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The effect of bone marrow mesenchymal stem cells on recovery of skeletal muscle after neurotization surgery in rat

Gholam Hossein Farjah [1]*, Farzaneh Fazli [2], Mojtaba Karimipour [3], Bagher Pourheidar [1], Behnam Heshmatiyan [4], Maryam Pourheidar [2]

[1] Neurophysiology Research Center, Department of Anatomy, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran
[2] Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran
[3] Department of Anatomy, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran
[4] Neurophysiology Research Center, Department of Physiology, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran

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ABSTRACT

Objective(s): When the nerve is injured near its entrance to the muscle belly, we cannot perform conventional methods. One useful method in such a situation is neurotization surgery. In this study, Bone marrow mesenchymal stem cells (BMSCs) implanted into the paralyzed muscle after neurotization surgery. These cells can stimulate axon growth and motor endplate formation, also prevent muscle atrophy.

Materials and Methods: Thirty-six adult male Sprague-Dawley rats were randomized into six groups: intact group, sham surgery group, control group, DMEM group, cell+DMEM group, denervated group. The motor nerve of the lateral head of gastrocnemius muscle was cut, and the proximal portion of the severed nerve was transplanted to the proximal third of the muscle paralysis. BMSCs with/or DMEM was injected into the site of injury. All animals were evaluated by withdrawal reflex latency (WRL), electromyography, muscle weight, histology and immunohistochemistry.

Results: The WRL difference between the control and cell+DMEM groups at weeks 4 and 12 post-operation was statistically significant ($P<0.05$). The mean number of motor end plates in cell+DMEM group was more than control group ($P<0.05$). At 12 weeks post-operation, the difference of the mean nerve conduction velocity (NCV) between cell treated group and sham surgery groups were not statistically significant ($P>0.05$). In weeks 4 and 12 post-operation, the mean fiber diameters in cell+DMEM group were more than control group ($P<0.05$).

Conclusion: The results of this study demonstrate that transplantation of BMSCs after neurotization surgery, prevent muscle atrophy and improve muscle function.

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Introduction

Peripheral nerve regeneration is a serious clinical problem (1). These injuries are the result of trauma, accident and surgery can cause paresis in human and therefore impact on beauty of person, employment opportunities and quality of life individuals (2). Researchers use many techniques like, epineurial suture, nerve autograft and nerve guidance channels for treatment of peripheral nerve injuries (3). Neurotization (reinnervation of muscular fibers) is one of the techniques used for to face various problems, including: repair of nerve, implantation of nerve in the muscle (neuro-muscular), and muscular neurotization (4). When the nerve is injured near its entrance to the muscle belly, conventional methods of nerve repair will be useless, because of the distal stump of the injured nerve was not observed. One of the best methods in such a situation is direct neurotization (5). In this method, the proximal end of severed nerve is directly placed into the paralyzed muscle and the recovery of muscle function assessed (6). Researchers use many neurotrophic factors (7) and extracellular matrix molecules to help axonal sprouting and establishment of motor end plate between nerve and muscle fibers in order to prevent muscle atrophy (8). In some studies, for inducing axon growth and neovascularization, researchers used cell transplantation method after neurotization surgery (7, 9). Mesenchymal stem cells (MSCs) are multi-potent progenitor cells (10) that scientists used for sciatic nerve (8), and spinal cord injury repair (11). BMSCs have a high potential in nerve regeneration via secretion of nerve growth factor, brain-derived growth factor and glial cell-derived growth factor (7, 12). Furthermore, these cells can secrete extracellular matrix molecules like laminin, fibronectin and collagen I and IV, which stimulate axon growth and motor end plate formation, also BMSCs prevent muscle atrophy

*Corresponding author: Gholam Hossein Farjah. Neurophysiology Research Center, Department of Anatomy, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran. Tel: +98-441-2770698; Fax: +98-441-2780800; Email: hfarjah@hotmail.com

after denervation (13, 14). In many studies, use of BMSCs and neurotrophic factors for peripheral nerve regeneration. Although, scientists use of direct neurotization in order to prevent muscle atrophy, but researchers do not study on the effect of BMSCs after direct neurotization. So this study designs for evaluating the impact of BMSCs on recovery of skeletal muscle after direct neurotization in the rat as a laboratory model.

