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Original Investigation

Protective Effect of Ad-VEGF-Bone Mesenchymal Stem Cells on Cerebral Infarction

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ABSTRACT

AIM: To understand the mechanism of intracerebroventricular transplantation of vascular endothelial growth factor (VEGF) gene-modified bone mesenchymal stem cells (BMSCs) in rats after cerebral infarction.

MATERIAL and METHODS: The middle cerebral artery occlusion ischemia/reperfusion (MCAO I/R) model was established in rats using the Zea-Longa suture method. A recombinant adenovirus (Ad-VEGF) was engineered to express VEGF. The rats were divided into 3 groups. Control BMSC infected with control adenovirus (BMSC-Ad), BMSC infected by Ad-VEGF (BMSC-Ad-VEGF), and phosphate buffered saline (PBS) suspension were injected into the intracerebroventricular system of the rats in groups 1, 2 and 3 respectively, 24 hours after middle cerebral artery occlusion (MCAO). The neurological function of rats was evaluated with the modified Neurological Severity Scores (mNSS). The infarct volume of brain in rats was determined using 2,3,5-triphenyltetrazolium chloride (TTC) stain at 14 days. GFAP and pGSK3β expression of ischemic penumbra was determined using immunohistochemical method. GFAP, pAKT, AKT, and pGSK3β expressions were determined with Western blot.

RESULTS: Functional improvement was accelerated in animals receiving BMSC-Ad, while improvement at all times between 7 days and 28 days post MCAO was significantly greater in animals transplanted with BMSC-Ad-VEGF than for other treated animals. The number of GFAP-labeled cells was prevented by post-ischemic BMSC-Ad-VEGF treatment; pMCAO activate the PI3K/AKT/GSK3β pathway to reduce reactive gliosis.

CONCLUSION: Our findings demonstrate that PI3K/AKT/GSK3β pathway could reduce reactive gliosis, ameliorate neurological deficit, diminish the percentage of cerebral infarction volume in rats, and facilitate angiogenesis.

KEYWORDS: Bone mesenchymal stem cell, Vascular endothelial growth factor, Ischemic stroke, Intracerebroventricular injection, Phosphoinositide-3-kinase/Akt /glycogen synthase kinase-3 β pathway

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INTRODUCTION

Despite the development of medical technology, cerebral vascular disease (CVD), approximately 70% of which is cerebral infarction, remains as the major cause of death and disability worldwide (20,23). The tissue-type plasminogen activator substance, which is used to treat cerebral infarction, is the only drug approved by the U.S. Food and Drug Administration. However, given the secondary bleeding risk induced by thrombolytic therapy, only 3% of the patients with cerebral infarction can be treated with thrombolytic agents (11, 24). Based on its multi-directional differentiation and proliferation ability, transplanting bone mesenchymal stem cells (BMSCs) for CVD treatment has attracted increasing attention. More importantly, these cells can secrete various cytokines and neurotrophic factors, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) that can promote ischemia brain functional recovery (4). Vascular endothelial growth factor (VEGF), which can promote endothelial cell proliferation and angiogenesis, is important in nerve cell protection (6, 7). Moreover, some studies have suggested that PI3K/AkT/GSK3β could affect neuronal death in rat cerebral ischemia models of ischemia reperfusion, but the mechanisms of how to improve the ischemic injury remains unclear (8, 14, 18). In the present study, we aimed to investigate the therapeutic potential of the VEGF-BMSCs in cerebral ischemia of the rats.

MATERIAL and METHODS

Experimental Reagents and Materials

Ethical approval for this study was obtained from the Ethical Committe of The Second Hospital affiliated to Soochow University. Rat mesenchymal stem cell osteogenic differentiation medium (CYAGEN, CHINA), β-glycerophosphate, vitamin C, dexamethasone (Sigma), inverted phase contrast microscope (NIKON, Japan), medical clean bench (Jiangsu Sujing, China), adenovirus vector carrying human VEGF (Li Huiyong cooperative construction), stereotaxic instrument (Clinical Medical College of Jiangsu University), 2,3,5-3-triphenyl tetrazolium chloride (TTC, Amresco), 10% chloral hydrate, fishing line, anti-rat VEGF antibody (SANTA), anti-rat GFAP antibody (SANTA), anti-rat AKT antibody (Wuhan Boster), anti-rat pAKT antibody (Santa), rat GSK (Cell Signaling), and rat pGSK (Cell attracted increasing attention Signaling) were used.

METHODS

Rat BMSCs Culture

Approximately 100 g femur and tibia of male Sprague-Dawley (SD) rats were removed from the epiphyseal side, and then the femoral and tibial bone marrow cavity was flushed with L-DMEM culture. Then, the suspension was directly inoculated in the culture bottle and cultured in L-containing 10% foetal bovine serum in DMEM (FBSL-DMEM) at 37 °C in a humidified atmosphere with 5% CO_2. The medium was first changed after 24 h, and changed again after 48 h to remove non-adherent blood cells. The medium was then changed every 3 d. The BMSCs were passaged at ratio 1:2. When passaged to the third generation cells, pure rat BMSCs were obtained.

