# HISTOMORPHOMETRIC STUDY OF THE SOLEUS MUSCLE UNDER CONDITIONS OF MODELING OF SPINAL CORD CONTUSION INJURY: EXPERIMENTAL MORPHOLOGICAL STUDY

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**Objective.** To conduct a morphometric analysis of the soleus muscle of rats after moderate spinal cord contusion injury.
**Material and Methods.** Experiments were performed on female Wistar rats aged 8–12 months, weighing 270–320 g. Animals of the experimental group (n = 25) underwent laminectomy at the T9 level under general anesthesia and modeling of spinal contusion injury of moderate severity. Intact rats constituted the control group (n = 10). Euthanasia was performed on the 5th, 15th, 30th, 60th, 90th, and 180th days of the experiment. Paraffin sections were stained with hematoxylin-eosin and Masson, the diameters of muscle fibers were determined by computer morphometry, and histograms of their distribution were obtained.
**Results.** In the soleus muscle, the signs of reversible reparative processes prevailed in response to neurotrophic damage. It was evidenced by a local increase in the diversity of myocyte diameters and the loss of polygonality of their profiles, focal destruction of muscle fibers, activation of the connective tissue component, disorganization of some intramuscular nerve conductors, and vascular fibrosis of perimysium. Nevertheless, the histostructure of an intact muscle prevailed in the course of the experiment, which was confirmed by the data of morphometric analysis. All histograms of the distribution of the muscle fiber diameters are unimodal with a mode in the range of 30–41 μm. On the 180th day, the maximum myocyte diameters in the histogram of the left limb muscle belonged to the range of 21–30 μm, which was typical for histograms in the intact group.
**Conclusion.** The nature of the plastic reorganization of the soleus muscle when neurotrophic control is impaired indicates compensatory regeneration of muscle tissue by the type of restitution, which opens up the possibility of predicting the rehabilitation period. It is advisable to take this into account when developing medical and social programs and therapeutic measures, where the most important role is played by superficial neuromuscular and functional electrical stimulation.
**Key Words:** spinal injury, soleus muscle, histomorphometry, restitution.
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It is a spinal injury that usually causes serious consequences for the neuromuscular system and often leads to cardiometabolic outcomes [1]. An in vivo study of the effect of spinal cord injury on the bioenergetics of the rats' hindlimb muscles by phosphorus magnetic resonance spectroscopy detected an acute oxidative metabolic defect in the paralyzed hindlimb muscle. This could promote the onset of motor dysfunctions observed after injury [2]. In patients with traumatic spinal cord injury, changes in the musculoskeletal system (skeletal muscle atrophy, increased sclerotization, adipose degeneration) are directly related to motor dysfunctions as a result of spinal cord injury [3]. The risk of heterotopic ossification in the muscles is 16–53 %. The main reason for this is severe neurotrophic disorders of metabolic processes in denervated tissues [4]. One third of the patients have ossifications of the 3rd-4th degree. The greatest barrier in mobilization of paralyzed limbs is ossifications in the joint area [5].

In experimental models, various types of spinal cord injuries are analyzed with an evaluation of their advantages and disadvantages to create an ideal model. This is essential for the development of treatment techniques in people with such injuries [6]. The effect of targeted stereotactic radiosurgery of the injured spinal cord was studied in rats. Locomotor function was assessed within 6 weeks after contusion injury using the Basso, Beattie, Bresnahan (BBB) locomotor rating scale [7]. Its indicators were considerably higher in the stereotactic radiosurgery group [8]. In guinea pigs, spinal cord compression injury is described under the concept of compression with a given force. The functional result correlated significantly with the survival rate of axons [9]. In models of spinal cord contusion injury, the gastrocnemius muscle [10], as well as the soleus and tibial muscles of rats were studied [11]. However, such studies are few and contradictory; additional research is required.

The objective is to conduct a histological and morphometric analysis of the soleus muscle of rats after moderate spinal cord contusion injury.

