Combined Transplantation of Neural Stem Cells and Olfactory Ensheathing Cells Improves the Motor Function of Rats with Intracerebral Hemorrhage[1]

ZHOU-PING TANG, XUE-WEI XIE, YUAN-HONG SHI, NA LIU, SUI-QIANG ZHU[2], ZAI-WANG LI, AND YUN CHEN

Department of Neurology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei, China

Objective To investigate the effects of combined transplantation of neural stem cells (NSC) and olfactory ensheathing cells (OEC) on the motor function of rats with intracerebral hemorrhage. **Methods** In three days after a rat model of caudate nucleus hemorrhage was established, NSCs and OEC, NSC, OEC (from embryos of Wistar rats) or normal saline were injected into hematomas of rats in combined transplantation group, NSC group, OEC group, and control group, respectively. Damage of neural function was scored before and in 3, 7, 14, 30 days after operation. Tissue after transplantation was observed by immunocytochemistry staining. **Results** The scores for the NSC, OEC and co-transplantation groups were significantly lower in 14 and 30 days after operation than in 3 days after operation ($P<0.05$). The scores for the NSC and OEC groups were significantly lower than those for the control group only in 30 days after operation ($P<0.05$), while the difference for the NSC-OEC group was significant in 14 days after operation ($P<0.05$). Immunocytochemistry staining revealed that the transplanted OEC and NSC could survive, migrate and differentiate into neurons, astrocytes, and oligodendrocytes. The number of neural precursor cells was greater in the NSC and combined transplantation groups than in the control group. The number of neurons differentiated from NSC was significantly greater in the co-transplantation group than in the NSC group. **Conclusion** Co-transplantation of NSC and OEC can promote the repair of injured tissue and improve the motor function of rats with intracerebral hemorrhage.

Key words: Stem cell; Olfactory bulb; Cell transplantation; Intracerebral hemorrhage; Motor function

INTRODUCTION

Intracerebral hemorrhage (ICH) is a severe type of acute vascular disease with a high rate of morbidity, mortality, and disability. It is a currently active research field to reduce the disability rate of ICH at acute and convalescent stages, although to date little progress has been made. With the development of methods for the isolation and culture of stem cells, many researchers have attempted to treat ICH with stem cell transplantation. Jeong *et al.* reported that treatment of ICH with neural stem cell (NSC) transplantation in rats can improve neural function[1]. An *et al*. have also reported similar results[2]. In the present study, olfactory ensheathing cells (OEC) and NSC were co-transplanted to explore the effect of co-transplantation on the motor function of rats with ICH.

MATERIALS AND METHODS

Experimental Materials

Experimental animals Healthy male Wistar rats at the age of 1-11 weeks, weighing 250-300 g, were purchased from the Experimental Animal Center, Tongji Medical College, Huazhong University of Science and Technology, China. All procedures involving animals, approved by the Animal Experimentation Ethics Committee of Huazhong University of Science and Technology, were consistent with the Chinese Laws and Regulations of Practice for the Care and Use of Animals for Scientific Purpose.

Main immunohistological reagents 5-bromodeoxyuridine (Brdu), mouse anti-Brdu monoclonal antibody, and rabbit anti-mouse P75 antibody were purchased from Sigma, USA. Mouse

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[2]Correspondence should be addressed to Sui-Qiang ZHU, Tel: 86-27-83662418, Fax: 86-27-83662418. E-mail: ddjtzp@163.com

Biographical note of the first author: Zhou-Ping TANG, male, born in 1969, MD, majoring in clinical and experimental research with intracerebral hemorrhage.

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anti-nestin polyclonal antibody was bought from Chemicon, Australia. 3, 3′-diaminobenzidine (DAB) staining kit, biotin-labeled goat anti-mouse II antibody, and horseradish peroxidase (HRP)-labeled avidin were obtained from Zhongshan Company, China. Mouse anti-glial fibrillary acidic protein (GFAP) IgG1, mouse anti-2′, 3′-cyclic nucleotide 3′-phosphodiesterase (CNP) IgG1, and mouse anti-tubulin-III IgG1 were purchased from Neo Market Company, USA. Avidin-labeled Cy3 was bought from KPL Company, USA. Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG1 was purchased from Serotec Company, USA.

