TÜBİTAK

Original Article

Turk J Med Sci
2012; 42 (1): 127-136
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E-mail: medsci@tubitak.gov.tr
doi:10.3906/sag-1009-1174

Transplantation of undifferentiated bone marrow stromal cells improves sciatic nerve regeneration and functional recovery through inside-out vein graft in rats

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Aim: To study the effect of undifferentiated bone marrow stromal cells (BMSCs) on peripheral nerve regeneration using a rat sciatic nerve regeneration model. In recent years, cell transplantation has become the focus of attention, and reliable outcomes have been achieved in regeneration of the sciatic nerve.

Materials and methods: A 10-mm sciatic nerve defect was bridged using an inside-out vein graft (IOVG) filled with undifferentiated BMSCs (2 × 107 cells/mL). In the control group, the vein was filled with phosphate buffered saline alone. The regenerated fibers were studied 4, 8, and 12 weeks after surgery. Assessment of nerve regeneration was based on functional (walking track analysis), histomorphometric, and immunohistochemical (Schwann cell detection by S-100 expression) criteria.

Results: The functional study confirmed significant recovery of regenerated axons in the IOVG/BMSC group ($P < 0.05$). Quantitative morphometric analyses of regenerated fibers showed that the number and diameter of myelinated fibers in the IOVG/BMSC group were significantly higher than in the control group ($P < 0.05$).

Conclusion: This study demonstrates the potential of using undifferentiated BMSCs in peripheral nerve regeneration without limitations of donor-site morbidity associated with isolation of Schwann cells. It also reduces costs due to the simplicity of laboratory procedures compared to those for differentiated BMSCs and the reduction in the interval from tissue collection until cell injection.

Key words: Sciatic nerve, peripheral nerve regeneration, undifferentiated bone marrow stromal cells, vein graft, rat

Introduction

In spite of the presence of various nerve coaptation materials and techniques, the achieved level of functional peripheral nerve regeneration is still inadequate (1). Traumatic nerve injury resulting in peripheral nerve gap often requires a graft to bridge the defect. Autologous nerve grafting is still the method of choice for bridging peripheral nerve gaps. However, it has the disadvantage of the sacrifice of a functional nerve. Numerous surgical techniques are performed each year for peripheral nerve regeneration (2). Veins have been used experimentally as conduits, with advantages including lack of donor morbidity, ease of harvesting and transplanting, availability, affordability, and lack of foreign body reactions (3,4).

Received: 26.09.2010 – Accepted: 16.12.2010

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In the last few years, cell transplantation, especially that of Schwann cells, has become the focus of attention, and reliable outcomes have been achieved in the regeneration of the sciatic nerve. It has been shown that the transplantation of differentiated bone marrow stromal cells (BMSCs) into silicone tube and cut ends of nerves exerts a beneficial effect on peripheral nerve regeneration (5-8). However, to the best knowledge of the authors, there is still no report in the literature on the transplantation of undifferentiated BMSCs into an inside-out vein graft.

The objective of the present study was to elicit functional recovery in peripheral nerve injury over a 10-mm gap defect by undifferentiated fibroblast-like BMSC implantation into an inside-out vein graft and to evaluate its effectiveness on sciatic nerve regeneration in a rat model. Assessment of the nerve regeneration was based on functional (walking track analysis), histomorphometric, and immunohistochemical (Schwann cell detection by S-100 expression) criteria 4, 8, and 12 weeks after surgery.

Materials and methods

Experimental design

A total of 54 male white Wistar rats, each weighing approximately 270 g, were randomly divided into 3 experimental groups (n = 18): a sham-operated group (sham), a control group (IOVG), and an undifferentiated bone marrow stromal cells group (IOVG/BMSC). Each group was further subdivided into 3 subgroups of 6 animals each. The vein graft donors were 36 male white Wistar rats weighing 300-350 g. Vein-graft harvesting was carried out in all of the rats. Four rats were assigned to isolation and preparation of undifferentiated BMSCs. During the entire experiment, and for a 2-week period before the experiment commenced, the animals were housed in individual plastic cages with an ambient temperature of 23 ± 3 °C, stable air humidity, and a natural day/night cycle. The rats had free access to standard laboratory rodent food and tap water.

Isolation and culture of BMSCs

Isolation of adult BMSCs was performed using the donor animals immediately after the harvesting of the vein graft, according to the method described by Cuevas et al. (7). Rats were euthanized and femurs were dissected out. The marrow was then extruded with 10 mL of Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 20% fetal bovine serum and antibiotics (100 U penicillin and 100 mg/mL streptomycin, Gibco). The cells were incubated at 37 °C, 95% humidity, and 5% CO_2. After 48 h, the nonadherent cells were removed by replacing the medium. The cells were used for the grafting procedure after the cultures had reached confluence. The undifferentiated BMSCs were harvested with 0.25% trypsin and 1 mM EDTA (Gibco), suspended in phosphate buffered saline solution, and loaded in a 1-mL syringe at a concentration of 2×10^7 cells/mL (Figures 1A-1C).

