

## Remyelination improvement after neurotrophic factors secreting cells transplantation in rat spinal cord injury

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### ABSTRACT

**Objective(s):** Neurotrophic factors secreting cells (NTS-SCs) may be a superior cell source for cell-based therapy in neurodegenerative diseases. NTS-SCs are able to secrete some neurotrophic factors such as nerve growth factor and glia-derived neurotrophic factor. Our primary aim was to assess transplantation of neurotrophic factor secreting cells derived from human adipose-derived stem cells (hADSCs) into the damaged spinal cord rats and determine the potential of these cells in remyelination.

**Materials and Methods:** To this end, 40 adult male Wistar rats were categorized into four groups including: control, lysolecithin (Lysophosphatidylcholines or LPC), vehicle, and NTS-SCs transplantation. Local demyelination was induced using LPC injection into the lateral column of spinal cord. Seven days after the lysolecithin lesion, the cells transplantation was performed. The ultrastructure of myelinated fibers was examined with a transmission electron microscope to determine the extent of myelin destruction and remyelination 4 weeks post cell transplantation. Moreover, the presence of oligodendrocyte in the lesion of spinal cord was assessed by immunohistochemistry procedure.

**Results:** The results of current study indicated that in NTF-SCs transplantation group, the remyelination process and the mean of myelin sheath thickness as well as axonal diameters were significantly higher than other groups ( $P < 0.001$ ). Furthermore, immunohistochemistry analysis revealed that in NTF-SCs transplantation group more than 10 percent of transplanted cells were positive for specific markers of oligodendrocyte cells.

**Conclusion:** NTF-SCs transplantation represents a valuable option for cell-based therapy in the nervous tissue damages.

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### Introduction

Spinal cord injury (SCI) is defined by destruction of neural and glial cells such as oligodendrocytes, which leads to demyelination and its attendant disability as well as neurological disorders. So far, there is no proficient treatment for this pathological condition, but the present documents demonstrated that cell-based therapies (1) such as bone marrow stem cells transplantation have some beneficial effects in treating this condition (2). It has been proven that mesenchymal stem cells (MSCs) are able to induce high range of neurotrophic factors that are able to amend cellular function, tissue regeneration and promote cell differentiation (3), and regulate the development of nervous system by intracellular signaling via particular receptors (4).

The results of a previous study showed that brain-derived neurotrophic factor (BDNF from bone marrow MSCs (BMSCs) transplantation can induce functional recovery of SCI via enhancing axonal

remyelination (5). Moreover, it has been shown that transplantation of BMSCs derived Schwann cells can diminish the size of cystic cavity and increase axonal regeneration as well as functional recovery (6).

Adipose tissue compared to other stem cell sources seems a better cell source. Because, it is an abundant tissue and contains more stem cells than other sources (7) and has the potential to differentiate into other cells including neural and glial cells (8). The expression of Krox20 myelination master-gene and immunomodulatory properties are other characteristics of adipose-derived stem cells (ADSCs) (9). Of all the growth factors that are secreted by ADSCs, nerve growth factor (NGF), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), glia-derived neurotrophic factor (GDNF), hepatocyte growth factor (HGF), and neurotrophics (NT) such as NT-1 (10) have high range of performance, which with myelin shell components that are also secreted by ADSCs, may play an important role in promotion of remyelination process and

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maintenance of CNS functions (11).

It has been reported that transplantation of ADSCs with overexpressing the neurogenin2 (Ngn2-ADSCs) could promote the functional recovery of SCI through upregulated BDNF and VEGF and inhibited the glial scar formation (12). This result is consistent with recent reports where in the transplantation of ADSCs for SCI was shown to be free of serious adverse effects and can promote neurological functions (13).

NTF-SCs secrete significant amounts of neurotrophic factors (NTF) compared to MSCs (14), thus NTF-SCs transplantation can be an important option in the treatment of neurodegenerative diseases.

