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Pre-Differentiated Embryonic Stem Cell versus Olfactory Ensheathing Cell for Spinal Cord Regeneration after Compressive Injury in Rat

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ABSTRACT

Background: Transplantation approaches are interventions currently available to apply to the devastating problem of spinal cord injury (SCI). Olfactory ensheathing cell (OEC) and embryonic stem cell (ESC) are considered to be promising therapeutic strategies**.** In this study, we compared the potential use of OECs and neurally pre-differentiated ESCs in contusion spinal cord.
Methods: OECs were harvested from olfactory bulb of rats and labeled with Hoescht 33342. ESCs were generated by using feeder free GFP positive CGR8 mouse ESCs and neurally pre-differentiation was induced by retinoic acid (RA) and characterized by different antibodies. SCI was induced by fogarty catheter at T8-T9 level in adult rats. Transplantations were performed 9 days after the injury. Rats were randomly divided into 3 main groups (neurally pre-differentiated ESC, OEC and media as control group). The recovery of gross motor function was evaluated using Basso-Beattie-Bresnahan (BBB) locomotor rating scale on the ninth day post injury and once per week thereafter for 4 weeks after cell transplantation. At 28 days after transplantation, histological assessment including transplanted cell detection in tissue, tissue sparing and myelinated axons was performed.
Results: Following transplantation, a significant recovery of hindlimb function according to BBB scale was observed in rats in the transplanted groups compared to control and sham groups ($p<0.05$). There was no significant difference between transplanted groups four weeks after transplantation. OEC and ESC were found in the tissue after transplantation. In OEC group, many of OECs were detected around and within the cystic cavity that number of these cells was significantly higher in comparison with number of cells in ESC group ($p< 0.001$).
In the site of injury, several cavities were produced that disrupted portions of the gray and white matters. The extent of tissue damage was more severe in the sham and control groups than the other groups. Significantly more spinal tissue was spared in OEC and ESC groups ($P < 0.001$). No significant difference in percentage of spared tissue was found between sham and control groups or transplanted groups. The percentage of myelinated area was greater in OEC group than in three other groups ($p< 0.05$) (Figure 5, A). Although the percentage of myelinated area was more in ESC group in comparison with non-treated groups, but this difference was not significant.
Conclusion: It seems that using of combination of a myelinating cells like OEC or schwann cell and source of cells to replace dead cells like Mesenchymal or embryonic stem cells, better results can be obtained due to probable synergic effects of these cells.

Keywords: Embryonic stem cell; Olfactory ensheathing cell; Compressive injury; Rat; Spinal cord

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INTRODUCTION

Spinal cord injury (SCI) lead to cell death (neuron and glia) and axonal degeneration, and produces cavities and cysts may interrupt axonal tracts. There is some spontaneous repair after central nervous system (CNS) injury but it is incomplete. Repair of SCI will require a combination of effective and safe therapeutic interventions. The versatility of cellular implants to overcome many obstacles encountered after SCI makes them ideal therapeutic candidates for repair. Cell based approaches have two fundamental directions: regeneration of white matter long tracts and replacement of dead cells (neuronal or oligodendrocyte) in the injured tissue [1]. Transplantation of variety specific cell types of peripheral and central nervous system, immune cells and stem cells for SCI repair have reduced progressive tissue loss, promoted axon regeneration, facilitated myelination and improved functional outcome [2,3].

Although different cell types have been tested in SCI animal models, there is no consensus on which is the best candidate, in terms of functional outcome, to be used in clinical human trials.

One of the most frequently used cells for white matter regeneration is olfactory ensheathing cell (OEC) [4]. OEC ensheathes axons of olfactory sensory neurons, guiding their growth from the olfactory epithelium to the olfactory bulb, expresses growth factors such as NGF, NT4/5, NT3, and BDNF and cell adhesion molecules such as L1 and laminin [5-8]. OEC has been shown to promote white matter regeneration and to improve functional recovery [9-12]. However, the failure of OEC transplants to support regeneration has been reported [13-15].

In contrast with white matter regeneration, the rationale for neuronal replacement in the injured spinal cord is less defined. Restoration of spinal gray matter continuity may be important to the overall recovery process. One of the approaches to cell replacement is to use stem cells can differentiate into neural or glial cells are necessary for spinal cord regeneration [16]. Among these cells, it has been suggested that ESCs possess a great potential for the repair of the damaged spinal cord [17]. ESCs can be induced to differentiate into several cell types, such as neural progenitors [18] and glial cells like oligodendrocyte cells in vitro [17,19,20]. Moreover, neurally pre-differentiated ESCs can survive, integrate and help restore function following transplantation into paralyzed rats [21-23].

