ORIGINAL ARTICLE

Veterinary Research Forum. 2021; 12 (4) 451 – 457
doi: 10.30466/vrf.2019.109276.2593
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Histomorphometrical evaluation of extensor digitorum longus muscle in sciatic nerve regeneration using tissue engineering in rats

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Article Info

Article history:

Received: 09 June 2019
Accepted: 17 September 2019
Available online: 15 December 2021

Keywords:

Extensor digitorum longus muscle
Mast cell
Mesenchymal stem cell
Neural tissue engineering
Rat

Abstract

Skeletal muscle atrophy induced by denervation is one of the common disorders in traumatic nerve injuries. The aim of this study was the evaluation of histomorphometrical changes of extensor digitorum longus muscle after denervation and its regeneration by tissue engineering. Ninety adult male Wistar rats were randomly divided into six main groups (n = 15) in three time periods (2, 4 and 8 weeks; n = 5). Control group was treated without surgery, in transection (Tr) group left sciatic nerve was transected, in scaffold (S) group only collagen gel scaffold was used, in mast cell (MC) group mast cells were used, mesenchymal stem cell (MSC) group was treated with mesenchymal stem cells and in MC+MSC group, mast cells along with mesenchymal stem cells were used. In the cellular groups, the scaffold and cells were mixed and placed in the transected nerve gap. The average diameter of muscle fibers, ratio of the muscle fibers nuclei to the fibrocytes nuclei (mn/fn), ratio of the muscle fibers nuclei number to the muscle fibers number (mn/mf), the average ratio of blood vessels to muscle fibers number (v/mf) and muscles weight in Tr group were the lowest compared to the other groups; but, in cellular and S groups, amelioration was observed according to the time period. However, in MC+MSC group, there were the highest ameliorative results. This study revealed that simultaneous use of MCs and MSCs mixed with collagen gel scaffold can be considered as a suitable approach to improve denervated skeletal muscle atrophy associated with sciatic nerve injury.

Introduction

Every year, around 300,000 people in Europe sustain peripheral nerve damage,[1] and about 5.00% of people who go to the trauma treatment centers suffer from peripheral nerve injuries,[2] imposing a lot of expenses to the society. Complete transection of the sciatic nerve is a well-standardized and validated method in which nerve fibers and epineurium are completely transected and the neuromuscular junction is severed.[3] Once the peripheral nervous system is damaged, it results in disordered muscle movement, and the atrophic process ensues.[4] Therefore, the nervous system has a major role in muscular functions such as locomotion and posture.[5] The control of skeletal muscle function by the nervous system has been a topic of great interest for scientists.[6]

The nervous system has two mechanisms for controlling the skeletal muscles: 1) Neuromotor control, which causes sarcolemma depolarization and muscle contraction through neuronal impulses generation in the cortex or brain stem and 2) Neurotrophic control, in which some factors are secreted from the nerve terminals of motor neurons at the neuromuscular junction.[7] The damage to the denervated muscles in rats consists of 3 stages: In the first stage (lasting two months), in addition to rapid loss of function, weight loss and muscle atrophy may occur. The second stage (2^{nd}-7^{th} months) is characterized by severe atrophy and loss of sarcomeric structures. In the third stage (after a 7^{th} month), fibrosis of the interstitial tissue and adipose tissue is observed with a significant reduction in the number of muscle fibers.[8] Muscle atrophy is accompanied by a significant decrease in

