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Protective effect of bone marrow mesenchymal stem cells combined with erythropoietin therapy on spinal cord injury rat model

Peng Xie*, Wen−Hui Ruan

Department of Orthopaedics and Traumatology, Hanzhong Municipal Central Hospital

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ABSTRACT

Objective: To study the protective effect of bone marrow mesenchymal stem cells combined with erythropoietin therapy on spinal cord injury rat model. **Methods:** SD rats were selected as experimental animals, spinal cord injury rat model was built by striking spinal cord with Hatteras Instruments PCI3000, and model rats were divided into control group, bone marrow mesenchymal stem cells (BMSCs) group, erythropoietin (EPO) group and BMSCs combined with EPO group according to different treatment methods. Then number of apoptotic cells in spinal cord tissue, contents of neural markers and neurotrophic factors as well as expression of apoptosis and injury molecules was detected. **Results:** Number of apoptotic cells as well as mRNA contents of Caspase-3 and c-fos of BMSCs group, EPO group and BMSCs+EPO group was lower than those of control group, and number of apoptotic cells as well as mRNA contents of Caspase-3 and c-fos of BMSCs+EPO group were lower than those of BMSCs group and EPO group; mRNA contents of NF-200 and MBP as well as protein contents of NGF and BDNF in spinal cord tissue of BMSCs group, EPO group and BMSCs+EPO group were higher than those of control group, and mRNA contents of NF-200 and MBP as well as protein contents of NGF and BDNF in spinal cord tissue of BMSCs+EPO group were higher than those of BMSCs group and EPO group. **Conclusions:** Bone marrow mesenchymal stem cells combined with erythropoietin therapy can inhibit cell apoptosis in the injured spinal cord tissue, increase neurotrophic factor levels and inhibit apoptosis and injury molecule expression; it has protective effect on spinal cord injury.

1. Introduction

Spinal cord injury is a rather serious type of trauma. After injury, endogenous newborn neurons were quite rare, functional axon regeneration is extremely difficult and spinal cord function is difficult to effectively recover. Stem cell implantation is a treatment method for spinal cord injury rising in recent years. Bone marrow mesenchymal stem cells (BMSCs) are the most common type of stem cells used in stem cell implantation, and can directionally differentiate into neurons and gliacytes and repair injured spinal cord[1]. However, local injured spinal cord tissue contains inflammatory mediators, oxygen free radicals and multiple other injury factors that will affect the function of migration to BMSCs and thereby affect repairing effect of BMSCs on spinal cord[2]. Erythropoietin (EPO) has the function of protecting and nourishing nerves and can create favorable local environment for BMSCs to exert the repairing effect[3]. In the following research, spinal cord injury rat models were taken as research objects to analyze the protective effect of bone marrow mesenchymal stem cells combined with erythropoietin therapy on spinal cord injury.

*Corresponding author: Peng Xie, Department of Orthopaedics and Traumatology,Hanzhong Municipal Central Hospital.
Tel: 18691691919
E-mail: xiaoxie2002xp@sina.cn
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2. Materials and methods

2.1. Research materials

SD rats were purchased from Shanghai Jiesijie Experimental Animal Co., Ltd, both TUNEL kits and Elisa kits were from Roche Company, RNA extraction kits were from Invitrogen Company, and reverse transcription kits and PCR kits were from Beijing ComWin Bio Company. Fluorescent microscope was from Nikon Company, and multimode reader and PCR apparatus were from Bio-tek Company.

2.2. Establishing methods of spinal cord injury rat model

Rats received intraperitoneal injection of 10% chloral hydrate 300 mg/kg body weight, and after the onset, they were placed in the prone position on the operation table, intumescent part in the back was taken as center to make a longitudinal incision, centrum was exposed to clamp off T8 and T9 spinous process and lamina, spinal cord tissue was fully exposed, then Hatteras Instruments PCI3000 precision strike device was used to strike the spinal cord, and parameters were set as follows: speed 1.5 m/s, depth 3.0 mm and compression time 0.85 s. Strike was under real-time observation and stopped when rats showed local hematoma of spinal cord, twitching of bilateral hind limps and spastic swinging of tail.

2.3. Treatment methods

Control group didn't receive special treatment; BMSCs group received bone marrow mesenchymal stem cell implantation therapy, on the 1 and 7 d, injecting 5 μL of BMSCs suspension with concentration of 5×10^4/μL into the injured spinal cord with micro-injector, and intraperitoneal injection of corresponding volume of normal saline; EPO group received erythropoietin treatment, intraperitoneal injection of 5 000 U/kg EPO every day for consecutive 7 d, and injecting corresponding volume of normal saline 5 μL into the injured spinal cord; BMSCs+EPO group received both bone marrow mesenchymal stem cell implantation and erythropoietin treatment, on the 1 and 7 d, injecting 5 μL of BMSCs suspension with concentration of 5×10^4/μL into the injured spinal cord with micro-injector, and intraperitoneal injection of 5 000 U/kg EPO every day for consecutive 7 d.