Expression of Rat BMSC Markers

Second-generation of rat BMSCs were digested and passaged into 24-well plate species. After culturing for 3 d, the BMSCs were treated with CY3-labeled rabbit anti-mouse CD44, CD105, CD133, and vimentin. The cells were observed under the fluorescence microscope and photographed. Nuclei were labelled with Hoechst 33342. PBS was used as a negative control instead of primary or secondary antibody.

Osteogenic and Adipogenic Differentiation of Rat BMSCS

Osteogenic culture: Third-generation rat BMSCs were seeded at 2×104 cells/well in six-well plate under osteogenic conditions: DMEM (L) + 10% foetal bovine + 10 mmol/L β-glycerophosphate + 1×l0-6 mol/L dexamethasone + 50 mg/L ascorbic acid. The medium was changed every 3 d, induced for 14 d, and calcium nodules were observed with the use of alizarin red S staining.

Adipogenic culture: The third-generation rat BMSCs were seeded at 2×104 cells/well in 2 mL of induced lipid A medium and incubated for 3 d, and then changed to 2 mL of induced lipid B medium, which was changed after 1 d. This process was repeated thrice. The cells were stained with oil red O, washed thrice with PBS, and then observed under inverted microscope. The cells containing stained red fat particles were recorded as adipogenic-positive cells.

Transplantation Procedure

Approximately 24 hours after SD rat model establishment (Figure 1A, B), the rats with neurological score from levels I to III were randomly divided into 3 groups (each n=20) and received intracerebroventricular inoculation of phosphate buffered saline (PBS), BMSC-Ad or BMSC-Ad-VEGF: Group 1 received PBS, Group 2 received BMSC infected with control adenovirus (BMSC-Ad) and Group 3 received BMSC infected by Ad-VEGF (BMSC-Ad-VEGF). A total of 60 SD rats were anesthetized with chloral hydrate (3 mL/kg intraperitoneal) and transferred to a stereotaxic apparatus (Figure 1B). Using aseptic technique 1 cm incision was made in the scalp 1.5 mm lateral to the bregma. Using a dental drill a burr hole was made in the bone 1.5 mm lateral to the bregma and the following suspension were slowly injected over 20 minutes into the lateral ventricle at a depth of 4.5 mm from the surface of the brain: Group 1: 20 μL PBS; Group 2: BMSC- Ad (1×10^6 cells); Group 3: BMSC-Ad-VEGF (1×10^6 cells).

Measurement of Infarct Volume

2,3,5-triphenyltetrazolium chloride (TTC) staining was employed to measure infarct volume. Five rats from each group were taken 14 d after cell transplantation and then sacrificed by anaesthesia overdose. Whole brain was removed and placed in the freezer for 10 min at -20 °C. Infarct volume was calculated using the formula V= t(A1 + An) - (A1 + An)/2 (t is the slice thickness, and A is infarction area). The volume of contralateral cerebral of infarction was calculated using the same method. The results was then calculated as infarct volume divided by contralateral cerebral of infarction volume.

