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Transplantation of Olfactory Mucosa Improve Functional Recovery and Axonal Regeneration Following Sciatic Nerve Repair in Rats

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ABSTRACT

Background: Olfactory ensheathing glia (OEG) has been shown to have a neuroprotective effect after being transplanted in rats with spinal cord injury. This study was conducted to determine the possible beneficial results of olfactory mucosa transplantation (OMT) which is a source of OEG on functional recovery and axonal regeneration after transection of the sciatic nerve. **Methods:** In this study, 36 adult female Sprague-Dawley rats were used. The sciatic nerve was transected in 24 rats and immediately repaired by sciatic-sciatic anastomosis, and randomly divided equally into two groups. The experimental group received the OMT at the transected site and the control group received the respiratory mucosa transplant. In another twelve rats as sham-operated animals, the sciatic nerve was exposed but no transection was made. DiI retrograde tracing was injected in the gastrocnemius muscle two months after surgery to allow visualization of the extent of axonal regeneration. Functional recovery was also assessed at 15, 30, 45 and 60 days after surgery using walking track analysis and sciatic function index (SFI) calculations. **Results:** The total number of DiI labeled motorneurones in the ventral horn (L4-L6) and the SFI scores were significantly higher in the group of rats that received olfactory mucosa rather than respiratory mucosa. **Conclusions:** The outcome indicates that olfactory mucosa is a useful treatment to improve nerve regeneration in mammals with peripheral nerve injury. *Iran. Biomed. J. 12 (4): 197-202, 2008*

Keywords: Olfactory mucosa transplantation (OMT), Retrograde tracing, Olfactory ensheathing glia (OEG), Functional recovery

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INTRODUCTION

Peripheral nerve injuries (PNI) are one of the most challenging problems faced by surgeons [1]. They are estimated to occur to 2.8% of all trauma patients, many of them acquire permanent disabilities and neuropathic pain as a result [2]. Peripheral nerves have the capacity to repair after lesion, but permissive environment and trophic support are required for axonal outgrowth [3]. In order to improve the functional recovery and histological outcome after PNI, the rat-sciatic-nerve model is a mainstay in the evaluation of motor and sensory nerve function [4].

Several strategies have been developed to rebuild the PN defect, including Schwann cell transplantation [5], peripheral nerve allograft [6], fibroblast growth factor [7], bone marrow stromal cells [8], implantation of neural stem cells [9], and the use of a fibrin sealant containing neurotrophic factors [2]. Transplantation of olfactory ensheathing glia (OEG) is another existing strategy used in response to nerve injury [10]. These cells have the unique property of ensheathing the entire axonal path from olfactory mucosa in the peripheral nervous system (PNS) to the outer layer of olfactory bulb (OB) in the central nervous system (CNS), thus preventing exposure of olfactory axon to inhibitory molecules [11,12]. They share properties with both Schwann cells of (PNS) and astrocytes of the CNS [13]. Extracting these cells from OB in humans presents major difficulty, whereas the olfactory

*Corresponding Author; Tel. & Fax: (+98-21) 88058689; E-mail: nilohamdi@yahoo.com. **Abbreviations:** OEG, olfactory ensheathing glia; OMT, olfactory mucosa transplantation; RMT, respiratory mucosa transplant; SFI, sciatic function index; PNI, peripheral nerve injuries; PNS, peripheral nervous system; CNS, central nervous system; OB, olfactory bulb mucosa is a source of these cells by a simple biopsy through the external nares [14]. OMT induces a sustained expression of trophic factors at the lesion site in PNI [15]. The survival of some neurons that depends on the retrograde transport of trophic molecules, because such neurons die when this transport is interrupted [16]. Trophic factors of olfactory mucosa support neuron production and survival [15] and this may be the OEG work optimally in concert with connective tissue element [17]. However, PNI complete functional recovery does not occur in most cases, despite optimal surgical treatment [18]. In the present study, our purpose is to evaluate the effect of the OMT in the functional recovery and axonal regeneration of the sciatic nerve following transection.

MATERIALS AND METHODS

Animals. All animal experiments were performed according to the guidelines of the Iranian Council for the Use and Care of Animals Guidelines and were approved by the Animal Research Ethical Committee of Iran Medical University (Tehran, Iran). Adult female Sprague-Dawley rats (n = 36 and 200-250 g) were randomized and divided equally into three groups: OMT, respiratory mucosa transplant (RMT) and a sham group. The rats were maintained on a 12 hours light/dark cycle with free access to food and water.

