

Remyelination of the Corpus Callosum by Olfactory Ensheathing Cell in an Experimental Model of Multiple Sclerosis

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Abstract- Multiple Sclerosis (MS) causes loss of the myelin sheath, which leads to loss of neurons. Regeneration of myelin sheath stimulates axon regeneration and neurons' survival. In this study, olfactory ensheathing cell (OEC) transplantation is investigated to restore myelin sheath in an experimental model of MS in male mice. OECs were isolated from the olfactory mucosa of seven-day-old infant rats and cultured. Then, cells were evaluated and approved by flow cytometry by p75 and GFAP markers. A total of 32 mice (C57BL/6) were studied in four groups; 1) without any treatment (control), 2) Sham (receiving PBS), 3) MS model and 4) MS and OEC transplantation. MS was induced by adding Cuprizone in the diet of animals for six weeks. After the expiration of 20 days, histologic analysis was performed with approval of the presence of cells in the graft area and the removal of myelin and myelin regeneration with two types of luxal fast blue (LFB) staining and immunohistochemistry. The purity of the cells ensheathing the olfactory was 90%. There was a significant difference in Myelin percentage of PBS and OEC recipient groups ($P \leq 0.05$). MBP and PLP of the myelin sheath in the group receiving OECs were more than MS group. According to the findings, in MS model MBP and PLP of the myelin sheath is reduced. In the group receiving OECs, it was returned to a normal level significantly compared to the sham group received only PBS significant differences were observed. The OECs transplantation can improve myelin restoration.

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Keywords: Olfactory ensheathing Cell; Remyelination; Demyelination

Introduction

Multiple sclerosis (MS) is an autoimmune disease that effects on the central nervous system. This disease is characterized by three characteristics including inflammation, demyelination and scarring (1), and leads to sensory disorders, weakness, muscle cramps, visual disturbances, cognitive impairment, fatigue, tremor of limbs, disorders in passing urine and stool, sexual dysfunction, balance, amnesia, loss of hearing, numbness, blurred vision, double vision, speech disorder in some patients (2).

Demyelination models in experimental animals are the best way to explore to find solutions for myelin restoration. Among the demyelination toxic models, EB ethidium bromide and cuprizone models can change the mitochondrial DNA, leading to abnormal breathing. This disorder can cause demyelination and necrosis (3,4).

Demyelination means the destruction of myelin proteins placed around the axons of neurons in sheathing form. In the central nervous system, demyelination occurs by the direct attack to oligodendrocytes. On the other hand, the remyelination is a process in which the demyelinated axon is repaired. However, in remyelination, thinner and shorter myelin sheaths are produced; it can compensate mainly functional disorders. This process is the natural response of the body to the remyelination, but is disrupted in patient with MS. Therefore, axons remain in the demyelinated form in MS patients and they are susceptible to damage which can lead to neurological impairment. Although the reasons for the failure of remyelination in patients with MS are still uncertain, progresses in relation to understanding remyelination process was achieved (1).

During the past few years, researchers have explored the possibility of cell transplantation with capacity of

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migration, differentiation and ability regenerate myelin sheaths safely in target areas without any tend to become a tumoral form (4).

Recently, the researchers have focused on olfactory ensheathing cells (OECs) transplantation, obtained from the olfactory mucosa, to treat the MS disease. OECs were injected into rats with damaged in the spinal cord as a treatment solution. These cells caused remyelination and reduction of scar components. Thus, with the reconstruction of the spine, the improvement of sensation and movement are increased. In these cases, OECs created an environment for axon growth (2). OECs are easily accessible and potentially used from adult cells of autologous population with good characteristics for remyelination (5).

OECs are the type of glial cells, that show properties of astrocytes and Schwann cells. In this study, OEC cell transplantation was performed for the reconstruction of lost myelin areas after creating the demyelination. The evidence is based on histological analysis of transplanted cells remains in the lesion area and causes myelin repair (6).

