Transplantation of bone marrowderived stromal cells to chronic cerebral ischemia rats on the influence ofCognitive function and proteins Nogo-A and NgR expression in the hippocampus.

Yanlin Wang, Songbo Yang, Huili Zhang, Guangming Gong, Qin Jie, Yuming Xu

Department of Neurology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan province, 450052, P. R. China. xuyuming@zzu.edu.cn

Abstract: TO study the transplantation of bone marrowderived stromal cells (mesenchimal stem cells,MSC) to chronic cerebral ischemia rats on the influence of proteins Nogo-A and NgR expression in the hippocampus and explore the mechanism of its function in improving the Cognitive function. BMSCs were harvested and purified by flCOLLC density gradient centrifugation. The chronic cerebral ischemia models were produced by permanent occlusion and snip of bilateral common carotid arteries. Thirty male rats were randomly divided into three groups: model group ,BMSCs transplantation group (Brdu marker)and sham operate group.All of the rats were trained in Morris Water Maze to find the changes of spatial learning and memory ability. The expression of Nogo-A and NgR in the hippocampus were measured by immunohistochemical technique. The model group exhibited serious spatial learning and memory deficits in both navigation test and spatial probe test. In the former test，the mean escape latency of BMSCs transplantation group was significantly shorter than that of model groups and the frequency of crossing the former platform was significantly higher. The expressions of Nogo-A and NgR in BMSCs transplantation group were significantly lower than those of model groups and significantly higher than sham operated group, which was accordant with the change of spatial learning and memory ability. BMSCs transplantation effectively promote spatial learning and memory ability for the rats of chronic cerebral ischemia, which maybe induced by the decrease of proteins Nogo-A and NgR.

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Keywords: BMSC; Chronic cerebral ischemia; Nogo-A ; NgR ; Cognitive function

Introduction

Chronic cerebral ischemia is cognitive impairment caused by the vasospasm, stenosis, occlusion of carotid artery system or vertebral - basilar artery system caused by various factors and long-term cerebral blood flow hypoperfusion, causing tissue ischemia and hypoxia, and further leading to impaired nerve function [1]. A series of pathophysiological changes caused by injury of nerve will lead to the release of a large number of inhibitory proteins against axon regeneration, which is not conducive to the functional rehabilitation of nerve [2]. Nogo-A is currently the strongest known inhibitory proteins against axon regeneration, with strong axonal growth inhibitory function after myelin sheath injury [3]. NgR is its receptor, expressing the inhibitory action of nerve growth, and is an important pathway affecting nerve repair [4]. Research has shown that Nogo-A and NgR protein expression both increase after rat ischemia and hypoxia, strongly inhibiting the regeneration and connection of nerve fibers and resulting in subsidence of nerve ability [5].

BMSCs is a kind of stem cells with multidifferentiation potential, a variety of sources, easy collection, preparation and preservation. It can avoid immunological rejection and at the same time is not related to social, ethical and legal issues, etc. It has extensive foreground of clinical application. Research has shown that BMSCs transplantation can bring down nerve injury through reducing Nogo-A and NgR expression of rats of spinal cord injury model and cerebral infarction model [6] [7]. In addition, studies have shown that BMSCs can improve rat's cognition dysfunction from chronic cerebral ischemia model, but it is not yet known that whether the improvement of cognitive status is related to the decreased NgR and Nogo-A expression.

The chronic cerebral ischemia models were produced by permanent occlusion and snip of bilateral common carotid arteries ,the changes of spatial learning and memory ability were detected by BMSCs transplantation ,the influence of proteins Nogo-A and NgR expression in the hippocampus were measured by immunohistochemical technique in our research to explore the mechanism of its function in improving the Cognitive function .

Materials and methods

1.1 Animal Grouping and Model Preparation

30male SD rats of clean grade at the age of 12-14 months, purchased from Experimental Animal Center of Zhengzhou University, were randomly divided into sham operation group, model group and BMSCs

transplantation group, 10 rats for each group. Sham operation group: just separate the bilateral common carotid artery without ligation after anesthetizing rats. Model group: after fasting and water deprivation and anesthesia, take the median incision in cervical part of rats, the proximal end and distal end of the bilateral common carotid artery were separated and double ligated and then cut, keeping the rectal temperature at 37℃. BMSCs transplantation group: after seven days of model preparation, rats were injected 1ml BMSCs single cell suspension, which contains 1×10^7 cells, by caudal vein.