Materials and Methods

Animal

Thirty-six adult male Wistar rats (200-250 g) were selected and kept in the same condition with 12 hr light and 12 hr darkness. Animals randomly divided into six groups: intact group, sham surgery group, control group (direct neurotization without injection), DMEM group (direct neurotization+DMEM injected), cell+DMEM group (direct neurotization and BMSCs+DMEM injected), denervated group. The experimental procedures were approved by the Ethical Committee of Urmia University of Medical Sciences.

Isolation and culture of bone marrow stem cells

Isolation and culture of BMSCs were, according to a previously published method (15). Briefly, from 1-month- old Wistar rats by flushing femurs and tibias under sterile conditions with Dulbecco Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS) and 1% penicillin streptomycin (Sigma Aldrich). Cells were then plated in 75 cm^2 culture flasks. After 3 days, nonadherent cells were removed along with the culture medium, and only adherent cells were further cultured in complete medium for four passages. Then the cells from several flasks were pooled, and number of cells was estimated by counting in a Burker's chamber. The final concentration was adjusted to about 1×10^6 cells/50 µl. In order to identify BMSCs into the muscle, 72 hr before transplantation, Bromodeoxyuridine (Brdu, sigma, 3 µg/ml) were added to the medium of the flask. The percentage of BrdU-labeled BMSCs was 95%. BMSCs identity is confirmed on the basis of morphological criteria and plastic adherence.

Surgical procedure

Rats were anesthetized using intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). A longitudinal incision was made on the posterior surface of the left thigh, and the tibial, common peroneal, and sural nerves were identified. The operation was terminated at this point in the sham-surgery group. The motor nerve of the lateral head of gastrocnemius muscle was cut, and the proximal portion of the severed nerve was transplanted to the proximal third of the muscle paralysis. A single 10/0 nylon suture was used to stabilize the epineurium of nerve stump to epimysium (5). In the control group, direct neurotization were done without injection. In DMEM group, direct neurotization were done and 50 µl DMEM were injected into the lateral head of the gastrocnemius muscle. In cell+DMEM group, direct neurotization was done and BMSCs+DMEM (approximately 1×10^6 cells in 50 µl) were injected into the lateral head of gastrocnemius muscle (16). In denervated group, the nerve of the lateral head of gastrocnemius muscle cut and the entrance of gluteus maximus muscle. After neurotization surgery, the cells that marked with Brdu were injected into the lateral head of the gastrocnemius muscle.

Neurobehavioral study

Four and 12 weeks post-operation, sensory functional recovery was evaluated by measuring withdrawal reflex (WRL). WRL was defined as time that rat withdraw hind paw because of heat. For this purpose, each rat was wrapped in a surgical towel, and then the affected hind paw positioned on a hot plate (DID SABZ Instrument Inc, Model DS8310) set in 56 °C, the time was measured from the onset of contact to withdrawal of the hind paw, with a stopwatch. The maximum time for this neurobehavioral test was 12 sec to avoid skin damage to the foot (17).

Electromyography

After WRL evaluation, the animals in each group were subjected to electromyograpy studies using Narco bio-system (USA). During the test, their body temperature was kept constant between 36.5-37 °C using a temperature control unit (Narco, USA). Under intraperitoneally urethane anesthesia (1 g/kg), the left sciatic nerve (operated side) was re-exposed by incision of the previous surgical site in the mid-thigh level. Stimulating electrodes were placed in the tibial nerve, and a recording electrode was inserted into the gastrocnemius muscle. The difference in electromyography latency, amplitude and distance between the proximal and distal stimulation sites was measured to calculate nerve conduction velocity (18).

Muscle weight

After neurobehavioral and electromyography assessment, gastrocnemius muscle completely detached from the bone, the macroscopic appearance of neurotized muscle was evaluated, and wet weight of muscle was measured by digital scale (19).

