

C₆₀ FULLERENE RESTORES GASTROCNEMIUS CONTRACTILE ACTIVITY IN A RAT MODEL OF NEUROGENIC MUSCLE ATROPHY

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Restoration of the motor function of the musculoskeletal system impaired due to innervation damage is an important clinical problem. In the study the potential therapeutic effect of C₆₀ fullerene application was estimated in the Wistar rat model of neurogenic muscle atrophy caused by nervus ischiadicus injury. The animals were divided into the following: control, injury, injury+C₆₀ groups. C₆₀ fullerene aqueous solution was administrated orally for 30 days after ischiadicus injury at a daily dose of 1 mg/kg. Biomechanical parameters of gastrocnemius muscle contraction and biochemical indices (creatinine, lactate, reduced glutathione content as well as creatine phosphokinase, lactate dehydrogenase, catalase and superoxide dismutase activity) in the blood of rats were estimated on day 30 after nerve transection. It has been found that muscle strength response in the injury+C₆₀ group was significantly enhanced, in particular, the muscle force impulse was increased by more than 30 ± 2% compared to the injury group. The studied biochemical indices of the muscle fatigue and oxidative stress in the blood of experimental animals had a pronounced tendency to increase after the initiation of the muscle neurogenic atrophy, while under the influence of C₆₀ fullerene they decreased compared with the injury group. In our opinion, C₆₀ fullerene prevented significant dysfunction of the gastrocnemius muscle after neurogenic atrophy by exerting an antioxidant effect and improving its contractile activity.

Key words: C₆₀ fullerene, muscle gastrocnemius, neurogenic atrophy, muscle contraction, biochemical indicators.

Impaired regeneration of damaged nerves is a significant clinical problem [1], primarily associated with a delay in the restoration of motor function of the musculoskeletal system [2]. This physiological process is slower than sensory recovery due to the proliferation of sensory axons, which interferes with the growth of motor axons [3]. Peripheral nerves undergo complex pathological changes (Wallerian degeneration) after nerve injury, during which distal axons degenerate and Schwann cells proliferate, restoring axonal growth and remyelination [4]. It should be noted that nerve repair (even 30 days after injury) causes a decrease in the amount of activating transcription factor (ATF3) in the nerve [5], which slows down the processes of

adequate transmission of downward motor neuron activity.

Denervation of skeletal muscle leads to a rapid loss of muscle size and functional activity – muscle atrophy. The molecular mechanisms that control the imbalance between the synthesis and degradation of muscle proteins during atrophy remain controversial [6]. For example, studies [7] have linked this process to muscle cell apoptosis. Denervated skeletal muscle atrophies in a two-stage time course: rapid loss of muscle mass during the first two weeks and its gradual decrease over the next two weeks [8]. This is followed by a long-term muscle recovery process. This duration depends on many factors, one of which is inflammation [9]. Therefore, anti-inflam-

matory therapy is often used to effectively reduce its development [4, 6, 10].

One of the most powerful artificial antioxidants is the carbon nanoparticle of C_{60} fullerene [11, 12]. The C_{60} molecule is characterized by a high regenerative capacity, which makes it an effective free radical scavenger in biological systems. In previous experiments, it was shown that the use of water-soluble C_{60} fullerenes at low doses helps to recover muscle contraction dynamics and physiological state after ischemic injury [13], fatigue [14], mechanical trauma [15], and atrophy caused by prolonged immobilization [16] of rat skeletal muscle. Thus, the aim of this study was to evaluate the effect of water-soluble C_{60} fullerenes on the restoration of rat muscle *gastrocnemius* contraction, in particular, muscle force impulse value, and blood biochemical indicators, namely concentrations of creatinine, creatine phosphokinase (CPK), lactate (LA), lactate dehydrogenase (LDH) and reduced glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) activities, after neurogenic atrophy caused by sciatic nerve injury.