Many types of restorative strategies have been used for optimal recovery, but the feasibility of each approach differs. In the present study the potential use of OEC and neurally prediferentiated ESCs for functional and histological improvements of spinal cord injuries as compared were evaluated.

MATERIALS AND METHODS

Isolation and culture of primary OEC

For the establishment of OEC, we used previously published method [22]. In brief, 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) for 30 minutes at 37°C in 5% CO2. The cell suspensions were plated into plastic cell culture flask in DMEM/F12 containing 10% fetal bovine serum (FBS; Sigma, USA), 2mM 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% CO2 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, USA) flasks. After seeding, the OEC attached to plate within 48 hours. Cultures continued until appropriate cell number was achieved. The medium was changed every 3 days.

For characterization of OECs, expression of the 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). In brief the cells were fixed by incubation in 4% paraformaldehyde (Sigma, USA). Fixed cells were rinsed with PBS and permeabilized with 0.1% Triton X-100 (Sigma, USA) in PBS, incubated with blocking buffer (1 mg/ml BSA (Sigma, USA) and 10% goat serum (Gibco, USA)) followed by incubation with primary antibodies overnight at 4°C. Cells were then rinsed with PBS and incubated with a species-specific secondary antibody. Cultures were washed three times with PBS; nucleus was stained by DAPI and visualized by fluorescent microscope

Before transplantation, the cells were incubated for 30 minutes at 37°C in the dark in cultured medium containing 10 ug/ml Hoescht 33342 (Sigma, USA) for labeling [24]. They were rinsed several times with DMEM without serum, resuspended in the same medium and then transplanted with defined concentration into the spinal cord.

ESC culture and neural differentiation

ESCs were generated by previously published method using feeder free GFP positive CGR8 mouse ESCs (Gift from Stem Cells Technology Research Center, Tehran, Iran). ESCs were grown on gelatinized plate in ES culture medium (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% FBS (Gibco, USA) and 1000 u/ml leukemia inhibitory factor (LIF; ESGRO, Chemicon)). ESC colonies were dissociated and cultured in ES medium without LIF and FBS but supplemented with 10% Knockout Serum Replacement (Gibco, USA) to form Embryoid Body (EB). Medium was replaced by previous medium supplemented with retinoic acid (1 µM) (Sigma, USA) and EBs were cultured for 4 days. EBs were partially trypsinized and seeded on gelatin coated flasks in serum-free DMEM/F12 medium supplemented with 1% N2 (Gibco, USA). For longer culture, they were transferred after 3 days to Neurobasal medium plus 2% B27 supplement (Gibco, USA) and 10% FBS. RA-induced differentiation was assessed by immunocytochemistry analysis. The expression of microtubule-associated protein 2 (MAP2) in differentiated mouse ESCs was visualized by staining the cells with primary monoclonal mouse anti MAP2 (Sigma, USA) and secondary phycoerythrin conjugated anti-mouse IgG antibody (Abcam, USA) with protocol that above was described.

Semi-quantitative RT–PCR was performed to evaluate altering in gene expression after neural induction with RA. Briefly, 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, USA) according to the manufacturer's instructions. Polymerase chain reactions were performed at 94°C for 1 minute, 30 cycles 94°C for 30s, 55-63°C for 30s, 72°C for 30s and 72°C for 10 minutes. Amplified DNA fragments were electrophoresed on 1.5% agarose gel. The gels were stained with ethidium bromide (10ug/ml) and photographed on a UV transilluminator (Uvidoc, UK).

Induction of spinal cord injury

Adult female wistar rats aged 6–8 weeks (n=21, 250–300g) were obtained and maintained on a 12-hour dark-light cycle at 20°C. Spinal cord injury was induced using Vanicky methods [25]. In brief, animals 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 was removed and the muscles and skin were 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 gentamicin. Autophagia was treated with acetaminophen 1-2 mg/ml in drinking water to prevent self-mutilation.

