ORIGINAL ARTICLE

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Histopathological Changes of Extensor Digitorum Longus Muscle Following Sciatic Nerve Transection: The Role of Mast Cells and Mesenchymal Stem Cells

Zahra Bakhtiary[1,2], Rasoul Shahrooz[1], Rahim Hobbenaghi[3], Saeed Azizi[4], Farhad Soltanalinejad[1], Ali Baradar Khoshfetrat[5], Saeede Amani[6]

[1] *Department of Basic Sciences, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran*
[2] *Department of Histology, Faculty of Veterinary Sciences, Ilam University, Ilam, Iran*
[3] *Department of Pathobiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran*
[4] *Department of Surgery and Diagnostic Imaging, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran*
[5] *Department of Chemical Engineering, Sahand University of Technology, Tabriz, Iran*
[6] *Department of Veterinary Basic Science, Karaj Branch, Islamic Azad University, Karaj, Iran*

CORRESPONDING AUTHORS:
Zahra Bakhtiary; Rasoul Shahrooz
Department of Basic Sciences
Faculty of Veterinary Medicine
Urmia University
SERO Street
Urmia, Iran
E-mails: z.bakhtiary@ilam.ac.ir;
rasoul_shahrooz@yahoo.com

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(Animal study)

SUMMARY

Objectives. Damage caused by the sciatic nerve transection leads to histopathological changes of related skeletal muscles such as atrophy, necrosis, etc. In this study, these changes and ameliorative effects of using mast cells and mesenchymal stem cells along with collagen gel scaffold at the nerve's transection site were evaluated.

Methods. For this purpose, 90 male rats (180-200 gm) were randomly placed into 6 groups (n = 15) and each group included 3 time periods (2, 4, and 8 weeks, n = 5). The groups include; 1-control: no surgery, 2-nerve transection (Tr): sciatic nerve transection (at a distance of 8 mm) inside a silicone tube, 3-scaffold (S): using collagen gel, 4, 5 and 6-mast cell (MC), mesenchymal stem cell (MSC) and mast cell along with mesenchymal stem cell (MC+MSC): 3×10^4 for each cell mixed with collagen gel. Animals euthanized and sampled at weeks 2, 4 and 8 for muscle histomorphometry and histopathological evaluations.

Results. The mean number of mast cells and percentage of sarcoplasmolized fibers in the Tr group increased significantly ($p < 0.05$) and the MC+MSC group had the lowest average. Also, the highest percentage of necrotic fibers was observed in the Tr group which decreased in the MC+MSC group. This study showed that all parameters of each group significantly recovered in the eighth week compared to other time periods ($p < 0.05$).

Conclusions. The simultaneous use of mesenchymal stem cells and mast cells in the denervation place and the duration of time significantly reduced the histopathological changes of the denervated muscle tissue.

KEY WORDS
Histopathology; extensor digitorum longus muscle; mast cells; mesenchymal stem cells; rat.

INTRODUCTION

Complete transection of peripheral nerves causes neuro-muscular communication disorder (1) and following that, muscle movement disorder and the beginning of the atrophy process (2). Atrophy of skeletal muscles is accompanied by thinning of muscle fibers and the replacement of fibrous connective tissue and fat instead of muscle tissue (3). If there is no re-innervation, the necrosis of myofibers and muscle fibrosis will occur (4). Muscular atrophy in rodents starts 1-2 weeks after denervation, which was reported to be 14 days long in the study conducted on the hind limbs of rats (5).

Mast cells have high biological activities in the body (6) and

their most important role is to participate in allergic reactions and the body's immune system against pathogens and wound healing (7). These cells are scattered in all tissues, but they are especially seen in the vicinity of blood vessels and nerve fibers (8). Mast cells and nerves have a mutual relationship so that the secretions of mast cells stimulate nerves (9) and facilitate axonal reflexes (10).

