Acute Transplantation of Human Olfactory Mucosa-Derived Olfactory Ensheathing Cells Fails to Improve Locomotor Recovery in Rats

Mina Afhami[1,3], Mansoureh Hashemi[2], Khashayar Afshari[1], Jamal Chenari[4], Seyed Mohammad Tavangar[5], Mahmoudreza Hadjighassem[1,6], Ahmad Reza Dehpour[1,7], Ahad Muhammadnejad[8], Arvin Haj-Mirzaian[7,9], Vafa Rahimi-Movaghar[1,10,11]

[1] *Brain and Spinal Cord Injury Research Center, Neuroscience Institute, Tehran University of Medical Sciences, Tehran, Iran*

[2] *Department of Neuroscience, Functional Neurosurgery Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran*

[3] *Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran*

[4] *Department of Anatomical Sciences, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran*

[5] *Department of Pathology, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran*

[6] *Department of Neuroscience, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran*

[7] *Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran*

[8] *Cancer Biology Research Center, Cancer Institute of Iran, Tehran University of Medical Sciences, Tehran, Iran*

[9] *Experimental Medicine Research Center, Tehran University of Medical Sciences, Tehran, Iran*

[10] *Sina Trauma and Surgery Research Center, Tehran University of Medical Sciences, Tehran, Iran*

[11] *Department of Neurosurgery, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran*

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Abstract- Olfactory ensheathing cells-based therapy for spinal cord injury (SCI) repair has been a possible treatment for clinical study because of their safety in autologous transplantation and potential regenerative capability. However, there are contradictory reports on the results after transplantation in animal models. The purpose of this research was to investigate the effect of acute transplantation of human mucosa-derived olfactory ensheathing cells (OECs) on the repair of the spinal cord. Human olfactory ensheathing cells were isolated from the human mucosa and cultured under supplemented neuronal cell culture medium. They were characterized by immunocytochemistry for olfactory ensheathing cell markers. We induced spinal cord injury at T8-T9 of rats by aneurysm clips and simultaneously injected two million OECs into subarachnoid space of spinal cord. Sensory and motor behaviors were recorded by tail-flick reflex (TFR) and BBB scores, respectively every week for seven weeks after injury. Morphology and S100-beta antigen expression in olfactory ensheathing cells of the human olfactory mucosa was confirmed by immunostaining**.** OECs transplantation did not recover inflammation, neuronal vacuolation, hemorrhage, and cyst formation. These findings suggest that OECs transplantation in this experimental setting did not lead to tissue regeneration to enhance locomotion. These results broaden current knowledge and are additions to the science and literature.

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Introduction

Spinal cord injury (SCI) is a neurological disorder characterized by demyelination and degeneration of spinal cord axons which may lead to paraplegia or quadriplegia. Paralyzed patients cannot control urination and defecation. Basic studies might speed the development of new therapeutic methods for patients suffering from SCIs. Cellular transplantation is one of important ways to regenerate and remyelinate spinal cord. Endogenous remyelination was restricted in human. However, transplantation of the Schwann cells has been shown to be able to play a role in repair of the demyelinated axons in animals and reestablish impulse conduction (1). Different studies have examined the therapeutic potential of various cells such as neural stem cells (2), embryonic stem cells (3), mesenchymal stem, cells (4) and Schwann cells (5) for regenerating spinal cord. Cell transplantation has limited potential according to safety and efficacy for clinical uses (6). Investigations

