Original Article

Effects of Transplanted Olfactory Ensheathing Cells on Functional Improvement and Axonal Regeneration in Acute and Delayed Spinal Cord Injury in Rats: A Comparative Study

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Abstract

Introduction: Cell therapy is known as one of the most common methods used for treating a spinal cord injury (SCI); particularly, olfactory ensheathing cells (OECs), which have attracted much more attention among scholars due to their properties such as promotion of axonal regeneration, remyelination, and angiogenesis. Thus, the present study compared the effects of transplanted OECs on functional improvement and axonal regeneration of contused rats during acute and delayed phases. **Material and Methods:** For this purpose, a total of 56 male Wistar rats were randomly divided into eight groups including sham, control, three vehicle groups (immediately, 3 and 7 days after injury), and three cell transplantation groups (immediately, 3 and 7 days following injury). The sham group had experienced only laminectomy and other groups had undergone the SCI. The olfactory mucosa of the 7-day-old rat pups was also used for cell culture. The cell type was then confirmed by immunocytochemistry. In the vehicle and cell transplantation groups, the cell culture medium was injected by itself or accompanied by cells, respectively. Subsequently, the motor function was evaluated. Finally, luxol fast blue (LFB) staining was used for histological assessment. **Results:** Motor test results showed an increase in the Basso, Beattie, and Bresnahan locomotor scale scores immediately after injury in the transplantation group compared to those in other two cell-treated groups; but it was not significant. **Discussion and Conclusion:** Based on the LFB staining results, the regeneration rate in the transplantation group immediately after injury was considerably higher than that in two other treated groups. Therefore, considering these findings, it seemed that cell transplantation immediately after injury was better than that 3 and 7 days following injury.

Keywords: *Cell transplantation, contusion, olfactory ensheathing cells, spinal cord injury*

Introduction

A spinal cord injury (SCI) is known as a life-disrupting disease with high mortality rates and it has been estimated that between 250,000 and 500,000 people are affected with this problem annually.[1,2]

In this respect, it has been reported that the transplantation of olfactory ensheathing cells (OECs) provides a promising treatment for neurodegenerative diseases.[3] These cells have properties similar to Schwann cells, but unlike the oligodendrocytes and Schwann cells, they are observed both in peripheral and central regions.[4-6]

The transplantation of OECs also enhances neural regeneration by stimulation of axonal regeneration and remyelination, angiogenesis, and migration into injured sites.[7] In addition, they promote neurotrophic factors, leading to axonal conduction and regeneration.[8] Moreover, the OECs remove residues from the axonal degradation, thereby modulating inflammatory responses and leading to a secondary regeneration.[9,10] The OECs from olfactory mucosa (OM-OECs) also provides a good source for autologous transplantation because of their easy access through biopsy.[11,12]

Therefore, the present study compared the effects of OM-OECs on the improvement of locomotor function and neuronal regeneration in acute and delayed phases of the contused rats.

Material and Methods

Animal grouping

In this study, a total of 56 male Wistar rats weighing approximately 210 ± 10 g were divided into eight different groups:

How to cite this article: Heydarizadi S, Abbasi N, Asadollahi K, Rezaee S, Moradipour A, Azizi M. Effects of transplanted olfactory ensheathing cells on functional improvement and axonal regeneration in acute and delayed spinal cord injury in rats: A comparative study. J Anat Soc India 2019;68:1-6.

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Access this article online

Website: www.jasi.org.in

DOI: 10.4103/JASI.JASI_21_19

Quick Response Code:

- Control (n = 7): the SCI was performed without any treatment
- Sham (n = 7): only laminectomy was performed
- Three vehicle groups (each one n = 7): only 10 µl of culture medium was injected immediately, 3 and 7 days after injury
- Three transplantation groups (each one n = 7): animals in these groups received 10^6 cell/10 µl Dulbecco's modified Eagle's medium (DMEM) immediately, 3 or 7 days after injury, respectively.

All the injections were performed using a 10-µl Hamilton syringe at a distance of 0.5 mm from the middle line and 1.5 mm from the level of the dura mater in the caudal and rostral areas of the injury (each 5 µl).

