Effect of olfactory ensheathing cells (OECs) transplantation on functional recovery in acute phase of spinal contused rats

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Abstract

Introduction: Spinal cord injuries (SCI) lead to permanent irreversible functional deficits. Poor prognosis of patients is the motivation of searching a treatment for the chronic injury. Planting stem cells provides us with a promising strategy. In the meanwhile, the use of olfactory ensheathing cells (OECs) has shown very good results. This study aims at evaluating the effect of transplanted OECs on functional recovery of acute SCI in rats.

Materials and methods: In this study, eighteen adult male Wistar rats weighting approximately 210 ± 10 gr were used to study spinal cord injury. They were randomly divided into four groups: Sham (n = 3), Control (n = 6), Vehicle (n=3) and Treatment (n=6). In the Sham Group, only laminectomy was performed in the area of T9 spinal cord. In three groups of Control, vehicle and Treatment, after laminectomy, spinal cord contusion model was performed using the Weight drop technique. Immediately after the injury, 10µl Dulbecco's Modified Eagle Medium (DMEM) alone or with 10^6 cells were injected to vehicle and Treatment Group animals. For culturing cells, the olfactory mucosa of 7-day-old male Wistar rats was used. Motor function of animals in all groups, was evaluated in the first 48 hours daily and then weekly for eight weeks.

Results: Comparing the results of the second to eighth week of the study showed significant differences in the group receiving the OECs with the control group ($P<0.05$).

Conclusion: The results indicate a positive influence of the olfactory ensheathing cells in functional improvement of spinal cord injury in the acute phase of injury.

Keywords: Spinal cord injury, Olfactory ensheathing cells

Introduction

Spinal cord injury (SCI) is a serious clinical problem that often leads to spinal cord damage, dysfunction and disability (1). Before Year 2009 the total number of patients with spinal cord injury had been reported about eighty nine thousand people (2). Ten thousand new patients in the United States of America are annually added to the population of patients with SCI (3). Although deaths from these losses have been reduced to less than 5%, but the important fact is that the main victims of this loss are healthy young people who suffer from a long-term disability (2). Contusion that due to vertebral fracture during mechanical events causes a severe blow to the spinal cord is the most common clinical lesion model in humans

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that cover about 40% of patients with spinal cord injury. As a result, it is the most commonly used animal model to study SCI (4). In this model, the spinal cord tissue is inflicted to spinal cord tissue, a central contusion, necrosis, hematoma and bleeding out which in turn can lead to pain, inability to control the bladder and bowel and the loss of muscle function (5).

After spinal cord injury, neuronal deaths lead to permanent and irreversible functional deficits in patients together with inability in regeneration of axons of injury site. Among the factors responsible for the lack of recovery after SCI, the following cases can be pointed out:

- Astrocyte cells activity that creates scar tissue at the site of injury and dysfunction of the distal axonal injury.
- The lack of growth-promoting factors (6).
-The poor ability of neurons in the central nervous system(CNS) in recovery response after injury (7).
-The presence of inhibitory factors in myelin released after the destruction of myelin from oligodendrocytes.

These factors include myelin- associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMGP), and Nogo (8).

Treatment of spinal cord injuries usually involves surgery to stabilize the vertebral column, administration of high-dose steroids, rehabilitation and using therapeutic protections such as methyl prednisolone and interleukin-10 (IL-10). However, with using these methods, significant improvements have been made in survival of affected people but trying to restore function has been ineffective yet (1). Considering the limited reconstruction nature of the damaged central nervous system, various experimental strategies are required to rebuild the axons environment. Some of these treatments include organ transplantation, the administration of neurotrophic factors, gene therapy, blocking factors inhibiting the formation of myelin and cell therapy. Among the cells used in the treatment of spinal cord injuries, Schwann cells in the peripheral nerves and OECs are all taken into consideration (9). Olfactory nerve is the only cerebral nerve that its olfactory neurons has unlimited lifetime and are renewed by stem cells in the lifetime (10). This ability in the olfactory nerve is due to OECs. OECs are high-capacity stem cells observed throughout olfactory nerve whether in peripheral or central area unlike oligodendrocytes and Schwann cells that are only found in the CNS and peripheral nervous system (PNS) respectively. Following by transplanting OECs to spinal cord injury site, promoting axonal regrowth, remyelination, increased angiogenesis, migration from within astroglial scar, acceleration of the regrowing axons and modifying glial scar formation was observed that leads to improvement lesions in a variety of rodent models (11). Less stimulating astrocytes and thereby less glial scarring after transplantation of these cells are from other benefits of OCEs (12, 13). There are also other factors such as Glial fibrillary acidic protein (GFAP) and other proteoglycans released in the injury site that cause scar formation and the lack of regeneration of axons in the spinal lesion site. But OECs transplantation in injured spinal cord leads to reduced expression of these factors and proteoglycans. Verdu et al used OECs to treat photochemical spinal cord injury confirm the results (14).

