muscle for 1 min with a clamp under pressure of 2.5 (1st degree of severity), 3.5 (2nd degree of severity) and 4.5 (3rd degree of severity) kg/cm².³⁴ The applied crush syndrome led to a systemic manifestation of pathological changes due to the destruction of muscle cells, in particular, the release of muscle cell components (creatine kinase, myoglobin) into the extracellular environment, which served as a marker of a muscle injury severity.

It should be noted that the applied dose of water-soluble C₆₀ fullerenes of 1 mg/kg was chosen as the most effective when studying their antioxidant effect in other models of muscle pathologies.^{22–26} In addition, it is significantly lower than the LD₅₀ value, which was 600 mg/kg in the case of oral administration to rats³² and 721 mg/kg in the case of intraperitoneal administration to mice.³⁰ An aqueous solution of C₆₀ fullerene was administered intraperitoneally daily throughout the experiment, starting on the first day immediately after the initiation of muscle injury.

Animals were anesthetized by intraperitoneal injection of nembutal (40 mg/kg). The rat *soleus muscle* was freed from the surrounding tissues. Its tendon part was cut across the distal part of the muscle, which was connected to the force sensors. Efferents were stimulated with electrical pulses of 2 ms duration, generated by an ADC (analog-to-digital converter) pulse generator through platinum electrodes.^{22–26}

Biomechanical and Biochemical Analysis

In the process of analyzing the biomechanical results, the following parameters were used: minimum and maximum muscle contraction forces when performing 6 s of stimulation pool and integrated muscle power (calculated area under the force curve), which is an indicator of its overall performance.^{22–26}

The content of creatinine, CPK, urea, and hydrogen peroxide, CAT, and SOD activity in the blood of experimental animals as muscle and kidney dysfunction markers were determined using clinical diagnostic equipment - biochemical analyzers RNL-200 and JN-1101-TR2 (Netherlands).^{22–26}

GFR was determined by the clearance of endogenous creatinine:³⁵ $GFR (\mu L/min) = (V \cdot Cr_U) / Cr_B$, where V is urine volume ($\mu L/min$; minute diuresis), Cr_U ($\mu mole/l$) and Cr_B ($\mu mole/l$) are the concentrations of creatinine in urine and blood, respectively.

The FE_{Na} value (the ratio between glomerular filtration of sodium and its reabsorption) was used to assess sodium transport in the kidneys:³⁶ $FE_{Na}(\%) = 100\% \times (Cr_B \times Na_U) / (Na_B \times Cr_U)$, where Na_U and Na_B are the concentrations of sodium in urine and blood, respectively.

Histopathological Analysis

The samples of a kidney and a muscle were separated and fixed in 10% formalin, embedded in paraffin, cut into 5 μm sections, and stained with hematoxylin and eosin (H&E) for general histopathological analyses.³⁷ Sections of the muscle were additionally stained with hematoxylin and picrofuchsin by van Gieson for detecting connective tissue and muscle fibers. Digital microphotographs of stained sections were taken at $\times 100$ and $\times 400$ magnification using a computer-assisted image analyzing system (consisting of Olympus BX41 microscope and Olympus C-5050 Zoom digital camera). The histopathological profiles of the muscles and kidneys were determined by light microscopy observation. Then, the muscle fiber diameters and the area occupied by connective tissue in the muscle bundles were measured using ImageJ software. In addition, the cross-sectional area of renal glomeruli and diameter of renal tubes were measured in the kidneys using ImageJ software.

Statistical Analysis

Statistical evaluation of the results was performed using the procedure of analysis of variances (ANOVA) with mixed design. Two between-group factors were supposed: 1) injury (three levels – 1st, 2nd, and 3rd degree of severity); 2) C₆₀ fullerene treatment (two levels – no and use of C₆₀). The factor of time was supposed a within-group with four levels (1, 3, 6, and 9 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 by the Bonferroni *post-hoc* test. The differences between the groups were considered significant at $P < 0.05$. The statistical evaluation was performed by the software package Statistica 8.0 (Dell, USA).

Results and Discussion

Biomechanics of Muscle Contraction

Figure 1 shows the generation curves of *muscle soleus* contraction force on days 1, 3, 6, and 9 after muscle injury initiation of 1, 2, and 3 degrees of severity. Thus, on the first day after the injury, integrated muscle power decreased to $69 \pm 3\%$, $43 \pm 2\%$, and $24 \pm 1\%$ of control values at 1, 2, and 3 degrees of severity, respectively (Figure 1A and C). On the third day, these indices amounted to $29 \pm 1\%$, $17 \pm 1\%$, and $11 \pm 1\%$, respectively, in comparison with the control (Figure 1A and C), which confirms the data on the maximum dysfunction of the injured muscle on the third day after the injury.^{38,39} On the sixth day, a significant increase in the integrated power of the muscle was recorded up to $62 \pm 2\%$, $55 \pm 3\%$, and $41 \pm 3\%$ at 1, 2, and 3 degrees of severity, respectively (Figure 1A and C). Finally, on the ninth day, the value of this parameter was $83 \pm 5\%$, $79 \pm 3\%$, and $75 \pm 3\%$, respectively, relative to the control (Figure 1A and C), which is consistent with the data on the recovery of muscle function on the ninth day after injury. It should be emphasized that during a long period (2–3 months) the performance of the injured muscle does not fully recover:^{40,41} in our case, its level on the ninth day after injury initiation was $75\text{--}80 \pm 3\%$ of the control and remained so during 1 month.

The injections of C₆₀ fullerene aqueous solution increased integrated muscle power by $18 \pm 1\%$, $13 \pm 1\%$, and $14 \pm 2\%$ at severity grades 1, 2, and 3, respectively, on the first day after injury and by $23 \pm 2\%$, $18 \pm 2\%$ and $11 \pm 1\%$, respectively, on the third day (Figure 1B and C). On the sixth and ninth day, these parameters did not change significantly (Figure 1B and C) relative to the injury group. It should be noted that there was a slight difference in the positive effect of C₆₀ fullerene between the different severity levels of muscle injury on both the first and ninth days of the experiment. Thus, C₆₀ fullerene aqueous solution injections increased the level of integrated muscle power by $15\text{--}25 \pm 2\%$ relative to the injury group on the ninth day of the experiment.

When performing single-joint movements, such biomechanical indices as the generation levels of maximum and minimum force of muscle contraction are the main markers of muscle dysfunction in the development of pathological processes.^{22–26} The positive effect of C₆₀ fullerene injections on the level of minimal force (the most sensitive marker of muscle dysfunction) did not exceed $10 \pm 2\%$ relative to the injury group on the first day of the experiment (Figure 2A). On the third day (the day of most maximal dysfunction of the injured muscle), this index increased significantly and was $29 \pm 2\%$, $73 \pm 3\%$, and $75 \pm 4\%$ at severity grades 1, 2, and 3, respectively (Figure 2B). On the sixth and ninth day, it amounted to $45\text{--}50 \pm 2\%$ relative to the injury group (Figure 2C and D). The change in the level of maximal force of muscle contraction at injections of C₆₀ fullerene amounted to 12–