## Material and Methods

Experiments were performed on 35 female Wistar rats aged 8–12 months, weighing 270–320 g. Experimental group: n = 25, control group: n = 10 (intact animals). Experiments and maintenance of animals were conducted in accordance with the Rules for Conducting Research Using Experimental Animals (annex to the Order of the Ministry of Health of the USSR as of August 12, 1977 No. 755) and the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986). The research was approved by the Ethics Committee of the National Ilizarov Medical Research Center for Traumatology and Orthopedics (Protocol No. 2 (57) as of May 17, 2018). Rats under general anesthesia (Rometar 2 % – 1–2 mg/kg, Bioveta, Czech Republic; Zoletil 100 – 10–15 mg/kg, Virbak Sante Animale, France) underwent laminectomy at the T9 level. The level of laminectomy was monitored radiographically (Premium Vet, Sedecal, Spain) in a lateral projection. A moderate spinal cord contusion injury was simulated without damage to the dura mater by means of an original impact device: a cylindrical load with a diameter of 1.8 mm, weighing 10 g, falling free from a height of 25 mm (registration of the application on March 10, 2021, registration No. 2021106172) [12, 13]. A complete closure of the surgical wound was made layer by layer. In the early postoperative period, animals were additionally heated and dehydration was prevented; antibiotic therapy and hemostatic therapy were administered [14, 15].

The animals were removed from the experiment by partial decapitation on 5th, 15th, 30th, 60th, 90th, and 180th day. After euthanasia, the heel cord of the triceps surae was cut off from the calcaneal tubercle; the soleus muscle of both limbs was dissected; it was fixed in 10 % neutral formalin. Paraffin sections were stained with hematoxylin-eosin and Masson's Trichrome Stain. They were examined using AxioScope.A1 stereoscope and AxioCam digital camera (Carl Zeiss MicroImaging GmbH, Germany). In the VideoTest-Master software (Russia), computer histomorphometry was performed; the average diameter of muscle fibers was defined (from 700 to 2,000 values during the experiment).

*Statistical analysis.* Quantitative data were analyzed using nonparametric statistics methods in the AtteStat version 12.5 embedded in Microsoft Excel. Distribution histograms of myocytes by diameter were obtained by ranking the samples at a pitch of 10.0 μm [16]. The significance of differences between the intact and experimental groups, the muscles on the left and right, in the dynamics of the experiment was defined under the Wilcoxon signed-rank test; the differences were considered significant at $p < 0.05$.

## Results

In the intact group, the muscles were characterized by polygonal profiles of myosymplasts, minimal layers of endo- and perimysium, preserved intramuscular nerve trunks and neuromuscular spindles, vessels of the arterial bed in the perimysium with open lumen, without fibrosis of the middle and outer membranes (Fig. 1a).

On the 5th day in the experimental group of animals, normal structure prevailed in the muscles, neural conductors were without pathology, as well as perimysium vessels.

On 15th day, the histological picture of the left limb muscle was marked by the presence of fibers, the profiles of which lost their polygonality; the proportion of interstitial tissue with fibroblast cells increased (Fig. 1b). Contractures, signs of myophagy (Fig. 1c), internal nuclei were observed in single fibers; some intramuscular nerve trunks showed signs of reactive and destructive changes, such as disorganization of nerve fibers, their deformation, fibrosis of the space.

After a month of the experiment, an insignificant increase in endo- and perimysium layers was visualized in the muscles; muscle fibers, as a rule, were within normal; there were spasms in some vessels of the arterial bed; the tunica media had signs of fibrosis.

On the 90th day, myocytes, neaural conductors, perimysium vessels and neuromuscular spindles were without signs of pathology.

After 1.5 years, the muscles were characterized by minimal layers of endomysium and very few hypertrophied myocytes. In some intramuscular neural conductors, there were signs of fibrosis of the space between nerve fibers.

Morphometric analysis showed the following: the average diameter of the myocytes of the muscles of both limbs in the intact group was considerably less than the corresponding values in the experimental group in all the studied periods ($p < 0.05$), except for 180 days in the muscle on the left (Table). On the 30th and 180th days, the values of the parameter on the left and right differed significantly ($p < 0.05$).

All histograms of distribution of the diameters of soleus muscle fibers were unimodal.

In the intact group, the number of classes in both muscles is 4; ranges with a minimum number of fibers can be ignored (Fig. 2a). On the left, the diameters are more uniform. In classes 1–20, 21–30, 31–40, 41–50 μm, there were 7.7 and 11.1 %; 52.9 and 44.5 %; 28.4 and 36.0 %; 4.9 and 7.1 % of myocytes on the left and right, respectively.