1.2 The Separation and Culture of the BMSCs

Separate the thighbones and shankbones of the rats on aseptic condition after they were anesthetized. The cell suspension was obtained by repeatedly flushing the bone marrow cavity with DMEM-F12 (Gibco 11330). Mononuclear cells were obtained by adopting flCOLLC density gradient centrifugation method and were incubated in the incubator (37℃, 5%CO2 saturated humidity). After one night, the suspended growth hematopoietic stem cells were removed. The liquid was changed every three days. When the cell density reached 80%～90%, cells were transferred of culture according to 1:2 to 1:3. Tested the BMSCs surface antigen CD34(-), CD44(+), CD45(-) and CD105(+) of rats with flow cytometry. Dissolved 10mg Brdu(Solarbio b8010) into 15ml distilled water. Took 50ul Brdu solution into 5ml cell culture solution at 48 hours before transplantation and cultured it in dark for 48 hours. Counted cells before transplantation and the number of the transplanted cells was about 1×10^7.

1.3 Ethology Test

After one month of modeling, all rats were performed by the Morris (8) water maze test . place navigation test: before training, made rats swim in the pool for two days, two minutes for each day to adapt them to the pool environment. Rats of each group had five days. There were nine periods for swimming training and four trainings for each period. Everyday, threw rats into the pool from different quadrants and recorded the time they found the platform, which was recorded as the escape latency. The longest time limit was 120 seconds. If rats couldn't find the platform in 120 seconds, they would be guided to the platform by the experimental personnel and the escape latency would be 120 seconds. On the fifth afternoon, rats were thrown into the pool from the same quadrant with the one of the first day, to test and record the escape latency. The spatial probe trial: removed the platform after recording the escape latency, and recorded the times that they crossed the platform in 120 seconds.

1.4 Specimens Collection and Tissue Section Preparation:

One month later after the behavioral test, take ten rats from each group, expose the heart upon excessive anesthesia, intubate the left ventricular and incise the right auricle and perfuse 200ml of 37℃ sterile saline through the left ventricular within 10 - 20min, then perfuse 250ml of PBS stationary liquid of 4℃, 40g/L paraformaldehyde and finish this perfusion within 1 - 2h, then decollate and take the brain. Take brain tissue in hippocampal region at the point 3mm after the optic chiasma, embed it with paraffin, then do continuous coronal sections with the thickness of 3 μm each, and do HE stain and Nogo-A and NgR immunohistochemical stain respectively.

1.5 Test Brdu with Immunofluorescence Stain and Test Nogo-A and NgR Protein Expression with Immunohistochemical Stain

(1) Take the paraffin sections for immunostaining, conventional dewaxing to water progressively. Enclose peroxidase in 3％ hydrogen and conduct microwave antigen retrieval for 15min, and enclose peroxidase in goat's blood serum for 20min. Add rabbit anti-Brdu FITC antibody (Beijingboaoshen bs-0489R) (1:100) respectively, and keep them at 4℃ overnight. Drop FITC-marked Brdu fluorescence secondary antibody upon PBS cleaning and drying and incubate for 3h at 37°C. After cleaning with PBS for three times, observe under 200 magnified visual field of fluorescence microscope that the ones with green fluorescence are Brdu-marked bone marrow derived mesenchymal stem cells.

(2) Take the paraffin sections for immunostaining, conventional dewaxing to water progressively. Enclose peroxidase in 3％ hydrogen and conduct microwave antigen retrieval for 15min, and enclose peroxidase in goat's blood serum for 20min. Rabbit anti-Nogo-A antibody (Beijingboaoshen bs-0134R) and rabbit anti-NgR antibody (Beijingboaoshen bs-0129R, 1:100), kept at 4℃ overnight. Drop goat anti-rabbit secondary antibody and horseradish peroxidase-marked strepto-avidin respectively upon PBS incubate for 3h at 37°C, dehydrate it after 3min DAB coloration and mount upon lucidity. Replace primary antibody with PBS for negative control. Observe with optical microscope (Germany, LEICA) that brown granules are immunoreactive cells. Choose 5 visual fields for each section under 400 times magnified visual field and do optical density analysis on them by image analysis system.