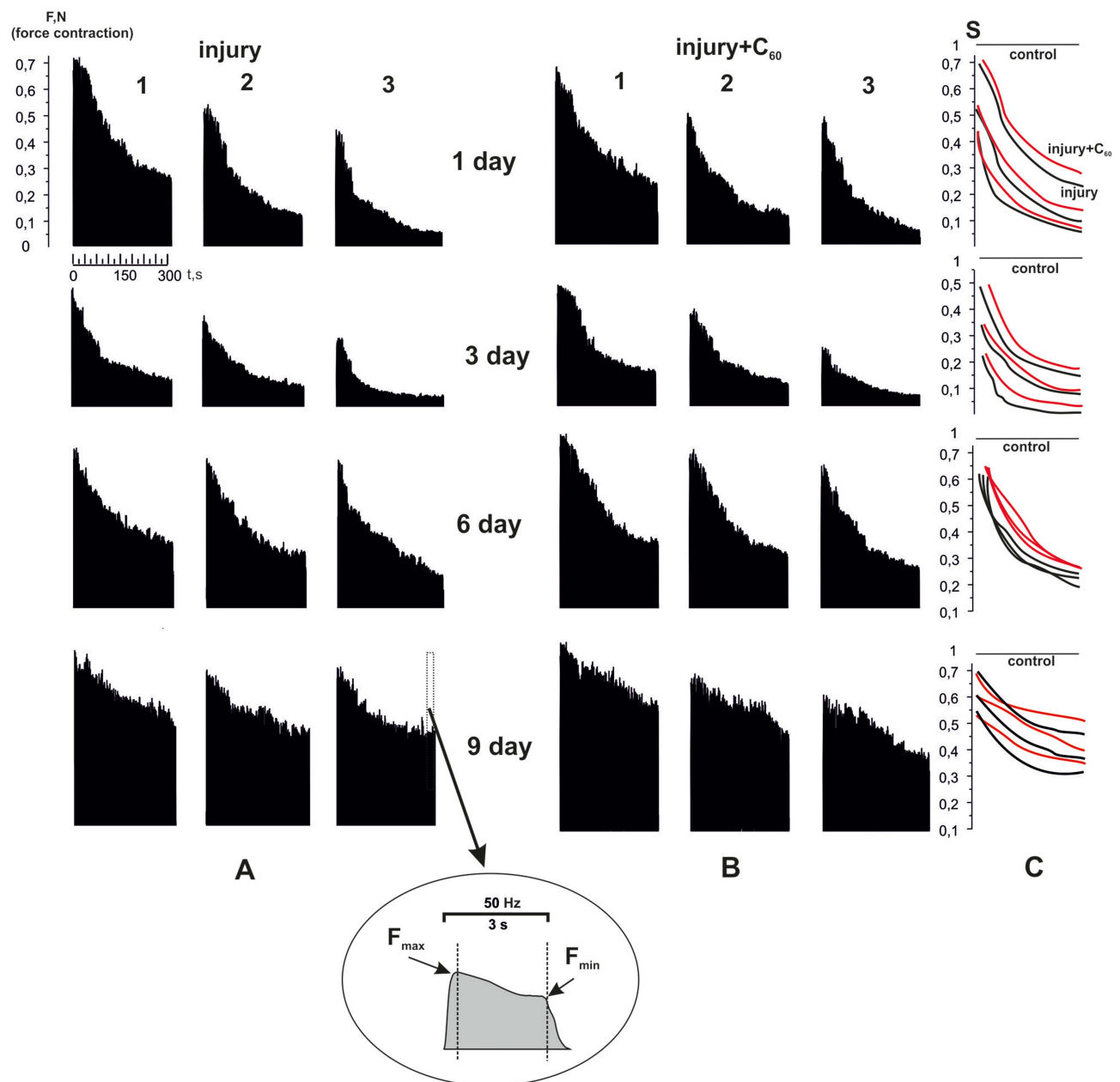


Figure 1 Muscle soleus contraction force after the injury initiation of 1, 2 and 3 degrees of severity on the 1st, 3rd, 6th and 9th day of the experiment without C₆₀ fullerene aqueous solution administration (A) and on the background of its injection (B) at stimulation by a signal of 50 Hz frequency with duration of 3 s for 300 s; F_{max} and F_{min} - maximum and minimum forces of single muscle contraction; S - integrated muscle power (in relative units) (C).

16 ± 1% relative to the injury group at all time periods of the experiment (Figure 2). The obtained data testify to the suppressive effect of the aqueous solution of C₆₀ fullerene on free-radical processes in the injured muscle, thus promoting myocytes to generate the minimum force at a higher level. The insignificant positive effect of C₆₀ fullerene on the level of maximal force generation in the injured muscle may be related to the free-radical processes that occur inside the injured myocytes and prevent actomyosin interactions.

Thus, biomechanical studies show that C₆₀ fullerene aqueous solution injections significantly reduce the level of development of pathologic processes in injured muscle, which, in turn, may influence the level of ARF-initiated rhabdomyolysis.

