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| Original Article |

Neurotrophin-3-Over-Expressing Mesenchymal Stem Cell Transplantation Improves Functional Recovery and Axonal Outgrowth in a Rat Model of Spinal Cord Injury

Kyung Hee Lee[5†], Jin soo Oh[2,3†], Yoon Ha[2,4], Bae Hwan Lee[1,3], William A. Pennant[2], Do Heum Yoon[2,3,4], and Keung Nyun Kim[2,4*]

[1]*Department of Physiology,* [2]*Department of Neurosurgery,* [3]*Brain Korea 21 project for Medical science,*
[4]*Spine and spinal cord institute, Yonsei University College of Medicine, Seoul, Korea*
[5]*Department of Dental Hygiene, Division of Health Science, Dongseo University, Busan, Korea*

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Abstract: We investigated whether the transplantation of neurotrophin-3 gene transfected mesenchymal stem cells (NT-3-MSC) could promote hind limb functional recovery and axonal outgrowth in a model of spinal cord injury. We found that NT3-MSC transplantation in the contused spinal cord significantly improved hind limb function, axonal outgrowth, and MSC survival, suggesting that gene therapy combined with MSC treatment may represent a useful strategy for treatment of spinal cord injury.

Key words: *spinal cord injury, human mesenchymal stem cell, neurotrophin-3, transplantation*

1. Introduction

Several therapeutic strategies have been developed to promote functional recovery and axonal outgrowth after spinal cord injury (SCI). The application of neurotrophic factors, such as the neutrophins, has been shown to improve axonal outgrowth after injury.[1-5]

Recent studies have reported that mesenchymal stem cells (MSCs) promote some functional recovery after grafting into sites of injury in the spinal cord.[6-9] Grafted MSCs that survived in the site of injury formed cell bridges in the resulting cavity. The transplantation of genetically modified cells expressing neurotrophic factors has been investigated as a method to improve axonal regeneration after SCI.[10-12] Neurotrophin (NT-3)-engineered fibroblasts applied acutely[13] and chronically[14] to lumbar motoneurons showed improvement in the intracellular recorded synaptic response. Transplanted NT-3 expressing mesenchymal stem cells (NT3-MSCs) have been shown to increase axonal growth in a model of spinal cord injury.[15]

In this study we investigated whether the transplantation of NT3-MSCs after a spinal cord contusion injury promoted hind limb function and axonal outgrowth, possibly offering gene therapy with MSCs as a future treatment paradigm.

†These authors contributed equally to this work
*Tel: +82-2-2228-2808; Fax: +82-2-393-9979
e-mail: knkim@yuhs.ac (Keung Nyun Kim)

2. Materials and Methods

2.1 Separation and Culture of MSC

Approximately 5 ml of bone marrow aspirates, recovered from human iliac crest during bone marrow harvesting for future allografic transplantation, were centrifuged through a density gradient for 30 minutes at 400 *g*. Cells were plated in 100 mm dishes containing DMEM supplemented with 10%FBS, 100 units/ml of penicllin, and 100 μg/ml of streptomycin (Gibco BRL). When the cells reached confluency, they were detached using 0.25% trypsin and 0.02% EDTA in PBS. Cells were then passed 3-4 times in all experiments. MSCs were infected with either a GFP adenovirus (100 MOI) or NT-3-GFP adenovirus (100 MOI), with approximately 3,000 cells/cm^2 plated and maintained overnight.

2.2 Construction of NT3-Ad and Transfection of MSC

The NT3 coding sequence was amplified from rat brain tissue by RT-PCR using the primers 5'gctggtaccatgtccacatcaacatc3' and 5'tcggatccttaatgcttgtagaagct3'. Rat NT3 cDNA was sub-cloned into pShuttle-IREShrGFP2 between the CMV early promoter and the SV40 polyadenylation site using the NotI and SalI sites. The subsequent clone was transformed into E. coli BJ-5183, along with pAdeasy1, to recombine NT3 cDNA with the

adenoviral genome according to the manufacturer's instruction (Stratagen). Recombinant DNA was then transfected into 293 cells for adenovirus generation. Viruses from a single plaque were amplified in 293 cells, and viral stocks were formed by the repeated freezing and thawing of cells prior to ultrasonication. Viral stocks were kept in aliquots at -80°C until use.