Grafting procedure

Animals were anesthetized by intraperitoneal administration of ketamine-xylazine (ketamine 5%, 90 mg/kg; xylazine 2%, 5 mg/kg). The procedures were carried out based on the guidelines of the ethics committee of the International Association for the Study of Pain (9). The University Research Council approved all experiments. A 15-mm segment of the right external jugular vein was harvested in a tube after the donor animals had been anesthetized, shaved, and prepared aseptically. Grafts were washed in physiological solution and left at room temperature for 30-40 min. A subtle retraction of 1 mm was expected. Each graft was inverted inside-out to prevent any potential branching of axons through the side branches during regeneration (6).

Following surgical preparation, in the sham-operated group, the left sciatic nerve was exposed through a gluteal muscle incision; after careful hemostasis, the muscle was sutured with resorbable 4/0 sutures and the skin with 3/0 nylon. In the IOVG group, the left sciatic nerve was exposed through a gluteal muscle incision and transected proximal to the tibioperoneal bifurcation, where a 7-mm segment was excised, leaving a gap of about 10 mm due to the retraction of nerve ends. Proximal and distal stumps were each inserted 2 mm into the graft, and 2 nylon sutures (10/0) were placed at each end of the cuff to fix the graft in place and assure a 10-mm gap between the stumps. The conduit was filled with 10 µL of phosphate buffered saline solution, and sterile

