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Chitosan-collagen porous scaffold and bone marrow mesenchymal stem cell transplantation for ischemic stroke

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Abstract

In this study, we successfully constructed a composite of bone marrow mesenchymal stem cells and a chitosan-collagen scaffold *in vitro*, transplanted either the composite or bone marrow mesenchymal stem cells alone into the ischemic area in animal models, and compared their effects. At 14 days after co-transplantation of bone marrow mesenchymal stem cells and the hitosan-collagen scaffold, neurological function recovered noticeably. Vascular endothelial growth factor expression and nestin-labeled neural precursor cells were detected in the ischemic area, surrounding tissue, hippocampal dentate gyrus and subventricular zone. Simultaneously, a high level of expression of glial fibrillary acidic protein and a low level of expression of neuron-specific enolase were visible in BrdU-labeled bone marrow mesenchymal stem cells. These findings suggest that transplantation of a composite of bone marrow mesenchymal stem cells and a chitosan-collagen scaffold has a neuroprotective effect following ischemic stroke.

Key Words: *nerve regeneration; ischemic stroke; chitosan-collagen scaffold; bone marrow mesenchymal stem cells; cell transplantation; cell differentiation; neurological function; neural regeneration*

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Introduction

The central nervous system can activate the proliferation, migration and differentiation of bone marrow mesenchymal stem cells (BMSCs) through an innate brain repairing and/or reshaping (brain plasticity) mechanism to recover the stability of the internal environment and to promote the repair of neurological function (Bjorklund and Lindvall, 2000). Our team observed local proliferation in the subventricular zone from the first day after damage in the adult rat models of brain injury, which reached a peak on day 7 (Zhang et al., 2006). However, endogenous neural stem cells in the brain with poor ability to migrate and differentiate into functional neurons only proliferated during a certain period after brain tissue damage (Li et al., 2015).

Some studies have shown that multiple hormone and neurotrophic factors secreted by BMSCs can promote the growth, differentiation and protection of endogenous neural precursor cells, which contribute to nerve repair and regeneration (Cantinieaux et al., 2013; Xia et al., 2013). Chitosan-collagen is a common tissue-engineered material for scaffolds and has been widely used in transplantation because of its advantages, including strong biodegradability, low antigenicity, good biocompatibility and lack of pyrogen reaction (Dash et al., 2011; Ji et al., 2011). In this study, we sought to observe the effects of transplantation of BMSCs and an absorbable chitosan-collagen porous scaffold on ischemic stroke.

Materials and Methods

Separation, purification, identification and labeling of BMSCs

Six healthy male Wistar rats aged 3 weeks and weighing 100 g were purchased from the Environment Institute of the Academy of Military Medical Sciences in China (License No. SCXK (Jing) 2012-003). The experiment was conducted in the Center Laboratory of Shaanxi Provincial People's Hospital of China. The whole bone marrow adherent culture method (Song et al., 2014) was used to obtain primary cultured BMSCs. Briefly, after rats were sacrificed under anesthesia with an intraperitoneal injection of chloral hydrate (0.4 g/kg; Sigma), the femur and tibia were separated under sterile conditions, and the medullary canal was rinsed

with serum-free Dulbecco's modified Eagle's medium/F12 (DMEM/F12) medium (HyClone, Waltham, MA, Amityville, NY, USA), collected in sterile tubes and centrifuged. Subsequently, the fat and supernatant were discarded, and the resuspended cells at a density of 1×10^6/mL were incubated in a 50 mL culture flask with DMEM/F12 medium containing 10% fetal bovine serum. The flask was placed in a CO_2 incubator (NAPCO Company, Amityville, NY, USA) with 5% CO_2 at 95% humidity and 37°C for 48 hours. After the culture medium was changed, non-adherent cells were removed, and the medium was changed every 2–3 days. When the cultured cells reached 80% confluence, cells were digested with 0.25% trypsin and subcultured for three passages. Flow cytometric analysis (Song et al., 2014) was used to detect CD29 and CD45 for the purity identification of the third passage cells. As a member of the integrin family, CD29 is strongly expressed in BMSCs and is a common surface marker of BMSCs. Simultaneously, CD45 is a marker for hematopoietic stem cells, and is not usually expressed in BMSCs (Kuhn and Tuan, 2010; Yang et al., 2014). Forty-eight hours before transplantation, 10 μM 5-bromo-2′-deoxyuridine (BrdU; Sigma, St. Louis, MO, USA) was added to the mesenchymal stem cell medium and replaced the nucleotide thymine in the DNA of replicating cells.

