Bone marrow stromal cells transplantation impact spatial learning and memory and the expression of BDNF and P75NTR in rats with chronic cerebral ischemia

Huili Zhang[1], Bo Song[1], Guangming Gong[2], Yanlin Wang[1], Jie Qin[1], Yingke Yang[1], Jing Qi[1], Avinash Chandra [1], Yuming Xu[1]

1. The Third Department of Neurology, First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, China
2. Department of Microbiology and Immunology, College of Basic Medicine, Zhengzhou University, Zhengzhou, Henan 450001, China
xuyuming@zzu.edu.cn

Abstract: Chronic cerebral ischemia gradually generates cognitive impairment associated with modifications in the hippocampus, a brain structure that is largely involved in learning and memory processes. Such alterations have been attributed to the damage of neuronal plasticity of hippocampus. Numerous of studies demonstrated that bone marrow stromal cells (BMSCs) transplantation improved neural function of animal models with neurological diseases, including chronic cerebral ischemia. The effects of BMSCs transplantation attributed to modulation the express of Brain-derived neurotrophic factor（BDNF）and P75 neurotrophin receptor (P75NTR) are uncertain. To investigate the potential mechanisms of BMSCs transplantation for treating chronic cerebral ischemia, we established animal model by permanent occlusion of bilateral common carotid arteries and administrated BMSCs with green fluorescent protein (GFP) via tail vein at 48 hours after surgery. We found that the deficiency of spatial learning and memory caused by chronic cerebral ischemia were improved compared with vehicle-injected group. Meanwhile, results of immunohistochemistry in the CA1 region of hippocampus showed that the expression of BDNF was up-regulated and P75NTR was down-regulated. Our research suggested that BDNF and P75NTR could be influenced by BMSCs transplantation which is associated with the spatial learning and memory improvement.
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1. Introduction

Chronic cerebral ischemia is the common pathological process of the development of various diseases, such as Vascular Dementia, Alzheimer's disease and Binswanger disease, and eventually cause cognitive impairment(Jian et al., 2012). The deficiency of spatial learning and memory ability is one manifestation of cognitive dysfunction. Researches demonstrated that chronic cerebral ischemia can induce pathological changes of dendrite, axon and myelin(Sozmen et al., 2012) which are the foundation of the function of spatial learning and memory.

Brain-derived neurotrophic factor（BDNF）is one of the neurotrophins which play an important role in many aspects of CNS function. BDNF has pleiotropic effects on modulating activity-dependent forms of synaptic plasticity, neuronal survival, neuronal development, dendritic arborization and axon growth (Cowansage et al., 2010, Tanaka et al., 2008), which underlie circuit formation and cognitive function.P75 neurotrophin receptor (P75NTR) is a transmembrane receptor that is identified as a low-affinity receptor for mature neurotrophins which includes nerve growth factor (NGF), BDNF, neurotrophin-3(NT-3) and neurotrophin- 4(NT-4). P75NTR plays as a negative regulator that involves in activity- dependent forms of synaptic plasticity (Teng et al., 2005) when it bands with BDNF. Studies also showed that P75NTR was required for the signaling pathway of myelin-associated inhibitors (Nogo-A, MAG, OMgp) that inhibited neurite outgrowth (Domeniconi et al., 2005).

Numbers of studies demonstrated that bone marrow stromal cells (BMSCs) may provide an adequate source for individualized cell transplantation and can circumvent problems which come from ethics and immunogenicity. BMSCs were demonstrated to improve from the function of spatial learning and memory of rats with chronic cerebral ischemia(Yanlin Wang 2012). The study of Zhang J et al(Zhang et al., 2008) have found that BMSCs could protect oligodendrocytes from injury of oxygen -glucose deprivation though reducing the expression of P75NTR.Whether the pan-neurotrophin receptor, p75NTR, might play critical roles in the pathogenesis of chronic cerebral ischemia or its express could be influenced by BMSCs transplantation was unclear.

In the present study, first we induced a rat chronic cerebral ischemia model by permanent occlusion of bilateral common carotid arteries. Then we transplanted BMSCs into the rat model and estimated the protective effects of BMSCs against the deficiency of spatial learning and memory ability caused by chronic cerebral ischemia. We also observe the changes of the expression of BDNF and P75NTR in the CA1 region of hippocampus of rats in different groups. The mechanism of recovery of spatial learning and memory may be relevant to BMSCs transplantation by mediating the expression of BDNF and P75NTR.