Following the occurrence of nerve injuries, the interaction between mast cells and nerves can lead to the mechanisms of nerve tissue regeneration (11). Also, by secreting neuropeptides (12) and synthesizing, storing, and secreting nerve growth factor (NGF) in rodents (13) as one of the neurotrophin factors, mast cells maintain and develop the population of central and peripheral nerve cells (14, 15). In addition, mast cells and their main chemical mediator, histamine, have the potential to multiply neural precursors and neurogenesis (16). Another cell that has beneficial effects on nerve tissue regeneration in studies is mesenchymal cells (17-19). These cells have the ability to differentiate into neuron-like and Schwann-like cells in the laboratory environment and the body, *in vivo* and *in vitro* (20-22). Also, researchers believe that the cooperation of these cells in nerve regeneration is related to the secretion of NGF, brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), glial cell neurotrophic factor (GDNF), and neurotrophin-3 (NT-3) (23, 19, 24).

In fact, they facilitate the process of nerve regeneration through the expression of neurotrophins at the site of injury and increase the number of Schwann cells and ultimately increase axonal growth (25-27). In the study of Lopez *et al*., the use of MSC inside the collagen tube at the site of sciatic nerve injury increased the number of Schwann cells and myelinated fibers (28).

On the other hand, mast cells cause the proliferation of bone marrow stem cells with CD34 origin (29) and increase the proliferation of mesenchymal stem cells (30), in one study, mast cells injected into the heart of mice that had an infarct significantly increased cell proliferation and the number of MSCs (30). Therefore, this study was conducted with the aim of evaluating the regenerative power of mast cells and mesenchymal cells mixed with collagen gel at the site of sciatic nerve transection by examining the histopathological changes of the extensor digitorum longus muscle.

MATERIALS AND METHODS

Experimental design and animals

In this study, 90 adults male Wistar rats with an approximate weight of 150-180 grams were used in 6 equal groups and 3 time periods (2, 4, and 8 weeks). The animals were acclimatized for one week before the start of the experiment, and 14 hours of light and 10 hours of darkness with free access to standard rodent laboratory food and water were applied. All procedures were carried out according to the guidelines of the Veterinary Ethics commission of the Veterinary Faculty of Urmia University, Urmia, Iran (Reference No.: IR-UU-AEC-1221/PD/3 – date of approval: September 04, 2022).

All groups respectively:

1. Control group: No surgery was performed on the animals.
2. Transection group (Tr): The left sciatic nerve was transected by creating an 8 mm gap inside the silicone tubes.
3. Scaffold group (S): Collagen gel scaffold was placed inside silicone tubes.
4. Mast cell group (MC): 3×10^4 mast cells (31) mixed with collagen gel was placed inside silicone tubes.
5. Mesenchymal group (MSC): 3×10^4 mesenchymal cells (31) mixed with collagen gel were placed inside silicone tubes.
6. Mast cell-mesenchyme group (MC+MSC): In this group, 3×10^4 mast cells and 3×10^4 mesenchymal cells were injected into silicone tubes mixed with collagen gel.

*The silicone tubes used had an internal cavity with a diameter of 2 mm.

Surgical procedure

The rats were anesthetized by intraperitoneal injection of ketamine hydrochloride 5%, 90 mg kg^{-1} (Ketaset 5%; Alfasan, Woerden, The Netherlands), and xylazine hydrochloride 2%, 5 mg kg^{-1} (Rompun 2%, Bayer, Leverkusen, Germany) and after preparing the surgical section (left thigh). The sciatic nerve was taken out before its bifurcation into the tibial and peroneal nerves and was transected with an 8 mm gap in all groups except the control (**figure 1**).

In the end, after placing the cell or scaffold according to the treatment group, the incision site of the gluteal muscle was sutured with 40 polyglycolic acid thread and the skin of the area was sutured with 4-0 nylon thread.