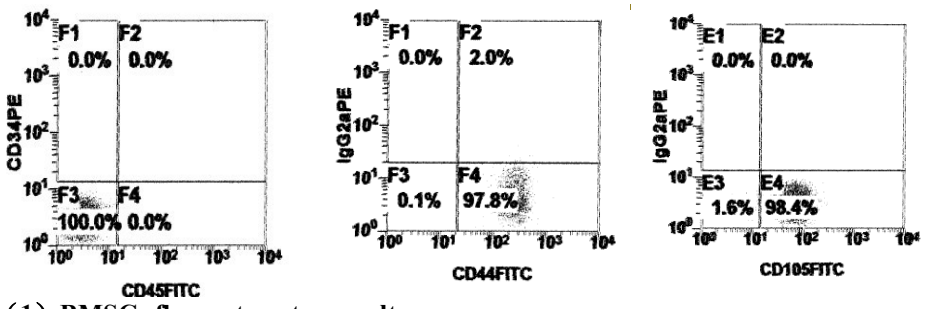
1.6 Data Analysis and Processing

Data is shown with $\overline{x} \pm s$. Firstly, conduct normality test and test of homogeneity variance for the data, do statistical analysis with SPSS17.0 software, T test and single factor analysis of variance are conducted for pairwise comparison between groups, take $P<0.05$ as difference and of statistical meaning.

2. Results:

2.1 BMSCs flow cytometry result

Tested the BMSCs with flow cytometry, the CD34(-) was 100%, CD44(+) was 97.8%, CD45(-) was 100% and CD105(+) was 98.4%.



Figure（1）**BMSCs flow cytometry result**

2.2 The Morris water maze result

The model group exhibited serious spatial learning and memory deficits in both navigation test and spatial probe test. In the former test,the mean escape latency of BMSCs transplantation group was significantly shorter（P<0.01）than model groups and the frequency of crossing the former platform was significantly higher（P<0.05）. In navigation test ,the mean escape latency of model groups was significantly longer（P<0.01）than sham-operated group and the frequency of crossing the former platform was significantly lower（P<0.01）.

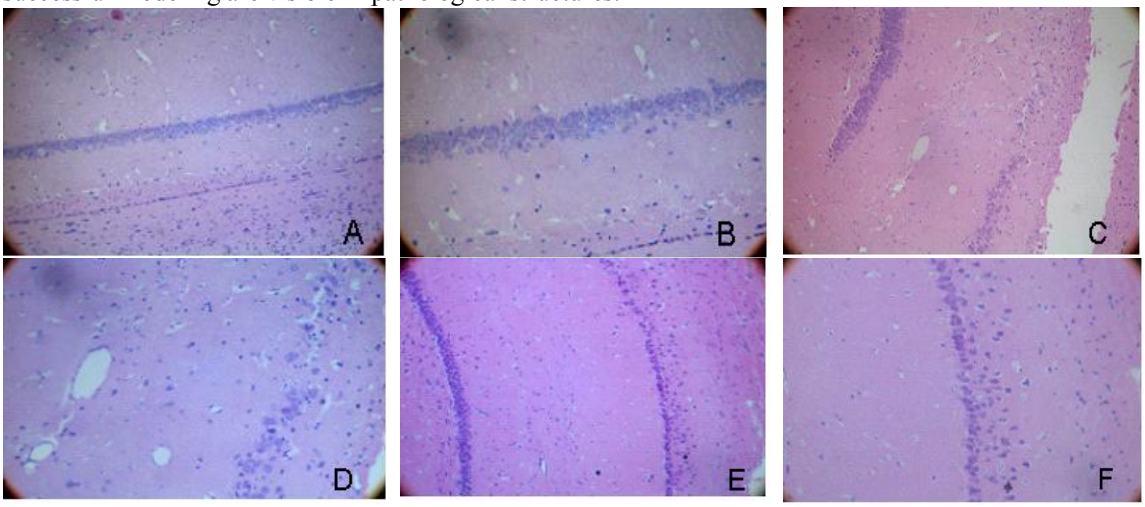
Table 1. The Morris water maze result

GROUPS	escape latency（S）	cross times
Model	53.10±1.85*	2.90±1.29★
BMSCs	32.20±1.32△	4.70±1.49▲
Sham	15.20±2.15□	7.20±1.93■

*compare with△*P*<0．01；*compare with□*P*<0．01；△compare with□*P*<0．01；★compare with ▲*P*<0．05；★compare with■*P*<0．01；▲compare with■*P*<0．01；