2. Materials and Methods

2.1. Isolation and culture of BMSCs

Bone marrow was obtained under sterile conditions from an 8-week-old rat according to the improved method that our laboratory described previously(Yanlin Wang 2012). This procedure was approved by the Ethics Committee of Zhengzhou University, Zhengzhou, China.

The femurs were separated and both ends were cut. Then, the marrow was flushed with 5 ml medium (Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12 (DMEM / F-12) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids (NEAAs), and 100IU/ml penicillin/streptomycin) (all from Invitrogen). Cells were collected by centrifugation, resuspended in 5 ml medium, plated in a 25 cm^2 tissue culture flask and incubated at 37°C and 5% CO2. The non-adherent cells were removed by changing the medium after 48 h. The medium was changed every 2 days. When the cells achieved 80% confluence, the cells were passed with 0.25% trypsin / EDTA at a ratio of 1:3. In this study, the cells were passed four times and were used for subsequent experiments. The levels of expression of BMSCs surface antigen CD34, CD44, CD45 and CD105 (Biolegend) were evaluated by flow cytometry.

2.2. Establishment of animal model

All experimental procedures were carried out under the Institutional Animal Care and Use which approved by the Ethics Committee of Zhengzhou University, Zhengzhou, China. Adult male Sprague-Dawley (SD) rats weighing between 270 and 300 g were used in this study. After 1 w of adaptive feeding, Morris water maze were used to select qualified experimental rats via excluding rats which found the platform within 2s or which did not find the platform within 120s.

The qualified experimental rats were randomly divided into sham group, vehicle-injected group and BMSCs-transplanted group. Each group had 10 rats. To establish chronic cerebral ischemic model, a surgery of bilateral common carotid arteries occlusion (BCCAO) was taken according to previous report(de la Torre and Aliev 2005). Rats were anesthetized with 10% chloral hydrate by intraperitoneal injection. Through a midline incision, the bilateral common carotid arteries were carefully separated and ligated permanently. Rats in the sham group were given a sham operation, in which their carotid arteries were merely separated carefully. The rats were kept under conditions of controlled temperature and humidity with free access to food and water.

2.3. Morris water maze

The spatial learning and memory ability of rats of each group was evaluated using Morris water maze experiments 1 month after surgery. Water maze experiments were performed in a large quiet room with a number of extra-maze visual cues including geometric images of squares, triangles, circles hung on the wall. The Morris water maze consisted of a black circular pool (diameter: 160 cm, height: 70 cm) filled to a depth of 30 cm of water which maintained at 22.0±1.0 °C and was virtually divided into four equal quadrants. An invisible platform of 10 cm in diameter was submerged approximately 1.0 cm below the surface of the water and placed in the center of a quadrant.

In hidden platform task, rats were given 4 trials per day for 5 consecutive days to locate and climb on to the hidden platform. A trial was initiated by placing the rat in the water directly facing the pool wall in one of the 4 quadrants. The time which they took to climb on to the platform was recorded as the escape latency. Each rat was allowed to rest for 20 s on the platform when they found it. The trial finished when the rat found the platform or when 60 s had elapsed. If the rat did not found the platform within 60 s, it was guided to the platform and allowed to rest for 20 s on it, meanwhile the escape latency was recorded as 60 s. The mean number of seconds (±SEM) spent in four trials was recorded as the mean escape latency of the day.

The day following the last hidden plat form trial, the platform was removed from the pool and probe trials (120 s in length) were performed. The number of crossings over the previous platform location and the time spent in each quadrant were measured.

2.4. Transplantation of GFP-labeled BMSCs

The BMSCs were transfected with lentiviruses which carried the cDNAs of GFP 48 hours before transplantation. GFP-labeled BMSCs cells were collected and resuspended with 0.01 mM PBS. Cells (2×10^7/mL) with 90–95% viability which was assessed by trypan blue exclusion were used for transplantation. At 48 h post-BCCAO, rats of the BMSCs- transplanted group were injected with 0.5ml cell suspension via tail vein. Rats in the vehicle- injected group were injected with an equal volume of PBS via the same vein.

