Original Article

The Effect of Fetal Olfactory Mucosa on Tissue Sparing and Locomotor Recovery after Spinal Cord Hemisection in Rats

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Abstract

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Objective: Olfactory ensheathing cells (OECs) has been shown to have a neuroprotective effect after transplanted in brain and spinal cord injury (SCI). This study was conducted to determine the possible beneficial results of transplantation of fetal olfactory mucosa (FOM) that was the source of OECs in the recovery of locomotor function and in spinal tissue sparing after spinal cord hemisection.

Materials and Methods: Forty-eight adult female Sprague-Dawley rats were spinally hemisected at the L1 level and were randomized into the three groups of 16 animals. The first group, immunosuppressed injured animals were received cyclosporine A (CsA) and FOM graft. The second group was received CsA and fetal respiratory mucosa (FRM) graft, and the control group; non-immunosuppressed rats were received saline and gel foam. Locomotor performance was assessed weekly for 8 weeks after lesion, using locomotive rating scale developed by Basso, Bresnahan and Beattie (BBB). After behavioral assessment, the spinal cord was examined by a histologist for spinal tissue sparing.

Results: From weeks 6-8, the functional recovery of the FOM rats significantly increased in comparison to the FRM, although a significant difference in tissue sparing was not apparent. From weeks, 2-8 the functional recovery of the FOM and FRM groups as well as tissue sparing of the FOM group increased significantly compared to the control group.

Conclusion: Thus, the FOM treatment may be effective to promote functional recovery and partially preserving tissue sparing.

Keywords: Ensheathing Glia, Fetal Olfactory Mucosa, Spinal Cord Hemisection, Transplantation

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Introduction

Following spinal cord injury (SCI) in adult mammals, regeneration of the axons and functional recovery are not successful. Many factors act as a physical and chemical barrier for axon regeneration, such as inhibitory molecules, an inhospitable extraneuronal environment, tissue scaring, cell death, glial cells and some meningeal cells (1, 2). Repeating words In order to reduce these barriers and to stimulate the remaining intrinsic plasticity of neurons to regenerate axons, external intervention is needed (1). Cell and tissue types from various sources have been considered and evaluated for therapy in the SCI, including peripheral nerve grafts (3), Shwann cells (4), and embryonic spinal cord tissue (5). In addition, transplantation of olfactory ensheating cells (OECs) into the injured spinal cord is another existing strategy. OECs have the unique property of enfolding olfactory axons along the entire axonal path from the olfactory mucosa (PNS) to the outer layer of olfactory bulb (CNS) (6) and prevention of their exposures to inhibitory molecules (7). The OECs is more accessible via the olfactory mucosa with a simple biopsy through the external nares than through the olfactory bulb (OB) (8). OECs from the olfactory lamia propria (OLP) have an increased rate of mitotic activity rate and migration than OECs from the OB (9). Functional improvement has been reported after transplantation of lamina pro-

pria into the complete thoracic spinal (10), and transplantation of olfactory mucosa in clinical trials have shown promising results (11). This study illustrates the feasibility of therapies designed to target FOM, to preserve tissue, and to improve functional outcome following spinal cord hemisection injury.

Materials and Methods

Surgical procedure and transplantation

All experiments were performed according to the guidelines of the Iranian Council for Use and Care of Animals Guidelines and were approved by the Animal Research Ethical Committee of Iran Medical University. Forty-eight Spruge-Dawley rats (2-3 months old, female) weighing between 200- 250 g were used in this study. Anesthesia was induced via intraperitoneal injection of a combination of ketamine (80 mg/kg) and xylazine (10 mg/kg). Under an operating microscope, a dorsal laminectomy of the T12 vertebrate was performed and the superior articular joint of the left side was removed. The L1 spinal segment was identified. Hemisection on the left side was performed by using a pair of iridectomy scissors, effectively disrupting all major unilateral descending pathways (12). Median sagittal vein was used as an anatomical landmark to demarcate the median plane. The FOM and FRM were removed from E18 as described previously. (8) One piece (0.5 -1mm) of OM or RM was gently situated between the rostral and caudal sites of the lesion and another piece (1 mm) was placed over the lesion site and covered with gel foam. Sixteen rats were randomly chosen to receive FOM, another 16 rats to receive FRM, and a final 16 to receive gel foam as the control group. (Two rats from the FRM group died as a result of anesthetic complications during preparation for transplantation). The overlying muscles were sutured in layers, and the skin was closed with wound clips. The FOM and FRM groups received intraperitoneal injection of cyclosporine A (Sandimmune, Sandoz, Basel, Switzerland) at a dose of 10 mg for every kg of body weight to prevent rejection of the graft (13) and the control group received saline. Cyclosporine and saline injections started 2 days before the transplantation procedures and continued throughout the experiment, daily. Otherwise, all three groups following surgery were maintained under the same conditions with free access to food and water. Postoperative treatments included saline (1.0 cc s.c.) for rehydration and penicillin-G (0.35ml/kg i.m) as a prophylactic antibiotic. Their bladders were manually expressed twice a day for the first 3 days.