2.4. TUNEL staining methods

Spinal cord tissue was taken, TUNEL kits were used for staining, TUNEL reagents were used to label apoptotic cells, DAPI reagents were used to label nuclei, 100 cells of 5 random view fields were observed under microscope, and number was apoptotic cells were counted.

2.5. ELISA detection methods

Spinal cord tissue was taken, added to PBS, fully grinded and centrifuged to get protein suspension, and rat Elisa kits were used to detect NGF and BDNF contents.

2.6. RNA extraction and PCR amplification methods

Spinal cord tissue was taken, added to Trizol lysate and then fully grinded, then kits were followed to extract RNA in tissue, kits were used for reverse transcription of RNA into cDNA; cDNA specimens were taken for PCR amplification, amplified genes included NF-200, MBP, Caspase-3 and c-fos, and mRNA contents of genes were semi-quantitatively analyzed.

2.7. Statistical processing methods

Detected data was input by SPSS21.0 software. Comparison among groups was by completely randomized analysis of variance, comparison among different points in time within group was by repeated measure analysis of variance. Differences were considered to be statistically significant at a level of $P< 0.05$.

3. Results

3.1. Number of apoptotic cells in spinal cord tissue

Number of apoptotic cells was detected through TUNEL staining, and statistical analysis results were as follows: (1) comparison among different points in time within group showed that on the 3, 5 and 7 d after treatment, number of apoptotic cells of BMSCs group, EPO group and BMSCs+EPO group displayed decreasing trend; (2) comparison at same point in time among groups showed that number of apoptotic cells of BMSCs group, EPO group and BMSCs+EPO group was less than that of control group, and number of apoptotic cells of BMSCs+EPO group was less than that of BMSCs group and EPO group (Table 1).

3.2. Neural marker contents

Comparison among different points in time within group showed that on the 3, 5 and 7 d after treatment, mRNA contents of NF-200 and MBP of BMSCs group, EPO group and BMSCs+EPO group displayed increasing trend; comparison at same point in time among groups showed that mRNA contents of NF-200 and MBP of BMSCs group, EPO group and BMSCs+EPO group were higher than those of control group, and mRNA contents of NF-200 and MBP of BMSCs+EPO group were higher than those of BMSCs group and EPO group (Table 2).

3.3. Neurotrophic factor contents

Comparison among different points in time within group showed that on the 3, 5 and 7 d after treatment, protein contents of NGF and BDNF of BMSCs group, EPO group and BMSCs+EPO group

Table 1
Comparison of number of apoptotic cells in spinal cord tissue of four groups.

Group	3 d after treatment	5 d after treatment	7 d after treatment
Control group	62.21±7.59	59.91±6.92	54.58±6.22a
BMSCs group	45.62±6.52$^{(1)}$	36.22±4.12$^{(1)a}$	29.13±2.65$^{(1)ab}$
EPO group	43.98±5.28$^{(1)}$	37.13±3.99$^{(1)a}$	31.34±3.57$^{(1)ab}$
BMSCs+EPO group	34.42±3.49$^{(1)(2)(3)}$	27.18±2.94$^{(1)(2)(3)a}$	18.84±2.41$^{(1)(2)(3)ab}$

[a]: compared with the 3 d after treatment, there are differences, [b]: compared with the 5 d after treatment, there are differences; (1): compared with control group, there are differences, (2): compared with BMSCs group, there are differences, (3): compared with EPO group, there are differences.

displayed increasing trend; comparison at same point in time among groups showed that protein contents of NGF and BDNF of BMSCs group, EPO group and BMSCs+EPO group were higher than those of control group, and protein contents of NGF and BDNF of BMSCs+EPO group were higher than those of BMSCs group and EPO group (Table 3).

3.4. Apoptosis and injury molecules

Caspase-3 is apoptosis-executing molecule that can induce neuron apoptosis; c-fos is sensitive to injury factors and external trauma will increase its expression. mRNA contents of Caspase-3 and c-fos in spinal cord tissue were detected and analyzed, and results showed that mRNA contents of Caspase-3 and c-fos of BMSCs group, EPO group and BMSCs+EPO group were lower than those of control group, and mRNA contents of Caspase-3 and c-fos of BMSCs+EPO group were lower than those of BMSCs group and EPO group (Figure 1).

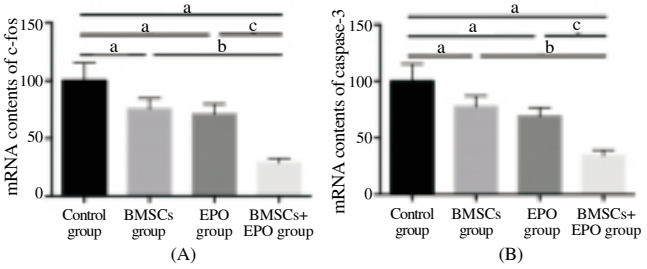

Figure 1. Comparison of mRNA contents of Caspase-3 and c-fos in spinal cord tissue.

