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Neural stem cell transplantation in a double-layer collagen membrane with unequal pore sizes for spinal cord injury repair

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Abstract

A novel double-layer collagen membrane with unequal pore sizes in each layer was designed and tested in this study. The inner, loose layer has about 100-μm-diameter pores, while the outer, compact layer has about 10-μm-diameter pores. In a rat model of incomplete spinal cord injury, a large number of neural stem cells were seeded into the loose layer, which was then adhered to the injured side, and the compact layer was placed against the lateral side. The results showed that the transplantation of neural stem cells in a double-layer collagen membrane with unequal pore sizes promoted the differentiation of neural stem cells, attenuated the pathological lesion, and significantly improved the motor function of the rats with incomplete spinal cord injuries. These experimental findings suggest that the transplantation of neural stem cells in a double-layer collagen membrane with unequal pore sizes is an effective therapeutic strategy to repair an injured spinal cord.

Key Words: nerve regeneration; spinal cord injury; collagen; scaffolds; neural stem cells; cell transplantation; nerve repair; neural regeneration

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Introduction

An increasing number of people sustain spinal cord injuries every year. The functional impairment that can occur following spinal cord injury results from damage to or severance of axons, loss of neurons and glia, and demyelination (Teng et al., 2002; Okano et al., 2003; Kwon et al., 2005; Pallini et al., 2005). Secondary pathological processes following a spinal cord injury including ischemia, anoxia, free-radical formation, and excitotoxicity may occur over hours to days after the injury. Many methods to decrease spinal cord functional deficiencies and promote axon regeneration have been reported, such as blocking inhibitory factor and antagonists of secondary injury, substitution of Schwann cells or olfactory ensheathing cells into the myelin sheath, and the application of neural growth factor or other cell factors (Döbrössy and Pruszak, 2012; Yin et al., 2014). However, there is still no truly effective treatment for spinal cord injury.

Neural stem cells are a group of pluripotent cells. Under the appropriate conditions, they can be induced to form neurons, astrocytes, and oligodendrocytes (Teng et al., 2002; Okano et al., 2003). After transplantation, they can migrate, differentiate, and integrate in the host to achieve different effects. Neural stem cell transplantation has emerged as an efficacious experimental treatment for central nervous system disorders, including spinal cord injuries (Maslov et al., 2004; Nakamura et al., 2005; Paspala et al., 2009). A scaffold for cell delivery can assist the neural stem cell transplantation (Teng et al., 2002; Farrell et al., 2006; Harley et al., 2008). Double-layer collagen membrane scaffolds with unequal pore sizes are traditionally fabricated by freeze-drying, where the freezing rate and final freezing temperature can be manipulated to alter the scaffold pore size and uniformity (Torres et al., 2000; O'Brien et al., 2004; O'Brien et al., 2005; Farrell et al., 2006; Harley et al., 2007; Harley et al., 2008; Haugh et al., 2010). In the novel double-layer collagen membrane created here, the inner, loose layer has about 100-μm-diameter pores that can hold a large amount of neural stem cells for transplantation. The outer, compact layer has < 10-μm-diameter pores, which can limit the invasion of surrounding blood cells and connective tissue to provide an appropriate local survival environment for the neural stem cells. The chemical composition and degradation kinetics of this novel collagen membrane scaffold were specifically optimized to prevent platelet accumulation and organized wound contraction (Harley et al., 2008). The collagen membrane can be degraded gradually after being transplanted into the body (Wrathall et al., 1985; McDonald et al., 1999; Akiyama et al., 2001). In this study, a novel double-layer collagen membrane with unequal pore sizes was designed as a scaffold for neural stem cells to determine the effects of scaffold-based neural stem cell transplantation into rats with a spinal cord injury.