Transplantation

Prior to implantation, GFP positive ESCs and Hoechst labeled OECs were harvested from culture via trypsinization, then cells were centrifuged, resuspended in fresh DMEM/F12 and counted. Appropriate aliquots of cells were kept on ice prior to surgery. Transplantations were performed 9 days after the injury, the rats were anesthetized and a small hole was made in the vertebral arches of T8 and/or T9 using a micromotor. The tip of a 20µl Hamilton syringe connected to a microinjector was inserted through the intact dura into the center of the developing lesion cavity, (penetration depth of 1.0 mm at an angle of 40–45º past perpendicular). The rate and volume of injection was 1µl/min and 5 µl volume respectively. Rats were randomly divided into 3 groups. In group I, 1×10^6 ESCs (n=6) and in group II, 1×10^6 OECs (n=6) were injected into the lesion site. In the third group serving as control, 5 µl of medium with no cells was injected as above (n=6). Three of the animals did not receive any media or cells. All animals received cyclosporine (10 mg/kg, SC) on day 8 for immunosuppression and continued for the duration of survival**.** After transplantation, muscles and skin were sutured and rats received antibiotic.

Behavioral assessment

The recovery of gross motor function was evaluated using Basso-Beattie-Bresnahan (BBB) locomotor rating scale on the ninth day post injury and once per week thereafter for 4 weeks after cell transplantation. BBB rating scale covers a range from complete paralysis (score 0) to normal gait (score 21) [26]. Locomotion was analyzed by two blind researchers.

Histological evaluation

After the final behavioral assessment at 28 days

post transplantation, 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 Tissue-Tek OCT compound (Sakura, USA). Serial 12- µm thick longitudinal sections were cut on a cryostat and mounted on Poly L-Lysine-coated slides.

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 Hoescht labeled and GFP positive cells in OEC and ESC groups using fluorescent microscope. Every sixth slides were selected, and the cell count was performed on 50 slides in each animal.

Every tenth longitudinal section was used to determine the spared tissue and myelinated area. The slides were stained by cresyl violet-Luxol fast blue (Merck, Germany). In each section, the pixel number in the damaged and myelinated area and the total number of pixels in spinal cord segment were measured. Measurements for 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 number of pixels in the area of damaged tissue and the number of pixels in the whole segment [27].

Statistical Analysis

All statistical analyses were performed on standard statistics computer software (SPSS 11.5). One-way ANOVA followed by Tuckey's post hoc test was used to determine the statistical differences between the spared tissue and myelin ratio in each group. BBB scores were compared between groups using nonparametric analysis (Kruskal–Wallis followed by Mann–Whitney U test). Cell numbers in spinal cord in transplanted groups were compared with Independent T test. A statistically significant difference was accepted at $p < 0.05$.

RESULTS

OEC & ESC characterization

OECs present low affinity NGF receptor (P75) on their cell surfaces. To verify the purification of the enriched population, cells were stained with anti-P75 antibody. Merged images showed that majority of the cells (>95%) were positive for this marker (Figure 1, A-C).

ESCs were detected in the palate using light microscope to find EBs. As previously described, these cells were GFP positive (Figure 1, D-F).

Neurally pre-differentiated ESCs characterization

Two days after partial trypsinization of treated EBs, many of aggregates gave off extensive outgrowth of neuritis. Neurite outgrowth occurs at many points along the perimeter of aggregate forming a dense, tangled