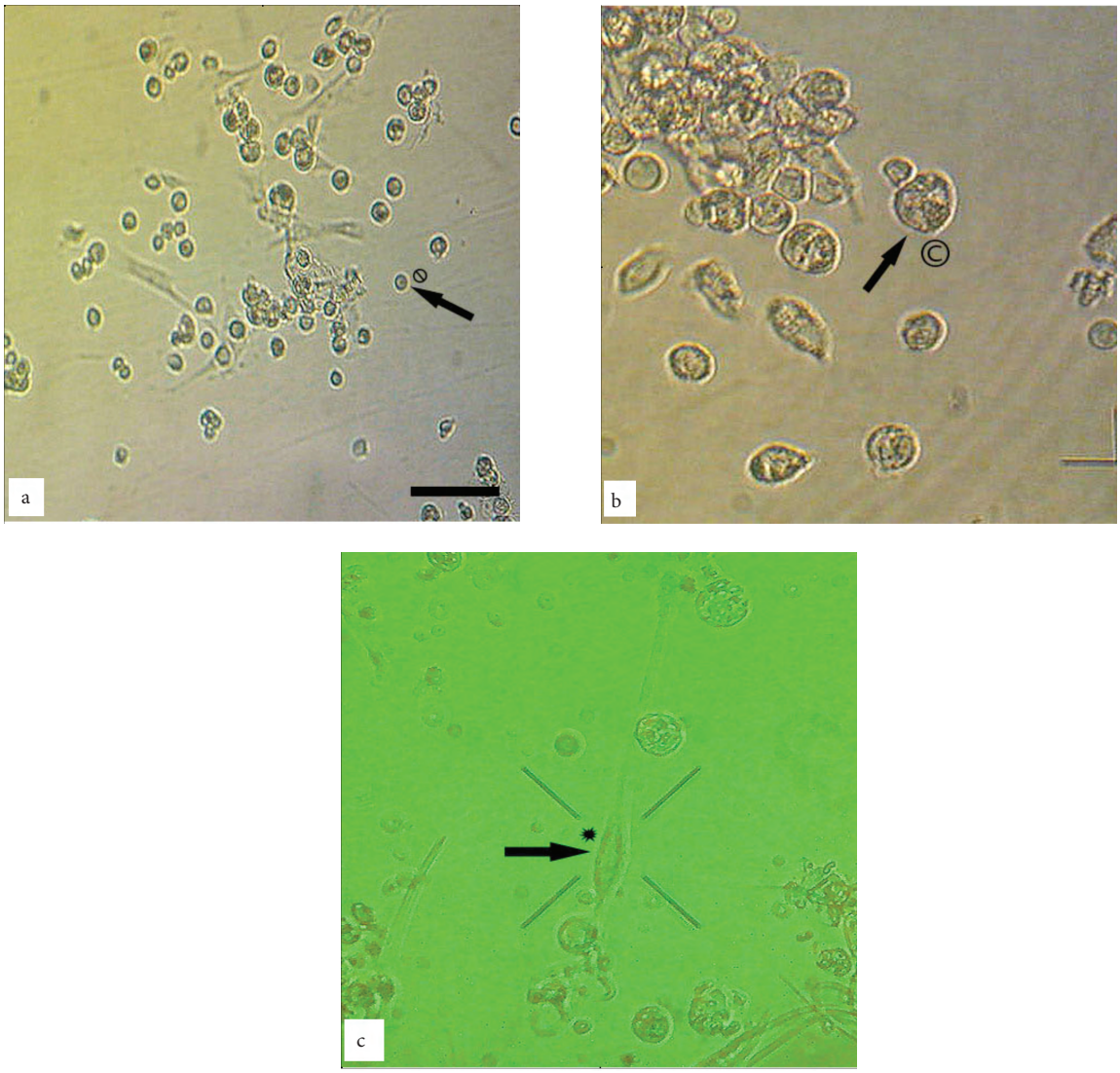

Figure 1. Morphological characteristics of cultured BMSCs. Cultured BMSCs had A) small and rounded and B) large and flattened morphology in the first passage of initial culture and C) fibroblast-like morphology in the confluent culture; representative cells with small and rounded, large and flattened, and fibroblast-like morphology are indicated by Ø, ©, and *, respectively. Scale bar: 100 μm.

Vaseline was used to seal the ends of the tubes to avoid leakage. In the IOVG/BMSC group, the inside-out vein graft was filled with 10 μL of aliquots (2×10^7 cells/mL).

The animals were anesthetized (see above) and euthanized with transcardial perfusion of a fixative containing 2% paraformaldehyde and 1% glutaraldehyde buffer (pH = 7.4) at 4 (n = 6), 8 (n = 6), and 12 weeks (n = 6) after surgery.

Functional assessment of nerve regeneration

Walking track analysis was performed 4, 8, and 12 weeks after surgery based on the methods of Bain et al. (10). The lengths of the third toe to its heel (PL), the first to the fifth toe (TS), and the second toe to the fourth toe (IT) were measured on the experimental side (E) and the contralateral normal side (N) in each rat. The sciatic function index (SFI) in each animal was calculated with the following formula:

SFI = −38.3 × (EPL− NPL)/NPL + 109.5 × (ETS − NTS)/NTS + 13.3 × (EIT − NIT)/NIT − 8.8.

In general, the SFI oscillates around 0 for normal nerve function, whereas values around −100 represent total dysfunction. The SFI was assessed based on the IOVG group, and the normal value was considered to be 0. The SFI had a negative value, and a higher SFI value meant better function of the sciatic nerve.

Muscle mass

Recovery assessment was also indexed using the weight ratio of the gastrocnemius muscles 12 weeks after surgery. Immediately after sacrificing the animals, the gastrocnemius muscles were dissected and harvested carefully from intact and injured sides, and they were weighed, while still wet, using an electronic balance. All measurements were made by 2 independent observers unaware of the analyzed group.

Histological preparation and quantitative morphometric studies

Graft middle cables from the sham, IOVG, and IOVG/BMSC groups were harvested and fixed in 2.5% glutaraldehyde. The grafts were then embedded in Paraplast paraffin, cut into sections of 5 μm, and stained with toluidine blue. Morphometric analysis was carried out using image analyzing software (Image-Pro Express, version 6.0.0.319, Media Cybernetics, Silver Springs, MD, USA). Equal opportunity and systematic random sampling and 2-dimensional dissector rules were followed in order to cope with sampling-related, fiber-location-related, and fiber-size-related biases (11).

Immunohistochemical analysis

In this study, anti-S-100 (1:200, Dako, Glostrup, Denmark) was used as a marker for axons and myelin sheath. Prior to immunohistochemistry, specimens were postfixed with 4% paraformaldehyde for 2 h and embedded in paraffin. After nonspecific immunoreactions were blocked, sections were incubated in an S-100 protein antibody solution for 1 h at room temperature. They were washed 3 times with PBS and incubated in biotinylated antimouse rabbit IgG solution for 1 h. Horseradish peroxidase-labeled secondary antibody was developed by the diaminobenzidine method. The results of the immunohistochemistry were examined under a light microscope.

Statistical analysis

Experimental results were expressed as means ± SD. Statistical analyses were performed using PASW 18.0 (SPSS Inc., Chicago, IL, USA). Model assumptions were evaluated by examining the residual plot. Results were analyzed using factorial ANOVA ($P < 0.05$) with 2 between-subject factors. The Bonferroni test for pairwise comparisons was used to examine the effect of time and treatments.

Results

Recovery of sciatic nerve function

Figure 2 shows SFI values in the experimental groups. Prior to surgery, SFI values in all groups were near 0. After the nerve axotomy, the mean SFI decreased to −100 due to the complete loss of sciatic nerve function in all animals. The mean SFI was −74.4 ± −1.28 in the IOVG/BMSC group 4 weeks after surgery, compared to −92.8 ± −1.24 in the IOVG group. Improvement in SFI was observed in animals in the IOVG/BMSC group (−59.8 ± −1.14) at 8 weeks after surgery; this was significantly higher than in IOVG animals (−74.6 ± −1.44; $P < 0.05$). After 12 weeks, animals in the IOVG/BMSC group achieved a mean SFI value of −41.9 ± −1.12, an approximate improvement of 58%, whereas in the IOVG group, a mean value of −64.1 ± −2.10, an approximate improvement of 35%, was found. The statistical analyses revealed that the recovery of nerve function was significantly different between the IOVG/BMSC and IOVG groups ($P < 0.05$), and BMSCs drastically promoted functional recovery.

