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Research Article

Bone Marrow Stromal Cells Associated with Poly L-Lactic-Co-Glycolic Acid (PLGA) Nanofiber Scaffold Improve Transected Sciatic Nerve Regeneration

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Background: Although peripheral nerves show capacity for regeneration after injury to a certain extent, the extent of regeneration is not remarkable. Previous studies have suggested that through the production of growth factors or extracellular matrix components, mesenchymal stem cells may enhance nerve regeneration.
Objectives: In the present study, the therapeutic potency of the Bone Marrow Stromal Cells (BMSCs) associated with Poly L-lactic-co-glycolic acid (PLGA) nanofiber Scaffolds on rat sciatic nerve repair was evaluated.
Material and Methods: Thirty adult male Wistar rats (220-250 g) were divided randomly into six groups, including control 1 (transected sciatic nerve), control 2 (transected sciatic nerve and stitched), Sham, PLGA, BMSCs, and PLGA+BMSCs. Functional recovery was evaluated at the end of 2nd, 4th, 6th, and 8th weeks after surgery using sciatic functional index (SFI) and hot water test. After killing all rats at the end of 8th week, their sciatic nerves were removed, fixed, and processed for the histological examination and analysis by the Motic software.
Results: A significant recovery of the sciatic nerve function was observed in the PLGA+BMSCs transplanted group at the 8th week after surgery as demonstrated by SFI and hot water findings. Histological examinations also showed a significant improvement in the PLGA+BMSCs group compared to the control 1, 2, Sham, PLGA and BMSCs groups.
Conclusion: BMSCs associated with PLGA nanofiber scaffold might be useful for improving the functional peripheral nerve repair having some clinical outcome.

Keywords: Poly L-lactic-co-glycolic acid (PLGA), Repair, Scaffold, Sciatic nerve.

1. Background

Peripheral nerve damage is among the most prevalent human disabilities. The rate of peripheral nerve injuries in the developed countries was assessed to be between 13 and 23 per 100,000 population per year (1, 2). Although these injuries are not life-threatening but impose a high financial burden on the patients and the health care systems (3).

Nerve damages are divided into neuraproxy, axonotmesis, and neurotmesis (4, 5). A relatively common type of traumatic injury affecting the peripheral nervous system is neurotmesis. Cell therapy, as a nerve repair strategy creates a favorable environment in the peripheral nervous system (6, 7). Since application of the embryonic stem/germ cells is likely to be limited due to the ethical, genetics, as well as other considerations, many investigators have focused their works on the adult stem cells as the potential source for the regenerative medicine (8, 9). Among the adult stem cell reservoirs, bone marrow has long been used as a source for deriving mesenchymal stem cells, but, notably changes such as reduction in the available cell number with an increase in the patient's age, longer doubling times, and lower differentiation potential have also been reported as the limiting factors *in* (10, 11). Polymers could be used as the scaffolds for helping differentiated cell adhesion and maintenance of their function without preventing proliferating cells' organization and growth as well as aiding the task of the extracellular matrix. As the cells' and tissues' transporters, scafolds can be constructed by

both natural and artificial polymers (12). These scaffolds must be biodegradable, nonpoisonous, mechanically similar to the tissue that will be substituted, and capable of binding to the other molecules (13). In this regard, natural polymers have been studied for biomaterials application. Poly L-lactic-co-glycolic acid (PLGA) is known as a biodegradable and biocompatible polymer. In addition, PLGA nanofibers could be used as the scaffold alongside bone marrow cells in the tissue engineering (14). In a study on the feasibility of PLGA (10:90) polymer, it was shown that PLGA could be considered as a potential candidate for the repair or regeneration in the CNS (11). These polymers have recently received much attentions in the tissue engineering (15).