Materials and Methods

Isolation, proliferation, differentiation, and identification of neural stem cells

After anesthesia, the cerebrum was harvested from 14-day-old female Sprague-Dawley rats (specific pathogen free (SPF) grade; Beijing Laboratory Animal Research Center, Beijing, China; license No. SYXK (Jing) 2006-0024). The meninges were dissected, removed, and mechanically dissociated in D-Hanks solution. Next, the recovered cells were filtered through a cell sieve in 0.25% trypsin at 37°C for 10–15 minutes and centrifuged at 500 r/min for 4 minutes. The cells were cultured in complete culture medium (1:1 DMEM/F12 with 2% B27, 10 ng/mL fibroblast growth factor, and 10 ng/mL epidermal growth factor; Sigma, St. Louis, MO, USA) for 2 weeks, and were then observed on an inverted microscope (Olympus, Tokyo, Japan). The cultured cells were identified by immunohistochemical staining with rabbit anti-rat nestin monoclonal primary antibody (1:100 dilated with PBS; Sigma). After the isolation, the neural stem cells were cultured in a culture flask with DMEM/F12 medium. The neural stem cells were expanded for further use. This study was approved by the Ethical Committees of Beijing Jishuitan Hospital, Beijing, China.

Preparation of double-layer unequal-hole collagen membrane

Fresh porcine tendons (SPF grade, male pig; Beijing Laboratory Animal Research Center) were cut into pieces and disinfected with 0.05% chlorhexidine solution. After rinsing in isotonic saline solution (0.85% NaCl), 0.1% ethylic acid was added to from a tissue homogenate. The homogenate was centrifuged at 1,000 r/min for 30 minutes, and the liquid supernatant, which was the desired collagen solution, was collected, frozen, dried, and stored in an ice box at 4°C (Torres et al., 2000; O'Brien et al., 2005; Harley et al., 2008).

The collagen was dissolved into 0.05% acetic acid at a mass concentration of 0.3%. The mixture was stirred to ensure that the collagen was fully dissolved. Then, 20% 6-chondroitin sulfate C was added to the mixture at a mass concentration of 9% of the collagen mass. The final solution was poured into templates and dried at 25°C to form a 0.3–0.5 mm thick collagen membranes. The collagen membranes were cross-linked with a 0.25% glutaraldehyde solution. Additional collagen solution was added to the membranes to a thickness of 2–3 mm. The solution was dried at the temperature of 25°C and –80°C separately to control the two different pore diameter sizes to achieve the double-layer collagen membranes with unequal pore sizes. The collagen membranes were soaked and fully rinsed in PBS, sterilized by ^{60}Co, and stored in an ice box at 4°C or equilibrated in distilled water or DMEM/F12 nutrient solution before cell culture use. The double-layer collagen membranes with unequal pore sizes were observed on a scanning electron microscope (Olympus).

Modeling of incomplete spinal cord injury

A total of 30 female adult Sprague-Dawley rats (SPF grade, 10 weeks old, 250–300 g; Beijing Laboratory Animal Research Center) were housed in 20°C, 50% humidity, dark and quiet environment, and underwent a surgery to create the spinal cord injury model. All the rats were anaesthetized with pentobarbital sodium. After positioning using X-rays, the vertebral lamina of $T_{9–10}$ was exposed through a midline incision. A laminectomy was performed to expose the spinal cord, and a spinal cord hemisection of the right side was performed. A 3-mm spinal cord defect was made to establish the spinal cord injury model (Choi et al., 2012).

Neural stem cell transplantation

After the spinal cord injury was induced, the rats were randomly divided into transplantation (n = 20) and model (n = 10) groups. The rats in the transplantation group were transplanted with collagen scaffolds seeded with neural stem cells. Before transplantation, each collagen scaffold was wetted with 10 μL containing 1×10^7 neural stem cells. The loose layer of the collagen scaffold was placed inside the spinal cord defect site, with the compact layer facing outside. Rats in the model group received no treatment. Postoperatively, artificial urination was performed twice per day for both groups until the rats regained their micturition reflex. The general condition and activity level of all the rats were observed. The rats were sacrificed at 2 and 4 weeks after surgery (10 rats from the transplantation group and 5 rats from the model group at each time point). The injury sites of the spinal cords were extracted for histological observation.

Behavioral assessment

The general condition and activity level of all the rats were observed, and any abnormal signs were recorded. Postoperatively, the changes in motor function of each rat were observed and recorded. The Basso, Beattie and Bresnahan (BBB) scale was used to quantify changes in the motor function of the rats in both groups at 2 and 4 weeks. The BBB scale ranges from 0 to 21, with lower scores indicating worse functional recovery.

Histological determination

The rats were sacrificed at 2 and 4 weeks postoperatively. The injury sites of the spinal cord were extracted, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. The stained specimens were observed with an inverted microscope.