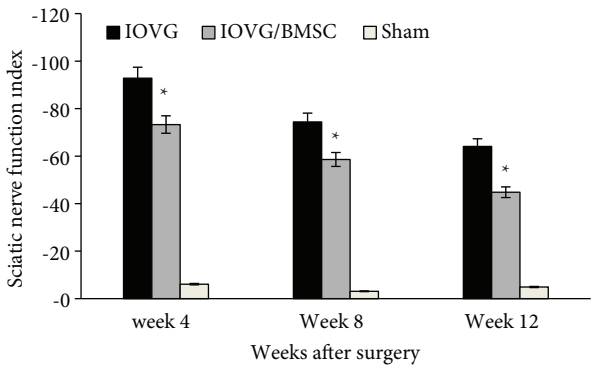

Figure 2. Diagrammatic representation of effects on the sciatic nerve function index (SFI). Transplantation with BMSCs gave better results in functional recovery of the sciatic nerve than in the IOVG group. Data are presented as mean ± SD; *$P < 0.05$ versus IOVG group.

Muscle mass measurement

The mean ratios of gastrocnemius muscle weight were measured. There was a statistically significant difference between the muscle weight ratios of the IOVG/BMSC and IOVG groups ($P < 0.05$). The results showed that in the IOVG/BMSC group, the muscle weight ratio was larger than in the IOVG group, and weight loss in the gastrocnemius muscle was ameliorated by BMSC transplantation (Figure 3).

Histological and morphometric findings

The Table shows the quantitative morphometric analyses of regenerated nerves for each of the experimental groups. The IOVG/BMSC group presented significantly greater nerve fiber, axon diameter, and myelin sheath thickness 4 weeks after surgery, compared to IOVG animals ($P < 0.05$). Although the IOVG group presented regeneration patterns, the morphometric indices in the IOVG/BMSC group, after both 8 and 12 weeks, were significantly higher than in IOVG. Using factorial ANOVA analysis with 2 between-subject factors (group × time), axon diameters did not show a significant difference between 8 and 12 weeks ($P > 0.05$) in the IOVG group. Thickness of the myelin sheath showed an interaction across time in the IOVG and IOVG/BMSC groups. The increase in the mean thickness of the myelin sheath did not show a statistical difference between 8 and 12 weeks in the IOVG and IOVG/BMSC groups ($P > 0.05$) (Figures 4-8).

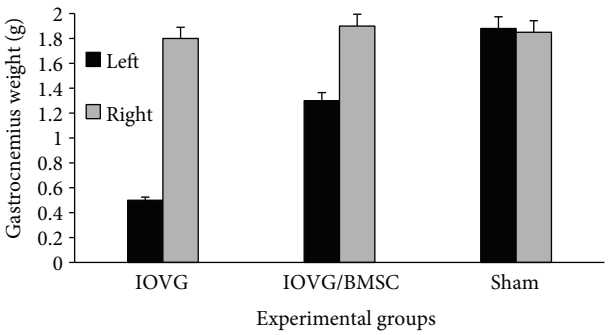

Figure 3. Measurement of gastrocnemius muscle. The gastrocnemius muscle of both sides (injured left and intact right) was removed and weighed in experimental groups 12 weeks after surgery. Data are presented as mean ± SD; *P < 0.05 versus IOVG group.

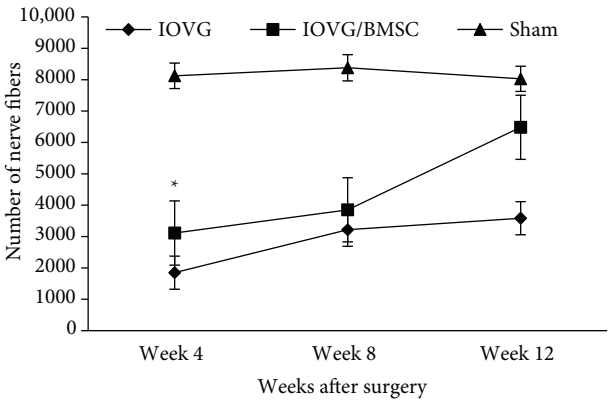

Figure 4. Line graph of the quantitative results of fiber counting. Both the IOVG and IOVG/BMSC groups showed a lower number of fibers than the sham-operated group, even at the end of the study. From 4 to 8 weeks, the IOVG/BMSC group had significantly more nerve fibers than the IOVG group, and this significant difference had increased in favor of the IOVG/BMSC group at the end of the study period. *P < 0.05, IOVG/BMSC group compared with IOVG group.

Table. Morphometric analyses of regenerated nerves for each of the experimental groups; values are given as mean ± SD.

