

ORIGINAL ARTICLE

EFFECT OF BONE MARROW ASPIRATE IN DENERVATION-INDUCED SKELETAL MUSCLE ATROPHY

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ABSTRACT

The aim: To evaluate muscle changes after sciatic nerve damage with the injection of bone marrow aspirate cells.**Materials and methods:** 36 rabbits underwent sciatic nerve cross-section and neuroraphy, bone marrow aspirate cells were injected directly or 7 weeks after neuroraphy. Changes in skeletal muscle morphology (photomicrographs of histological sections were analyzed for morphometric analysis of collagen region, quantitative analysis of collagen density and measurement of muscle fibers diameter) and biochemical parameters (catalase activity, superoxide dismutase and glutathione peroxidase measurements and level of TBARS was determined) at 8, 12, and 16 weeks were examined.**Results:** There is atrophy of muscle fibers in denervated muscles, and it has a negative tendency between 8 and 12 weeks. Delayed bone marrow aspirate cells injection into the muscles at 7 week – delayed atrophy and formation of TBA reactive substances. But bone marrow aspirate cells injection into the muscles directly after neuroraphy increased collagen formation, and development of fibrosis in areas of atrophy.**Conclusions:** Sciatic nerve injury results in atrophy of muscle tissue, which is partially delayed after delayed bone marrow aspirate cells injection at week 7. Muscle atrophy was characterized by a sharp increase in TBARS levels at 12 and 16 weeks and catalase activity at 12 weeks, and changes in biochemical parameters were partially normalized after the use of cell aspirates, to a greater extent with delayed injection.**KEY WORDS:** muscles, denervation, atrophy, fibrosis, bone marrow aspirate cells

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INTRODUCTION

Innervation play a critical role in skeletal muscle function. Denervation leads to muscle atrophy, dramatically impairs quality of life and has a poor prognosis for recovery [1]. Atrophy can be associated with the weight loss of individual fibers, without reducing the total number of fibers, or muscle fibers atrophy and even necrosis with prolonged denervation and severe muscle damage [2]. In damaged muscles (atrophy, denervation, or ischemia) number of muscle fibers and their diameter decreases within 9 months, and the amount of intramuscular adipose and connective tissue increases, at this time size of the muscle itself can both to decrease or doesn't change [3]. At the same time, a significant percentage of thin muscle fibers in the period from 6 months gives hope for the emergence of new fibers, ie the manifestations of recovery [3]. Unfortunately, in long-term reinnervation, which is often unlikely after severe nerve injury, regenerative effects associated with new muscle fibers formation by satellite cells are short-lived, focal, and not significant enough to compensate for skeletal muscle atrophy.

Metabolic processes in muscles on the background of atrophy are also not unidirectional, in particular on the

background of protein amount reducing in muscle revealed accumulation of peroxidation products (malonic dialdehyde, diene conjugates, protein carbonyls) and 3-fold decrease in NO-synthase activity (NOS) [4], but enzymes activity of antioxidant system on the contrary increases, or their activity is multidirectional (increase in SOD and reduction of GR, GPx) [5]. Morphometric characteristics of muscles and metabolic processes are criteria for assessing the state of denervated muscles and the dynamics of atrophy.

Large number of ways to use mesenchymal cells from bone marrow to stimulate regeneration of damaged nerves and muscle reinnervation are described in literature. These cells have a trophic effect on tissues, activate nerve fibers regeneration, secrete growth factors, stimulate synthesis of extracellular matrix proteins, in particular collagen, fibronectin. Injection of these cells is considered as a potential way of denervated muscles trophic support and reinnervation. [6,7].

Injection of bone marrow mesenchymal stem cells in m.gastrocnemius has already been studied and even some encouraging results have been obtained [8], however, little is known about delayed muscle changes that occur in denervated muscles and how muscle tissue responds to cell injection.

THE AIM

To evaluate muscle changes after sciatic nerve damage with the injection of bone marrow aspirate cells.