Statistical analysis

All the data were analyzed using SPSS 16.0 software (SPSS, Chicago, IL, USA). Data are expressed as mean ± SEM. One-way analysis of variance and Student-Newman-Keuls tests were used to assess the differences between the two groups. P-values < 0.05 were considered significant.

Results

Neural stem cell morphology

At 1 day of *in vitro* culture, a subset of cells began to adhere. Most of the cells remained floating in the nutrient fluid and showed different sizes and shapes. At 3 days of *in vitro* culture, most of the cells were adhered to the wall. The cells aggregated and formed small free-floating spheres, and those

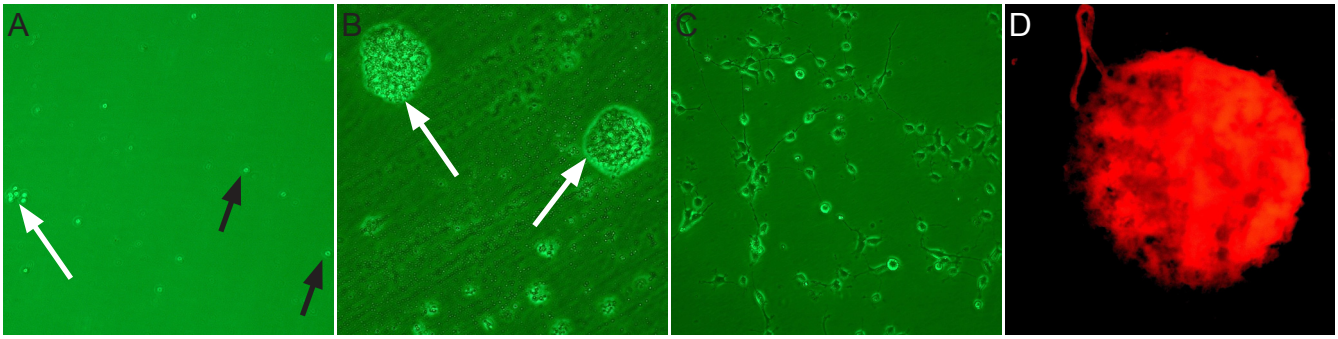

Figure 1 Morphology of neural stem cells with an inverted microscope.
(A) Neural stem cells adhered to the wall (black arrows). Cells started to aggregate as small, free-floating spheres (white arrow) after 3 days in culture (× 100). (B) Three neural stem cell spheres (white arrows) were observed after 1 week in culture (× 100). (C) Neurites were observed around the neural stem cells after 2 weeks in culture (× 100). (D) Neural stem cells clumped into spheres and were nestin-positive after 1 week in culture (× 400).

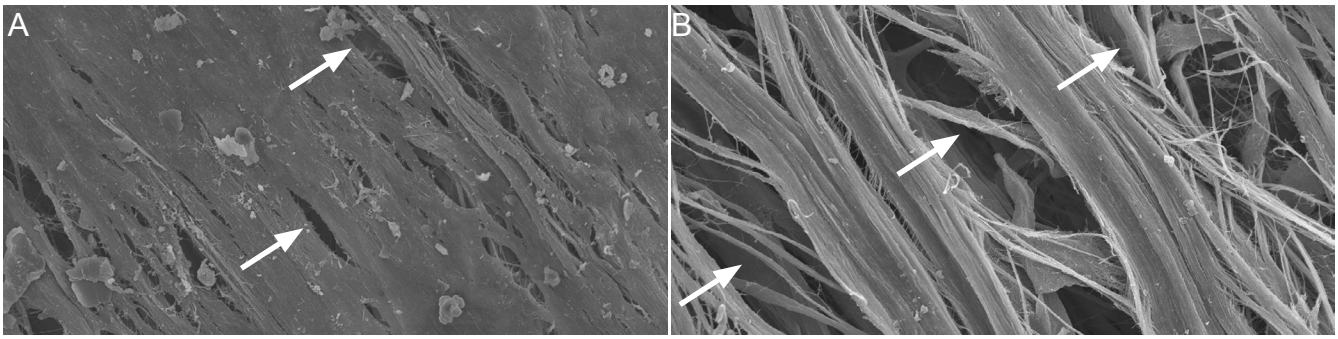

Figure 2 Morphology of the double-layer collagen membrane with unequal pore sizes by scanning electron microscopy (× 500).
(A) The compact layer of the collagen membrane. The arrows point to the smaller pores. (B) The loose layer of the collagen membrane. The arrows point to the larger pores.

