Subarachnoid Space Transplantation of Schwann and/or Olfactory Ensheathing Cells Following Severe Spinal Cord Injury Fails to Improve Locomotor Recovery in Rats

Mohsen Nategh¹, Masoumeh Firouzi¹, Mehdi Naji-Tehrani¹, Leila Oryadi Zanjani¹, Zahra Hassannejad¹,², Mohammad Hosein Nabian¹, Shayan Abdollah Zadegan¹,², Mehrbod Karimi³, and Vafa Rahimi-Movaghar²,⁴

¹ Tissue Repair Lab, Institute of Biochemistry and Biophysics (IBB), University of Tehran, Tehran, Iran
² Sina Trauma and Surgery Research Center, Tehran University of Medical Sciences, Tehran, Iran
³ Pathology Lab, Atie Hospital, Tehran, Iran
⁴ Brain and Spinal Injuries Research Center (BASIR), Tehran University of Medical Sciences, Tehran, Iran

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Abstract - Treatment of spinal cord injury by exogenous cells has brought both successful and unsuccessful results. Olfactory ensheathing cells and Schwann cells have been widely used for transplantation purposes. In this study, we investigated the effects of these cells on contused spinal cord by introducing cells into subarachnoid space. Fifty thousand Schwann cells or olfactory ensheathing cells or a mixture of both cell types were transplanted one week after a 3-second clip compression injury at T-9 spinal cord level in rats. Starting from the day one of spinal cord injury, animals were assessed for six months by BBB test and then were sacrificed for immunohistochemistry labeling of the spinal cord injury site. There was no locomotor recovery in any of the treatment groups including controls. Immunohistochemistry assessment indicated positive labeling of P75 and S100 markers in the cell-transplanted groups compared with control. Our data suggest that transplantation of Schwann cells and/or olfactory ensheathing cells into the subarachnoid space does not improve motor recovery in severely injured spinal cord, at least with the number of cells transplanted here. This, however, should not be regarded as an essentially negative outcome, and further studies which consider higher densities of cells are required.

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Introduction

Transplantation of Schwann cell and Olfactory Ensheathing Cell (OEC) has shown promising results in animal models of spinal cord injury (1-3). These cells have functions such as secretion of growth factors, axon guidance, myelination and structural support (4). Previous studies suggest that these two cell types behave differently in vitro or in vivo when transplanted into the demyelinated CNS (5). Transplantation of Schwann cell in injured spinal cord has yielded beneficial results in the functional and structural recovery of spinal cord lesions (6-10). Other studies provide evidence that transplantation of OECs improves motor function either in contusion (11,12) or transection models (13,14). OEC also improves functional properties of lesioned dorsal column in an animal model of spinal cord injury (15). However, these cells are not always beneficial for regeneration purposes (5,16-19). Despite contradicting results in the use of Schwann cell and OEC in experimental spinal cord injury models, they have been amongst best candidates for the purpose of cell therapy (20).

The idea of a combination of Schwann cell and OEC for spinal cord injury transplantation is pertinent to their specifications. For example, OECs have a better ability for migration; and when co-cultured with astrocytes in vitro, do not disintegrate from them, as is the case for Schwann cells (5). Schwann cells, on the other hand, have been shown superior in promoting axonal growth and myelination (4). There are a few studies which used
OEC and Schwann cell in combination (8,21,22). It has been reported that OEC exerts beneficial effects on Schwann cells when transplanted in combination (23).

We have previously shown that transplantation of Schwann cell through subarachnoid route improves motor performance in moderate spinal cord contusion in rats (24). Yet our human study has not yielded such improvement in transplanted cases (25). In order to consider very severe injuries of spinal cord compared to our previous study (24), we investigated subarachnoid transplantation of OEC and Schwann cell, alone or in combination, in contused spinal cord of rat; and evaluated behavioral recovery in long-term.

Materials and Methods

Study design
A total number of sixty female inbred Wistar rats weighing 180-200 g were randomly divided into seven groups of Control (n=8), sham-operated (n=4), DMEM (n=8), OEC (n=10), Schwann (n=10), Mixed (OEC+Schwann, n=10) and fibroblast (n=10) based on injected suspension. All procedures were carried out according to the guidelines of ethics committee of Tehran University of Medical Sciences. Behavioral recovery of animals was assessed by BBB locomotor rating scale for six months (26). Animals were then sacrificed and prepared for histology and immunohistochemistry assessments.

