Original

A Study on Acute Ischemia-Reperfusion Models in Rats Treated by Bone Mesenchymal Stem Cells Grafting via Arteries and Veins

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Abstract: To investigate differences in curative effect of bone mesenchymal stem cells grafting via arteries and veins on 6-hour ischemia-reperfusion. A 2-hour middle cerebral artery ischemia-reperfusion model was established in 27 SD female rats. BMSCs were isolated and cultured using the whole bone marrow adherent technique, identified by flow cytometry. The expression of surface antigen CD90, CD29, CD106, CD11b, CD34 and CD45 on BMSCs was 95.7%, 97.3%, 52.7%, 6.01%, 2.95% and 2.26%, respectively. When compared with two PBS groups, the neurological severity was ameliorated significantly and expression of GFAP and Bcl-2 in infarction border zone were further up-regulated in two BMSCs groups. Moreover, the levels of TNF-α in serum and TUNEL staining in brain tissue were significantly reduced after transplantation. BMSC grafting via caudal veins was the best approach during the early periods of ischemia-reperfusion due to minor trauma and the rich source of BMSCs. It provides a possible way to future clinical treatment of cerebral infarction by stem cell infuson to patients.

Key words: BMSCs, Ischemia-reperfusion cerebral infarction, Transplantation apoptosis

Introduction

Cerebral infarction is the most common cerebral vascular disease. At present, only thrombolysis and interventional therapy can effectively restore revascularization. However, due to narrow treatment time windows, only few patients receive these benefits through the abovementioned therapies. Therefore, there is an urgent need to develop some new therapeutic measures. Some studies have shown that stem cell grafting can greatly improve neurologic impairment1 and widen treatment time windows2. Therefore, stem cell grafting is expected to provide new thoughts to the treatment of cerebral infarction.

Bone mesenchymal stem cells (BMSCs) are capable of multi-directional differentiation, self-renewal and paracrine, and these cells are divided into nerve cells across germ layers3. BMSCs can be obtained from a wide variety of sources, and can be autologous-supplied with convenience, safety and low immunogenicity. Autologous and heterogenous grafting does not cause immunoinflammatory responses4, allowing this to be easily accepted by patients. BMSCs have unique advantages for treating cerebral infarction.

A number of experiments have found that mesenchymal stem cell (MSC) grafting could significantly improve the neurological function of patients with cerebral infarction5-7. Its potential mechanism include cell replacement8, nutritional support9, immune inflammation regulation10, acceleration in the formation of new vessels11, and acceleration in endogenous neurogenesis12. However, its specific mechanism remains controversial. Common grafting pathways for MSCs in treating cerebral infarction include the venous pathway, arterial pathway, and stereotactic pathway. There are pros and cons in all grafting pathways13-15, but the best technique remains controversial. Hence, this experiment focused on the differences in curative effects between trans-arterial and trans-venous BMSC 6-hour grafting, and its specific mechanisms were also preliminarily explored.

Materials and Methods

Isolation, culture, amplification and identification of BMSCs

Sprague-Dawley (SD) rats (3-4 week old) were sacrificed by dislocation and immersed in 75% medical alcohol to sterilize for 10 minutes. The femurs and tibias of these rats were separated on a super clean bench, residual tissues were washed by sterile phosphate buffered saline (PBS), and the epiphysis at two ends was removed. The marrow cavity was repeatedly washed by Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) Media containing 10% fetal bovine serum (FBS) with a sterile syringe. The bone marrow was blown even, inoculated in a 25 cm² cell culture bottle, and placed at 37°C in a 5% CO₂ cell incubator. After 24 hours, half of the amount of the medium was replaced, and the full amount of the medium was replaced after two days. The medium was replaced every 2-3 days. When growing to approximately 90% confluence, the cells were subcultured at a ratio of 1: 2.