Materials and Methods

Cell culture OEC

Nash *et al.*, method (7) with modifications was used

for cell extraction and cell culture (8). For cell culture and isolation of OEC, seven-day-old rat infants were used. All animals were anesthetized by ketamine (75 mg /kg) and xylazine (10 mg /kg) intraperitoneally, and then heads were separated. The olfactory mucosa was separated from two sides of Vomer bone and after washing twice with 10 cc PBS containing antibiotics; lamina propria was separated with insulin needle and divided into smaller parts. Then several pipettes were poured into 15 falcon and 1cc trypsin (1%) was added. It was incubated for 5 hours at 37° C and 5% CO₂. Then it was centrifuged at 1200 RPM for 10 minutes to separate the cells from the tissues. The collected cells were placed in the bottom of the Falcon. The cell suspension was poured into the non-coat flask and it incubated for 18 hours at 37°C and % 5 CO₂. During these times, the majority of fibroblasts were attached to the floor of the flasks. The supernatant was transferred to another uncovered flask, and incubated for 36 again. During this time, astrocytes were attached to the floor but the OECs were located in supernatant fluid. Then the supernatant fluid was transferred to the flask coated with poly-L-lysine and OEC cells attached to the floor of the plate for 48 hours (Figure 1:a). Flowcytometry was performed for confirmation OECs by using P75 and GFAP markers. According to the results, the purity of the cells was approximately 90% (8) (Figure 1:c).

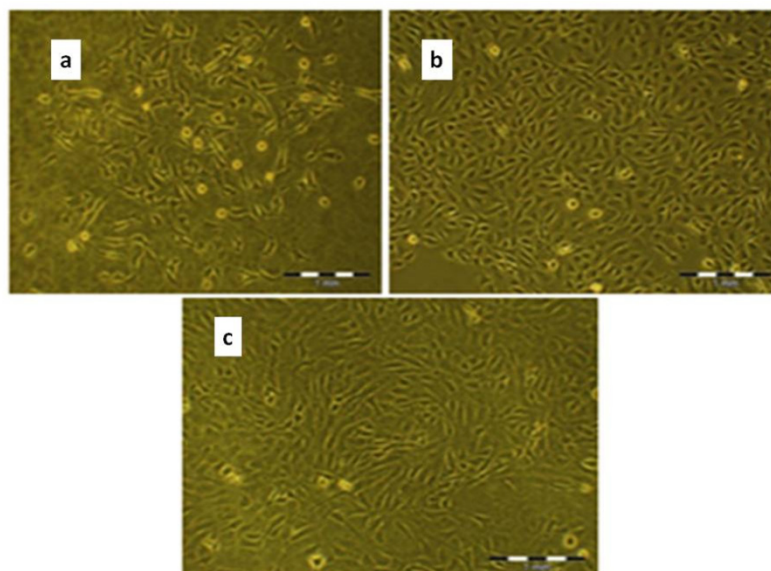


Figure 1. a: Adherent cells 18 h after cell suspension culture. b: Cell culture supernatants after 48 h of culture Flasks coated with poly-L-lysine transport c: Cells cultured in DMEM / F12 medium

Tracing by DIL

When the cells completely covered the floor of dish, they were separated by trypsin 0.25%. Then centrifuge

was done and 5µg/ml DIL solution was added to the pellets (9) and incubated for 5 min at 37°c temperature. Then the cells were prepared for transplantation.

Induction of MS model

A total of 32 mice (C57BL /6) were studied in 4 groups; 1) without any treatment (control), 2) Sham (receiving PBS), 3) MS model and 4) treatment group: MS and OECs transplantation. MS model was induced by toxic substance Cuprizone, mixed (0.2%) with ordinary animal diet for six weeks (9). Feature of this approach is that it causes demyelination of the corpus callosum and upper cerebellum peduncle (Figure3b) (10)

Cell transplantation

About 0.5 μ L of OEC suspension contains one hundred thousand cells injected by Stereotaxic method and intraparenchymal technique into corpus callosum of mice. In this method, after six weeks, animals were anesthetized by IP injection of ketamine (ketamine hydrochloride) and xylazine, then placed on the stereotaxic apparatus, and fixed. After opening the skull and identification of Lambda and bregma, (11) the injection site was determined. Then OECs were injected by a Hamilton syringe into the corpus callosum. PBS was injected to six other mice (12).

Histological evaluation of the brain

After 20 days of cells injection, brain fixation was performed. At first, animals were anesthetized with ketamine and xylazine and then perfused with paraformaldehyde 4% (Sigma, USA) soluble in PBS 0.1 M and glutaraldehyde. Then, the mice brains were removed and placed in the same fixative for one night. After that, it was placed in 10% sucrose soluble for other one night and it was ready for tissue preparation. Serial transverse sections were performed by microtome and with 7 μ m thickness of each slice was micrometers (13).