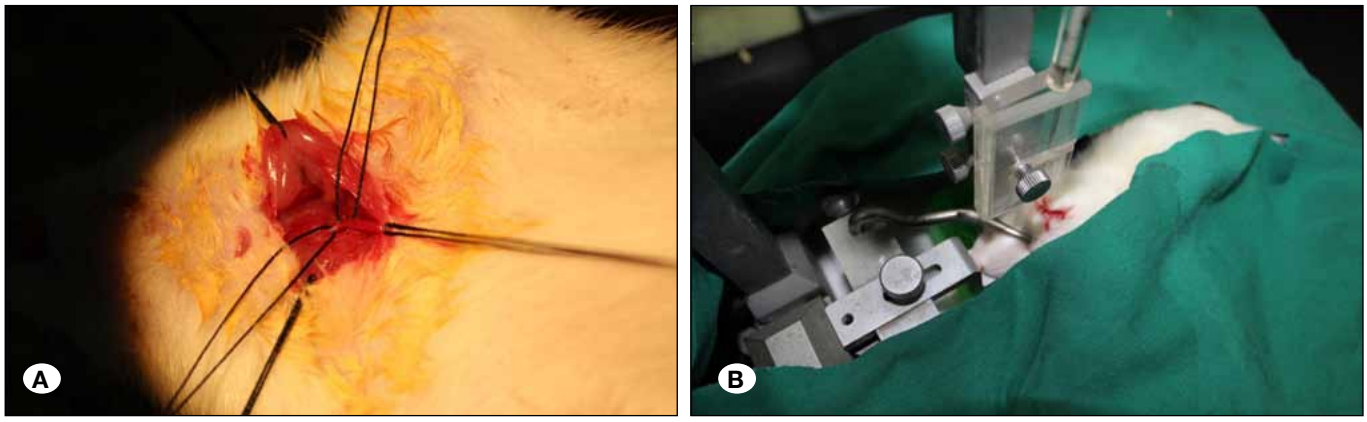

Figure 1: A) Establishment of MCAO I/R injury in rat using Zea-Longa suture method. **B)** Inoculation of PBS, BMSC-Ad and BMSC-Ad-VEGF using stereotactic method.

Behavioral Testing

Nerve function of each rat group was evaluated using modified neurological Severity Score (mNSS) (16) over 4 weeks (Figure 2A-F). The mNSS is a composite of motor (muscle status, abnormal movement), sensory (visual, tactile, proprioceptive), reflex, and balance tests. Neurological function is graded on a scale of 0 to 18, 0, normal; 1 to 6, mild injury; 7 to 12, moderate injury; 13 to 18, serious damage; and 18, most severe neurological deficits.

Histological Analysis

Five rats from each group were taken 14 days after cell transplantation and anaesthetised with the use of 10% chloral hydrate. The whole brain was removed and postfixed in 4% paraformaldehyde. Streptavidin-peroxidase was used to detect the GFAP and pGSK3β expression. Rabbit anti-rat GFAP and pGSK3β (1:400) antibodies were incubated overnight at 4 °C, and then washed thrice with PBS, added with DAB, stained with haematoxylin, dehydrated, sealed in transparent and neutral gum, and then observed under light microscope.

Western Blotting

For in vitro detection, the transfected and untransfected cells were collected, washed with PBS, and 50 μL lysis buffer added. After protein quantification using BCA method, buffer was added into the sample. The protein samples were boiled for 5 minutes to 10 minutes and then stored at -20 °C after aliquots were obtained.

For in vivo detection, the brains were removed, followed by left penumbra separation (100 g), which was placed in the grinder. Then 1 mL lysis buffer was added, and the penumbra was ground and treated with equal volume of sample buffer after protein quantification using BCA. The protein samples were boiled for 5 minutes to 10 minutes and stored at -20 °C after aliquots were obtained. After electrophoresis, the proteins were transferred to polyvinylidene fluoride membrane, and then sealed with 5% BSA for 1 hour at room temperature. Antibody was added first, and the samples were incubated overnight, followed by washing with TBST. The horseradish peroxidase-labelled secondary antibodies were then added, incubated for 1 hour, and washed by TBST and exposed to X-ray film.

Statistical Analysis

All data were expressed as means ± standard deviation (SD). Data were evaluated with repeated measures of analysis of variance (ANOVA), and the unpaired Student's t-test. Where data were normally distributed the Kolmogorov–Smirnov test was applied; in other cases the Mann–Whitney U-test was applied. The significance level was set at $P < 0.05$.

■ RESULTS

The Expression of VEGF in BMSC and Its Marker Expression and Induction of Osteogenesis and Adipocyte in Vitro

Immunofluorescence of 3 days cultured primary BMSCs showed positive CD44, CD105, CD133, and vimentin expression (Figure 3A). After adipocytic differentiation and oil red O staining, red fat particles were found in cells, which may be determined as fat cells. The cells became square or irregular in shape after osteogenic differentiation and then gradually transformed into osteoblasts, followed by round mineralised nodule formation. Meanwhile, calcium nodule formation was confirmed as alizarin red staining was positive (Figure 3B (a,b)). The results indicated that BMSCs with different functions were obtained.

Western blotting of BMSC-Ad-VEGF revealed a specific protein band at 46 kDa consistent with the predicted size of the VEGF protein. Little VEGF band was detected either in Ad-infected or uninfected cells. This experiment indicates that Ad-VEGF infects BMSC and efficiently directs the expression of VEGF protein (Figure 3C).

mNSS

There were no significant differences among the groups in NSS scores before MCAO or at day 1 after MCAO. Behavioral deficits showed progressive recovery in all groups from week 1 to week 4 after MCAO. At 14 days to 28 days after

