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# Repair of Spinal Cord Injury by Co-Transplantation of embryonic Stem Cell-Derived Motor Neuron and Olfactory Ensheathing Cell

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## ABSTRACT

**Background:** The failure of regeneration after spinal cord injury (SCI) has been attributed to axonal demyelination and neuronal death. Cellular replacement and white matter regeneration are both necessary for SCI repair. In this study, we evaluated the co-transplantation of olfactory ensheathing cells (OEC) and embryonic stem (ES) cell-derived motor neurons (ESMN) on contused SCI. **Methods:** OEC cultured from olfactory nerve rootlets and olfactory bulbs. ESMN was generated by exposing mouse ES cells to retinoic acid and sonic hedgehog. Thirty female rats were used to prepare SCI models in five groups. Control and medium-injected groups was subjected to induce lesion without cell transplantation. OEC or ESMN or both were transplanted into the site of the lesion in other groups. **Results:** The purity of OEC culture was 95%. Motor neuron progenitor markers (Olig2, Nkx6.1 and Pax6) and motor neuron markers (Isl1, Isl2 and Hb9) were expressed. Histological analysis showed that significantly more ($P<0.001$) spinal tissue was spared in OEC, ESMN and OEC+ ESMN groups but the OEC+ ESMN group had a significantly greater percentage of spared tissue and myelination than other groups ($P< 0.05$). The numbers of ESMN in co-transplanted group were significantly higher than ESMN group ($P<0.05$). A significant ($P<0.05$) recovery of hindlimb function was observed in rats in the transplanted groups. **Conclusion:** We found that the co-transplantation of ESMN and OEC into an injured spinal cord has a synergistic effect, promoting neural regeneration, ESMN survival and partial functional recovery. *Iran. Biomed. J. 13 (3): 125-135, 2009*

*Keywords:* Spinal cord injury (SCI), Embryonic stem (ES) cell, Motor neuron, Olfactory ensheathing cell (OEC)

## INTRODUCTION

The long-term effects of spinal cord injuries (SCI) often persist for the life of the patient. The main goal of basic SCI research is to develop new therapeutic interventions which can be applied to prevent or reduce disability. One of the major strategies to regenerate spinal cord is cellular transplantation. The aim of cell therapy is to replace dead cells, remyelinate axons and also create a favorable environment for axon regeneration. Numerous studies have evaluated the therapeutic potential of cellular transplants of various types for white matter regeneration [1-3]. Olfactory ensheathing cell (OEC) is a specialized glial cell which associates with the olfactory nerve, surrounding the sensory axons and express an array of trophic factors, transcription factors, and extracellular matrix molecules [4-6]. Combination of these properties made OEC a candidate for spinal cord repair [7-9].

The degeneration of motor neurons after SCI is encouraging for cell replacement strategies using stem cell-derived motor neurons [10, 11]. Embryonic stem (ES) cells have several features that include replicating indefinitely without aging, pluripotency, giving rise to all different cell types in the body [12]. A multitude of *in vitro* differentiation protocols aimed at deriving specific neuronal and glial subtypes from ES cells have been developed.

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Spinal motor neurons and interneurons can be both generated efficiently by exposing mouse ES cells to retinoic acid (RA) and sonic hedgehog (Shh). Some studies have evaluated the potential of ES cells to arrest spinal motor neuron degeneration and restore function to animals with SCI [2, 6]. However, embryonic stem (ES) cell-derived motor neurons (ESMN) transplantation alone is not sufficient for spinal cord repair because the majority of the ESMN engrafted into the spinal cord will not survive. Therefore, in the present study, for the first time, we investigated the potential use of co-transplantation of OEC and ESMN as a therapeutic strategy in spinal cord injured rats.

## MATERIALS AND METHODS

***Animals.*** Adult female Wistar rats (n = 40, aged 6-8 weeks and 250-300 g) were obtained from the Razi Vaccine and Serum Research Institute, Karaj, Iran. The animals were maintained on a 12 hour dark/light cycle at 20°C. Food and water was proportioned ad libitum. All experimental procedures were approved by the Animal Care and Ethics in Tehran University of Medical Sciences, Tehran, Iran. For inducing SCI, we used 30 rats in the following five groups (6 rats in each group): control group, lesion induction without cellular transplantation; medium-injected group, lesion induction and medium injection; OEC group, lesion induction and transplantation of OEC; ESMN group, lesion induction and transplantation of ESMN, and finally OEC + ESMN group, lesion induction and co-transplantation of OEC and ESMN. We also used 10 rats for OEC culture.