Preparation and detection of BMSCs and chitosan-collagen porous scaffold

The chitosan-collagen porous scaffolds 3.0 mm × 3.0 mm × 2.0 mm were prepared using the freeze-drying method (Zhang et al., 2013). After disinfection in alcohol, the scaffold was co-cultured with BrdU-labeled BMSCs at a cell concentration of 1×10^6/μL *in vitro* for 48 hours. A scanning electron microscope (Hitachi, Tokyo, Japan) was used to observe the growth of BMSCs on the scaffolds.

3-(4,5-Cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay for the growth of BMSCs on the chitosan-collagen porous scaffolds

The third passage cell suspension was uniformly inoculated into a 96-well plate. A 200-μL suspension containing 10,000 cells per well was inoculated onto the scaffolds in the experimental group and with DMEM/F12 complete medium containing 10% fetal bovine serum in the control group, respectively. From day 3 after inoculation, 15 μL of MTT (Amresco Inc., Solon, Ohio, USA) liquid (5 g/L) was added into four wells in each group. After 4 hours of culture, the supernatant was discarded. Then, 150 μL of dimethyl sulfoxide was added per well to completely dissolve formazan. Optical density value at 490 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA). Average values from four repeated tests were obtained for each of 5 consecutive days to construct a cell growth curve.

Establishment of animal models of cerebral ischemia

Twenty-one male specific-pathogen-free Wistar rats aged 10–12 weeks and weighing 280–320 g were provided by the Environment Institute of the Academy of Military Medical Sciences of Chinese PLA. Rats were raised separately and kept on a 12-hour light/dark cycle, with free access to normal food and water in a cage. Rats were acclimatized for more than 1 week. Wistar rats were randomly divided into a model group, a BMSCs group and a co-transplant group, with seven rats in each group.

According to the modified Zea-Longa suture method, first described by Longa et al. (1989) and revised by Ma et al. (2006), intraperitoneal anesthesia was given with 10% chloral hydrate (0.33 mL/100 g). After inserting the blunt end of a sterilized carbonline wire (5 cm in length, 0.26 mm in diameter) into the right internal carotid artery, the micro-artery clamp was released. The wire was slowly pushed upward until it was approximately 1.8 ± 0.1 cm from the middle cerebral artery bifurcation. It was slightly withdrawn if there was any resistance. The wire was fixed on the external carotid artery leaving approximately 6 mm outside. The temperature was maintained at 37°C during the operation. Immediately after the operation, 2% Evans blue (7 mL/kg) was intraperitoneally injected to dye the ischemic brain tissue blue. After the operation, ipsilateral Horner's syndrome on the ischemic side soon appeared (ipsilateral drooping eyelids, small eyelid fission). Two hours after the operation, the carbon wire was extracted and the suture was withdrawn 10 mm for reperfusion. Model establishment was considered successful with the appearance of contralateral hemiplegia (limb disturbance such as flexion and adduction) and contralateral circling while walking. When the rats were fully awake, they were sent back to the animal rooms for feeding and observation. Rats with Neurological Severity Scores (Wang et al., 2014) of 1–3 points were included and some were randomly added; those scoring 0 or 4 points, and those that died, were excluded.

BMSCs/scaffold co-transplantation

Twenty-four hours after surgery, craniotomy decompression on the ipsilateral side was performed after anesthesia. By craniotomy, the BMSCs/scaffold composite was directly covered in the ischemic and infarct areas (Evans blue-stained zone) in the co-transplant group. Rats were placed on a stereotaxic frame, and 10 μL of BMSC suspension (1×10^6/μL) was injected under stereotactic guide using a micro syringe into the tissues around the infarct area in the BMSC group. DMEM/F12 complete medium (10 μL) was separately injected into the same brain area in the model groups.