On the 5th day in the experimental group both histograms were shifted to the right by 1 class compared to the intact one, the number of classes was 3 (Fig. 2b). On the left, the histogram belonged to the normal type; and it was asymmetric in the muscle of the right limb. In classes 21–30, 31–40, 41–50 μm, respectively, 24.1 and 33.3 %; 53.0 and 55.6 %; 21.0 and 8.3 % of myocytes were located on the left and right.

On the 15th day of the experiment, the number of classes in both histograms grew to four: the variety of diameters increased (Fig. 2c). 20.8 and 23.9 %, 41.8 and 34.4 %, 24.5 and 27.4 %, 8.2 and 10.8 % of fibers belonged to the classes

21–30, 31–40, 41–50 and 51–60 μm, respectively, on the left and right.

Within one month of the experiment, the histogram on the left visually did not differ much from the histogram for the previous period; the number of classes was 4; the distribution was of a normal type (Fig. 2d). The number of classes on the right was reduced to three. There were 25.0 and 35.8 %, 37.9 and 47.7 %, 28.9 and 13.3 %, 5.2 and 0.7 % of myocytes belonging respectively to the classes 21–30, 31–40, 41–50, 51–60 μm in the muscles on the left and right.

On the 90th day, the number of small myosymplasts in both muscles increased; the number of classes decreased to three on the left and increased to four on the right (Fig. 2e). There were 2.3 and 8.7 %, 35.9 and 23.3 %, 48.8 and 46.2 %, 12.5 and 20.2 % of fibers in classes 11–20, 21–30, 31–40, 41–50 μm, respectively.

After 1.5 years, in the muscle on the left in the histogram, the mode shifted towards smaller diameters; the number of classes was 4; 44.9 % of fibers belonged to the range of 21–30 μm, which was found in the intact group (Fig. 2f). In the histogram on the right, the maximum was shifted by 2 classes towards large diameters; only 11.7 % of myocytes were located in the class of 21–30 μm. There were 39.1 and 38.5 %; 8.3 and 40.4 %; 5.0 and 6.9 % of muscle fibers belonging to the classes 31–40, 41–50, 51–60 μm on the left and right, respectively.

## Discussion

*Musculus soleus* consists mainly of type 1 oxidative myocytes. There are many mitochondria in its cytoplasm; it has developed capillary network and a high ability to accumulate lipids [17]. Phenotypic diversity is typical: 4 types were identified, depending on the morphology of the central tendon, medial and lateral aponeurosis, and the pennation angle of the fibers. Nevertheless, there were no differences in the distribution of types on the left and right or gender [18].

It follows from the presented data that a slightly higher injuring effect was exposed to the muscle of the left limb, especially in the early experimental period. This may be explained by an eccentric hit of the load in the spinal cord. Previously, it was found that changes in the viscoelastic properties of vessels are more pronounced on the left in 42 % of observations, on the right – only in 25 % [19]. In the first two weeks, hemodynamics in the tissues of distal parts of pelvic limbs developed according to the hyperkinetic type due to an increased tonus in the large-bore arterial vessels. By the end of the 1st month, vasoconstriction of the vessels was replaced by their vasodilation [19].

The histograms of myocyte diameters with unimodal distribution were obtained; the mode in the intact group was in the range of 21–30 μm. In the experimental group within the studied periods, except for the 180$^{th}$ day, the maximum in the histograms belonged to the class of 31–40 μm, shifting in the increasing diameters by a class compared with the histograms of the intact group. On the 180th day in the muscle on the left, the histogram took the form similar to an intact histogram. The maximum number of fibers was again in the class of 21–30 μm. This, along with the absence of a significant difference between the average diameter of myocytes in this muscle and the intact parameter, indicated the potential possibility of restoring the histological structure of muscle tissue in the distant future.