Cells at second generation were digested by pancreatic enzyme containing 0.25% EDTA, and then centrifuged at 1,000 rpm at 4°C, and rinsed by PBS solution after the liquid supernatant was discarded. Afterwards, cell density was adjusted to 1×10⁶ by PBS, and surface antigen CD29, CD90, CD106, CD34, CD45 and CD11b (Biolegend, CA, USA) were incubated in dark, and cells were centrifuged at 1,000 rpm. Next,
Establishment of the middle cerebral artery occlusion (MCAO) model

Clean 250-280 g SD female rats (provided by Hunan SJA Laboratory Animal Co., Ltd., Changsha, China) underwent intraperitoneal anesthesia with 10% chloral hydrate at the dosage of 0.3 ml/100 g. Rats were placed in the spinal position, and the four limbs and head were fixed. An incision was performed in the middle of the front neck, and a blunt dissection was made in the common carotid artery, internal carotid artery, external carotid artery and vagus nerves. The common carotid artery and internal carotid artery were clipped, and the external carotid artery was ligatured and cut off. A nylon thread (Beijing Sino Biological Technology Co., Ltd., Beijing, China) with a diameter of 0.26 mm and a head diameter of 0.36 mm was used and inserted into the internal carotid artery from the stump of the external carotid artery at a depth of approximately 18 mm until the thread was blocked. The thread was slowly removed two hours after allowing blood to flow through the middle cerebral artery, in order to relieve obstruction and recirculate blood flow.

Grafting

Rats in the MCAO model were divided into four groups based on a random number table: carotid arterial BMSCs group (n = 8), carotid arterial PBS group (n = 6), and caudal vein PBS group (n = 6). Rats in the carotid arterial BMSCs group and carotid arterial PBS group were treated as follows: rats underwent intraperitoneal anesthesia with 10% chloral hydrate, and the original incision was opened. Blunt separation was made in the common carotid artery and internal carotid artery. A PE-50 catheter was inserted into the internal carotid artery from the stump of the external carotid artery, and 500 μl of cell suspension containing 1×10^6 BMSCs or PBS was injected. The incision was pressed by cotton swabs and sutured. Rats in the caudal vein BMSCs group and caudal vein PBS group were treated as follows: rats were fixed and the tails of these rats were immersed in warm water, transplanted under relatively sterile conditions, and were not treated with antibiotics.

Detection of neurological impairment

Six hours after ischemia-reperfusion and on the 7th day after transplantation, the modified neurologic severity score (mNSS, including evaluation of motor, sensory, balance and reflex functions) was measured. The severity of neurological function was positively correlated with the scores.

Detection by immunohistochemistry

On the 7th day after transplantation, rats were sacrificed, and blood was extracted from the cardiac apex. The brain was taken out and fixed with 4% paraformaldehyde overnight at 4°C. The site of cerebral infarction was cut into 2 mm thick slices through the coronal view. These slices were dehydrated and embedded with paraffin. The paraffin block was cut into 4 μm thick slices, dewaxed and repaired by sodium citrates. These slices were inactivated by 3% H₂O₂ and then the primary antibodies against glial fibrillary acidic protein (GFAP; 1: 3,000, Abcam, Cambridge, UK), B-cell lymphoma 2 protein (Bcl-2; 1: 100, Abcam, Cambridge, UK) and BCL2-associated X protein (Bax; 1: 100, Abcam, Cambridge, UK) were added onto the specimen and incubated overnight at 4°C. Then after PBS washing, the specimens were incubated with secondary antibody at room temperature for 60 min. After that, the sections were counterstained with hematoxylin and incubated with DAB solution and were observed under a light microscopy. Finally, these slices were dehydrated to be transparent, and sealed.

Detection of cytokines

Blood was extracted from the cardiac apex, placed in room temperature for two hours, and centrifuged at 2,000 rpm for 10 minutes. Sub-packed serum was frozen at -80°C, and was unfrozen at room temperature before testing. The detection was performed according to the instructions of the ELISA kits (Xitang, Shanghai, China).

Image collection and analysis

Light microscope (Olympus, Tokyo, Japan; 40 objective lens) and Image Pro Plus6.0 software were used to collect and analyze the images. Each slide carried six images of ischemia penumbra sites, and the mean optical density and positive cell count were calculated.

Statistical treatment

All data were expressed as mean ± standard deviation (SD). One-way analysis of variance was performed using SPSS 19.0 statistical software. In case of homogeneity of data variance, the least significant difference (LSD) method was used to compare the means. In case of heterogeneity of variance, the Dunnett’ T3 method was used to analyze data. P<0.05 was considered statistically significant.