Stem cells are cells with a high potential for differentiation as characterized by their capability for unlimited self-renewal, tissue-specific precursors, and differentiation (16). Bone marrow stromal cells (BMSCs) could differentiate into hepatocytes, skeletal muscle, cardiomyocytes, and blood cells. The potency of the BMSCs to induce neurogenesis might constitute a potential for assisting the functional recovery of the damaged CNS. Remarkably, recent *in vitro* studies have shown that BMSCs can differentiate into the neural cells depending on the growth conditions. BMSCs might represent a potential alternative for substituting the embryonic stem cells. BMSCs are cells of choice for application in cell the therapy by replacing the defunct neurons. These cells are capable of producing various types of growth factors and cytokines (17). BMSCs are among the cells which can appropriately grow and proliferate on the scaffolds (15).

2. Objective

The present study has aimed to evaluate the effects of BMSCs on PLGA nanofiber scaffolds for repairing of the transected sciatic nerve in the rat.

3. Materials and Methods

3.1. Fabrication of the PLGA Scaffolds by Electrospinning

The PLGA nanofibrous (Sigma, USA) scaffolds were fabricated by electrospinning process (Fanavaran, Iran). Dimethylformamide (Merck, Germany) and chloroform (Ghatranshimi, Iran) (1:3) were used to dissolve PLGA. For electrospinning, the solution was fed into a 5 mL plastic syringe with a needle diameter of 0.4 mm. A high voltage (20 kV) was attached to the tip of the needle to generate the electric field when a fluid jet was ejected at the speed of 1 $mL.h^{-1}$ A positively charged jet was formed from the Taylor cone and was sprayed to the grounded aluminum foil target (12).

3.2. Scanning Electron Microscope (SEM)

The nanofibers were observed with Scanning Electron Microscopy (SEM; HITACHI S-4800, Japan) after gold coating. XRD patterns of all samples were obtained at room temperature with a BRUKER D8 diffractometer (Cu Kα radiation, $\lambda = 1.5406$ A°) with the scanning rate of $5°.min^{-1}$. One hundred different fibers were selected randomly in SEM images and the diameter mean was presented.

3.3. Isolation and Culture of BMSCs

Bone marrow was extracted from the femurs of 3 Wistar rats. Briefly, the rat femurs were aseptically removed under deep anesthesia using ketamine and xylazine (Alfasan, Holland) intraperitonealy. The femurs were transected and bone marrow aspirated by a syringe containing 1mL of culture medium α-MEM (Gibco, UK). The content of the syringe was cultured in α-MEM medium containing 1% penicillin/streptomycin (Gibco, UK) in addition to 10% Fetal Bovine Serum (FBS) (Gibco, UK) and incubated at 37 °C and 5% CO_2. After 24 hours, the BMSCs cell culture medium was removed and the cells were rinsed with the sterile phosphate-buffered saline (PBS). Subsequently PBS was replaced by addition of fresh medium. Once the cells were attached and grown to 70-80% of the confluency, the cells were passaged using 0.25% Trypsin (Sigma, USA) and 0.04% Ethylenediaminetetraacetic acid (EDTA) (Sigma, USA) (18, 19).

3.4. BMSCs Culturing on the PLGA Nanofiber Scaffold

PLGA nanofibrous scaffolds were sterilized with 70% ethanol for 30 min and UV ray for 24 h and seeded with the rat third passage of BMSCs in the α-MEM medium. The constructs were cultured for 2 weeks with the medium being replaced once every 3 days. The cell adhesion efficiency was expressed as the number of cells attached to the scaffold as the percentage of seeded cells. At the end of the culture, the BMSCs on PLGA were fixed and stained with Hematoxylin and Eosin H&E.

3.5. Animals and Surgical Procedure

All experiments including animals and surgical procedures were approved by the Ethics Committee of Baqiyatallah University of Medical Sciences. In this research, right sciatic nerve transected in midpoint of thigh in 30 adult male rats and were divided randomly into six groups: 1) Control 1 without any intervention; 2) Control 2 immediately after transected sciatic nerve

stitched; 3) Sham-treated with culture medium on the injured nerve; 4) PLGA treated with a thin PLGA nanofiber around the injured nerve; 5) BMSCs treated with transplantation of 100000 BMSCs around the injured nerve, and 6) PLGA+BMSCs treated with a thin PLGA nanofiber contain attached BMSCs around the injured nerve.