2.3 HE Stain Result

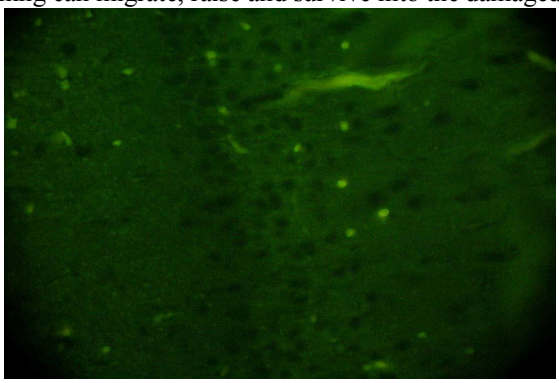
HE stain result in hippocampus tissue of model group shows that cone cells are arranged disorderly, and even shows a linear fracture, the original structure is damaged, a large number of cell necrosis and apoptosis and vacuolization, cone cells of hippocampus in transplantation group are more chaotic arranged, but we can also see a large number of nuclear hyperchromatism and vacuolization, cells in sham-operated group are arranged normally, the successful modeling are visible in pathological structures.



Figure（2）**HE Stain Result :**hippocampus tissue of sham operation group in A(×100) and **B**(×200); hippocampus tissue of model group in **C**(×100) and **D**(×200); hippocampus tissue of BMSCs transplantation group in **E**(×100) and **F**(×200);

2.4 Brdu Immunofluorescence Stain Result

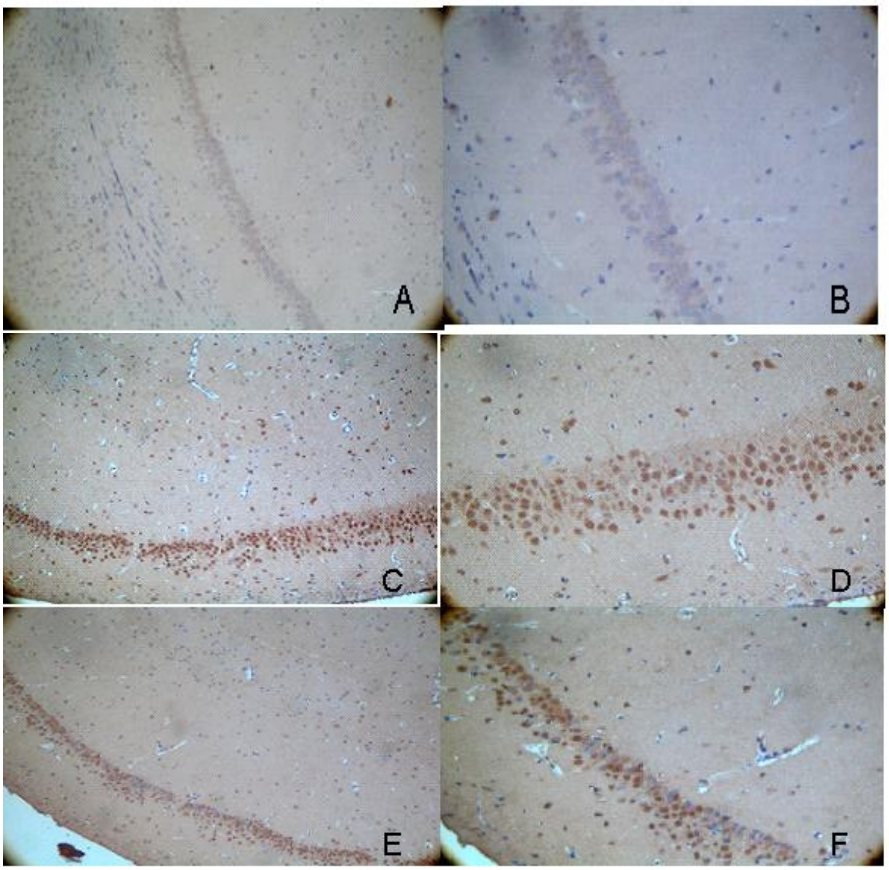
As shown in Figure 3 in the hippocampal tissue of BMSCs transplantation group, it can be observed that positive BMSCs of Brdu fluorescent staining can migrate, raise and survive into the damaged area of cerebral ischemia.