Weeks	Sham			IOVG			IOVG/BMSC		
	4	8	12	4	8	12	4	8	12
N	8124 ± 385	8379 ± 446	8028 ± 404	1849 ± 297†	3217 ± 307†	3584 ± 264†	3112 ± 264†	3852 ± 234†	6483 ± 285†
D	12.01 ± 0.01	11.93 ± 0.17	12.06 ± 0.23	3.24 ± 0.69†	7.49 ± 0.37†	7.94 ± 0.49†	7.35 ± 0.48†	9.87 ± 0.75†	10.06 ± 0.6†
d	7.03 ± 0.02	6.97 ± 0.39	7.06 ± 0.46	2.22 ± 0.47†	3.87 ± 0.25†	4.05 ± 0.02†	3.61 ± 0.32†	5.02 ± 0.23†	5.86 ± 0.19†
T	2.56 ± 0.01	2.48 ± 0.02	2.53 ± 0.01	0.51 ± 0.03†	1.82 ± 0.34†	1.9 5 ± 0.24†	1.94 ± 0.05†	2.24 ± 0.17†	2.11 ± 0.21†

N: Number of fibers, D: diameter of fibers (μm), d: diameter of axon (μm), T: thickness of myelin sheath (μm),
†: results were significantly different from those of sham-operated animals ($P < 0.05$).

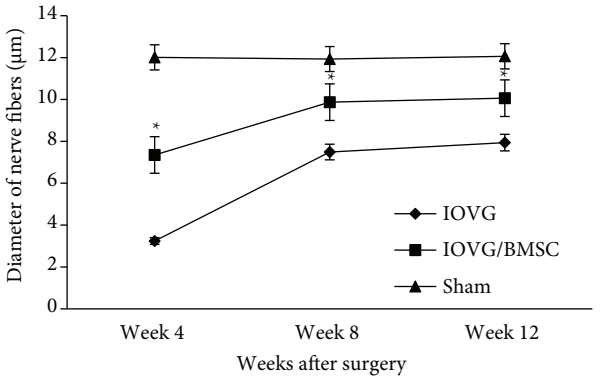

Figure 5. Line graph of the quantitative results of mean diameter of nerves fibers. Both IOVG and IOVG/BMSC groups showed a lower mean diameter of nerve fibers than the sham-operated group, even at the end of the study. From 4 to 8 weeks, the IOVG/BMSC group had significantly more mature nerves than the IOVG group. *P < 0.05, IOVG/BMSC group compared with IOVG group.

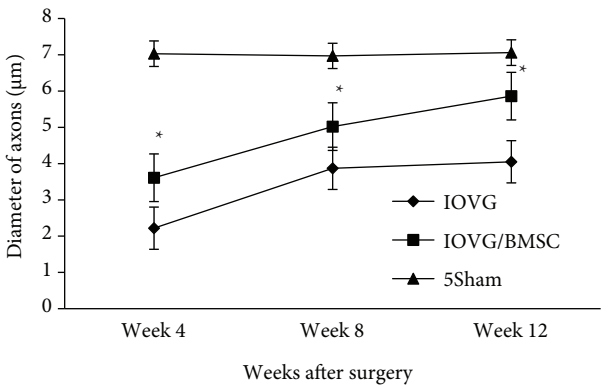

Figure 6. Line graph of the quantitative results of mean diameter of axons. Both IOVG and IOVG/BMSC groups showed a lower mean axon diameter than the sham-operated group, even at the end of the study. From 4 to 8 weeks, the IOVG/BMSC group had significantly more mature axons than the IOVG group, and this significant difference increased in favor of the IOVG/BMSC group in the later period. The diameters of axons in the IOVG group did not increase after 8 weeks; *P <0.05, IOVG/BMSC group compared with IOVG group.

Immunohistochemistry

Immunoreactivity to S-100 protein was extensively observed in the cross-sections of regenerated nerve segments. The expression of the S-100 protein signal was located mainly in the myelin sheath. The axon also showed a weak expression, indicating that a Schwann cell-like phenotype existed around the myelinated axons (Figure 9). In both groups, the expression of S-100 and the findings resembled those of the histological evaluations.

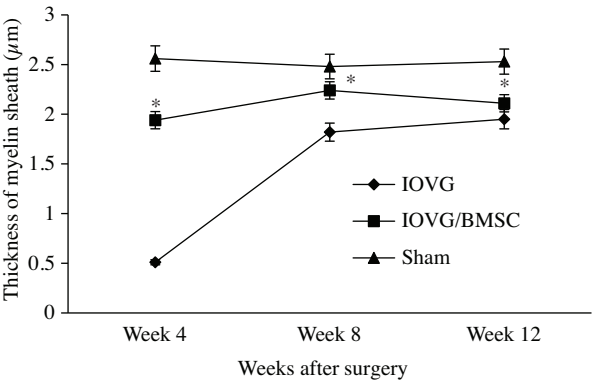

Figure 7. Line graph of the quantitative results of myelin thickness. Both IOVG and IOVG/BMSC groups showed a lower mean myelin thickness than the sham-operated group, even at the end of the study. From 4 to 8 weeks, the IOVG/BMSC group had significantly lower mean myelin thickness than the IOVG group, and this significant difference increased in favor of the IOVG/BMSC group in the later period. Mean myelin thickness in the IOVG and IOVG/BMSC groups did not show a significant increase after 8 weeks; *P < 0.05, IOVG/BMSC group compared with IOVG group.