3.6. Sciatic Functional Index (SFI) Assessment

Functional recovery was assessed by SFI at the end of 2nd, 4th, 6th, and the 8th weeks after surgery. In order to determine the SFI index, the hind limbs were stained with ink to be printed footprints on a white paper. The lengths of the third toe to its heel (PL), the second toe to the fourth toe (IT), and the first to the fifth toe (TS) were measured on the contralateral normal side (N), and the experimental side (E) in each rat. SFI was computed using the following modified formula:

SFI= |-38.5 (EPL-NPL/NPL) + 109.5 (ETS-NTS/NTS) + 13.3 (EIT-NIT/NIT)-8.8|

In this study, SFI equal to -100 indicates a significant impairment, whereas, an SFI oscillating around 0 is considered to reflect normal function (20).

3.7. Assessment of Sciatic Nerve Sensory Functional Index; Hot Water

At the end of above defined times, the hot water behavioral test was done; a method for evaluation the sensory function of the sciatic nerve. After the water temperature reached to 50 °C, the injured leg was entered to the warm water bath as long as the animals were sensing and dragging their leg out of the water. The amount of time required for the animal to retract its limb from the water was considered as the "reaction time". We repeated this test 3 times with 10 minutes intervals for each animal separately (21).

3.8. Histological Analysis

At the end of the 8th week after surgery, all right rat's sciatic nerves were removed, fixed. and processed for the histological evaluation. Sciatic nerves were transversely sectioned at 5µm thickness, stained with hematoxylin-eosin (H&E) (Merck, Germany), and hematoxylin-Van Gieson stain (Merck, Germany). The number of nerve fibers waere counted from at least 5 randomly selected areas (magnification:1000×) in different categories based on the diameter (D) (including D> 6 µm, D= 4-6 µm, and D< 4 µm) using the Motic software. In addition, the surface area occupied by blood vessels (per unit area=100000 μm^2) were also measured.

3.9. Statistical Analysis

All data were expressed as mean±SEM and were analyzed using one-way analysis of variance (ANOVA) followed by Post Hoc Tukey with the SPSS 22.0 software package. $p< 0.05$ was considered as an indication of a significant difference. All data showed in mean ± SEM.

4. Results

4.1. SEM Evaluation

PLGA porous electrospun nanofibers were formed with various thicknesses, folds, layers, and different directions. In SEM imaging, the diameter of these fibers was estimated between 100 and 270 nm.

4.2. BMSCs Proliferation on the PLGA Nanofibers

Our previous study showed that the cultured BMSCs, as the mesenchymal stem cells, have high purity, and are available in sufficient amount (13,14). Light microscope and SEM evaluation of BMSCs seeded on the PLGA nanofibers showed that nanofibers retain their structure, the rate of degradation was negligible, as well as appropriate interaction with cells. The cells proliferated and closely attached to each other. SEM imaging showed no major differences between the cell morphology in nanofibers groups (Fig. 1). The BMSCs grown on these scaffolds had a more flattened morphology with multiple poses and seeded cells attached to the nanofiber surface and penetrated into the pores of PLGA nanofiber scaffold.

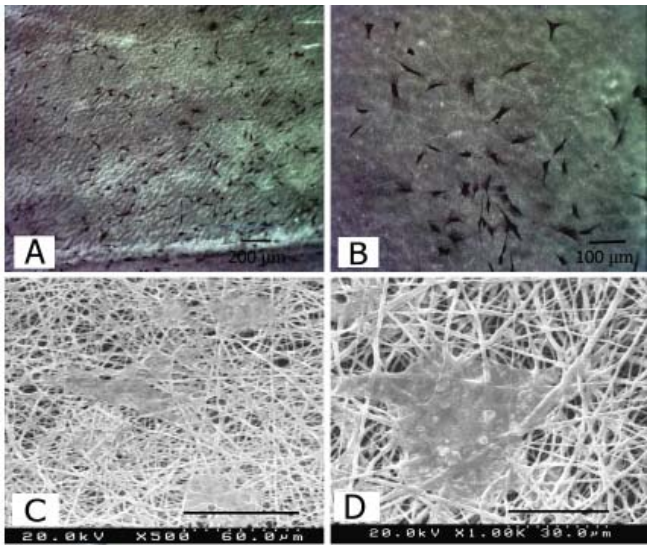

Figure 1. Micrographs of the BMSCs cultured on the PLGA nanofibrous scaffold. Panels A and B show BMSCs stained with H&E (magnifications 40× and 100×). Panels C and D show the SEM images of BMSCs.