Behavioral assessment

Neurological Severity Scores were determined before surgery (day 0) and 1 (before transplantation), 7, and 14 days after surgery. Neurological Severity Scores were assessed as follows: normal neurological function, no nerve defect signs (0 points); mild nerve defect, unable to fully flex the left forearm while lifting the tail (1 point); moderate nerve defect: rotating to the left (2 points); dumping to the left (3 points); no spontaneous walking, consciousness impairment (4 points); ischemia-related death (5 points).

Hematoxylin-eosin staining and immunohistochemical staining

Brain tissues taken out at days 7 and 14 after transplantation

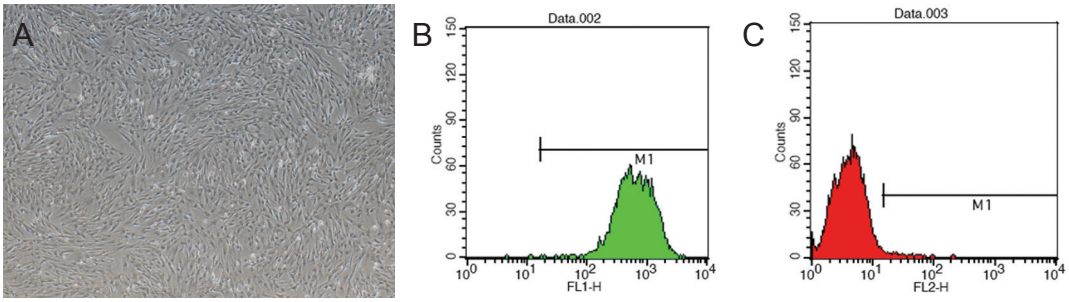

Figure 1 Morphology and identification of BMSCs.
(A) Morphology of the tenth passage BMSCs (× 40). (B, C) Surface antigen CD29 (99.97%) and CD45 (0.89%) of the third passage BMSCs identified by flow cytometry. BMSCs: Bone marrow mesenchymal stem cells.

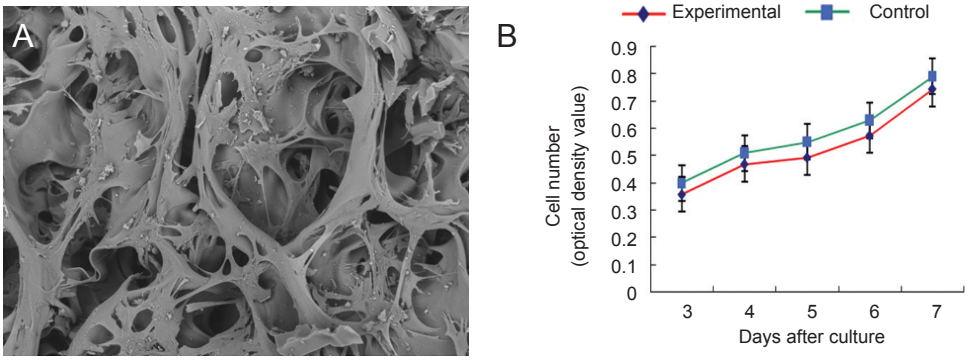

Figure 2 Compatibility of BMSCs with the chitosan-collagen porous scaffold in three-dimensional co-culture.
(A) Coculture of the chitosan-collagen porous scaffold and BMSCs for 48 hours under a screening electronic microscope (× 300). (B) Growth curve of BMSCs in the experimental group (three-dimensional culture) and the control group (conventional culture) (MTT assay). Data are expressed as the mean ± SD. The experiment was repeated in triplicate. The paired *t*-test and one-way analysis of variance were used for data analysis within and between groups. BMSCs: Bone marrow mesenchymal stem cells; MTT: 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