Immunostaining for identification of OECs in fetal olfactory mucosa

The FOM and FRM were removed from E18 and immediately transferred to separate 35 mm Petri dishes containing 4 % paraformaldehyde in 0.1 M PB, (pH=7.4, 4°C) for two hours. The tissues were then cryoprotected (in 30% sucrose) overnight and embedded in tissue freezing medium. Transverse sections (20 µm – thick) of the tissues were made with a freezing microtome (leica cryostat, CM 3000), mounted onto gelatin-coated glass slides, and immunostained with anti- p75 (Mouse anti-nerve growth factor- receptor monoclonal antibody; cat number MAB365, Chemicon) 1:100, overnight. The following day, the sections were washed in PBS and incubated with a secondary anti-body 1:200 (molecular probes, Eugen, Oregon, USA, Alexa Fluor 488nm wave length) in PBS for one hour at room temperature. The slides were then washed with PBS, cover slipped and viewed with a fluorescence microscopy (Fig 1). Control sections without the primary antibody were prepared for each tissue as well.

Assessment of hindlimb motor function

Hindlimb motor function was assessed based on the Basso, Beattie, and Bresnahan (BBB) Locomotor Rating Scale (14) at one day post-surgery and weekly for a total of 8 weeks. For BBB assessment, the rats were allowed to move individually for 5 minute on a smooth, nonslip floor in an open field (200x100 cm). Hindlimb motor function was scored from 0 to 21 based on the performance of the ipsilateral hindlimb by an observer who was not a participant in the experiment. Expectedly spinal cord hemisection of the thoracolumbar region resulted in pronounced paralysis on the ipsilateral hindlimb (impaired hindlimb), with the partially recovery by 3 weeks (15). Hemisections were considered complete when they included the dorsal column, Lissaur's tract, lateral and ventral column, and gray matter.

Estimating the amount of tissue sparing

following behavioral assessment, five rats from each of the groups were deeply anesthetized with an overdose of ketamine (200 mg/kg body weight) and xylazine (20 mg/kg body weight), perfused through their hearts with 0.9% NaCl in distilled water (200 ml) followed by 4% paraformaldehyde in 0.1 M PB, pH 7.4 (4°C, 500 ml). A 5-mm-long spinal cord segment with epicenter of the transplanted tissue were removed, fixed in the same solution for 24 hours, cryoprotected in 30% sucrose overnight and cut into 50µm serial horizontal sections by a leica cryostat. Every fourth horizontal section from rostral to the caudal of the segment, mounted onto gelatin-coated glass slides, was stained with cresyl violet or H&E dehydrated and cover slipped. To determine the volume of spared spinal tissue, low-power magnification (x4) images of the spinal cord sections were taken with the aid of a digital camera (DP 11) attached to the microscope (Olympus Ax70). By using an image analysis computer system (olysia) on every fourth horizontal section of a 5-mm- long spinal segment, the total area of the left spinal cord and the area of damaged spinal tissue (cavity) in left half of the spinal cord were determined. The border of the damaged area was defined as an obvious discontinuity in density of small cells and the absence of healthy-looking spinal neurons (16). As

previously described (16), the measurements of each section were summed per rat and multiplied by 4 to give the total area of the left half and total area of the damaged tissue of a 5-mm-long spinal segment. This value was expressed as a percentage of the total volume of the left half of a 5-mm-long segment from the same cord level from uninjured rats (n=5).