***OEC cell culture.*** For establishment of OEC, we used Nash [13] methods with some modifications [14]. In brief, rats were anesthetized with overdose of ketamine and xylazine and killed by decapitation. A skin incision was made in the scalp extending from the tip of the nose to the occipital bone of the skull and the nasal and frontal bones were removed. The olfactory nerve rootlets and olfactory bulbs were detached and placed in chilled calcium and magnesium free Hank's balanced salt solution (HBSS, Sigma). The tissue was divested of all meninges and blood vessels. The outer two layers of the olfactory bulb and the olfactory nerve were dissected and retained. The tissues were minced and incubated within a solution of 0.25% collagenase type IA and dispase II (2.4 U/ml in Puck s solution; Roche Diagnostics, Mannheim, Germany) in DMEM/F12 (Gibco, USA) in 5% $CO_2$ at 37°C for 30 minutes. The tissue was then triturated mechanically, after which the dispase and collagenase activity was inactivated using HBSS. The cell suspensions were then centrifuged at 300 ×g for 5 minutes and plated into plastic cell culture flask in DMEM/F12 (Gibco, USA) containing 10% fetal bovine serum (FBS, Sigma), 2 mM L-glutamine (Gibco, USA), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco, USA). Cultures were kept at 37ºC in a humidified atmosphere containing 95% air and 5% $CO_2$ for 18 hours. Most of the fibroblasts and fibroblast-like cells attached to plate during this first incubation period. The supernatants from the culture were removed and plated in a second set of uncoated culture flasks. After 36 hours of incubation, most of the OEC remained in the supernatant. This supernatant was used to seed a set of poly-L-lysine (Sigma) flasks. After seeding, the OEC attached within 48 hours. Cultures continued until appropriate cell number was achieved and the media were changed every 2 days.

***ES cell culture and differentiation.*** ESMN generated by previously published method using feeder-free GFP-positive CGR8 mouse ES cells (a Gift from Stem cells Technology, Tehran, Iran) [10]. ES cells were grown on gelatinized plate in ES culture medium including KoDMEM (Gibco, USA) supplemented with 1% nonessential amino acids (Gibco, USA), 0.1 mM 2-mercaptoethanol (Sigma, USA), 2 mM L-Glutamine (Gibco, USA), 1% Penicillin/Streptomycin (Gibco, USA), 10% ES FBS (Gibco, USA) and 1000 μ/ml LIF (ESGRO, Chemi-Con). Also, ES cell colonies were dissociated and cultured in DFK5 medium [DFK5 medium was prepared by mixing DF medium (DMEM/F12 supplemented with 2 mM L-Glutamine, 1%Penicillin/Streptomycin, 0.1 mM 2-mercapto-ethanol, 1%Insulin-Trasferrin-Selenium Supplement (Gibco, USA)) in a 1:1 ratio with ES medium (ES cell medium without LIF and FBS but supplemented with 10% Knockout Serum Replacement (Gibco, USA)] to form Embryoid Body (EB). Medium was replaced by DFK5 supplemented with RA (1 μM) and Shh (300 nM, both from Sigma, USA) and EB were cultured for 4 days. EB were trypsinized and seeded on gelatin coated flasks in serum-free DMEM/F12 medium supplemented with 1% N2 (Gibco, USA). For longer culture, they were



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transferred after 3 days to Neurobasal medium plus 2% B27 supplement (Gibco, USA) and 10% FBS.

***Immunocytochemistry.*** The cells were fixed by incubation in 4% paraformaldehyde (Sigma, USA). The fixed cells were rinsed with PBS and permeabilized with 0.1% Triton X-100 (Sigma, USA) in PBS, incubated with blocking buffer (PBS, 1 mg/ml BSA (Sigma, USA) and 10% goat serum (Gibco, USA) followed by incubation with primary antibodies at 4°C overnight. Cells were then rinsed with PBS and incubated with a species-specific secondary antibody. Cultures were washed with three changes of PBS and nucleus was stained by 4',6-diamidino-2-phenylindole and visualized by fluorescent microscope. The expression of p75 low-affinity nerve growth factor receptor in OEC culture was visualized by immunostaining the cells with primary monoclonal mouse anti-p75 NGFR antibody (Sigma, USA) and secondary FITC-conjugated anti-mouse IgG antibody (Abcam, USA). The expression of microtubule assassination protein 2 (MAP2) and tubulin βIII in differentiated mouse ES cells was visualized by immunostaining the cells with primary monoclonal mouse anti-MAP2 and tubulin βIII (Sigma, USA) and secondary phycoerythrin-conjugated anti-mouse IgG antibody (Abcam, USA).

***RNA extraction and RT-PCR analysis of gene expression.*** Total RNA was isolated from cells using the RNX plus kit (Cinnagen, Iran). Standard RT was performed using the Revert Aid$^{TM}$ H Minus First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. PCR were performed at 94°C for 1 minute, followed by 30 cycles of 94°C for 30 s, 55-63°C for 30 s, 72°C for 30 s and 72°C for 10 minutes. Amplified DNA fragments were electrophoresed on 1.5% agarose gel and the gels were stained with ethidium bromide (10 μg/ml) and photographed on a UV transilluminator (Uvidoc, UK). The primers are listed in Table 1.

***Surgical procedures.*** SCI was induced using Vanicky's method [15]. In brief, adult female Wistar rats were anesthetized with intraperitoneal injections of ketamine (50 mg/kg) and xylazine (5 mg/kg). The spinous processes of vertebrae T10-T11 were removed and a small hole (2 mm diameter) was drilled in vertebral arch of T10. A 2-French Fogarty catheter (Percuse, France) was inserted into the epidural space and advanced cranially so that the center of the balloon rested at T8-T9 level. The balloon was rapidly inflated with 20 μl saline for 5 minutes. Catheter removed and the muscles and skin sutured. All rats received antibiotic (cefazolin 50 mg/kg) and analgesic (acetaminophen 1-2 mg/ml in drinking water). Bladder expression was performed two times per day. Urinary tract infections were treated with suspension of cefazolin and of 12 mg/kg gentamicin. Autophagia was treated with acetaminophen (1-2 mg/ml) in drinking water to stop self-mutilation.