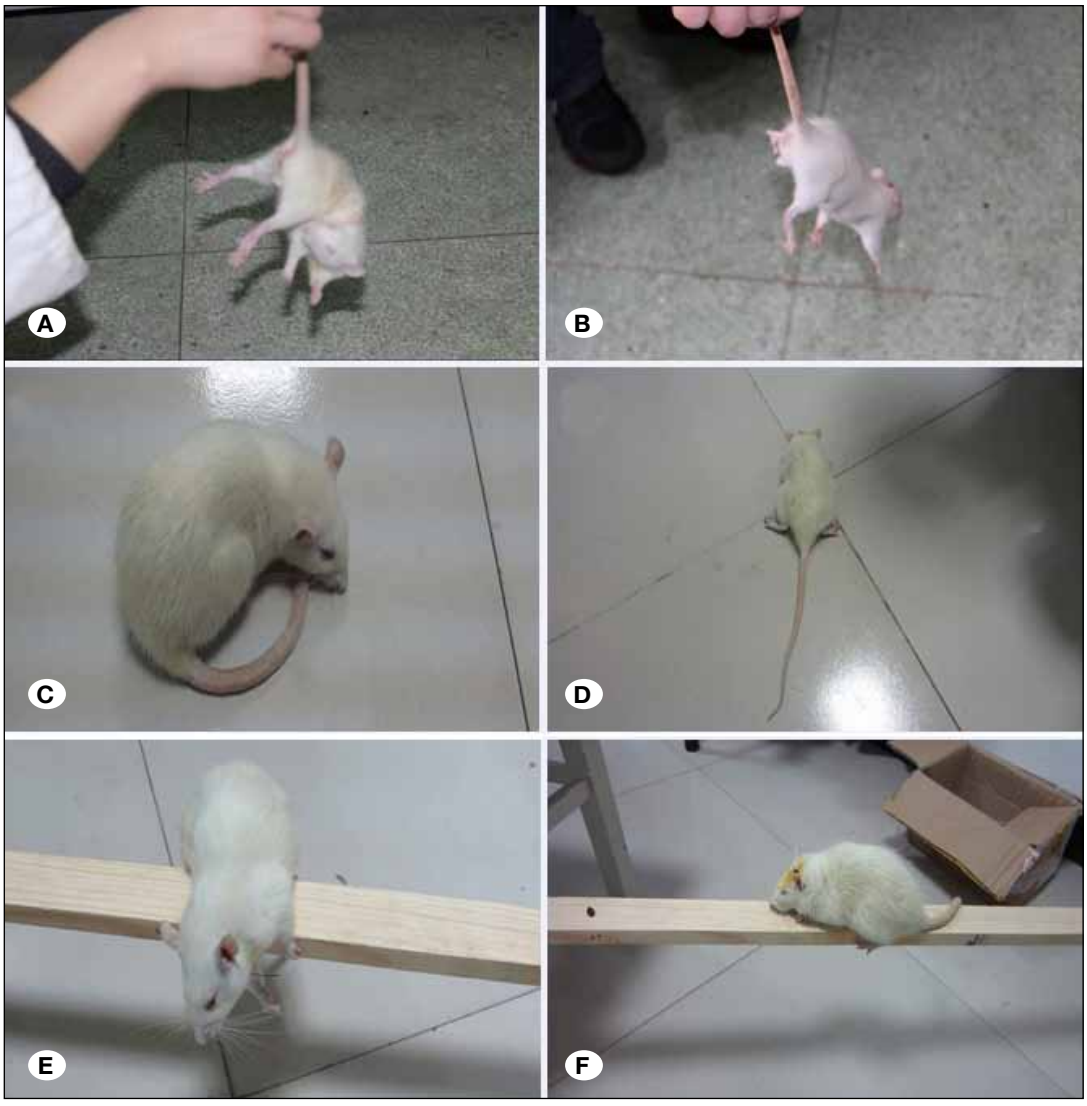

Figure 2:
A, C, E) Neurological assessment of the rats after MCAO I/R injury. **B, D, F)** After the injection of BMSC-Ad-VEGF.