Corresponding Author: V. Rahimi-Movaghar
Sina Trauma and Surgery Research Center, Tehran University of Medical Sciences, Tehran, Iran
Tel: +98 21 66757010, Fax: +98 21 66757009, E-mail address: v_rahimi@sina.tums.ac.ir

have reported that transplantation of olfactory ensheathing cells seems to improve the function of patients with SCI (7). OECs are the glial-like cells in the olfactory mucosa and bulb that surround the axons of the olfactory neurons from the olfactory mucosa to olfactory bulb (8). These cells have unique properties such as secretion of different neurotrophic factors and adhesion molecules and long-distance migration (9). Moreover, they work similar to Schwann cells in regenerative and myelination processes in SCI. Suitable effects of OECs transplantation have been reported on axonal regeneration and neuronal viability after SCI (10). But, some studies have been reported opposite results (11). The exact mechanism of regeneration of OECs has not been understood yet. Au *et al*., 2007 indicated that OECs could secrete proteins which indirectly lead to neurite growth and recovery of spinal cord function (12). Meanwhile, OECs are easy accessible cells available in the human olfactory mucosa. The aim of the present study was to evaluate whether OECs isolated from the human olfactory mucosa could recover injured spinal cord in rats.

Materials and Methods

Isolation of olfactory ensheathing cells from human olfactory mucosa

This study was confirmed by the ethics committee of the Tehran University of Medical Sciences. Biopsy provided endoscopically from a patient under general anesthesia from superior turbinate of the human nose. The patient gave written consents before the operation. A biopsy was delivered into a sterile tube filled with HBSS (Gibco/Invitrogen) supplemented with 10-15% antibiotics (Antibiotic/Antimycotic). Tissue incubated with dispase II solution (2.4 IU/ml) for 1 hour at 37° C. Dissection of lamina propria from the epithelium was performed under an inverted light microscope. Lamina propria was divided into small pieces and incubated with collagenase IA for 20 min at 37° C. After being centrifuged, the resulting pellet cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with neuronal growth factor (NGF, 50 ng/ml), 1 % (100 U/ml) antibiotic/ antimycotic and incubated in 5%CO2 at 37° C for 3-4 weeks. The medium changed every five days. The phenotype of OECs was evaluated by the marker of S100 beta via immunocytochemistry.

Immunocytochemistry

OECs (density of 5×10^3 per well) were seeded in a 24 well culture plate. Cells fixed with 4% paraformaldehyde in PBS for 20 min. After washing with PBS, cells permeabilized with 0.2% Triton X-100 for 15 min and non-specific proteins blocked with 10% albumin bovine serum for 1 hour. Cells were incubated with primary antibody of rabbit anti-S100beta (1:100, Sigma-Aldrich, USA), overnight at 4° C. After washing three times, cells incubated with fluorescent secondary antibody of 1:500 Alexa Fluor 560 goat anti-rabbit (Invitrogen, USA) for two hours at room temperature (13).

Spinal cord compression

Ethical guidelines for experimental investigations in animal cares of Tehran University of Medical Sciences were followed. Twenty-one adult male Wistar rats (7 rats per group) weighing 180-250 g were randomly divided into three groups of SCI: Control that was injured without further treatment; injured and received medium (DMEM); injured and received the OECs immediately. To induce compression injury, rats were intraperitoneally (i.p.) injected anesthetized drugs of ketamine (100 mg/kg) and xylazine (10 mg/kg). Laminectomy was performed at T8 or T9 vertebrate. The spinal cord was compressed for 3 seconds by aneurysm clip which induced the closing force 119 g. In the cell transplanted group, OECs was delivered immediately after injury into subarachnoid space of lesion epicenter in a volume of (2 million cells/10 µl) at a rate of 1 µl/minute.

DMEM medium was injected with the same procedure into the spinal cord. To suppress immune reactions in cell transplanted rats, cyclosporine a (15 mg/kg) was subcutaneously injected from the day before the injury to the end of the experiment only in OEC-injected group. All animals received an antibiotic mixture containing Gentamycin (1mg/kg) and Cefazolin (75 mg/kg) i.p. for one week to reduce the risk of infection. Normal saline (10 cc/12h) was subcutaneously injected for three to seven days after surgery. The bladder was expressed manually two to three times daily throughout the study period. The picric acid solution was carefully applied to the abdomen, hind limbs and tails of all rats to prevent autophagia. Tail-flick reflex (TFR) and locomotor function were evaluated weekly for seven weeks to check neurological signs. After the end of the experiment, spinal cords were removed and were processed histologically.