**Table 1.** Specific primers used for PCR amplification.

| Genes | Primer sequences | Accession Number |
|---|---|---|
| β2M | F: TTC AGT CGC GGT CGC TTC AGT C<br>R: CAA TGT GAG GCG GGT GGA ACT G | NM 009735.3 |
| OCT4 | F: TGT GGA CCT CAG GTT GGA CT<br>R: CTT CTG CAG GGC TTT CAT GT | NM 013633.2 |
| Olig2 | F: CTA ATT CAC ATT CGG AAG GTT G<br>R: GGA CGA TGG GCG ACT AGA C | NM 016967.2 |
| Pax6 | F: CTG GAG AAA GAG TTT GAG AGG<br>R: CTG CTG CTG ATA GGA ATG TG | NM 013627.2 |
| Lim1 | F: TTT ACG GAG ATT ACC AGA GTG AG<br>R: CAG ATG ATG GCA CAA AGG GTA G | NM 008498.2<br>NM 177693.3 |
| Lim2 | F: GAC CGA GAG CAT CGC ATA TTG<br>R: ACT CCA GTG TAA ATA GCC AAG G | NM 021459.3 |
| ISL1 | F: CTG AGG GTT TCT CCG GAT TT<br>R: TGA AGC CTA TGC TGC ACT TG | NM 027397.2 |
| ISL2 | F: CAG ATC CAC GAC CAG TTT ATC C<br>R: GAT GCC GAA CAG CCT GAC G | NM 144955.2 |
| NKX6.1 | F: CAG GTC AAG GTC TGG TTC C<br>R: TTG TTG TAA TCG TCG TCA TCC | NM 019944.1 |
| HB9 | F: CGC TCA TGC TCA CCG AGA CT<br>R: TTC TCC TCA CTG CCG CCT TT | NM 019409.1 |
| OMG | F: GAC GAC ATT ATG GGC TTT GAT GG<br>R: GTT TCT GCC TGA ACA GTC CAC | NM 010277.2 |

***Hoechst labeling.*** OEC were incubated at 37°C for 30 minutes in the dark in cultured medium containing 10 μg/ml Hoechst 33342 (Sigma, USA) before transplantation [1, 16]. Then, they were rinsed several times with DMEM without serum, resuspended in the same medium and transplanted into the spinal cord.

***Transplantation.*** Transplantations were performed 9 days after the injury [17]. All animals were received cyclosporine (10 mg/kg, SC) on day 8 for immunosuppression and continued for duration of survival [18]. Rats received DMEM medium in medium-injected group, transplants of OEC ($1 \times 10^6$) in OEC group, ESMN ($1 \times 10^6$) in ESMN group, and OEC and ESMN (each $1 \times 10^6$ ) in OEC + ESMN group by means of 5 μL Hamilton syringe. The ESMN and OEC suspension (5 μl) or DMEM medium (5 μl) was injected into the center of the syrinx at T9 level over a 5-minute period. After transplantation, muscles and skin was sutured and rats received antibiotic.



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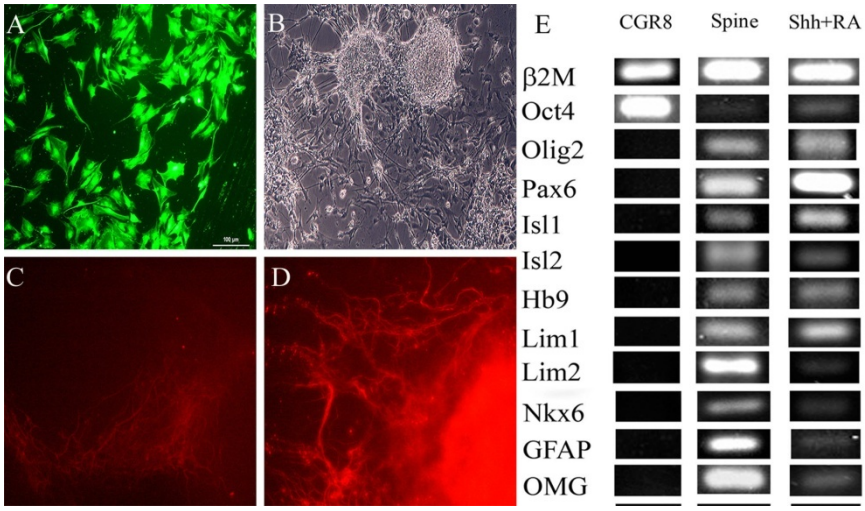


**Fig. 1. (A)** Olfactory ensheathing cells expressing p75; **(B)** multiple neurites and neuronal cells emerging from aggregate. Immunostaining of aggregates was performed using antibody against Tubulin βIII and **(C)** microtubule assassination protein 2 **(D)**. **(E)** The expression of several neuronal genes in undifferentiated ES cells as negative cell control (CGR8), mouse spinal cord as positive control and RA + Shh treated.

***Locomotor function.*** Locomotor function was evaluated using the Basso, Beattie and Bresnahan (BBB) open-field locomotor test using two independent observers [19]. BBB assessments were performed 3 days after injury and prior to transplantation and 7, 14, 21 and 28 days post transplantation.