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the creatine phosphokinase enzyme levels [9] providing the muscle cells energy. In the denervated gastrocnemius muscle, using mesenchymal stem cells (MSCs) following sciatic nerve transection leads to an increase in this enzyme.[10] Satellite cells are stem cells stored in skeletal muscles inducing muscle hypertrophy by proliferation.[4] There is a direct relationship between muscle atrophy and satellite cells, [11] in a way that muscle disuse reduces these cells proliferation and differentiation. In rodent models, the muscle atrophy following denervation has been occurred over 1-2 weeks in the hind limbs of female rats.[12] Mast cells (MCs) are recognized by their secretory granules containing histamine, tryptase, kinase, tumor necrosis factor-α, serotonin, heparin, proteoglycan and vascular endothelial growth factor (VEGF).[13] Interactions between MCs and nerves are mutual; therefore, MCs secretions have the ability to stimulate the nerves[14] and to facilitate axon reflexes.[15] This interference between MCs and nerves leads to nerve tissue reconstruction mechanisms following injury[16] due to neuropeptides secretion by MCs.[17] Evaluation of the relationship between MCs and MSCs indicates the impact of MCs on MSCs differentiation, proliferation and migration in *in vitro* and *in vivo* environments.[18] The MCs injected into the infarcted heart of mice dramatically increased cell proliferation and MSCs count.[18] Investigations on damaged nerve tissue have showed that nerve regeneration takes place when tube conduits are filled with materials such as collagen fibers and sponge collagen.[19] Additionally, a study has shown the healing effects of MSCs inside different types of tubular prosthesis on the damaged sciatic nerve.[20]

The aim of this study was to evaluate the histological changes in extensor digitorum longus muscle as a result of sciatic nerve transection and its repair using simultaneous application of MCs and MSCs within collagen gel scaffold.

Materials and Methods

Experimental design and animals. Experiments were conducted in 90 adult male Wistar rats weighing 150-200 g randomly divided into six main groups (n=15) and each group was subdivided into three subgroups of five animals each in three time periods (2, 4 and 8 weeks).[21] They were housed at 23.00 ± 3.00 ˚C and 12-hr light/dark cycle with free access to standard rodent laboratory food and water and kept for two weeks before being used in the surgery. All surgeries were performed under anesthesia at least distress of the animals.

Surgical procedure. All procedures were carried out in accordance with the guidelines of the Veterinary Ethics Committee of Faculty of Veterinary Medicine, Urmia University, Urmia, Iran (No.: AECUU-268-2019). Animals were anesthetized by intraperitoneal (IP) administration of ketamine hydrochloride (90.00 mg kg^{-1}; Alfasan, Woerden, The Netherlands) and xylazine hydrochloride (5.00 mg kg^{-1}; Bayer, Leverkusen, Germany) at sterile conditions as described by Rigon *et al.*[22] In the control group, surgery was performed without nerve transection; but, in the other groups, complete left sciatic nerve transection was done. For nerve transection, the left sciatic nerve was exposed, fixed by silk ligature on epineurium to silicone conduit and transected. Then, free (distal) end was ligated on the other side of conduit with an 8.00 mm distance between two ends. In the transection (Tr) group only nerve transection was performed. In the scaffold (S) group, collagen gel scaffold (10.00 µL) was placed without any cells in the silicone tube. In the MC group, 3.00×10^4 MCs were added to the scaffold. In the MSC group, 3.00×10^4 MSCs along with collagen gel scaffold were added to silicone tube. Also, in the MC+MSC group, MCs (3.00×10^4) along with MSCs (3.00×10^4) in collagen gel were placed in a silicone tube. After sciatic nerve surgery, the muscle was sutured with 4/0 Vicryl (Ethicon, Norderstedt, Germany) and the skin was sutured with 3/0 nylon (B/Braun, Melsungen, Germany).

Histological analysis. At 2, 4 and 8 weeks after surgery, animals were euthanized using an overdose of ketamine-xylazine (150 mg kg^{-1} of ketamine (Alfasan) and 10.00 mg kg^{-1} of xylazine (Alfasan) intraperitoneally. Extensor digitorum longus muscles were sampled and fixed in 10.00% formaldehyde buffer. Then, the paraffin sections of muscles were prepared (5.00-7.00 µm) using a rotary microtome (Microm GmbH, Hessen, Germany) and histomorphometrical studies were performed after staining with hematoxylin and eosin.[23]

Mice MCs isolation and culture. Mast cells were derived from the bone marrow of male mice according to a method previously described.[24] Obtained MCs purity (> 90.00%) was determined by flow cytometry (Fig. 1).

Rat MSCs isolation. Based on the proven method, [25] MSCs were obtained from rats' femur and tibia bone marrow. Mesenchymal stem cell purity (> 90.00%) was determined by flow cytometry (Fig. 1).