Surgery procedures and transplantation. Rats were anesthetized with an intraperitoneal injection consisting of a combination of ketamine (80 mg/kg) and xylazine (10 mg/kg). The left sciatic nerve was exposed and transected by means of sharp microscissors near the obturator tendon at midthigh [19]. The proximal stump was then immediately microsurgically reconnected to the distal stump with two 11-0 atraumatic sutures (Ethicon EH 7438G, Ethicon, Norderstedt, Germany). The olfactory mucosa and RM were provided as described previously [14] from same old adult Sprague-Dawley rats. Either the olfactory mucosa or RM was gently laid over the sutured epineurium in experimental and control groups, respectively. The muscles were then sutured in layers and the skin was closed. The rats were returned to their cages with access to water and food ad libitum. In the sham-operated animals, the sciatic nerve was exposed by separating the surrounding muscles in the same manner as performed in the grafted animals, but the sciatic nerve was not transected.

Footprint recording and analysis. Motor functional assessment was performed 15, 30, 45 and 60 days after injury using walking track analysis [20]. The hind feet of the rats were dipped in dilute China ink and the animals were allowed to walk down an 11 × 45 cm corridor into a darkened box. The floor of the corridor was covered with a sheet of paper to record the footprints. The print length (PL), toe spread from the first to the fifth toe (TS), and intermediary toe spread (IT) from the second to the fourth toe were measured on the experimental (EPL, ETS, and EIT) and normal sides (NPL, NTS, and NIT). The sciatic functional index (SFI) was calculated according to the following formula [20]:

$$SFI = -38.3 \frac{(EPL - NPL)}{NPL} + 109.5 \frac{(ETS - NTS)}{NTS} + 13.3 \frac{(EIT - NIT)}{NIT} - 8.8.$$

A SFI of zero indicates normal nerve function and -100 represents complete dysfunction.

Retrograde tracing and histological procedures. Two months after sciatic nerve transection and behavioral assessment, six rats from each group were used for retrograde tracing with 1, 1´-dioctadecyl-3, 3, 3´, 3´–tetramethylindocarbocyanin perchlorat (DiI) from Molecular Probes (Leiden, The Netherlands; cat. No, D-282). The animals were anesthetized and the gastrocnemius muscles on the left side were exposed by an incision through the overlying skin. DiI (8-9 µl, in 170 mg/ml DMSO) was diluted 1:10 in saline and injected into 5 locations on the body of the muscle [21] using a 10-µl Hamilton syringe. The skin was sutured and the rats were allowed to recover. Two weeks after injection [22], all animals were transcardially perfused with 0.9% NaCl in distilled water followed by fixation with 4% paraformaldehyde (0.1 M phosphate buffer, pH 7.4) under deep anesthesia. The embedded spinal cord (L4-L6) was dissected out and cryoprotected in 30% sucrose overnight. Serial 20 µm -thick transverse sections of the segment were made on a freezing microtome (Leica cryostat). Every second section was mounted onto gelatin-coated glass slides, cover slipped and searched for labeled neurons using fluorescent microscopy (Olympus Ax70). As previously described [2] in each spinal segment the number of labeled motorneurons of each section was summed together to give the total number of motorneurons for each rat.

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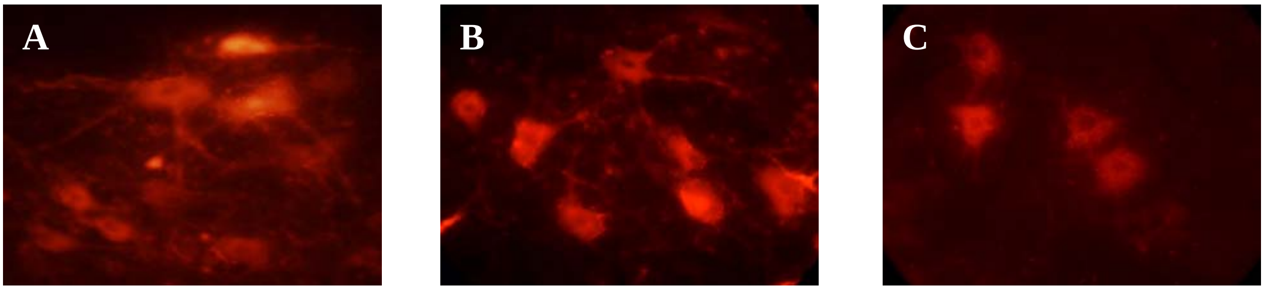

Fig. 1. Fluorescence photomicrographs of retrogradely DiI labeled motorneurons in the left ventral horn at the level of L4-L6. The presence of the retrograde tracing in the motorneurons is indicative of regeneration of the some transected axons into OMT **(B)** and the respiratory mucosa transplant **(C)** compared to the sham group **(A)**. The number of DiI labeled motorneurons in OMT rats was significantly higher than RMT animals. n = 6, $P<0.05$ and scale bar, 50 µm.