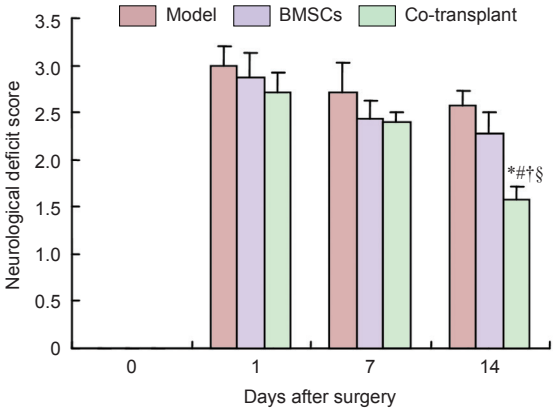

Figure 3 Effect of BMSC/scaffold co-transplantation on neurological function in ischemic stroke rats.
Data are expressed as the mean ± SD ($n = 7$). The paired *t*-test and one-way analysis of variance were used for data analysis within and between groups. *$P < 0.05$, *vs.* model group; #$P < 0.05$, *vs.* BMSCs group; †$P < 0.05$, *vs.* 1 day; §$P < 0.05$, *vs.* 7 days. BMSCs: Bone marrow mesenchymal stem cells.

were processed into 6-μm coronal paraffin sections (Lecia, Heidelberg, Germany). The tissue was fixed, dehydrated through graded ethyl alcohol, and embedded in paraffin. Subsequently, sections were conventionally dewaxed, stained with hematoxylin and eosin, and mounted. The hippocampus, cortex and striatum (Paxinos and Watson, 2005) on the ischemic side were observed under an optical microscope (Chongqing Optical Instrument Factory, Chongqing, China).

Immunohistochemical staining for nestin and vascular endothelial growth factor (VEGF), as well as double staining for BrdU/neuron-specific enolase (NSE) and BrdU/glial fibrillary acidic protein (GFAP), were used to indicate migration and differentiation of the transplanted mesenchymal stem cells. In brief, following a series of procedures, including xylene dewaxing, gradient ethanol hydration and running water washing, the slices were placed in citrate buffer (pH 6.0) in an oven at 65°C for 2 hours. Then, the slices were washed with PBS twice and incubated in 2 M HCl at 37°C for 30 minutes. The glass slices were cooled to room temperature and rinsed three times with PBS. The sections were incubated with sheep anti-BrdU antibody (1:100; Abcam, Shanghai, China), mouse anti-rat VEGF monoclonal antibody (1:3,000; CST, Shanghai, China), mouse anti-GFAP

antibody (1:200; CST), mouse anti-nestin antibody (1:200; CST) or mouse anti-NSE antibody (1:200; CST) at 4°C overnight. The coronal sections were washed with PBS and incubated with horseradish peroxidase-labeled rabbit anti-sheep-BrdU IgG antibody (ready to use; EarthOx Life Sciences, Millbrae, CA, USA) or sheep anti-mouse-GFAP and NSE antibody (ready to use; Zhongshan Jinqiao, Beijing, China) as secondary antibodies for BrdU-GFAP and BrdU-NSE. Each slice was supplemented with 100 μL of enhanced fresh 3,3′-diaminobenzidine (Fuzhou Maxim Biotech, Fuzhou, Fujian Province, China) solution. The sections were rinsed with running water, counterstained with hematoxylin, rinsed with running water, hydrated by gradient ethanol, permeabilized with xylene, and mounted with neutral resin. The region around the ischemic focus was observed under an optical microscope (Chongqing Optical Instrument Factory) with 200× magnification.

For double immunohistochemical staining, the sections were incubated with sheep anti-BrdU antibody, mouse anti-GFAP antibody and mouse anti-NSE antibody, simultaneously, at 4°C overnight. The sections were then washed with PBS and incubated with horseradish peroxidase-labeled rabbit anti-sheep-BrdU IgG antibody or sheep anti-mouse-GFAP and NSE antibody as secondary antibodies for BrdU-GFAP and BrdU-NSE. Other procedures were performed as for single immunohistochemical staining.

Statistical analysis

Data are expressed as the mean ± SD, and were analyzed using SPSS 13.0 (SPSS, Chicago, IL, USA) statistical software package. Paired *t*-test and one-way analysis of variance followed by Student-Newman-Keuls test were used for data analysis within and between groups, respectively. A value of $P < 0.05$ was considered statistically significant.