Neurological assessments

TFR and locomotor function were carried out every

week for seven weeks to evaluate the neurological status of animals after surgery. TFR was monitored by pinching the rats' tail. Withdrawal reflex was counted as a positive or negative response (14).

Locomotor activity was measured by using Basso, Beattie, and Bresnahan (BBB) scale every week until the end of the experiment. We considered BBB score of zero as inclusion criteria. Hind limbs movements were assessed during 4 minutes in the open field area and were scored from 0 (without noticeable locomotion) to 21 (normal movements) by two blinded observers. In this scoring system, individual joint movement, sweeping, weight support, plantar stepping, limb coordination and gait coordination were scored and an average of right and left hind limb scores was expressed as a final score for each animal (15,16).

Histology procedure

After the behavioral assessments, all animals were anesthetized by i.p. injection of Ketamine (100 mg/kg) and xylazine (10 mg/kg) and perfused transcardially with 150 ml of phosphate buffer saline (pH 7.4), followed by 250 ml of 4% paraformaldehyde (PFA). The spinal cord was dissected, post-fixed in 4% paraformaldehyde for 72 hours in PFA solution.

Spinal cord sections were stained with hematoxylin and eosin (H and E) to study pathological changes. Three sections from each animal were initially deparaffinized in 70º C temperature for 20 min and xylene solution for 3 min, then rehydrated (2 min) and finally rinsed in tap water. The slides were rinsed with hematoxylin solution (Sigma) for 15 min to stain nuclei. After rinsing in tap water, sections were stained in eosin for 2 min followed by rinsing in 90%, 96%, and 100% ethanol for 2 minutes. Then, sections immerse in xylene and mounting medium (Merck). Sections were scored for quantitative analysis by two blinded pathologists. The scores were expressed from 0 to 3 based on four factors including inflammation, hemorrhage, axonal vacuolation, and cyst formation (17).

Immunohistochemistry

Immunostaining for S100-beta protein was performed on the adjacent sections used for H and E for each spinal cord. After antigen retrieval by boiling the sections in citrate buffer (pH 6) for 5-7 minutes using the microwave in 110º C, endogenous peroxidases were quenched with 3% hydrogen peroxide (H_2O_2) in absolute methanol. Then, slides dipped in Tris-buffered saline washing buffer for 5 minutes. Sections were incubated with primary antibody S100-beta (mouse monoclonal, ready-to-use dilution, Biocare PM089 AA, H) for 30 min and P75 (RA0249-C.5, SYTEC, 1:200) for 50 minutes at room temperature, followed by three times rinse in wash buffer. Then, sections were incubated with anti-mouse and anti-rabbit secondary antibodies for 40 minutes at room temperature (DAKO, KS, 2007). The staining was developed using diaminobenzidine (DAB) as a chromogen, then counterstained with hematoxylin (18).

Statistical analysis

BBB scores were compared using a mixed model repeated measures ANOVA followed by Tukey's post hoc test. Histological analysis was performed by nonparametric Kruskal-Wallis test followed by Mann-Whitney test for between-groups comparisons with Bonferroni correction. All data are presented as mean±standard error of the mean. $P<0.05$ were considered to be the statistically significant difference.

Results

Morphology and phenotypic characteristics of human olfactory mucosa OECs

Cell survival, movement and morphology were evaluated before injection. The morphology of cells was spindle-shaped and bipolar with long processes and small cytoplasm and multipolar form with short processes and plentiful cytoplasm such as stellate morphology (Figure 1a). Immunocytochemistry of S100-beta antigen was confirmed by using fluorescence (Figure 1b).