Materials and Methods

To obtain C_{60} fullerene aqueous solution (C_{60} FAS), we used a method based on the transfer of C_{60} molecules from toluene to water, followed by sonication [17]. The resulting C_{60} FAS at a maximum concentration of 0.15 mg/ml remains stable for 12-18 months at a temperature of +4-25°C. It is a typical nanocolloid containing both single C_{60} molecules as well as their nanoaggregates [18].

The experiments were performed on male Wistar rats aged 1 to 2 months (at the end of the experiment) weighing $110-170 \pm 5$ g. The animals were kept in an air-filtered and temperature-controlled ($21 \pm 1^\circ\text{C}$) 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 ($n = 7$); injury group ($n = 7$); 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; $n = 7$).

The choice of the above dose of C_{60} FAS is based on its high effectiveness in the treatment of various muscle pathologies *in vivo* [13-16]. In addition, the total dose of 30 mg/kg in the experiment is significantly lower than the LD_{50} value, which was 600 mg/kg in the case of oral administration to rats [19] and is, therefore, safe for biotesting.

Animals were anesthetized by intraperitoneal injection of Zoletil (40 mg/kg). The sciatic nerve (*nervus ischiadicus*) of the experimental animals in the left hindlimb was exposed and cut 10 mm proximal to the tibial and peroneal nerve branches (injury model [20]). To study the effect of nerve repair on muscle atrophy, rats were re-anesthetized, and biomechanical studies of muscle *gastrocnemius* activity were performed on day 30 after nerve transection. It should be noted that such studies are based on the fact that for up to 30 days, the processes of atrophic degeneration do not occur in the muscle [21].

Muscle *gastrocnemius* was isolated from surrounding tissues in the area of the hamstring fossa. All branches of the muscle, except for those innervating it, 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.

A 12-bit analog-to-digital and digital-to-analog converter (ADC-DAC) was used to record electrophysiological signals. The output pulses of the DAC were formed by a pulse generator (DS2A, Digitimer, USA), which stimulated the nerves (the electrical pulses with a duration of 2 ms, a frequency of 50 Hz and a voltage of 7 V). The input signals were fed to the ADC through an amplifier (Brownlee, USA) and recorded at 10 kHz. The force of contraction of the muscle *gastrocnemius* was recorded using strain gauges to which the tendon of the muscle under study was attached. The output signals of muscle contraction force were optimized using an Aurora Scientific ASI 402A (USA) amplifier. During the operation and the experiment, the heart rate and ECG amplitude of the animals were monitored.

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. 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 [13-16].

The blood plasma concentrations of creatinine, CPK, LA, LDH and GSH, CAT and SOD activities were determined in the experimental animals on the 30th day after neurogenic atrophy of the *muscle gastrocnemius* caused by sciatic nerve injury, as markers of muscle damage [22], 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 to be: 1) injury; 2) C₆₀FAS treatment (two levels – no and use of C₆₀FAS). 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 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 the software package Statistica 8.0 (Dell, USA).

Results and Discussion

On the 30th day after the nervus ischiadicus injury, a decrease in the force of contraction of the experimental skeletal muscle caused by neurogenic atrophy was recorded (Fig. 1, a). Thus, the muscle force impulse was $79 \pm 4\%$ and $29 \pm 1\%$ at the 1st and 20th contractions, respectively, compared to the control (Fig. 1, b). This confirms the literature data that neurogenic skeletal muscle atrophy is characterized by a decrease in muscle strength, increased fatigue and reduced muscle exercise capacity [8, 9, 14, 23]. The use of C₆₀FAS increased the strength response of the muscle gastrocnemius: the muscle force impulse was $92 \pm 5\%$ and $61 \pm 3\%$ at the 1st and 20th contractions, respectively, compared to the control (Fig. 1, b). Thus, the positive effect of C₆₀FAS treatment was $13 \pm 1\%$ and $32 \pm 2\%$ at the 1st and 20th contractions, respectively, indicating a significant reduction in the severity of neurogenic atrophy of the muscle gastrocnemius during contractile activity.