***Histological evaluation.*** After the final behavioral assessment post transplantation at 28 days, rats were deeply anesthetized and perfused transcardially with 0.1 M PBS followed by 4% paraformaldehyde and 2.5% glutaraldehyde in PBS. The spinal cords were dissected, postfixed and transferred to 10% sucrose. The cords were embedded into TissueTek OCT compound. Serial 12 μm thick longitudinal sections were cut on a cryostat and mounted on Poly-L-Lysine coated slides. The slides were stained by cresyl violet-Luxol fast blue and silver nitrate (both from Merck, Germany). For morphometry and cell counting, sections were captured by digital camera. The identified areas in individual sections were measured using image analysis software (Image J, NIH). To perform cell counts, we counted the number of transplanted cells in the longitudinal sections as Hoechst-labeled and GFP-positive cells in OEC, ESMN and OEC+ ESMN groups by using fluorescent microscope. Every other six slides were selected and the cell count was performed on 50 slides in each animal. Every ten longitudinal section was used to determine the spared tissue and myelinated area. In each section, the number of pixel in damaged and myelinated area and the total number of pixels in spinal cord segment were measured. Measurements from each section were summed per rat and corrected for the total number of sections. Spared spinal tissue was considered to be the difference between the numbers of pixels in the area of damaged tissue and the number of pixels in the whole segment [20].

***Statistical analysis.*** All statistical analyses were performed on standard statistics computer software (SPSS 11.5 for Windows). One-way ANOVA followed by Tukey's post hoc test was used to determine statistical differences between the spared tissue and myelin ratio in each group. BBB scores were compared among groups using non-parametric analysis (Kruskal-Wallis followed by Mann-Whitney U test). Cell numbers in spinal cord in transplanted groups (OEC vs co-transplant and ESMN vs co-transplant) were compared with independent t-test. A statistically significant difference was accepted at $P<0.05$.

## RESULTS

***OEC cell culture.*** As p75 is a receptor found on the external surface of the plasma membranes of OEC, the purity of the cell cultures and number of OEC obtained were determined by counting the immunofluorescently labeled cell types. The purity of OEC culture by this separation procedure was 95% (Fig. 1A).



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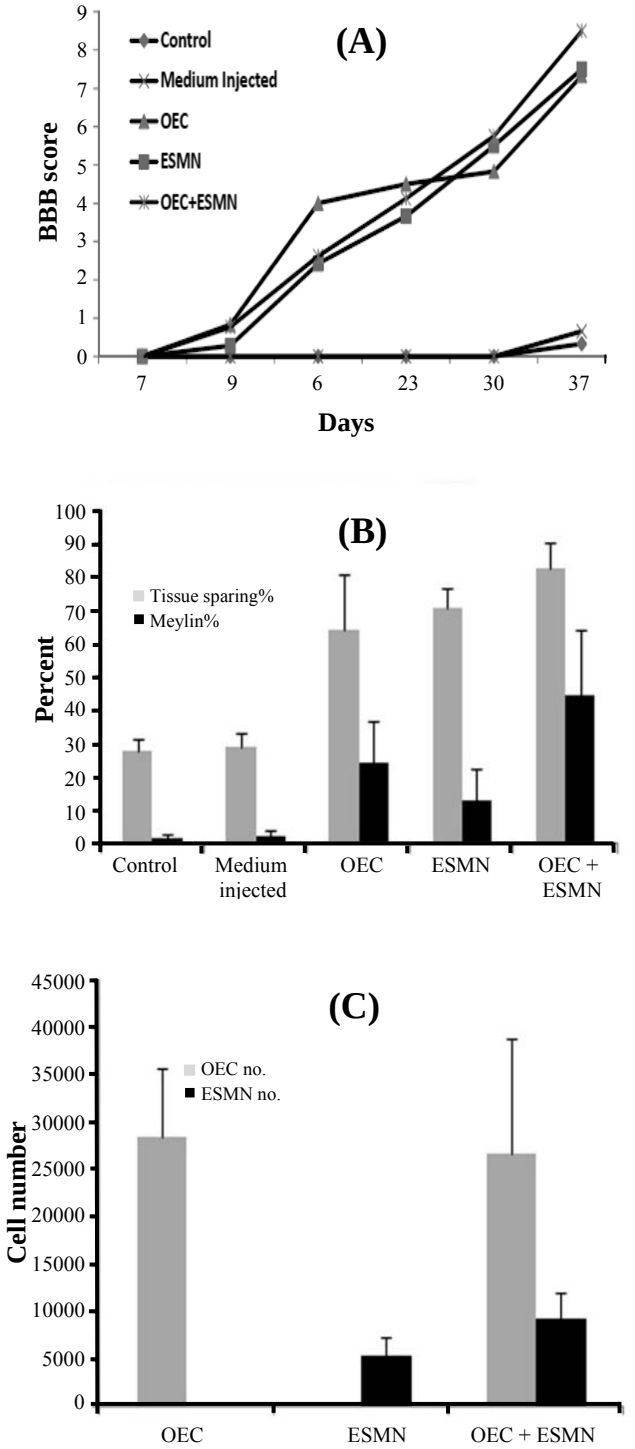


**Fig. 2. (A)** BBB scores for five groups. Animals received contusion injury on Day 0. Transplants were made on Day 9. BBB scores of groups receiving transplants were significantly different from control and medium-injected groups. **(B)** Percentage of spared tissue and myelinated area in five groups. The OEC + ESMN group had a significantly greater percentage of spared tissue and myelinated area than other groups. **(C)** Bar graphs show the cell counts. The number of GFP-positive cells in the OEC + ESMN groups was significantly larger than that in the ESMN group.