Results

Identification of BMSCs

BMSCs appeared as fusiform spindles and adhered to the wall. Cell surface antigen identification results: The expression of CD90, CD29, CD106, CD11b, CD34 and CD45 was 95.7%, 97.3%, 52.7%, 6.01%, 2.95% and 2.26%, respectively. Various types of cells exist in the narrow cavity of the epiphysis, and we proved that the majority of the cells we obtained were BMSCs.

Measurement and comparison of mNSS

Before transplantation, the mNSS score in the carotid arterial BMSCs group, caudal vein BMSCs group, carotid arterial PBS group and caudal vein PBS group was 9.35±0.92, 9.14±1.06, 9.00±1.2 and 9.17±0.75, respectively. The difference between these four groups was not statistically significant. On the 7th day, the mNSS score was significantly lower in the carotid arterial BMSCs group (5.13±0.64) than in carotid arterial PBS group (6.57±0.58), and in the caudal vein BMSCs group (5.14±0.69) than in caudal vein PBS group, indicating the significant neural protection of BMSCs grafting. Differences in mNSS scores were statistically significant.
between the carotid arterial BMSCs group and caudal vein BMSCs group were not statistically significant. In addition, sample size calculation was conducted based on the mNSS score after BMSCs grafting, and the suggested sample size was 7, 6, 7, and 6, respectively, for the carotid arterial BMSCs group, caudal vein BMSCs group, carotid arterial PBS group, and caudal vein PBS group, proving that the number of animals in each group was reasonable.

1. The number of positive GFAP cells was significantly higher in the carotid arterial BMSCs group (45.38±7.11) than in the carotid arterial PBS group (35.33±3.61), as well as in caudal vein BMSCs group and caudal vein PBS group (47.00±6.27 vs. 34.00±4.34). Differences in the number of positive GFAP cells between the carotid arterial BMSCs group and caudal vein BMSCs group was not statistically significant. 

2. The mean optical density value (222.90±22.63) of Bcl-2 was significantly higher in the carotid arterial BMSCs group than in the carotid arterial PBS group (195.26±12.94). This was also the same between the caudal vein BMSCs group and caudal vein PBS group (224.36±17.24). The mean optical density value of Bcl-2 between the carotid arterial BMSCs group and caudal vein BMSCs group was not statistically significant. 

3. The mean optical density value of Bax in the carotid arterial BMSCs group, caudal vein BMSCs group, carotid arterial PBS group and caudal vein PBS group was 215.09±36.81, 218.29±42.65, 231.47±46.23 and 230.10±52.17, respectively. The difference between these four groups was not statistically significant. However, the mean optical density value of Bax gradually decreased in the carotid arterial BMSCs group and caudal vein BMSCs group, compared with that in the carotid arterial PBS group and caudal vein PBS group (Figs. 1, 2 and 3).

Figure 1. The number of cells showing positive IHC staining of GFAP was significantly higher in the carotid arterial BMSCs group than in the carotid arterial PBS group. A1: carotid arterial BMSCs group, A2: caudal vein BMSCs group, A3: carotid arterial PBS group, A4: caudal vein PBS group. Scale bar = 100 μm.

Figure 2. The mean optical density value of IHC staining of Bcl-2 was significantly higher in the carotid arterial BMSCs group than in the carotid arterial PBS group. This was also the same between the caudal vein BMSCs group and caudal vein PBS group. B1: carotid arterial BMSCs group, B2: caudal vein BMSCs group, B3: carotid arterial PBS group, B4: caudal vein PBS group. Scale bar = 100 μm.

Figure 3. The mean optical density value of IHC staining of Bax gradually decreased in the carotid arterial BMSCs group and caudal vein BMSCs group, compared with that in the carotid arterial PBS group and caudal vein PBS group. C1: carotid arterial BMSCs group, C2: caudal vein BMSCs group, C3: carotid arterial PBS group, C4: caudal vein PBS group. Scale bar = 100 μm.