It is important to note that with the development of achillotenotomy, the magnitude of the muscle force impulse changes significantly during 15-20 non-relaxation contractions [16]. In the future, with an increase in the number of muscle contractions, this parameter changes quite slowly.

Changes in the chemical composition of the blood of experimental animals during prolonged non-relaxation contractions of atrophic skeletal muscle reflect biochemical disorders that occur as a

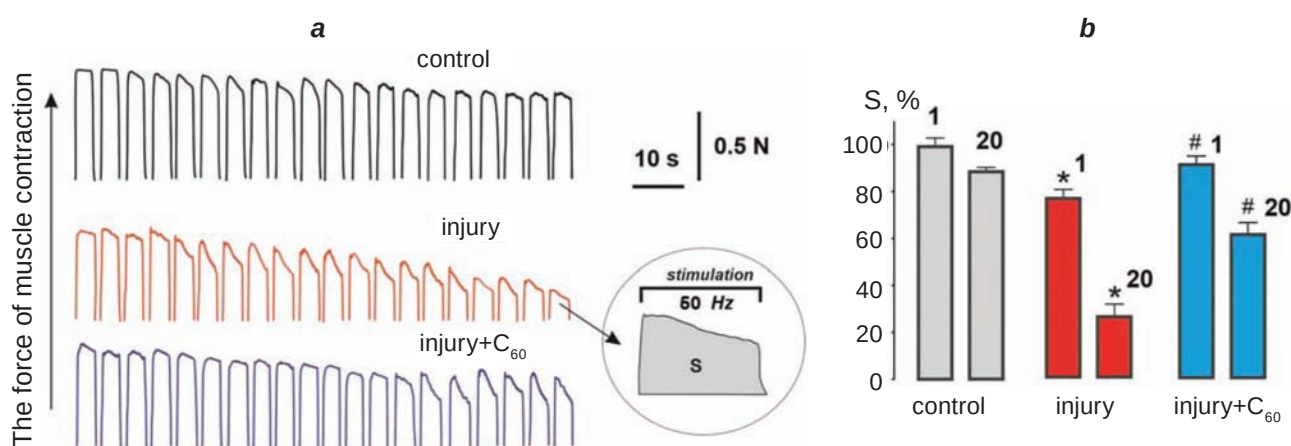


Fig. 1. Mechanograms of 20 consecutive contractions of the rat muscle gastrocnemius elicited by 6 s of non-relaxation stimulation pools in control (uninjured nerve), 30 days after nervus ischiadicus transection (injury) and after C₆₀FAS administration (injury+C₆₀; daily dose 1 mg/kg) (a). Calculated muscle force impulse at the 1st and 20th contractions of the muscle gastrocnemius (relative to the control, which was taken as 100%) (b). * $P < 0.05$ relative to the control group; # $P < 0.05$ relative to the injury group

result of the development of fatigue processes in it. Our studies have shown (Fig. 2) that the biochemical markers of physiological disorders of muscle tissue selected by us [24] have a pronounced tendency to increase after the initiation of neurogenic atrophy of the muscle gastrocnemius. This indicates that the muscular system performs work that is too intense for its physiological level, followed by the development of muscle fatigue.

The concentration of creatinine, a product formed as a result of damage to intramuscular structures during intense contractions, increased from $53 \pm 1 \mu\text{M}$ in the control group to $152 \pm 5 \mu\text{M}$ in the injury group. The use of C_{60} FAS reduced its concentration to $101 \pm 4 \mu\text{M}$ (Fig. 2). Thus, the positive effect of C_{60} FAS was $34 \pm 2\%$. In our opinion, this is due to the antioxidant effect of C_{60} fullerenes in the early stages of the pathological process by protecting myocyte membranes from nonspecific free radical damage.