Results

Morphological observation and identification of the third passage BMSCs

Primary cultured BMSCs were isolated from four limbs of rats and cultured with the whole bone marrow adherent culture method for 8 days until cells reached confluence in whirls. The majority of cells were spindle shaped. The cells after subculture with pancreatic enzyme grew more rapidly so that subculturing was repeated 4 to 6 days later. Even after passage 10, the cells with a uniform morphology grew rapidly (**Figure 1A**). The high purity of the third passage BMSCs was detected using flow cytometry. Rates of positivity for CD29 and CD45 were 99.97% and 0.89%, respectively, meeting the requirements for cell transplantation (**Figure 1B**, **C**).

Compatibility of BMSCs with the chitosan-collagen porous scaffold in three-dimensional co-culture

After 48 hours of co-culture, under a scanning electron microscope, spindle-shaped BMSCs could be seen to have grown well in the three-dimensional culture environment, were widely distributed on the surface of the scaffolds and wells, and secreted extracellular matrix on the scaffolds with pseudopodia extending into the materials, indicating a good biocompatibility of cells with scaffold (**Figure 2A**). A growth curve based on MTT assay showed that, at 3 days after coculture, BMSCs were active in the logarithmic phase, and then entered a plateau phase. Proliferation declined. The morphology was similar to that of cells growing in conventional culture (**Figure 2B**).

BMSC/scaffold co-transplantation improved neurological function of ischemic stroke rats

There was no significant change in the neurological deficit scores in rats between the model and BMSCs groups on days 7 and 14 ($P > 0.05$). In the co-transplant group, the neurological deficit score on day 14 was significantly lower compared with that on days 1 and 7 ($P < 0.05$), while there was no significant difference in the reduction of neurological deficit score on days 1 and 7 ($P > 0.05$). On day 14, the neurological deficit score of rats in the co-transplant group was significantly lower compared with that of rats in the model and BMSCs groups ($P < 0.05$). There was no significant difference in the neurological deficit scores of rats between the model and BMSCs groups ($P > 0.05$; **Figure 3**).

BMSC/scaffold co-transplantation improved pathological changes in the brains of ischemic stroke rats

At 14 days after transplantation, hematoxylin-eosin staining showed cell degeneration in the hippocampus, cortex and striatum on the ischemic side in the brains of rats in the model group. Cell degeneration was reduced at corresponding sites of rats in the BMSCs and co-transplant groups (**Figure 4**). Immunohistochemical staining results showed that the level of VEGF expression at the transplanted area and its surroundings, the hippocampal dentate gyrus and the subventricular zone was significantly higher in the co-transplant group than in the BMSCs group, and only low-level VEGF expression was found in the above-mentioned areas of the model group. The number of nestin-positive neural precursor cells in the ischemic area, transplanted area, dentate gyrus and subventricular zone was significantly higher in the co-transplant group than in the model and BMSCs groups (**Figure 4**).

Double immunohistochemical staining experiments showed an increase in the number of BrdU-positive BMSCs in the ischemic area and its surroundings over time after transplantation. Some BrdU/NSE-positive cells had already migrated to the site surrounding the infarcted area or even into normal brain tissues, maximally to approximately 2 mm in distance. These cells were round and gathered together. In the cortex and striatum, no NSE or GFAP was observed in BrdU-labeled BMSCs during a short period after transplantation. On day 7, GFAP was only weakly detected and NSE was not detected in BrdU-labeled BMSCs; on day 14, the levels of GFAP and NSE had increased in BrdU-labeled BMSCs (**Figure 5**).