2.5. Histological analysis

At the end of behavioral testing, each animal was

anesthetized and perfused through the heart with ice-cold saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were rapidly extracted, post-fixed overnight in the same fixative at 4 °C. The brain tissues of hippocampal region were then embedded in paraffin, sectioned at a thickness of 3μm in the coronal plane and mounted on slides coated with poly-lysine. The sections were processed for fluorescence observation, HE staining and immunohistochemistry.

After incubation in 3% H_2O_2 followed by 4% normal goat serum (Boster), sections were incubated with a primary antibody against BDNF (1:100, Santa) or P75NTR (1:100, Santa) at 4 °C overnight for immunohistochemistry. Sections were then incubated with biotinylated goat anti-rabbit IgG(1:200, ZSGB-Bio) for 2 h. Detection of the bound antibodies was performed using a standard peroxidase-based method (ABC-kit, ZSGB-Bio), followed by DAB staining. Quantification of BDNF and P75NTR positive cells of CA1 region of the hippocampus was carried out in 2 sections bilaterally per rat. The sections were visualized under a 40× objective in a microscope (Leica) and five sample areas were obtained for each section. Image J software was used for gray scale analysis of each sample area, and the mean value (±SEM) was recorded as the gray scale value of the rat.

2.6. Statistical analysis

Data are presented as mean ± SEM. One-way analysis of variance (ANOVA) followed Fisher's LSD post hoc test by was used for multiple comparisons. A P-value < 0.05 was considered statistically significant.

3. Results

3.1. Results of flow cytometry

Flow cytometry was used to identify whether the cells isolated and cultured were BMSCs. MSCs are identified by the expression of many molecules including CD105 and CD44 and are negative for the hematopoietic markers CD34and CD45.In our research, the BMSCs had a high purity, CD105 and CD44 double-positive rate was 99.8%, while the negative rate of CD34 and CD45 was 100% (Figure.1).

3.2. Green fluorescence observed after virus transfection

BMSCs exhibited typical morphology of fusiform. When the cells achieved 80% confluence, they present morphology like swirling or fish stock. 48 h after viral transduction, there was not any significant changes in cell morphology and green fluorescence could be observed when the BMSCs were visualized under a fluorescence microscope.

3.3. Assessment of spatial memory in the Morris Water Maze experiment

After the surgery, the vehicle-injected group showed significant disorder of spatial leaning and memory function in the hidden platform task and probe trials. In the hidden platform task, the vehicle-injected group showed a longer escape latency than the sham group (P<0.01). Interestingly, the escape latency of the BMSCs- transplanted group decreased gradually during the testing period compared with the vehicle- injected group (P<0.01) (Figure.2).In the probe trials, the mean number of seconds (±SEM) spent in target quadrant and the total number of crossings over the previous platform area were both compared. Statistic results showed that the time spent in target quadrant and number of crossings of the vehicle-injected group decreased significantly than the sham group (P <0.05). Compared with the vehicle-injected group, the BMSCs- transplanted group obtained an increase in the time spent in target quadrant and number of crossings (P <0.05)(Table 1). These data indicate that the spatial learning and memory ability of rats of the BMSCs-transplanted group has improved.

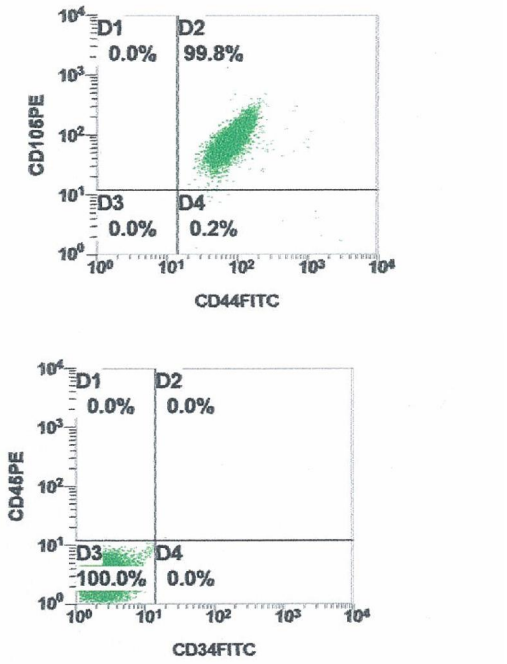

Figure 1. Results of flow cytometry

3.4. Migration of BMSCs

Under fluorescence microscopy, the GFP- positive cells could be seen in bilateral hippocampus of the rat of BMSCs-transplanted group. However, few GFP-positive cells could not be found in other brain regions. This result demonstrated that BMSCs could survival and migration in the chronic ischemic regions of rat brains 1 month after tansplantation.