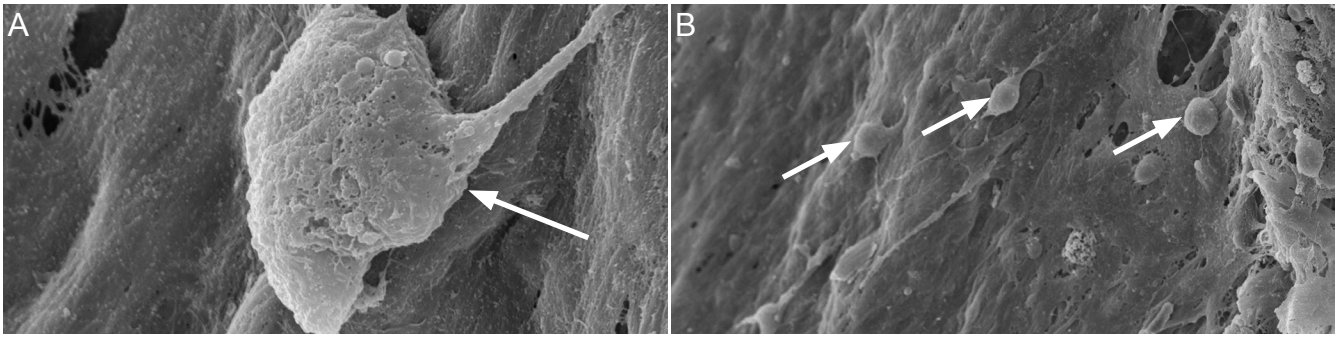

Figure 3 Neural stem cells attached to the double-layer collagen membrane as seen by scanning electron microscopy.
(A) The arrow points to a neural stem cell with a neurite (× 2,500). (B) The arrows point to neural stem cells (× 500).

cells were small and round. At 6 days of *in vitro* culture, the number and size of the cell spheres had increased significantly, and only cell bodies were observed in the spheres, no neurites were found. Immunohistochemical staining for nestin was positive. The cells were then cultured in culture media that contained serum for another 6 days. The cell sphere size increased continuously over time, and apparent neurites were observed around the cell spheres. Immunohistochemical staining for glial fibrillary acidic protein, tubulin, and nestin revealed many glial fibrillary acidic protein-positive cells and a few tubulin-positive cells, as well as a small number of nestin-positive cells (**Figure 1**). This evidence indicated that the purified cells were neural stem cells. After these cells were cultured in serum-free culture medium, most of the cells stained positive for glial fibrillary acidic protein, indicating that they were astrocytes. A few cells stained positive for tubulin, suggesting that they were oligodendrogliomas. Neural stem cells have multi-directional differentiation potential and differentiate along different lineages depending on their culture conditions.

