Transplantation of Schwann Cells Differentiated from Adipose Stem Cells Improves Functional Recovery in Rat Spinal Cord Injury

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Abstract

Background: When the spinal cord is damaged, medical procedures are vital to prevent of improvement of the lesion. Because of poor regeneration ability of central nervous tissue, the most injuries are irreversible. One of encouraging interventions for treatment of spinal cord injury is Schwann cell transplantation. However, isolation of Schwann cell for clinical interventions is complicated approach with low cells yield and purity. Thus, easily accessed sources like Adipose mesenchymal stem cells have been taken notice. Therefore, this study was planned to assess the effect of adipose stromal cell-derived Schwann cell transplantation in functional recovery after lateral hemisection in adult rats.

Methods: After isolation, adipose stem cells were differentiated to Schwann cells. The differentiation was verified by immunocytochemistry and reverse transcription-polymerase chain reaction (RT-PCR). Then, we loaded the cells into collagen scaffolds with parallel aligned canals and transplanted into rats with 3 mm lesions at T9 – T10 level. Motor and sensory improvement were evaluated by open field locomotor scale, narrow beam, and tail flick tests for 60 days. Subsequently, conventional histology and immunohistochemistry were performed.

Results: In vitro results revealed that mesenchymal stem cells after differentiation gained Schwann cells morphology and markers. Schwann cell-grafted group had significantly higher locomotor and sensory scores in comparison with the control and scaffold without cell groups. Histological observations showed differentiated cells have the ability to improve axonal regeneration and remyelination.

Conclusion: Our study proved that adipose tissue- derived Schwann cells can change the rough environment of damaged spinal cord and support axon regeneration and enhance functional recovery, and possibly be helpful for people suffering from spinal cord injuries.

Keywords: Hemisection, mesenchymal stem cells, narrow beam, rat, scaffold

Cite this article as: Zaminy A, Shokrgozar MA, Sadeghi Y, Noroozian M, Heidari MH, Piryaei A. Transplantation of Schwann cells differentiated from adipose stem cells improves functional recovery in rat spinal cord injury. *Arch Iran Med.* 2013; **16**(9): 533 – 541.

Introduction

F ollowing the spinal cord trauma, in the most of cases, people are faced with problematic neurological status, can cause the complete palsy of the lower and upper limbs. Unfortunately, a standard and effective medical procedure is not available yet. Lately, some encouraging outcomes have been gained in rodents by recruit of stem cells, nerve and spinal cord segments, growth factors and scaffolds.¹⁻⁴ Results of these expriments showed that regeneration of spinal nerve fibers across the spinal cord defect is possible. One approach to SCI restoration is transplantation of Schwann cells (SCs).⁵

Schwann cells are categorized as peripheral nervous system cells which have an active and effective role in myelin formation and peripheral nerves regeneration after injury.⁶ Therefore, these cells probably have the capacity to make a better environment for axonal regeneration of the central nervous system (CNS) after an accident. In fact, improvement of axonal regeneration

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Accepted for publication: 17 July 2013

of injured spinal cord has been reported by some studies in rats.⁷ But in reality, the isolation adequate number of Schwann cells for clinical practice is confronted with donor morbidity and low cell yield. So that, several groups are being evaluated the capability of different type of stem cells into SCs. Stem cells are a type of cell found in multicellular organisms, that the capacity to divide and differentiate to different specialized cell types.⁸

Mesenchymal stem cells (MSCs) are multipotent stromal cells that can differentiate to various type cells. On the other hand, they are a easily accessed source with high growth rate. These properties make them an interesting source for cell therapy. They can differentiate to all mesodermal lineage cells.^{9–11} Furthermore, MSCs can transdifferentiate into functional neural cells.^{12–14} In addition, these cell have low immunogenicity. Taken all together, MSCs is considered as a brilliant cell for central nervous system treatment.¹⁵ Schwann cells that differentiated from MSCs enhanced neurite outgrowth.^{16,17} In vivo studies revealed that these cells support axonal surviving and remyelination.^{18,19} Besides, our perviouse study showed bone marrow derived schwann cells can improve functional recovery, as well as axonal regenration in injured rats.²⁰

Therefore, in this study, we intended to evaluate whether adipose stem cell derived Schwann cells can be an alternative source for Schwann cells in spinal cord injury. In addition, we compared functional recovery outcomes in our present and previous studies.