Spinal cord injury
Aseptic surgical procedures were performed under anesthesia induced by a mixture of Ketamine (100 mg/kg, ip) and Xylazine (10 mg/kg, ip). Following anesthesia, assessed by corneal reflex, an incision was made through skin spanning T4-T12 thoracic vertebra. Laminectomy was performed at the T9-T10 level under loupe magnification. A curved aneurysm clip with closing force of 1.23 N was used to compress the cord for three seconds, with lower and upper blades of the clip compressing ventral and dorsal surfaces of the cord. Flanking muscles and skin were then closed with 4-0 chromic catgut and silk sutures respectively. Normal saline solution was injected after the surgery (5 ml, ip) and animals were transferred to recovery cages. Aspirin was dissolved in drinking water (100 mg/L) and supplied for three days as an analgesic. Special care included twice-a-day bladder expression and subcutaneous injection of Cefazolin (150 mg/kg) and Gentamicin (5 mg/kg) for up to two months in cases where animals were still incapable of urination or when urinary tract infection was observed.

Cell preparation
OEC and Schwann cell cultures were prepared from the olfactory bulb and sciatic nerve of neonatal rats inbred with recipient animals, respectively. Schwann cells were purified by a protocol described previously with some modifications (24,27). Briefly, sciatic nerves were extracted from 3- to 5-day-old rats; the epineurium was removed, and the nerve was split into several explants where subsequently placed on poly-L-lysine coated plates filled with culture media (DMEM+10% FCS). In order to reduce fibroblast contamination, explants were subcultured 3 to 4 times during four weeks. The cold jet technique was engaged to increase the purity of the Schwann cells. In this way, our cultures had a purity of about 95% determined by labeling with the anti-S100 antibody (28). Since fibroblast contamination always exists, we added a seventh group to evaluate the effect of pure fibroblast transplantation. Purification of OEC was according to the method of Nash et al., (29). Briefly olfactory bulbs from neonates were extracted, and the nerve fiber layer was dissected away and trypsinized. Cell suspension incubated for 18 hours, and then the supernatant was transferred to a second plate which incubated for 36 hours. The remaining supernatant was transferred into poly-L-lysine coated plates and maintained until cells reached a confluency of 70-80%.

Cell transplantation
One week after spinal cord injury, injury site was re-exposed for cell transplantation. Scar tissue was detached, and care was taken not to damage the exposed dura mater. The suspension for injection of either OEC or Schwann cells contained 5×10^4 cells of each in 5 μL of DMEM, and for the mixed suspension, a half concentration of each was prepared. Fibroblasts, with a similar concentration, were also prepared. Cell suspensions were injected into the subarachnoid space within 10 seconds using a Hamilton syringe with a fine 30-gauge needle bent in the middle to minimize unwanted tissue damage. The needle tip was inserted into the dura mater 3-4 mm caudal from the injury epicenter in order to compensate its bent portion distance. After injection, the needle was kept still for 10 seconds to minimize leakage of the suspension and then carefully withdrawn.

Behavioral assessment
Assessment of hindlimb function using Basso,
Beattie, Bresnahan (BBB) locomotor rating scale was performed every week for nine weeks and afterward, twice or once a month until 160 days post-injury, by two examiners blinded to the animal treatment (26,27). For this purpose, animals were placed in a round plastic container with a diameter and wall of 85 and 30 centimeters, respectively.