Olfactory ensheathing cells cultivating

The OM-OECs are potent, and they also have greater populations and longer proliferation time.[12] Moreover, they seem to be immature or have more progenitor populations.[13] Strong migration nature and mitosis have been further reported for OM-OECs compared to olfactory bulb (OB)-OECs.[14]

In this study, the cells were cultivated as those in previous studies with some slight modifications.[15] To this end, the animals were anesthetized using ketamine/xylazine (60/6 mg/kg). The OM from one-third posterior of the nasal septum of 7-day-old rat pups was then isolated and digested with trypsin (0.25%, 10 min) in the incubator. The enzyme activity was subsequently stopped by adding fetal bovine serum (FBS), and the resulting soup was centrifuged (2000 rpm, 10 min). After discarding the supernatant, the cells were resuspended in the DMEM-containing FBS 5%, antibiotic 1%, and 5 µM of Forskolin; incubating at 37°C in the presence of 5% CO_2.[15]

It should be noted that the cells from the second passage were used in all treatment groups.

Spinal cord injury induction

The contusion model was induced by dropping a weight of 10 g from a height of 25 mm on the T10 segment of the spinal cord.[16]

Immunocytochemistry double staining

Some of the markers used for identification and verification of OECs were p75 and S100.[17,18] The simultaneous double immunostaining was also used for p75/S100, and only double-positive stained cells were considered as OECs. The primary and secondary antibodies employed included anti-p75 (Sigma, rabbit, 1:200), anti-S100 (Sigma, mouse, 1:200), Alexa fluor 488 goat anti-mouse, and Alexa fluor 568 goats anti-rabbit (Sigma, 1:1000).

The simultaneous immunocytochemistry was performed as follows: 5 × 10^4 cells were cultured on a coverslip impregnated with poly-L-lysine (50 mg/ml, invitrogen) for 48–72 h.

The primary fixation of the cells was then performed by adding paraformaldehyde solution 4% (0.5 ml, 2 min) followed by secondary fixation with paraformaldehyde solution (2%, 10 min). For the purpose of the penetration of primary antibodies into the cells, the fixative solution was discarded, and a solution of Triton X-100 (0.25%, 10 min) was added followed by washings with phosphate buffer (PB) solution for three times (0.1M, pH = 7.4, each one for 5 min). To block the nonspecific staining of the secondary antibodies, the specimens were subjected to goat serum (3%, an hour). In the next step, the samples were exposed to the primary antibody overnight at 2°C–8°C. Then, the cells were exposed to secondary antibodies for an hour in a dark room. Afterward, differential staining of the nucleus was performed with 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI). It should be noted that washing with PB solution (each one for 10 min) was performed for three times in the interval of each step after adding the primary antibody. Finally, the specimens were mounted and the images were taken by a fluorescence microscope (Olympus AX70). The immunocytochemistry staining was also repeated three times, and the results were reported as a mean ± standard deviation (SD). The means of the cell count (calculated by ImageJ Software (free java software provided by the national institute of health, Maryland, USA)) in five different fields of the positive immunostained cells were reported by 89% ± 0.7%. The experiment was also performed in triplicate. Furthermore, the flow cytometry results of previous studies had exhibited that 87.9 ± 2.4 of the cultured cells were OECs.[19]

Locomotor assessment

The Basso, Beattie, and Bresnahan (BBB) locomotor scale method was used to evaluate animals' locomotor.[20] Thus, the locomotion in all groups was evaluated in the first 48 h after developing the injury daily and then weekly (once a week) for 8 weeks by two individuals in a completely blinded and separated manner. The final score was then reported as the mean scores given by two individuals.

Histological assessment

The luxol fast blue (LFB) staining was used for myelin evaluation in the groups at the end of the study. Therefore, 5-µm paraffin sections of the spinal cord were prepared after tissue processing.

Ethical considerations

The present study was approved by the Ethics Committee of Ilam University of Medical Sciences with the following code number: EC/93/A/110. This study was also in accordance with the Declaration of Helsinki in 1975.

Statistical analysis

The data were analyzed statistically using Minitab Statistical Software Version 17.0 (Release 11.12, Minitab Inc., State

College, PA, USA) and were reported as mean ± SD. The difference between groups was also considered statistically significant if value of $P \leq 0.05$. The intergroup comparisons were further performed through one-way analysis of variance.