Statistical analysis

All data are expressed as mean ± SD. One-way ANOVA was used for data analysis, followed by the Tukey test for post hoc analysis. A P-value<0.05 was considered to be statistically significant.

Results

Olfactory ensheathing cells are present in the lamina propria of fetal olfactory mucosa

The lamina propria beneath the olfactory epithelium contains olfactory nerve bundles surrounded by OECs which identified immunochemically with an anti-body to P75 NGFR (Fig 1C). The OM removed by its posterior position on the nasal septum and identified from RM by semicircular line (Fig 1A).

The FOM transplants improve locomotor recovery

One day after operation, the hemisected animals displayed loss of ipsilateral hindlimb function with no observable movement. Of the 46 rats that received left hemisection, the six rats showed consistent weight supported plantar steps and consistent forelimb – hind limb (FL-HL) coordination (BBB= 15). For one week after operation, subsequent histological evaluation showed that in these cases the full hemisection of the L1 segment was incomplete and they were thus excluded from the study. Of the remaining 40 rats, the 4 rats were killed and horizontal and parasagittal section confirmed the full hemisection (Fig 2). The inflammatory reactions were observed (have seen delete) at the lesion epicenter one week after lesion (Fig 2A). Then it followed by infarction and cysts formation four weeks after the hemisection (Fig 2B).

The following BBB scores increased considerably for the FOM, FRM and control groups, respectively, on the 8th day post surgery (mean±SD): 10.41±1.31, 10.16±1.85 and 8.75±1.76 (Fig 3). From weeks 2-8, the FOM and FRM rats demonstrated a significant increase in the movements of their hind limbs compared to the control group ($p<0.05$). The FOM and FRM rats recovered at similar rates during the 5th week post-injury but from weeks 6-8, the FOM treated rats slightly improved their walking behaviors in comparison to the FRM group. Furthermore, the FOM group showed consistent plantar stepping and consistent forelimb-hindlimb (FL-HL) coordination, whereas FRM demonstrated limited FL-HL coordination.