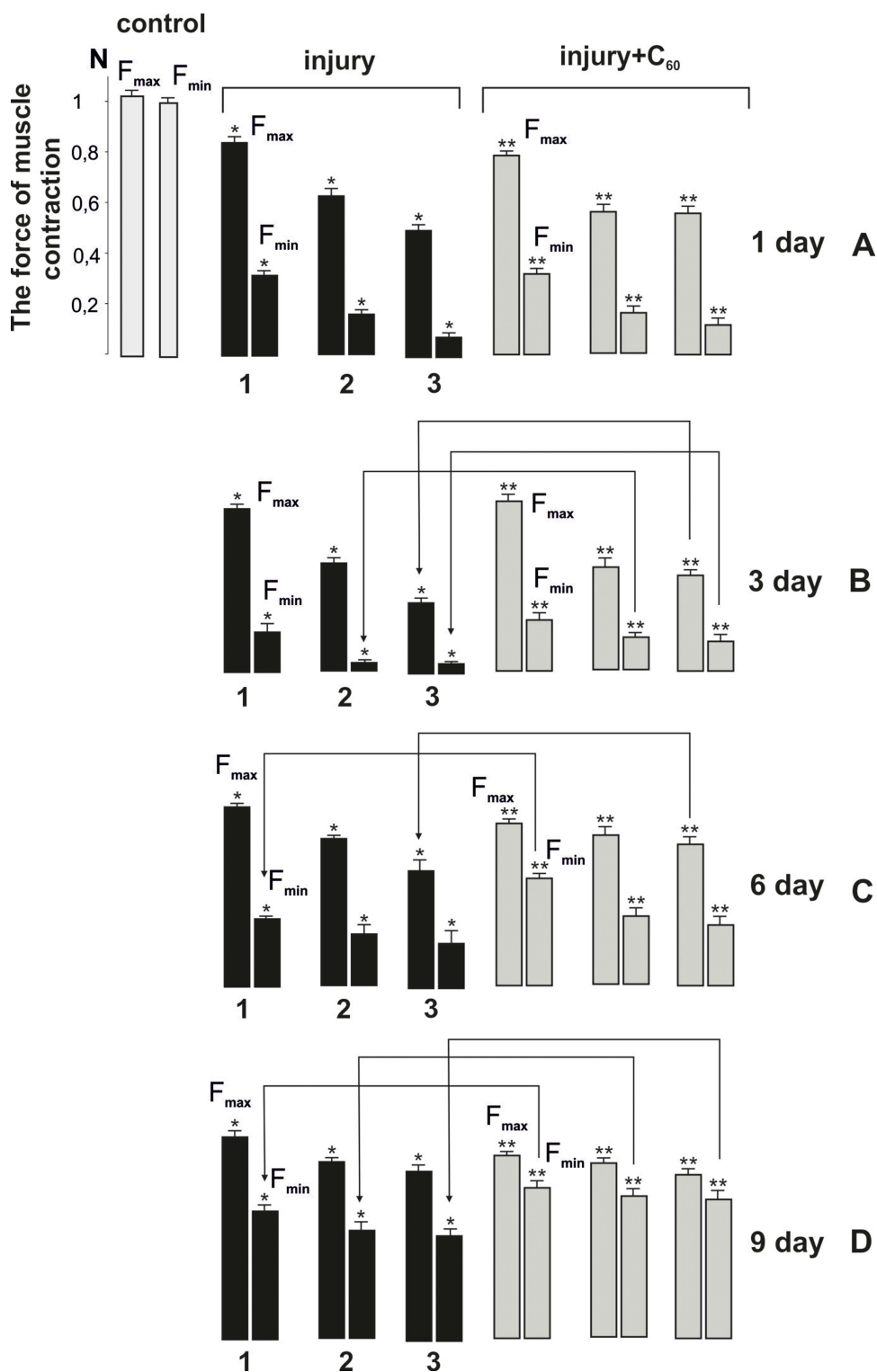


Figure 2 The biomechanical parameters of *muscle soleus* contraction after the injury initiation of 1, 2, and 3 degrees of severity on the 1st (A), 3rd (B), 6th (C) and 9th (D) day of the experiment without C₆₀ fullerene aqueous solution administration (injury) and against the background of its injections (injury + C₆₀); F_{max} and F_{min} - maximum and minimum forces of single muscle contraction; *P < 0.05 relative to the control group; **P < 0.05 relative to the injury group. Arrows indicate the most significant differences between injury and injury+C₆₀ groups.

Biochemical Analysis

Blood creatinine level is an important marker of muscle injury as well as ARF.⁴² In rhabdomyolysis' ARF, it can increase 2 (with 1 degree of injury severity), 3 (with 2 degree of injury severity), 4 times or higher (with 3 degree of injury

severity) from baseline.² The data obtained (Figure 3A) confirm the significant increase in creatinine level with increasing severity of muscle injury. However, on the third day of the experiment, the creatinine level decreased compared to the first day after injury initiation by $11 \pm 1\%$, $14 \pm 1\%$, and $23 \pm 1\%$ at 1, 2, and 3 degrees of severity, respectively, relative to the control. In our opinion, this is due to the cessation of the release of intracellular myocyte contents into the circulating blood. On the sixth and ninth day after the injury, creatinine levels increased dramatically by

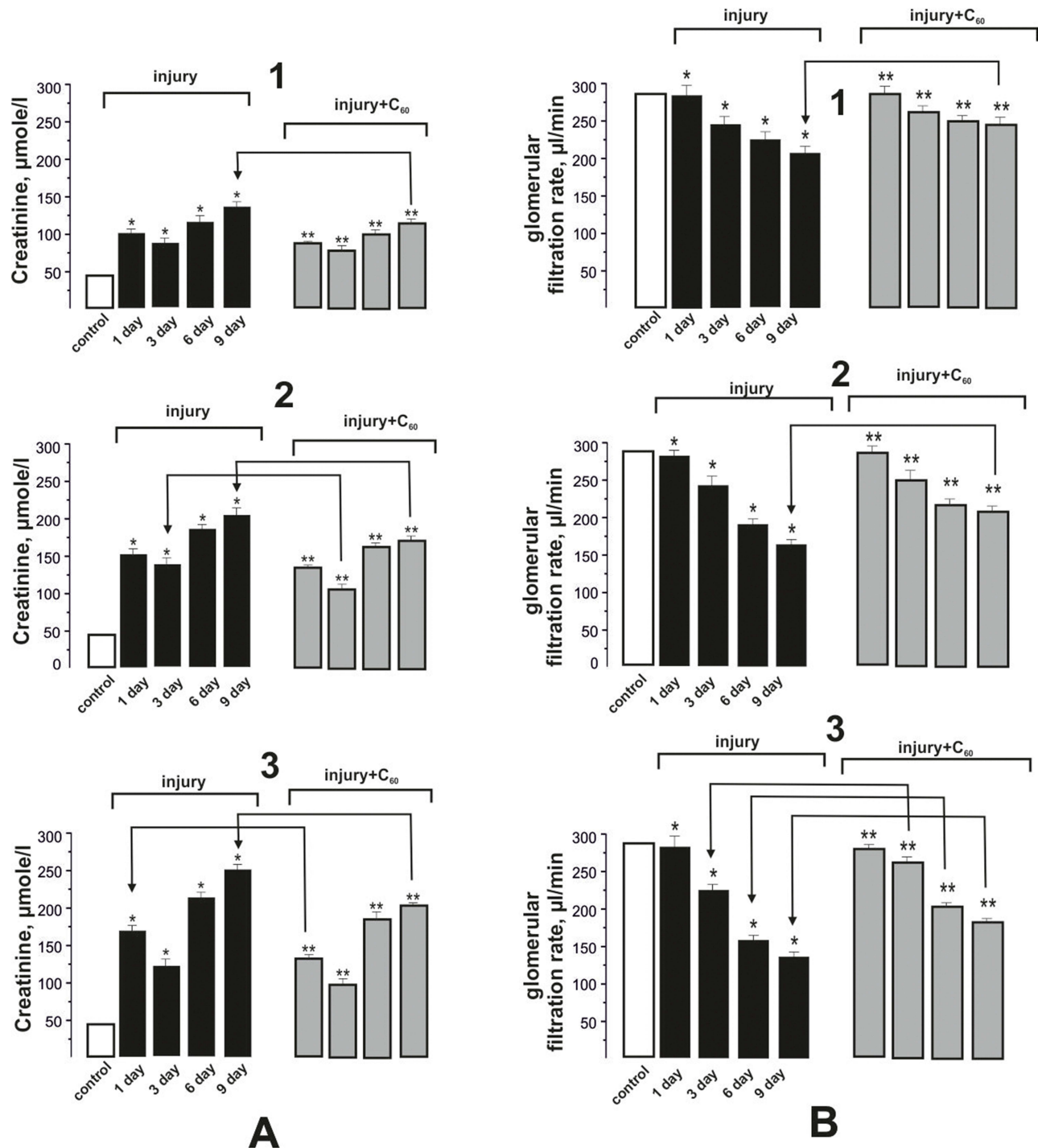


Figure 3 Blood creatinine (A) levels in rats and GFR (B) with rhabdomyolysis kidney injury: injury and injury + C₆₀ - indices after initiation of muscle injury of 1, 2, and 3 degrees of severity on the 1st, 3rd, 6th, and 9th day of the experiment without C₆₀ fullerene aqueous solution administration and on the background of its injection, respectively; *P < 0.05 relative to the control; **P < 0.05 relative to the injury group. Arrows indicate the most significant differences between injury and injury+C₆₀ groups.

150–170 ± 5% and 300–450 ± 5% at 1 and 3 injury severity levels, respectively. Such an increase indicates the development of ARF caused by rhabdomyolysis.

C₆₀ fullerene aqueous solution injections lead to a 14–22 ± 1%, 21–24 ± 2%, and 30–40 ± 2% decrease in the level of creatinine at the 1st, 2nd, and 3rd degree of injury severity, respectively, relative to the injury group during the experiment (Figure 3A).