2.3 Spinal Cord Injury

Male Sprague-Dawley rats (Daehan Biolink, Chungbuk, Korea), weighing 300-350 g, were used. All animal experiments were approved by the Animal Care and Use Committee of Yonsei University College of Medicine. Animals were anesthetized using sodium pentobarbital (20 mg/kg), followed by the contusion at the T9 level of the spinal cord using the NYU weight-drop device, producing a moderately contused spinal cord injury model.

2.4 Transplantation of MSC

For the transplantation study, animals were randomly assigned to one of three groups: the media treatment group, the MSC treatment group, and the NT3-MSC treatment group (n=8 for each group). A week after spinal cord contusion, using an electrode microneedle, 5 μl (1×10^5 cells/μl) of MSCs, NT3-MSCs, or media were injected through a small hole in the dura into the lesion space. All animals daily received Cyclosporine A (10 mg/kg) for 8 weeks after transplantation.

2.5 Behavioral Assessment After SCI

To measure functional recovery of the hind limbs, each animal was tested as previously described.[16] Briefly, rats were placed into an open field and allowed to adapt. Once a rat walked continuously, two examiners conducted a 5 minute, pre-operative testing session using the BBB locomotor rating scale. Post-operative (p.o.) open-field testing was carried out at least once a week for 8 weeks after experimental treatment.

2.6 Immunohistochemistry

To evaluate the cellular characteristics of the transplanted cells *in vivo*, immunhistochemical analysis was performed. Eight weeks after experimental treatment, rats were perfused with PBS and 4% paraformaldehyde (PFA). Spinal cords were removed and fixed in 4% PFA for 4 hours, followed by overnight submersion in 30% sucrose in PBS. Serial, longitudinal sections of the spinal cord were obtained using the cryostat (12 μm thick; Microm/HM500V, CE, Germany). The following day, sections were fixed with 4% PFA for 10-15 minutes and rinsed with PBS. Sections were blocked for 1 hour at room temperature, followed by primary anti-body exposure, which consisted of anti-GFP (1:500, Chemicon), anti-human specific mitochondria (1:50, Chemichon), anti-neurofilament (1:1000, DAKO), and anti-VEGF (1:50, Chemicon). Texas red (1:250, vector) or FITC (1:250, vector) was applied as the secondary anti-body for one hour. Sections were then mounted on slides using fluorescent mounting medium (Vectorshield, Vector, USA), followed by observation under a florescence microscope (BX51, Olympus, Tokyo, Japans) or confocal microscope (LSM 510, Zeiss, Germany).

2.7 Real-Time Polymerase Chain Reaction (RT-PCR)

To measure NT-3 *in vivo*, real-time PCR was performed using the 7500 real-time PCR system (Applied Biosystems, Cheshire, UK) 1 week after transplantation (n=3 for each group). Twenty microliters of reaction solution (12.5 ng of cDNA, 0.5 μM reverse primer, and SYBR green PCR master