NTF-SCs by secreting a family of proteins that are essential for neuronal viability and development, could pave the way for the treatment of patients suffering from neurodegenerative diseases (15). Consistent with our previous works, which indicated that ADSCs can secrete nerve growth factors (16, 17), NTF-SCs were also able to produce several nerve growth factors (14), thus these cells can serve as vehicles for delivering NTFs into nervous tissue. Therefore, in present study we evaluated the effects of *in vivo* transplantation of NTF-SCs differentiated from hADSCs in demyelinated spinal cord rat.

## Materials and Methods

### Cell isolation and culture

All materials that were used in this study, except specified ones, were purchased from Sigma-Aldrich, St Louis, MO, United States of America. In addition, all stages of experiment were approved by the Ethics Committee of Isfahan University of Medical Sciences. After receiving informed consent of female donors (with age range: 20–40 years), hADSCs were isolated from their superficial abdominal fat and expanded as previously described (18). Briefly, in order to eliminate the contaminated debris, tissue samples were washed twice with phosphate-buffered saline (PBS), then incubated in 0.075% collagenase type I (30 min at 37°C). After enzyme neutralization and centrifuging, the cellular pellet was resuspended and cultured at standard condition in Dulbecco's Modified Eagles Medium (DMEM) (Gibco BRL, Paisley, UK) supplemented with 10% FBS and 1% penicillin/streptomycin. At about 80 % confluency, the cultured cells were passaged using 0.25% Trypsin and 0.02% ethylene diamine tetra acetic acid (EDTA).

### Characterization of human ADSCs

For characteristic confirmation of hADSCs, the isolated cells (at three passages) were examined by Flow cytometry (Becton Dickinson). These cells were harvested and then incubated with Fluorescein isothiocyanate (FITC) or phycoerythrin conjugated antibodies against CD90, CD44, CD105, CD34, CD14, and CD45 (Chemicon, Temecula, CA, USA) for 30 min.

In addition, nonspecific FITC-conjugated IgG was used for isotype control.

### HADSCs induction into NTF-SCs cells

In order to NTF-SCs induction, according to the previous study (17), hADSCs ( $1 \times 10^6$ ) were cultured in DMEM/F12 (SPN, L-glutamine) supplemented with 20 ng/ml human basic fibroblast growth factor (hbFGF), 20 ng/ml human epidermal growth factor (hEGF), and N2 supplement for 3 days. After this time, the pre-differentiation medium was changed to DMEM/F12 (SPN, L-glutamine) containing 1 mM dibutyryl cyclic AMP (dbcAMP), 0.5 mM isobutyl methyl xanthine (IBMX), 5 ng/ml human platelet derived growth factor (PDGF), 50 ng/ml human neuregulin 1-b1/HRG1-b1 EGF domain (R & D Systems) and 20 ng/ml hbFGF for 3 days.

### Cell viability assessment

NTF-SCs viability assessment was performed using MTT assay. MTT (5 mg/ml) was added into the culture medium at 1:10 dilution and the plates were incubated for 4 hours. After aspiration of medium and addition of 200  $\mu$ l dimethyl sulfoxide (DMSO) into each well, the absorbance of solution was detected by a microplate reader (Hiperion MPR 4+, Germany) at 540 nm.

### NTF-SCs labeling with PKH26

NTF-SCs were labeled with PKH26 according to manufacturer's procedure. Briefly, after cell washing with serum free medium, in order to obtain a loose cell pellet, the cells were centrifuged (5 min, 400  $\times$  g), and then a cell suspension containing  $1 \times 10^6$  NTF-SCs in 1 ml of diluent was prepared and incubated for 1–5 min. After stopping PKH-26 staining with bovine serum albumin (1%) and cell washing, in order to define effectiveness of labeling, the stained cells were examined by fluorescence microscope (Olympus BX51, Japan). In addition, a few of labeled cells were cultured in standard condition to confirm cell viability.

### Induction of demyelination in lateral column of spinal cord

Forty male Wistar rats (weight 200–250 g) were purchased from Pasteur Institute (Tehran, Iran) and were communally housed on a 12-hr light/dark cycle with free access to water and standard dry diet. All animal experiments were approved by the Animal Ethics Committee of the University of Isfahan (No: 189067).