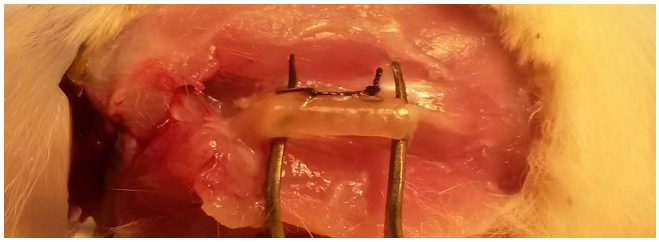

Figure 1. Fixation of transected nerve in silicone tubes.

Sampling and preparation of sections

Animals were euthanized 2, 4, and 8 weeks after surgery and the extensor digitorum longus muscle were sampled. Then the samples were fixed in the 10% formal saline solution and paraffin sections (5-7 µm) were prepared using a rotary microtome (Microm, GmbH, Germany). Then they were stained with hematoxylin-eosin (H&E) and toluidine blue to study the percentage of sarcoplasmolized and necrotic fibers and the average number of mast cells respectively. The sections were examined under a light microscope (CX41, Olympus, Japan) equipped with a digital camera (DP25, Olympus, Japan). In each treatment, 5 areas in a fixed microscopic field (1 mm^2) were counted and the resulting data were compared statistically.

Isolation and culture of mice mast cells

Following the mice were euthanized using an overdose of ketamine-xylazine (3 × anesthesia dose), non-adherent cells were isolated from bone marrow and differentiated into mast cells using mitogens obtained from spleen cell culture (32). According to the results of flow cytometry, a purity percentage of mast cells were reported to be more than 90% (33).

Isolation of mesenchymal stem cells from rat

After euthanizing the rats, bone marrow mesenchymal stem cells were isolated from the tibia and femur of rats according to standard scientific methods (34). The results obtained from the flow cytometry of these cells reported the purity percentage above 90% (33).

Preparation of collagen gel scaffold

In order to, a 1% solution of collagen isolated from the rats tail (2.16 mg mL^{-1} protein in 0.60% acetic 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 (33).

*Mast cells and mesenchymal stem cells (3 × 10^4 cells of each) were implanted in the collagen gel scaffold and 10 µl of it was placed inside the silicone tubes at the site of nerve transected.

Statistical analysis

The results were analyzed by SPSS software (version 20, SPSS Inc., Chicago, IL, USA), one-way ANOVA, and Tukey post hoc test. Results were evaluated as mean ± SE and significant differences between all groups were set at $p < 0.05$.

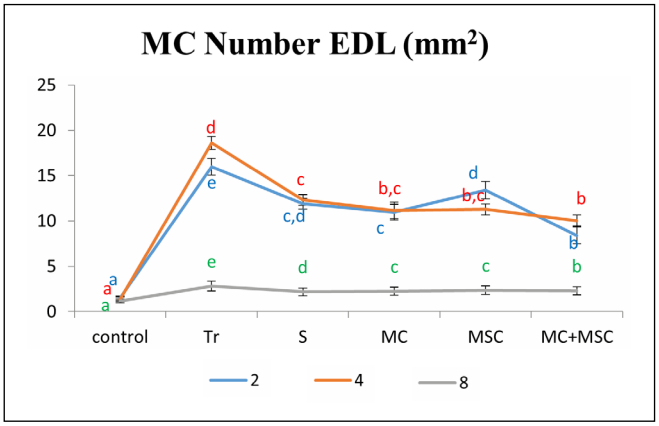

Figure 2. The average number of EDL muscle mast cells among different groups.

Non-similar letters in similar colors indicate significant differences between the groups ($p < 0.05$).

RESULTS

Histopathological results of extensor digitorum longus (EDL) muscles in different groups and time periods

The mean number of mast cells

In the results obtained, the lowest average in all time periods was related to the control group, which had a significant difference from all the treatment groups ($p < 0.05$). The highest average obtained in all time periods was also observed in the Tr group, and this increase was significantly reported with other groups ($p < 0.05$). The lowest mean number after the control group was reported in the MC+MSC group in all time periods, so in weeks 2 and 8, the reduction was significant with other treatment groups ($p < 0.05$). In the fourth week, all the cell and scaffold groups showed a significant decrease compared to the Tr group ($p < 0.05$) (**figures 2** and **3**).