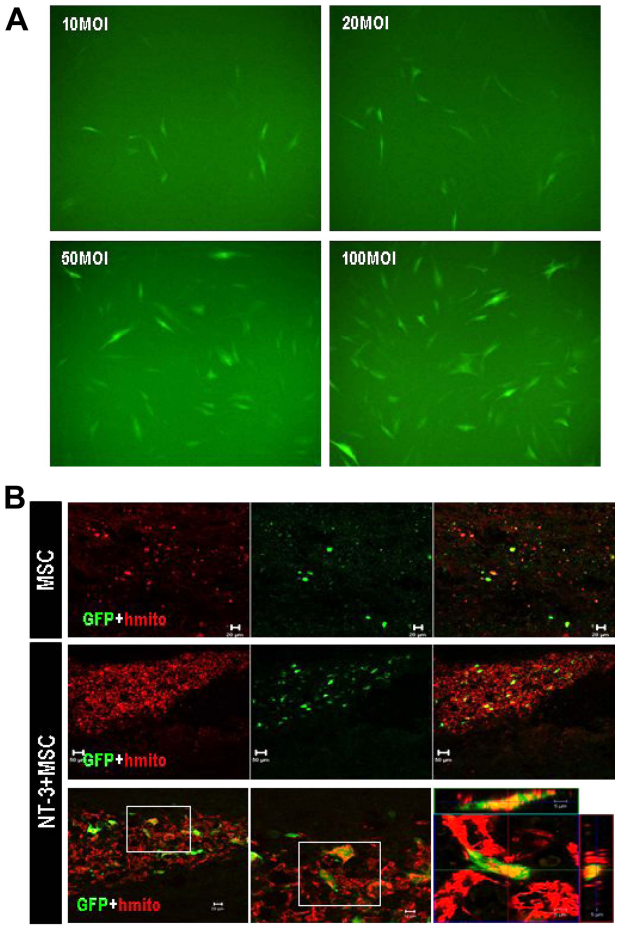

Figure 1. MSCs transplanted into the injured spinal cord. (A) Adenovirus GFP was introduced into MSC. The number of GFP expressing-MSC increased with MOI dependently. (B) Double staining for human mitochondria and GFP was performed at 8 weeks after transplantation. The number of human mitochondria-positive cells was greater in NT-3-MSC transplanted group.

mix) were used for PCR as follows: 50°C for 2min, 95°C for 10min, and 45cycle for 15s at 95°C and 1min at 55°C. The following primers were used (NT-3: forward primer, 5′-CTC CCT GCT CTG GTT CTC TG -3'; reverse primer, 5′-CCA GGC GGA TAT CTT GAA AA-3'. For GAPDH: forward primer, 5′-AAG GGC TCA TGA CCA CAG TC -3′; reverse primer, 5′-GGA TGC AGG GAT GAT GTT CT -3′).

2.8 Statistical Analysis

Data are expressed as mean±standard error. One-way ANOVA followed by Dunnett's post-hoc multiple comparison tests was conducted to determine statistical differences between MSC, NT3-MSC, and media-treated groups.

3. Results

3.1 Transplanted MSCs

MSCs were transfected with adenovirus GFP or NT3-GFP. GFP expression was observed using a fluorescence microscope. Infection efficiency increased MOI in a dependent manner (Fig 1A). About 50% of all cells were transfected at 100 MOI. To confirm the existence of transplanted MSCs, surviving MSCs were visualized through human mitochondria and GFP staining 8 weeks after transplantation (Fig 1B). Immunoreactivity to human-specific mitochondria antibodies was observed in the site of injury, indicating the mesenchymal stem cell transplantations were successfully grafted into the lesion site after transplantation. MSC survival was increased in the NT3-MSC transplanted group compared to the MSC group.

3.2 Expression of NT-3 and Vascular Endothelial Cell Growth Factor (VEGF)

One week after transplantation, NT-3 expression was measured in normal, untreated injured, MSC transplanted, and NT3-MSC transplanted rats (Fig 2A). NT-3 expression in the MSC group, when compared to the untreated (media injected) group, was not significant, but also it was not showed the consistent expression pattern. In contrast, NT-3 expression in NT3-MSC transplanted animals was higher than in the media control groups ($p < 0.01$), it's consistently showed the high expression level compare with MSC group. To confirm whether injected MSCs can express other growth factors, double-staining with anti-GFP and vascular endothelial cell growth factor (VEGF) was performed, confirming that MSCs transplanted into the injured spinal cord indeed express VEGF.

3.3 Axonal Outgrowth and Tissue Restoration

To investigate whether NT3-MSC tranplants could improve axonal growth, anti-neurofilament (NF) immunostaining was performed 8 weeks after transplantation (Fig 3A). While axonal outgrowth in the MSC transplanted group was greater than the media injected group, such growth was more prominent in the NT3-MSC transplanted group. In addition, cavity formation was routinely observed in the media treated group, whereas the incidences of cavity sightings were lower in the groups that received cell transplants.