Archives of Iranian Medicine, Volume 16, Number 9, September 2013 533

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Materials and Methods

Adipose- derived stem cells Isolation

Adipose- derived stem cells (ADSCs) were isolated from adult male Wistar rats (weight 250 - 300 g, n = 5) euthanized by diethyl ether. Epididymal fat was enzymatically dissociated for 60 min at 37 °C by 0.1 % (w/v) collagenase type I (Sigma-Aldrich). After centrifugation, the stromal cell pellet was resuspended in Dulbecco's Modified Eagle's Medium (DMEM) /F12 (Sigma-Aldrich) containing 10 % (v/v) FBS and 1 % (v/v) penicillin/ streptomycin solution. Cultures were maintained at subconfluent levels in a 37 °C incubator with 5 % CO₂ and passages with trypsin/EDTA (Gibco) when required. Cells at passage 3 or below were used for experimentation.

Cell characterization

Multilineage differentiation

Osteogenic and adipogenic differentiation were performed to ascertain multipotency of isolated cells. The cells cultured in differentiation media for 3 weeks. After that, the cultures were stained by Von kossa and Oil Red O for osteogenic and adipogenic differentiation, respectively.^{10,21}

Flow cytometry

Multipotent mesenchymal stem cell markers were measured by flow cytometry. For this purpose, Phycoerythrin (PE) antibodies against rat CD73 (Biocampare), CD45, CD34 and CD44 (eBioscience) were used. Analysis were performed by Flow cytometer (Becton Dickinson, USA).

Glial induction

For glial differentiation, a 3 step protocol was used. First, cells were cultured in basic medium that contained 1 mM β -mercaptoethanol for 24 hrs. Next, previous medium were replaced with a medium supplemented with 35 ng/ml all-trans retinoic acid for a further 72 hrs. For last step, the cells were treated with glial differentiation medium contained 5 ng/ml platelet-derived growth factor (PDGF), 10 ng/ml basic fibroblast growth factor (bFGF), 14 μ M forskolin, and 200 ng/ml β -heregulin (all Sigma-Aldrich) for eight days.^{22,23}

Differentiation confirmation

Immunocytochemistry

To confirm of differentiation, we evaluated the expression Schwann cell markers in the differentiated cells. For this, following antibodies were used to investigate glial markers: fluorescein isothiocyanate (FITC) - conjugated mouse monoclonal antibodies against low-affinity neural growth factor (NGF) p75 receptor (1 : 500; Abcam) and rabbit polyclonal antibodies against s100 protein (1 : 500; Abcam). After primary antibody incubation, Texas Red (TR) - linked secondary goat antirabbit antibodies (1: 1000; Abcam) were applied for s100 primary antibody. After staining by antibodies fluorescent signals were photographed with a Nikon DXM 1200 digital camera attached to a Leitz Aristoplan microscope.

RT-PCR

Total RNA of differentiated cells was extracted by RNeasy TM mini kit (QIAGEN Ltd., UK). The One-Step RT-PCR kit (QIAGEN Ltd., UK) was used for all RT-PCRs with primers which synthesized by Sigma-Aldrich (Table1). A Techen Genius thermocycler was used for all reactions.

Scaffold preparation

For cell transplantation, we used atelocollagen honeycomb (KOKEN Inc., Tokyo, Japan) scaffolds which had a unidirectional porous pattern with pore diameter between $250 - 400 \,\mu\text{m}$. 20×10^4 cells were loaded into each scaffold 24 hrs before implantation. For checking of cell existence on scaffolds, scanning electron microscope (TESCAN, Vega II, Czech).

Spinal cord injury and transplantation

Forty male Wistar rats were used for this experiment. The experimental procedures were treated in accordance with the guidelines approved by the Animal Care and Use Committee of Pasteur Institute of Iran. Animals (250 g - 300 g) were anesthetized with an intraperitoneally injection of 60 mg/kg of ketamine HCl (Alfasan, WOERDEN, HOLLAND) and 5 mg/ kg xylazine HCl (Alfasan, WOERDEN, HOLLAND). Surgeries were performed under microscope at the T9 - T10 level. By an incision, 3.0 mm of left half of spinal cord tissue was cut out. Animals were divided into four groups (10 rats in each group): the first group (scaffold+ SC-ADSCs group), SC-ADSCs filled scaffolds were implemented into lesioned site. In second group (scaffold group), a collagen scaffold without any cells were put into spinal cord. The third group (control group), only a gap was created in spinal cord. The fourth group (laminectomy group), rats were just subjected to laminectomy without any damage. For five days all animals were received antibiotics and analgesics. All groups were evaluated for 60 days.