Discussion

BMSCs can proliferate and differentiate into cells to replace

those lost in the damage zone under the stimulation of the local microenvironment, which may repair the function of ischemic neurons through multiple channels (Chen et al., 2014). Achieving long-term survival, differentiation and proliferation of transplanted cells is the key to successful cell replacement therapy. However, different methods of stem cell transplantation may produce different efficacies (Lu et al., 2007).Continuous developments in tissue engineering and stem cell transplantation have led to scaffolds that successfully create a three-dimensional environment favorable for neural regeneration (McMurtrey, 2015). A formed cell/scaffold composite can now be directly placed in the damaged brain area. This orthotopic transplantation method could greatly increase the viability and quantity of stem cells retained in the necrotic area compared with the conventional intravenous injection and targeted transplantation, therefore reducing the loss of cells. Lu et al. (2007) placed a suitable collagen scaffold with BMSCs in the ischemic zone and found that the memory and motor functions of the rats improved, injury volume reduced, and BMSCs moved to the edge of the ischemic zone. The three-dimensional structure of the biomaterial scaffold not only provides a place for nutrition and growth metabolism for the cells, but also reduces glial scar formation (Hsieh et al., 2007; Bettahalli et al., 2013). Glial scar formation after neuronal necrosis could prevent axon regeneration into the injury zone and synaptic connection with normal nerve cells. Therefore, tissue reconstruction in the injury zone helps promote nerve cell regeneration.

In this study, hematoxylin staining showed the integration of the transplant composite with the host at approximately 10 days after transplantation, and plenty of cells were seen at the transplant point and the partial areas of the degraded chitosan-collagen porous scaffolds. A large number of BrdU-positive cells were found in the transplanted area and these migrated a short distance (≤ 2 mm) toward the surrounding area, which indicated a good integration of the transplant composite with the host. The implanted BMSCs in the brain microenvironment could survive and showed active proliferation and migration, but the cell migration distance did not increase with prolonged time, consistent with previous results, indicating that the migration occurred within a specific time window – only during cell division or just after division of transplanted cells (usually within 1–3 days after transplantation) – when precursor cells were undergoing active migration. Additionally, some transplanted cells may undergo apoptosis. No BrdU-positive cells were found in the hippocampal dentate gyrus and subventricular zone of rats, indicating that transplanted BMSCs were unable to migrate to the hippocampus and subventricular zone. The increase in the number of nestin-positive cells in the hippocampal dentate gyrus and subventricular zone after transplantation indicated that implanted BMSCs could increase the plasticity of neuronal receptors in the central nervous system by producing specific cytokines. However, the number of nestin-positive cells did not increase with time. Thus, we speculated that BMSCs transformed into neural precursor cells after transplantation and differentiated into neuron-like cells and glial cells at a certain time. Simultaneously, the BMSCs stimulated self repair of rat tissues. In addition, VEGF antibody staining revealed that BMSC/scaffold co-transplantation increased the number of VEGF-positive cells, which probably promotes the proliferation of blood vessels and improves blood supply.

The functional improvements in the BMSCs or co-transplant groups on day 7 partly benefited from the mobilized progenitor cells. Like endothelial cells, BMSCs can contribute to nerve growth, differentiation, and nutritional factors to protect neurons and, therefore, to simulate progenitor cells *via* neural receptors. Importantly, on day 14, neurological deficit scores were apparently decreased in the co-transplant group compared with the model and BMSCs groups, suggesting the obvious advantages of promoting the recovery of neurological function in the co-transplant group.

This study has several limitations. First, we did not include a middle cerebral artery occlusion + scaffold control group, because preliminary experimental results showed no treatment effect. Thus, we only focused on the model, BMSCs and co-transplant groups. Second, the number of animals was small. We will consider the above problems in our future studies.

In summary, BMSCs could differentiate into neuron-like and astrocyte-like cells as demonstrated by double immunohistochemical staining, and these cells were mainly distributed in the surroundings of the ischemic area. Apparently, the three-dimensional microenvironment augments the treatment effect of BMSCs. Coculture of BMSCs and tissue-engineered scaffolds may be a promising and feasible therapeutic strategy for ischemic stroke.