Results

Olfactory ensheathing cells cultivation

In the OECs culture, two different phenotypes of the attached cells were observed: spindle-shaped cells (Schwann-like) and astrocyte-like cells with their flat appearance and multiple redundancies [Figure 1].

Immunocytochemistry

The immunocytochemistry staining was repeated three times, and the results were reported as mean ± SD. The results showed about 89% ± 0.7% of the cells were the OECs. This simultaneous staining also demonstrated the OECs-positive p75 cells as red, S100-positive cells as green, and their nucleus as blue [Figure 2].

Locomotor test

The scores of BBB test showed a significant difference between three transplantation groups compared to those in the control group at the end of 1st week after the injection until the end of the study ($P = 0.009$, $P = 0.018$, and $P = 0.015$ for immediately, 3 and 7 days after injury, respectively). However, the locomotor test did not reveal any significant difference between the three transplantation groups during the whole study period [Figure 3]. Furthermore, a significant difference was observed between the sham and other groups with SCI from the beginning to the end of the present study ($P < 0.05$). Comparing the vehicle and control groups with cell-transplanted groups, using their BBB scores, revealed a significant difference from the 5th week of the study until its end ($P < 0.05$) [Figure 3].

The luxol fast blue staining

The results of staining in the sham group showed healthy spinal cords, whereas a hollow cystic cavity was observed in the control group [Figure 4]. In the all three cell-receiving groups, some of the myelinated neural fibers were observed inside the cavity, but the extent of the myelinated areas in the immediate transplantation group was more than other two transplantation groups. Moreover, the extent of the myelinated area in the 7-day transplantation group was reported ≥3-day transplantation one measured through Image j software (free java software provided by the national institute of health, Maryland, USA) [Figure 4].

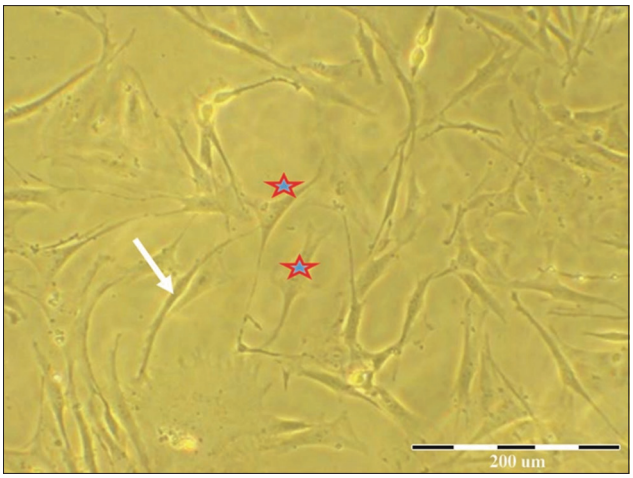

Figure 1: Postculture phase contrast photo of olfactory ensheathing cells. The arrow mark indicated spindle-shaped cells (Schwann-like cells) and the asterisk represented astrocyte-like cells with flat appearance and multiple redundancies

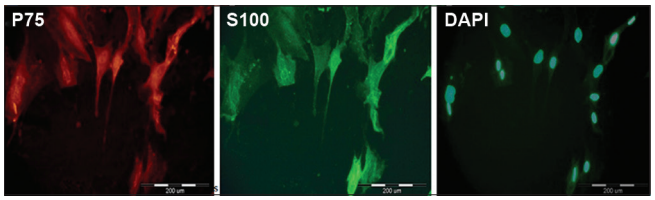

Figure 2: A simultaneous double immunostaining for p75 and S100 markers of olfactory ensheathing cells and differential staining of the nucleus (counter stain) performed using DAPI. p75+/S100+ was double-positive olfactory ensheathing cells for p75 and S100 simultaneously, and p75-positive cells (p75+), S100-positive cells (S100+), and their nucleus were seen in red, green, and blue; respectively