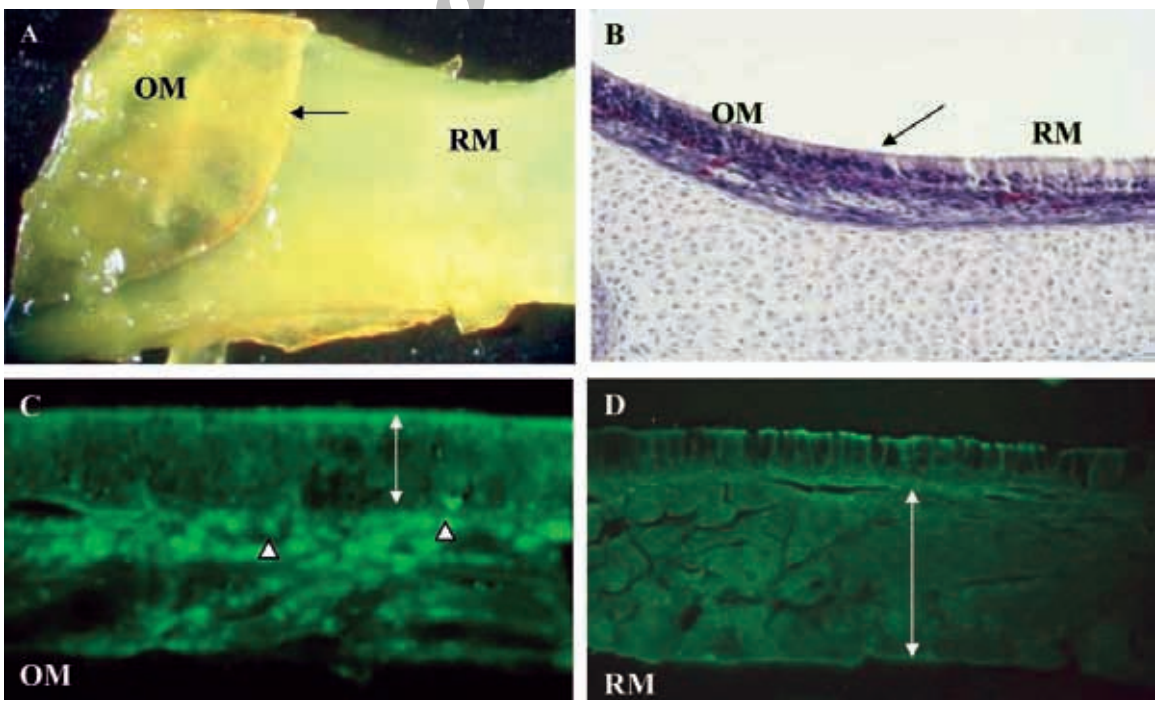

Fig 1: (A) Photograph of the nasal septum of a fetal (E18) rat indicating the locations of the OM and the RM separated from each other by a semicircular line (arrow). (B) H&E staining of the nasal septum: arrow indicates the transitional zone between OM and RM. (C) Anti-p75 immunoreactivity (light green) indicates olfactory ensheathing cells lying into the lamina propria of the OM (arrowhead): the olfactory epithelium and lamia propria of the RM are indicated by double arrow. (D) The lamia propria of the RM contains no p75 immunoreactivity. Scale bar: 50μm (B, C, D).
OM, olfactory mucosa; RM, respiratory mucosa.

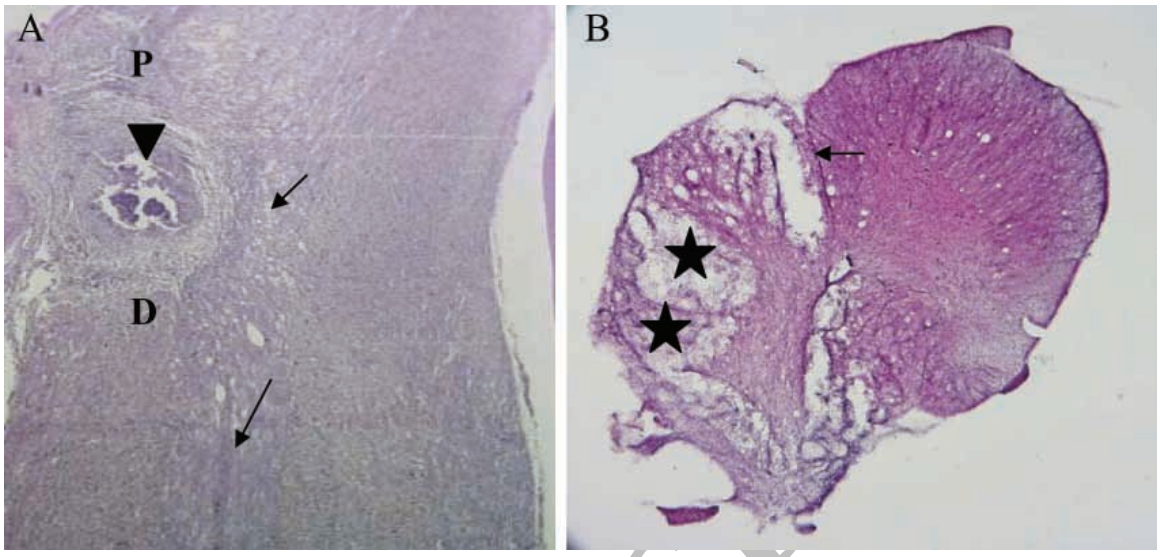

Fig 2: (A) Cresyl violet stained, 20 µm thick sections, sagittal sections through the injury site one week after injury. Inflammatory reaction (arrowhead) formed at the lesion epicenter. (B) H&E stained, 20 µm thick horizontal of L1 lumbar spinal cord 28 days after lesion. Stars indicate cystic cavities. Arrow indicates median fissure; D, distal; P, proximal; Scale bar: A, 100 µm; B, 200 µm.

On the 8th week, the FRM animals achieved a plateau score of 15±1.27, whereas the FOM rats reached an average score of 16.5±0.90. One-way ANOVA results showed significant difference between FOM and FRM groups during the weeks 6 to 8 ($p < 0.05$).