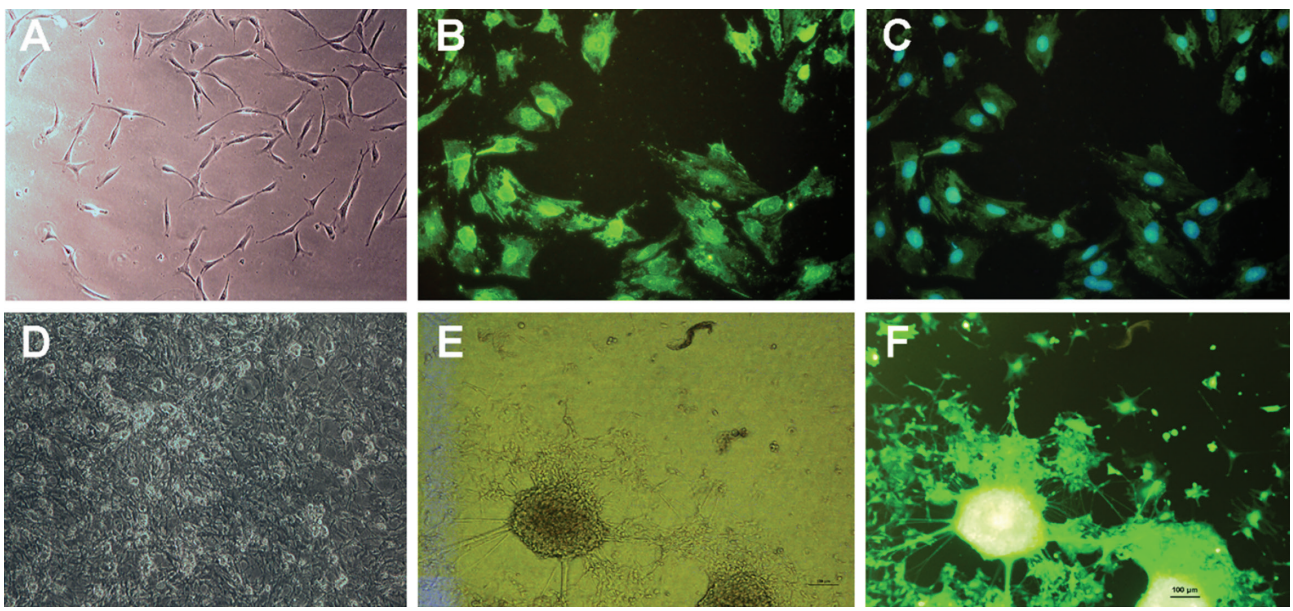

Figure 1. OEC characterization A-C; Light microscopy of OEC (A), P75 staining (B). Merged images of DAPI and P75 staining revealed >90% purification. ESC characterization D-F; Light microscopy of ESC (D), magnified image of EB (E). All ESCs were GFP positive (F)

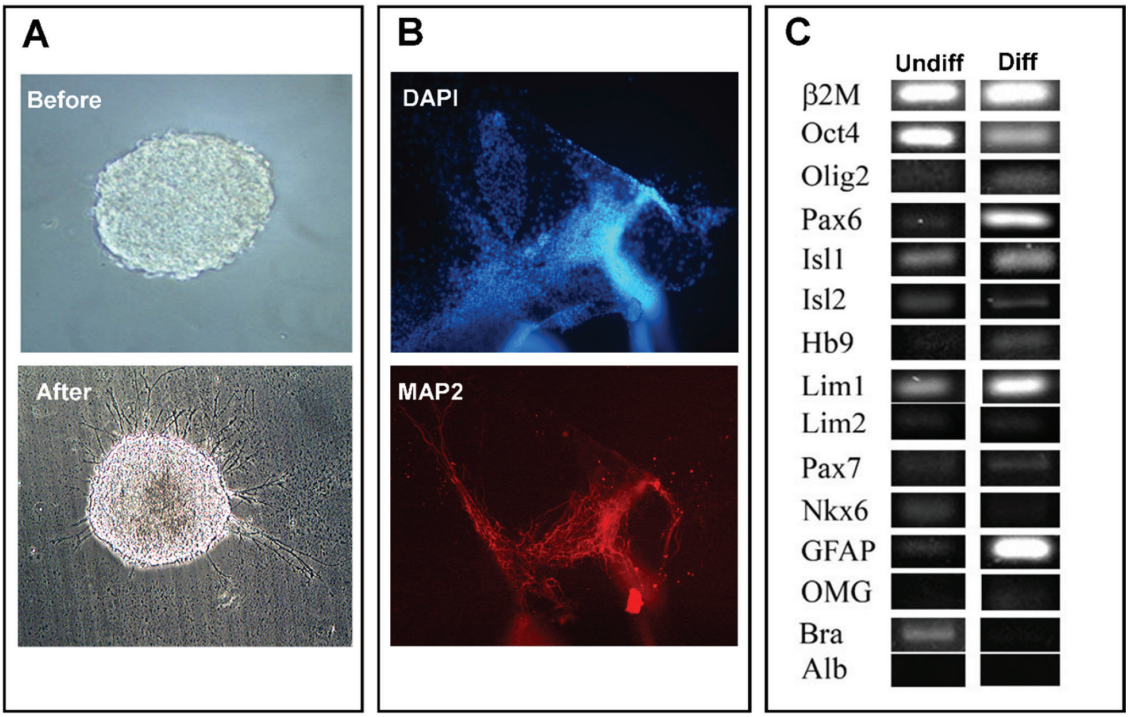

Figure 2. Neurite outgrowth occurs at many points along the perimeter of aggregate forming a dense, tangled collection of neuritis after neuronal differentiation (A). MAP2 staining after differentiation (B). Expression of neuronal gene in differentiated ES cultures (C).