The average number of mast cells in each group at different periods

According to the results of this study, the lowest average number of mast cells among the three periods in different groups is related to the eighth week. The highest average in the Tr group was in week 4 and in the MSC group was in week 2, and a significant difference was seen between the second and fourth weeks ($p < 0.05$). In other groups, no significant difference was observed between weeks 2 and 4 (**figure 4**).

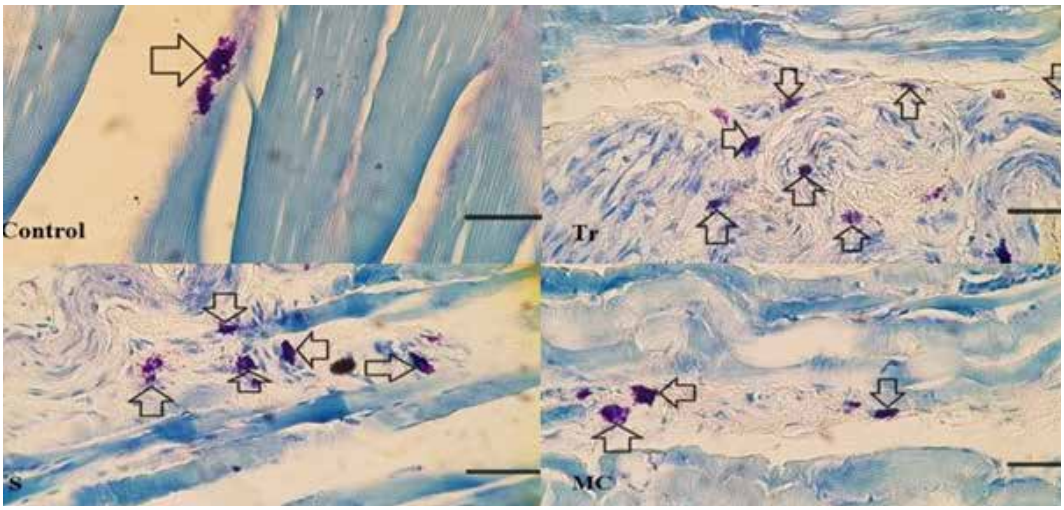

Figure 3. Mast cells stained by Toluidine Blue of the EDL muscle (arrows) in different groups. Scale bar = 40 µm.

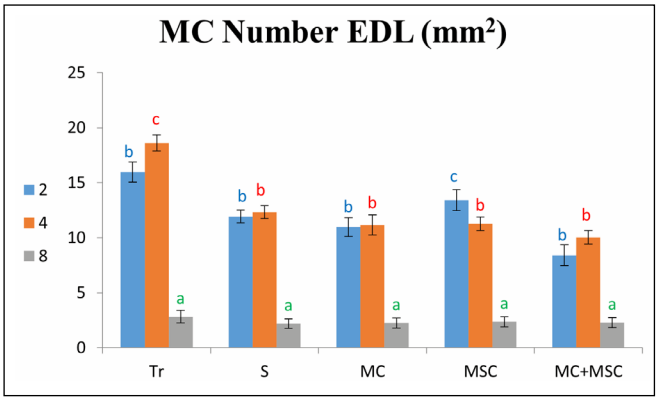

Figure 4. The average number of EDL muscle mast cells in each group separately at different periods.

Non-similar letters in similar colors indicate significant differences between the groups ($p < 0.05$).