3.4 Behavior Tests

Animals with low scores and correspondingly paretic hind

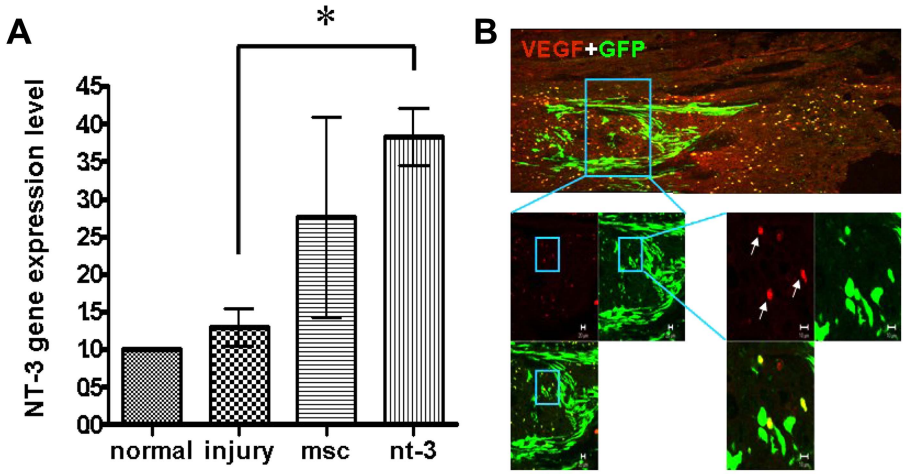

Figure 2. Expression of NT-3 and VEGF after transplantation. (A) NT-3 expression was confirmed by real time PCR one week after transplantation. NT-3 expression in NT-3-MSC was significantly greater than that of the media injected group. NT-3 expression in NT-3-MSC consistently increased over time compared to NT-3 expression in MSCs. *:significant difference compare to media group (ANOVA, $p < 0.05$). Data are shown as mean±SD. (B) Expression of the endogenous growth factor one week after transplantation. MSCs were well grafted into the injured spinal cord, and transplanted MSCs also expressed endogenous VEGF. Scale bar= 20 um.

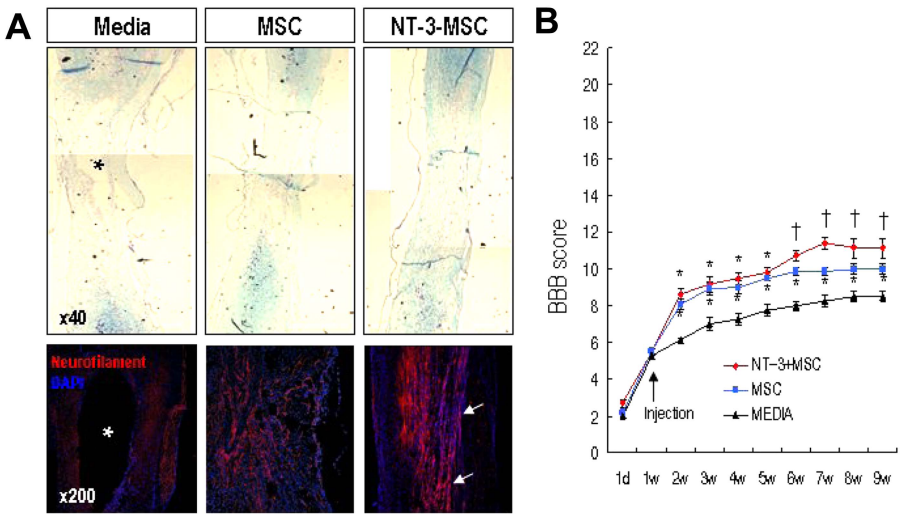

Figure 3. Axonal outgrowth, tissue restoration, and functional recovery (A) Transplantation with MSC or NT-3-MSC showed better tissue restoration compare with media treatment. Specifically, NT-3-MSC transplantation was associated with greater axonal outgrowth 8 weeks after treatment. (*:cavity formation) (B) Transplantation with MSC or NT-3-MSC improved hind limb performance compared with that of media treatment, an effect present from one week after transplantation. Functional recovery in the NT-3-MSC transplantation group significantly improved compared to the MSC transplanted from 5 weeks after treatment. (↑:transplantation time; †:significant difference compare with both MSC and Media group, $p < 0.05$; *:significant difference compare with media group, $p < 0.05$). Data are shown as mean±SEM.