On the 15th day, signs of reactive and destructive changes were observed in the muscle of the left limb: smoothing of polygonality of myocyte profiles; their focal disruption; increase in diameter diversity, disorganization of part of intramuscular neural conductors; fibrosis of perimysium vessels, and activation of connective tissue component. This is a response to neurotrophic muscle damage and suggests the transition of muscle tissue into a state of structural transformation. Smoothing of polygonality of myocyte profiles and reduction of their diameters was observed in the gastrocnemius muscle of rats with spinal cord contusion injury. This is one of the signs of its denervation [10]. It should be noted that

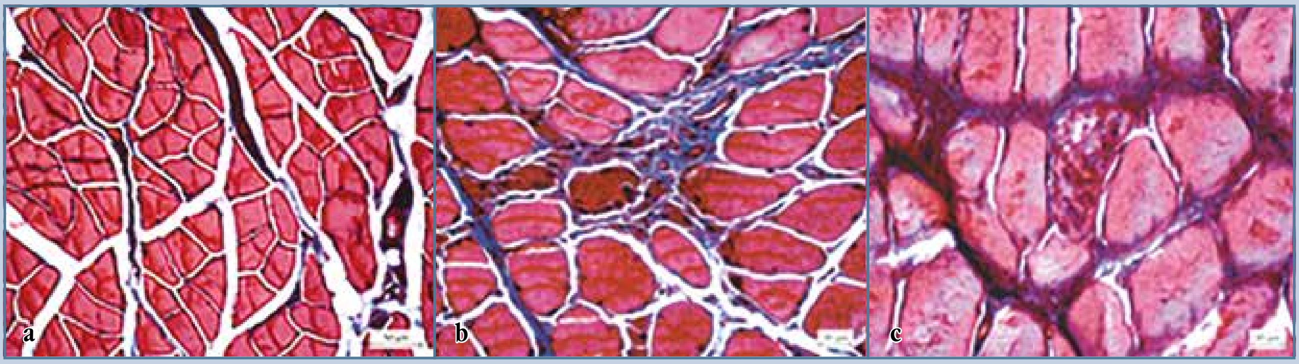


**Fig 1**
Fragments of transverse paraffin sections of *m. soleus* of rats: **a** – intact group; **b**, **c** – on the 15$^{th}$ day of the experiment; Masson stain; mag.: **a** – 400; **b**, **c** – 630

Hyun et al. [10] observed the restoration of the myocyte profiles polygonality after electrostimulation of the sacral nerve.

After 3 months the average diameter in the muscle of the left limb was considerably smaller relative to the previous period of experiment. In a study by Lin et al. [11], in the third month after spinal cord contusion injury, the cross-sectional area of myocytes reduced in soleus muscle and tibial muscles; the locomotor function index on the BBB scale decreased, which was explained by motor dysfunction. The third month of posttraumatic denervation was characterized by the adaptive histogenesis of connective tissue. There were considerable layers filled with loose connective tissue between the bundles of myocytes [20]. It is believed that the connective tissue component development provides the integrity and mechanical safety of the muscle. It reflects its adaptive properties as a phylogenetically older and less organized tissue. Meanwhile, the main mass of myocytes maintains its histologic structure [20]. According to Kim et al. [21], it has been shown that after spinal cord contusion injury in rats, a combination of exercises on the treadmill with bone marrow stromal cell transplantation can accelerate protein synthesis and hypertrophy of the soleus muscle due to activation of the signaling pathway: insulin-like growth factor-I/mammalian target of rapamycin (mTOR) regulates cell growth and survival. Other authors have illustrated how treadmill training of injured rats affects the remodeling of microcirculatory vessels in the tibial muscle. Weight-bearing sessions play an important role in maintaining the oxidative phenotype of muscles. The angiogenic response to training improved the distribution of capillaries in the muscles, increasing their oxygenation [22]. In the impact study of sclerostin antibodies (Scl-Ab) or the administration of testosterone enanthate (TE), an agent with known myotrophic effects, on the atrophy of the soleus muscle of rodents after spinal cord injury, the following was revealed: the mass of *m. soleus* reduced by 31 %, the cross-sectional area – by more than 50 % compared with the control group [23]. It was concluded that Sci-Ab does not prevent atrophy of the soleus muscle after injury. The expression of androgen receptors affects variable myotrophic reactions following TE administration. It has been proven that spinal cord injury can cause oncological diseases of muscle tissue. For example, in *m. soleus*, 2–3 times higher expression of the p53 protein, which acts as a suppressor of the formation of malignant tumors, was determined after 2 weeks, 1 and 2 months compared with markers of secretory phenotypes associated with aging [24].