Tissue processing and immunohistochemistry

Two months after transplantation, all animals were scarified and spinal cords were prepared for histology. After cutting samples blocks into 5 μ m thick sections, they were incubated with primary antibody at its optimal dilution, rabbit polyclonal antibody against neurofilament 200 kD (1 : 100; Abcam). After that, sections were subjected to biotinylated goat polyclonal secondary antibody to rabbit IgG (1 : 200; Abcam) and then incubated with streptavidinhorseradish peroxidase (HRP) complex. For HRP reaction, we used diaminobenzidine kit (DAB histochemistry kit, Molecular probes, Invitrogen) according to kit manufacture's instructions. For myelination, tissue samples were stained with Luxol fast blue (Sigma-Aldrich).

Sensory-motor recovery evaluation *Behaviour assessment*

Hind limb motor function was measured by using the Basso, Beattie, and Bresnahan (BBB) scale. Locomotor ability was assessed in an open area by observing each animal for 5 min, before injury and one, two, four, six, and eight weeks after injury.²⁴

Narrow beam test

In addition to BBB test, We performed another assay to measure the locomotor ability of injured rats by narrow beam test.²⁵ Rats were trained to cross a narrow pathway (4 cm wide, 80 cm height) before to surgery. Then, the assessment scores were obtained from the second week post injury for eight weeks. The ability of rats to cross the beam was scored from 0 to 3; score 0 for complete inability to walk, 0.5 was assigned when an animal passed half

Tail flick test

After surgery, we assessed the pain response in animals. A standard sensory assay, tail flick test, was used for sensation recovery after trauma.²⁶ For this, on the tail dimeter infrared beam was focused on tail and latency of tail with drawal was measured (P. antacutoffr, Ugo Basile, Comersoo, Italy). The cut-off t. me was set to the test to avoid tissue damage. For each rat, test was repeated three times.

Statistical analysis

One-way analysis of variance (ANOVA) followed by post hoc Tukey test was used to determine statistical differences between the experimental groups. Data were expressed as the mean \pm standard deviation (SD).

Results

Isolation, expansion, and characterization of MSCs

Adipose derived stem cells were isolated and cultured in flasks to grow. About one week after isolation, they formed confluent fibroblast-like monolayer (Figure 2A). Two methods were used for characterizing of isolated cells: Flow cytometry and multilineage differentiation. For flow cytometric analysis, we examined mesenchymal stem cell markers (Figure 1). As expected, they were negative for hematopoietic stem cell marker, CD 45. On the other hand, the isolated stromal cells strongly expressed mesenchymal stem cells; CD 90, CD73 and CD 44. The other method we performed to find out whether the isolated cells were multipotent, was multilineage differentiation. The cells were subjected to osteogenic and adipogenic media. Von kossa staining confirmed that isolated cells produced calcium salts (Figure 2B). Adipogenic differentiation was verified by Oil Red O staining of lipid droplets (Figure 2C). These data confirmed that the cultured cells are multipotent stem cell.

Differentiation and characterization MSC derived SCs for transplantation

Next step after multipotency confirmation, was differentiation to Schwann like cells. Cells were induced by the protocol that mentioned earlier. During differentiation, the cells lost their fibroblastic features and gained a spindle shape with two or



Figure 1. Flow cytometric characterization of ADSCs. CD marker analysis of MSCs at passage3 revealed a similar positivity of indicated surface markers between isolated cells from adipose tissue and MSCs.



Figure 2. Multilineage differentiation. Undifferentiated ADSCs, under phase contrast microscopy, display a flattened fibroblast-like morphology; A) Scale bar 100µm. Von Kossa staining of mineralized tissue; B) Scale bar 10µm and Oil Red O positive intracellular lipid droplets; C) Indicates that these cells can differentiate into osteoblasts and adipocytes. Scale bar 100µm.



Figure 3. ADSCs post-differentiation, show a bipolar, spindle-shaped morphology with two to three processes. Differentiated MSCs; A) Immunofluorescence staining of differentiated MSCs, Anti-s100-Texas red staining; B) anti-p75-FITC staining; C) Scale bar 100µm.