collection of neuritis (Figure 2A). In order to determine whether the neuron-like cells and process contain neuron-associated proteins, cultures were stained with antibodies to MAP2 (Figure 2B). 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 (Figure 2C). The results revealed that the expression of motor neuron progenitor (Olig2 and Nkx6.1) and motor neuron (Hb9) markers were low. Interneuron markers (Lim1 and Lim2) were expressed and Lim1 had a higher expression. Astrocyte (GFAP) and oligodendrocyte (OMG) markers were expressed, as well (Figure 2C).

Recovery of Hindlimb Function

BBB score was measured nine days following injury and at weekly intervals starting on day 9. On the same day, the animals were assessed and selected for transplantation. The criterion for inclusion in this study was that the animal had to score 0–2 on day nine. Figure 3 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 the 4 groups ($p > 0.05$). Following transplantation, a significant recovery of hindlimb function was observed in rats in the transplanted groups compared to control and sham groups ($p<0.05$). The mean recovery scores in the OEC and ESC groups 4 weeks after transplantation were 7.33 ± 1.03 and 7.2 ± 0.44, respectively. The corresponding scores in the control and sham were 0.66 ± 0.57 and 0.4 ± 0.53. There was no significant difference between transplanted groups four weeks after transplantation (Figure 3).

Histological evaluation

To perform cell counts, we counted the number of transplanted cells in the longitudinal sections as Hoescht labeled cells and GFP positive (Figure 4). In OEC group, many of OECs were detected around and within the cystic cavity, and the average number of cells was 28524 ± 7287. In ESC group, the number of GFP positive cells was 4818 ± 1881 (Figure 4). There was a significant difference between cell number in OEC and ES groups ($p< 0.001$).

In the site of injury, several cavities were produced that disrupted portions of the gray and white matters. The extent of tissue damage was more severe in the sham and control groups than the other groups (Figure 5, A). Spinal tissue sparing was calculated from the damaged area (including degenerate tissue and transplanted cells) of spinal cord segment. In the sham and control groups of the spinal cord, 27.95 ± 3.52 and 29.33 ± 4.04 of the

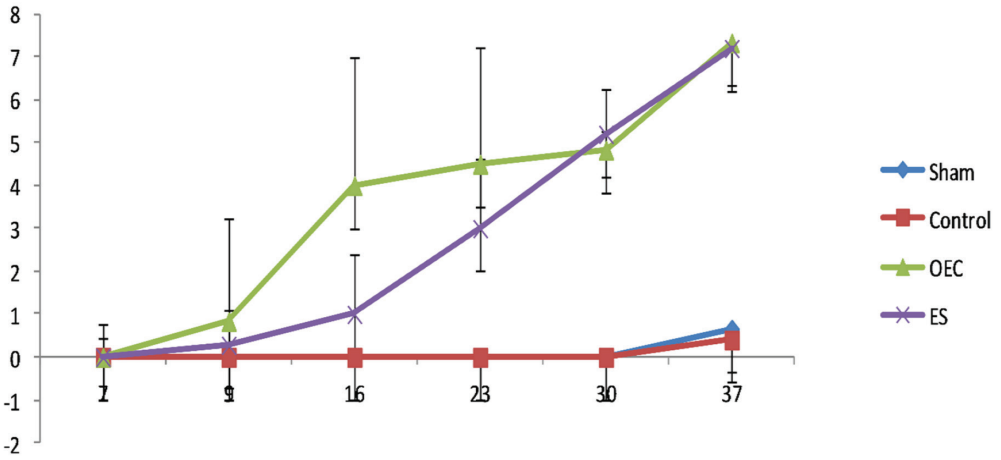

Figure 3. Average BBB scores across the post-injury and post-transplantation period. Following transplantation, a significant recovery of hindlimb function was observed in rats in the transplanted groups compared to control and sham groups ($p<0.05$). There was no significant difference between transplanted groups four weeks after transplantation.