In this study, adult male Wistar rats were categorized into four groups each with 10 rats including: control (only laminectomy), LPC (laminectomy and demyelination), vehicle control (laminectomy, demyelination and injection of 2  $\mu$ l basal medium), and NTF-SCs transplantation

(laminectomy, demyelination and injection of 2  $\mu$ l medium containing PKH-26 labeled NTF-SCs). After animal anesthetizing with ketamine/xylazine (100/10 mg/kg, IP), laminectomy was carried out at the level of the T9/11 vertebra. In order to demyelination of white matter of spinal cord, 2  $\mu$ l solution of 1% LPC (Aburaihan Pharmaceutical, Tehran City, Iran) in saline, pH=7.4 was injected into the lateral column of the rat spinal cord through a glass micropipette (Supa, Tehran, Iran) (Outer-tip diameter 50  $\mu$ m). In order to avoid of LPC backflow, micropipette was kept 2 min further in the site of injection. Finally, laminectomy site was sutured and 15 mg/kg Gentamycin (Hakim Pharmaceutical, Tehran City, Iran) with lactated ringers' solution (5 ml, IP) were given to prevent infection.

### Cells transplantation into the lysolecithin-treated spinal cord

NTF-SCs transplantation was performed one week after inducing of demyelination, according to Boido *et al* protocol (19). Briefly, following general anesthesia and re-exposing the laminectomy site, in cell transplantation group, 10  $\mu$ l medium containing  $1 \times 10^6$  PKH26-labeled NTF-SCs was injected slowly (over about 1 min) into the lateral column nearly at the same level of LPC lesion by a glass micropipette. While, for the control and LPC groups only laminectomy site was re-exposed and for the vehicle control group 10  $\mu$ l medium was introduced gradually into the spinal cord lesion.

### Electron microscopic examination

Rats were transcardially perfused with 2.5% glutaraldehyde and 2.5% Paraformaldehyde (PFA) in 0.1 M PBS. Dissected spinal cords were fixed in 1% osmium tetroxide. Then, embedded samples were sectioned and stained with uranyl acetate and lead citrate, subsequently observed with a transmission electron microscope (LEO 906 Germany, 100 kV). The myelin sheath thickness and axonal diameters in different groups were assessed with image J software.

### Immunohistochemistry analysis

At the end of experiment, following deep anesthesia and fixative perfusion, rat spinal cord was dissected and processed for serial frozen sections (10  $\mu$ m thick) using a cryostat microtome. In order to examine the presence of myelinating oligodendrocyte, immunohistochemistry staining was carried out with specific antibody (16). Briefly, after sample permeabilization, incubation was performed in primary antibodies; mouse monoclonal anti-Major basic protein (MBP) (1:1000) (Abcam, Cambridge City, MA, USA) and mouse monoclonal anti-Olig2 (1:1000, Abcam), and subsequently in secondary antibody; Goat anti-mouse FITC - (1:2000, Abcam).

Finally, nuclear counterstaining was performed using 4', 6-Diamidino-2-Phenylindole, Dilactate (DAPI). The cells were examined using a fluorescence microscope (Olympus BX51, Tokyo City, Japan) for at least five sections of each sample. The ratio number of labeled positive cells to the number of nuclei was calculated for each antigen by Image J, a public domain, Java-based image processing program developed at the National Institutes of Health (NIH).

### Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) and independent sample *t*-test. Data are presented as mean  $\pm$  SEM, and  $P < 0.05$  was considered to be statistically significant.

## Results

### HADSCs and NTF-SCs characterization

As shown in Figure 1a, hADSCs in primary culture exhibit a typical fibroblast-like morphology. Moreover, flow cytometric results of hADSCs within passage 3 showed that hADSCs were positive for CD44, CD90, and CD105, but the results were negative for hematopoietic markers including CD14, CD34, and CD45 (Data was not shown).