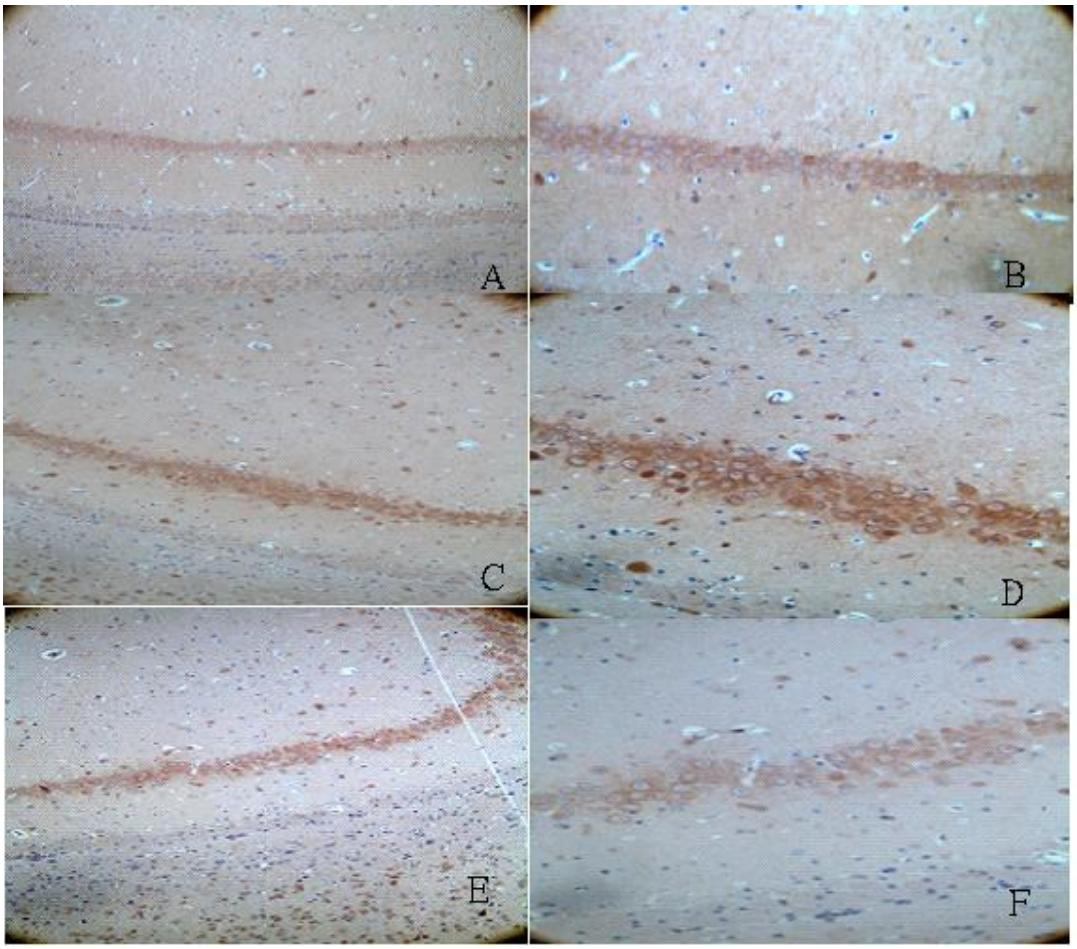
Figure（**3**）Brdu fluorescent staining positive cells are BMSCs;

2.5 Nogo-A and NgR Immunohistochemical Stain Result in Various Hippocampal CA1 Regions

Brown granules can be seen in neurons cell membrane of hippocampal CA1 region, indicating that there are both Nogo-A and NgR expression; The expressions of Nogo-A and NgR in BMSCs transplantation group and model group were significantly higher（$P<0.05$） than those of sham operated group;tThe expressions of Nogo-A and NgR in BMSCs transplantation group were significantly lower（$P<0.05$）than those of model group ;



Figure（**4**）Nogo-A Immunohistochemical Stain Result：A(DAB×100) and **B**(DAB×200) : the expression of Nogo-A in sham operation group ; **C**(DAB×100) and **D**(DAB×200): the expression of Nogo-A in model group ; **E**(DAB×100) and **F**(DAB×200) :the expression of Nogo-A in BMSCs transplantation group ;



Figure（**5**）NgR Immunohistochemical Stain Result：A(DAB×100) and **B**(DAB×200) : the expression of NgR in sham operation group ; **C**(DAB×100) and **D**(DAB×200): the expression of NgR in model group ; **E**(DAB×100) and **F**(DAB×200) :the expression ofNgR in BMSCs transplantation group;

Table 2. Optical density resuit

GROUPS	Nogo-A	NgR
Model	170.15±4.41*	178.56±7.56★
BMSCs	145.76±3.94△	136.29±3.56▲
Sham	125.50±3.02□	115.62±4.45■