Figure 4. Expression pattern of several genes in, transdifferentiated MSCs, at mRNA level. For product sizes, see Table 1.

Table1. Primer sequences for RT-PCR			
Rat primer	Accession number	Sequence	Product size (bp)
S100	NM_013191.1	5′-ATAGCACCTCCGTTGGACAG-3′ 5′-TCGTTTGCACAGAGGACAAG-3′	132
B-actin	NM_031144.2	5'-CACCCGCGAGTACAACCTTC-3' 5'-CCCATACCCACCATCACACC-3'	207
PMP22	NM_017037.1	5'-TCTCACGGTCGGAGCATCA-3' 5'-CAGTCCTTGGAGGCACAGAAC-3'	97
BDNF	NM_012513.3	5'-GTCATTGGTAACCTCGCTCATTC-3' 5'-ATAGATTTACGCAAACGCCCTC-3'	90
NT-3	NM_031073.2	5'-CGGCAACAGAGACGCTACAA-3' 5'-CCTCCGTGGTGATGTTCTATTG-3'	132
NGFr(p75)	NM_012610.2	5'-GGTCTATTCTGATGGAGTCAAGCTAAG-3' 5'-ACCAAGAATGAGCGCACTAACAG-3'	123
NGF	XM_003753645.1	5'-GGACGCAGCTTTCTATCCTG-3' 5'-GTCCGTGGCTGTGGTCTTAT-3'	496

three processes (Figure 3A). Antibodies against of Schwann cell markers, $p75^{NGFR}$ and $s100\beta$ were used to check glial differentiation. Immunocytochemistry showed that induced MSCs were positive for s100 and p75, widely recognized as markers for Schwann cells (Figure3). Approximately 45 % – 50 % of the cells were positive for s100 marker. In addition of immunocytochemistry, RT-PCR analysis was performed after SC differentiation to the command of the Galileo gene expression such as p75, s100, NGF, BDNF, NT-3, and PMP22. The RT-PCR analysis confirmed that after two week induction, MSCs were differentiated to Schwann- like cells (Figure 4). After differentiation confirmation, cells were loaded into scaffolds 24 hours before implantation. Scanning electron microscope images were shown the presence of cells inside the scaffolds (Figure 5).

Animal surgery

Inability to move of ipsilateral hind limb after surgery indicated the procedure was successful, otherwise excluded from the field. Nearly 30 % of the animals died after surgery due to ulcer and autophagia. The other reason of rats death was a major weight loss. Losing weight in the first month of after surgery happened almost in all animals, but most of them recovered. In this study, scaffolds were implemented in the acute phase and during surgery. No tumor formation was observed at transplantation site during the study.

Histologic findings

Eight weeks following transplantation, spinal cord tissues were evaluated histologically (Figure 6). In the transplanted groups, we did not observe any boundaries and gaps between scaffolds and host tissues and spinal cord tissue had structural continuity with the implanted scaffolds. The central portion of the lesion site was occupied by a cystic cavity in the cell implanted groups. A narrow structural continuity, composed of connective tissue, was found in some of the control group at the eighth week postoperatively. In the transplantation group, the tissue surrounding was filled with a large number of cells.



Figure 5. Scanning electron microscopy of scaffold showed presence of SC-MSCs in scaffolds before implantation, the upper surface; A) Inside of scaffold pores; B) Indicated by arrows. Scale bar 200µm A, 50 µm B.



Figure 6. Spinal cord sections at eighth week after injury and following implantation of graft. H&E staining of longitudinal sections, control; A) Scaffold; B) SC-ADCSs; C) Groups. Scale bar 200µm.



Figure 7. Myelination of some axons were shown by Luxol fast blue staining, bare scaffold; A) SC-ADSCs; B) indicated by arrow. Scale bar 100µm. Immunohistochemistry staining for neuronal fibers, positive axons for NF200 were detected at the lesion site as black spots, indicated by arrows. Bare scaffold; C) SC-ADCSs; D) Scale bar 100µm.