Histomorphometrical studies. Diameter of muscle fibers was measured randomly using a calibrated graded objective lens in 20 fibers from each tissue sample. To determine the ratio of muscle fibers nuclei to the fibrocytes nuclei (mn/fn), the nuclei were counted and calculated in a fixed microscopic field (0.0625 mm^2). To evaluate the ratio of muscle fibers nuclei number to the muscle fibers number (mn/mf), a fixed number of muscle fibers cross-sections (10 per cross section) in each tissue sample was estimated. To calculate the average ratio of blood vessels to muscle fibers number (v/mf), a fixed number of muscle fibers cross-sections (10 pcs) in each tissue sample was counted and calculated.

Collagen gel preparation. In order to prepare the collagen gel scaffold, a 1.00% solution of collagen isolated from the rats tail (2.16 mg mL^{-1} protein in 0.60% acetic

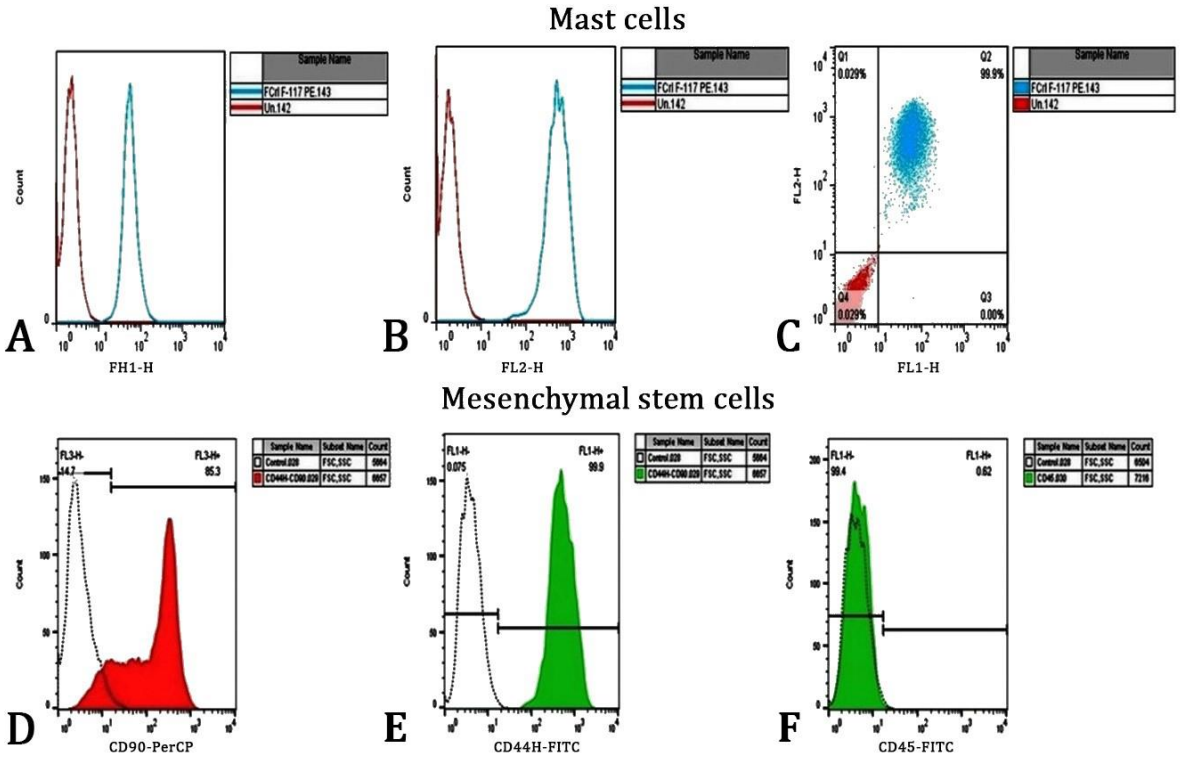

Fig. 1. Flow cytometry analysis of mouse bone marrow-derived mast cells. **A)** Positive cells for CD117 (c-kit); **B)** Positive cells for FCεRI; **C)** Double-positive cells (99.90%). Flow cytometry analysis of rat bone marrow-derived mesenchymal stem cells; surface markers of mesenchymal stem cells were labeled with IgG1 (Isotype control), CD45, CD44 and CD90 antibodies. The CD90 (85.30%; **D**) and CD44 (99.90%; **E**) were positive; but, they did not express the CD45 (**F**) marker.