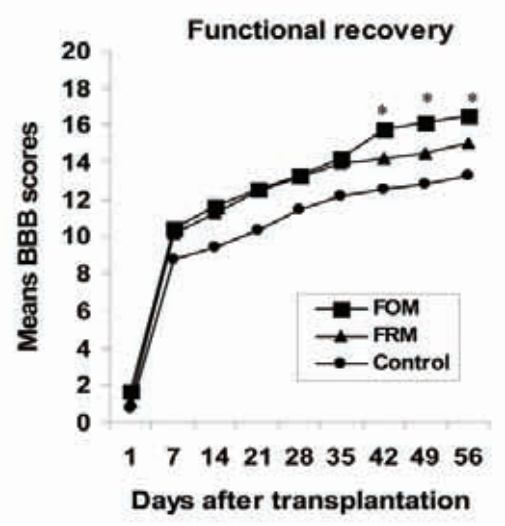

Fig 3: The Means BBB scores of the different groups following hemisection. During the two weeks post-injury all groups recovered nearly at similar rates, but from weeks 2-8, the FOM and FRM rats demonstrated significant performance improvement over the control group. Values represent means ± SD. n=12, *p<0.05: compared to FRM group.

Histomorphology of the grafts and lesion site after eight weeks

Histological evaluation of the spinal cord from the epicenter of the lesion site revealed that the FOM grafts, survived, grew and proliferated to fuse with the parenchyma of the host tissue. There were no obvious glial boundaries at the host–graft interface (Fig 4, A, D). In the FRM rats, there were a few cavities at the host–graft interface (Fig 4, B, E). In the both group, a clear boundary consisting of a different cytoarchitecture was observed at the host–graft interface (Fig 4, A, B). In the control rats, there were large cysts that replaced all of the area previously occupied by gray matter and white matter (Fig 4, C, F). In some of the rats from the control group, fibrous tissue had replaced the lesion site (Fig 4C).

Transplantation of olfactory mucosa improve partially spinal tissue sparing

To ensure consistency in the analyses of tissue sparing, all sections were seen through the same illumination level, and microscope and digital camera settings were maintained constant throughout the image capturing sessions. The volume of the left half of a 5-mm-long spinal cord segment of a normal uninjured rat at the L1 level was (mean±SD): 11.85±0.12 mm^3. In contrast, the spared tissue of the left half in the FOM, FRM, and control groups were 5.65±0.54 mm^3, 5.0±0.94 mm^3, and 4.25±0.11 mm^3, respectively (Fig 5). Although an ANOVA revealed no significant difference in the percentage of spared tissue between the FOM and FRM rats, there was a modest increase in tissue sparing as a result of FOM treatment in comparison to the control group ($p<0.05$). Whereas there was no significant difference in the percentage of spared tissue between the FRM group and control rats, indicating that the FOM grafts enhanced tissue sparing better than FRM after hemisection.