Preparation of rat models of caudate nucleus ICH Rats were injected intraperitoneally with 6% chloral hydrate (5 mL/kg) and then mounted on a stereotaxic frame. Following skin preparation and disinfection, a 1 cm-long incision in the scalp was made along the midline, followed by drilling of a burr hole (coordinates: 3.2 mm lateral and 1.4 mm posterior to the bregma). A 50-μL gauge needle was inserted vertically through the burr hole into 5.6 mm of the caudate nuclei. Collagenase type IV (0.4 U in 2 μL) was injected over a period of 10 min, with the needle left in place for an additional 4 minutes after injection. The needle was gently removed and the wound was sutured.

Preparation of transplanted cells (1) Preparation of neural cells: the subventricular zone from Wistar rat embryos was triturated and filtered. The harvested cells were cultured and passaged. The NSC thus obtained were labeled with Brdu and repeatedly centrifuged at 800 rpm for 5 min. The pelleted cells were adjusted to a concentration of 4×10^4/μL with D-Hanks solution. (2) Preparation of OEC suspension: the olfactory bulb from Wistar rat embryos of 16D was digested with trypsin. The harvested cells were cultured and passaged. The OEC thus obtained were labeled with rabbit anti-mouse P75 antibody and repeatedly centrifuged at 800 rpm for 5 min. The pelleted cells were adjusted to a density of 4×10^4/μL with D-Hanks solution.

Stereotaxic cell transplantation Three days after establishment of the caudate nucleus ICH model, the rats were weighed, intraperitoneally anesthetized with chloral hydrate, and then placed in the stereotaxic frame. After disinfection, an incision was made in the scalp along the midline to expose the cranial bone. A cell suspension was microinjected into 6.6 mm brain tissues at a rate of 1 μL/min under the guidance of a stereotaxic device (coordinates: 3.2 mm lateral and 1.4 mm posterior to the bregma). Five minutes after approximately 5 μL of cell suspension was injected, the microinjector was withdrawn 2.0 mm and another 5 μL of cell suspension was subsequently injected. After 5 min, the needle was gently removed and the wound was sutured.

Experimental groups The rats with ICH were randomly divided into 4 groups, 16 in each group. Rats in group A and control group were subjected to microinjection of 10 μL normal saline without cell transplantation. Rats in group B were subjected to microinjection of 10 μL NSC suspension. Rats in group C were subjected to microinjection of 10 μL OEC suspension. Rats in group D were subjected to co-transplantation of OEC (4×10^4/μL) and NSC (4×10^4/μL) at a total volume of 10 μL.

Behavioral testing The neurological deficits were scored using a Zea-Longa's scoring system as previously described[3] (0=no deficit, 1=failure to extend the left forepaw fully, 2=circling to the left, 3=falling to the left, 4=no spontaneous walking with a depressed level of consciousness, and 5=dead). The neurological deficits were scored before and 3, 7, 15, and 30 days after transplantation.

Histological examination One day after establishment of the models, rats in each group were killed. The injured tissues were serially cut into 10-μm thick sections, observed, and photographed. After 3, 7, 14, and 30 days of transplantation, paraffin-embedded sections of tissues taken from the transplantation site were prepared Survival, migration and differentiation of the transplanted NSC and OEC were observed by immunohistochemical staining according to the instructions of kit used. Differentiation into neurons, astrocytes, and oligodendrocytes was monitored by immunofluorescence double staining with Brdu/tubulin, Brdu/GFAP, and Brdu/CNP, respectively, according to the manufacturer's instructions.

Assessment of staining results (1) Number of neural precursor cells: ten randomly selected, non-overlapping fields in the periphery of each caudate nucleus hematoma section were counted (magnification, ×200). (2) Number of neural cells: the total number of cells and the number of positive cells were counted in 10 randomly selected, non-overlapping fields of 4 hematoma sections (on days 3, 7, 14, and 30) for each parameter (magnification, ×800). The proportion of positive cells was calculated and presented as percentages.

Statistical Analysis

All data are expressed as $\bar{x} \pm s$. Statistical analysis was performed using SPSS 10.0 software. $P<0.05$ was considered statistically significant. Analysis of the number of neural precursor cells and neurons was performed using t and χ^2 test, respectively. Analysis of variance and q test were used in behavioral testing.