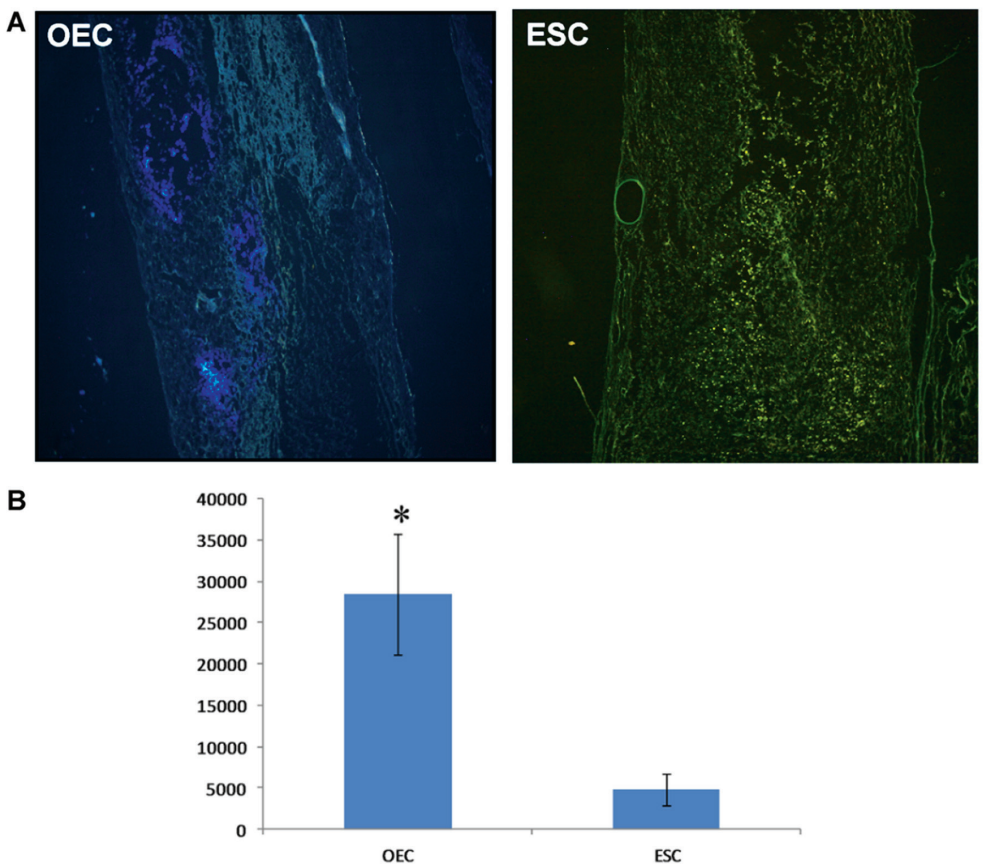

Figure 4. Counting the number of transplanted cells in the longitudinal sections as Hoescht labeled cells and GFP positive (A). In OEC group, many of OECs were detected around and within the cystic cavity. In ESC group, the number of GFP positive cells was significantly lower than OEC ($p< 0.001$).

segment examined was spared respectively. Significantly more spinal tissue was spared in OEC (63.95 ± 17.09) and ESC (68.93 ± 3.08) groups ($P < 0.001$) (Figure 5, B). No significant difference in percentage of spared tissue was found between sham and control groups or transplanted groups.

The percentage of myelinated area was greater in OEC group than in three other groups (24.43 ± 12.69) ($p< 0.05$)