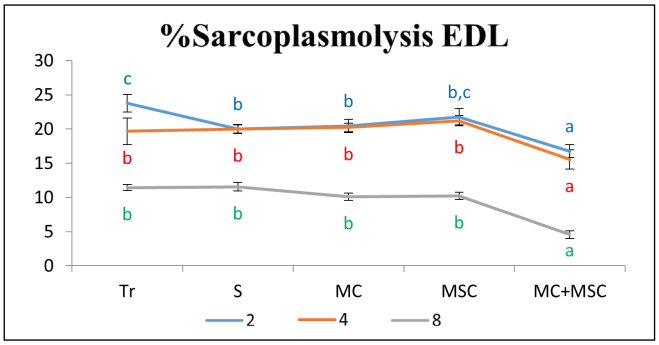

Figure 5. The average percentage of sarcoplasmolized muscle fibers of the EDL muscle among different groups.

Non-similar letters in similar colors indicate significant differences between the groups ($p < 0.05$).

The average percentage of sarcoplasmolyzed muscle fibers

The results of this study showed that the average percentage of sarcoplasmolized muscle fibers in the Tr group had a significant difference with the MC+MSC group in all the investigated weeks ($p < 0.05$), while there was no difference with the other groups in the fourth and eighth weeks. In addition, the Tr group had a significant difference with S and MC groups in the second week ($p < 0.05$) (**figures 5** and **6**).

The average percentage of sarcoplasmolyzed muscle fibers of each group at different time periods

In this study, the average number of sarcoplasmolized fibers in week 8 in all experimental groups was lower than in the

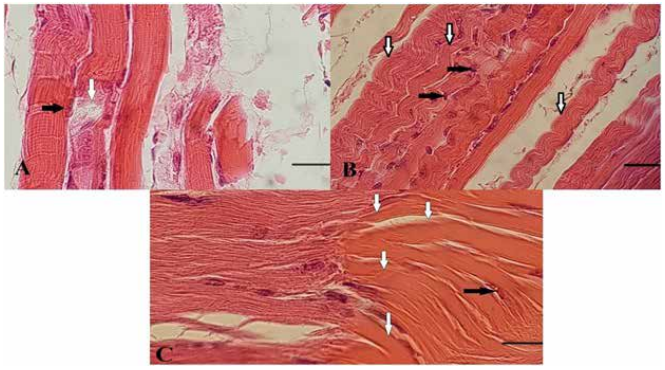

Figure 6. **(A)** White arrows indicate sarcoplasmolized muscle fibers and black arrows indicate euchromatin nucleus; **(B,C)** White arrows indicate necrotic curly muscle fibers and black arrows indicate pyknosis nuclei.

Hematoxylin-eosin staining; scale bar = 40 µm.

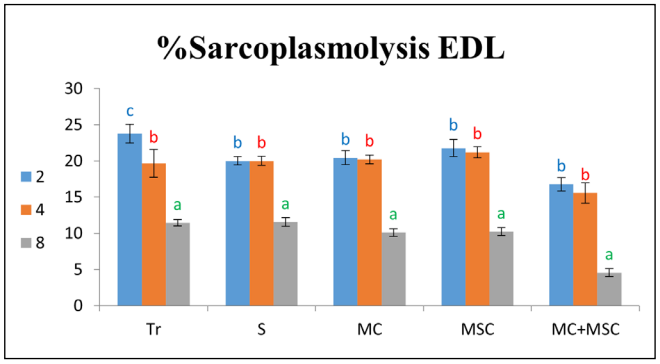

Figure 7. The average percentage of sarcoplasmolized muscle fibers of the EDL muscle of each group separately at different times.

Non-similar letters in similar colors indicate significant differences between the groups ($p < 0.05$).