MATERIALS AND METHODS

EXPERIMENTAL GROUPS

Experimental studies were performed on rabbits weighing 3-4 kg. Animals were divided into 5 groups of 3 animals per group (for each experimental period):

- 1) Control group - intact animals;
- 2) Group of sham-operated animals – only surgical approach to sciatic nerve and wound suturing;
- 3) Group 1 – surgical approach, neurotomy and nerve suture at the hip level, wound suturing;
- 4) Group 2 – same as Group 1, and bone marrow aspirate harvest from femur, with its injection into m.gastrocnemius, wound suturing;
- 5) Group 3 - same as Group 1, and at the beginning of muscle reinnervation (7 weeks) - bone marrow aspirate harvest from femur, and its injection into m.gastrocnemius.

SCIATIC NERVE INJURY

Two end-to-end nerve sutures by EHICON PROLENE® 7/0 (Johnson's Johnson Init) using microsurgical techniques was applied. In order to worsen conditions of reinnervation, nerve suture is applied through the entire thickness of sciatic nerve, with significant tension, and without adaptation of nerve stumps. Skin was sutured with COROLENE® 2/0 (Peters SURGICAL). Operating field was treated three times with Sterillium® Classic Pur disinfectant (BODE Chemie GmbH, Germany).

BONE MARROW ASPIRATE

Animals of group 2 after performing a nerve suture was injected with purified bone marrow aspirate in m.gastrocnemius. Animals of group 3 purified aspirate was injected 7 weeks after nerve suturing.

0.2 ml of 4% ACD-A solution was collected in a 5 ml syringe. A 1.2 mm diameter needle was used to puncture the skin in the projection of greater trochanter of femoral bone. Drilling of the greater trochanter outer cortical layer was performed, and immersion of needle to the inner cortical layer. Using a 0.9 mm thick conductor, needle is cleaned of bone debris. Syringe was attached and 2 ml of bone marrow aspirate was harvested. Needle was removed, puncture site was treated with Sterillium® classic pur disinfectant (BODE Chemie GmbH, Germany). Bone marrow aspirate was purified of spongy bone particles using a Tulip® Emulsifier™ subcutaneous fat aspirate filter. A 0.6 mm thick injection needle is connected to a syringe with a purified bone marrow aspirate. Purified bone marrow aspirate was inserted into m.gastrocnemius.

SKELETAL MUSCLE HISTOLOGY

Skeletal muscle (m.gastrocnemius) was fixed in a 10% solution of neutral formalin. After fixation, samples were

embedded in paraffin via isopropanol-paraffin method and 8µm microsections were made on a Thermo Microm HM 360 microtome (Thermo Scientific, USA). Dewaxed sections were stained for 30 min at 25° red Sirius (0.5 g Direct Red 80 (Magnacol Ltd, UK) in 500 ml of saturated picric acid solution) [9], dehydrated, enclosed in balm (Merck, Germany). Photomicrographs were obtained on an Olympus BX51 microscope. Photomicrographs of histological sections were analyzed using ImageJ software (Wayne Rasband, USA). Microphotographs (lens magnification ×10) were selected for morphometric analysis of collagen region. Quantitative analysis of conducted collagen density is presented as a percentage (%).

To measure muscle fibers diameter, 10-15 microscopic fields were randomly selected for each sample to examine at least 200 fibers. Diameter was measured using Carl Zeiss AxioVision SE64 Rel.4.9.1 software as a linear projection between short ends of a single fiber.

BIOCHEMICAL RESEARCH

Homogenates were obtained from muscle samples using an Glas-Col electric homogenizer (USA) (100 mg tissue mass, 1 ml chilled 0.05 M phosphate buffer, 0.1 mM EDTA, pH 7.6). Homogenates were centrifuged at 10,000g for 20 min to obtain supernatants. Protein levels were measured by Lowry O.H. method [10]. Indicators were determined by spectrophotometric methods using a spectrophotometer µQuant, Bio-Tek, (USA). Catalase activity was determined by Aebi H. method [11], superoxide dismutase was determined by Mirsa method [12]. Glutathione peroxidase was measured by decrease in NADPH levels in conjugated glutathione reductase reaction [13]. Level of TBARS was determined by method described in the article published by Uchiyama M. [Uchiyama M.].

STATISTICAL RESEARCH

Distribution normality of data samples was carried out according to Kolmogorov-Smirnov criterion. Intergroup differences were assessed using one-parameter variance analysis of ANOVA variations with Bonferroni correction. Difference between the groups was considered significant at $P < 0.05$. Correlation analysis was performed according to Spearman's criterion. Statistical studies were conducted using OriginPro software ver. 8 (OriginLab Corporation, USA) and StatPlus ver. 7.3.0. (AnalystSoft Inc. USA). Data are presented as mean and mean error (Mean ± SEM).