RESULTS

Behavioral Observation and Neurological Deficit Scores

Four hours after collagenase injection into the caudate nuclei, the neurological deficit scores for all rats were greater than 2. However, the neurological deficit scores for rats in the 4 groups exhibited a decreasing tendency after cell transplantation (Table 1). The scores for rats in the control group were obviously lower in 30 days than in 3 days after operation ($P<0.05$). In contrast, the scores for rats in the NSC, OEC, and combined transplantation groups, significantly lower in 14 and 30 than in 3 days after operation. The scores for rats in the NSC and OEC groups were markedly lower in 30 days after operation than those for rats in the control group ($P<0.05$). The scores for rats in the combined transplantation group, were significantly lower in 14 and 30 days after operation than those for rats in the control, NSC, and OEC groups ($P<0.05$). No significant difference was found in scores for rats in the NSC and OEC groups at different time points ($P>0.05$).

TABLE 1

Neurological Deficit Scores for Rats after Operation ($\bar{x} \pm s$)

Group	*n*	Neurological Deficit Scores at Different Time Points			
		Day 3	Day 7	Day 14	Day 30
A	16	2.76 ± 0.20	2.47 ± 0.22	2.32 ± 0.15	1.96 ± 0.12
B	16	2.75 ± 0.20	2.33 ± 0.20	2.20 ± 0.22	1.60 ± 0.13
C	16	2.74 ± 0.21	2.32 ± 0.21	2.21 ± 0.20	1.50 ± 0.21
D	16	2.75 ± 0.21	2.28 ± 0.11	1.33 ± 0.14	1.01 ± 0.11

Changes of Grafts in ICH Rats

Immunocytochemical studies of NSC and OEC Immunohistochemical analysis demonstrated that almost all neurospheres were positive for the neural stem cell marker nestin. The majority of cells produced from differentiating neurospheres were immunoreactive for 5-bromodeoxyuridine (Brdu). Counterstaining of cell nuclei with DAPI confirmed the presence of Nestin-negative cells in the culture, which documented the presence of several cell phenotypes (Fig. 1). OEC were identified in culture by their characteristic morphology (bi- or multipolar cells with long processes, small nuclei, and reduced cytoplasm and expression of p75-LNGFR or GFAP (Fig. 2).

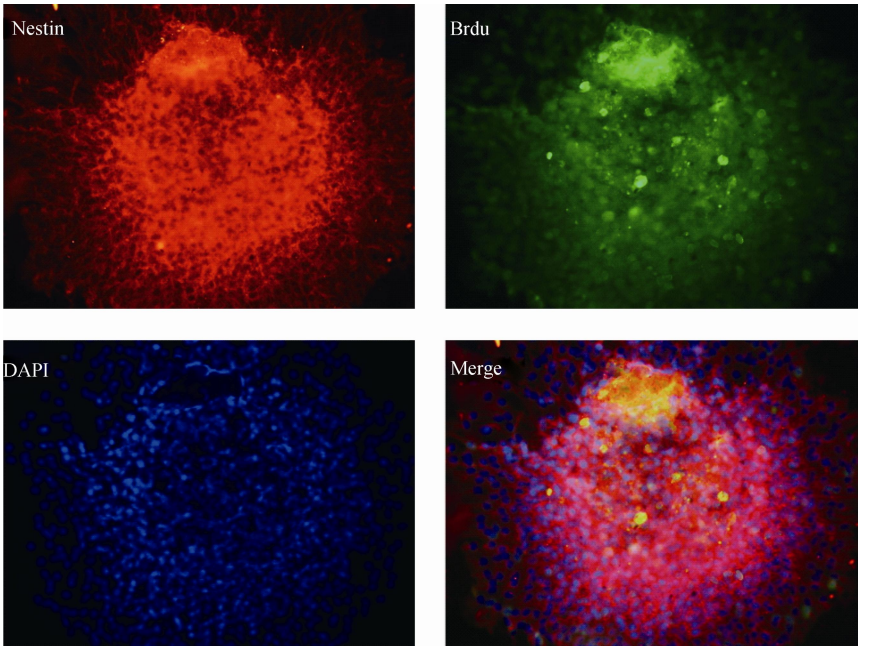

FIG. 1. Immunofluorescence analysis of NSC features showing expression of neural stem cell markers nestin and proliferating marker Brdu.