Immunocytochemistry

Incubated cells were fixed in 4% paraformaldehyde (Sigma, USA). Fixed cells were washed with PBS and permeabilized with 0.1% Triton X-100 (Sigma, United States of America) in PBS, in blocking buffer (PBS, 1 mg/ml BSA (Sigma, USA) and goat serum 10% (Gibco, USA) and followed was placed by incubation with primary antibodies one night at 4°C. Then cells were washed with PBS and incubated with the species-specific secondary antibody .

Culture with three changes of PBS, and nuclei were stained with diamidino-2-4-6 phenylindole and observed by fluorescent microscopy and were washed. Expression of nerve growth factor receptor p75 low-affinity OEC culture were detected by immunostaining of cells with anti-p75 primary monoclonal antibodies in mice NGFR (Sigma, USA) and IgG antimouse FITC conjugated secondary antibody (Abcam, USA). Protein expression of terror with microtubule 2 (MAP2) BIII-tubulin in mouse ES cells were differentiated primary cells visualized by immunostaining Mouse monoclonal anti-MAP2 and tubulin β II (Sigma, USA) and phycoerythrin conjugated secondary anti-mouse IgG antibody (Abcam, USA).

Results

The histological results in the studied groups

The remyelination was measured with the olisabioreport software and reported in quantitative form. To confirm luxal fast blue (LFB) staining, immunohistochemistry was performed and reported in qualitative form (Figures 1-3).

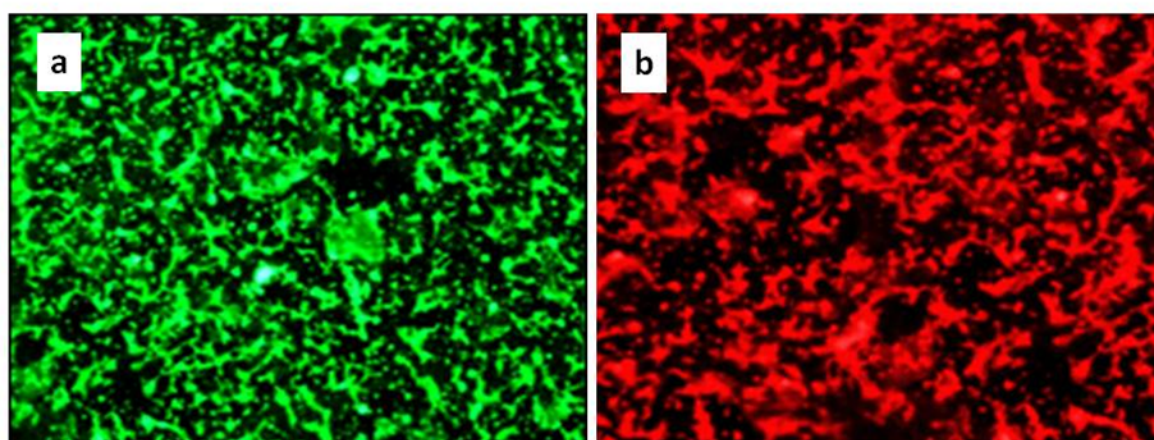


Figure 2. Immunocytochemistry Olfactory ensheathing cells expressing p75(A) and GFAP(B)