During active contraction of skeletal muscle, there is a significant depletion of cellular energy substances, especially ATP, which leads to a sharp disturbance of homeostasis, loss of the ionic gradient through cell membranes and, accordingly, the accumulation of LA. A decrease in ATP production inhibits the activity of Na^+ , K^+ -ATPase, which leads to an increase in the concentration of intracellular Na^+ and an increased content of K^+ ions and causes a delay in the generation of the action potential [25]. Thus, ionic changes impair the ability of muscles to respond to electrical impulses, impede the development of excitation, and cause a decrease in muscle strength. Therefore, LA concentration is an important marker for assessing the performance of atrophic muscle.

LA concentration increased from $7.9 \pm 0.6 \text{ mM}$ in the control to $13.5 \pm 1 \text{ mM}$ in the injury group. The use of C_{60} FAS reduced its concentration to $11.3 \pm 1 \text{ mM}$ (Fig. 2). Thus, the positive effect of

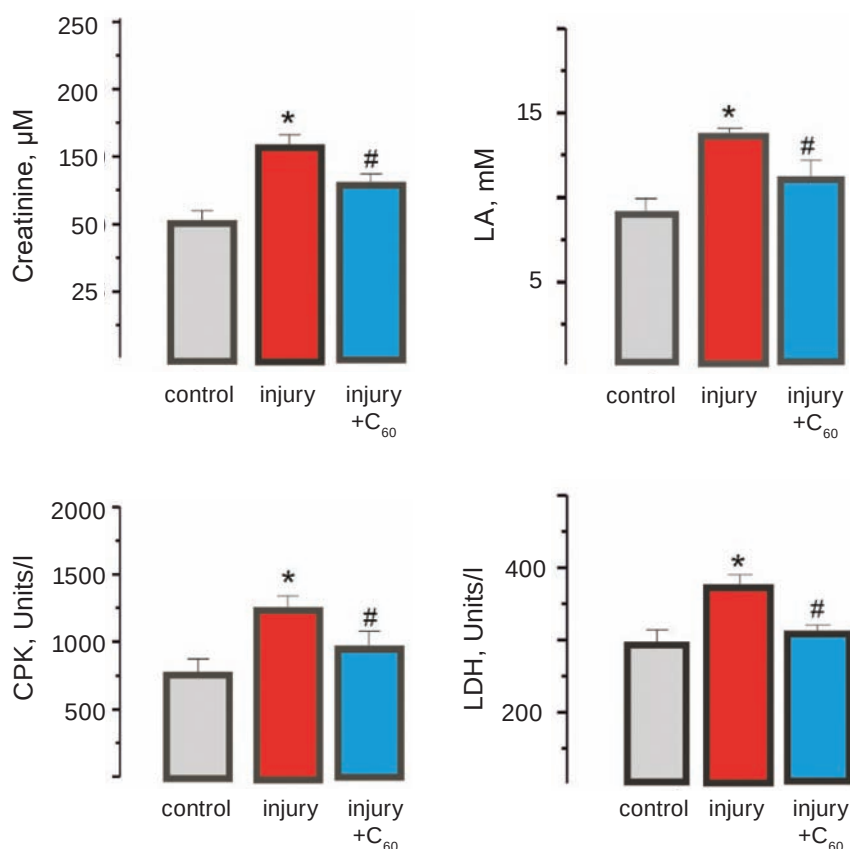


Fig. 2. Blood plasma concentrations of creatinine, LA, CPK and LDH in rats after initiation of neurogenic atrophy of the muscle gastrocnemius: control, injury and injury+ C_{60} – control group, 30 days after nervus ischiadicus transection (injury) and after the administration of C_{60} FAS (injury+ C_{60} ; daily dose 1 mg/kg). * $P < 0.05$ compared to the control group; # $P < 0.05$ compared to the injury group

C₆₀FAS administration was $16 \pm 1\%$, which indicates the ability of C₆₀ fullerenes to restore LA levels in an active muscle.