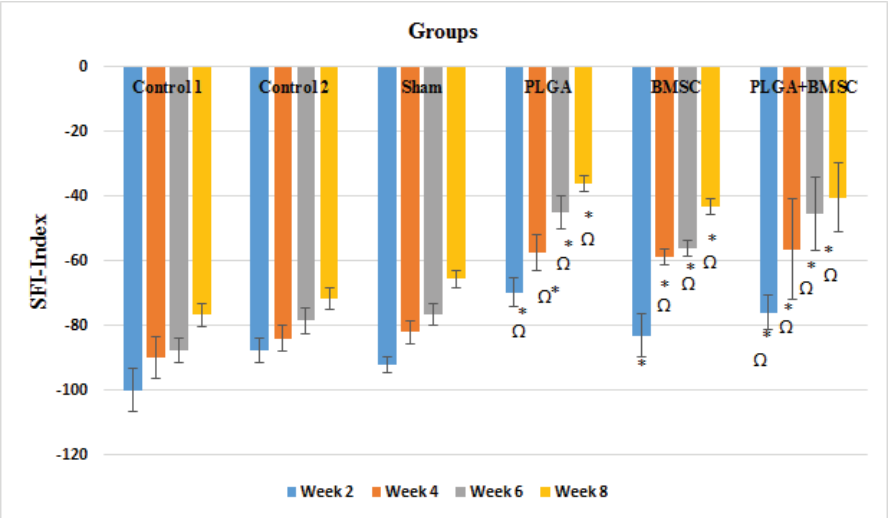

Figure 2. Comparison of the SFI means functional recovery in different groups ($p< 0.05$). Control 1 without any intervention; Control 2 immediately after transected sciatic nerve stitched; Sham treated with the culture medium on the injured nerve; PLGA treated with a thin PLGA nanofiber around the injured nerve; BMSCs treated with transplantation of BMSCs around the nerve, and PLGA+BMSCs treated with a thin PLGA nanofiber containing attached BMSCs.
* Significantly different from the control 1 group.
Ω Significantly different from the control 2 and Sham groups