***ES cell differentiation.*** Two days after partial trypsinization of treated EB, many of aggregates gave off extensive outgrowth of neuritis. Neurite outgrowth occurs at many points along the perimeter of aggregate forming a dense, tangled collection of neuritis (Fig. 1B). In order to determine if the neuron-like cells and process contain neuron-associated proteins, cultures were stained with antibodies to class III β-tubulin and MAP2 (Fig. 1C and D). To characterize expression of neuronal gene in differentiated ES cultures, we analyzed the expression of Olig2, Pax6, Nkx6.1, Isl1, Isl2, Hb9, Lim1, Lim2, GFAP and OMG by RT-PCR (Fig. 1E). The results revealed that motor neuron progenitor markers (Olig2, Nkx6.1 and Pax6) were expressed and the expression of Pax6 and Olig2 was higher than that of Nkx6.1. Motor neuron markers (Isl1, Isl2 and Hb9) were expressed, and expression of Isl1 and Hb9 was higher than that of Isl2. Interneuron markers (Lim1 and Lim2) were expressed and Lim1 had a higher expression. Astrocyte (GFAP) and oligodendrocyte (OMG) markers were expressed in low levels.

***Recovery of hindlimb function.*** BBB score was measured three and nine days post injury and at weekly intervals, starting on day 9. In the same day, animals were assessed and selected for transplantation. The criteria for inclusion in this study were that the animal must score 0-2 on day nine. Figure 2A illustrates average BBB scores across the post injury and post transplantation period. There was no significant difference in average BBB scores prior to transplantation among 5 groups ($P>0.05$). Following transplantation, a significant recovery of hindlimb function was observed in rats in the transplanted groups compared with medium-injected and control groups ($P<0.05$). The mean recovery score in OEC, ESMN and OEC + ESMN groups 4 weeks after transplantation were 7.33 ± 1.03 (range 7-9),7.5 ± 0.83 (range 7-9), 8.5 ± 1.2 (range 7-10), respectively and the corresponding score in the control and medium-injected groups were 0.4 ± 0.53 (range 0-1) and 0.66 ± 0.57 (range 0-1). There was no significant difference between transplanted groups four weeks after transplantation.

***Histological evaluation.*** Longitudinal sections of the injured animals showed edema, inflammation and loss of normal tissue architecture. In the site of injury, several cavities were produced that disrupted portions of the gray and white matters, surrounded by scar tissue. In this study, axonal damage and



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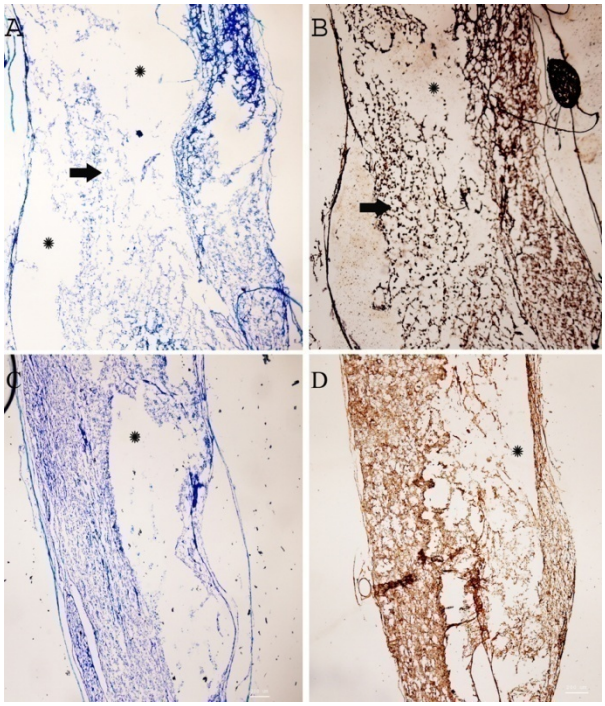


**Fig. 3.** Photomicrographs of longitudinal sections of the spinal cord in the control group (**A** and **B**) and in the medium-injected group (**C** and **D**) 4 weeks after second surgery. Slides were stained using Luxol fast blue (**A** and **C**) and silver nitrate (**B** and **D**). Large cystic cavities formed in the injured area (Stars). The white matter injury was not stained with Luxol fast blue. Arrows show damaged area.