Figure 4. The number of positive TUNEL cells was significantly lower in the carotid arterial BMSCs group than in the carotid arterial PBS group, as well as in the caudal vein BMSCs group and caudal vein PBS group. D1: carotid arterial BMSCs group, D2: caudal vein BMSCs group, D3: carotid arterial PBS group, D4: caudal vein PBS group. Scale bar = 100 μm.
Comparison of TUNEL test results

The number of positive TUNEL cells was significantly lower in the carotid arterial BMSCs group (60.75±16.27) than in the carotid arterial PBS group (77.50±10.6), as well as in the caudal vein BMSCs group and caudal vein PBS group (58.70±17.16 vs. 76.67±15.65). Differences in the number of positive TUNEL cells between the carotid arterial BMSCs group and caudal vein BMSCs group was not statistically significant (Fig 4).

Serum TNF-α

Serum TNF-α concentration was significantly lower in the carotid arterial BMSCs group (31.81±4.72 pg/ml) than in the carotid arterial PBS group (55.3±8.35 pg/ml), as well as in the caudal vein BMSCs group and caudal vein PBS group (35.36±9.57 vs. 49.02±9.67). Differences in serum TNF-α concentration between the carotid arterial BMSCs group and caudal vein BMSCs group were not statistically significant. Furthermore, serum TNF-α concentration gradually increased in the carotid arterial PBS group, compared with the caudal vein PBS group; but the difference was not statistically significant (Table 1).

Discussion

Our findings indicate that administration of BMSCs via artery and vein is an effective therapy for middle artery occlusion in rat model. Two delivery routes reveal equally curative effect for promoting neurological recovery, ameliorating brain tissue damage and facilitating the protection mechanisms.

Cerebral infarction is a cascade of deficiencies caused by ischemia-hypoxia, which is featured by the irreversible necrosis of infarct central cells and the apoptosis of damaged cells in the ischemia and penumbra. High-level excitatory toxic substances after ischemia-reperfusion, calcium overload, the activation of free radicals and peripheral circulating inflammatory cells induces secondary damage caused by accelerated cell infiltration and apoptosis in the ischemia penumbra, which promote the further enlargement of the infarct area. Preliminary experiments in this population have proven that continuously high-level inflammatory mediators after ischemia-reperfusion are closely correlated with cell apoptosis in the ischemia-reperfusion band and accelerate neurologic impairment. It can be observed that repairing the ischemia penumbra is the key to improve the prognosis of cerebral infarction.

Serum TNF-α was reduced after BMSCs grafting in rat with acute ischemia reperfusion

After ischemia reperfusion, TNF-α is mainly secreted by activated microglial cells and mononuclear/macrophages. As a very important proinflammatory factor, TNF-α can participate in the damage and repair of cerebral infarction-induced inflammation through multiple mechanisms. Furthermore, TNF-α may destroy the blood brain barrier (BBB) to induce the upregulated expression of the adhesion molecule in vascular endothelial cells and facilitate the infiltration of invasive lesions in peripheral circulating inflammatory cells, thereby aggravating damage. Steiner et al. found that a decrease in TNF-α content can effectively reduce the infarct volume. Research results revealed that when BMSCs and activated-macrophages were co-cultured in vitro, BMSCs significantly decreased the secretion level of macrophage-sourced TNF-α through various factors, including TGF-1 with the regulatory effect on the inflammation, and prostaglandin E2 during paracrine. In the experiment, it was shown that the transplantation of BMSCs contributed to the significant decrease in TNF-α content, thereby improving neurological function.

The protective astrocytes were activated after BMSCs grafting in rat with acute ischemia reperfusion

After the cerebral infarction, activated astrocytes exhibited a high expression of nestin, vimentin and glue fibrin with particular forms and function changes. Hence, these astrocytes can adjust the K+ concentration and PH value, and absorb excess glutamate, in order to withstand glutamate neurotoxicity and improve the local microenvironment of lesions for remediation. In addition, paracrine featured a variety of nutrient and growth factors including brain derived neurotrophic factor (BDNF), nerve growth factor (NGF) and vascular endothelial growth factor (VEGF), in order to provide nutrition support for damaged cells, thereby realizing neurogenesis and revascularization. Our result showed that after the transplantation of BMSCs, the number of astrocytes increased. However, further investigation is required to clarify the specific mechanism. In addition, previous research results also showed that the transplantation of BMSCs can induce activated astrocytes to secrete glial cell line derived neurotrophic factor (GDNF), thereby strengthening the nutrition support of astrocytes.
**TUNEL staining was reduced and Bcl-2 expression was upregulated after BMSCs grafting in rat with acute ischemia reperfusion**