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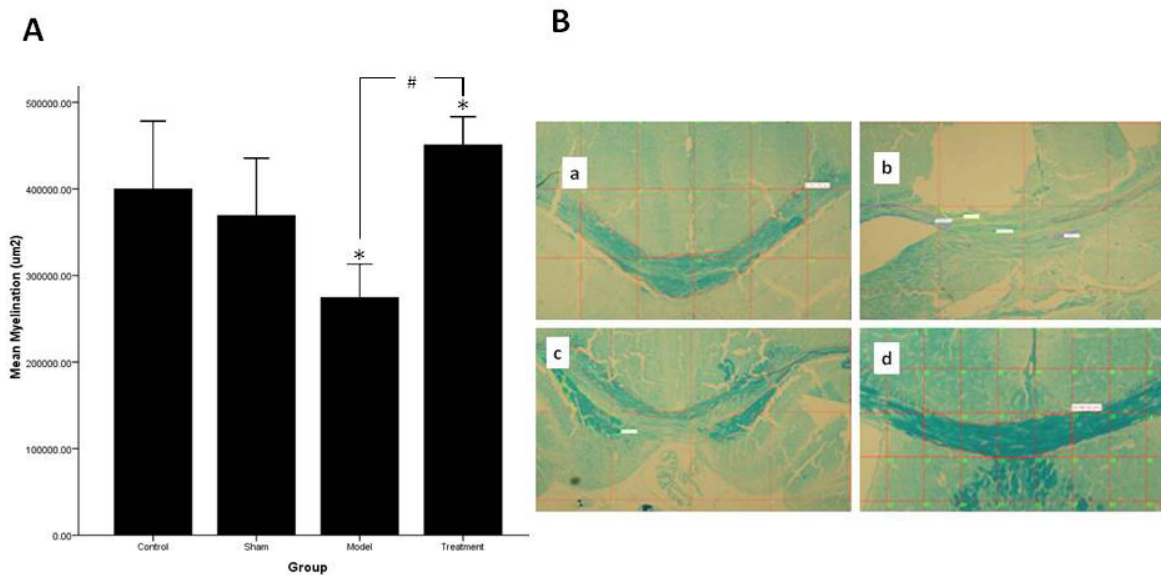


Figure 3. A: OEC effects on remyelination in MS model of mice

*significant difference between control and other groups (Model and treatment) ($P \leq 0.05$), #significant difference between model and treatment group ($P \leq 0.05$). **b:** effects on remyelination in MS model of mice. **a:** control, **b:** model, **c:** sham, **d:** treatment (luxol fast blue)

In a model group, demyelination was present in the affected area and the amount of remyelination was few or not seen (10). OECs were present at high levels in the damaged nerve cells. Remyelination was observed at the injection site and its surroundings (14). Myelinated

fibers were observed in LFB staining of neurons (15). A large myelin repaired area was observed in the treatment group by specifying color filter of DIL (Figure 6). There was a significant difference in Myelin percentage of PBS and OEC recipient groups ($P \leq 0.05$).

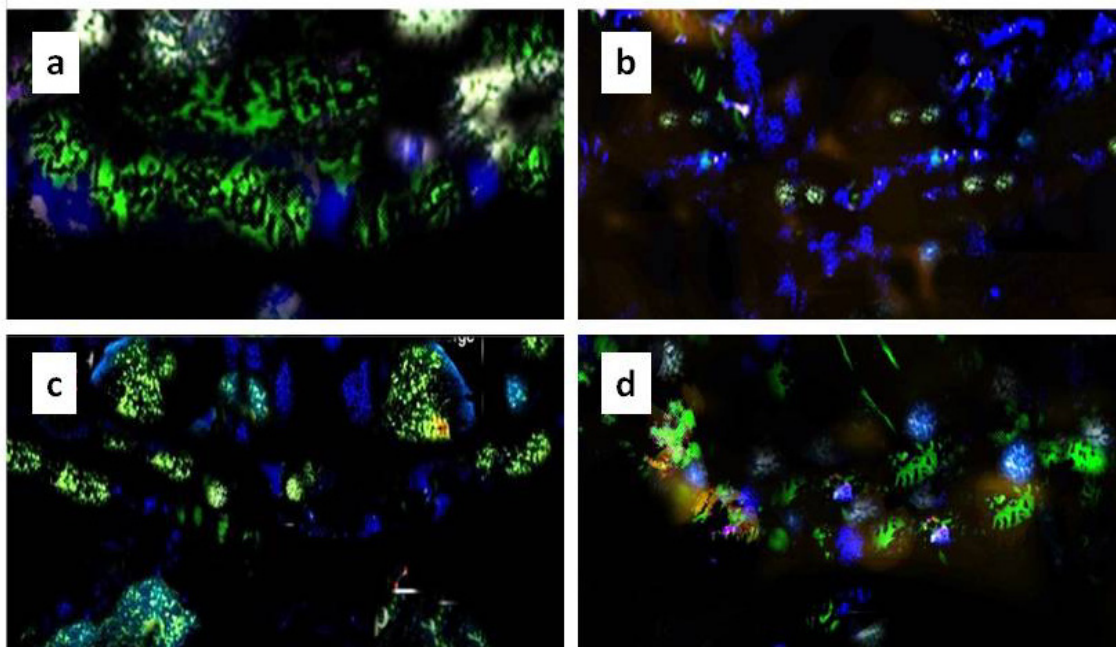


Figure 4. Immunohistochemistry OEC cells with antibodies MBP. OEC effects on remyelination in MS model of mice.