acid; First Link Ltd., Birmingham, UK), in a phosphate-buffered saline solvent (pH= 7.50-8.00), was prepared on a shaker in a refrigerator (4.00 ˚C) overnight, sterilized by chloroform solution (0.10 solution volume) and evaluated by scanning electron microscope (Fig. 2).[26]

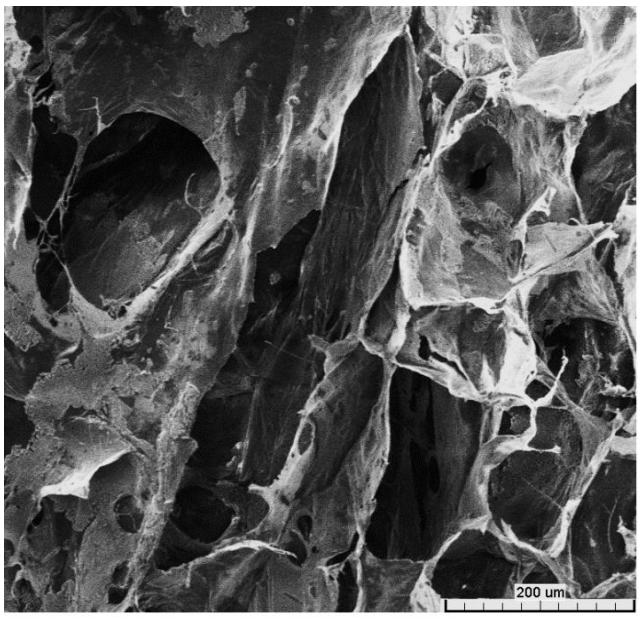

Fig. 2. Scanning electron microscopy of collagen gel scaffold.

Muscles weight. The extensor digitorum longus muscle samples were weighted using digital balance (A&D Co., Tokyo, Japan) and the data were analyzed with Duncan test.

Statistical analysis. Data were analyzed by SPSS software (version 20.0; IBM Corp., Armonk, USA), one-way ANOVA and Tukey post-hoc test. All results were expressed as mean ± SE and significant differences between experimental groups were set at $p < 0.05$.

Results

Muscle fibers diameter. The mean diameter of muscle fibers in the 2nd week showed a significant difference in control group compared to the other treatment groups ($p < 0.05$). In MC+MSC group, the mean diameter of muscle fibers was not significantly different from control group. In the 4th week, Tr group was significantly different from the other treatment groups, which the muscle fibers in Tr group were at the lowest mean diameter ($p < 0.05$). Also, the highest diameter was observed in the control and MC+MSC groups, which was significantly different from other groups ($p < 0.05$). In the 8th week, the diameter of muscle fibers in the Tr group was significantly different in comparison with control and MC+MSC groups ($p < 0.05$); but, it was not significantly

different from the other therapeutic groups. It was also found that there was no significant difference between S, MC and MSC groups compared to MC+MSC and control groups (Fig. 3).

The ratio of the muscles nuclei number to the fibrocytes nuclei number. The average of mn/fn in Tr group in the 2nd week showed a significant decrease compared to other groups ($p < 0.05$); but, between the control and cellular groups, there was not any significant difference. In the 4th week, the lowest mean was observed in the Tr group, which had a significant difference with the other groups ($p < 0.05$); but, S group increased the mean same up to the control group. In the 8th week, there was a significant decrease in mn/fn in Tr group compared to control and S groups ($p < 0.05$); but, in the cell receiving groups, in spite of increase, there was no significant difference with Tr group (Fig. 3).

The ratio of muscle nuclei number to the muscle fibers number. The results of the mn/mf in the 2nd week showed a significant decrease in this parameter in the Tr group compared to other groups ($p < 0.05$); while, in the 4th and 8th weeks, in S, MC and MC+MSC groups this ratio increased up to the extent that, there was no significant difference with the control group (Fig. 3).