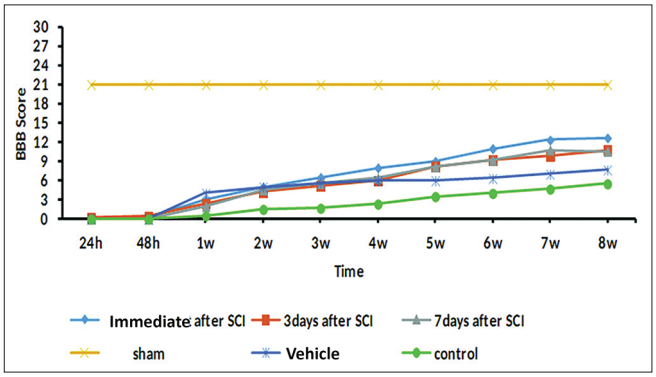

Figure 3: Basso, Beattie, and Bresnahan locomotor rating scale for different groups from onset to end of the study (8th week). The results were expressed as mean ± standard deviation. They also showed a significant difference between the control and the treatment groups, while the difference between the three treatment groups was not reported significant although Basso, Beattie, and Bresnahan score in the cell-transplanted group immediately after the injury was higher

Discussion

In the present study, the BBB results suggested a significant difference in the treatment groups compared to those in the control and vehicle-treated groups at the end of the study. Despite an increasing value in the BBB scores of the immediately-treated group, no significant differences were observed between three transplantation groups, but the LFB staining results indicated that the myelination rate in the immediately transplanted group was higher than the two other groups.

In a study, transplanted OECs isolated from the OM as well as the lamina propria of the OM of the mature rats were

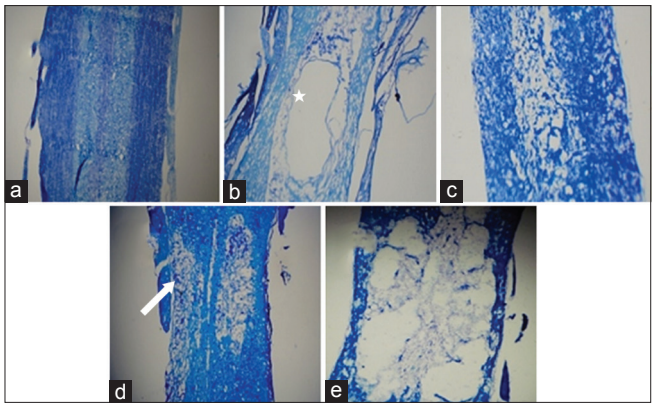

Figure 4: Luxol fast blue staining of the spinal cord sections in the T10 segment in the sham (a), control (b), and three cell transplantation groups: immediately (c), 7 days (d) and 3 days (e) after injury. (a) The tissue image of the sham group illustrated healthy spinal cord tissue and absence of local injury. (b) The tissue image of hollow cystic cavity (control group) was marked with stars. (c) The tissue images of immediately-transplanted group showed smaller size of the cystic cavity and higher amount of myelin compared to the other two transplantation groups. (d) The 7-day transplantation group also showed greater amount of myelin (arrow marks) and smaller size of the cystic cavity compared to the 3-day transplantation group (e)

applied to treat the complete transection model of SCI in the T10 segment 4 weeks after injury. They also reported a significant motor improvement from the 4th week until the end of the 8th week of the study in both groups receiving the transplanted OECs and the lamina propria components. In addition, at the end of the 8th week, the BBB score remained about 2 in the control group, but 8 in the two groups receiving OECs and the lamina propria.[4] However, in the present study, all three transplantation groups showed a significant improvement in the locomotor from the end of the 1st week to the end of the 8th week. Moreover, at the end of the 8th week, the BBB score was 6 in the control group while that was 12 in the animals in the treated groups with OECs. The reason for this discrepancy in both studies was probably due to differences between the types of injuries and the times selected for cell injection.

In another study by López-Vales *et al*., using OB-OECs to treat SCI in T8 at three times (immediately, 30 min and 7 days after injury), the BBB scores increased up to 45 days after follow-up.[21] These results were in agreement with the findings in the present study, in which the BBB scores in all three transplantation groups were also improved until the end of the 8th week after the treatment.