Histological examination

The gastrocnemius muscles were fixed in 10 % formalin solution, and cross sections of the reinnervated muscles stained with Hematoxylin-Eosin (H-E). The diameter of muscle fibers was measured with calibrated eyepiece. Methylene blue staining were used to examine and count the number of motor end

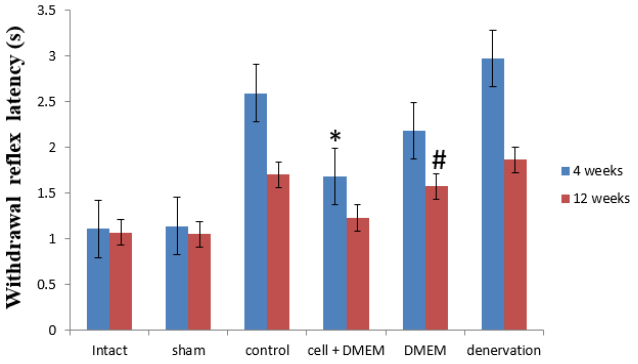

Figure 1. Withdrawal reflex latency (s) at 4 and 12 weeks after surgery, (± SEM)
* The significant statistical difference between cell+DMEM and control groups at 4 and 12 weeks post-operation, (P<0.02)
# The significant statistical difference between DMEM and cell+DMEM groups at 12 weeks post-operation, (P<0.05)

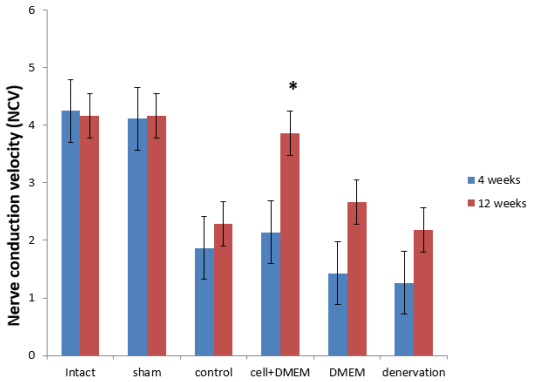

Figure 2. Nerve conduction velocity (NCV) at 4 and 12 weeks post-operative, (± SEM)
* The significant statistical difference between cell+DMEM in comparison with control, DMEM, and denervation groups at 12 weeks post-operation, (P<0.05)

plate. So, that, sections were taken to include the transplanted nerve and attached muscle such that the nerve branches and nerve terminals were parallel with along axis of the muscle (20).

Immunohistochemistry examination

Immunohistochemical staining was applied to detect cells derived from MSCs at 4 and 12 weeks post-operation. Briefly, a series of adjacent 6 µm thick sections (25 µm interval) from each block were incubated in 50% formaldehyde (Merck, Germany), 2×SSC (standard sodium citrate: 0.3 M NaCl, and 0.03M sodium citrate) at 65 ˚C for 2 hr, washed for 10 min with 2×SSC at room temperature. They were then incubated in 2 M HCl (Merck, Germany) at 37 ˚C for 30 min, rinsed in 0.1M boric Acid (Merck, Germany) for 10 min, washed in PBS, and incubated with mouse anti-BrdU monoclonal antibody (Sigma Aldrich) at 4 ˚C overnight. After rinsing 3 times in PBS for 10 min, the sections were incubated overnight in the dark at 4 ˚C with Rhodamine conjugated secondary antibody (1: 100), washed in PBS, covered with a cover slip, and were studied under a light microscope (21).

Statistical analysis

All data were analyzed by using a one-way analysis of variance (ANOVA) followed by the Turkey's test. P<0.05 were considered to be statistically significant. All calculations were conducted using SPSS 16.0 software.

Results

Two rats died in the perioperative period (probably because of overdosing of the anesthetic drug).

Neurobehavioral study

Withdrawal reflex latency (WRL) was significantly lower in the cell transplantation group than in the denervation group 4 and 12 weeks post-operative and the difference were significant (P<0.05). Four Weeks post-operative, WRL was significantly improved in the cell transplantation group, while the difference between cell transplantation and DMEM groups was not identified (P>0.05). However, all WRL values were significantly more than those seen in the intact and sham surgery groups (P<0.05). At 12 weeks post-operation, although WRL in the intact and sham surgery groups were lower than cell+DMEM group, but the difference was not statistically significant (P>0.05) (Figure 1).