The ratio of blood vessels number to muscle fibers number. The v/mf in the 2nd and 4th weeks was the lowest in Tr group compared to the other groups ($p < 0.05$); but, it increased in cell receiving or S groups, not up to the control group, and there was a significant difference between these groups with control group ($p < 0.05$). In the 8th week, the cell receiving groups increased the v/mf up to the control group and there was no significant difference with this group; while, in the S group, this ratio was almost same as Tr group (Fig. 3).

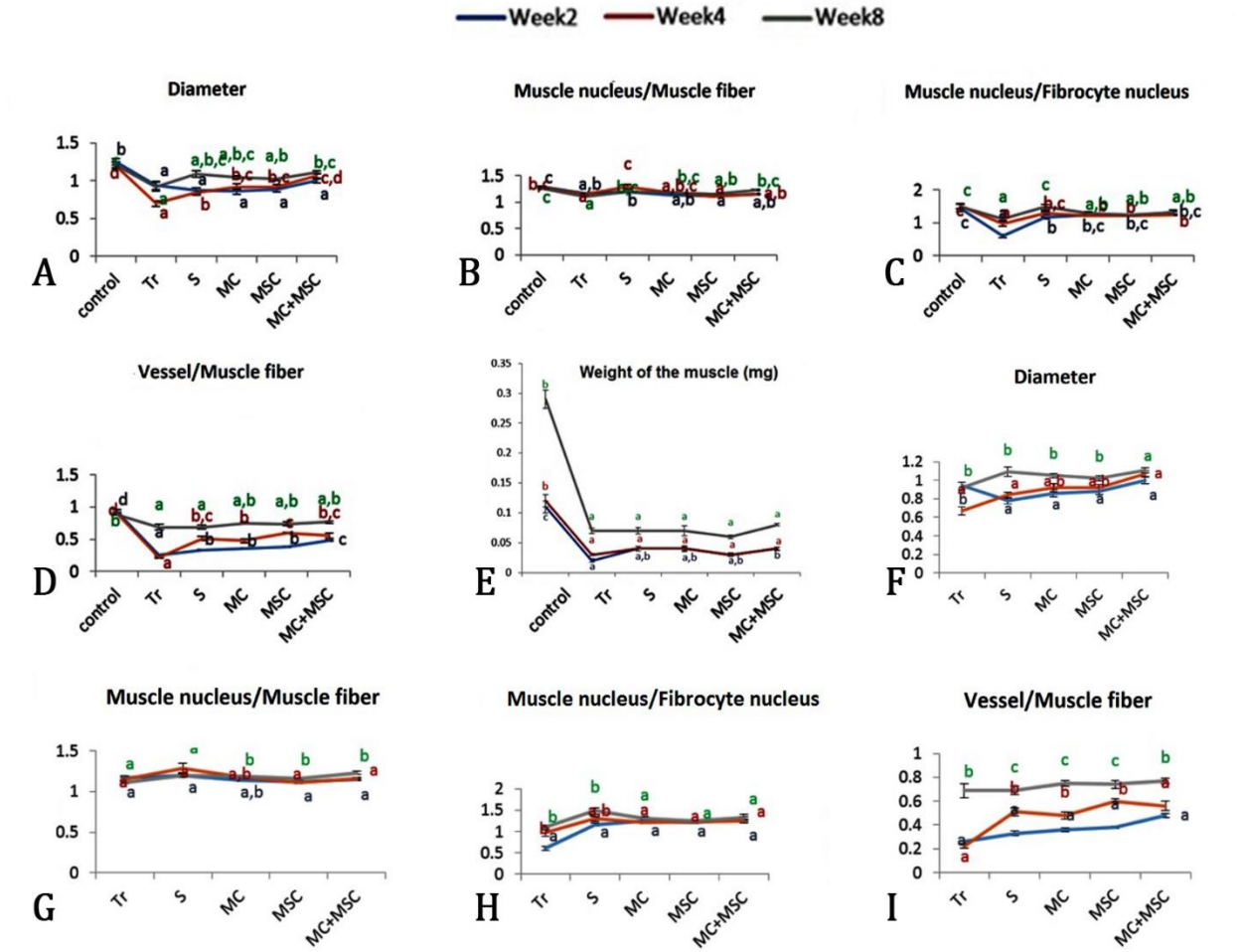

Fig. 3. Changes of muscle histomorphometrical parameters between groups. **A)** The average diameter of muscle fibers; **B)** Ratio of the muscle nucleus to the muscle fibers; **C)** Ratio of the muscle fibers nucleus number to the fibrocytes nucleus; **D)** The average ratio of blood vessels to muscle fibers number; **E)** Average weight of the muscle in different groups. abc Non-similar letters in the row indicate significant difference among groups ($p < 0.05$); Changes of muscle histomorphometrical parameters according to the time periods. **F)** The average diameter of muscle fibers; **G)** Ratio of the muscle fibers nucleus to the muscle fibers; **H)** Ratio of the muscle fibers nuclei to the fibrocytes nucleus; **I)** The average ratio of blood vessels to muscle fibers number. abc Non-similar letters in the columns indicate significant difference for parameters separately ($p < 0.05$).

Muscles weight. The evaluation of muscle weight in 2, 4 and 8 weeks in Tr group showed the dramatically decrease up to 3.50-4.20 times lesser than the control group; but, using of scaffold or cell led to increase in this parameter, that it was significant only in MC+MSC group compared to Tr group ($p < 0.05$). Furthermore, the highest weight between therapeutic groups was seen in MC+MSC group, especially in 8th week. Generally, cellular intervention improved the muscles weight in 8th week compared to the other time periods (Fig. 3).