BIOETHICS

All manipulations with animals were carried out in accordance with European Convention for Protection of Vertebrate Animals (Strasbourg, March 18, 1986) and recommendations of Bioethics Commission of the SI "Institute of Traumatology and Orthopedics of National Academy of Medical Sciences of Ukraine".

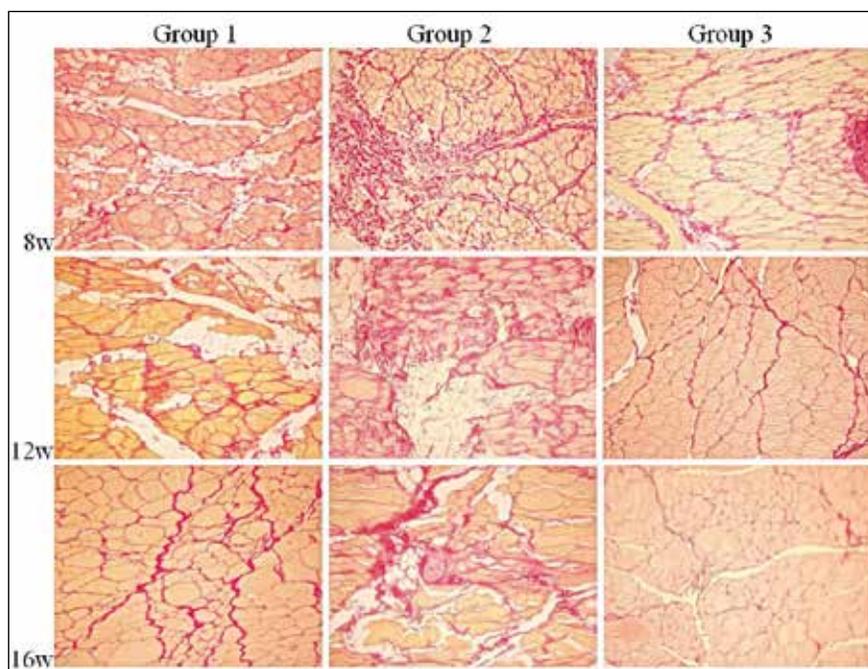


Fig. 1. Photomicrograph of m.gastrocnemius after picosirius red staining with muscle fibers (yellow) and collagen (red).

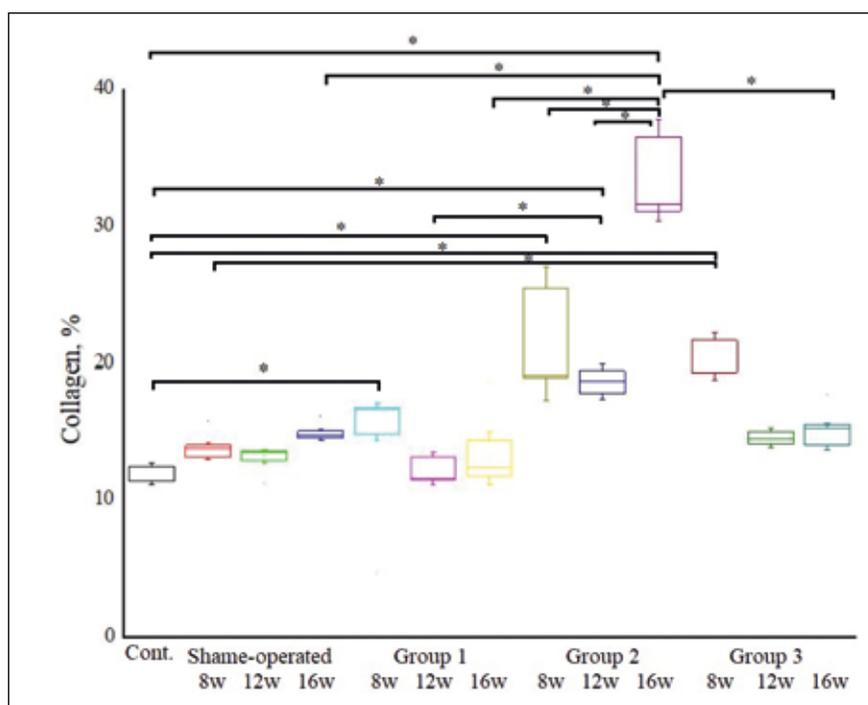


Fig. 2. Graph of collagen quantitative measurement in m.gastrocnemius histological micropreparations after staining with picosirius red. * P < 0.05