GFR is an important indicator that characterizes the ability of the kidneys to perform their main function - to clear plasma and produce urine.⁴³ During the development of rhabdomyolysis' ARF outside the proximal convoluted tubule (in the thick ascending part of the loop of Henle), urinary myoglobin combines with Tamm-Horsfall protein to form a precipitate. pH-dependent precipitate forms tubular casts that obstruct the distal tubules.⁴⁴ Obstruction is thought to increase intra-canalicular pressure above interstitial pressure, reducing vascular inflow and perfusion, promoting inflammation, and directly reducing GFR by altering Starling forces, and the effect of antioxidants on this process may reduce ARF development.^{43,44}

Decrease in GFR value was recorded starting from the third day after injury initiation (Figure 3B). On the ninth day, the difference from control values was 21 ± 1%, 43 ± 4%, and 67 ± 4% at 1, 2, and 3 degrees of injury severity, respectively. When applying C₆₀ fullerene injections, this index increased approximately by 17 ± 1%, 21 ± 1%, and 27 ± 1% at 1, 2, and 3 degrees of injury severity, respectively, relative to the injury group. At the same time, it should be noted a slight difference in the GFR values (within the error) on the sixth and ninth day of the experiment (Figure 3B).

When ARF develops, the concentration in the blood of nitrogenous slag, which can only be excreted by the body with urine, increases significantly, which is a sign of deteriorating kidney function. Urea is one of the end products of protein metabolism that contains nitrogen. The result of a blood urea test is one of the main indicators of the quality of glomerular filtration in ARF development.

An increase in urea levels was observed starting from the third day of the experiment and amounted to 83–156 ± 5%, 89–211 ± 5%, and 203–340 ± 7% of the control at 1, 2, and 3 muscle injury severity degrees, respectively, on days 3–9 of the experiment (Figure 4A). C₆₀ fullerene injections decreased urea concentration by 17–18 ± 1%, 20–22 ± 1%, and 24–27 ± 2% at 1, 2, and 3 degrees of injury severity, respectively, during the experiment (Figure 4A).

Also, one of the important quantitative indicators of the degree of ARF development is sodium transport assessment, since the mechanism of free-radical inhibition of reabsorption in rhabdomyolysis is associated with the inhibition of sodium-potassium ATPase transport system, and the action of antioxidants can reduce the development of cascade pathological reactions.⁴⁵ On the third day of the experiment, the FE_{Na} value increased by 31 ± 1%, 98 ± 3%, and 141 ± 4% relative to the control at 1, 2, and 3 injury severity, respectively (Figure 4B). On the sixth and ninth day, this index increased by 45–271 ± 5% and 128–387 ± 7% relative to the control at 2 and 3 injury severity, respectively. Injections of C₆₀ fullerene aqueous solution reduced FE_{Na} value by 13–15 ± 1%, 20–23 ± 1%, and 25–32 ± 2% at 1, 2, and 3 muscle injury severity, respectively, relative to the injury group during the experiment (Figure 4B).

Thus, the application of C₆₀ fullerene demonstrated significant (15–35 ± 2%) positive effects on the main markers of ARF induced by rhabdomyolysis. Note that their most significant reduction is observed in severe forms of rhabdomyolysis on the ninth day after muscle injury initiation.

Recently, CPK concentration measurement in blood has become a clinical test to determine the level of rhabdomyolysis development due to its rapidity and cheapness.⁵ The relationship between CPK level increase and ARF development in patients with muscle injuries has been established.⁴⁶ CPK is an enzyme from the energy supply system of skeletal muscle cells, which catalyzes the transfer of phosphate group from ATP to creatinine molecule to form the high-energy compound creatinine phosphate. In case of mechanical muscle damage, there is a release of this enzyme from the cells and, accordingly, an increase in its concentration in the blood.

CPK levels on the first day after injury rose by 112 ± 3%, 234 ± 7%, and 414 ± 7% compared with control values at 1, 2, and 3 degrees of muscle injury severity, respectively (Figure 5A). CPK levels decreased on the following days of the experiment and on the ninth day were 13–25 ± 1% of control values, despite an increase in ARF development on the sixth and ninth days.

C₆₀ fullerene injections significantly reduced CPK levels, which were 24 ± 1%, 31 ± 2% and 34 ± 2% and 17 ± 1%, 36 ± 2% and 42 ± 2% at 1, 2, and 3 degrees of muscle injury severity, respectively, on the first and third day of the

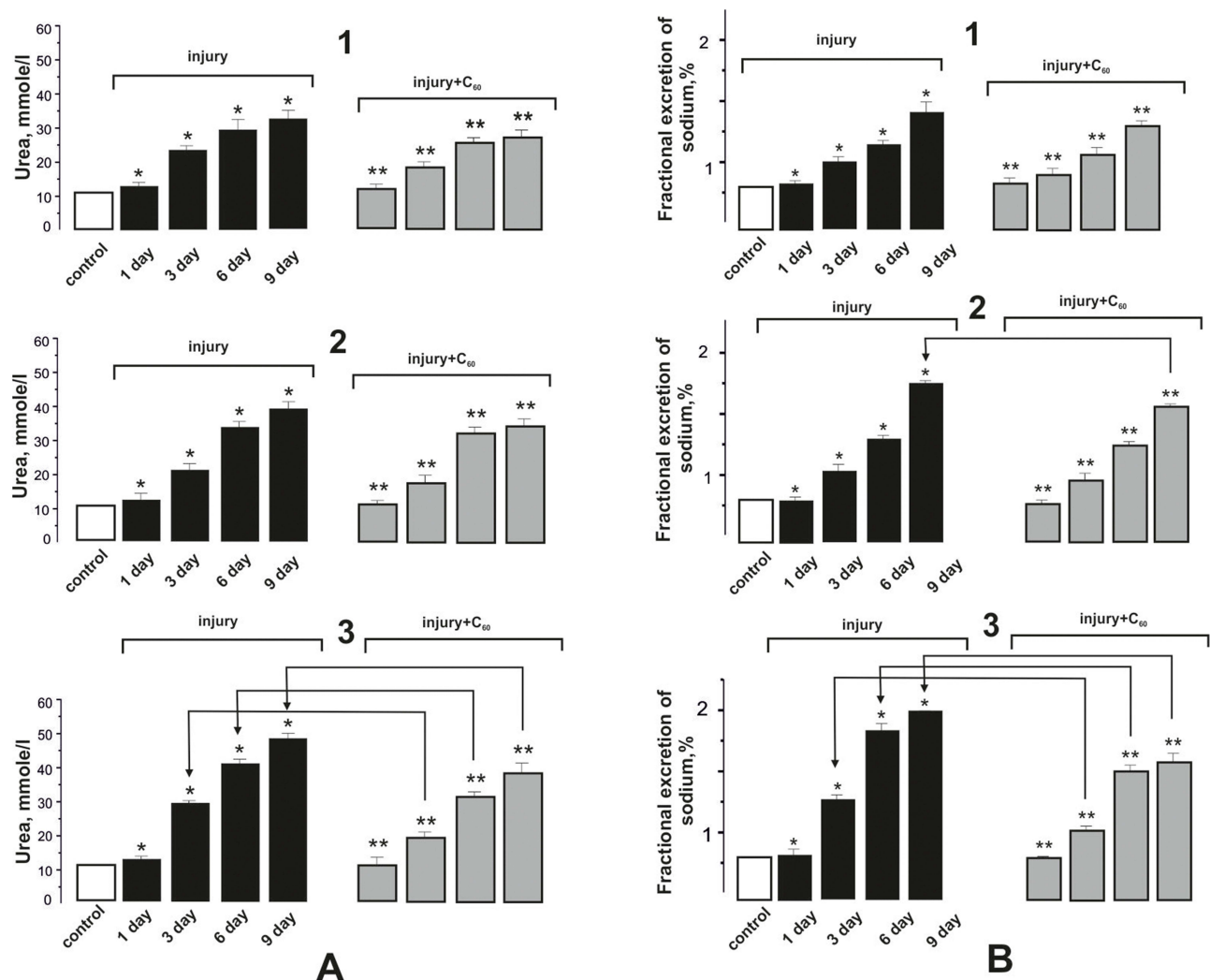


Figure 4 Blood urea (A) levels in rats and FE_{Na} (B) in rhabdomyolysis kidney injury: injury and injury+C₆₀ - indices after muscle injury initiation of 1, 2, and 3 degrees of severity on the 1st, 3rd, 6th, and 9th day of the experiment without C₆₀ fullerene aqueous solution administration and on the background of its injection, respectively; **P* < 0.05 relative to the control; ***P* < 0.05 relative to the injury group. Arrows indicate the most significant differences between injury and injury+C₆₀ groups.