Data analysis. Significant differences among groups were determined by two-way ANOVA. Data are presented as the mean ± S.E.M. $P<0.05$ between any two groups was considered significant according to the Bonferroni procedure.

RESULTS

DiI-labelled motoneurons in ventral horn. Two months after sciatic nerve transection, the axon exhibited regeneration past the transected site, and retrograde tracing DiI had been transported to the motorneurons in the ventral horn. The average number of counted retrogradely labeled motoneurons in the left ventral horn (operated side) in the sham group that had not received transection of the sciatic nerve was (mean ± SEM) 132.07 ± 4.2 (Figs.1 and 2). This number was less in the OMT and RMT groups: 84.76 ± 4.5 and 53.23 ± 5.61, respectively at the corresponding level (L4-L6). Furthermore, a Two-way ANOVA test followed by Bonferroni's test showed that there was a significant difference in the ratio of the number of labeled motorneurons in OMT rats compared to the RMT animals, n = 6 and $P<0.05$. This result shows that OMT to the transected sciatic nerve improves nerve regeneration.

Functional assessment. In sham operated animals, the hind foot toes completely spread with normal gait, with a mean SFI (-7.36 ± .8) at 15 days and (-6.22 ± .7) at 60 days. The OMT and RMT groups showed an adduction of the toes and foot drop, and were unable to bear weight. The mean SFI for the OMT rats was -86 ± 2.3 and for RMT group was -89 ± 1.7 at 15 days. At 15, 30, and 45 days, there were no statistically significant differences between the OMT and RMT groups (Fig. 3). The mean SFI scores increased at 60 days in the OMT group when (-69 ± 0.4) compared with a mean of (-80 ± 0.8) for the RMT group (Table. 1). At this time Statistical analysis by SFI showed that there was significant differences ($P<0.05$).

DISCUSSION

In the present work, the mature olfactory mucosa engraftment enhances axonal regeneration after sciatic nerve transection. The increase of SFI scores and DiI labeled motorneurons in L4-L6 spinal level in the OMT rats compared to control group (the RMT rats) demonstrates that OMT to the transected sciatic nerve enhances nerve regeneration and

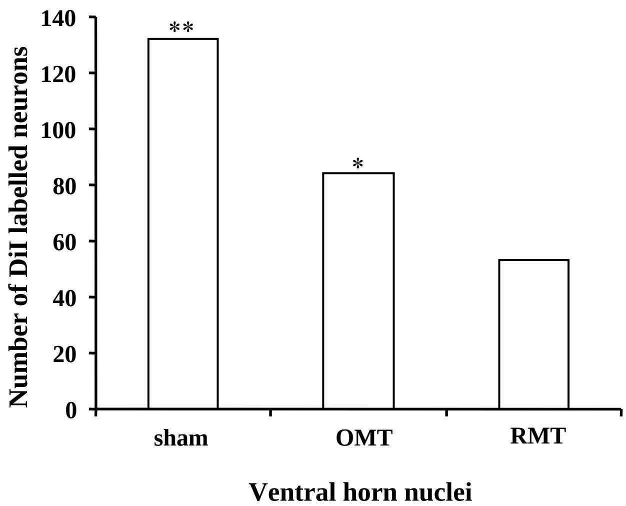

Fig. 2. The total number of DiI labeled motorneurons in each group. Single asterisks indicate a significant difference between OMT and RMT, double asterisks indicate a significant difference between the sham group and both transplanted group. n = 6 and $P<0.05$.

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Table 1. SFI scores for sham group, olfactory mucosa transplantation and respiratory mucosa transplantation.