**Table**

The average muscle fiber diameter of the soleus muscle in rats, μm

| Duration of the experiment | Left limb | Right limb |
|---|---|---|
| Intact group | 29.6 ± 0.3 | 29.3 ± 0.2 |
| Injury on the 5th day | **34.5 ± 0.3** | **32.4 ± 0.2** |
| Injury on the 15th day | **37.8 ± 0.4** | **37.5 ± 0.2** |
| Injury on the 30th day | **39.3 ± 0.2 Δ** | **32.5 ± 0.2** |
| Injury on the 90th day | **32.5 ± 0.2*** | **33.1 ± 0.2** |
| Injury on the 180th day | 31.0 ± 0.3 Δ | **39.6 ± 0.2*** |

The values that differ significantly between the intact and experimental groups are highlighted in bold; Δ — significant differences between the muscles on the left and right;
* in the dynamics of the experiment; $p < 0.05$.

In clinical practice, the issue of rehabilitation of patients with spinal injury is discussed mainly in the sense of neuromuscular electrostimulation [1, 25–30]. Emergency surgery performed on a patient with spinal cord injury using noninvasive percutaneous electrical stimulation of the spinal cord and mechanical stimulation of the supporting surface of the foot provided regression of neurological disorders, contributed to earlier recovery of support and gait skills [27]. Neuromuscular electrical stimulation resistance training (NMES-RT) has been developed as a strategy to weaken the process of skeletal muscle atrophy, reduce ectopic obesity, improve mitochondrial functions and enhance insulin sensitivity [1]. Functional electrical stimulation (FES-LEC) allows activating 6 muscle groups to cause greater metabolic and cardiovascular adaptation [28]. FES of the central and peripheral nervous systems can use intact neuromuscular systems for therapeutic exercises to provide functional recovery and manage complications after spinal injury. The strategy of a long-term use of the combined NMES-RT and FES-LEC protocol is designed to preserve the integrity of the musculoskeletal system. FES devices are cost effective and may be used at home as part of a rehabilitation plan [1, 28]. Training on a step ergometer supplemented with FES-LEC show an increase in the Hauser Ambulation Index in 50 % of cases by at least one level; in 60 % – an improvement in the motor parameters of the lower limbs [29]. In the study of quadriceps biopsies, it was found that even 5 years after spinal injury, atrophic myofibrils with cluster reorganization of muscle nuclei respond to FES-LEC using specially designed stimulators and electrodes. While denervated myofibrils quickly lose their ability to resist high-frequency contractions, they respond to very long pulses. The latter may contribute to the re-occurrence of tetanic contractions [30]. In addition, long-term denervation/reinnervation events occur in the elderly and are part of the mechanisms responsible for muscle aging. It is worth noting that in this case, too, FES proved to be useful in slowing down the aging process. Neurotechnology is proposed to restore control over paralyzed muscles in spinal cord injury. It is an implanted pulse generator with the ability to run in real time. The device delivers a series of spatially selective stimulation of the lumbosacral region of the spinal cord with synchronization coinciding