In addition, In-vitro studies conducted on OECs, it is shown that these cells can promote neurotrophic factors that these factors themselves can improve lesion and axon guidance. These factors include vascular endothelial growth factor (VEGF), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and Glial cell-derived neurotrophic factor (GDNF), etc (15). The source of OECs can be the olfactory mucosa or olfactory bulb. Cells originating from olfactory mucosa have more population and power and a longer time to reproduce and isolating

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them is even more comfortable than the cells of the olfactory bulb origin (16). The OECs in the olfactory mucosa are probably more immature or have a more progenitor population (10). Also, studies have shown that OECs originating from olfactory mucosa have more migration and mitosis power than cells derived of olfactory bulb (17). Therefore, in the present study, the cells taken from the olfactory mucosa were used and their impact was investigated on functional recovery in spinal cord contusion injury model.

Materials and methods

In this study, eighteen adult male Wistar rats weighting approximately 210 ± 10 gr were used to study spinal cord injury. They were randomly divided into four groups:

1. The control group (N = 6): spinal cord injury contusion mode was created, but no remedial action was taken.
2. Sham group (N = 3): in this group, only laminectomy was performed and no lesion was in the spinal cord.
3. Vehicle (N = 3): in this group one week after creating the injury, 10µl Dulbecco's Modified Eagle Medium (DMEM) solution with no cells was injected in the caudal and Rostral area of lesion site (each 5µl).
4. Treatment Group (N = 6): in this group, immediately after the lesion, 10^6 cells / 10µl DMEM were injected to caudal and Rostral areas of lesion (each 5µl).

It should be noted that all injections were done in different groups using a 10ml Hamilton syringe at a distance of 0.5mm from midline and 1.5 mm from the surface of Dora in the caudal and Rostral area of the lesion was performed.

Culturing olfactory ensheathing cells (OECs) from the olfactory mucosa: To isolate cells with high proliferation, olfactory mucosa lamina propria of 7-day-old rat pups was used. To this end, after anesthesia using a mixture of ketamine / xylazine (60/6 mg / kg) and removal of the lower jaw and hard palate, with entry to the area of the nose, nasal septum was separated (Figure1). Then, it is washed several times with a new Phosphate Buffered Saline (PBS) containing 1% of antibiotics and then washed in a solution without antibiotics. Then one third of the posterior septum that contains the olfactory mucosa consisting of olfactory epithelium and lamina propria was separated and transferred to a sterile Petri dish. After crushing the lamina propria into small pieces, using 0.25% trypsin enzyme, enzyme digestion was done after incubation for 10 minutes. Then adding fetal bovine serum (FBS), enzyme activity was halted. Then obtained soup was centrifuged for 10 minutes at 2000 rpm. After discarding the supernatant, supernatant cells were suspended again in 4 ml of culture medium containing FBS 5% and antibiotic 1% and forskolin mitogenic factor 5µM and after transfer to the flask, they were stored in an incubator at 37 ° C and carbon dioxide 5%. To remove fibroblast cells, the flasks were replaced 48 hours after cultivation. After 48 hours, the cells begin to stick to the bottom of the flask with two different appearances: Spindle-shaped schwann-like cells and astrocyte-like cells with a flat appearance with multiple redundancies. After 48 hours, the flask culture was replaced. After ten to twelve days of culture, when the bottom of flask was completely filled with proliferated cells, Cell Passage was performed (18).



Figure1. Nasal septum of 7-old-day rat pups. Olfactory epithelium isolated from 1/3 posterior part with yellowish appearance.

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Spinal cord injury induction: Spinal cord injury in the type of contusion was created in animals as follows. After anesthesia, using a mixture of ketamine / xylazine (60/6 mg / kg), shaving the hair at the back of the animal and disinfecting the site, a midline incision was created. Muscles and vertebral lamina of T9 was removed without damaging the dura mater. Then contusion injury was created in T10 segment with dropping a ten-gram-weight from a height of 25mm (Figure 2). Then muscles and skin was stitched, and to prevent dehydration of animal, 10 mg ringer serum was injected intra-peritoneally. Moreover, cefazolin (10 mg / kg) was injected to two days after surgery. The bladder was emptied twice daily until establishing the urinary reflexes. After the loss of motion test, animals were assessed two consecutive days and the animals that two days after the injury had BBB test score higher than 3 were excluded from the study (19).