Discussion

In the present study, we analyzed and compared the effectiveness of undifferentiated BMSCs on sciatic nerve regeneration. The results of the present study showed that BMSCs, when loaded into a vein graft, enhanced sciatic nerve functional recovery. As the posterior tibial branch of the sciatic nerve regenerates into the gastrocnemius muscle, it will regain its mass proportional to the amount of axonal reinnervation (12,13). In the present study, 12 weeks after surgery, muscle mass was found in both experimental groups. However, the IOVG/BMSC group showed significantly greater ratios of mean gastrocnemius muscle weight than the IOVG group, indicating indirect evidence of successful end-organ reinnervation.

In the histological studies, quantitative morphometric indices of regenerated nerve fibers showed a significant difference between experimental groups, indicating that BMSCs drastically improved sciatic nerve regeneration.

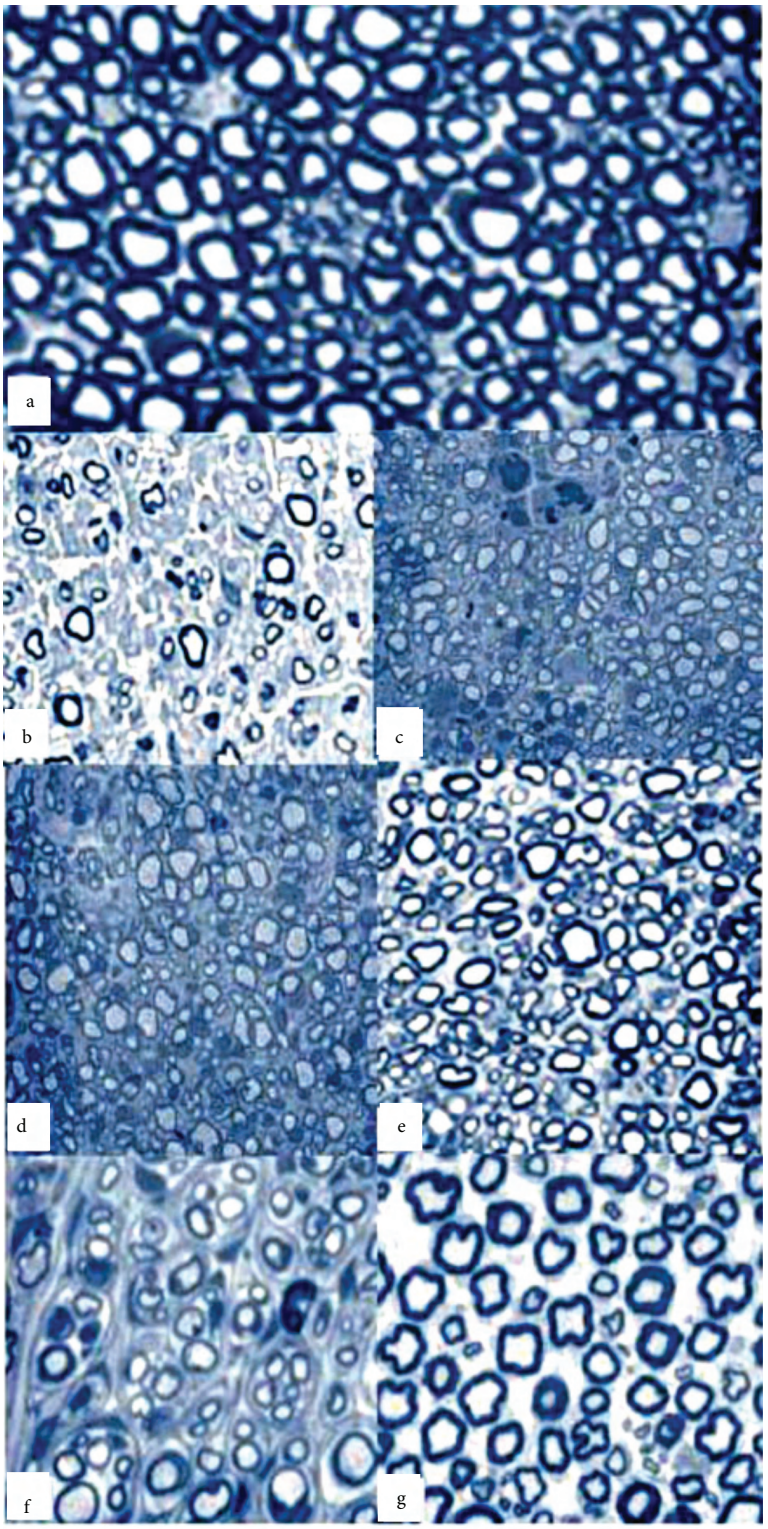

Figure 8. Light micrographs of representative cross-sections taken from: A) midpoint of normal sciatic nerve of sham group, B) middle cable of IOVG group, and (C) middle cable of IOVG/BMSC group 4 weeks after surgery; D) middle cable of IOVG and E) IOVG/BMSC groups 8 weeks after surgery; F) middle cable of IOVG and G) IOVG/BMSC groups 12 weeks after surgery. Toluidine blue, × 400.