Behavior assessment

Functional recovery was observed in all rats and assessed with the BBB functional test every week. We demonstrated different trends, but there were not significant alterations among injured group and groups receiving OEC and DMEM. Although in the first week, motor recovery was slightly greater in animals receiving OECs in comparison with control animals, in the following weeks, locomotor recovery slightly reduced in OECs treated group compared to the other groups, (Figure 2). TFR was positive in all experimental groups during the study.

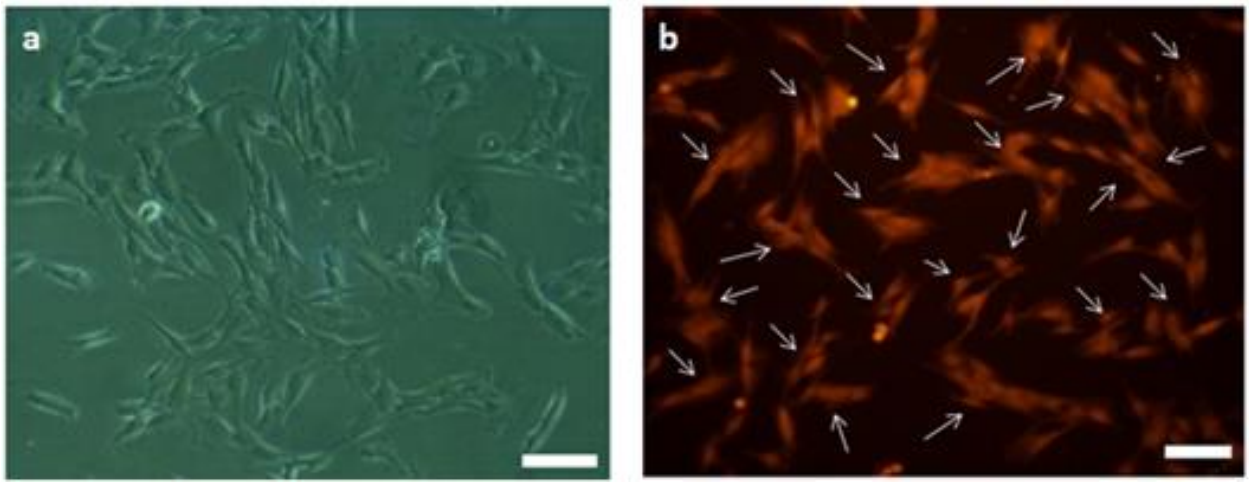

Figure 1. Morphology and S100-beta antigen expression in olfactory ensheathing cell (OEC) of the human olfactory mucosa. (a), Observation of isolated OECs of human olfactory mucosa tissue under phase contrast microscopy. OECs observed in two forms: long fusiform bipolar and astrocyte-like morphology. (b) Cells were expressed S100-beta antigen in OECs (Scale bar: 100 µm)

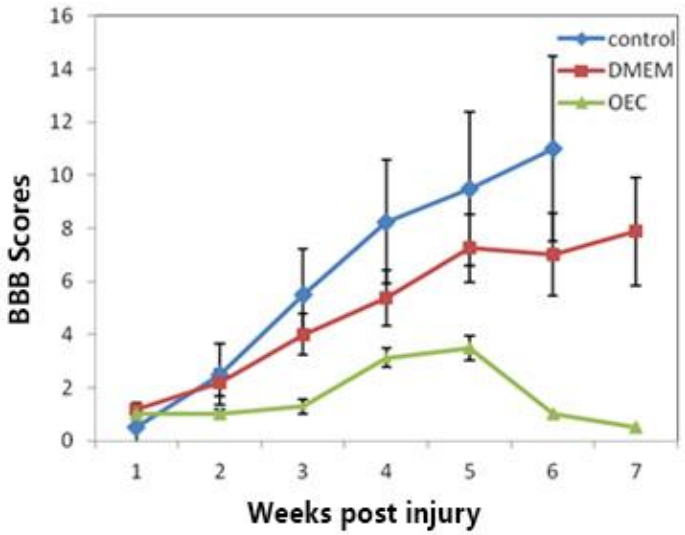

Figure 2. Assessment of locomotor recovery by BBB locomotor rating scale. The significant improvement was not visible between groups after Olfactory Ensheathing Cells (OECs) transplants. The values are presented as mean±SEM