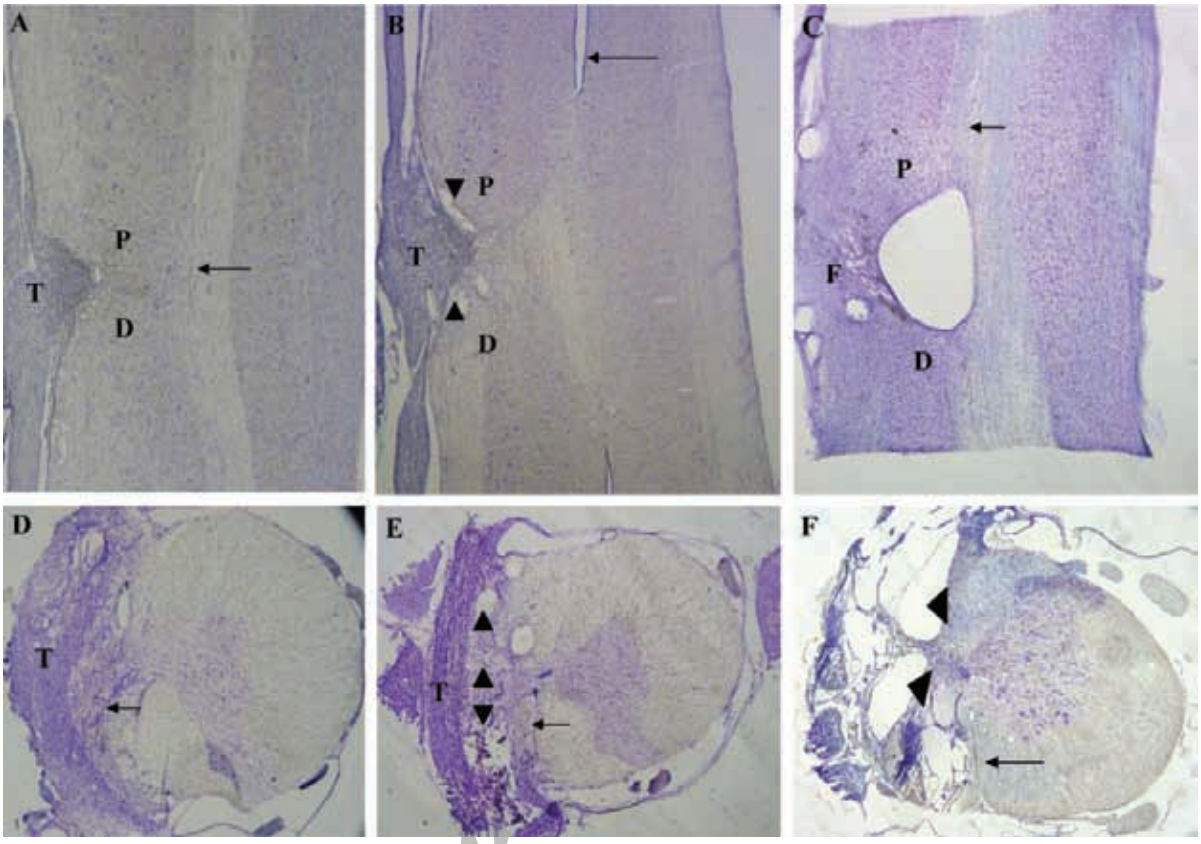

Fig 4: Parasagittal and transverse sections (50 µm) at the lesion epicenter at the L1 level, after a hemisection injury. In the FOM rats, the grafts (A, D) fused with the host tissue and formed a seamless continuous spinal cord, whereas in some of the FRM animals, (B, E) there were a few cavities at the host–graft interface (arrowhead) at the 8th week after transplantation. At the lesion epicenter, the control group showed characteristic necrosis and cavitations of the spinal parenchyma, (C, D). Arrows indicate central canal or median fissure, arrowheads indicate cavity, T, transplant; F, fibroses tissue; P, proximal; D, distal; scale bar: A, 200 µm; B, C, 300 µm; D, 100 µm; E, 200 µm; F, 400 µm.

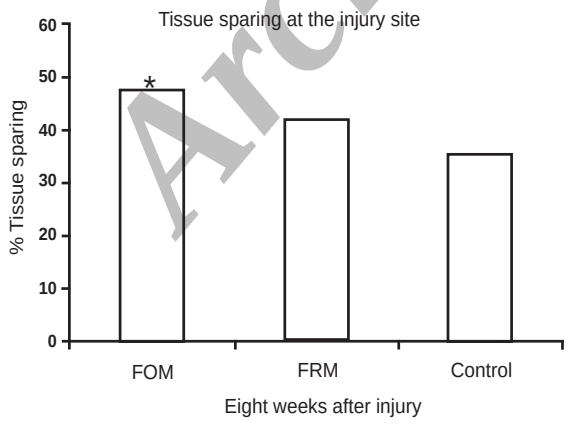

*Fig 5: Graph representing the spared spinal tissue in the left half of the spinal cord at the L1 level at the lesion epicenter in different groups. FOM showed a significant difference in the percentage of spared tissue compared to the control groups. Values represent means±SD, n=5.*p<0.05 compare to the control group.*