gliosis were visible. The extent of tissue damage was more severe in the control and medium-injected groups than other groups (Fig 3-6). Spinal tissue sparing was calculated from the damaged area (including degenerate tissue and transplanted cells) of spinal cord segment. In the control (27.95 ± 3.52) and medium-injected groups (29.33 ± 4.04) of the spinal cord, the segment examined was spared. In contrast, significantly more spinal tissue ($P<0.001$) was spared in OEC (63.95 ± 17.09), ESMN (70.93 ± 5.62) and OEC + ESMN (82.07 ± 7.83) groups (Fig. 2B). No significant differences in percentage of spared tissue were found between control and medium-injected groups or OEC and ESMN groups. The OEC + ESMN group had a significantly greater percentage of spared tissue than other groups ($P<0.05$). The ESMN group had a greater percentage of myelinated area than control and medium-injected groups, but there was no significant difference. The percentage of myelinated area was greater in OEC group than control, medium-injected and ESMN groups ($P<0.05$). The OEC + ESMN group had a significantly greater percentage of myelinated area than other groups ($P<0.05$) (Fig. 2B). To perform cell counts, we counted the number of transplanted cells in the longitudinal sections as Hoechst-labeled cells (Figs. 4A and 6B-D) and GFP-positive (Figs. 5A and 6A, 6C and 6D). We observed surviving transplant GFP-positive and Hoechst-labeled cells within the cavity and spared tissue of the spinal cord. In OEC and OEC + ESMN groups, many of the OEC were detected around and within the cystic cavity, and the average numbers of cells were 28524 ± 7287 in OEC and 26702 ± 12120 in OEC + ESMN groups. In ESMN and OEC + ESMN groups, the numbers of GFP-positive cells were 5348 ± 2125

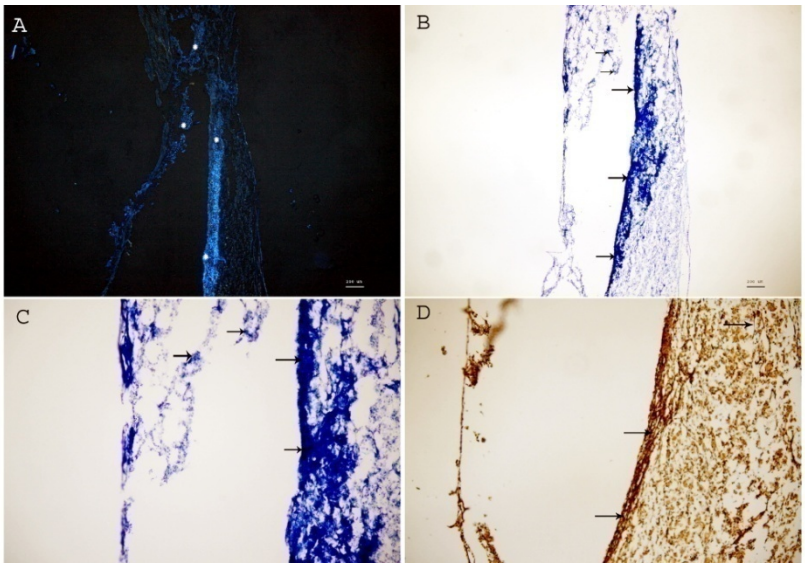


**Fig. 4.** Photomicrographs of longitudinal sections of the spinal cord in the OEC group 4 weeks after OEC transplantation. Hoechst-labeled OEC (**A**, stars) produced myelin (**B** and **C** stained by Luxol fast blue, arrows). Presence of neuronal processes in silver nitrate staining (**D**, arrows).



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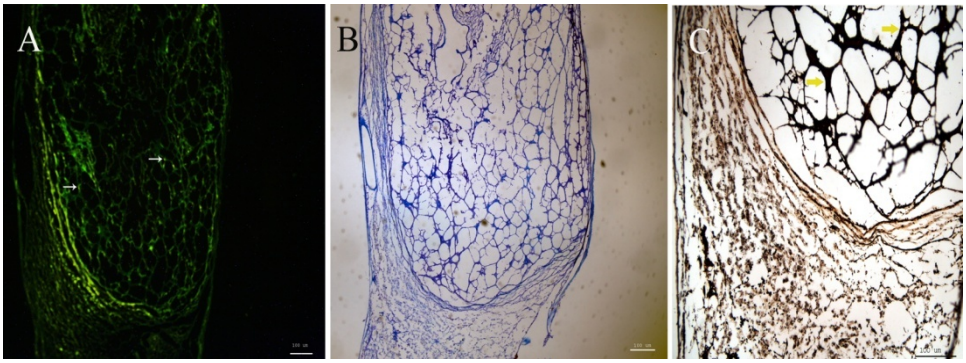


**Fig. 5.** Photomicrographs of longitudinal sections of the spinal cord in the ESMN group 4 weeks after transplantation. GFP-positive cells (**A**, arrows) survived in spinal cord and established neuronal network. Myelinated area was lower than OEC and OEC + ESMN groups (**B**, stained by Luxol fast blue). Neuronal network stained by silver nitrate (**C**, arrows).

and 9362 ± 2783, respectively (Fig. 2C). There was a significant difference between cell number in OEC and ESMN groups ($P<0.001$). The Hoechst-labeled cells in OEC and OEC + ESMN were not significantly different ($P>0.05$). The numbers of GFP-positive cells in OEC + ESMN were significantly higher than those of ESMN group ($P<0.05$).

## DISCUSSION

Most SCI research focuses on restoration of white matter long tracts (regeneration) and cellular replacement (neuronal or oligodendrocyte). The most frequently used cell for white matter regeneration is OEC. The results of this study demonstrated that OEC promote histological and functional recovery after transplantation.