In recent years, many scholars have conducted the transplantation of BMSCs through various methods, in order to treat cerebral infarction. Although these treatments have effectively improved neurological impairment, the specific mechanism remains elucidated(30,31). Deng et al.(32) found that after the transplantation of BMSCs via caudal veins, cell apoptosis was significantly decreased in the infarction-embraced cortex. In the experiment, it was found that the transplantation of BMSCs decreased apoptosis and improved neurologic impairment. According to the further research, Bcl-2 was upregulated after the transplantation of BMSCs. Bcl-2 can stabilize the mitochondrial outer membrane, regulate calcium overload and inhibit pro-apoptotic protein Bax, thereby restraining apoptosis progression. This anti-apoptosis effect may be one of the curative mechanisms of BMSCs. Although a literature has verified the effect of BMSCs on the downregulated expression of Bax(33), in the present research, there was no difference between the BMSCs groups (carotid arterial BMSCs group and caudal vein BMSCs group) and PBS groups (carotid arterial PBS group and caudal vein PBS group) in terms of the expression of Bax, regardless of the decreasing tendency. The reason may be that the observed sample was relatively less and observation time was slightly shorter. In addition, we need to use more tools and provide more evidence for the reduced apoptosis and its mechanism in future studies.

**No difference was found in the curative effect of BMSC grafting on cerebral infarction between the carotid artery pathway and caudal vein pathway**

At present, the best transplantation approach of BMSCs for cerebral infarction remains to be determined. The stereotactic pathway can provide BMSCs for lesions to the largest extent, in order to reduce the loss of stem cells. However, it is difficult to apply such method for some factors including complicated operations, severe impairment and complications, and side effects such as increased intracranial pressure and edema oppression in the grafting site. On the contrary, the arterial pathway and venous pathway features stronger operability, less trauma, as well as easy to be clinically performed and accepted. Therefore, in the present research, a comparison was carried out to determine the differences in the curative effects of BMSC grafting on 6-hour ischemia-reperfusion via the trans-carotid artery and trans-caudal vein.

On one hand, the most important advantage of the carotid artery pathway is the avoidance of the first pass elimination effect from peripheral organs. Hence, the quantity of cells entering the lesions was more than those by the caudal veins(34). On the other hand, the disadvantage is that the grafted cells tend to form cell embolus, which obstructs the microvessel in the corresponding blood supply area. This restricts the increase in the number of transplanted cells and is a potential risk. The advantages of the caudal vein pathway is minor trauma and the larger quantity of transplantable cells, while its disadvantage is the retention of many cells among peripheral organs with few cells entering the lesions(35). Considering the above comparisons of advantages and disadvantages, Ruan et al.(36) found that the carotid artery pathway is superior to the caudal vein pathway.

However, in the research, it was found that there was no difference in the curative effect of BMSC grafting on cerebral infarction via the carotid artery pathway and caudal vein pathway. The reasons may be as follows: (1) Grafting via the artery pathway led to differences in the number of stem cells in the lesions but was not the factor to the difference in curative effect. (2) Stem cells grafted via the veins could continue to migrate to the lesions and played a curative role with the help of the effect of the destruction of the BBB and chemotactic factor, thereby offsetting the impact of the first pass elimination. (3) Stem cells could exert therapeutic effects by means of the anti-inflammatory medium and nutritional factors from the paracrine in the peripheral region(37), and it was not necessary for it to enter the bad lesions in the microenvironment.

In conclusion, BMSCs grafting via the carotid artery or caudal vein pathways at six hours after ischemia-reperfusion improved the symptoms of neurologic impairment, which was probably by reducing cell apoptosis in the ischemic penumbra. However, grafting via the caudal vein was the best transplantation option for the treatment of cerebral infarction during the early period, with advantages of minor trauma and the rich source of BMSCs. Therefore, BMSC grafting via the caudal vein can be a potential method to investigate the mechanisms related to the neural protection of BMSCs in rodent models of cerebral ischemia-reperfusion. Also, it provides a possible way to future clinical treatment of cerebral infarction by stem cell infusion to patients.

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**Competing Interests**

The authors declare that they have no competing interests.

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