One of the main markers of muscle fatigue is a change in the concentration of CPK, an enzyme that catalyzes the transfer of a phosphate group from ATP to the creatine molecule to form creatine phosphate. During intensive muscle function, this enzyme is released from the cells into the extracellular space due to atrophic changes in myocytes and incomplete recovery from nerve injury, and, accordingly, there is an increase in the level of CPK in the blood.

CPK concentration increased from 758 ± 28 Units/l in the control to 1252 ± 36 Units/l in the injury group. The use of C₆₀FAS reduced its concentration to 929 ± 21 Units/l (Fig. 2). Thus, the positive effect of C₆₀FAS was $26 \pm 1\%$, which is evidence of the direct protective effect of the drug.

The concentration of LDH, an enzyme that catalyzes LA oxidation, allows us to assess the condition of atrophic muscle after prolonged activation.

The increase in LDH concentration from 285 ± 11 Units/l in the control group to 342 ± 15 Units/l in the injury group is evidence of the development of significant dysfunctions in the neuromuscular system. The use of C₆₀FAS reduced its concentration to 292 ± 8 Units/l (Fig. 2). Thus, the positive effect of C₆₀FAS administration was $15 \pm 1\%$. This is evidence that C₆₀ fullerenes are able to increase the energy capacity of a functioning muscle both by reducing mechanical damage to muscle fibers and the concentration of LA in the muscle system as a whole.

During the development of muscle atrophy, changes in the pro- and antioxidant balance in the

blood of experimental animals determine the level of physiological disorders of myocytes. An increase in oxidative stress is a pathophysiological feature of neurogenic atrophy associated, in particular, with dysfunction of the ubiquitin-proteasome, autophagic lysosomes, and mTOR systems [26].

SOD activity increased from 1.8 ± 0.1 Units/ml in the control to 3.1 ± 0.2 Units/ml in the injury group. The use of C₆₀FAS reduced its value to 2.1 ± 0.1 Units/ml (Fig. 3). Thus, the positive effect of C₆₀FAS was $32 \pm 2\%$.

The activity of CAT increased from 0.70 ± 0.06 mM/min in the control to 1.8 ± 0.1 mM/min in the injury group. The use of C₆₀FAS reduced its value to 1.1 ± 0.1 mM/min (Fig. 3). Thus, the positive effect of C₆₀FAS was $39 \pm 2\%$.

GSH concentration increased from 2.2 ± 0.1 μ M in the control to 2.6 ± 0.1 μ M in the injury group. The use of C₆₀FAS reduced its value to 2.3 ± 0.1 μ M (Fig. 3). Thus, the positive effect of C₆₀FAS was $12 \pm 1\%$.

Thus, reducing the level of oxidative stress with the use of antioxidant therapy during denervation leads to a decrease in the effects of atrophic degeneration of muscle cells, which is in good agreement with the data [9].

Conclusions. It has been shown that the use of water-soluble C₆₀ fullerenes at a daily oral dose of 1 mg/kg during the 30-day process of recovering the functional activity of the rat muscle gastrocnemius after neurogenic atrophy caused by sciatic nerve injury significantly increased its strength response, in particular, the muscle force impulse increased by more than $30 \pm 2\%$ compared to the injury group. At the same time, the biochemical indicators of the