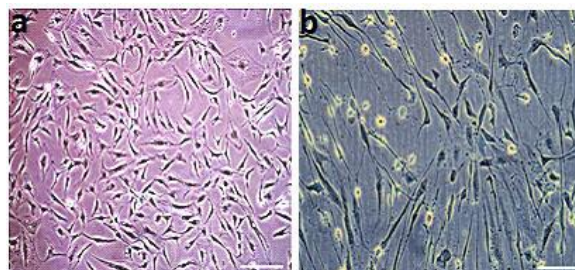
In the end-stage of differentiation, NTF-SCs display a satellite-like morphology (Figure 1b). Moreover, in our pervious study using real time-PCR and enzyme linked immunosorbent assay (ELISA) the ability of differentiated cells in the production of neurotrophic factors was demonstrated (17).

### Cell viability

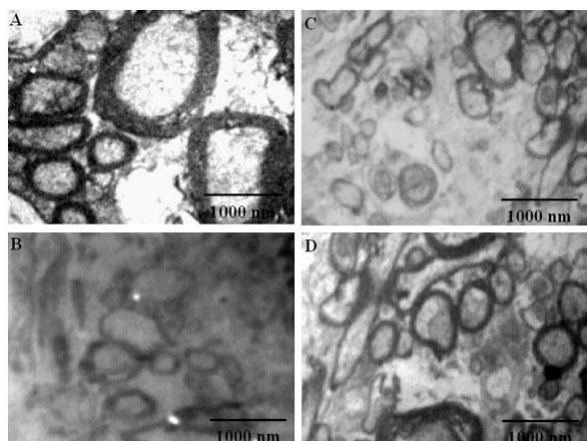
We examined the viability of NTF-SCs by MTT assay. The mean absorbance of NTF-SCs ( $0.7 \pm 0.18$ ) was significantly increased as compared to hADSCs ( $0.41 \pm 0.12$ ) ( $P < 0.05$ ).

### Ultrastructural evidence of remyelination

Electron microscopy of the cell transplanted rat spinal cord showed myelinated axons in the lesion by their multilayered myelin feature. Multilayered compact myelin sheaths in NTF-SC transplantation group were higher as compared to other groups at 4 weeks post cell transplantation (Figure 2).

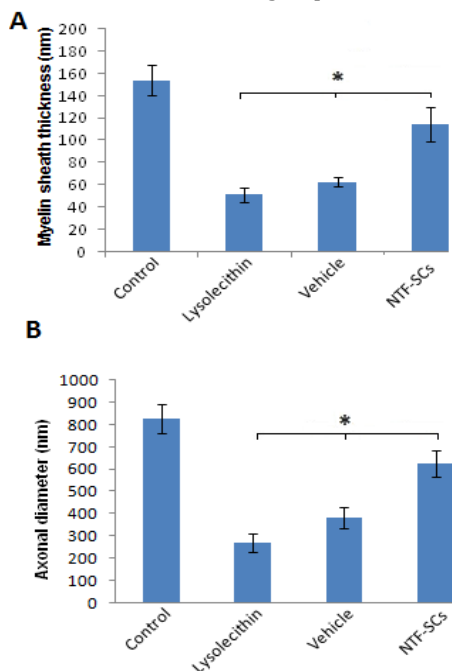


**Figure 1.** Phase contrast images of human adipose-derived stem cells (hADSCs) differentiation to neurotrophic factor secreting cells (NTF SCs). Cultured hADSCs in passage three (a), hADSCs induced to NTF-SCs differentiation at the end of differentiation process (b). Scale bars = 150  $\mu$ m