RESULTS

MUSCLE FIBROSIS

In skeletal muscles of group 1 at 8 weeks revealed a higher density of positively stained regions with picosirius red compared to control (difference $4.38 \pm 1.63\%$, $P < 0.05$), which is the result of an increase in the amount of connective tissue (Fig. 1). In group 2, density of Sirius Red-positive regions was significantly higher relative to control group and group of pseudooperated animals by 8 (difference $10.2 \pm 2.75\%$, $P < 0.05$), 12 (difference $6.71 \pm 1.98\%$, $P < 0.05$) and 16 (difference $22.1 \pm 1.97\%$, $P < 0.05$) weeks (Fig. 2). There was a statistical difference relative to group 1, namely

an increase in positively colored regions by 12 (difference $6.33 \pm 1.78\%$, $P < 0.05$) and 16 (difference $21.0 \pm 2.21\%$, $P < 0.05$) weeks. These data indicate that injection of bone marrow aspirate after neuroraphy causes cellular reactions of connective tissue with collagen synthesis, stimulates collagen genesis. In group 3, increased collagen density was detected at 8 weeks relative to control (difference $8.56 \pm 2.15\%$, $P < 0.05$). At 12 and 16 weeks, no difference between control group and group of pseudooperated animals was detected; collagen density was higher relative to group 1 at 8 (difference $2.24 \pm 0.96\%$, $P < 0.05$) and 12 (difference $5.61 \pm 1.64\%$, $P < 0.05$) weeks. Indicator was significantly lower than in group 2 at 16 weeks (difference $17.5 \pm 2.94\%$,

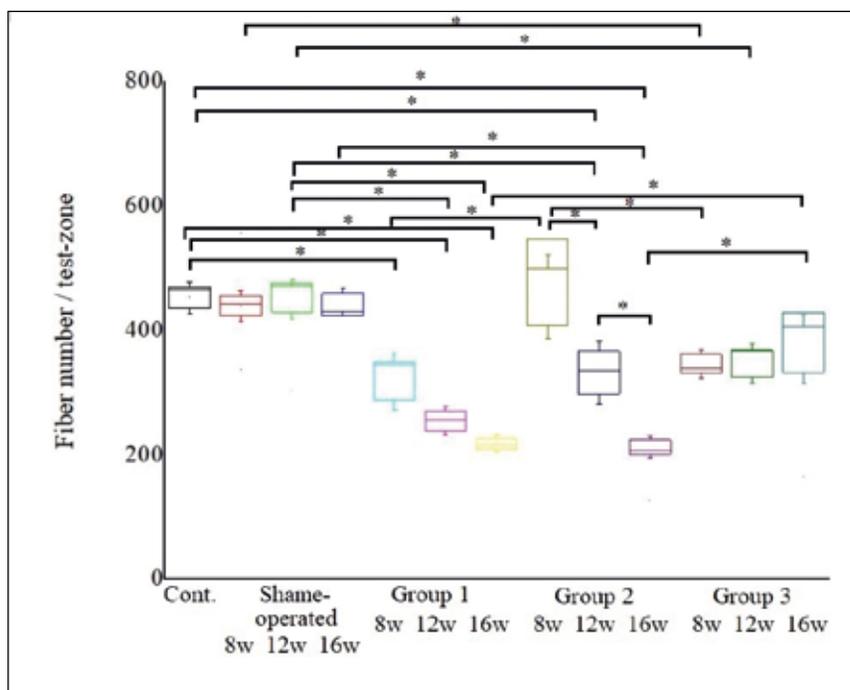


Fig. 3. Muscle fibers number in m.gastrocnemius, at the background of denervation. * P < 0.05