Muscle weight

4 weeks post-operative, the mean weight of muscles in all experimental groups reached its lowest value but there were significant statistical differences between the cell+DMEM group (1.6 ±0. 27) and denervation group (0.8 ± 0.22) (P<0.001). The result showed that there was a significant difference between the intact and sham surgery groups in comparison with other experimental groups (P<0.01). Twelve weeks post-operative, although the muscle mass in the cell+DMEM (2.1±0.31) and DMEM (1.89±0.97) were lower than the intact and sham surgery group (2.3±0.34, 2.3±0.33, respectively), but the difference was not significant (P>0.05).

Electromyography study

Electromyography stimulation studies indicated that the nerve conduction velocity (NCV) of the animals in the cell+DMEM group improved over time. Foure weeks post-operation, there was a significant difference between the intact and sham surgery groups in comparison with other experimental groups (P<0.002). Twelve weeks post-operative, the differences were identified between cell+DMEM in comparison with DMEM, denervation, and control groups (P<0.05). In the cell+DMEM group, the measured values were lower than those measured in the intact and sham surgery groups, but the differences were not statistically significant (P>0.05) (Figure 2).

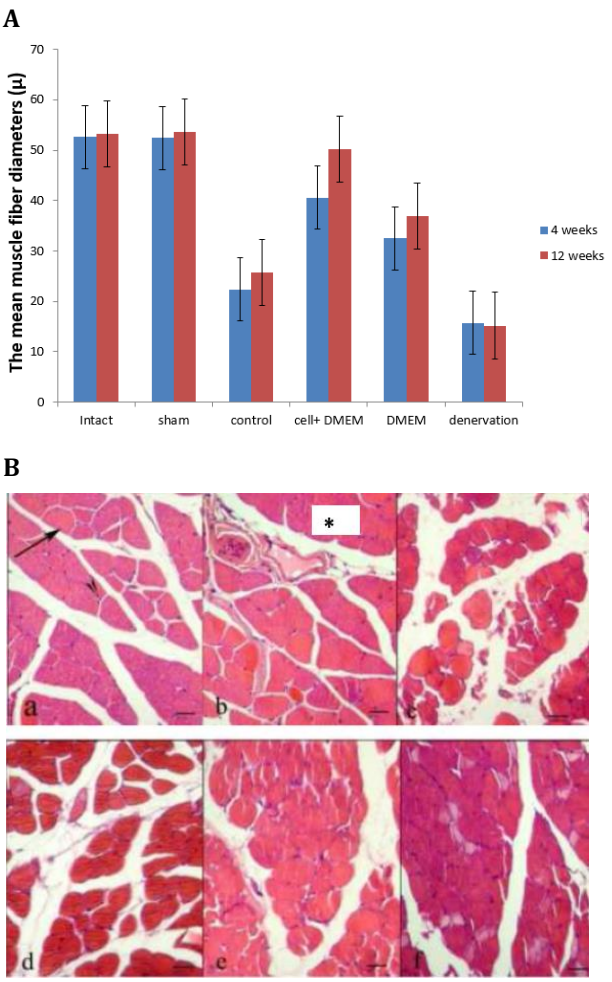

Figure 3. A. The mean muscle fiber diameters (µM) at 4 and 12 weeks post-operation, (±SEM). *The significant statistical difference between cell+DMEM in comparison with control, and denervation groups at 12 weeks post-operation, (P<0.05). **B.** Cross-section of the rat gastrocnemius muscle stained with hematoxylin and eosin (H&E), showing fiber morphology after reinnervation. (a): normal group, (b): sham surgery group, (c): denervation group, (d): control group, (e): DMEM group, (f): cell+DMEM group. Muscle fiber diameter and nucleus of muscle fiber (black arrows). The section was taken from the lateral head of gastrocnemius muscle 12 weeks afrer neurotization surgery (Scale bar 50 µm)