*compare with△P<0. 01；*compare with□P<0. 01；△compare with□P<0. 01；★compare with▲P<0. 05；★compare with■P<0. 01；▲compare with■P<0. 01；

3. **Discussions**

In this study, cone cells of hippocampus in transplantation group are more chaotic arranged than model group in HE stain result. The mean escape latency of BMSCs transplantation group was significantly shorter（P<0.01）and the frequency of crossing the former platform was significantly higher （P<0.05） than those of model groups in the Morris water maze ,the model group and BMSCs transplantation group both exhibited cognitive function deficits than sham operated group, but BMSCs transplantation group was better than model group. Brdu fluorescent staining positive cells were observed in hippocampal tissue of BMSCs transplantation group, indicates that the transplanted BMSCs can migrate, raise, and survive into the damaged area of cerebral ischemia. Indicates that BMSCs transplantation effectively promote cognitive function for the rats of chronic cerebral ischemia.

The damaged central nervous system is difficult to form effective regeneration, which may be related to inadequate secretion of nerve growth factor and over-expression of nerve growth inhibitory factor, the vast majority of stem cell transplantation researches

currently are focused on promoting nerve growth factor expression, while there are few studies on nerve growth inhibitory factor. Nogo-A has been confirmed to be the strongest axonal growth inhibitory factor, through p75NTR and NgR receptor, it plays inhibitory effect [8]. After integrating Nogo-A and NgR-p75 complex, acting on Rho-kinase-RhoA pathway increases the level of RhoA, leading to retraction of growth cone cell body, reducing simultaneously the levels of the other two members of the Rho family, namely, Rac1 and Cdc42 level, can retract the filopodium and pseudopodia on growth cones, NgR also can make the Rac1 and Cdc42 change to the RhoA, and thus leading to growth cone collapse [9]. Studies found that the usage of neutralizing Nogo-A antibody or NgR antagonist effectively reduce the Nogo-A and NgR expression after nerve injury, and induce long-distance axonal regeneration, increasing structural plasticity and promoting functional recovery [10,11]. The above study suggests that reducing Nogo-A and N gR expression can promote synapse regeneration and reduce nerve injury, with the neuroprotective effect.

In the paper, it can be seen that expression of Nogo-A and NgR protein in hippocampal nerve cells of model group increased. This may be involved in the rat's cognitive dysfunction, which is coincident with the few documentary reports [12] [13]. In this study, Nogo-A and NgR protein expression in hippocampal of transplantation group was less than those of model group, indicating that BMSCs transplantation may promote the rebirth of axon of damaged nerve cells and then improve the cognitive functions by lowering down Nogo-A and NgR protein. Possible mechanism of the lower down of Nogo-A and NgR protein expression after BMSCs transplantation may lie on those: 1: BMSCs may inhibit relating inhibitive factor of myelin sheath by adjusting the expression of MMP-1, for example, the generation of Nogo-A [14]. 2: BMSCs may also be able to improve the expression of synaptophysin, which will make the feedback to inhibition to the generation of Nogo-A [7]. In the spinal cord injury model and cerebral infarction model, BMSCs transplantation may protect nerve function, and it also can be seen that it reduced the expression of Nogo-A and NgR [6] [7] at the same time, which is similar to the result of this study.

Suppression and regeneration mechanism of central nervous system is complicated. There are lots of factors which may promote the regeneration or inhibit the growth of nerve cells in the central system, and specific mechanism affecting the learning and memory activities is still not completely clear. And the promoting effect of bone marrow derived mesenchymal stem cells to cognitive function may be a result of co-working of various approaches, Nogo-A and NgR is just one of them, and there are still many factors affected by bone marrow derived mesenchymal stem cells in the upstream and downstream. As for how bone marrow derived mesenchymal stem cells acts on Nogo-A and NgR and whether it brings into play via other factors, further study is still in need.

Conclusion:

We have for the first time to expole whether improvement of cognitive function by BMSCs transplantation is related to decreased NgR and Nogo-A expression.We find that BMSCs transplantation effectively promote spatial learning and memory ability for the rats of chronic cerebral ischemia,which maybe induced by the decrease of proteins Nogo-A and NgR.

Corresponding author:
Dr. Yuming Xu, xuyuming@zzu.edu.cn.
Mailing address:1 East Jianshe Road，Zhengzhou, Henan province, 450052, P. R. China

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