3.5. Morphological changes of hippocampal neurons

The sections of hippocampal region were stained with HE in all three groups to observe the morphological changes of hippocampal neurons. The

neurons of hippocampal region of the sham group which had original morphology arranged normally. Any pyknosis, vacuolization, degeneration, edema or necrosis was found. In vehicle-injected group, hippocampal neurons arranged disorderly , and showed obvious swelling, vacuolization, degeneration and loss. These pathological characteristic demonstrated a successful model. Compared with the vehicle- injected group, the neurons of hippocampal region of the BMSCs-transplanted group arranged more orderly, and the number of pathological cells was reduced.

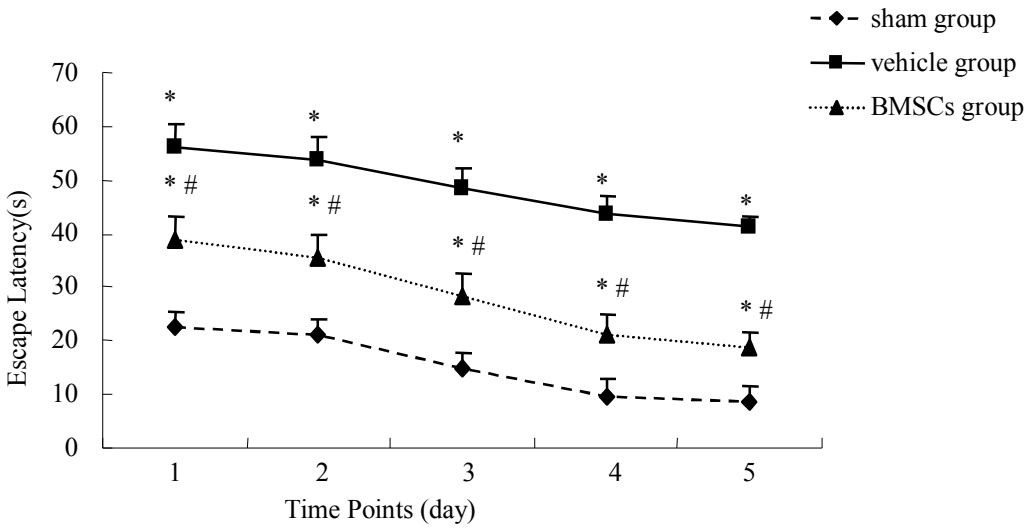

Figure 2. Average escape latency of all three groups at each time point (n=10). * P < 0.05 vs sham group, # P < 0.05 vs vehicle-injected group.

Table 1. Results of the probe trials ($\overline{X} \pm S$, n=10)

Groups	Mean time spent in target quadrant (s)	Mean number of crossings over the previous platform area
sham group	57.86±2.33	3.50±1.65
vehicle-injected group	21.74±3.46*	1.00±1.05*
BMSCs- transplanted group	37.97±2.72#*	2.20±1.61#*

* P < 0.05 vs sham group;# P < 0.05 vs vehicle-injected group.

3.6. Expression of BDNF and P75NTR in CA1 region of hippocampus

BDNF and P75NTR immunoreactivity was detected in all three groups. BDNF immunostaining was less observed in the vehicle-injected group throughout the hippocampal area. In contrast, BDNF immunoreactivity was more prominent in the BMSCs-transplanted group and the sham group. Furthermore, the intensity of immunoreactivity in BDNF-positive cells was obviously higher in another two groups than the vehicle-injected group (P <0.05).P75NTR immunoreactivity was most evident in the vehicle-injected group, while was rarely detected in the sham group. In the BMSCs-transplanted group, P75NTR immunostaining was less prominent compared with the vehicle-injected group (P <0.05) (Figure 3).