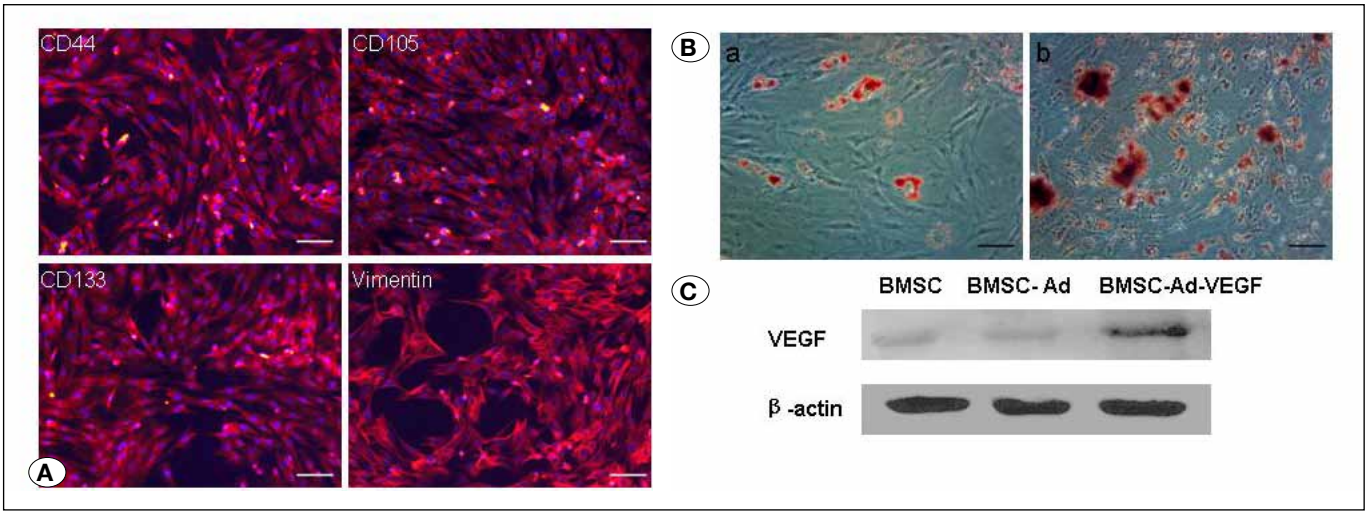

Figure 3: A) The surface markers of Bone marrow-derived mesenchymal stem cell, **B) a:** Adipogenic differentiation of BMSCs at 14 day. **b:** Osteogenic of differentiation BMSCs at 14 day. Scale bar equals 20μm. **C)** The VEGF protein differences between BMSC-Ad-VEGF and BMSC-Ad,PBS.

cell transplantation, compared with the PBS group, rat NSS scores of BMSC-Ad-VEGF and BMSC-Ad groups were significantly low (P <0.05). However, rats receiving BMSC-Ad-VEGF showed greater functional recovery than rats receiving BMSC-Ad (P <0.05, Figures 2A-F, 4) (Video 1 and 2).

Measurement of infarct and HE Staining

At 7 days after cell transplantation, the area percentage of infarct in BMSC-Ad-VEGF group (28.12 ± 1.73) was significantly lower than those of the PBS (40.46 ± 1.80) and BMSC-Ad group (35.30 ± 1.60) (P <0.05, Figures 5A, B).

At 7, 14, and 21 days after cell transplantation, brain tissue sections were prepared in each group followed by H&E staining. Brain tissues in each group showed oedema and disorderly morphological structure after 7 days. Moreover, a large number of inflammatory cell infiltrations were found. Inflammatory cells were found after 14 days, which had greater remission, and tissue morphology was restored to normal. No significant difference in tissue morphology was found after 14 and 21 days (Figure 5C).

BMSC-Ad-VEGF Activate pAkt/Akt and pGSK3β and Inhibit reactive Gliosis

Our findings are consistent with previous reports (9), in which the Western blot results showed that the activity of pAkt was upregulated after MCAO in BMSC-Ad-VEGF group compared with those in the other groups (Figure 6B).

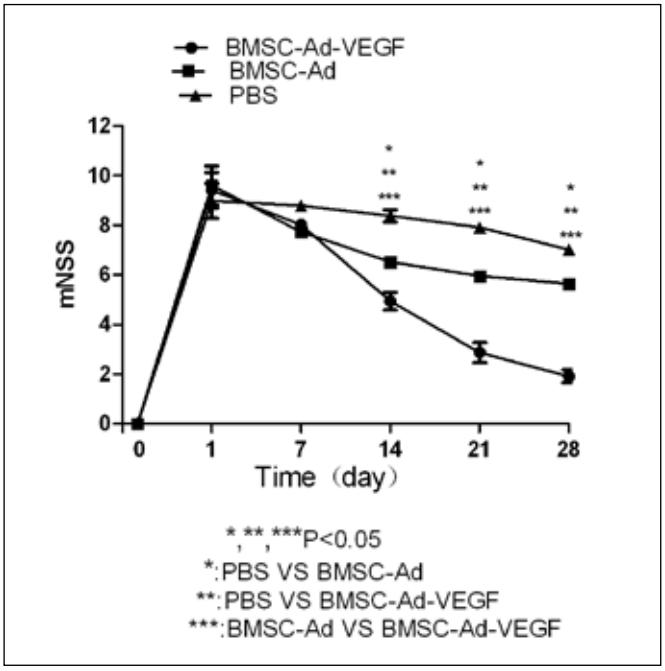

Figure 4: Nerve functions were evaluated through modified Neurological Severity Scores (mNSS). Compared with the other group, the rats' mNSS scores of BMSC-Ad-VEGF group were lower, the recovery of nerve function was the best (P <0.05).