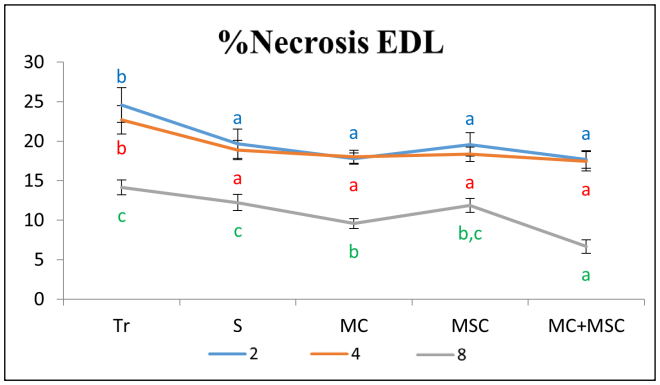

Figure 8. The average percentage of necrotic EDL muscle fibers among different groups.

Non-similar letters in similar colors indicate significant differences between the groups ($p < 0.05$).

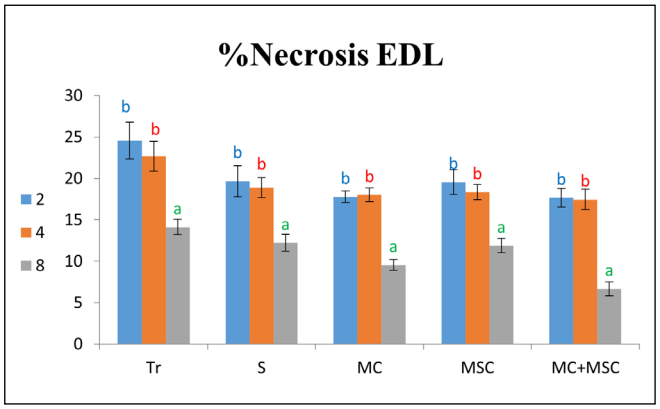

Figure 9. The average percentage of necrotic EDL muscle fibers of each group separately at different times.

Non-similar letters in similar colors indicate significant differences between the groups ($p < 0.05$).

second and fourth weeks, and there was a significant difference between those time periods ($p < 0.05$). It was also shown that there is no significant difference between weeks 2 and 4 in all groups except the Tr group. In the Tr group, all three periods had significant differences ($p < 0.05$), and the highest average was observed in the second week ($p < 0.05$) (**figure 7**).

The mean percentage of the number of necrotic muscle fibers

The results of these parameters showed that the highest number of these fibers was in the Tr group and the lowest in the MC+MSC group in all three time periods. Also, in weeks 2 and 4, there was a significant difference between the Tr group and other groups ($p < 0.05$), but in the eighth week, a significant difference was observed only with the MC and MC+MSC groups ($p < 0.05$) (**figures 8** and **9**).

The mean percentage of the number of necrotic muscle fibers of each group in different periods

In this study, the lowest average number of necrotic fibers in all groups is related to the 8th week, which had a significant difference with the 2nd and 4th weeks ($p < 0.05$). Also, no significant difference was observed in any of the groups between weeks 2 and 4 (**figure 9**).

DISCUSSION

During the damage muscle that occurs after denervation, the muscle fibers are completely decomposed due to myolysis (35). This happens due to the disruption of the sarcolemma and calcium imbalance, and as a result, proteases are activated and muscle is broken down (36). However, in the parts of the muscle fibers that have undergone sarcoplasmolysis, the sarcoplasm of the fibers becomes eosinophilic (hyaline) and striation is removed, but the sarcolemma is preserved (37).

In fact, the myofibrils are destroyed and an empty space surrounded by sarcolemma is created (37). This complication, which is also called myocytolysis, has been observed following the toxic effects of furazolidone in the heart of poultry and potassium deficiency in rats (37). The findings of this study showed that the mentioned parameter was more or almost equal in the Tr group compared to other groups but in relation to the group of combined cells, it decreased significantly, which shows the synergistic effect of mesenchymal cells and mast cells in nerve regeneration and the reduction of the percentage of sarcoplasmolysis in this group compared to other

intervention groups, especially Tr. Also, in the evaluation of the eighth week of each group, a lower percentage of sarcoplasmolized fibers were seen than at other periods. Therefore, the time parameter has been very effective in improving the conditions.