Histomorphometrical changes of each group at different time periods. Measurement of the muscle fiber diameter showed an increase in all treatment groups at the 8th week ($p < 0.05$). Only in MC+MSC group, this increase was not significantly different from the 2nd and 4th weeks and in the S, MC and MSC groups, there was a significant difference with the 2nd week ($p < 0.05$; Fig. 3). The average of mn\fn in the cellular groups was not significantly different from any time periods; however, it increased non-significantly in the 8th week. Also, in the Tr and S groups, there was a significant increase in the 8th week compared to the 2nd week ($p < 0.05$; Fig. 3). The mn/mf in Tr and S groups was not significantly different in any time periods; but, it increased in the cell receiving groups in the 8th week, which this increase in MC and MC+MSC groups was significantly different in comparison with the 2nd and 4th weeks ($p < 0.05$); but, in the MC group it was not significantly different from the 2nd and 4th weeks (Fig. 3). The v/mf in all groups showed a significant increase in the 8th week compared to the 2nd and 4th weeks ($p < 0.05$; Fig. 3).

Discussion

Discontinuity or injury of the peripheral nerves inducing impaired or loss of muscle movement often results in the atrophy and structural changes in muscle tissue. These changes are mainly related to the diameter and number of muscle fibers nuclei or satellite cells, muscle tissue fibrosis and blood circulation alterations. The aim of this study was to investigate the muscle tissue changes after cutting of the sciatic nerve and its repair by tissue engineering using cell therapy in three-time intervals. Former study has shown that motor neurons damage in the skeletal muscle has a trophic role and in the absence of neural activity, it reduces muscle mass and diameter and a number of muscle fibers.[27]

When the muscle loses its stimulatory action as a result of motor neuron damage, it quickly becomes atrophic. Thus, muscle mass and diameter decrease, apoptosis occurs in myofibers[28] and the number of muscle fibers reduces.[29] Previous studies have shown that the diameter of muscle fibers decreases seven days after nerve transection in rats[30] and mice.[31] The degeneration process in axon after the nerve injury is known as Wallerian degeneration.[32]

In the present study, changes in the diameter of the muscle fibers affected by sciatic nerve transection indicated that application of cells especially in MC+MSC group in the 8th week resulted in a better recovery compared to the previous weeks being not significantly different compared to the control group in some cases.