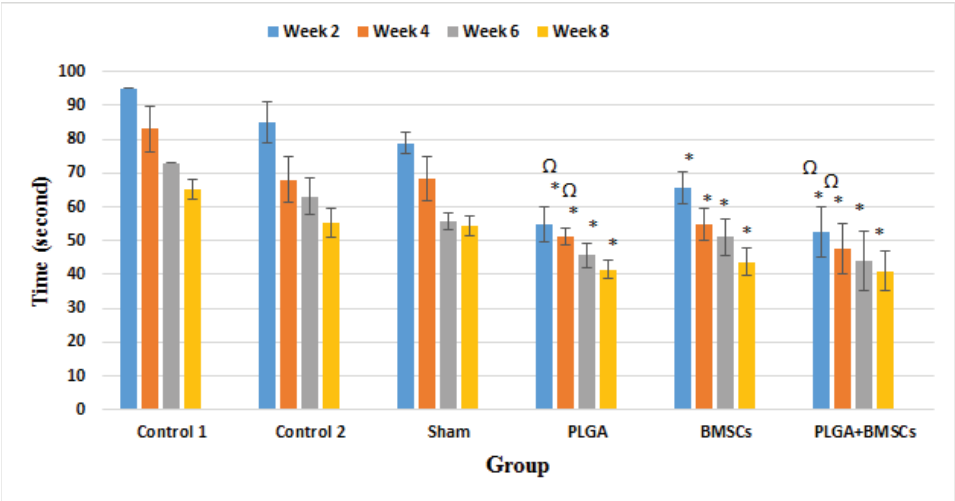

Figure 3. Sciatic nerve sensory evaluation in different groups. Control 1 without any intervention; Control 2 immediately after transected sciatic nerve stitched; Sham treated with the culture medium on the injured nerve; PLGA treated with a thin PLGA nanofiber around the injured nerve; BMSCs treated with the transplantation of BMSCs around the nerve, and PLGA+BMSCs treated with a thin PLGA nanofiber attached to the BMSCs.
* Significantly different from the control 1 group.
Ω Significantly different from the control 2 and Sham groups

4.3. SFI Results

The mean of SFI indexes after surgery in all groups were significantly decreased and tended to -100. After 2nd, 4th, and 6th weeks of surgery, SFI mean was increased in all groups. Our results showed that the mean SFI in PLGA, BMSCs, and PLGA+BMSCs groups was significantly increased compared to controls and Sham groups (Fig. 2).

4.4. Sciatic Nerve Sensory Evaluation; the Hot Water

At the 8th week after surgery, the reaction time in the hot water test was significantly decreased in PLGA, BMSCs groups, and substantially in the PLGA+BMSCs group compared to the controls and Sham groups (Fig. 3).

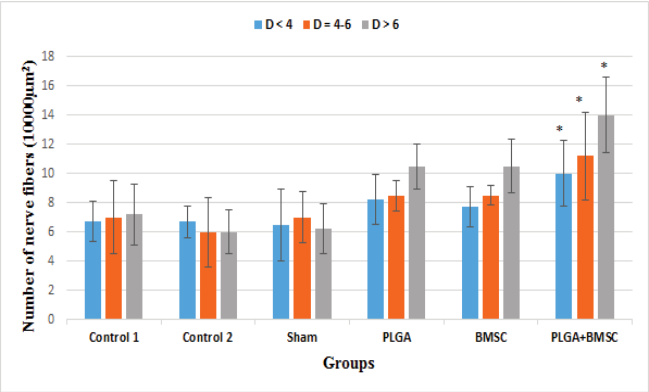

Figure 4. The number of nerve fibers per 100000 µm^2. The mean of nerve fibers in different categories based on diameter (p< 0.05). Control 1 without any intervention; Control 2 immediately after transected sciatic nerve stitched; Sham treated with culture medium on the injured nerve; PLGA treated with a thin PLGA nanofiber around the injured nerve; BMSCs treated with the transplantation of BMSCs around the nerve, and PLGA+BMSCs treated with a thin PLGA nanofiber containing attached BMSCs.
* Significantly different from the controls 1, 2 and Sham group.

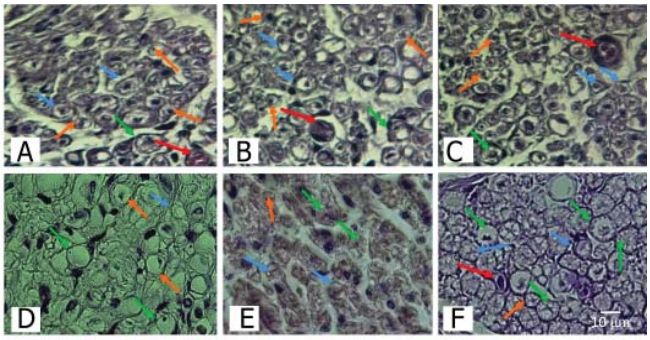

Figure 5. The cross sectional photomicrographs through distal part of transsected sciatic nerve at the 8th week after surgery in the different treatment groups (A) Control 1 without any intervention, (B) Control 2 immediately after transected sciatic nerve stitched, (C) Sham treated with culture medium of the injured nerve, (D) PLGA treated with a thin PLGA nanofiber around the injured nerve, (E) BMSCs, and (F) PLGA+BMSCs treated with a thin PLGA nanofiber containing the attached BMSCs groups. Green arrows indicate the diameter of the nerve fibers with more than 6 µm. Blue arrows are showing the diameter of nerve fibers between 4-6 µm, and orange arrows represent the diameter of nerve fibers less than 4 µm. (magnifications 1000×, H&E stain).