4. Discussion

In this research, we investigated the effects of BMSCs transplantation and the expression of BDNF and P75NTR of rats suffering the chronic cerebral ischemic injury or sham operation. A rat chronic cerebral ischemia model was established by occluding bilateral common carotid arteries permanently. Morris water maze, the most favorite and significant tool for detecting the level of learning and memory of laboratory animals, was used to estimate differences of the spatial learning and memory among the three groups.30 days post chronic cerebral ischemic injury,

the rats in the vehicle-injected group showed a longer escape latency in the hidden platform task, a shorter time spent in the target quadrant and number of crossings over the previous platform area compared with rats in the sham group. This demonstrated that chronic cerebral ischemia could cause dysfunction of spatial learning and memory. While the results of morris water maze of rats in the BMSCs- transplanted group were significantly superior to that of rats in vehicle-injected group. These behavior results indicated that BMSCs transplantation in some ways improved the cognitive function of rats suffering ischemic injury, which was similar to the results of study of Perasso L et al (Perasso et al., 2010).

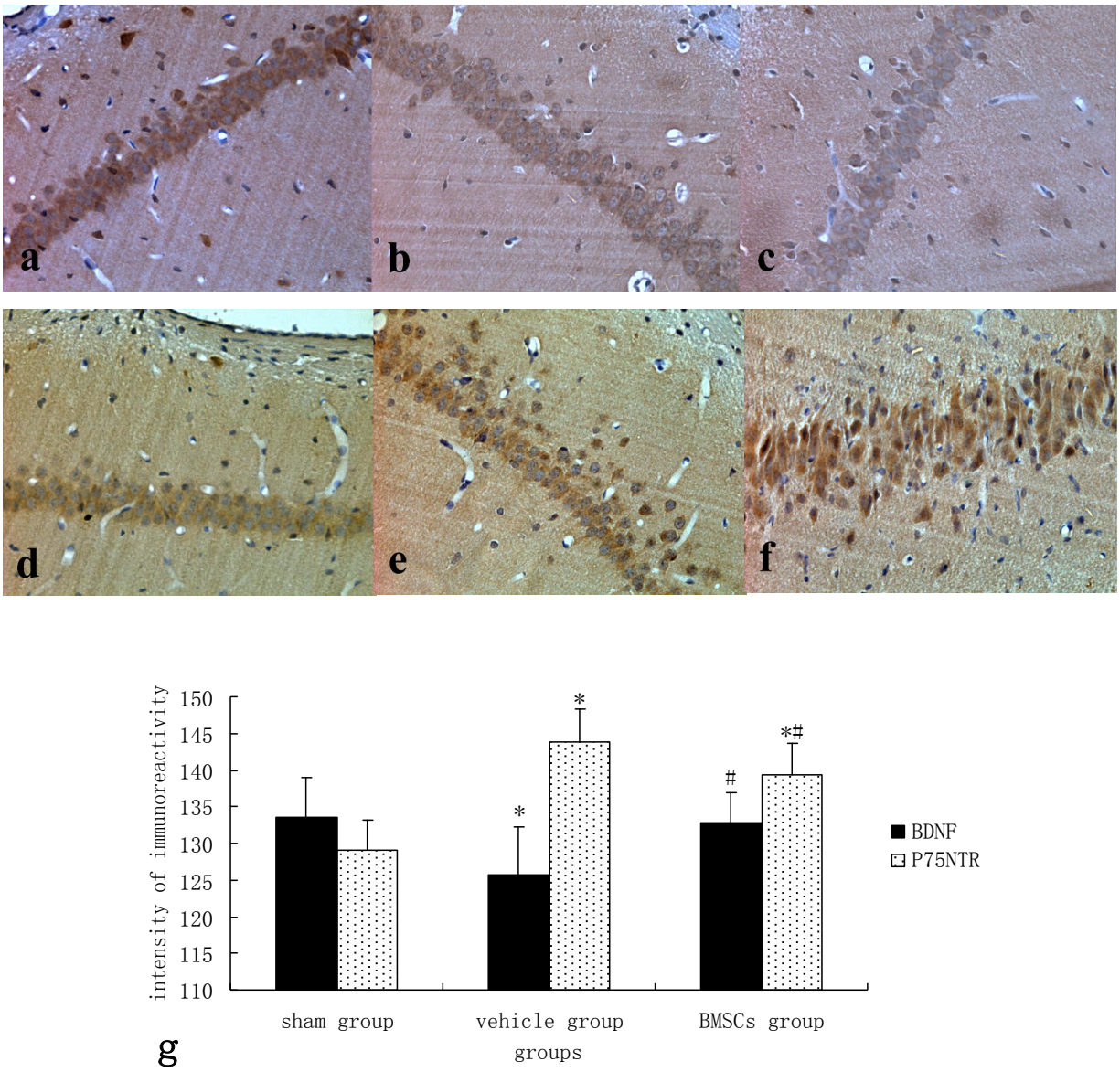

Figure 3. Expression of BDNF and P75NTR in CA1 region of hippocampus. a-f are figures of immunohistochemistry,400×. a, b and c were the results of BDNF immunostaining. d, e and f were the results of P75NTR immunostaining. a and d: sham group. b and e: BMSCs-transplanted group. c and f: vehicle-injected group. g is the intensity of immunoreactivity, * $P < 0.05$ vs sham group;# $P < 0.05$ vs vehicle-injected group.

Hippocampus is largely involved in learning and memory processes and its alterations were associated with the decline of cognitive functions. BDNF belongs to the neurotrophins family and the highest level of BDNF in the CNS is found in the hippocampus. BDNF is involved in the survival of neurons, modulating synaptic structure and quantity, promoting the maturation of new synapses and the growth of dendritic spines and axons (Cowansage et al., 2010, Tanaka et al., 2008). In such ways BDNF impacts the learning and memory ability. Most neuronal effects of BDNF are mediated through high-affinity receptors, tyrosine kinase B receptors (TrkB) (Nagahara and Tuszynski 2011). Nevertheless, BDNF also binds to the low-affinity receptor P75NTR, which activates ceramide turnover, c-Jun kinase(JNK) cascade and caspases(Ibanez and Simi 2012), mediating functions opposite of TrkB like inducing neuronal apoptosis and negatively regulating the complexity of dendrite of mature hippocampal neurons. P75NTR can also activate the small GTPase RhoA which leads to growth-cone collapse and inhibition of axonal growth.