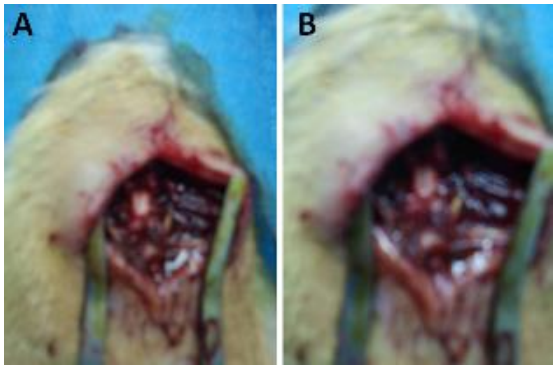


Figure 2. Laminectomy (A) and creating spinal cord injury after laminectomy with dropping ten-gram weight from a height of 25mm on the spinal cord in T9 vertebra, which is equivalent to the T10 segment of the spinal cord (B), shiny appearance of spinal cord is indicative of Dura matter safe.

Functional recovery assessment: To evaluate the mobility, BBB test (Basso, Bresnahan and Beattie) with a score of zero to twenty-one was used. In this case, the assessment within the first 48 hours after spinal cord injury as daily and then on a weekly basis (once a week) Amount of motion was evaluated separately by two individuals in all groups for eight weeks. The final score was reported equal to the average of the scores given by the two individuals. Animals in the 48 hours after the injury had a motor score of 3 or higher, were excluded from further study (20).

Statistical analysis

Statistical analysis of the data was conducted using the software Minitab 17. Data have been reported as Mean ± Standard Deviation in the significant level $P<0.05$. The difference between the groups, was stated when $P<0.05$. Groups were compared using one-way ANOVA with the significance level $P<0.05$.

Result

Cell culture: In order to isolate and culture cells with high proliferation, olfactory mucosa lamina propria of seven-day old rat was used, because with increasing age of the animal, although the number of cells remains constant but their proliferation potential significantly reduces.

Cells showed two different appearances in cell culture process after sticking to flask bottom and passage of 48 to 72 hours :

1. Spindle-shaped cells which were similar to Schwann cells.
2. Astrocyte-like cells with the broad and multiple redundancies (Figure 3).

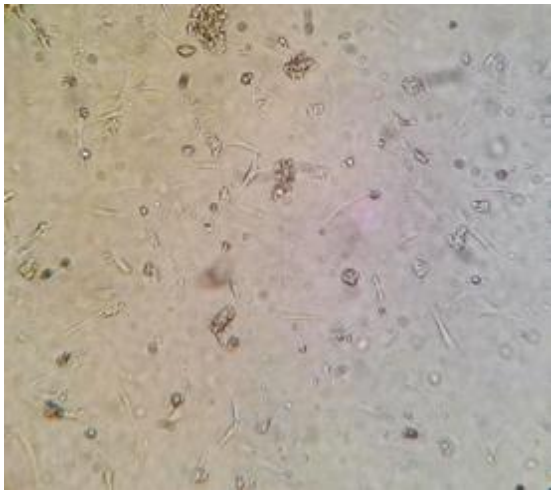


Figure 3. OEC cultured from olfactory mucosa (OM-OECs) of 7-day old rat pups, after four days of cell culture. Magnification is 4X.

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Assessment of Functional recovery: Comparing BBB test results of groups during the study period shows a significant difference of Sham Group with other groups containing spinal injury, from the beginning to the end of the study (P<0.05). Also in comparing the locomotor test of the group receiving the OECs with the control group, although the difference between their scores at 24 and 48 hours of study was pointless (P>0.05), but this difference was significant at the end of the first week (P = 0.018). Comparing the results of the second to eighth weeks of the study also showed significant differences in the group receiving the OECs with the control group (P<0.05) (Figure 4). About vehicle group, differences in BBB scores with control group was meaningless from the beginning of study until the end of the second week (P>0.05), in the third and fourth weeks of the study, a significant difference was observed between the study group and the control group and the vehicle group (P<0.05) and the difference after the fifth week until the end of the study was pointless again (P>0.05). The difference between the group receiving the OECs with vehicle Group was pointless from the beginning to the end of the fifth week of the study (P>0.05) but a significant difference was observed from the sixth week until the end of the study (P<0.05) (Figure 4).