experiment relative to the injury group. On the ninth day, the positive effect was insignificant due to the small level of CPK increase relative to the control during this period (Figure 5A).

Pathological inflammatory processes occurring immediately after injury are a source of ROS and contribute to the intensification of LPO processes.^{38,47} During the physiological recovery of muscle tissue, oxygen entering the tissues initiates xanthine and hypoxanthine oxidation by xanthine oxidase, which leads to the formation of a large amount of superoxide anion radical and hydrogen peroxide. In addition, inflammatory processes increase the expression of adhesive molecules on the endothelium. Activated neutrophils attracted to the focus of damage also release hydrogen peroxide and provoke pathological free-radical processes.

Throughout the experiment, an increase in the level of H₂O₂ was recorded, which is associated with the muscle fiber destruction and the development of inflammatory processes. The increase in H₂O₂ concentration on the first and third day was 24–28 ± 1%, 42–79 ± 2%, and 98–124 ± 3% at 1, 2, and 3 injury severity levels, respectively, relative to the control (Figure 5B). Further increases in H₂O₂ concentration of 10 ± 1%, 14 ± 1% and 19 ± 1% and 15 ± 1%, 26 ± 2% and 33 ± 2% on the sixth and ninth day of the experiment, respectively, indicated the development of rhabdomyolysis' ARF.

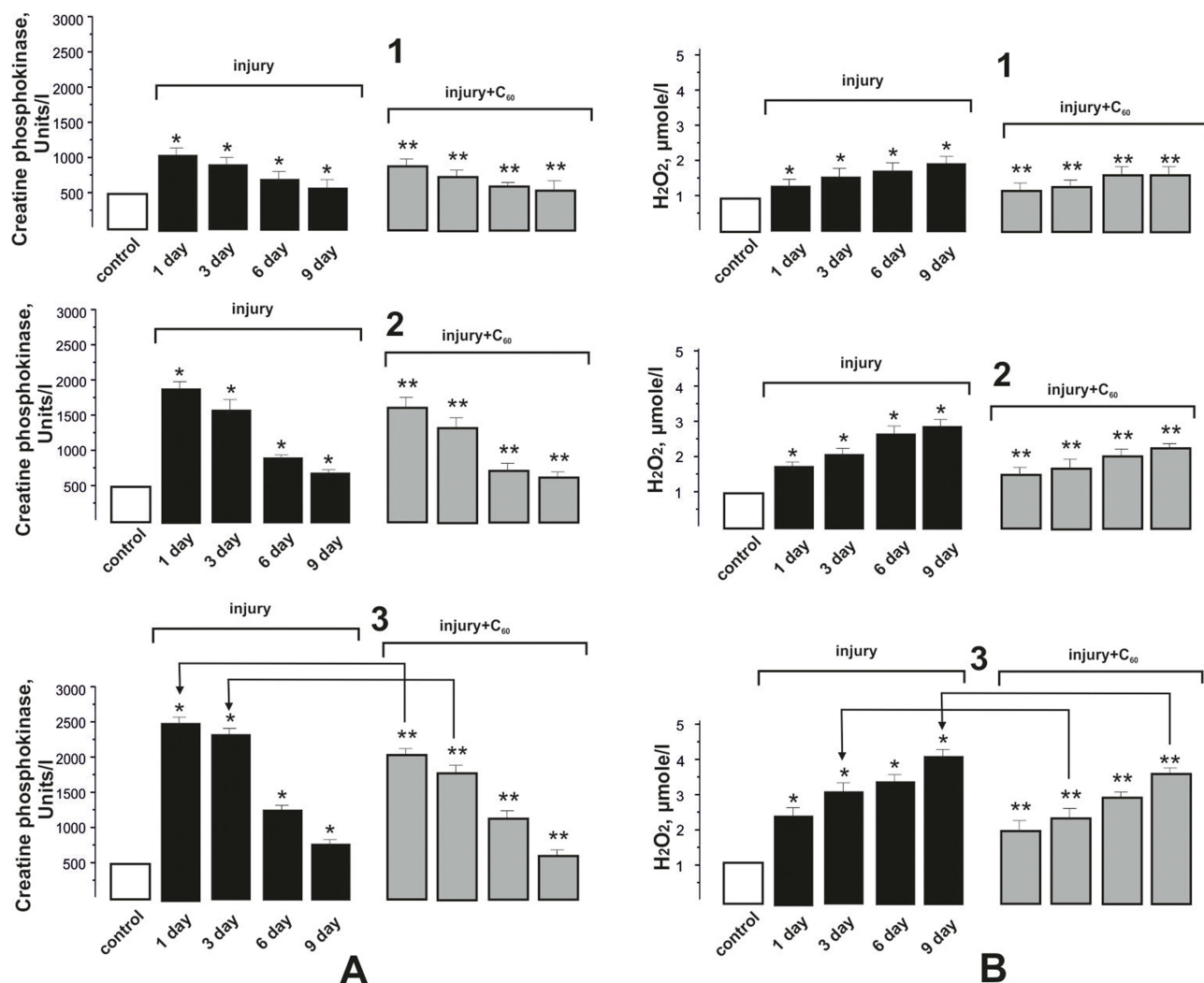


Figure 5 Levels of CPK (A) and H₂O₂ (B) in the blood of rats at rhabdomyolysis kidney injury: injury and injury + C₆₀ - indices after muscle injury initiation of 1, 2 and 3 degrees of severity on the 1st, 3rd, 6th and 9th day of the experiment without C₆₀ fullerene aqueous solution administration and on the background of its injection, respectively; *P < 0.05 relative to the control; **P < 0.05 relative to the injury group. Arrows indicate the most significant differences between injury and injury+C₆₀ groups.

At C₆₀ fullerene aqueous solution injections there is a decrease in H₂O₂ concentration by 11–12 ± 1%, 15–18 ± 1%, and 22–29 ± 1% at 1, 2, and 3 degrees of muscle injury severity, respectively, relative to the injury group during the experiment (Figure 5B).

The increase in H₂O₂ level during muscle fiber injury leads to an increase in CAT activity, which performs a protective antioxidant function by catalyzing the decomposition of hydrogen peroxide into water, oxygen and SOD. The most powerful natural antioxidant and enzyme of the first link of antioxidant defense is SOD, which performs the dismutation reaction of superoxide anion radicals and transforms them into less reactive hydrogen peroxide molecules.