There is a relationship between human muscle atrophy and the change in the number of satellite cells.[11,33] Reportedly, short-term hospitalization (5 days) in elderly adults (69.00 ± 2.00 years) causes fast muscle atrophy.[33] Muscle atrophy after denervation is associated with the activity of satellite cells, which their electrical stimulation increases the number of muscle fibers and formation of embryonic myosin heavy chain isoform fibers (an indicator of muscle regeneration) and accelerates the differentiation potential of satellite cells. According to Xing *et al*., this increase in differentiation improves muscle mass.[34] The mn/fn, in the 2nd week, showed that the muscle atrophy occurred in the Tr group and this reduction was also observed in the following weeks. Thus, this finding showed that along with the muscle tissue atrophy, fibrotic status and increased connective tissue occurred in the denervated muscles. In the cell receiving groups, due to the time-dependent nerve repair, this trend improved and this ratio condition progressed toward control group as there wasn't a significant difference with the control group in some cases. It was also found that in the group receiving only scaffold without cells, the aforementioned ratio increased dramatically compared to the Tr group, which may be due to the settlement of scaffold as a matrix in which peripheral cells of nerve transection site migrated into it, accelerating the recovery of the nervous system and improving the muscle mass condition.

Liu *et al*. have showed that the decreased number of myonuclei (derived from satellite cells) in the post-synaptic region of SC-depo mice is probably associated with the onset of gene expression needed for neuro-muscular junction regeneration.[35] In the present study, the nm/mf was evaluated to indicate the extent of improvement in muscle tissue. In the Tr group, this ratio was significantly lower than other groups and it was dependent on the time period. However, in the 8th week, the value of this ratio for S, MC and MC+MSC groups was recovered up to the control group. The reason that the combined cell group acts better than other cell groups can be related to the role of MCs in angiogenesis around the restored neural fibers[36,37] as well as the neurogenesis of these cells[38] and their cooperation with MSCs in differentiation to the Schwann cells.[18]

In this study, the improvement of muscle conditions as a result of the application of regenerative factors at the site of nerve transection was basically dependent on the amount of blood circulation. In this regard, the present study showed that the v/mf in the Tr group was lower than that in other groups; while, in the 4th week, in the

group receiving the scaffold, the value of the mentioned ratio was improved to a level equivalent to that in the control group. It was also found that in the 8th week, this ratio increased in cell groups. This finding could also be related to the improvement of the neural impulse and its interconnection with the blood vessels of the muscle tissue causing a better result of cell therapy. The histological changes of the muscles were also investigated in relation to the nerve repair, time-dependently in separate groups. Most vessels produce signals such as artemin and neurotrophin-3; so, the axons are placed in the vessels direction. The nerves also produce signals such as VEGF to stimulate vascular growth.[39] Thus, when the nerves are damaged; the number of vessels in their path is also reduced. On the other hand, there is a strong relationship between the muscle and the capillary beds in skeletal muscle;[40] so that, capillary death follows muscles atrophy.[41]

In the present study, sciatic nerve transection induced rapid muscle weight loss during 2 weeks. Considering that muscle weight compensation happens gradually, even with tissue engineering method return to normal conditions takes time.[42]

In general, the present study revealed that sciatic nerve damage caused extensor digitorum longus muscle atrophy. Furthermore, the mn/mf, mn/fn and v/mf decreased. However, use of MCs and MSCs and a combination of both cell types with scaffold accelerated nerve repair and improved the muscle condition. In addition, it was found that use of both types of cell at the site of sciatic nerve transection was better than other treatments.

Acknowledgments

This study was financially supported by Urmia University, Urmia, Iran, as a thesis grant and council for development of stem cell scientific and technologies. We wish to thank Dr. Esmaeil Mortaz, from the Immunology Laboratory of the Massih Daneshvari Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran, Dr Ali Karimi from Histology Laboratory, Mr. Aliasghar Aliari, from the Immunology Laboratory, Mr. Mohammad Asgary and Mr. Hossein Aghayarlou at the Faculty of Veterinary Medicine, Urmia University, Urmia, Iran, for their generous technical supports.

Conflict of interest

The authors have no conflict of interest to declare regarding the research described in this article and the manuscript preparation.

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