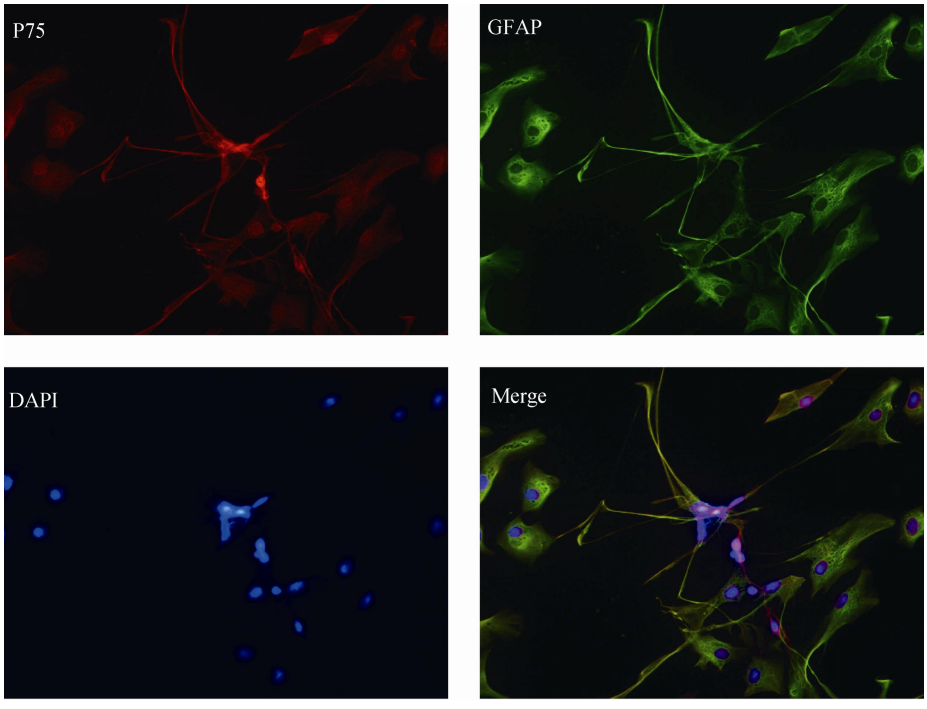

FIG. 2. Immunofluorescence analysis of OEC features showing expression of p75-LNGFR and GFAP.

Immunohistochemical detection of brdu-labeled NSC In three days after operation, accumulation of a large number of round Brdu-labeled NSC was observed at the periphery of hematoma (Fig. 3). Brdu-positive cells were also found around the needle entry site and at the center of hematoma loci (Fig. 4), indicating the active migration of NSC. The greatest migration distance of transplanted NSC was up to 2 mm in 7 and 14 days after operation. The longer the time after transplantation was, the greater the number of Brdu-positive cells in the perihematomal areas and hematoma loci was, suggesting that the transplanted NSC can divide and proliferate (Fig. 5).

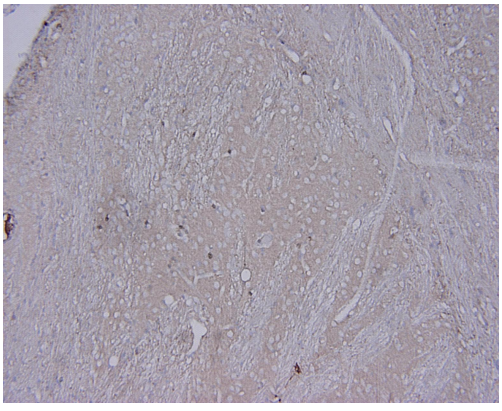

FIG. 4. Brdu positive cells in hematoma loci (×200).

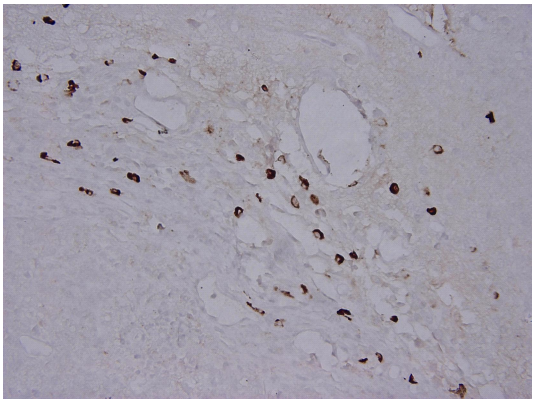

FIG. 3. A large number of Brdu-labeled NSC around hematoma loci (×400).