limbs were selected for experimentation. The media treated group (n=8) had a score of 2 in both legs one day after injury, with the scores gradually increasing over the course of 8 weeks after treatment. In contrast, just one week after treatment, the MSC (n=8) and NT3-MSC (n=8) transplanted groups exhibited improved hind limb performance when compared to the media controlled group. From 5 weeks after transplantation onward, the NT3-MSC transplanted group showed significant hind limb improvement over the MSC treated group ($p < 0.05$).

4. Discussion

Mesenchymal stem cells have been shown to have positive effects in the environment of the injured spinal cord. NT-3, a growth factor that has been shown to enhance the sprouting of injured axons, has potential to positively affect long tract regeneration in the injured spinal cord[15] and cell survival.[17] As such, the combination of MSCs with NT-3 delivery provides an attractive method for treating the injured spinal cord.

In this study, we showed that the transplantation of MSCs or NT3-MSCs into the contused spinal cord could facilitate the promotion of functional improvement. Previous reports suggest that MSCs promote functional recovery when grafted in spinal cord contusion models.[7-9,18] As we report, hind limb performance in the present experiment was modestly improved in cell transplanted groups. Using immunohistochemistry, human-specific mitochondria-positive cells were observed in as well as around the injury epicenter, raising the idea that MSCs may migrate short distances from the epicenter into host tissue.[11] Transplanted cell survival was more profound in the NT3-MSC group than in the MSC group, reinforcing the understanding that NT-3 has a neurotrophic effect[17], it can reasonably be explained that the presence of NT-3 allowed for the MSCs to survival longer in the hostile environment present in SCI. While one study demonstrated that transplantation of NT3-MSCs into the injured spinal cord led to neuronal differentiation *in vitro*,[19] we did not observe this phenomenon in either of the cell transplanted groups.

Analyzing the PCR result, there was no significant difference in the expression of NT-3 between the NT3-MSC and MSC transplanted groups one week after treatment. However, NT-3 expression in GFP-MSCs (control) was not significant compared to the PBS group, also NT-3 expression was not consistently increased. In contrast, NT-3 expression was consistently high in the NT3-MSC transplanted group over the course of the experiment. Yet, it showed a similar pattern of increase as seen in the control GFP-MSC group. This leads us to think that the increase in functional recovery and axonal outgrowth is consistent with increased NT-3 expression.

In a previous study of ours, we confirmed that MSCs can

secrete paracrine factors such as VEGF,[20] and that such factors may contribute to neural protection.[21,22] In this study, MSCs transplanted into the site of injury exhibited VEGF expression, pointing toward the idea that paracrine factor release by MSCs possibly accelerates functional improvement.

5. Conclusion

In this study, the transplantation of NT3-MSCs into the spinal cord after contusion injury greatly improved functional outcome and axonal outgrowth. MSCs may represent a useful resource for combinatorial approaches to SCI repair, including *ex vivo* gene therapy.