Histological analysis and immunohistochemistry

Longitudinal and horizontal sections were examined by H and E staining to study pathological changes at the lesion site and surrounding area. The presence of inflammatory cells, hemorrhage, tissue degeneration and the extent of cyst formation were quantified. There was no significant difference between groups regarding pathological factors (Figure 3a). Tissue degeneration and cyst formation were evident in all groups in longitudinal sections (Figure 3b).

The spinal cord sections were not immunoreactive for P75, and S100 staining and no positive labeling was evident in the stained groups (Figure 3 c,d).

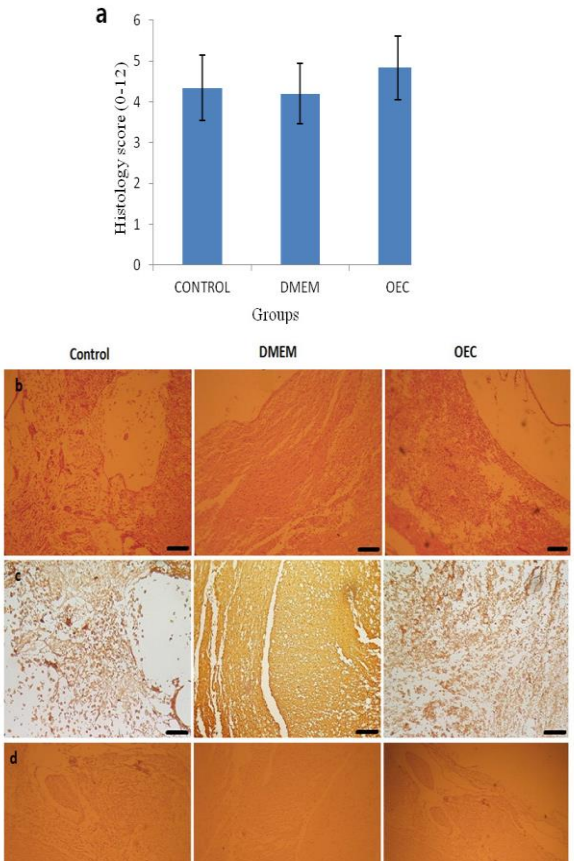

Figure 3. Quantitative analysis of pathological features of spinal cord longitudinal sections stained with H and E and immunostaining of S100-beta and P75 proteins. Cumulative scores of 0-12 were assigned to each group. Spinal cord injury (SCI) initiated pathological pathways with immediate hemorrhage followed by inflammatory cell invasion, neuronal vacuolation, and cyst formation. Olfactory Ensheathing Cells (OECs) transplantation did not diminish hemorrhage, inflammation and neuronal vacuolation and extent of the cyst (a) Representative images of three groups were shown (b). No positive staining was observed in experimental groups for S100-beta (c), and P75 (d) staining observed using DAB substrate. The data represented as Mean±SEM. (Scale bar: 100 µm)

Discussion

We assessed the potential use of acute transplantation of the human olfactory mucosa-derived OECs for locomotor function and histological alteration after SCI. We found that acute transplantation of human OEC into subarachnoid space of injured rats did not lead to the recovery of the locomotor function and pathological improvement. We injected cells into subarachnoid space to prevent more tissue damage in case of direct injection into lesion site. Moreover, in acute phase, spinal cord is filled with inflammatory components and cavity formation has not yet progressed to be used for injection (19). Our findings were consistent with our previous study representing no locomotor recovery after OEC transplantation into this model of SCI which used fifty thousand Schwann cells or olfactory ensheathing cells (18). Similar findings showed no locomotor recovery after transplantation of OEC transplant alone in SCI rats (20). OECs transplantation for repair of degenerated nervous system has inspired scientists for cell therapy-based treatments due to their safe and effective transplant in a clinical trial. There are few reports about the reparative capacity of OECs. Fouladi *et al.*, in 2003 found that delayed transplant of OECs might induce functional improvements after corticospinal tract lesion in rats (21). Another study showed that cells from olfactory system restored the function of sensory pathways (22). It has been reported olfactory ensheathing glia (OEG) regenerated motor axons and led to ameliorated functional deficits (23) and promoted functional recovery (24). However, findings of the current study did not support the previous reports on the regenerative potential of OECs. The conflicting results might be due to the following reasons: BBB evaluation has to be