SOD activity on the first day of the experiment increased by 25 ± 1%, 39 ± 4%, and 45 ± 2% at the 1st, 2nd, and 3rd degree of muscle injury severity, respectively, relative to the control (Figure 6A). On the third day, this index decreased by 11–14 ± 1%, which seems to be associated with a decrease in inflammatory processes in the muscle tissue. However, on the sixth and ninth day, SOD activity increased dramatically by 23–29 ± 1%, 39–41 ± 2%, and 56–59 ± 2% at injury severity grades 1, 2, and 3, respectively, compared to the third day of the experiment, which is most likely due to the development of ARF caused by rhabdomyolysis.

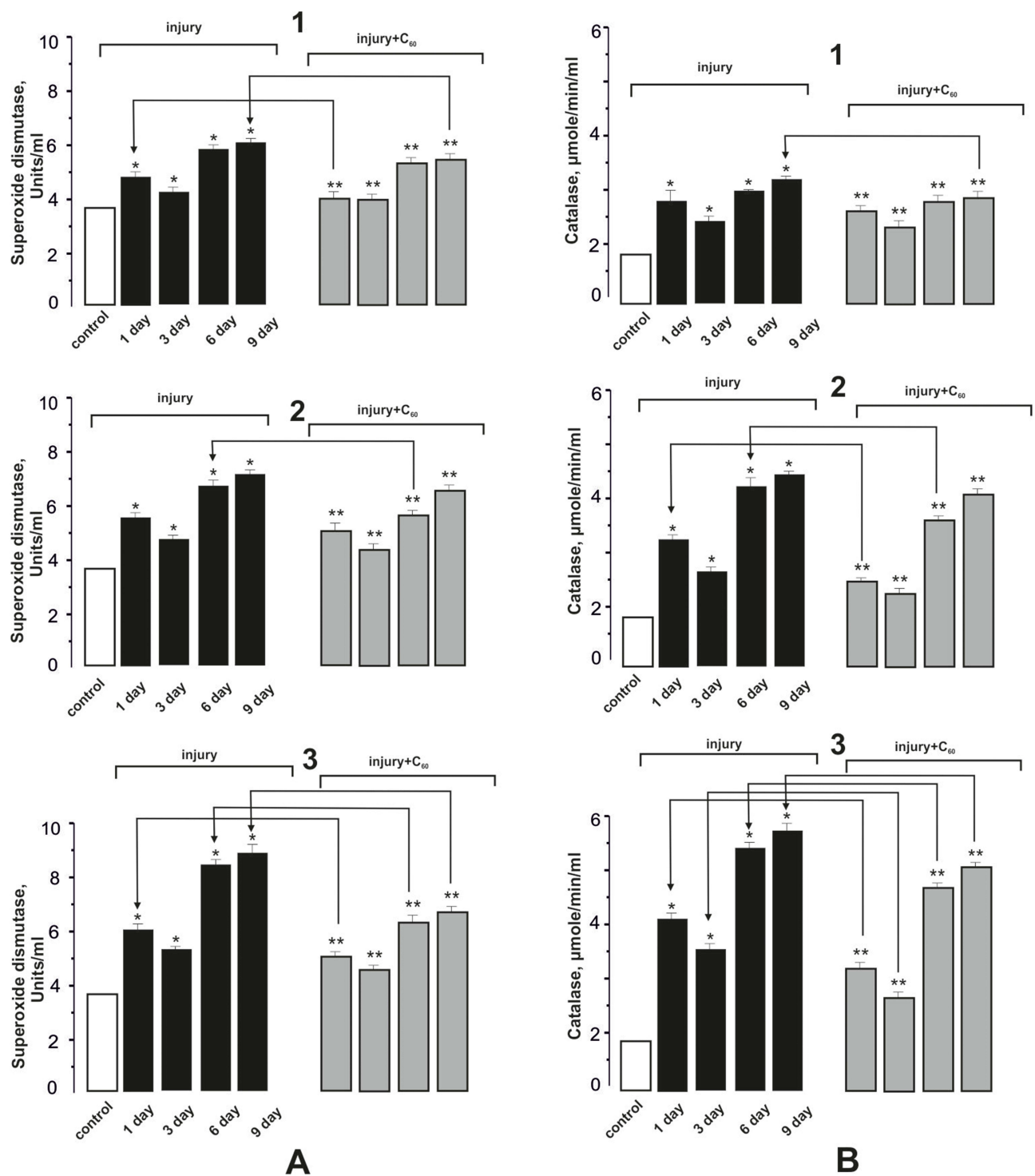


Figure 6 Activity of SOD (A) and CAT (B) in the blood of rats at rhabdomyolysis kidney injury: injury and injury + C₆₀ - indices after muscle injury initiation of 1, 2, and 3 degrees of severity on the 1st, 3rd, 6th, and 9th day of the experiment without C₆₀ fullerene aqueous solution administration and on the background of its injection, respectively; *P < 0.05 relative to the control; **P < 0.05 relative to the injury group. Arrows indicate the most significant differences between injury and injury+C₆₀ groups.

C₆₀ fullerene injections decreased SOD activity by $12 \pm 1\%$ and $15 \pm 1\%$ relative to the injury group on the first and third day of the experiment. On the sixth and ninth day, the positive effects of C₆₀ fullerene injections were $10 \pm 1\%$, $15 \pm 1\%$, and $29 \pm 2\%$ at 1, 2, and 3 degrees of muscle injury severity, respectively, relative to the injury group (Figure 6A).

CAT activity on the first day of the experiment increased by $111 \pm 6\%$, $123 \pm 6\%$, and $215 \pm 8\%$ at 1, 2, and 3 degrees of muscle injury severity, respectively, relative to the control (Figure 6B). On the third day, this index, as well as SOD activity, decreased by $15 \pm 1\%$, $27 \pm 2\%$, and $34 \pm 6\%$, which seems to be associated with a decrease in inflammatory processes in muscle tissue. On the sixth and ninth day, CAT activity increased sharply by $27\text{--}31 \pm 2\%$, $65\text{--}79 \pm 3\%$, and $81\text{--}94 \pm 3\%$ at 1, 2, and 3 injury severity levels, respectively, compared to the third day of the experiment.

The positive therapeutic effect of C_{60} fullerene aqueous solution injections on CAT activity was within the changes of SOD activity in all investigated time ranges and degrees of muscle injury (Figure 6A and B). So, C_{60} fullerene injections decreased CAT activity by $11 \pm 1\%$, $24 \pm 1\%$, and $33 \pm 2\%$ relative to the injury group on day one and by $10 \pm 1\%$, $11 \pm 1\%$, and $14 \pm 1\%$ on day three of the experiment. On the sixth and ninth day, the positive effects of C_{60} fullerene injections were $10 \pm 1\%$, $12 \pm 2\%$ and $16 \pm 2\%$ and $10 \pm 1\%$, $11 \pm 1\%$ and $14 \pm 1\%$ at 1, 2, and 3 degrees of muscle injury severity, respectively, relative to the injury group (Figure 6B).

Histopathological Analysis

Muscles

No deviations from the normal histological structure of the muscle were observed in the control group. Groups of muscle fibers are visible, separated by thin layers of connective tissue. Inside each muscle fiber, myofibrils are clearly visible (Figure 7A).