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Author contributions: *FY and WY performed statistical analysis, data interpretation and manuscript preparation. YLZ contributed to study concept, design and supervision. GCM, KG and ZXZ performed literature research and data extraction. YJZ and HL were responsible for quality control of data and accuracy. All authors wrote the manuscript and approved the final version of the paper.*

Conflicts of interest: *None declared.*

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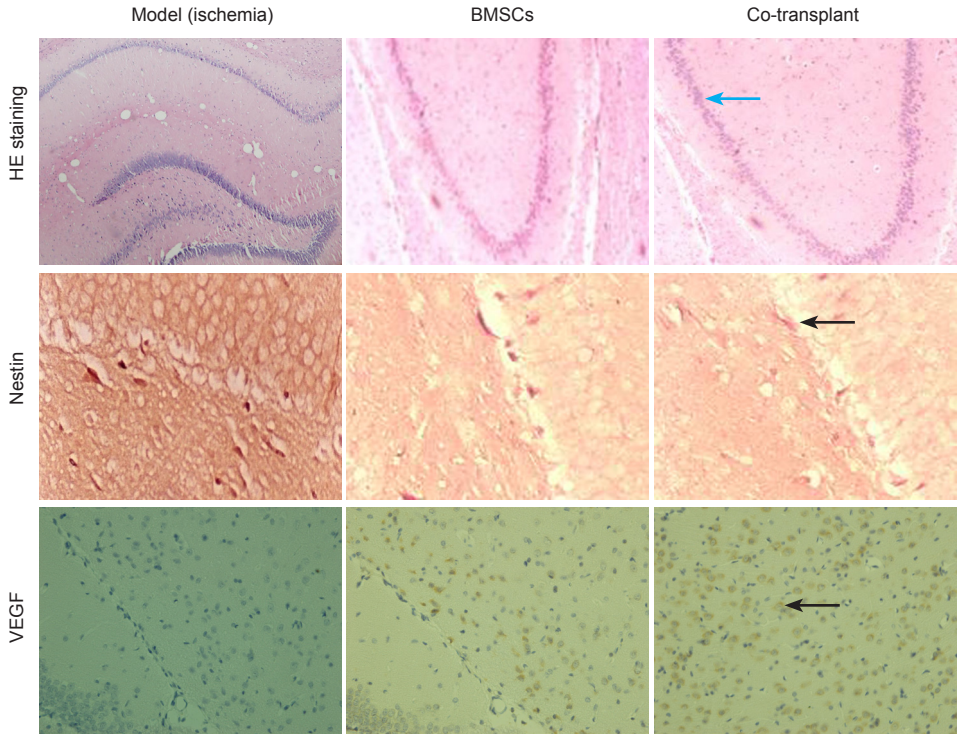

Figure 4 Effect of BMSC/scaffold co-transplantati on pathological change in the hippocampi of ischemic stroke rats at 14 days after transplantation (× 100).
Cell degeneration in the rat hippocampus (blue arrow) was decreased after BMSC transplantation alone or after BMSC/scaffold co-transplantation, and VEGF- (black arrow) and nestin (black arrow)-positive cells were distinct after BMSC/scaffold co-transplantation. BMSCs: Bone marrow mesenchymal stem cells; HE: hematoxylin-eosin; VEGF: vascular endothelial growth factor.

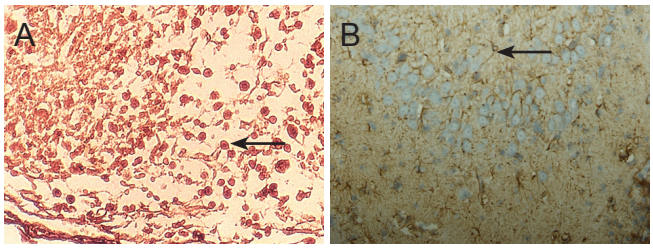

Figure 5 BMSCs migrated and differentiated on day 14 after transplantation.
(A) Double staining with BrdU/NSE (arrow) in the ischemic area of ischemic stroke rats after BMSC/scaffold co-transplantation (× 300); (B) double staining with BrdU/GFAP (arrow) in the ischemic area of ischemic stroke rats after BMSC/scaffold co-transplantation (× 200). BMSCs: Bone marrow mesenchymal stem cells; BrdU: 5-bromo-2-deoxyuridine; NSE: neuron-specific enolase; GFAP: glial fibrillary acidic protein.

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