a:control, **b:**model, **c:**sham, **d:**treatment MBP

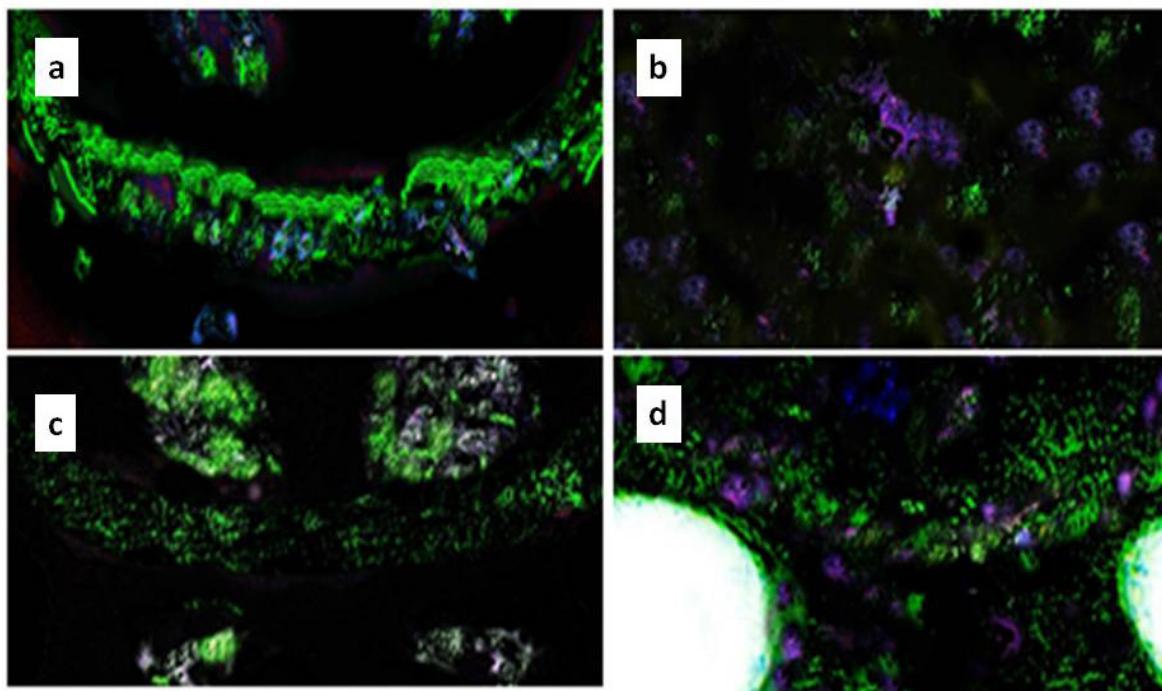


Figure 5. Immunohistochemistry OEC cells with antibodies PLP. OEC effects on remyelination in MS model of mice.
a:control, **b:**model, **c:**sham, **d:**treatment PLP