Group	15 d	30 d	45 d	60 d
Sham	-9.46 ± 4.04*	-8.72 ± 2.81*	-8.61 ± 5.11*	-7.34 ± 6.12*
OMT	-98.21 ± 7.11	-86.41 ± 10.71	-81.64 ± 11.62	-79.34 ± 8.14**
RMT	-96.51 ± 9.42	-83.23 ± 6.52	-74.14 ± 4.91	-65.41 ± 11.17

*$P<0.05$, sham group compared to OMT and RMT groups; **$P<0.05$, OMT group compared to RMT group.

improves motor performance. Autograft is the most common strategy of peripheral nerve gap [23] and olfactory mucosa is a readily accessible source of OEG for autologous grafting from the olfactory system and is not an additional burden to the immune system [14, 17]. In this study, olfactory mucosa provided an appropriate microenvironment for induction and enhancement of nerve regeneration. Axonal regeneration is probably influenced by the growth factors released by OEG in the olfactory mucosa [15]. Neurotrophic factors are regulatory proteins that modulate neuronal survival, axonal growth, synaptic plasticity and neurotransmission [24]. The olfactory neuro-epithelium undergoes lifelong repair by progenitor cells, which are capable of replacing both neuronal and supporting cells [25]. Extracellular matrix proteins unable to reduce the number of axonal branches on the facial nerve injury of the rats [26], whereas OMT minimizes axonal branching after facial nerve repair in rats [15]. The improved motor recovery seen after 8 weeks post-transplantation in our study correlated with improved motor axon regeneration exemplified by the retrograde tracing in the OMT rats. Immunostaining for trophic factors at the lesion site of olfactory mucosa -transplanted rats in the transected facial nerve showed increased expression of NGF, BDNF, and FGF-2 [15].

Thus, the trophic factors within the graft act as a nerve guide and direct the outgrowing nerve fibers towards the distal nerve stump. Growth factors such as NGF and NT3 retrogradely transported in the sciatic nerve [27] and NT3 plays a role in the conveyance of trophic signals from organs to neurons such as motoneurons and proprioceptive sensory neurons in adult rats [27]. The increase of the SFI scores at 60 day after operation in the OMT rats in the present study shows that the olfactory mucosa may provide a weaker but long-lasting secretion of neurotrophins and bFGF at the lesion site [15].

In the olfactory system, OEG can promote axonal regeneration across the PNS: CNS boundary and perhaps may become a prime candidate for cell-mediated repair following different CNS lesions [28, 29]. OEG have differential expression in the PNS and CNS and they are comprised of a heterogeneous population of cells [10]. OEG in the Lamina propria of the olfactory mucosa are responsible for histological outcome and promotion of functional recovery. Also, they exhibit a higher mitotic rate, migratory and reduced cavity and lesion site compared with OEG from the OB in the lesion spinal cord [30.]. Although axonal regeneration is influenced by the growth factors that are provided by reactive Schwann cells and macrophages, the number of these cells is low in both olfactory

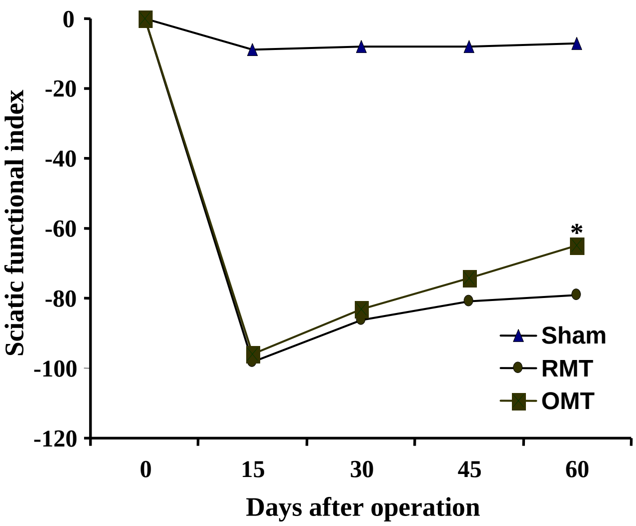

Fig. 3. Walking track analysis is showing the SFI in each group at 15, 30, 45 and 60 days after surgery. The SFI scores of OMT was significant compare to the RMT at 60 days. *$P<0.05$.

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mucosa and RM [14]. It is likely that not only OEG but also neuroprogenitor cells of the olfactory neuroepithelium are responsible for axonal regeneration and promotion of recovery. Multipotent progenitor cells in the adult olfactory epithelium could give rise to neurons and non-neural cells [31]. These cells in the adult human olfactory epithelium can assist spinal cord regeneration and promote functional recovery [32]. A pilot clinical study has shown that autograft of olfactory mucosa is fairly safe and feasible and may possibly promote functional recovery in chronic, severe spinal cord injury in humans [33]. Thus, OMT, including its lamina propria and olfactory neuroepithelium, is a feasible means of achieving functional recovery of peripheral nerves via axonal regeneration.

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