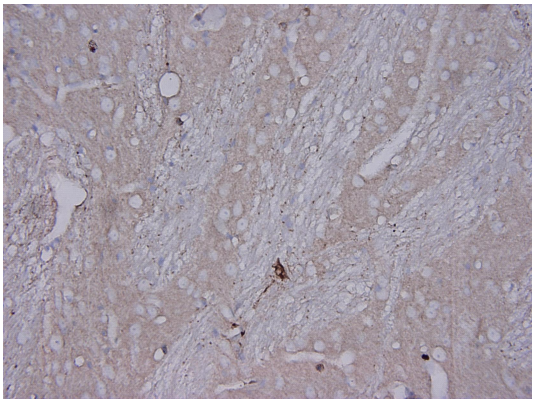

FIG. 5. Increasing Brdu positive cells in hematoma loci 14 days after operation (×400).

Immunohistochemical detection of nestin In seven days after operation, the number of nestin-positive cells at the periphery of hematoma was significantly greater in the NSC and combined transplantation groups than in the control and OEC groups. Thus, the number of nestin-positive cells was significantly increased at the same sites at which Brdu-positive cells were observed (Fig. 6). The number of nestin-positive cells was somewhat reduced in 14 days than in 7 days after operation in the NSC and combined transplantation groups, suggesting that the transplanted NSC can survive at the transplanted site and promote repair of the injured tissues in rats, and that the surviving NSC have differentiated into neurons and gliocytes.

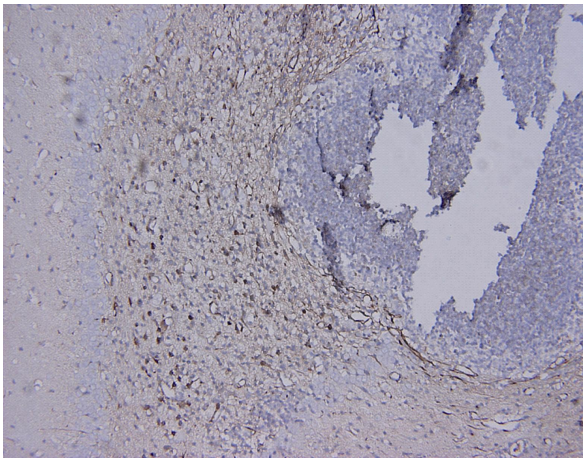

FIG. 6. Significantly increasing nestin positive cells in the combined transplantation group 7 days after operation (×200).

Detection of Brdu/tubulin, Brdu/GFAP, and Brdu/CNP In order to investigate the differentiation of transplanted NSC, Brdu/tubulin, Brdu/GFAP, and Brdu/CNP were monitored using the immunofluorescence double-labeling method to study the growth and differentiation of Brdu-labeled NSC in rats at different time points after operation. Cells positive for Brdu+/tubulin+, Brdu+/GFAP, and Brdu+/CNP+ staining indicated that the transplanted NSC were differentiated into neurons (Fig. 7), astrocytes (Fig. 8), and oligodendrocytes (Fig. 9), respectively.

Differentiation of transplanted NSC into neurons in NSC and combined transplantation groups The number of neurons differentiated from NSC was significantly greater in the combined transplantation group than in the NSC group (Table 2, χ^2=154.79, P<0.01).

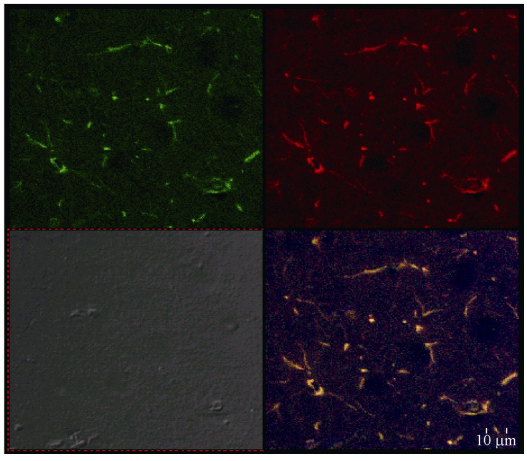

FIG. 7. Differentiation of Brdu-labeled NSC into neurons (Brdu+/tubulin+, Confocal laser scanning microscopy×800).