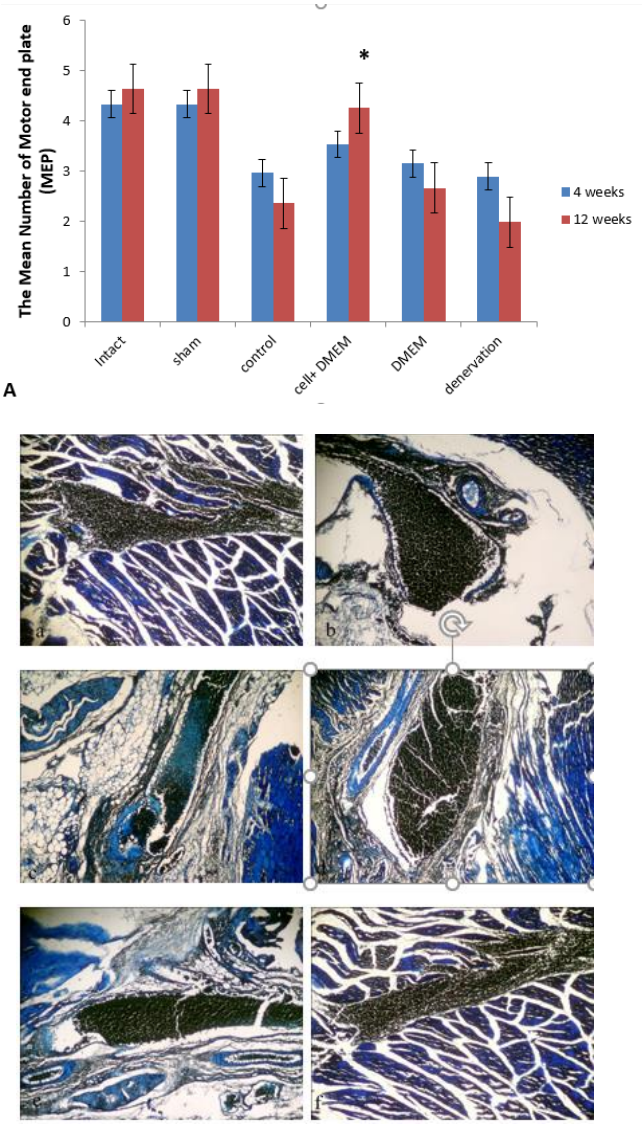

Figure 4. A. The mean number of motor end plate (NEP) at 4 and 12 weeks post-operation, (±SEM). * The significant statistical difference between cell+DMEM in comparison with control, DMEM, and denervation groups at 12 weeks post-operation, (P<0.05). **B.** Longitudinal-section of the rat gastrocnemius muscle stained with methylene blue, showing motor end plate after reinnervation. (a): normal group, (b): sham surgery group, (c): denervation group, (d): control group, (e): DMEM group, (f): cell+DMEM group. Muscle fiber diameter and nucleus of muscle fiber (black arrows). The section was taken from the lateral head of gastrocnemius muscle 12 weeks after neurotization surgery

Histological and immunohistochemical assessment

The mean muscle fiber diameters were measured 4 and 12 weeks post–operative in a cross-section. Some of them rounded, others polygonal shaped. Small branches of nerve were evident in the cross-section of the muscles. 4 weeks post–operative, the result indicated that the mean muscle fiber diameters in all experimental groups were lower than intact group and the differences were statistically significant (P<0.02) (Figure 3A). In the later period of reinnervation (12 weeks post-operation) most of the fibers were large and oval shaped in the cell+DMEM group. The result showed that preservation of muscle structure with less fiber atrophy in the cell+DMEM group, and the mean muscle fiber diameters in cell+DMEM group in comparison with control and denervated groups were significant differences (P<0.05). Although the mean muscle fiber diameters in cell+DMEM group were more than the DMEM group, but this difference was not significant (Figure 3-A). Twelve weeks post-operation, in the denervated muscle group can observe significant atrophy of muscle, marked by a reduction in the fiber size and an increase in connective tissue (Figure 3B).