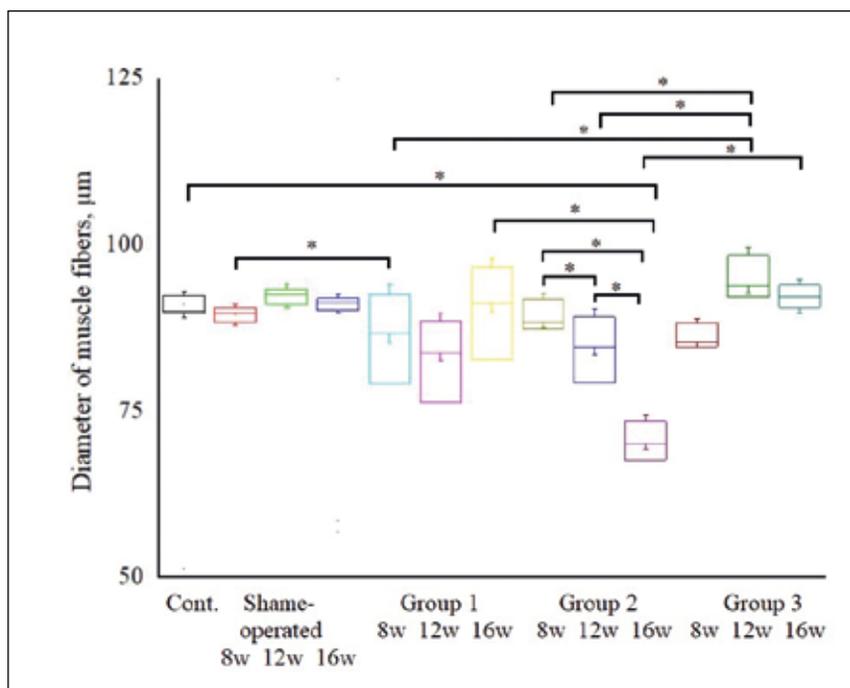


Fig. 4. Muscle fibers diameter in m.gastrocnemius, at the background of denervation. *P<0,05

P < 0.05). Difference between groups 1, 2 and 3 indicates that injection of bone marrow aspirate cells after neuroraphy stimulates more collagen formation than after injection of aspirate cells at 7 weeks after neuroraphy.

MUSCLE ATROPHY

Analysis of muscle fibers number in m.gastrocnemius shows a significant decrease in group 1 and group 2 (Fig. 3). Dynamics of change in groups 1 and 2 was similar, but in group 2 difference between the terms of 8 and 12 weeks (difference 27.5%; P < 0.05), 12 and 16 weeks (difference

27.7%; P < 0.05) was reliable. At 8 weeks, muscle fibers number in group 2 was significantly higher than in group 1 (difference 27.5%; P < 0.05), at 12 and 16 weeks the difference in change was within the statistical error. This may indicate that injection of bone marrow aspirate cells immediately after injury partially prevents atrophy, but in subsequent stages of change progress.

In group 3, muscle fibers number was statistically lower compared to group of pseudooperated animals. Rate between groups 1 and 3 did not differ at 8-12 weeks, but statistically significant difference was found at 16 weeks of the experiment (P < 0.05). Compared with group 2, figure

Table I. Results of biochemical studies of m.gastrocnemius, at the background of denervation

Group	TBARS nmol×mg ⁻¹	CAT μmol×min ⁻¹ ×mg ⁻¹	GPx nmol×min ⁻¹ ×mg ⁻¹	GR nmol×min ⁻¹ ×mg ⁻¹	SOD U×min ⁻¹ ×mg ⁻¹
8 weeks					
Shame	0.62±0.16	5.14±0.43	2.15±0.15	2.15±0.24	4.16±0.40
Group 1	1.11±0.17	3.88±0.31	3.88±0.37	3.17±0.35	4.95±0.54
Group 2	1.51±0.34	5.66±0.39*	4.03±0.72	3.40±0.46	5.18±0.46
Group 3	1.58±0.51	4.06±0.38^	3.60±0.27	4.19±0.30	4.66±0.35
12 weeks					
Shame	0.75±0.07	3.42±0.02	3.39±0.56	4.59±0.26	4.77±0.52
Group 1	4.33±0.78#**	5.17±0.29#	4.04±0.39	3.22±0.34	5.42±0.16
Group 2	0.97±0.29*	2.47±0.17*	2.15±0.21*#	2.56±0.62	4.10±0.29
Group 3	2.04±0.78*	2.98±0.20*	2.86±0.14	3.55±0.15	4.58±0.18
16 weeks					
Shame	0.66±0.13	2.16±0.37#	1.94±0.14	2.34±0.14	4.67±0.47
Group 1	5.89±1.94#**	3.62±0.41	3.3±0.50	3.85±0.43	5.37±1.02
Group 2	3.05±0.12*,**	4.37±0.17@**	3.05±0.37	3.46±0.23	4.01±0.22
Group 3	0.43±0.23*	2.29±0.32^#	1.96±0.24*#	2.10±0.28*#	5.22±0.31