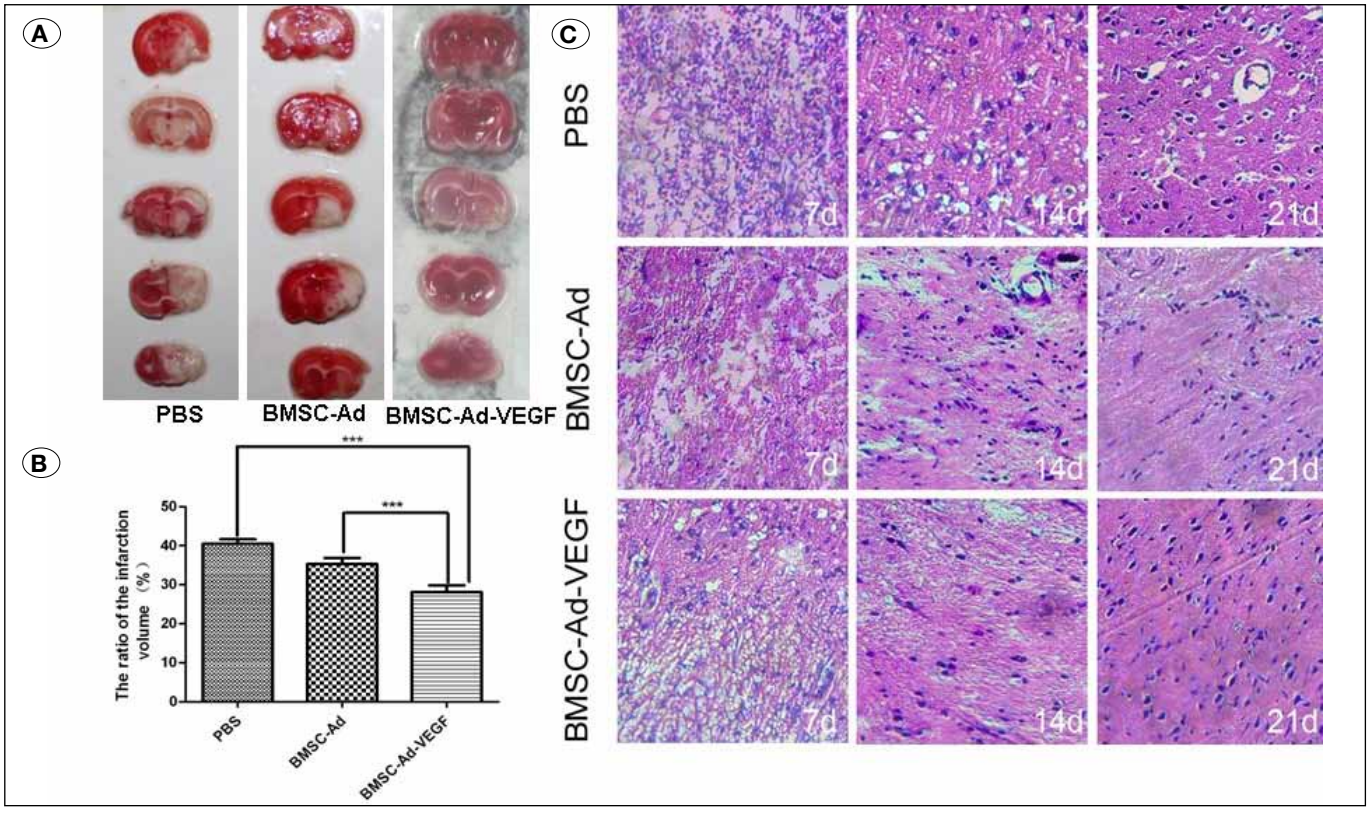

Figure 5: A) The infarct volume was measured by TTC; **B)** The histogram showed: Compared with the other group, The infarct volume about BMSC-Ad-VEGF group were lower (P <0.0001). **C)** The PBS, BMSC-Ad group, BMSC-Ad-VEGF group at 7d, 14d and 21d respectively.

After MCAO, glial cells were activated, followed by reactive gliosis, which is mainly dependent on the degree of damage (1). To evaluate the effects after treatment of MCAO by intracerebroventricular injection of BMSC-Ad-VEGF, GFAP was used to label and count the microglia and astrocytes. GFAP-positive cells are astrocytes, stellate-shaped, protrusion slender, and deeply stained. These cells gradually increased from the central ischemic area to the ischemic penumbra, and counted under 200× magnification microscope. The results are as follows: BMSC-Ad-VEGF group (36.89±2.60), BMSC-Ad group (48.51±1.18), PBS group (63.84±4.04). Statistically significant difference was found between the BMSC-Ad and PBS groups when compare with the BMSC-Ad-VEGF group ($p < 0.0001$, Figure 6A).

pGSK3β and GFAP protein expression after 1, 7, and 14 days were analysed using Western blot. No significant difference was found between each group after 1 day. However, pGSK3β protein expression was upregulated and GFAP protein expression was downregulated after 7 and 14 days in BMSC-Ad-VEGF group compared with those in the other groups (Figure 6B).