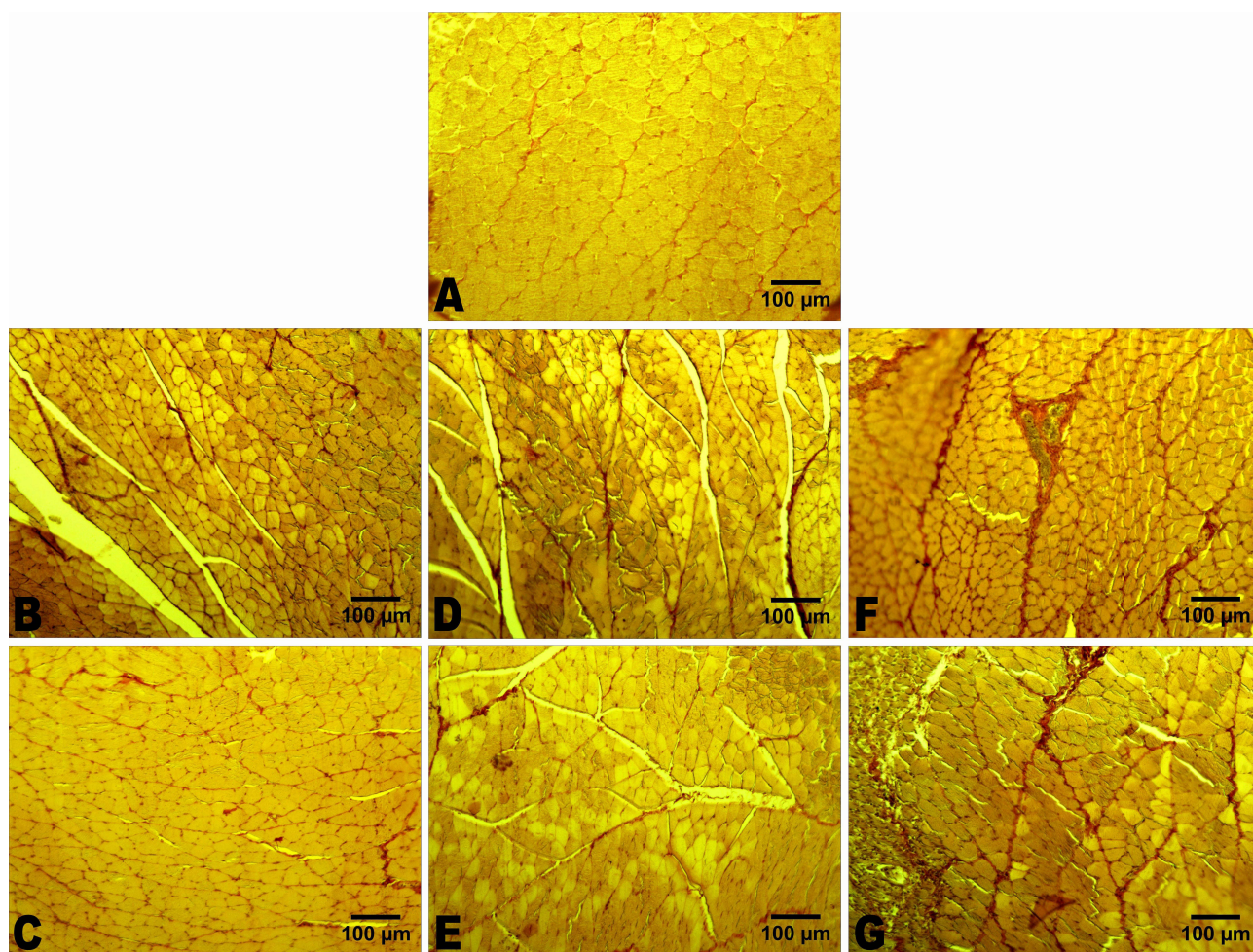


Figure 7 Representative histological images of the *muscle soleus*: (A) – control group; (B) – muscle injury (1st degree of severity); (C) – muscle injury (1st degree of severity) + C_{60} ; (D) – muscle injury (2nd degree of severity); (E) – muscle injury (2nd degree of severity) + C_{60} ; (F) – muscle injury (3rd degree of severity); (G) – muscle injury (3rd degree of severity) + C_{60} . Hematoxylin and picrofuchsin staining by van Gieson. Scale bars – 100 μ m.

In rats with muscle injury, muscle fibers are destroyed and connective tissue is heavily proliferated at the site of injury (Figure 7B, D and F). In other parts of the muscle, the normal histological structure of the muscle fibers is disturbed. Some of the fibers are hypochromatic, with the signs of edema. Some fibers are hyperchromatic, with the signs of destruction. The structure of myofibrils inside the muscle fibers is disturbed. There is a disturbance in the transverse striation of the fiber. Fibers may decrease in diameter. Connective tissue also grows: the area occupied by connective tissue increases in all study groups (Table 1). In cases of the 1st and the 2nd degrees of muscle injury severity (Figure 7B and D), the increase in connective tissue volume is mainly due to the matrix ground substance of connective tissue, and in the case of the 3rd degree (Figure 7F) – mainly due to the growth of collagen fibers. The severity of the above pathological and morphometric changes varies in different experimental groups (Table 1).

The area occupied by connective tissue in rats with the 1st degree of muscle injury severity is $80\text{--}110 \pm 7\%$ higher than in the control group. The diameter of muscle fibers is $16 \pm 2\%$ smaller compared to the control group in rats that did not receive water-soluble C₆₀ fullerenes but does not differ from the control values in rats that received them (Table 1).

In rats with the 2nd degree of muscle injury severity, which received water-soluble C₆₀ fullerenes, the area occupied by connective tissue is $20 \pm 2\%$ smaller and the diameter of muscle fibers is $30 \pm 2\%$ larger (and does not differ from the control) compared to rats that did not receive them (Table 1).

In rats with the 3rd degree of muscle injury severity, which received water-soluble C₆₀ fullerenes, the area occupied by connective tissue is $31 \pm 2\%$ smaller and the diameter of muscle fibers is $12 \pm 1\%$ larger compared to rats that did not receive them (Table 1).

Thus, in rats treated with water-soluble C₆₀ fullerenes, connective tissue does not grow so much, and the diameter of muscle fibers does not decrease (in cases of the 1st and 2nd severity of muscle injury), or decreases to a lesser extent (in case of the 3rd severity of muscle injury). The degree of destructive pathological changes in muscle tissue is also less (Figure 7C, E and G).

Kidney

In control rats, the kidneys have a normal histological structure (Figure 8A). In rats with muscle injury (Figure 8B, D and F), retraction of glomerular tuft is observed in part of the renal glomeruli. As a result, the cross-sectional area of the renal glomeruli decreases. In addition, in cases of 3rd degree of muscle injury severity (Figure 8F), a slight hemorrhage is observed in some renal glomeruli. The diameter of the renal tubes may decrease (Table 1).