4.5. Histomorphometric Analysis

Based on nerve diameter, the histomorphometric results showed that at the 8th week after surgery, the number of nerve fibers in the different categories was significantly increased in the PLGA+BMSCs group compared to the control 1, 2, and Sham groups, respectively (p<0.05) (Fig. 4, 5).

The amount of collagen fibers in the epineurium and perineurium was decreased in the PLGA+BMSCs group compared to the control 1, 2, and Sham groups, as well (p<0.05) (Fig. 6).

Also our results showed that area engaged in the blood vessels in the PLGA+BMSCs group was significantly increased compared to the other groups, as well (Fig. 7).

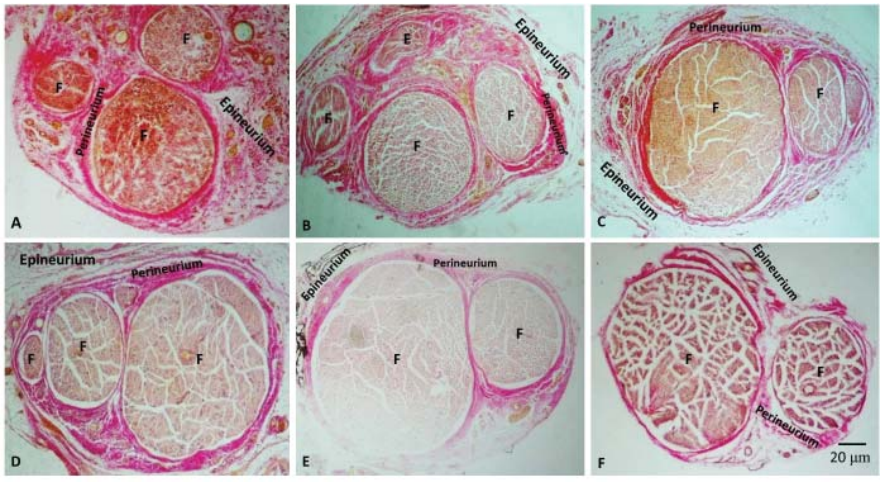

Figure 6. Photomicrographs of the sciatic nerve. (A) Control 1 without any intervention, (B) Control 2 immediately after transected sciatic nerve stitched, (C) Sham treated with the culture medium on the injured nerve, (D) PLGA treated with a thin PLGA nanofiber around the injured nerve, (E) BMSCs, and (F) PLGA+BMSCs treated sciatic nerve with a thin PLGA nanofiber containing the attached BMSCs. The formation of collagen fibers in Epineurium and Perinurium of PLGA+BMSCs group has increased copmpared to the control 1 group (F: Nerve Fascicle). The magnification was set at 40× and haematoxylin-Van Gieson's was used for tissue staining.

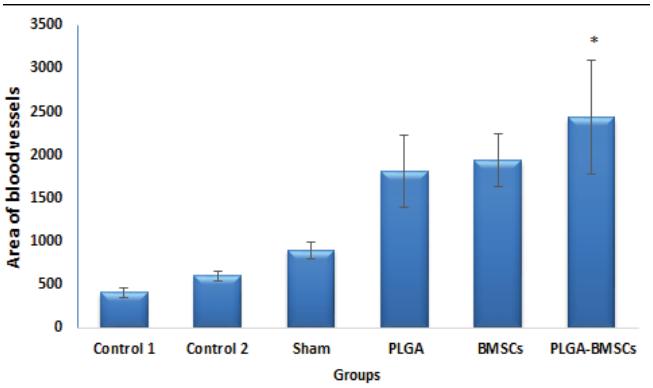

Figure 7. The estimated blood vessels' area as per unit area of 100000 μm^2. Area of blood vessels was significantly increased in PLGA+BMSCs group compared to the control 1, 2, and Sham groups*($p< 0.05$).