In OEC group, the sparing and myelination of spinal tissue increased and cavity formation decreased. The mean BBB score in OEC group was higher than control and medium-injected groups. Several studies have indicated that OEC transplantation holds promise as a potential therapy for SCI, although other studies have suggested that OEC may not always be beneficial [21, 22]. Comparison of studies is made difficult by a

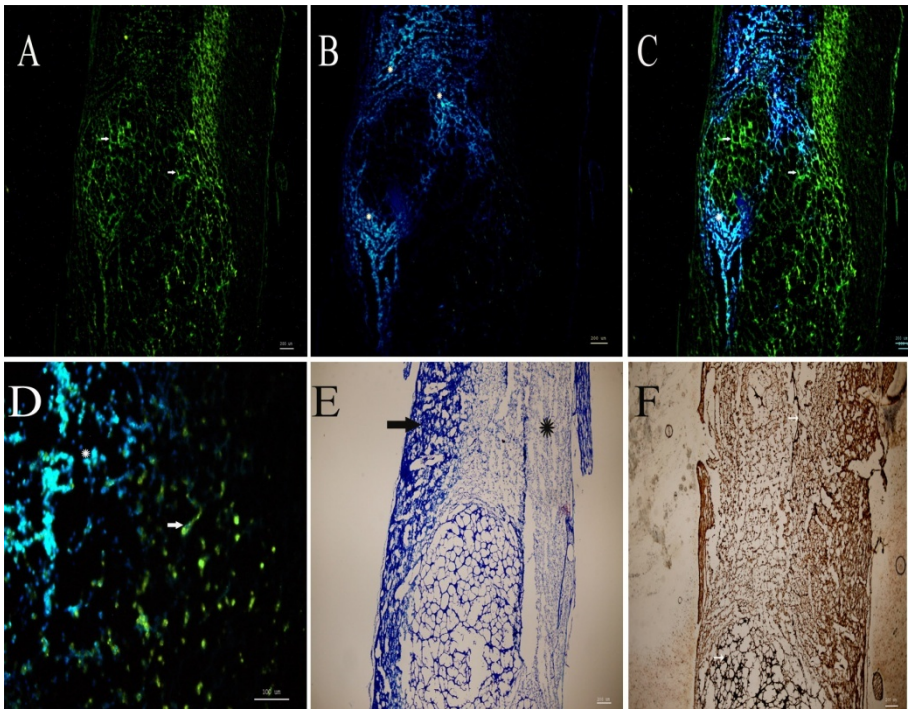


**Fig. 6.** Photomicrographs of longitudinal sections of the spinal cord in the OEC + ESMN group 4 weeks after transplantation. GFP-positive cells (**A**, **C** and **D**, arrows) and Hoechst-labeled OEC (**B**, **C** and **D**, stars) survived in spinal cord. E stained by Luxol fast blue (myelinated [arrow] and demyelinated [star] areas) and by silver nitrate (**F**, neuronal process, arrows).



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multitude of approaches by different research groups; for example, the various purification strategies and criteria used for defining OEC [5]. In the most successful experiments, adult rats that received OEC transplants recovered climbing ability and sensorimotor reflexes after complete spinal cord transaction [23], recovered respiratory function after cervical hemisection [24] and bladder control after lumbosacral dorsal root transaction [25]. This functional recovery correlated with anatomical and activity-based regeneration in the associated axon tracts, including long distance regrowth of some axons [5, 6, 23, 25, 26].

Improvement of BBB score in previous researches and present research showed that OEC has profound effects on corticospinal and other tracts involved in rat hindlimb movement. The effects of OEC on SCI are due to nature of this cell. OEC express several molecules that are known to promote axonal growth including NGF, BDNF, GDNF, and NT-4/5 [5, 6]. Reports suggested that OEC were unique in their ability to promote axonal regeneration not only within a transplant, but also beyond it into the white matter tracts of the host spinal cord. Such regeneration was reported for several fiber types including corticospinal, dorsal column and monoaminergic fibers [6]. The increase in tissue sparing in the present study might be due to the promotion of axonal regeneration and extracellular matrix production after OEC transplantation. Improvement of BBB score not only needs regeneration of axons but also requires establishment of new synaptic connections and attaining myelin sheath to enable efficient propagation of action potentials. OEC transplanted from rats into demyelinating lesions in the rat spinal cord promoted remyelination of axons and restored impulse conduction [7, 27, 28]. Sasaki *et al.* [29] showed that transplantation of OEC expressing green fluorescent protein indicates that transplanted cells account for about 50% of the peripheral-type myelin around regenerating axons, while the remainder is attributable to Schwann cells infiltrating the transplant [29].

After SCI, most of the affected cells die within 12 hours; however, a delayed phase of cell death persists for several days or weeks. Cell death occurs in the gray matter of the cord first, where the bodies of nerve cells reside [30]. Therefore, we used differentiated ES cells for replacement of dead cells. In the present study, we committed ES cells to a motor neuron fate before transplantation and evaluated the expression of motor neuron progenitor, motor neuron and interneuron markers before transplantation. The result of this study showed that the motor neuron progenitor markers (Pax6, Nkx6.1 and olig2) and ventral motor neuron markers (HB9, Isl1and Isl2) were expressed. Our results revealed that interneuron markers Lim1 and Lim2 were expressed in differentiated cells. This is in accordance with the results of previous researchers [10]. In the presence of RA, ES cells express class I neural gene like Pax6, irx3, dbx1 and Pax7 in high levels and class II like Nkx6.1 and Olig2 in low levels. This profile of transcription factor expression is characteristic of interneuron progenitors [31].