■ DISCUSSION

In recent years, more and more researches have interesting in the transgenic stem cell treatment of cerebral ischemia. Neural and embryonic neural stem cells, as well as BMSCs, are candidates for transgenic stem cells. BMSCs are particularly preferred as transgenic carriers for treatment of cerebral ischemia because of its accessibility, low immunogenicity and no ethical conflict.

In the current experiment, intracerebroventricular injection of BMSC-Ad-VEGF was used to treat cerebral infarction of rats. Large amounts of inflammatory mediators and chemokines

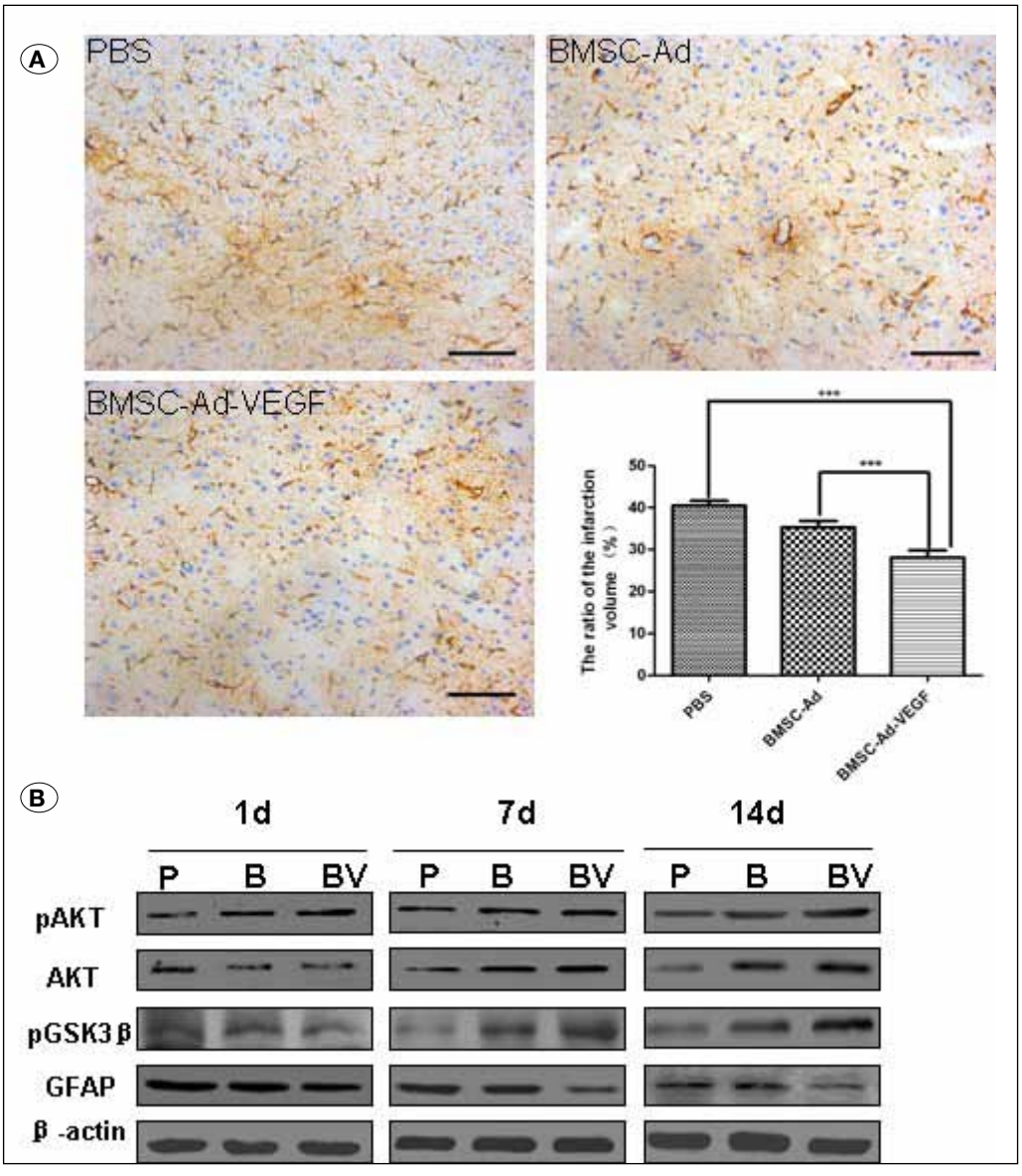

Figure 6: A) The immunohistochemical analysis showed that the reactive gliosis were attenuated after the treatment of BMSC-Ad-VEGF group (x200). The histogram showed the number of GFAP-positive cells. Compared with the other group, the number of GFAP-positive cells were lower after the treatment of BMSC-Ad-VEGF group ($p<0.0001$). Scale bar equals 100 μm. **B)** The expression of pAKT, AKT, pGSK3β and GFAP were detected by western blotting. The results showed that the proportion of pAkt/Akt was increased on BMSC-Ad-VEGF group. There was no significant difference in the expression of pGSK3β and GFAP in 1 day. Otherwise, we founded that the expression of pGSK3β was increased, but the expression of GFAP were lower after the treatment of BMSC-Ad-VEGF group at 7, 14 days (**P:** PBS; **B:** BMSC-Ad; **BV:** BMSC-Ad-VEGF).