Discussion

This experiment shows that the fetal olfactory mucosa has neuroprotective effects that improve locomotor recovery, axonal regeneration and attenuation of lesion size compared to the cyclosporine and fetal respiratory mucosa. Our behavioral and histological data demonstrate that the animals used in this study had completely hemisected spinal cords (Fig 2). The ascending and descending tract were interrupted after transection of the spinal cord and spinal tissue undergo apoptosis and inflammatory reaction as observed at the first week of the post-injury (17). In this study, we confirm following L1 hemisection in rats, the ipsilateral hindlimb is initially paralyzed and partially recovers within 2 to 3 weeks (18). The functional recovery and promotion of axonal regeneration by OECs from the olfactory bulb have been studied in various adult rat spinal cord injury models (19-22). Unlike rats, the olfactory bulb in humans is comparatively small and relatively inaccessible (23). Present study shows the transplantation of

FOM into the spinal cord injury is safe and feasible. The FOM rats showed that grafts entirely filled the lesion site between the rostral and caudal host tissue and formed an interface without cysts or scare (Fig 4, A, D). The absence of scar formation may reflect the mild host immune reaction attributable to immunosuppression by CsA (24). The scar tissue was visible in nonimmunosuppresive rats. OECs transplants did not prevent glial scar formation (19). Importantly, our data indicate that the FOM treatment was important to decrease damaged area and cavity formation following a hemisected lesion. Morphometric analyses indicated that the FOM rats had a 48% of preservation tissue around the injury site compared to a similar segment in the uninjured rats; this value was significantly greater than the volume of the spared tissue in the control group (35%). It has been documented that small changes in tissue sparing can significantly impact locomotor function (25). Thus, a 6% increase in tissue sparing in the FOM rats in comparison to the FRM group, and a similar 12.5% increase in comparison to the control group may indeed contribute to improve functional recovery. The improvement of functional recovery in FRM compared to the control group may be the effect of CsA, rather than FRM. The fibroblast and endothelials cells in FRM are not reported to assist spinal cord regeneration (8). Although, Schwann cells and macrophages can assist axonal regeneration but they are present in low number in both olfactory and respiratory lamina propria (8, 21). Whereas cyclosporine A promotes axonal regeneration in rats submitted to transverse section of the spinal cord (26) and reduces delayed motor neuron death after spinal cord ischemia in rabbits (27). Cyclosporin-A promotes neuroprotection by diminishing both demyelination and neuronal cell death, resulting in a better motor outcome after spinal cord injury (24). We cannot rule out that the growth factor in connective tissue of the grafts may facilitate endogenous repair (28). Also, there are reports that the extra cellular matrix (ECM) are neuroprotective and assist spinal cord repair (29, 30).

The protection effect of trophic factors of transplanted olfactory mucosa at the lesion site (31) may benefit functional recovery. Intrathecal injections of bFGF alone could preserve more spared tissue and enhance the functional recovery after spinal cord contusion (32). Immunostaining for trophic factors at the lesion site of OM-transplanted rats in the transected facial nerve showed increased expression of NGF, BDNF, and FGF-2 (31). The increase of the BBB scores on the 42nd day after the operation in the FOM 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 (31). In other experiments, OECs from LP transplantation into the cervical dorsolateral funiculus crush provides the greatest opportunity to promote regeneration or reconnect local circuitry (9). Transplantation of olfactory lamina propria promotes locomotor recovery after delaying transplantation into the transected spinal cord (8) and promotes partial recovery in paraplegic rats (14). It may be the OECs work optimally in concert with other cells or connective tissue element (23). In this study, not only OECs and CsA but also neural progenitor cells in the olfactory epithelium are probably responsible for tissue sparing and promotion of recovery in the FOM rats. Adult olfactory epithelium contains multipotent progenitor cells that give rise to neurons and non-neural cells (33). Human adult olfactory neural progenitor cells rescue axotomized rodent rubrospinal neurons and promote functional recovery (34). Thus, FOM have the potential to provide an efficient and renewable source of cells for autologous transplantation studies for spinal cord injury.

Conclusion

This study shows that transplantation of fetal olfactory mucosa with its lamina propria and of olfactory neuroepithelium results in promotion of tissue sparing and in functional recovery in mammals with partial spinal cord injury. Whatever the eventual outcome of these investigations, further work on the cell biology of the FOM and its behavior following transplantation will advance our understanding to the development of ways of spinal cord repair.

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