* P < 0.05 to group 1; # P < 0.05 before 8 weeks; ** P < 0.05 to the group of pseudooperated; ^ P < 0.05 to group 2; @ P < 0.05 to 12 weeks

was significantly lower at 8 weeks, no difference at 12 weeks, and muscle fibers number was significantly higher than in groups 1 and 2 at 16 weeks. This indicates that injection of bone marrow aspirate cells at 7 weeks after injury reduces dynamics of muscle fibers atrophy in the following terms.

Negative correlation was found between muscle fibers number and level of fibrosis in m.gastrocnemius. In group 1, a high negative dependence was found at 12 ($r = -0.75$; $p = 0.001$) and 16 ($r = -0.84$; $p = 0.002$) weeks; in group 2 high dependence on 8 ($r = -0.73$; $p = 0.05$), weak and medium on 12 ($r = -0.26$; $p = 0.34$) and 16 ($r = -0.54$; $p = 0.03$) weeks; in group 3 weak and medium correlation strength by 8 ($r = -0.36$; $p = 0.18$), 12 ($r = -0.55$; $p = 0.03$) and 16 ($r = -0.33$; $p = 0.22$). These results suggest an association of muscle fiber malnutrition with collagenogenesis in m.gastrocnemius, especially in group 1.

Difference in m.gastrocnemius morphometry was not limited to atrophic changes in individual muscle fibers. Additionally, analysis of muscle fibers diameter and fibers distribution by diameter (Fig. 4 and 5) was made. At the background of denervation, two manifestations of changes in muscle fibers were detected simultaneously: appearance of atypically thin, atrophic fibers (less than 40 microns in diameter) and hypertrophy of individual fibers (more than 160 microns in diameter). As can be seen from the diagrams (Fig. 5), in group 1 <5% of fine fibers were found in all terms of the experiment, and hypertrophied $\geq 6\%$. In the group of 2 ~ 5% of fine atrophic fibers were detected at 16 weeks, and hypertrophied fibers - 2.3%, 7.5% and 1.9% according to the terms of experiment. In group 3, relative number of atypically thin muscle fibers was <2%, and hypertrophied were recorded at 12 and 16 weeks (10.7% and <2%). That is, dynamics are similar. Analysis of muscle fiber distribution patterns indicates that in group 3, malnutri-

tion develops at a slower rate than in groups 1 and 2, and hypertrophy is likely to be both a compensatory response to denervation and to injection of bone marrow aspirate cells. It should be noted that appearance of hypertrophied fibers was reflected in the morphometry of studied muscle samples, so the conclusions about action of aspirate cells were formed based on the assessment of several indicators (number, diameter and distribution of muscle fibers).

RESPONSE OF MUSCLE ANTIOXIDANT SYSTEM

Analysis of changes in thiobarbituric acid reactive substances (TBARS) was selected as an indicator of metabolic changes in muscle during injury. As can be seen from table I, level of TBARS increased relative to control at all times of the experiment. Marked trend of TBARS accumulation at 8 weeks increased to a statistically significant difference at 12 and 16 weeks (difference of 3.9 and 5.3 times; $P < 0.05$). In group 2, level of TBARS differed from pseudooperated animals at 16 weeks (difference 4.5 times; $P < 0.05$) and was significantly lower than group 1 (difference 48.1%; $P < 0.05$). In group 3, the level of TBARS was significantly lower than in group 1 at 12 and 16 weeks and did not differ from pseudo-operated animals. These data indicate that lipid peroxidation products accumulate during muscle atrophy, which is an indicator of cell damage, and they decrease after injection of bone marrow aspirate cells, to a greater extent in group 3.