The induction of motor neuron progenitors depends on Shh activity [10]. Adding Shh to EB increases class II genes but reduces the expression of Pax7. After transplantation of differentiated ES cells, the tissue sparing and BBB score increased and cavity ratio decreased compared to control and medium-injected groups. There was no significant difference between tissue sparing, cavity ratio and BBB score in OEC and ESMN groups but myelin ratio in OEC group was higher. Our results showed that many ESMN die after transplantation, but some of them fill the cavity and remained in spinal cord and established neuronal network. McDonald and Howard [32] showed the same result after transplantation of ES derived neuronal cells. Other researchers used ESMN for brain damage and found that the grafted cells not only remained close to the implantation site, but also exhibited substantial migration, penetrating into the damaged lesion in a directed manner up to the cortical region. They suggested that the transplanted neurons interacted with the recipient neurons to form a neural network [33]. Partial recovery from paralysis after transplantation of mES cells treated with Shh and RA was reported [2]. However, other researchers found that transplantation of apoptosis resistant differentiated cells derived from KD3 line into the injured spinal cord did not improve locomotor recovery and led to tumor-like growth of cells, accompanied by increased debilitation, morbidity and mortality.

Our results suggested that increase in tissue sparing might be due to: 1) transplanted motor neuron and other cell types in cell mixture that produced neural network, 2) endogenous neural progenitors and 3) extracellular matrix produced by progenitors derived from ES cells [30]. Previous researchers showed that unlike neural progenitors that are endogenous to the central nervous system, progenitors derived from ES cells produce



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considerable quantities of extracellular matrix like laminin and fibronectin [30]. The myelin ratio was greater in the ESMN group compared with control and medium-injected groups, but there was no significant difference. The low levels of myelination might be caused by small number of oligodendrocyte differentiated by action of RA on ES cells. The previous reports demonstrate that ES cell-derived oligodendrocytes could myelinate injured spinal cord [30].

We have found that the mean of Hoechst-labeled cells in OEC group were significantly higher than GFP-positive cells in ESMN group. The reduction of GFP-positive cells might be due to the injured environment and rejection after transplantation. Many ESMN die after transplantation, but some of them remained and established neuronal network. Previous researchers estimated that only few of ESMN extended axons into the peripheral nervous system and also the survival rate of these cells in spinal cord were very low [10, 11]. Shukla et al. [34] showed that transplantation of neural stem cell (from the mesencephalic region of brain) derived dopamine neurons is associated with low survival of cells, limited host-graft interaction and poor axonal outgrowth. They evaluated the co-transplantation of OEC and neural stem cell (from the mesencephalic region of brain) derived dopamine neuron and suggested that in the presence of OEC, significantly enhanced survival of neural stem cell-derived dopamine neurons, axonal fiber outgrowth and restitution of motor function recovery were evident in the co-transplanted group.

In the present study, we evaluated the effect of co-transplantation of ESMN and OEC. We hypothesized that the lost neurons would be functionally replaced by ESMN, while the OEC would increase the survival of transplanted ESMN through released NTF and cell adhesion molecules. The results showed that tissue sparing and myelin ratio were higher than other groups. This finding might be due to the synergistic effects of two cell types in repairing of spinal cord. The survival rate of GFP-positive cells in co-transplanted group was higher than in ESMN group. This is the first report of positive effects of OEC on survival rate of ESMN. This enhanced GFP-positive cells in the spinal cord suggests the existence of functional graft at transplantation site which could be due to the higher survival of ESMN neurons in graft itself as well as possible resprouting of intrinsic neurons in response to various NTF including NGF, secreted by OEC as well as contact-mediated support of OEC to residual axonal fibers. Since NGF, has dual activity of providing NTF support along with properties of anti-oxidant [35], anti-apoptotic [36], and neurorescue activity. OEC derived from olfactory bulb, release NTFs such as NGF, GDNF, BDNF, NT-4/5, CNTF and FGF2 [5, 6]. NGF reported to enhance the number of dopamine neurons in lesioned nigral region and graft [37], increase synapse formation and helping in neuronal path finding by enhancing the expression of cell adhesion molecules on nerve cells thus guiding axons in establishing connectivity with host cells [38]. We hypothesize that increased survival of transplanted cells in the co-transplanted group could be possibly due to the expression of neural cell adhesion molecules, L1 and growth associated proteins by OEC, thus helping in synapse formation and neuronal path finding by guiding axons in making connections [38]. The BBB score was not significant with ESMN and OEC groups. The authors suggest that transplanted neurons have not established more functional synaptic connections or the length of study was too short to show hindlimb improvement.

Therapeutic use of stem cells to regenerate motor neurons in the spinal cord requires a method which guarantees cell survival for extended periods of time while sending axons through the white matter toward muscle targets. We have found that the co-transplantation of ESMN and OEC into an injured spinal cord have a synergistic effect, promoting neural regeneration, ESMN survival and functional recovery. Therefore, we established a new strategy for treatment of SCI by co-transplantation of ESMN and OEC.

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