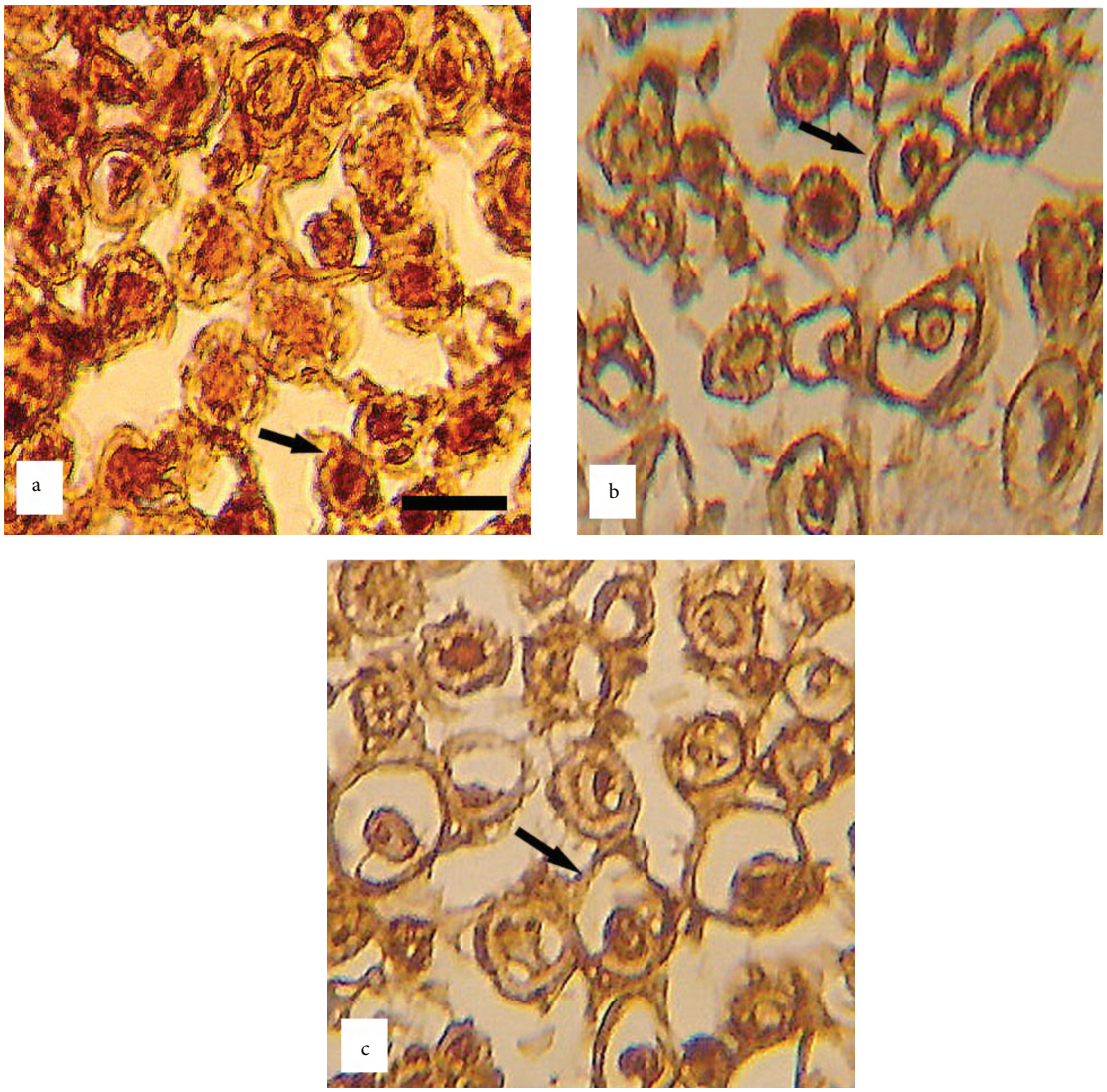

Figure 9. Immunohistochemical analysis of the regenerated nerves 12 weeks after surgery from middle cable of A) sham, (B) IOVG, and (C) IOVG/BMSC groups. There is clearly more positive staining of the myelin sheath-associated S-100 protein (arrow) within the periphery of nerve, indicating well-organized structural nerve reconstruction in BMSC transplanted nerves. Scale bar: 10 μm

In the immunohistochemistry, the expression of axon and myelin sheath special proteins was evident in the IOVG/BMSC group, which indicated a normal histological structure. The location of reactions to S-100 in the IOVG/BMSC group was significantly different from that of the IOVG group, further implying that both regenerated axon and Schwann cell-like cells existed and were accompanied by the process of myelination and the structural recovery of the regenerated nerves in the IOVG/BMSC group.