In our research, the express of BDNF declined in the brains of rats in the vehicle-injected group, which is consistent with the study of Sun H et al(Sun et al., 2010). A decrease in BDNF expression can cause neuronal death or axonal damage. Researches demonstrated that applying exogenous BDNF was effective in treating some neurological disorders (Nagahara et al., 2009). However, BDNF is a medium molecular protein with charge that poorly penetrates the blood–brain barrier and the brain parenchyma. Such characteristic make it a poor drug candidate for neurological diseases therapy. MSCs, an effective cell therapy which can improve the neural function, have been found to increase the level of BDNF of different animal models such as ischemic stroke, spinal cord injury, traumatic brain injury and so on(Honmou et al., 2012, Wright et al., 2011, Walker et al., 2012). P75NTR is rarely expressed in adult tissues, but in pathological conditions it is highly expressed. P75NTR was found upregulated in the hippocampus of rats with transient global cerebral ischemia (Soltys et al., 2003) and our study also found a similar result. In vitro study, BMSCs could reduce p75NTR expression in the oligodendrocyte subjected to oxygen-glucose deprivation (Zhang et al., 2008). In our study, the expression of P75NTR was decreased in hippocampus of the BMSCs-transplanted group than that of the vehicle-injected group. This indicated that BMSCs could down-regulate P75NTR expression of the CA1 region of hippocampus of rats subjected chronic cerebral ischemia.

P75NTR knockout mice showed that the dendritic complexity and dendritic spine density of hippocampal neurons were increased(Barrett et al., 2010). Meanwhile, spatial learning and hippocampal LTP deficits could be reversed by applying BMSCs. After BMSCs transplantation in our research, the GFP-positive cells could be observed survival and migration in bilateral hippocampus of rats. The neurons of hippocampal region of the BMSCs-transplanted group arranged more orderly compared with the vehicle-injected group and the extent of cellular edema and necrosis was significantly lightened. The spatial leaning and memory function was improved in the BMSCs-transplanted group to a certain extent. Our results suggested that the mechanisms of spatial learning and memory improvement caused by BMSCs transplantation may be correlated with down-regulation of P75NTR and up-regulation of BDNF which thereby mediated synaptic activity, promoted dendritic and axonal growth, improved self-repair of the nervous system.

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Corresponding author:
Yuming Xu, Ph.D, M.D.
Third Department of Neurology, First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052 Henan Province, China
Email: xuyuming@zzu.edu.cn

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