In this study, we found a decrease in catalase activity (CAT) in pseudooperated animals between 8 and 16 weeks. This muscle response has been evaluated as the response of tissues to surgical approach to sciatic nerve. In group 1, activation of CAT at 12 weeks and then decreased. In group 2, rate varied similarly to the group of pseudooperated

animals, but at 16 weeks a significant increase was found, which is probably a reaction in response to atrophy. In group 3, the dynamics is similar to those of pseudooperated animals, and the rate is statistically significantly lower than in group 2 at 8 and 16 weeks.

Glutathione peroxidase (GPx) activity did not differ significantly between groups. In group 2, difference compared to group 1 was observed at 12 weeks, and in group 3 at 16 weeks. In both groups, these values were probably lower than original. Glutathione reductase (GR) activity decreased only in group 3 at 16 weeks, as a manifestation of metabolic reactions restoration. Analysis of changes in superoxide dismutase (SOD) activity did not reveal an intergroup difference. Based on the analysis of described indicators, it was concluded that in group 3 metabolic changes are less striking compared to group 2 and especially group 1, both in the level of TBARS formation and response of the antioxidant system to surgery and denervation.

DISCUSSION

To date, some morphological and molecular mechanisms of skeletal muscle atrophy have been discovered [15]. Main morphological manifestation of muscle fiber atrophy is a decrease in its thickness, which in our study was estimated by fibers diameter. If skeletal muscle is affected at an early time of denervation and innervation is restored, stabilization of muscle morphology and even partial restoration of muscle fiber diameter can be achieved, as shown after muscle electrical stimulation [16]. However, amount of thin fibers can remain for a long time and this is a morphological manifestation of atrophy in denervated muscles.

Results show that both the percentage of fibrotic changes and atrophic muscle fibers are lower after injection of bone marrow aspirate cells 7 weeks after denervation, whereas early injection stimulate collagenogenesis. However, data on muscle fibers number at 8 weeks in groups 2 and 3 may indicate a delay in muscle atrophy in early stages of cell injection. Obviously, third observation period (16 weeks) is crucial and ultimately based on the comparison of muscle morphology, morphometry results and biochemical data, delayed route of administration can be considered as potentially favorable. We are not the first to find a similar relationship between the level of fibrosis and injection of bone marrow cells. Liu X, et al described a significant increase in fibrosis and muscle inflammation in contusive muscle injury [6]. Mesenchymal cell transplantation has caused infiltration of leukocytes and macrophages, increased levels of inflammatory cytokines and chemokines, matrix metalloproteinases and oxidative stress in muscles, ie processes associated with inflammatory muscle damage [6]. Under other conditions, injection of cells with fibrin did not have such consequences [17]. Obviously, opposite results in different works depend on the experimental model, for example, nerve transection and suturing or auto-grafting [18]. In our own research, we obtained similar results. Injection of aspirate cells immediately after nerve injury was characterized by increased fibrosis, while injection of cells 7

weeks after denervation had no such effect. However, it should be borne in mind that the reference point in experiments is the period after nerve injury, while the time difference between cells injection in groups 2 and 3 is 7 weeks. We do not rule out that fibrosis may increase in the future, so we consider the error of this assessment, but in general, bone marrow aspirate cells injection exacerbated fibrosis.

Dynamics of muscle atrophy can be assessed not only by morphological methods, although changes in structure are the most convincing indicator. We found that the level of TBARS, as a product of lipid peroxidation, increases at all times. After bone marrow aspirate cells injection, their level decreased, which can be assessed as a manifestation of their utilization and inhibition of formation. In response to the level of TBARS, catalase activity increased, as the antioxidant system is activated in response to oxidative stress. At the same time, protective mechanisms of this system were more pronounced in group 3. These results should be taken into account when using bone marrow aspirate cells in regenerative medicine and traumatology.

CONCLUSIONS

Sciatic nerve injury results in atrophy of muscle tissue, which is partially delayed after delayed injection of bone marrow aspirate cells. Direct injection of aspirate cells caused fibrotic changes in the areas of atrophy, more than after late injection and without it. Muscle atrophy was characterized by a sharp increase in TBARS levels at 12 and 16 weeks and catalase activity at 12 weeks, and changes in biochemical parameters were partially normalized after the use of cell aspirates, to a greater extent with delayed injection.

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The Authors declare no conflict of interest.

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