Axon regeneration and myelation

After Immunohistochemistry, regenerated axons were seen to cross the implant–spinal cord in the implant group. In the implanted groups, nerve axons were found to run parallel to the axis of the plant. A number of regenerated nerve axons were found to run parallel to the axis of the scaffold and spinal cord in the middle of the implant, mainly at its periphery (Figure 7A, 7B). Many myelinated axons were found to run through the implant in the implant groups, especially in cell- treated groups (Figure 7C, 7D). Nerve-free cysts occupied the central portion of the implants.

Functional recovery

Before the injury, bilateral hind limb BBB scores of all animals were 21. All scores decreased to 0 - 1 after of injury, except



Figure 8. Hind limb functional recovery. The BBB scale scores of the implanted group were significantly better than those of the control group at eighth week postoperatively. The results of our previous study (SC-BMSCs) is also presented. Laminectomy group is omitted. The data represent mean \pm SD. *P < 0.05.



Figure 9. Narrow beam test. This sensorimotor test showed recovery of function in the implanted group in comparison with the control group at eighth week. *P < 0.05.

laminectomy group. The behavioral effects (Figure 8) were collected every two weeks for two months. By the second week, all groups showed quite similar recovery, however, after that the score differences increased gradually. The BBB scores were in the range of 4.1 ± 0.64 in the control animal two months after SCI. At 8 weeks after transplantation, the scores of transplanted animals showed BBB scores significantly (P < 0.05) higher than that achieved by the control group. Cell transplanted group reached a score of 7.9 ± 0.55 by 8 weeks. There were statistically significant differences between the cell transplanted group and other groups (P < 0.05). In comparing to our previous study, bone marrow derived Schwann cell transplanted group reached a final average score of 7 ± 0.51 which was higher than other groups. However, no significant difference in functional recovery was seen between rats that received differentiated mesenchymal stem cells in our studies.

Figure 10. Tail flick test. Responses to thermal stimuli in tail flick showed hypersensitivity at second week post-transplantation. In the implanted group and cell implanted group, further recovery occurred toward normal values in comparison with the operated controls. *P < 0.05.

Narrow beam test

Functional recovery also was evaluated by narrow beam test (Figure 9). All rats were trained before surgery to cross narrow pathway without any errors. After injury test scores showed severe locomotion impairment. Recovery was too slow in all groups, however, some of the animals could cross the beam by eight weeks. No significant differences were found between groups after cell transplantation. Nevertheless, cell transplantation groups (our present and previous studies) showed significantly higher scores in comparison with the control and plain scaffold.

Tail flick test

The thermal sensitivity test consists of applying a low intensity heat stimulus to the plantar surfaces of the tail and measuring the latency to withdrawal. Scores showed hypersensitivity after injury in all groups (Figure10). The sensation was recovered gradually. After eight weeks, animals that had received cell grafts recovered closer to laminectomy uninjured group levels (6 s - 7 s), showed significant improvement over control grafts at eight weeks, but no significant difference was shown between the cell-treated groups.

Discussion

Our study showed adipose derived stem cells successfully differentiated to Schwann like cells. Then, the differentiated cells were transplanted into rats with spinal cord injury lateral hemisection model. For transplantation, collagen scaffolds with aligned canal were used. Our results revealed that ADSC derived Schwann cells have the capability to promote axonal regeneration and myelation after nervous tissue trauma. In vivo observations also showed that these cells have the potential to help with sensorymotor recovery. Besides, we compared the results of our previous experiment with new data. The findings showed both the source of mesenchymal stem cells is able to enhance functional recovery after injury. However, they have almost the same potential for healing of injured spinal cord.

Growth factors and extracellular matrix which is secreted by Schwann cells, have a key role to change unpleasant and rough environment after trauma and enhance axonal regeneration.^{27,28} However, their usage due to some limitations like low proliferation rate, contamination with other cells e.g. Fibroblast and donor morbidity, is confined. So, researchers have been encouraged to seek alternatives to SCs. Easily accessible sources like mesenchymal stem cells have attracted attention to them, especially there are some reports about their potential to trans-differentiation into cells with Schwann cells features and function.^{21,22,29,30} Nevertheless, there are many doubts about their operational capacities. Therefore, we designed this study to investigate that Schwann cell derived from adipose stem cells have the capacity to promote axonal regeneration, consequently in sensory-motor recovery after spinal cord injury in hemisection model. Meanwhile, we compared the results with our previous findings.