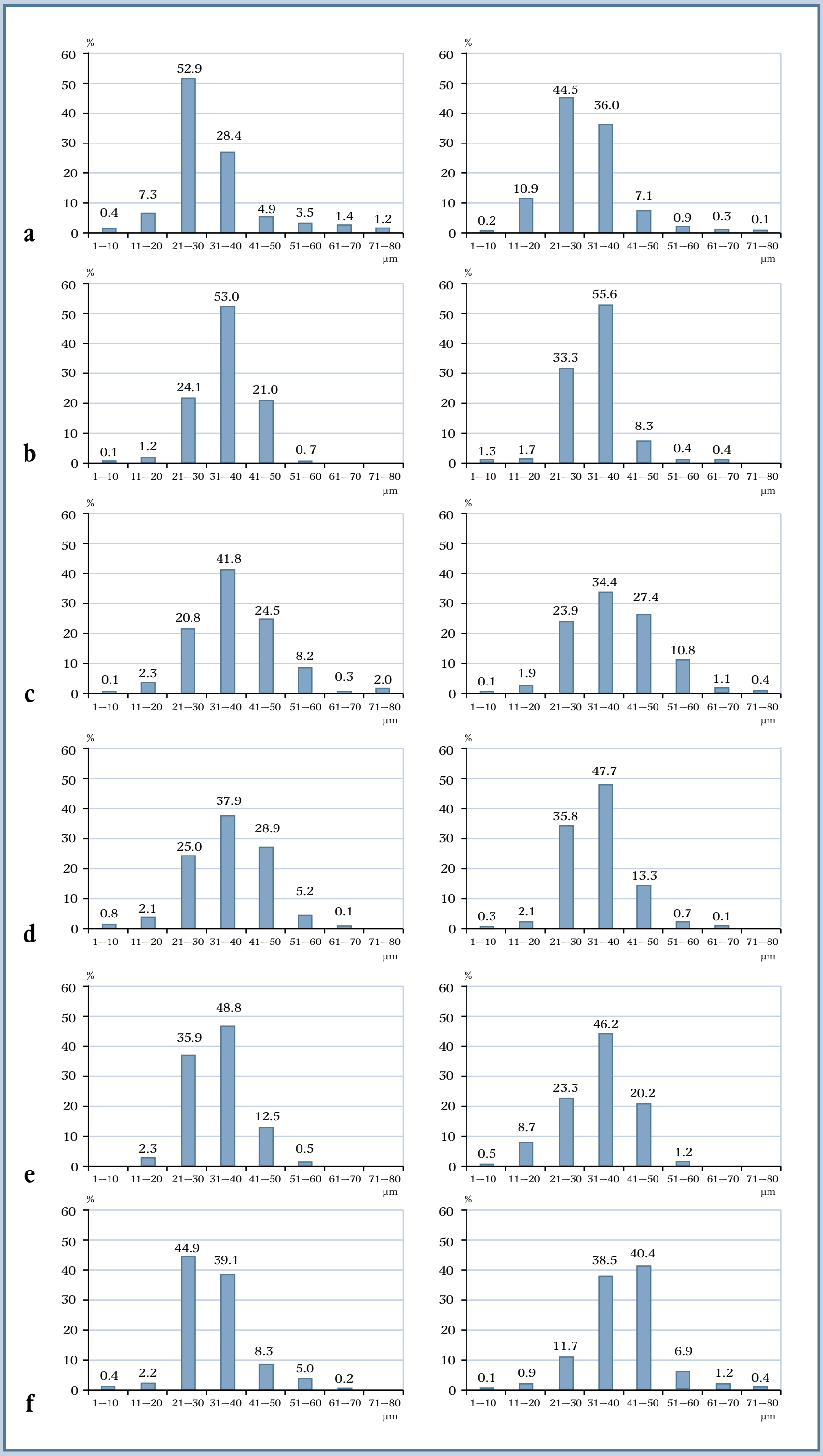


**Fig 2**
Distribution histograms of muscle fibers by diameter in the soleus muscle of rats of the left and right limbs; position in the table: on the left and right, respectively: **a** – intact group; **b** – 5th day of experiment; **c** – 15th day of experiment; **d** – 30th day of experiment; **e** – 90th day of experiment; **f** – 180th day of experiment

with the intended movement. After a few months, the study participants could walk and ride a bicycle [31].

## Conclusions

The signs of reversible reparative processes predominated in the soleus muscle in response to neurotrophic damage. This was proved by smoothing the polygonality of myocyte profiles, an increase in the diversity of fiber diameters, their contractures, myophagy, internal nuclei, and an increase in interstitial space. Disorganization of nerve fibers was noted in some intramuscular neural conductors; in perimysium: spasm of arterial vessels with signs of fibrosis of *t. media* and *t. adventiti*a. Nonetheless, the histostructure of the intact muscle prevailed during the experiment, which was proven by morphometric analysis data. All distribution histograms of the diameters of muscle fibers were unimodal. The maximum number of myocytes belonged to the range from 30 to 41 μm. In the long-term period of the study, the maximum of myocytes in the histogram of the left limb muscle was in the range of 21–30 μm, which was specific for histograms of the intact group.

The nature of the plastic reorganization in skeletal muscles of the pelvic limbs in spinal cord contusion injury indicates compensatory regeneration of muscle tissue by the type of restitution. It provides an opportunity of predicting the rehabilitation period. In this regard, it is advisable to take this into account when developing medical and social programs and therapeutic measures, where neuromuscular and functional electrical stimulation are of great significance.

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*The authors declare that they have no conflict of interest.*

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