Thus, noticeable histopathological changes in the kidneys are not observed after muscle injury. However, morphometric parameters may decrease (this is evidence of a decrease in the functional activity of the kidney). The severity of

Table 1 Morphometrical Features of Muscle and Kidney in Tested Rats

Groups	Morphometrical Features	Muscle		Kidney	
		Diameter of Muscle Fibers, μm	Area occupied by Connective Tissue, $\mu\text{m}^2/\mu\text{m}^2$	Cross-sectional Area of Renal Glomeruli, μm^2	Diameter of Renal Tubes, μm
Control		30.2 ± 1.0	0.10 ± 0.01	4848 ± 217	36.1 ± 1.3
muscle injury (1 st degree of severity)		$25.4 \pm 1.1^*$	$0.21 \pm 0.02^*$	$2723 \pm 149^*$	$32.2 \pm 1.1^*$
muscle injury (1 st degree of severity) + C ₆₀		$29.5 \pm 1.5^\wedge$	$0.18 \pm 0.01^\wedge$	$3681 \pm 205^\wedge$	$35.0 \pm 1.1^\wedge$
muscle injury (2 nd degree of severity)		$21.6 \pm 0.4^*$	$0.25 \pm 0.03^*$	$3853 \pm 163^*$	$32.8 \pm 1.0^*$
muscle injury (2 nd degree of severity) + C ₆₀		$28.0 \pm 0.9^\wedge$	$0.20 \pm 0.01^\wedge$	$3460 \pm 116^\wedge$	$34.3 \pm 1.0^\wedge$
muscle injury (3 rd degree of severity)		$23.2 \pm 0.6^*$	$0.22 \pm 0.02^*$	$3572 \pm 217^*$	$29.0 \pm 1.1^*$
muscle injury (3 rd degree of severity) + C ₆₀		$26.0 \pm 1.4^\wedge$	$0.15 \pm 0.01^\wedge$	$3629 \pm 228^\wedge$	$31.7 \pm 1.0^\wedge$

Notes: * $P < 0.05$ compared with the control group; $^\wedge P < 0.05$ compared with the muscle injury group at the same degree of severity.

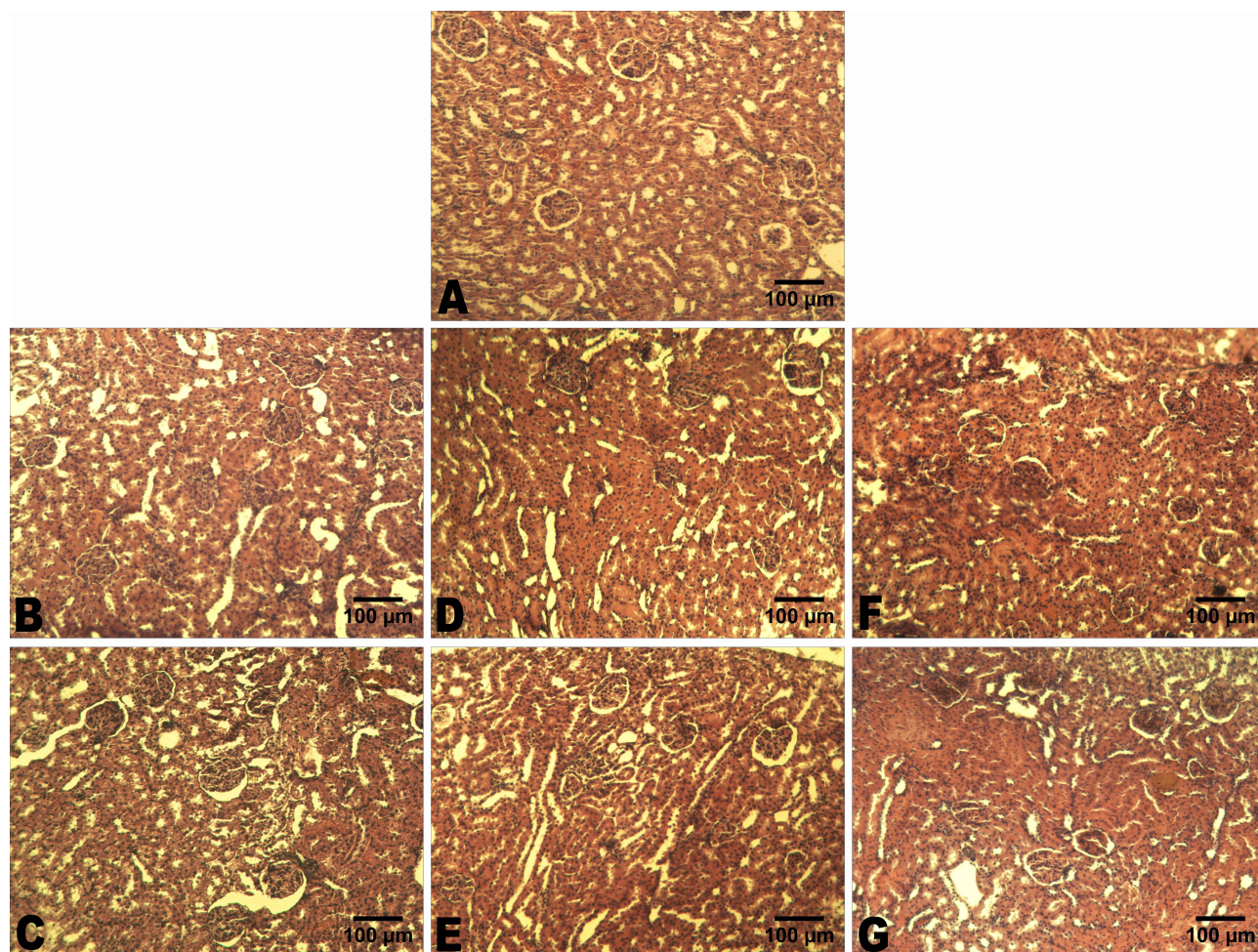


Figure 8 Representative histological images of the kidney: (A) – control group; (B) – muscle injury (1st degree of severity); (C) – muscle injury (1st degree of severity) + C₆₀; (D) – muscle injury (2nd degree of severity); (E) – muscle injury (2nd degree of severity) + C₆₀; (F) – muscle injury (3rd degree of severity); (G) – muscle injury (3rd degree of severity) + C₆₀. H&E staining. Scale bars – 100 µm.

these changes varies in the different experimental groups. For example, the diameter of renal tubules in rats with 3rd degree of muscle injury severity, which received water-soluble C₆₀ fullerenes, is bigger by $10 \pm 1\%$ compared to rats with injury (Table 1; Figure 8G), which did not receive them. In rats with 1st and 2nd degrees of muscle injury severity, which received water-soluble C₆₀ fullerenes, the diameter of renal tubules did not differ from the control values (Table 1; Figure 8C and E).

Summarizing, the above-described positive effects of exposure to C₆₀ fullerenes on the ARF caused by rhabdomyolysis are related precisely to their powerful antioxidant properties.^{25,26} C₆₀ molecules effectively inactivate the ROS, protecting nephron membranes from oxidation, and, thus, reduce the severity of ARF.

Conclusion

Thus, during the experiment, there is a positive change of the studied biomechanical and biochemical parameters by about $27\text{--}30 \pm 2\%$ with C₆₀ fullerene aqueous solution administration at a low therapeutic dosage. Besides, they were confirmed by the data of histopathological analysis. This indicates that there is compensatory activation by C₆₀ fullerenes of the endogenous antioxidant system of the organism in the process of posttraumatic changes of the *muscle soleus* caused by mechanical damage of different degrees of severity. Thus, C₆₀ fullerenes, as powerful antioxidants, reduce the development of both rhabdomyolysis and ARF induced by it. We can also assume the therapeutic effect of C₆₀ fullerenes on the acceleration of the process of repair of muscle structures damaged by rhabdomyolysis, which is confirmed by the

previously obtained data on their efficient action on the functions of the antioxidant system of the organism at various pathological processes.^{22,24,25}

Based on the obtained results, it can be suggested that the use of the aqueous solution of C₆₀ fullerene as a therapeutic agent is able to correct pathological conditions of the muscular and excretory system arising from skeletal muscle injuries.

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Disclosure

The authors declare no conflicts of interest in this work.

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