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References

1. N Bamber, H Li, X Lu, *et al.*, Neurotrophins BDNF and NT-3 promote axonal re-entry into the distal host spinal cord through Schwann cell-seeded mini-channels, *Eur J Neurosci*, **13**, 257 (2001).
2. J Bonner, A Blesch, B Neuhuber, *et al.*, Promoting directional axon growth from neural progenitors grafted into the injured spinal cord, *J Neurosci Res*, **88**, 1182 (2010).
3. J Coumans, T Lin, H Dai, *et al.*, Axonal regeneration and functional recovery after complete spinal cord transection in rats by delayed treatment with transplants and neurotrophins, *J Neurosci*, **21**, 9334 (2001).
4. P Johnson, A Tatara, A Shiu, *et al.*, Controlled release of neurotrophin-3 and platelet derived growth factor from fibrin scaffolds containing neural progenitor cells enhances survival and differentiation into neurons in a subacute model of SCI, *Cell transplant*, **19**, 89 (2009).
5. L Taylor, L Jones, M Tuszynski, *et al.*, Neurotrophin-3 gradients established by lentiviral gene delivery promote short-distance axonal bridging beyond cellular grafts in the injured spinal cord, *J Neurosci*, **26**, 9713 (2006).
6. Y Chiba, S Kuroda, K Maruichi, *et al.*, Transplanted bone marrow stromal cells promote axonal regeneration and improve motor function in a rat spinal cord injury model, *Neurosurgery*, **64**, 991 (2009).
7. M Chopp, X Zhang, Y Li, *et al.*, Spinal cord injury in rat: treatment with bone marrow stromal cell transplantation, *Neuroreport*, **11**, 3001 (2000).
8. C Hofstetter, E Schwarz, D Hess, *et al.*, Marrow stromal cells form guiding strands in the injured spinal cord and promote recovery, *Proc Natl Acad Sci U S A*, **99**, 2199 (2002).
9. K Lee, H Suh-Kim, J Choi, *et al.*, Human mesenchymal stem cell transplantation promotes functional recovery following acute spinal cord injury in rats, *Acta Neurobiol Exp*, **67**, 13 (2007).
10. A Blesch, M Tuszynski, Cellular GDNF delivery promotes growth of motor and dorsal column sensory axons after partial and complete spinal cord transections and induces remyelination, *J Comp Neurol*, **467**, 403 (2003).
11. P Lu, L Jones, M Tuszynski, BDNF-expressing marrow stromal cells support extensive axonal growth at sites of spinal cord injury, *Exp Neurol*, **191**, 344 (2005).
12. M Ruitenberg, G Plant, F Hamers, *et al.*, Ex vivo adenoviral vector-mediated neurotrophin gene transfer to olfactory ensheathing glia: effects on rubrospinal tract regeneration, lesion size, and functional recovery after implantation in the injured rat spinal cord, *J Neurosci*, **23**, 7045 (2003).
13. V Arvanov, B Seebach, L Mendell, NT-3 evokes an LTP-like facilitation of AMPA/kainate receptor-mediated synaptic transmission in the neonatal rat spinal cord, *J Neurophysiol*, **84**, 752 (2000).
14. V Arvanian, P Horner, F Gage, *et al.*, Chronic neurotrophin-3 strengthens synaptic connections to motoneurons in the neonatal rat, *J Neurosci*, **23**, 8706 (2003).
15. P Lu, LL Jones, MH Tuszynski, Axon regeneration through scars and into sites of chronic spinal cord injury, *Exp Neurol*, **203**, 8 (2007).
16. D Basso, M Beattie, J Bresnahan, Graded histological and locomotor outcomes after spinal cord contusion using the NYU weight-drop device versus transection, *Exp Neurol*, **139**, 244 (1996).
17. LN Novikova, LN Novikov, JO Kellerth, Survival effects of BDNF and NT 3 on axotomized rubrospinal neurons depend on the temporal pattern of neurotrophin administration, *Eur J Neurosci*, **12**, 776 (2000).
18. B Himes, B Neuhuber, C Coleman, *et al.*, Recovery of function following grafting of human bone marrow-derived stromal cells into the injured spinal cord, *Neurorehabil Neural Repair*, **20**, 278 (2006).
19. B Himes, M Goldberger, A Tessler, Grafts of fetal central nervous system tissue rescue axotomized Clarke's nucleus neurons in adult and neonatal operates, *J Comp Neurol*, **339**, 117 (2004).
20. JSoo Oh, ML Liu, HL Jin, *et al*., Effect of bone marrow derived mesenchymal stem cell conditioned media for neurite outgrowth in human Ntera-2 neurons, *Tissue Eng Regen Med*, **6**, 562 (2009).
21. KN Kim, JD Guest, JS Oh, *et al.*, Effect of primate bone marrow stromal cells on survival and neurite outgrowth, *Neuroreport*, **21**, 877, (2010).
22. SS An, HL Jin, KN Kim, *et al.*, Neuroprotective effect of combined hypoxia-induced VEGF and bone marrow-derived mesenchymal stem cell treatment, *Childs Nerv Sys*, **26**, 323, (2010).