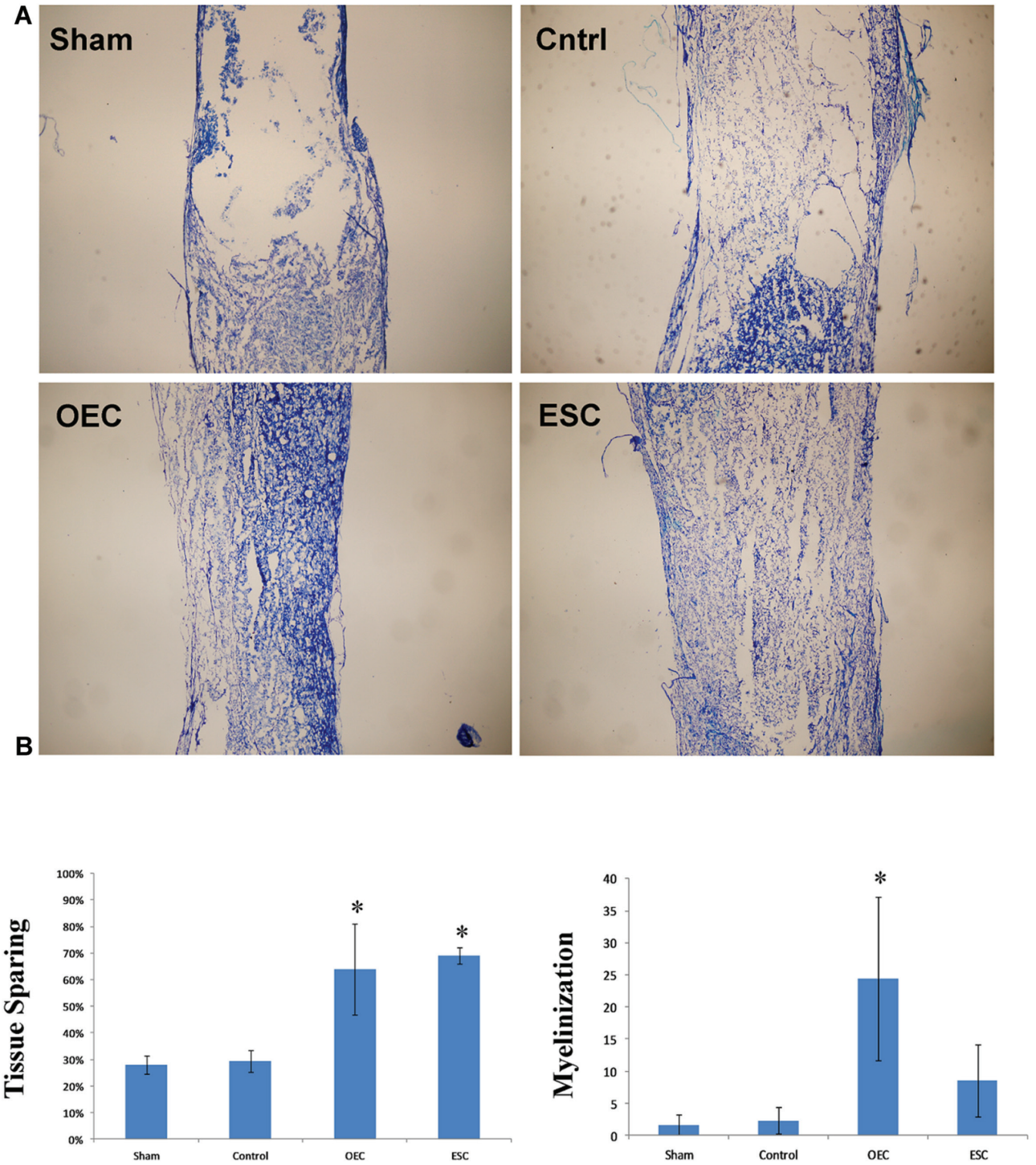

Figure 5. The extent of tissue damage was more severe in the sham and control groups than the other groups (A). Significantly more spinal tissue was spared in OEC and ESC groups compared to control and sham groups ($P < 0.001$) (B).

(Figure 5, A). Although the percentage of myelinated area was more in ESC group (% 8.48 ± 5.65) in comparison with non treated groups (% 2.33 ± 2.08 in sham group and % 1.66 ± 1.50 in control group), but this difference was not significant (Figure 5, B)

There was no significant correlation between tissue sparing and cell number in OEC and ESC groups ($P > 0.05$). Moreover, there was no significant correlation between myelinated area and cell number in OEC group ($P > 0.05$). However, there was a significant correlation

between BBB score in day 28 and tissue sparing in OEC group (($P< 0.05$), but such correlation was not found in ESC group ($P> 0.05$). Finally, there was no significant correlation between myelinated area and BBB score in day 28 in OEC and ESC groups ($P> 0.05$).