**Immunohistochemistry**

At the end of the study, all surviving animals were transcardially perfused with 4% paraformaldehyde. Two-centimeter segments of spinal cord containing the center of the lesion in the middle were then collected, embedded in paraffin and sectioned (3 µm). After rehydration, the sections underwent antigen retrieval: slides were placed in a humidified chamber and rinsed with a solution of hydrogen peroxide with methanol (1:9) for 20 minutes. Following washing with distilled water, slides were incubated in Tris solution (pH=6) at 120°C for 15 minutes. Blocking was performed by normal donkey serum (1:10) for 30 minutes. Anti-P75 (rabbit polyclonal, 1:500; Sigma) and anti-S100 (S2644; rabbit polyclonal, 1:500; Sigma) antibodies were used for 1 hour at room temperature. Sections were then washed in Tris/Tween 20 solution for 10 minutes. Then HRP-conjugated secondary antibody was used to incubate the sections for 30 minutes at room temperature. After another wash with Tris/Tween 20, diaminobenzidine tetrahydrochloride (DAB) was administered as the chromogen for up to 10 minutes. Finally, all specimens were counterstained with Hematoxylin. Slides were then dehydrated, mounted, and observed under light microscope. In a separate staining, every four sections were stained with Hematoxylin and Eosin (H and E) and observed by a pathologist for gliosis and inflammation.

**Statistical analysis**

Statistical comparisons of BBB scores between experimental groups were performed using two-way analysis of variances (ANOVA). A $P < 0.05$ was considered significant in this study.

**Results**

Eight animals died during the study, and their records were not included in the analysis of data. Prior to the injury, all animals were scored 21 according to the BBB scale. One day after injury BBB was evaluated, and seven animals which scored 1 or more in the scale were excluded from the study to assure that all of the animals sustain an intensive injury. Other animals, fulfilling injury criteria, were assigned into experimental groups. Assessment of BBB score started at one week post-injury and carried out for 21 weeks, revealed no significant difference between the treatment and control groups during this time (Figure 1).

![Figure 1. Mean of BBB scores (±SEM) of the all experimental groups. Animals were assessed one week prior to the injury, and all scored 21, which is considered normal locomotion. Except the higher score of fibroblast group at the third week (*$P<0.05$), there is no significant difference between the experimental groups during the study.](image-url)

The only exception was the fibroblast group, which showed a slight difference at the third week compared to control, DMEM, OEC, and Schwann cell/OEC groups, but not Schwann cell group. However, it’s BBB score declined at the fourth week and in following evaluations had no difference with any of the groups. Sham-operated...
animals showed no functional impairment one week after injury and thereafter. When analyzed for the time factor in each single group, BBB score showed literally no improvement even 21 weeks after the injury.

In H and E stained sections, fibrosis and gliosis were evident at the injury site in all groups; macrophages were rarely detected in some sections (data not shown). Labeling of the spinal cord with antibodies against P75 or S100 reveals positive staining in white matter areas of injury site in OEC, Schwann, and mixed groups, but not in the control and DMEM groups (Figure 2).

![Figure 2](image)

**Figure 2.** Injury site of spinal cord showing a portion of the white matter. Immunohistochemistry against P75 and S100 was visualized by the enzymatic HRP-tagged secondary antibody. Arrows show cells which stained positively for either P75 (B and C) or S100 (D). A: Anti-P75 was used in an animal from the control group. No positive staining is evident. B: Anti-P75 positively stains a cell in an animal which received OEC. C and D: Positive staining of both anti-P75 (C) and anti-S100 (D) in an animal which received Schwann cell. Scale bar: 10 µm.

**Discussion**

In this study, we found that transplantation of Schwann cell and/or OEC through the subarachnoid space did not improve motor function in rats with severe spinal cord injury. The only improvement in motor performance was seen in the fibroblast group at the third-week post-injury, which followed by an unexpected decline. Further studies are needed to clearly address the role of exogenous fibroblasts transplanted into the injured cord. Although motor recovery is the ideal goal of most transplantation studies, sensory improvement is also important which was not assessed in this study.

In several studies of Schwann cell or OEC transplantation, behavioral recovery is reported in treatment groups (8,12,31-34). On the other hand, it has been reported that transplanted Schwann cells fail to migrate and myelinate areas of demyelinated axons in CNS (16-19,35). Similarly, failure of OEC for improvement of hindlimb function and axon regeneration (29,36-39), and even unwanted hyperalgesic effects have been reported (40). A recent human study has reported improvement in three patients, which suggests that autologous OEC transplantation can be beneficial (41). In most transplantation studies which report improvement in motor function, the weight drop model of spinal cord injury were used, whereas we used severe clip compression which could be considered complete contusion (42). We have previously shown beneficial results in transplantation of Schwann cell in moderate spinal cord contusion by clip compression in rats (24).