In this respect, Verdú *et al*. employed the transplanted OB-OECs to treat the photochemical SCI in its acute phase (30 min after injury). Following 3 months of developing injury, the group receiving OECs showed only functional recovery with maintaining the spinal cord morphology compared to the group receiving cell-free medium.[22]

In the study by Verdú *et al*., the BBB scores at the end of the 1st week of the investigation in the control and cell transplantation groups were 11 and 16, respectively, while these scores were 1 in the control and 2 in the treatment groups in the present study. Presumably, this difference was due to the SCI severity, because the photochemical injury's severity used by Verdú *et al*. was reported milder than the contusional injury that occurred in the present study.

On the other hand, according to the study by Verdú *et al*., it seemed that the prolonged study duration for 3 months could result in more favorable outcomes because the SCI process could become stable after this period.[22]

Tharion *et al*. similarly used the transplanted OM-OECs in adult rats for contusional injury treatment and reported a significant difference in the BBB scores between the transplanted OECs and control groups up to 37 weeks following injury.[23]

In the present study, the highest BBB score was related to the cell transplantation groups, in which the animals were cared up to 8 weeks after the treatment.

In this line, Lu *et al*. used olfactory lamina propria (OLP) and respiratory lamina propria (RLP) to treat complete SCI in T10, 4 weeks after injury, and reported that the transplantation of OLP at this time had significantly increased the BBB scores.[12] In contrast, Steward *et al*. applied the OLP and RLP for the treatment of complete SCI in T104 weeks after injury and they did not report any significant differences, considering the BBB scores between groups at any time.[24] No significant difference was also found in the present study in terms of locomotor improvement between cell transplantation groups at different times, but a significant improvement was observed in motor function among these groups compared to the control group.

Richter *et al*. also used OM-OECs and OB-OECs to treat the SCI in the posterior funiculus scratch and then reported higher migration and mitotic potent of the OM-OECs. These cells also reduced the size of the cystic cavity and subsequently increased the number of neuronal redundancies and remyelination after the transplantation at the SCI sites.[14]

Furthermore, Mayeur *et al*. employed the transplanted OB-OECs and OM-OECs for the treatment of SCI and reported that the transplantation of both cell types had improved the growth of axons and subsequently reduced glial scar; nevertheless, the use of OM-OECs was preferable because of their easy access.[25] They also found that OM-OECs were able to survive for up to 60 days in the spinal cord.[25] Hence, the OM-OECs from 7-day-old rat pups were used in the present study.

Besides, according to the study conducted by Li and Lepski, it seemed that subacute phase was the best time for treatment and intervention because the glial scar had not yet been completely formed at this stage, unlike the chronic

phase. The problem of cell transplantation during the acute phase of the SCI was the presence of severe inflammatory reactions and cellular responses that could result in the loss of transplanted cells.[26]

In this respect, Nishimura *et al*. used neural stem/progenitor cells to treat the SCI in two acute and subacute phases and reported that cell transplantation in the subacute phase of the SCI had only induced motor function improvement in the rats.[27] In this study, cell transplantation in the subacute phase (9th day) to 42 days following transplantation led to functional and physiological improvements compared to those in the chronic phase (42 days). In the chronic phase of the injury, the limited distribution of the transplanted cells was observed around the injury sites, since the formed glial scar prevented the spread of the cells around the injury.[27]

López-Vales *et al*., using the OB-OECs for the treatment of SCI in T8 at 45 days after injury, also stated that delayed transplantation of OECs could not histologically reduce astrogliosis.[21] Histologically, the results of LFB staining in the present study showed that the transplantation of the OECs at all three times had resulted in the formation of myelin sheath at the SCI sites in the treated groups, and this was reported much more in the group receiving treatment immediately after injury than the other two-treated groups.

Conclusion

Based on the locomotor and histologic results of this study, it seemed that the immediate cell transplantation in the SCI was more appropriate than the subacute phase of the injury.

Acknowledgments

The authors, hereby, extend their gratitude to Ilam University of Medical Sciences for financially supporting this research project (Grant No. 45/931020). They also appreciate the Research Center for Medicinal Plants affiliated to Ilam University of Medical Sciences for its collaboration in this study.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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