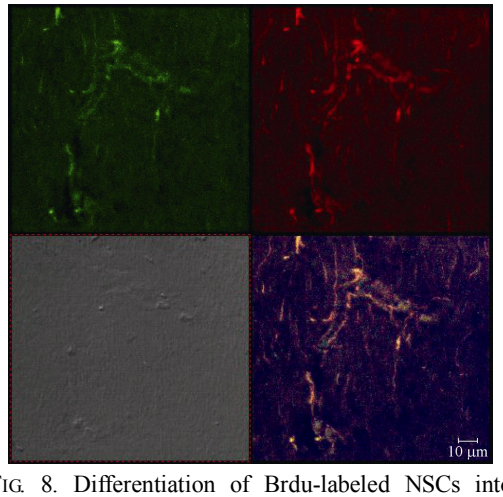

FIG. 8. Differentiation of Brdu-labeled NSCs into astrocytes (Brdu+/GFAP+, confocal laser scanning microscopy×800).

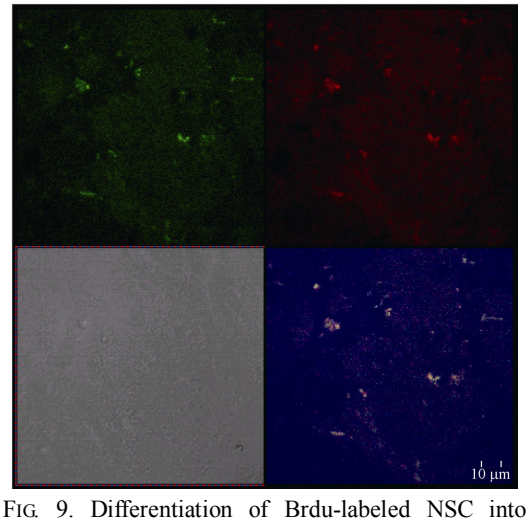

FIG. 9. Differentiation of Brdu-labeled NSC into oligodendrocytes (Brdu+/CNP+, confocal laser scanning microscopy×800).

TABLE 2

Number of Neurons in NSC and Combined Transplantation Groups

Group	Brdu+/tubulin+	Brdu+	Percentage of Neurons (%)
NSC	55	1 861	2.96
Combined Transplantation	312	2 198	14.19

DISCUSSION

In this study, transplanted NSC differentiated into a few neurons in the NSC group, suggesting that the majority of NSC can differentiate into gliocytes, which is consistent with the reported data[2]. This differentiation is probably induced by serum components derived from hematoma. *In vitro* serum-induced differentiation experiments showed that the higher the serum ratio, the greater the number of differentiated gliocytes[4], suggesting that the differentiation of NSC is related to the surrounding environment. Other studies also found that transplanted NSC can differentiate into cells similar to those in the adjacent areas of the host[5-8]. After co-transplantation of NSC and OEC, the number of neurons differentiated from NSC was significantly increased (Table 2), suggesting that OEC can promote the division and differentiation of NSC in brain tissues, which may be explained by the following mechanism by which NSC and OEC can change the microenvironment in the transplanted area by secreting growth factors and extracellular matrix during cell division, differentiation, and migration, thus providing favorable conditions for the regeneration of neural tissues, including inhibiting formation of glial scar, promoting growth of neurites, and building a "bridge" composed of OEC for the regeneration of neuritis[9-15].

The scores for neurological deficits in the control group were significantly lower in 30 days after operation than in 3 days after operation. However, the scores for neurological deficits in 3 transplantation groups were significantly decreased in 14 days after operation ($P<0.05$), particularly in the combined transplantation group, in which the scores for neurological deficits in 30 days after operation were lower than those in the other 3 groups ($P<0.05$), indicating that although the transplantation of OEC or NSC alone can promote the repair of injured tissues and restore motor function in rats with caudate nucleus ICH, the effects of co-transplantation of OEC and NSC are more pronounced.

In conclusion, following co-transplantation of NSC and OEC, OEC promote the proliferation and differentiation of endogenous neural precursor cells and exogenous NSC. OEC are involved in the regeneration and myelinization of neurites and subsequently promote the growth of host neurons. These properties can significantly increase the curative effect of cell transplantation in the treatment of ICH.

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