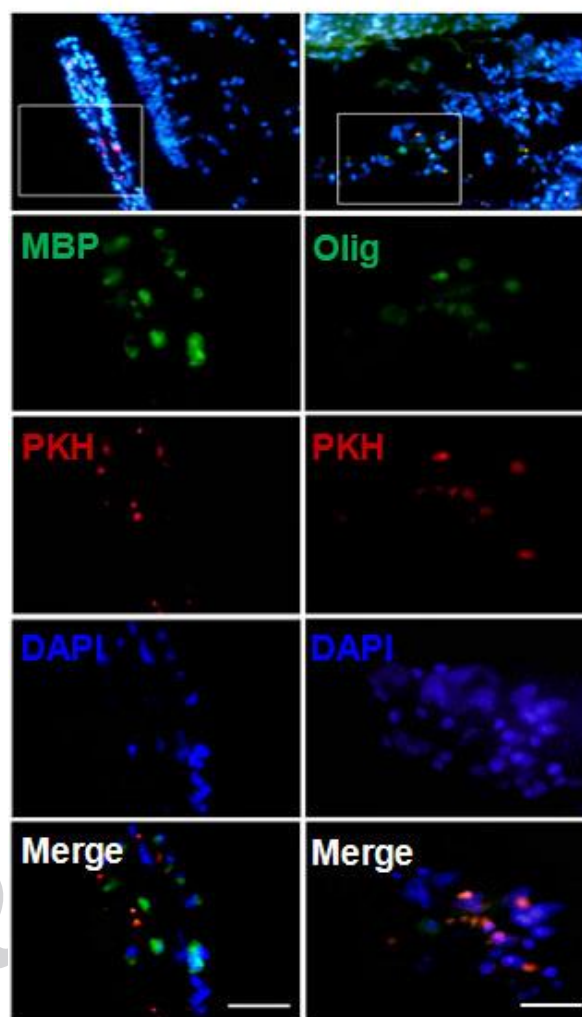
**Figure 2.** Ultramicrophotographic images of remyelination following engraftment of neurotrophic factor secreting cells (NTF-SCs). Representative electron micrographs show myelinated and demyelinated axons in the spinal cord sections before and four weeks post cell transplantation. Control (A), lysophosphatidylcholines (LPC) (B), vehicle (C), NTF-SCs (D). Scale bar = 1000 nm

The mean of myelin sheath thickness was significantly increased in NTF-SCs transplantation group ( $105.05 \pm 11.7$  nm) relative to the lysolecithin ( $51.06 \pm 6.33$  nm) and vehicle ( $62.66 \pm 4.14$  nm) groups ( $P \leq 0.05$ ) (Figure 3A). Also, the mean of axonal diameter was significantly increased in NTF-SCs transplantation group ( $596.4 \pm 60.90$  nm) than lysolecithin ( $271.66 \pm 40.55$  nm) and vehicle ( $383.8 \pm 48.76$  nm) groups ( $P \leq 0.001$ ) (Figure 3B). However, the differences of changes in mean axonal diameters and myelin sheath thickness in NTF-SCs transplantation group were not significant relative to control group.



**Figure 3** The mean of myelin sheath thickness (A) and the mean of axonal diameters (B) in different groups. Comparative study of the ultramicrographs show that the mean of axonal diameters and myelin sheath thickness was significantly increased in cell transplantation group relative to in lysolecithin and control vehicle groups ( $*P < 0.001$ )

**NTF-SC transplant group**



**Figure 4.** Longitudinal sections of lysolecithin lesions and immunohistochemistry staining four weeks after cell transplantation. Neurotrophic factor secreting cells (NTF-SCs) prelabeled with PKH26 (red) and counterstained with 4', 6-Diamidino-2-Phenylindole, Dilactate (DAPI) (blue). The PKH26 positive transplanted cells presented Olig2 and MBP markers. Scale bars represent 100  $\mu$ m

**Immunohistochemistry study of NTF-SCs transplantation**

Fluorescence microscopic analysis were showed that in cell transplantation group,  $10.8 \pm 0.9\%$  of the transplanted cells were MBP marker positive, a indicator for myelinating oligodendrocyte, and  $5 \pm 1.11\%$  of them were Olig2 marker positive, a indicator for immature oligodendrocyte (Figure 4).

**Discussion**

The SCI has been explained as an acute or chronic lesion due to trauma, loss of its normal blood supply, and compression from tumor or infection, which can lead to severe physical and neurological defects. The pathological hallmarks of SCI are macrophage

activation and aggregation of astrocytes in the SCI lesion (astrogliosis), which acts as regeneration barrier (20).