After methylene blue staining, the number of motor end plate with a reinnervated axon was counted. The

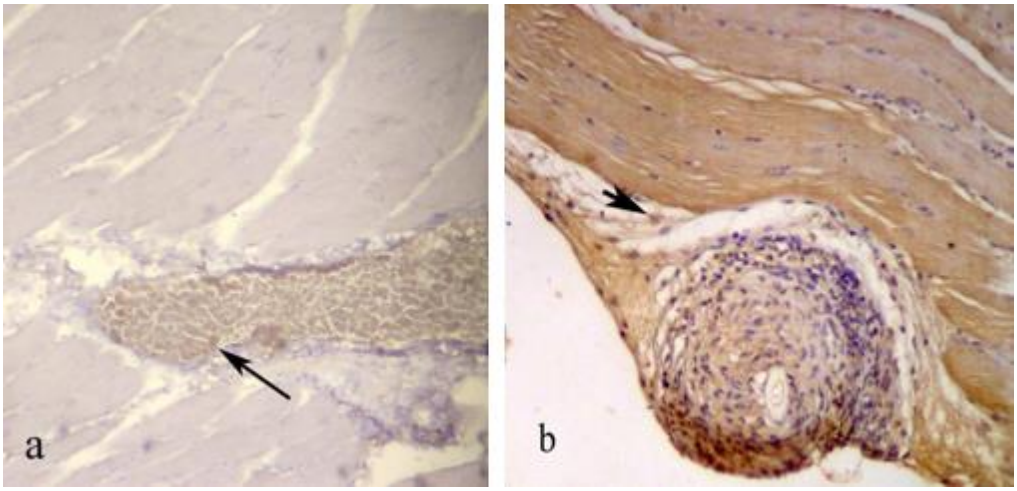

Figure 5. Immunohistochemical study of cross-section to the main axis of the gastrocnemius muscle 4 (a) and 12 (b) weeks post-operation. The scattered Brdu-positive cells were detected in muscle. The Most of the Brdu-positive cells were located near the motor end plate

result indicated that after 4 weeks, the number of motor end plate in experimental groups was significantly lower than intact groups (P<0.002). Twelve weeks post-operation, the mean number of motor end plates in cell+DMEM group was more than DMEM, control, and denervated groups and the differences were significant (P<0.03). The result showed that although the mean number of motor end plates in cell+DMEM group was lower than intact and sham surgery groups, but there were no statistical differences between these groups (P>0.05) (Figure 4). Immunohistochemical study showed that the scattered Brdu-positive cells were detected in muscle. The most of the Brdu-positive cells were located near the motor end plates (Figure 5).

Discussion

The finding of this study demonstrated that BMSCs significantly improved the recovery of the paralyzed muscle in rats with a direct nerve implantation.

Several studies have demonstrated that direct neurotization of an injured proximal nerve stump can grow into the paralyzed muscle and establish functional connections (motor end plates) (5). In our study, it seemed that the transplantation of adult BMSCs compared with DMEM, control, and denervated groups enhance nerve repair after direct neurotization. Bone marrow provides a source of hematopoietic and nonhematopoietic stem cells (22). The precursors of nonhemattopoietic tissues are referred to as BMSCs, their multipotentiality for differentiation, and their possible use for both cell and gene therapy (23). A previous study demonstrated that the intravenous injection of BMSCs significantly improved the recovery of hind limb motor function in rats with a spinal compression lesion (24). Several investigations about transplantation of adult BMSCs directly into the adult rat brain and spinal cord showed that reduces functional deficits associated with stroke, traumatic brain injury, and spinal cord injury (25).

BMSCs can differentiate into a variety of cell types including myoblasts (26), adipocytes (27), osteoblasts (28), and chondroblasts (29), when placed into different microenvironments. In addition, the adult rat and human BMSCs can differentiate into neuronal phenotypes *in vitro* (30), and leads to extensive remyelination (31).

In the present study, the mean weight of muscles was significantly greater for the cell+DMEM group *vs.* the control group, but this difference was not significant in the DMEM group. As for skeletal muscle, denervation due to nerve injury or surgical division (e.g. for free muscle transfer) leads to muscle atrophy, as evidenced by a reduction in the number and size of muscle fibers, with preservation of residual connective tissue (6). So that muscle weight would expect to correlate with the degree of innervations of a muscle (32). A denervated muscle mass can lose up to two-thirds of its mass within 1 month (33). On the other hand, in this study, the mean muscle fiber diameter (μm) was significantly greater for the cell+DMEM group *vs.* the control group, but this difference was not significant in the DMEM group. BMSCs produce muscle when implanted in the appropriate tissue *in vivo*, and may serve to replenish lost cells; moreover, they contain a variety of cells that secrete substances that may lead to recovery (26). In addition, DMEM contains amino acids and vitamins, as well as additional supplementary component that may lead to recovery, but the mechanisms or factors that promote reduced deficit are still unknown (34). BMSCs secrete cytokines such as interleukins (35), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and vascular endothelial cell growth factor (VEGF) (36). It has also been reported that BMSCs stimulate glial cells to produce neurotrophic factors (37).