One of the principal causes that disrupts the axonal regeneration after injury, is lack of conducting extracellular matrix that helps to neuronal fiber regrowth.³¹ The main factor that effects on axon regeneration, is environmental signals.^{32,33} Contact guidance is one of the signals that transected axons need to reconnect after spinal cord injury.³⁴ Therefore, the presence of a structure that connects two ends of a trauma zone, is necessary to guide regenerating axons³⁵ and help to reconstruct spinal cord tissue.³⁶

Different scaffolds with various patterns and substances have been investigated for axonal regeneration after nervous tissue trauma.^{37,38} It was shown that the pattern and structure of synthetic matrix have a strong effect on tissue regeneration.³⁹ Therefore, we used collagen scaffold with aligned and parallel pores, and expected this pattern could help the regenerated axons in the injured spinal cord in a specific and correct direction. For monitoring whether axons regrow after our interventions, we used antineurofilament 200 antibodies. The findings indicated that the scaffold had a positive influence and increased the number positive fibers at the lesion site. The cell transplantation increased the number of NF-positive fibers that entered the scaffold. Nevertheless, the regenerated axons mainly accumulated around the injured area.

Mesenchymal stem cell derived Schwann cell transplantation caused tissue formation in damaged site and increased locomotion activity. These cells also increased myelination in growing axons. Our data demonstrated that SC-MSCs are helpful and can increase recovery outcomes. The results of our experiment were consistent with previous reports.^{17,40} This might be achieved either by direct remyelination by SC-MSCs or by stimulation of endogenous precursor cells and protection from more cell loss resulting from the action of neurotrophic factors released fromechanisms.⁴¹ In our studies probably both mechanism were contributed in promotion of tissue repair mainly because when these cells used for peripheral nerves repair, they directly engaged in myelin sheath formation. In addition, they secreted growth factors such as NGF, BDNF, CTNF, and GDNF like peripheral Schwann cells. But it calls for further studies to reveal which one (endogenous or transplanted SCs) and how much percent involve in spinal cord injury repair.

The RT-PCR showed the cells produced various types of neurotrophic factors that were consistent with former studies.^{6,42} Growth factors enhance axon regeneration and functional recovery.^{43,44} Therefore, growth factor secretion can be one of the ways that SC-MSCs help to repair of spinal cord tissue after transplantation. They may stimulate axon regeneration and endogenous SCs migration.⁴⁵ The differentiated cells in addition to neurotrophic factors secrete vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF).⁴⁶ They help to neuronal division and axonal outgrowth,^{47,48} also involve in axon myelination and regeneration, neuronal survival, differentiation, and glial cell proliferation.^{49,50}

Our results showed functional recovery was poor and some recovery was observed in tasks that depend more on sensory information (narrow beam and tail flick). Recovery in sensory based tests probably was due to growth of dorsal root primary afferents which happened after spinal cord injury^{51,52} and in response of NGF secretion.^{53,54} Descending tracts also send collateral fibers to the injury site.^{55,56} These mechanisms may cause the recovery that observed in the hind limb after injury and for the greater recovery observed after transplantation.

Hypersensitivity generally happened after spinal cord injury.^{57,58} Some cell type after transplantation can increase this sensitivity.⁵⁹ This phenomenon may be partly because of that nociceptive dorsal root axons are sensitive to injury⁶⁰ and neurotrophic factors^{61–64} and grow in response to them. The sensitivity was observed in our studies after injury that recovered gradually by eight weeks.

The level of contribution of spinal cord pathways to recovery in the thermal sensitivity test was not assessed in our surveys, but it is necessary to investigate the potential for recovery mediated by these tracts to find new strategies for treatment of this occurrence. Therefore, more studies with different approaches and methods are needed for better understanding of axon regeneration and find the correct procedure for treatment of SCI.

In summary, implantation of scaffold improved functional recovery in the injured rats and the presence of Schwann like cells along with them significantly increased sensory-motor outcomes. The results revealed combination of the SC-MSCs and scaffold are important for greater recovery. Therefore, transplantation of Schwann cells derived from mesenchymal stromal cells is a potentially useful treatment for spinal cord injury. But before the clinical application Schwann-like cells, extensive laboratory tests are needed to establish a comprehensive approach for more effective functional recovery.

Acknowledgments

We would like to thank Martin Oudega (Pittsburg US) and Ales Hejcl (Czech Republic) for their generous scientific assistance.

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