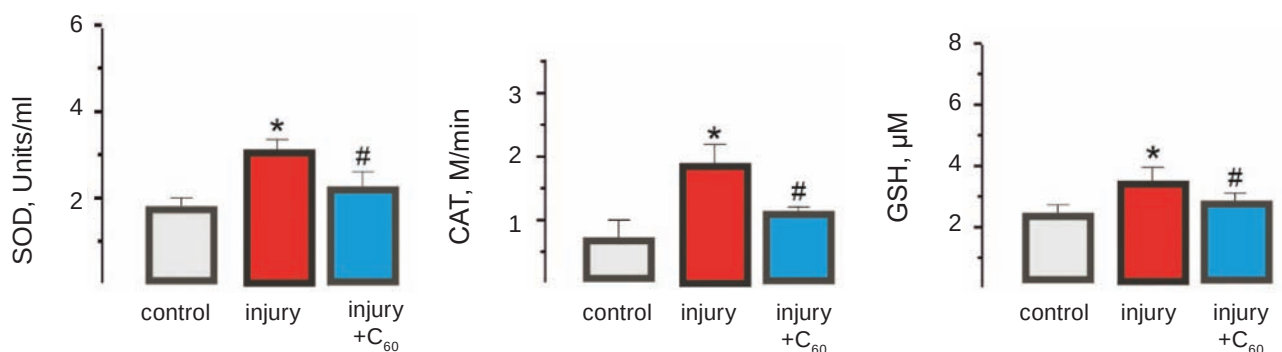


Fig. 3. Indicators of pro- and antioxidant balance (SOD, CAT and GSH) in the blood plasma of rats after initiation of neurogenic atrophy of the muscle gastrocnemius: control, injury and injury+C₆₀ – control group, 30 days after nervus ischiadicus transection (injury) and after administration of C₆₀FAS (injury+C₆₀; daily dose 1 mg/kg). * $P < 0.05$ compared to the control group; # $P < 0.05$ compared to the injury group

blood of experimental animals had a pronounced tendency to increase after the initiation of neurogenic atrophy of the muscle gastrocnemius and to decrease by $12-39 \pm 2\%$ compared with the injury group under the influence of water-soluble C_{60} fullerenes. In our opinion, C_{60} fullerenes, by affecting the activity of endogenous antioxidants, prevent the occurrence of significant dysfunction in the active muscle of rats and improve its contractile activity after neurogenic atrophy.

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

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C_{60} ФУЛЕРЕН ВІДНОВЛЮЄ СКОРОЧУВАЛЬНУ АКТИВНІСТЬ GASTROCNEMIUS У МОДЕЛІ НЕЙРОГЕННОЇ АТРОФІЇ М'ЯЗІВ ЩУРІВ

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Відновлення рухової функції скелетно-м'язової системи після іннервації є важливою клінічною проблемою. У цій роботі потенційний терапевтичний ефект C_{60} фулерену оцінювали на моделі нейрогенної атрофії м'язів щурів лінії Wistar, спричиненої пошкодженням сідничного нерва. Тварини були розподілені на такі групи: контроль, травма, травма+ C_{60} . Водний розчин C_{60} фулерену застосовували перорально упродовж 30 днів після пошкодження сідничного нерва у добовій дозі 1 мг/кг. Біомеханічні параметри скорочення *muscle gastrocnemius* та біохімічні показники (вміст креатиніну, лактату, відновленого глутатіону, а також активність креатинфосфокінази, лактатдегідрогенази, каталази та супероксиддисмутази) у крові щурів

оцінювали на 30 добу після пошкодження нерва. Встановлено, що силовий відгук м'яза у групі травма+ C_{60} значно підвищувався, зокрема імпульс сили м'яза збільшувався більш ніж на $30 \pm 2\%$ порівняно з групою травма. Біохімічні показники м'язової втоми та оксидативного стресу в крові піддослідних тварин мали виражену тенденцію до підвищення після ініціації нейрогенної атрофії м'язів, тоді як за дії C_{60} фулерену вони знижувалися порівняно з групою травма. На нашу думку, C_{60} фулерен запобігає значній дисфункції *muscle gastrocnemius* після нейрогенної атрофії, покращуючи його скорочувальну активність завдяки своїй антиоксидантній дії.

Ключові слова: C_{60} фулерен, *muscle gastrocnemius*, нейрогенна атрофія, скорочення м'яза, біохімічні показники.

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