were released because of ischemic brain tissue, which facilitate the migration of BMSCs into the lesion, delivery of VEGF, and ultimately the stimulation of VEGFR2 expression (13, 20). This process contributed to the formation and growth of new capillaries in ischemic tissue, which reduced neuronal apoptosis and infarct size as well as promoted recovery of neurological function. Meanwhile, some nutritional factors and cytokines (such as brain-derived neurotrophic factor, nerve growth factor) were secreted in the surrounding brain tissue through paracrine and autocrine (17), which promoted stem cells to differentiate into neural-like and glial-like cells (15), instead of damaged nerve cells, thereby increasing recovery of neurological function.

Zhang et al. (23) showed that VEGF facilitates the blood brain damages to become heavier in the early stage of cerebral infarction (1 hour infarction), increases vascular permeability, and aggravates cerebral oedema and neurological damage. However, after 48 hours of cerebral infarction, the ischemic penumbra neovascularisation and recovery of nerve regeneration could be promoted by VEGF. Therefore, intracerebroventricular injection of BMSC-Ad-VEGF was chosen for the treatment of cerebral infarction in rats after 48 hours. However, HE staining showed that oedema still existed in the BMSC-Ad-VEGF, BMSC-Ad, and PBS groups after 7 days. These phenomena may have been caused by further exacerbation of the brain damage followed by increasing oedema due to intracerebroventricular injection. Alleviated encephaloedema, however, the PBS group still showed oedema and disordered structure. As a result, the indexes were detected after 14 days.

The signalling pathway mechanism of BMSC-Ad-VEGF treatment of cerebral infarction was studied. We found that nerve injury after ischemia could be alleviated through activation of AKT signalling pathway. Our studies indicated that the reactive gliosis increased because of the ischemic injury. Intracerebroventricular injection of BMSC-Ad-VEGF could inhibit the GFAP expression, reduce the reactive glial proliferation, activate pAKT/AKT signalling pathway, and activate the expression of downstream genes pGSK3β. In addition, VEGF/VEGFR2/Flk1 and pAKT/AKT signalling pathway was affected by some nutritional factors secreted in the BMSC group (5, 11), which participated in neuroprotection. The mNSS showed significant recovery of nerve functions in the BMSC-Ad group compared with the PBS group. Significant difference was found between the BMSC-Ad and BMSC-Ad-VEGF groups. This result indicated that VEGF-BMSC combination therapy stimulated the VEGF/VEGFR2/Flk1 and pAKT/AKT signalling pathway and reduced reactive nerve gliosis, which is related to microgliastrocytes (2, 3, 12) and ischemic damage and recovery. Therefore, it can be considered as an evaluation indicator of cerebral ischemia. The number of microglia is believed to be related to the area of cerebral infarction (24).

pAKT, an important part of the PI3K signalling pathway, thereby enhancing cell survival after activation through GSK-3 and other downstream gene regulation. In various cerebral ischemia animal models, neuroprotective effects can be achieved after Akt activation. pAkt is mainly expressed in the peripheral region of the core damage, but less in severely damaged core area, which is related to inadequate energy to activate Akt. Glycogen synthase kinase 3 (GSK3), which is involved in glucose metabolism, inhibits glycogen synthesis. GSK3 is mainly divided into two subtypes, namely, GSK3α and GSK3β. GSK3β is mainly present in the nervous system.

Endo et al. (8) showed that in the transient cerebral ischemia rat model, GSK3β is expressed in the ischemic region. However, GSK3β expression is different in some reported animal model of cerebral ischemia, which may be associated with the degree of damage of the animal model. GSK3β can be activated by serious damage, which leads to apoptosis, but it can be inactivated by mild injury to promote cell survival. In the current study, the effects of PI3K/AkT/GSK3β signal pathway on BMSC-Ad-VEGF combined treatment of cerebral ischemia had been examined. Compared with the other groups, the p-AkT and p-GSK3β in the BMSC-Ad-VEGF group were significantly increased after treatment, and GFAP expression was reduced, followed by reduced gliosis, which resulted in nerve protection and promotion of recovery of neurological function. However, this process is only one protection mechanism against ischemic injury. Further studies on other protection mechanisms are necessary.

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