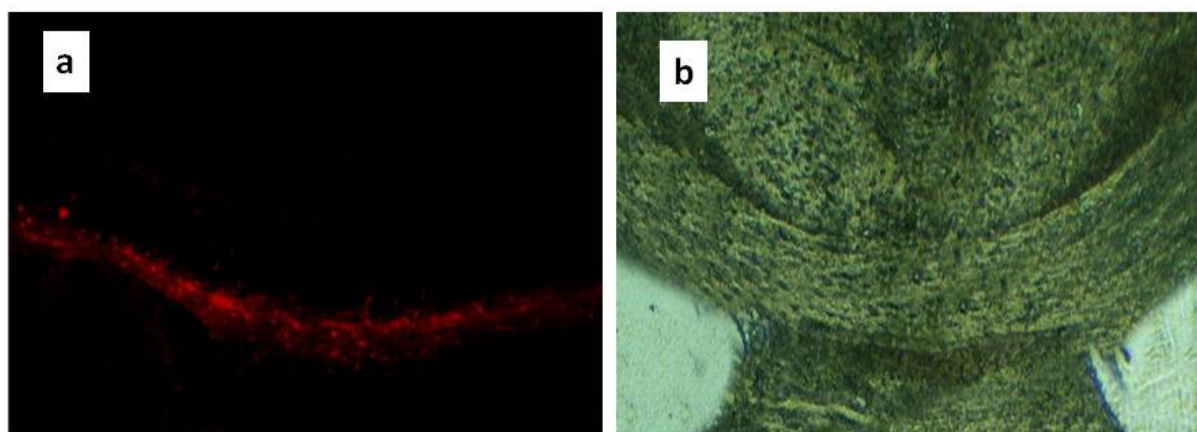


Figure 6. Tracing with DII

Discussion

T Sensitized cells can pass through the blood-brain barrier. Their task is to control the presence of CNS antigens and then leave the area. In MS, T sensitized cells remain in CNS and can help to infiltration of other factors that may lead to loss of the immune system. Attack to the immune system can cause inflammation. Inflammation can eliminate the myelin sheet (which normally covers the axon to speed the impulses along

the axon) and the cells that produce myelin in the CNS. Sclerosed plaques appear in the path of demyelinated axons and create more interruption in the transmission of impulses. MRI is considered the first diagnostic tool for detection of small plaques and evaluating the process of treatment .

There are two main goals in the treatment researches of MS disease.

- Myelin retrieval and regeneration
- The replacement of lost cells, such as

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oligodendrocyte

Different cells were used for replacement of lost cells, but one of the most important cells of myelin regeneration is OECs. The results of the research showed that the transplantation of OECs in MS models can restore the myelin sheath. In this study, the average myelin restoration in the treatment group was more than MS group.

OECs were isolated from the olfactory mucosa. During OECs isolation in the multi-stage process, different cell lines were obtained. Nash *et al.*, showed that 70% of the cells in the first phase of culturing, attached to the floor of the flask are fibroblasts (7). The supernatant was transferred to another uncovered flask and were incubated for 36 hrs. During this time, 67% of cells attached to the floor of the flask are astrocytes. But the OECs are located in the supernatant. Then supernatants were transferred to the covered flask with poly-L-lysine and within 48 hours, the OECs attached to the floor of the plate.

OECs were confirmed by the P75 and GFAP markers in flowcytometry. In the present study, the purity of OECs was approximately 90% (7,8). Cuprizone powders (mixed 0.2% with diet for six weeks) was used for MS induction (9). Then, demyelination of corpus callosum was confirmed by LFB and immunohistochemical (MBP (Figure 4) and PLP (Figure 5) markers) which was similar to previous studies (10). In this study, OECs were used for restoration of myelin and according to the results a large area of remyelination in the damaged area was observed in the treatment group.

Several studies have been performed to evaluate OECs and its effects on spinal cord injuries. Improvement has been observed just in some studies (16). The results of this study showed that the transplanted cells OEC can restore the myelin sheath in mice model of MS. In this study average myelin restoration in the treatment group were more than the sham group and the model that was similar to previous studies (10). However, some studies have reported that the OEC is unable to restore the myelin sheath (16). The reasons for these conflicting reports are not clear. But the some factors such as the type of injury, cells culture method of OECs, purity degree, and the time of transplantation can effect on the result of studies (17).

Despite, the majority of studies suggest axonal regeneration was improved. Feron *et al.*, (2005) transplanted OECs into the damaged spinal cord. Regeneration of Myelin in downside routes and significant improvement of locomotor function in animals

received transplantation were observed (18). Kato *et al.*, (2000) reported although transplanted Schwann cells can improve the myelin sheets in the injured area alone, transplantation Schwann cells with astrocytes had better results with much wider remyelination areas. Since the OECs shows properties of both cells astrocyte and Schwann like cell and they are able to restore axonal conduction without cotransplantation with astrocytes (19). Barnett *et al.*, showed that induction by OECs can myelinate axons in vitro four weeks (20). Then, further studies on the mechanism of OECs myelin regeneration indicated that this ability of OECs associated with age and the response of myelin occurs in fetal compared with adult OEC (21). Several studies have shown that OEC myelin causes spinal cord injury SCI (22). This myelin has a normal structure (23).

The present study demonstrated that the OECs transplantation can improve myelin restoration. This finding is consistent with the findings of other researchers. In MS model MBP and PLP of the myelin sheath are reduced. In the group receiving OECs, it was returned to normal level significantly compared to the sham group received only PBS

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