A major rate-limiting step in the induction of nerve regeneration across a gap is the proliferation and migration of Schwann cells between the nerve stumps. Therefore, formation of a properly aligned extracellular matrix scaffold is essential to enhance Schwann cell proliferation in a conduit, through which blood vessels and other cell types migrate and form the primordial assembly for the formation of a new nerve structure (14). We used a vein graft as a conduit to provide a scaffold for BMSCs and to facilitate Schwann cell migration. The vein as a conduit has been utilized to repair segmental nerve tissue loss, which proved to be a supportive conduit for peripheral nerve axonal regeneration and maturity (3,15,16).

Bone marrow stromal cells have become one of the most interesting targets for the study of tissue regeneration because of their plasticity. BMSCs are multipotential cells that contribute to the regeneration of tissues, such as bone, cartilage, fat, and muscle, and to the expression of many cytokines and cellular factors (17-21).

It has been demonstrated that BMSC-derived elements bear the capacity to localize in the murine peripheral nervous system in order to integrate in these tissues and assume the morphology of some resident cells (22). Given the pluripotency of BMSCs, the prospect of using them to elicit neuroprotection has been explored in peripheral nerve injury (7,8,23). Others have reported that the transplantation of neural stem cells into a peripheral nerve gap results in significant axonal regeneration; it has been suggested that neural stem cells might differentiate into Schwann cell-like supportive cells (24). Schwann cells have great importance in organizing the structure of the peripheral nerve because they produce a basement membrane containing extracellular matrix proteins that support axonal growth and form the endoneurial tubes through which regenerating axons grow (25). BMSCs have significant advantages over neural stem cells. Marrow cells are readily accessible, which overcomes the risks of obtaining neural stem cells from the brain. Recent studies have shown that differentiated BMSCs, when implanted into the cut ends of peripheral nerves, are capable of supporting nerve fiber regeneration (7,8,26). It has been reported that the potential for using BMSCs to promote axonal regeneration in peripheral nerve defects is strongly correlated with the production of trophic substances. Multiple mechanisms may exist in stem cell therapy. Stem cells could aid regeneration by transdifferentiation into Schwann cells or the release of soluble nerve growth factors such as brain-derived neurotrophic factor or angiogenic molecules, including vascular endothelial growth factor (27,28). It has been previously shown that only differentiated BMSCs, rather than untreated BMSCs, can stimulate nerve regeneration (8,27). In contrast, the present study indicated that undifferentiated BMSCs can also enhance regeneration and lead to improved motor function using a vein graft.

The value or necessity of isolating and proliferating specific adherent cell lines from each tissue source, and the impact of additional growth factors contained in bone marrow, are poorly understood. Additionally, regulatory authorities such as the FDA allow autologous minimally manipulated cell therapy when the procedures do not appreciably change the cells (i.e. differentiation), whereas more manipulative methods, such as differentiation and sorting, may require formal approval as a drug before clinical use (29). Undifferentiated BMSCs provide an alternative source of multipotent cells in the form of concentrated nucleated cell populations, many of which may be clinically relevant when compared with differentiated bone marrow (30). Thus, the choice of undifferentiated BMSCs as a more readily accessible and instant source of multipotent cells might seem more favorable than differentiation for cell therapy. Our results suggest that some form of transdifferentiation might occur in vivo as the result of local signals from injured Schwann cells and axons. However, the long-term effect of undifferentiated cells remains to be determined, and therefore we will continue to study the mechanisms of undifferentiated BMSCs in hope of generating clinically useful cells for the treatment of peripheral nerve injuries.

In conclusion, undifferentiated BMSCs could provide an injectable, instant, and more readily accessible source of cells than differentiated BMSCs. They enhanced sciatic nerve regeneration, which could be taken into consideration in the emerging field of regenerative medicine and surgery. The interval from tissue harvest to injection of the cellular product is shorter in undifferentiated BMSCs compared with results for differentiated BMSCs. It also reduces costs due to the simplicity of laboratory procedures compared to those for undifferentiated BMSCs and the reduction in the interval from tissue collection to cell injection. It could be considered clinically as a translatable route toward new methods to enhance peripheral nerve repair in clinical applications without the complications associated with differentiated BMSCs.

Acknowledgments

The authors would like to thank Dr Behfar, Dr Dalir-Naghadeh, and Mr Matin, Department of Clinical Sciences, and Mr Jaafary, Urmia Pathobiology Center, for their technical expertise.

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