Denervation can lead to irreversible structural damage to the muscle, including necrosis of muscle fibers, increase in connective tissue, and damage to muscle tissue (38). Necrosis of muscle fibers in the early stages can be seen in a wavy and curly shape in longitudinal sections (39) and then in the necrotic parts of the muscle, along with the loss of sarcolemma and pyknosis of nuclei, sarcoplasm and myofibrils become hyaline (37).

This hyaline degeneration, which is characterized by eosinophilia in the cytoplasm of muscle fibers, is a prominent feature in the necrotic phase of damaged muscles (40). In the continuation of this process, sarcoplasm becomes follicular and myofibers are decomposed (39). In examining the percentage of necrosis in the EDL muscle, the Tr group showed the highest percentage and the MC+MSC group showed the lowest percentage of necrosis, and in the eighth week, due to sufficient time for nerve regeneration and subsequent muscle repair, the effects in all groups Improvement was observed, and the highest improvement rate were related to the MC+MSC group.

This finding shows that, along with the time parameter, the use of cells that have synergistic cooperation with each other has an effective role in improving the conditions of muscle necrosis. Necrosis and muscle eosinophilia observed as a result of denervation in this study are also observed in other muscle disorders (41) such as Duchenne muscular dystrophy (40). On the other hand, denervation to the muscle, if it is accompanied by a recovery in the first 2 months after the injury, can lead to improvement in the functional status and muscle mass (42, 43), but if there is no recovery over time due to the increase of necrosis of muscle fibers and the proliferation of connective tissue, the muscles are less receptive to regenerated motor axons (44).

The results of this study showed that the Tr group showed the highest percentage of necrosis and the MC+MSC group showed the lowest percentage of necrosis, and in the 8th week, due to sufficient time for nerve regeneration and then muscle repair, recovery effects were observed in all groups that the highest recovery rate was in the MC+MSC group. This finding shows that, along with the time parameter, the use of cells that have synergistic cooperation with each other has an effective role in improving the conditions of muscle necrosis.

Physiologically, skeletal muscles have a number of mast cells in the epimysium, perimysium and to a very small extent in the endomysium (45). In fact, mast cells have a physiological role in muscle repair through the secretion of trophic and inflammatory factors such as vascular activating mediators (46). The number of these cells increases during inflammation and tissue damage and has the potential to break down myofibers through the secretion of proteases (47).

In the studies conducted on denervated muscles or myopathy, the accumulation of mast cells in the endomysium of the muscle has been confirmed (48). Also, in the evaluation of the extensor digitorum longus muscle in amyotrophic lateral sclerosis, an increase in the number of mast cells, especially in the endomysium, is evident (49). In this study, the control group had the lowest average number of mast cells, while the Tr group had the highest average number due to nerve damage. Also, in the S and cell groups, this average decreased compared to the Tr group, and the MC+MSC group had the lowest average of mast cells after the control group. This finding shows that the use of cells or scaffolds, especially combined cells, by accelerating the healing process of the transected nerve, has led to the modulation of cell reactions in the muscle tissue. In addition, this result was observed in all 3 time periods.

Also, the examination of the average of mast cells in different periods of time reported the lowest amount in the eighth week, which had a significant difference from other times. This condition also shows the effect of the passage of time on reducing the inflammatory conditions of the muscle tissue.

CONCLUSIONS

The results of the present study showed that the mast cells and mesenchymal cells, the synergistic effect of these two cells on each other, and the passage of time caused a faster repair of the nerve and subsequently the EDL muscle, which was accompanied by a significant reduction in histopathological changes of the muscle.

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DATA AVAILABILITY

All data generated and/or analyzed during the current study are included in this published article.

CONTRIBUTIONS

RS, ZB: conceptualization, investigation, histomorphometric studies, resources. ZB: cell-culture. SA, ZB: surgery. FS: anatomical works. ABKH: scaffold production. RHN: pathological studies. RS: supervision. ZB: writing – original draft. RS, SAM: writing - review & editing.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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