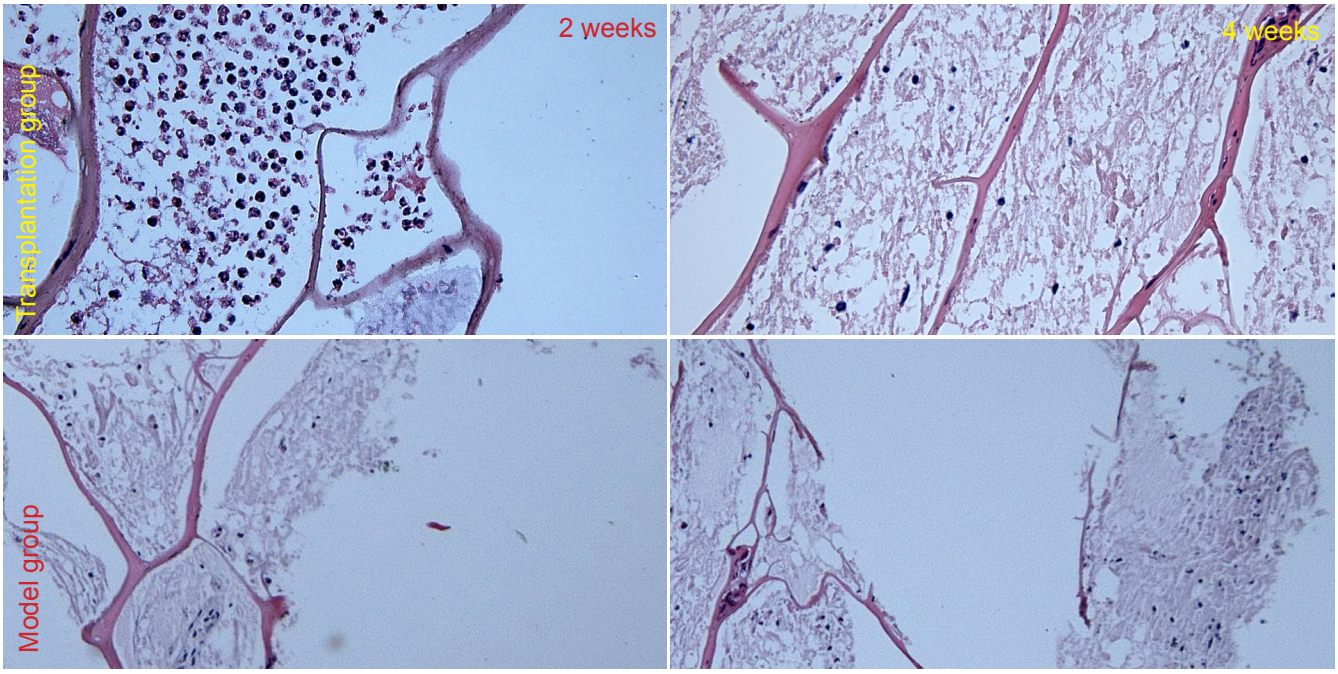

Figure 4 Histology of the double-layer collagen membranes with unequal pore sizes seeded with neural stem cells after transplantation (× 100).
Hematoxylin-eosin staining revealed that the pathological lesions at the spinal cord injury sites after transplantation of the double-layer collagen membranes seeded with neural stem cells were lighter than those of the model group.

Morphology of the double-layer collagen membrane with unequal pore sizes

The double-layer collagen membrane with unequal pore sizes was observed by scanning electron microscopy. The collagen membrane was composed of many collagen fibers, and there were many pores between the fibers. The prepared collagen membranes showed different structures in the outer and inner layers. The pores in the outer, compact layer were smaller, while the pores in the inner, loose layer were much larger (**Figure 2**).

Neural stem cell morphology on the double-layer collagen membranes

The neural stem cells cultured *in vitro* on the double-layer collagen membranes with unequal pore sizes were observed by scanning electron microscopy. The neural stem cells attached to the surface and inside the pores of the collagen membrane. Over time in culture, the number of neural stem cells increased. Neurites could be seen on partial neural stem cells. The double-layer collagen membranes maintained their structure during the entire *in vitro* culture period (**Figure 3**).

Histological changes at the spinal cord injury sites after transplantation of the double-layer collagen membrane seeded with neural stem cells

At 2 weeks postoperatively in the transplantation group, a large number of neural stem cells and a few fibers containing blood cells were found at the spinal cord injury sites, the partial neural stem cells had begun to differentiate, and blood cells were found outside of the compact collagen membrane layer, while neuronal tissue was found inside the layer. There was no neuronal tissue in the spinal cord injury sites of rats in the model control group. At 4 weeks postoperatively, the interfiber space of the transplantation group was full of neuronal tissue and had fused with the normal spinal cord at the injury sites, whereas the injury sites of rats in the model group were filled with irregular connective tissue or empty (**Figure 4**).

Transplantation of the double-layer collagen membrane with unequal pore sizes seeded with neural stem cells improved the motor function of rats after spinal cord injury

After transplantation with a double-layer collagen membrane with unequal pore sizes seeded with neural stem cells, the rats remained healthy. No systemic or local undesirable reactions were observed. All rats demonstrated reduced left lower extremity activities postoperatively, and no right lower extremity activity was observed. At 2 weeks after the operation, the left lower extremities had recovered normal activities, while the right lower extremities showed partial activities, with more activity observed in the transplantation group than in the model group. At 2 weeks after the operation, the average BBB score of the transplantation group was similar to that of the model group (1.5 ± 0.5 *vs.* 1.4 ± 0.5, $P > 0.05$). At 4 weeks after the operation, the right lower extremities of the transplantation group were able to step on the ground with plantar flexion, while those of the model group could not. The model group only recovered a narrow range of activities in three joints or more wide-ranging activities in a single joint. Four weeks after the operation, the average BBB score of the transplantation

group was higher than that of the model group (8.8 ± 1.1 *vs.* 4.4 ± 0.8, $P < 0.05$).