5. Discussion

Construction of a biodegradable scaffold for cell culture and application in tissue repair is one of the most important parameters in the tissue engineering. PLGA, as a synthetic, non-toxic, and biodegradable material has a high tensile strength is being used in the manufacturing biological scaffolds (22). In this study, the electron microscopic images of the PLGA nanofibers showed that these fibers with diameters ranging from 100 to 270 nm provide high porosity and suitable bonding capabilities for cultivation and cell growth such as BMSCs *in vitro*. This finding is somewhat in alignment with the results of Xu *et al*. (2007) as they could culture smooth muscle and endothelial cells on the nanofiber scaffold PLLA-CL (poly L-lactide-co-epsilon-caprolactone) (23). Formerly, Kazeminejad and his colleagues have shown the possibility of human bone marrow mesenchymal stem cells' proliferation and differentiation into hepatocytes on nanofibrous scaffold formed by Poly ($_{\varepsilon}$-caprolactone; PCL), collagen, and polyethersulfone (PES) (24). In the present study, the results of SFI at the 8th weeks after surgery has shown that the mean of SFI was significantly improved in the PLGA+BMSCs group compared to the control group. This finding is agreement with the results of Liu *et al*. (2011) and Zarbakhsh *et al.* (2012). Liu and coworkers have reported the significantly higher SFI in the group that had a biodegradable nerve conduit and stem cells derived from adipose tissue at the 8 weeks after surgery (25). In addition, Zarbakhsh and colleagues have reported that bone marrow mesenchymal stem cells have the ability to improve SFI following to a nerve injury (26). It seems SFI improvement in the transected sciatic nerve treated with the PLGA+BMSCs group is due to neurotrophins secretion from transplanted bone marrow mesenchymal stem cells (27) and increased PLGA nanofibers porosity, flexibility, and surface area. Hot water test results showed a significant sensory function improvement in the PLGA+BMSCs group compared to the control group. Gärtner *et al*. (2012) have demonstrated that chitosan membranes and human mesenchymal stem cells from Wharton jelly of the umbilical cord improve pain-reflexes; an observation in agreement with our results (28). It seems that in our study, functional improvement of the sciatic nerve in therapeutic groups is due to secretion of neurotrophic factors such as Brain Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3), Neurotrophin-4 (NT-4), Glial cell line-derived neurotrophic factor (GDNF), and platelet-derived growth factor (PDGF). It is well known that neurotrophins are a family of proteins that play a critical role, particularly in the cell growth and differentiation, as well as sensory neurons restoration; therefore, affecting pain reflexes (27). Histomorphometric results of this research showed that at 8th weeks after surgery, the number of nerve fibers has increased in the PLGA+BMSCs group compared to the control group. Liu and colleagues have reported that transplantation of the adipose-derived stem cells significantly increases the number of nerve fibers compared to DMEM group (25). It has been reported that VEGF directly improves axonal regeneration via stimulation of angiogenesis (29). It seems that improvement of transected sciatic nerve fibers could be due to angiogenesis. Our histological results have demonstrated that formation of collagen fibers in the epineurium has gradually decreased in the control group 1 to PLGA+BMSCs group, respectively.

Several possible mechanisms have suggested for explaining the effect of mesenchymal stem cells on peripheral nerve repair. Overall, mesenchymal stem cells affect the improvement of nerve regeneration by induction of cell transplantation, production of growth factors, extracellular matrix synthesis, release of anti-inflammatory, anti-apoptotic molecules as well as immune system modulators to create a favorable environment for the nerve repair (27).

In addition, previous studies have reported that the PLGA stimulates angiogenesis by endothelial sprouting and creating a suitable environment for the nerve growth and axons repair (30, 31) which are in agreement with the results of the present study. Altogether, these findings suggest that BMSCs along with the PLGA membrane could improve histomorphometric recovery of the transected sciatic nerves in the rat model.

6. Conclusion

Our results have suggested that transplantation of BMSCs seeded on the PLGA nanofiber scaffolds improve transected sciatic nerve regeneration.

Acknowledgements

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