The most important difference between our previous study and the current is the exact contusion model of the spinal cord. Here, the cord was compressed with a curved aneurysm clip, with the blades totally spanning and pressing dorsal and ventral surfaces, while in our previous study the clip was straight, and the blades compressed lateral surfaces. Hence, in our previous study, there was a possibility that the most ventral parts of the spinal cord remained intact; a possibility that was controlled in the current study. Another point of dissimilarity is the 0.07-N higher closing force than the previously-used straight clip. These two factors, position and the closing force of the clip, seems to be responsible for the greater injury seen in this study. Indeed, in the current study, the BBB scores of the control group are less than half of those of our previous study. There are factors that potentially affect the outcome of transplantation which vary from cell preparation and engraftment to routes of transplantation. The source of OECs was the olfactory bulb in this study, which is suggested not to be different from those of olfactory mucosa (43). Survival of transplanted cells is another determining factor. For example in the case of Schwann cell, it has been reported that most cell death occurs in the first 24 hours; but even seven days after transplantation, necrosis and apoptosis is observed (44). It has been documented that density of cell suspension is an
important factor in the outcome of transplantation in that high concentration of cells- 50,000 to 100,000 /µl-yields better results (45). Thus, a cell population of 50,000 in 5 µL in our study is relatively a sparse transplantation; however, it was effective in our previous study. Bladder expression and its timing before behavioral assessment have been implicated as a potential source of varied results (38). In neither the previous nor the current study, we performed bladder expression prior to BBB scoring.

Part of the recovery from spinal cord injuries is because of the plasticity in both spinal cord and higher motor centers (46). It has been suggested that a greater injury leaves less spared tissue. When less than 15 percent of the fibers are left, plasticity cannot take place in higher centers rostral to the site of injury (47). Compared to our current intensive injury, the milder injury in our previous study would, therefore, leave more spared tissue, raising the possibility for greater plasticity with the resultant tangible recovery.

Immunohistochemical labeling against P75 and S100 reveals positive labeling in animals received Schwann cell, OEC or both. Although Schwann cell and OEC express S100 and P75, these markers are not specific for them and thus positively labeled cells cannot be exclusively attributed to transplanted Schwann cells and/or OECs. It has been shown that P75 expression increases in oligodendrocytes following spinal cord injury (48); and on the other hand, gliosis is the well-known proliferation of astrocytes which express S100 (49). Nonetheless, as shown in figure 1, sparse positive staining of P75 and S100 in the white matter of injury center in cell-treated groups, suggests integration of transplanted cells into the injury site. Nevertheless, even this sparse labeling is absent in control group. Current methods do not specifically purify OECs from its mixture with Schwann cells (8,22,50,51). This is especially important when OECs are extracted from lamina propria or olfactory nerve layer, which may raise Schwann contamination (51,52). We purified OEC from the olfactory bulb to minimize Schwann contamination.

The timing of cell transplantation is also an important issue and acute transplantation usually gives better results (53,54). In the present study, transplantation was sub-acute and was done one week after injury. The first few hours following injury are extremely critical and after this time the spinal cord undergoes secondary injury cascades (55) and is difficult to repair by exogenous cell transplantation. Co-transplantation studies in acute phase have shown promising results (34,56).

This study suggests that after severe compression of the spinal cord, subarachnoid transplantation of Schwann cell and/or OEC does not improve motor performance. In other words, when the injury is severe, transplanted cells fail to exert functional motor recovery. However, it should not be concluded that OEC or Schwann cell are incapable of supporting regeneration and recovery under other models of spinal cord injury and transplantation, or higher concentration of cells. The injured spinal cord is one of the most hostile milieus for transplanted cells to survive. Regarding the low density of cells in injection bolus, higher densities should be applied especially because of the potential dispersion of cells in subarachnoid space. Due to the paucity of cell therapy studies through this route of transplantation, a definite conclusion cannot be made based on studies to date and further research is suggested in this field.

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