So far, there is no definitive treatment for SCI. Recently, MSC-based therapies are proposed as a new approach for treatment of neural defects due to their immune modulatory effects and neuroprotective potential (1, 21). Recently, Zurita *et al* demonstrated that after BMSCs transplantation into SCI, these cells produce a bridge between proximal and distal parts of nerve injury in spinal cord lesion, which provide support for regenerated axons, and express marker of neural proteins (22). In addition, another study revealed that isolated neural stem cells from spinal cord will have potential to differentiate into oligodendrocytes due to NTF production (23).

Fat tissues are an appropriate source of MSCs, which are known for their capacity to secrete bioactive growth factors *in vitro* as well as *in vivo* condition (24).

A wide range of NTFs have been applied in attempts to improve morphological and behavioral outcomes after SCI. For example, recent studies have shown that VEGF is able to reduce the symptom of the SCI via inhibiting the inflammation and increasing the autophagy function (25). It was demonstrated that ciliary neurotrophic factor -over expressing BMSCs can improve feature of SCI and can be used in the treatment of traumatic CNS injuries in the near future (26).

On the other hand, BDNF contributes to neuron survival, regulation of neurotransmitter releasing as well as outgrowth of dendrite and has a direct impact on synaptic transmission (27). In addition, NTF3 enhances the sprouting and local growth of corticospinal tract axons; GDNF promotes remyelination after SCI and elicits tropic effects on adult spinal axons and Schwann cells that lead to growth of damaged axon. Also, NGF has positive synergistic effect with BDNF, which can promote the biosynthesis of myelin, delay the onset of autoimmune encephalomyelitis lesions, diminish neuronal apoptosis of spinal cord and improve locomotors function (28).

Neurotrophic factor delivery method into CNS is also an important variable, which is performed in various ways, including injection of recombinant proteins, using osmotic mini-pumps and polymer release vehicles, using of gene therapy to modify neural cells and finally cell-mediated delivery (29).

NTFs have short half-life and when delivered exogenously, their efficacy in the CNS is reduced due to the blood-brain barrier, so cell-mediated NTFs delivery could be an ideal vehicle for delivering NTFs into the CNS.

HADSCs transplantation in the rat model of MS showed that these cells can improve myelination

either by differentiating into mature oligodendrocyte or by stimulating migration of oligodendrocyte precursor cells into the lesion (16). Hence, administration of these cells can ameliorate neural disorders.

Successful transplantation of NTF-SCs in a variety of neurodegenerative diseases has been performed by a number of researchers (15, 30, 31). Although the exact mechanism responsible for the therapeutic effect of NTF-SCs transplantation remains unclear, but it seems to be due to potential of cell migration and NTF secretion of these cells. Consistent with this hypothesis, Sadan *et al* demonstrated that NTF-SCs can migrate to induced lesion in the rat model of Huntington's disease (31).

Here, we had differentiated hADSCs into NTF secreting cells and transplanted in the rat model of SCI. We indicated that NTF-SCs can survive and migrate around the lysolecithin lesions, by means of immunohistochemistry confirmation.

The electron microscopic photograph demonstrated that transplantation of NTF-SCs reduced the areas of demyelination in the spinal cord and enhanced remyelination as well as improved myelin sheath thickness and axonal diameter. In addition, the transplanted cells in the lesion area, expressed specific markers of myelin and immature oligodendrocyte (MBP and Olig2). These results are consistent with other studies, which have also found that NTF-SCs can effectively ameliorate the symptoms of various neurodegenerative diseases (30, 31). However, the efficiency of NTF-SCs transplantation in remyelination of nerve fibers is higher than ADSCS transplantation (16) in spinal cord lesion induced by lysolecithin. One hypothesis for these events may be NTF-SCs secreted higher levels of NT factors, which could support the survival, growth and maturity of inherent oligodendrocyte progenitor cells that lead to promotion of remyelination process.

## Conclusion

Overall, our results showed that the NTF-SCs transplantation in LPC lesion can induce differentiation of oligodendrocyte precursor into mature oligodendrocyte cells and moderate remyelination. Hence, NTF-SCs transplantation may be an ideal cell source for cell-based therapy in neurodegenerative diseases.

## Conflicts of interest

The authors declare no conflicts of interest.

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