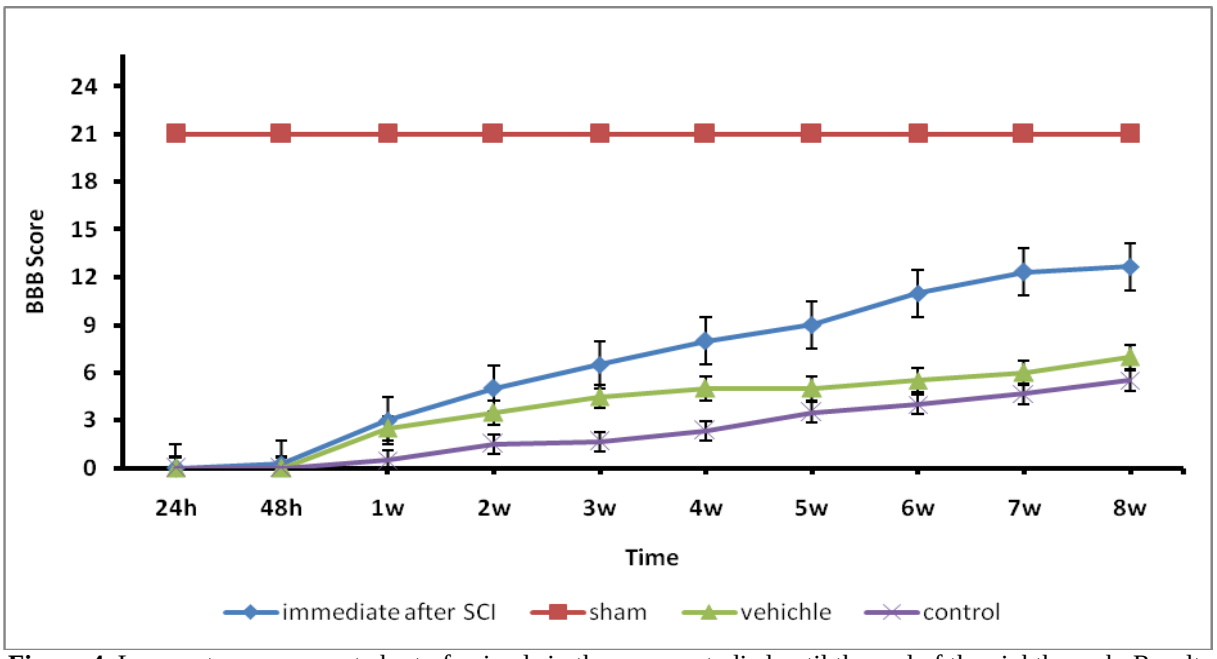


Figure 4. Locomotor assessment chart of animals in the groups studied until the end of the eighth week. Results are shown as mean ± SD and statistically significant difference has been expressed in the level of P<0.05.

Discussion

The results show that transplanted OECs immediately after a spinal cord injury can improve motor function in rats with contusion SCI. Similar studies show the impact of these cells in functional recovery and axonal regeneration after spinal cord injury.

Transplantation of OECs and fragments of lamina propria in olfactory mucosa in adult rats for the treatment of SCI after spinal cord injury in the segment T10, leads to a significant motor improvement in both groups who received OECs transplantation and fragments of lamina propria so that at the end of the eighth week BBB scores in the control group remained at around 2. But in both groups receiving the OECs and the lamina propria fragments it was about 8 (21). In this study, we also observed a significant motor recovery in the group receiving the OECs than the control group from the end

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of the first week to the end of the eighth week so that at the end of the eighth week of the study, mean BBB score in the control group and the group receiving OECs was about 6 and 13 respectively. The reason for the difference of scores at the end of the eighth week of the study can be the severity of spinal cord injury. Besides, the OECs, with olfactory bulb origin for treating complete spinal cord injury at T8, 45 days after the injury, increased BBB scores (22). Tharion et al in 2011 used OECs transplantation for the treatment of spinal cord injury and reported that BBB scores in the group receiving the OECs transplantation was significantly different from the control group. In this study, with maintaining one of the animals of treatment group up to 264 days after cell transplantation, highest score of motion (BBB = 17) was obtained which shows with increasing the duration of the study, we can be obtain more favorable results (23).Verdu et al study in 2003, used OECs transplantation for the treatment of photochemical spinal cord injury (shining halogen bulb for 5.2 minutes on the exposed spinal cord) in the acute phase of spinal cord injury. After three months, they declared that the group receiving the OECs compared to the group receiving cell-free culture medium shows only improved performance of spine morphology. At the end of the study, the BBB score was 18 in the control group and 19 in the group receiving the cell transplants, i.e., the difference was pointless (14). But in our study, a significant difference was observed from the first week until the end of the study and BBB score at the end of the eighth week was 13 in the group receiving cellular transplantation. The reason for this difference may be a difference in injection timing, severity of injury to spinal cord and the site of injury because the severity of photochemical injury in this study is much milder than contusion injury.

Conclusion

The results indicate a positive influence of the olfactory ensheathing cells in functional improvement of spinal cord injury in the acute phase of injury.

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