Discussion

Many studies have reported the successful effects of neural stem cell transplantation on the repair of spinal cord injuries (Okano et al., 2003; Kwon et al., 2005; Pallini et al., 2005; Döbrössy and Pruszak, 2012; Yin et al., 2014). A double-layer collagen membrane with unequal pore sizes, acting as a scaffold, can increase the adhesion area for neural stem cells within the loose layer. At the same time, it can prevent scar tissue growth into the compact layer. A collagen membrane seeded with neural stem cells demonstrated good biocompatibility after transplantation *in vivo* (Elias and Spector, 2012). Such membranes can provide an appropriate local environment to promote neural stem cell survival, proliferation, and differentiation, which can aid the recovery of remnant tissue and the partial repair of local defect neural tissue (Hatami et al., 2009).

In the present study, *in vitro* culture of the neural stem cells on the double-layer collagen membranes resulted in cellular growth. After the scaffolds were grafted *in vivo*, no systemic or local abnormal reactions were observed. This shows that the scaffolds were biocompatible with both the neural stem cells and the recipient rat tissue. Collagen membranes have been used for scaffolds by other researchers in similar studies (Farrell et al., 2006; Choi et al., 2012; Elias and Spector, 2012). However, the scaffold used here had a unique, two-layer structure that may increase the repair effects of transplanted neural stem cells on spinal cord injuries. The results after *in vivo* transplantation indicated that the neural stem cell-seeded scaffolds promoted recovery of the damaged spinal cord, as well as functional recovery, compared with no treatment in rats. Although there was no statistical difference in the BBB scores at 2 weeks postoperatively between the transplantation and model groups, there was a significant difference in the BBB scores at 4 weeks postoperatively between the two groups. The mechanism explaining this difference is unclear, but may be caused by neural stem cell differentiation. The differentiated neural cells may secrete neural growth factor or participate directly in the spinal cord repair (Bonner et al., 2011). For example, differentiated astrocytes can secrete neurotropin, which promotes axon growth (Paspala et al., 2009; Bonner et al., 2010; Döbrössy and Pruszak, 2012). The present study focused on observing the early effects of the transplanted scaffold seeded with neural stem cells. The transplantation of the neural stem cell-seeded scaffold was easy to perform, the scaffolds were able to hold a large number of neural stem cells for in their inner loose layers, and the scaffold covered the injury site to provide a suitable local micro-environment. Supplying enough neural stem cells and maintaining an appropriate local micro-environment can increase the effects of transplanted neural stem cells (Bregman et al., 2001; Kim et al., 2010; Xia et al., 2013; Yuan and Shaner, 2013).

This is a preliminary study on the use of double-layer collagen membranes with unequal pore sizes for neural stem cell transplantation. Our findings in this study focused on the feasibility of this novel scaffold for transplantation with neural stem cells. Further study will be conducted to clarify how the pore diameters and scaffold degradation rate influence the transplantation of neural stem cells. In addition, more control groups including neural stem cells and scaffolds transplanted individually should be performed. Longer time follow-ups after transplantation should also be done to determine the late-stage effects of transplantation of these neural stem cell-seeded scaffolds. Besides behavioral function, other tools such as electrophysiology could be used to evaluate the functional outcome (Bambakidis and Miller, 2004; Meng et al., 2008; Pan et al., 2008).

In summary, these novel double-layer collagen membrane scaffolds with unequal pore sizes were biocompatible with neural stem cells and the rat spinal cord. They were easy to seed with neural stem cells and to transplant into the injury site, making them theoretically and practically a suitable scaffold for neural stem cell transplantation.

Author contributions: *The design, implementation and evaluation of the study were carried out by all the authors. All authors approved the final version of the paper.*
Conflicts of interest: *None declared.*

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