In the present study, the withdrawal reflex latency was greater for the control and DMEM group *vs.* the cell+DMEM group. Our results are in agreement with the findings of Urdzíková *et al.* (2006) who found improved behavioral parameters after spinal cord injury in BMSCs group in comparison with the control saline treated group (24). In addition, BMSCs can differentiate into neuron-like cells and glial (38). The glial cells maintain synaptic structure and function and promote development of the neuromuscular junction *in vivo* (39).

There are several reasons supporting the use of MSCs in recovery of the paralyzed muscle with a direct nerve implantation: easy to isolate from the bone marrow that can be obtained under local anesthesia, capable of rapid proliferation in culture, may serve to replenish lost cells (34), act by immune modulation (40), have self-renewal capacity and multi-potentiality (41), capacity to pass through the blood-brain barrier (42), moreover, they contain a variety of cells that secrete substances that may lead to recovery (34).

In this study, the BMSCs were transplanted directly into denervated gastrocnemious muscle. Our results are in agreement with the findings of Hocking *et al.* (2010), who reported that, the direct application of BMSCs on wounds which has the potential advantage of promoting regeneration via MSCs differentiation and BMSCs paracrine signaling (43). So that BMSCs acts as a chemoattractant for specific cell types to the wound (44). In addition, they may also regulate cell migration in response to injury (45).

In the present study, the mean number of motor end plates in the cell+DMEM group was significantly larger than the control and DMEM groups. A previous study reported that, when grafted the motor branch of the peroneal nerve of rabbit into the "a neural" zone of the lateral head of the gastrocnemius muscle, the motor function had recovered and motor end-plates were found in histological sections (46), So that function after neurotization of denervated muscles as well as histological evidence of new motor end-plate formation in a neural zone of the muscle (47).

Also, we found small number BrdU reactive MSCs cells in the cell+DMEM group survive in the muscle near to end plate. Li *et al.* (2000) showed that a small number (~0.5 %) of BrdU reactive cells that survive in the normal mouse brain might be because of the possibility that infused cells divide rapidly and consequently the label below levels of detection of BrdU (48). Transplanted stem cells can either replace missing populations of cells or rescue cells in the injured area by the production of interleukins and stem cell factor (36), that induced tissue plasticity, and neuroprotective factors (49). It has also been reported that BMSCs stimulate glial cells to produce neurotrophic factors such as NGF and BDNF (35, 37). These cytokines are known to be essential factors for the survival and differentiation of neuronal progenitor cells (24). BMSCs has the ability to produce extracellular matrix proteins such as; collagen I, collagen IV, fibronectin, and laminin (5, 50). Therefore, there is good evidence to support the hypothesis that transplantation of BMSCs may repair peripheral nerve injuries (51). In addition, Stem cells may help create a microenvironment that promoted axon extension gap (52). However, the mechanisms or factors that can be generated motor end plate are still unknown.

In the other hand, motor nerves are rarely purely motor, likewise, sensory nerves may not be purely sensory (53). The sensory fibers are traditionally thought to innervate mainly the intrafusal muscle spindles and Golgi tendon organs, and not the motor end plates (6). Also, it has been suggested that neutrally released acetylcholine may act as a neuromuscular trophic agent, reducing muscle atrophy and fibrosis by suppressing muscle collagen biosynthesis and reduction of lysosomal proteolysis (54).

In this study, the mean nerve conduction velocity (m/s) in the cell + DMEM group was significantly greater than in the control and DMEM groups. The decreased NCV is the effect of the loss of larger diameter fibers, a phenomenon reported in other traumatic nerve lesions. NCV has been shown to be dependent on axon diameter, myelination, and intermediate distance (55).

Conclusion

BMSCs effectively enhance the recovery of the paralyzed muscle in rats with a direct nerve implantation. However, the mechanisms or factors that promote reduced deficit are still not fully unknown.

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