performed soon after bladder expression. If the time passes, reflexive spastic motion of lower limbs might be conflicted with real movements. In our study, the low BBB score in cell grafted group and to an extent in DMEM group might be due to iatrogenic damage during the surgical procedure. We considered the following risk factors: Cell injection by insulin needle which is thicker than Hamilton syringe; addition of intolerable volume on spinal cord produced by 10 µl cell volume injected in acute phase; and finally, enhanced inflammatory responses after human OECs transplantation.

In our study, SCI initiated histopathological responses such as inflammation, hemorrhage, cyst formation and neuronal vacuolation. OECs transplants did not diminish accumulated inflammatory cells, hemorrhage, and neuronal swelling and progression of cyst formation. This finding also is in line with the studies reported no axonal regeneration and reparative properties after grafting OEC (25) and no axonal sparing after 12 weeks (26). In contrast to our current results, the earlier evidence demonstrated that delayed engraft of OECs promoted tissue sparing and regenerated axons (27,28). OEC graft provided a better environment for axonal growth represented by functional and histological outcomes (28).

In this study, we conducted a moderate force clip compression with curved aneurysm clip which induces equal force in dorsal and ventral surfaces and examined acute transplantation of the mucosa-originated OECs which are more feasible for human studies. Lack of functional recovery after OECs transplant may indicate that there would not be axon and myelin regeneration to make connections in lesion site or activation of the intraspinal network (29). Our injection method and death of neurons during injection might be limiting factors preventing functional improvement. In addition, duration of functional recovery measurement might not be long enough to reveal changes in locomotor function. Unlike the study showed OEC transplantation modulate inflammatory response (24), our histological outcomes did not represent marked decrease in inflammatory cell infiltration, blood cell, and axonal vacuolation. Promoting effects of OECs in tissue regeneration and functional recovery may be caused by their capabilities in myelin regeneration and release of growth factors such as NGF and BDNF and cell adhesion molecules laminin which contribute to tissue regeneration and functional recovery (30-32). Immunostaining against P-75 and S100-beta proteins showed no positive stained cells in lesion center and epicenter, indicating that OECs could not survive long enough in a hostile environment after injury in the acute phase and therefore they were not detectable. OECs are sensitive to oxidative stress and cytotoxic elements after injury (26).

This might contribute to the death of cells in case of low cell density. Laboratory-dependent factors such as the origin of OECs from olfactory system, culture media and essential components for preservation and purification of cells, duration of cell maintenance can affect the study results. Furthermore, type of inducible SCI models, route and place of cell injection, the density of delivered cells and acute or delayed injection influence on the obtained results. Our results failed to demonstrate mechanism underlying functional and histological changes. We did not detect axon and myelin regeneration. Reliable evaluation of OEC survival and their migration to discriminate them from Schwann cells requires long-standing labeling of cells before injection which was limiting factor in our study.

Further studies need to be performed to cover these limitations and use a suitable molecular marker to characterize cells. It should focus on the efficacy of OECs transplantation on functional recovery in a long-term study. Our study raised the need to further investigate and replicate studies to see the possible effects of OECs transplantation following SCI.

Taken together, injection of OECs in the acute phase is not a safe time period according to cell survival and functional recovery.

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