DISCUSSION

Restoring function to the injured spinal cord represents one of the most formidable challenges in regenerative medicine**.** The present study was undertaken to assess promotion effects of OECs and neurally pre-differentiated ESCs to axonal regeneration and functional recovery as compared in contusion model of spinal cord injury in rat. The results demonstrated no significant recovery of hindlimb movements (BBB score) in sham and control groups. Therefore, these animals were suitable for assessing the effect of cell transplantation therapy. Data showed significant locomotor functional recovery in both transplanted groups and explained no significant difference in functional restoration capacity of them. The histological results showed significant increased tissue spared area in both transplanted groups in comparison with non treated groups. Also significant increase in myelinated area in OEC transplanted group compared to other three groups was seen.

OEC implantation is among the exciting strategies being investigated as potential therapies for spinal cord trauma [28]. OEC transplantation has a protective effect on the injured spinal cord by limiting cavitations and increasing tissue sparing [29]. OECs promote axonal regeneration in corticospinal tract [9,11],dorsal column [30] and monoaminergic fibers [9,31]. However several experiments have demonstrated that, under specific conditions, OECs cannot associate with axons regeneration in corticospinal tract [13,15,27], rubrospinal tract [32,33] and ascending dorsal column fibers [15,34]. Reasons for controversial reports in histological and functional outcome following OEC transplantation are likely differences in OEC biology as a result of harvest location, culture methods, purity, time in culture and transplantation paradigm, as well as the delay between injury and OEC application [7,35]. The results of the present study demonstrate that OEC transplantation promote partial recovery of motor functions. Our results showed that OEC transplants facilitate axon remyelination following damage to the spinal cord. Several studies have described the evidence supporting the myelinating capacity of OEC in vivo and in vitro [8,36]. It has been suggested that OEC must collaborate with additional cell types like schwann cell in order to promote beneficial effects on remyelinating axons within the spinal cord [37].

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 [7,8]. Reports suggested that OECs 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 [8]. Increasing of 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. Transplanted OECs into demyelinating lesions in the rat spinal cord promote remyelination of axons and restored impulse conduction [6,38,39]. Sasaki et al 2004 showed that transplantation of OECs 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 [36].

We also assessed the application of neurally pre-differentiated ESC transplantation to the spinal cord injury. Because, 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 [17]. We used pre-differentiated ESCs for replacement of dead cells with regards capability of these cells to differentiate into various cell types such as neural progenitors [18] and oligodendrocyte cells [40,41]. In the presence of RA, ESCs 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 [42]. The results presented here suggest that the transplantation of stem cell lead to functional improvement and increased tissue sparing with less amount of myelination. McDonald et al demonstrated that transplanted mouse pre-differentiated ESCs into the spinal cord myelinate axons and differentiate into astrocytes, oligodendrocytes and neurons; and produce improved locomotor function without forelimb–hindlimb coordination [17,19]. They stated that oligodendrocytes, oligodendrocyte progenitors and producing growth

factors by cells can be associated with remyelination. ESC derived oligodendrocytes, survived in the injured cord and improved recovery [43]. Several studies have shown ESCs to survive, propagate, and even provide some beneficial effects in the rodent CNS after experimental injury [17,19, 44-49].

Increase in tissue sparing might be due to: 1) transplanted neuronal cells that produced neural network, 2) endogenous neural progenitors and 3) extracellular matrix produced by progenitors derived from ESCs [17]. Previous researchers showed that unlike neural progenitors that are endogenous to the central nervous system, progenitors derived from ESCs produce considerable quantities of extracellular matrix like laminin and fibronectin [17]. The low levels of myelination might be caused by small number of oligodendrocyte differentiated by action of RA on ESCs. The previous reports demonstrate that ESC derived oligodendrocytes could myelinate injured spinal cord [17].

The transplantation of neurally pre-differentiated ESC alone may not recover demyelinated axons by a remyelinating activity but the beneficial effect of the transplanted these cells could be due to a neuroprotective mechanism [50] that is provoked by an immunomodulatory [51] or a suppression effect on T cells [50,52].

Another possible explanation for larger tissue sparing area and better functional outcomes in ESC treated group is that the embryonic stem cells could release neurotrophic factors that would either rescue axons from wallerian degeneration after injury or enhance axonal growth in surviving neurons [47].

In conclusion, the results of the present study demonstrate that both pre-differentiated ESCs and OEC transplantation into the contused spinal cord of adult rats promote axonal regeneration and partial recovery of motor functions.

We hypothesized by using of combination of a myelinating cells like OEC or schwann cell and source of cells to replace dead cells like Mesenchymal or embryonic stem cells, better results can be obtained due to probable synergic effects of these cells.

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