[a]: compared with control group, there are differences, [b]: compared with BMSCs group, there aredifferences, [c]: compared with EPO group, there are differences.

4. Discussion

Stem cell implantation is important means of treatment of spinal cord injury. BMSCs are important raw materials of stem cell implantation, can be induced to differentiate into neurons and gliacytes, thus migrating to the injured spinal cord and directly repairing nerve structures, and meanwhile can secrete a variety of neurotrophic factors and contribute to the repair of injured neurons[4]. In recent years, more and more studies have reported the positive value of BMSCs implantation in treatment of spinal cord injury and other nervous system diseases[5]. However, when spinal cord injury occurs, large amounts of inflammatory factors, oxygen free radicals and other molecules with injuring effect will be generated in local part, which causes that mortality of BMSCs migrating to the local part is high, number of cells directionally differentiating into neurons and gliacytes is less and contents of secreted neurotrophic factors are insufficient, and affects the effect of stem cell implantation[6,7].

How to reduce inflammatory factors and oxygen free radicals in local injured spinal cord and create favorable condition for the repairing effect of BMSCs is the research hotspot of spinal cord injury. EPO is a new type of cell protector discovered in recent years[8]. Previous study about EPS believes that EPO is merely an endocrine hormone that acts on hematopoietic cells and has the effect of enhancing hematopoietic function[9]. In recent years, more and more studies have proved that EPO has neuroprotective and neurotrophic effect, and can promote oligodendrocyte proliferation and remyelination, as well as induce directed differentiation of neural stem cells[10,11]. BMSCs combined with EPO therapy was adopted to treat spinal cord injury, and analysis of cell apoptosis degree in local spinal cord showed that both BMSCs and EPO treatment could decrease number of apoptotic cells and after BMSCs combined with EPO treatment, decrease of number of apoptotic cells was more significant, which indicated that BMSCs combined with EPO therapy could relieve spinal cord injury and promote spinal cord repair.

Neurons and axon structures contain NF-200, MBP and other marker molecules that can reflect the reconstruction and recovery of corresponding neuroanatomy structure function. NF-200 is the main component constituting cell body of neurons and cytoskeleton of axons, plays an important role in maintaining normal function of neurons, and local NF-200 content was positively correlated with

Table 2
Comparison of mRNA contents of neural markers in spinal cord tissue of four groups.

Group	NF-200			MBP		
	3 d after treatment	5 d after treatment	7 d after treatment	3 d after treatment	5 d after treatment	7 d after treatment
Control group	100.00±15.95	116.59±12.48[a]	130.29±16.13[ab]	100.00±13.48	118.39±15.03[a]	127.64±14.42[ab]
BMSCs group	136.49±16.59[(1)]	168.79±20.13[a(1)]	189.47±19.38[ab(1)]	140.19±15.52[(1)]	155.32±17.98[a(1)]	179.91±21.49[ab(1)]
EPO group	129.39±13.48[(1)]	155.54±16.13[a(1)]	179.87±21.34[ab(1)]	138.39±15.93[(1)]	161.12±16.56[a(1)]	183.49±21.48[ab(1)]
BMSCs+EPO group	156.48±17.65[(1)(2)(3)]	191.19±23.17[a(1)(2)(3)]	247.85±30.29[ab(1)(2)(3)]	173.32±20.12[(1)(2)(3)]	216.59±23.64[a(1)(2)(3)]	272.39±31.48[ab(1)(2)(3)]

[a]: compared with the 3 d after treatment, there are differences, [b]: compared with the 5 d after treatment, there are differences; [(1)]: compared with control group, there are differences, [(2)]: compared with BMSCs group, there are differences, [(3)]: compared with EPO group, there are differences.

Table 3
Comparison of protein contents of neurotrophic factor in spinal cord tissue of four groups (ng/mL).

Group	NGF			BDNF		
	3 d after treatment	5 d after treatment	7 d after treatment	3 d after treatment	5 d after treatment	7 d after treatment
Control group	57.12±6.12	62.52±8.18[a]	70.34±7.93[ab]	107.12±12.93	125.34±16.34[a]	141.32±14.17[ab]
BMSCs group	71.34±8.44[(1)]	98.34±10.24[a(1)]	134.29±14.82[ab(1)]	132.45±15.24[(1)]	158.78±20.12[a(1)]	177.36±19.32[ab(1)]
EPO group	69.39±7.12[(1)]	92.78±9.52[a(1)]	131.35±16.29[ab(1)]	128.72±13.48[(1)]	148.48±17.13[a(1)]	168.61±19.34[ab(1)]
BMSCs+EPO group	92.34±9.24[(1)(2)(3)]	134.45±16.34[a(1)(2)(3)]	213.12±22.49[ab(1)(2)(3)]	168.59±18.34[(1)(2)(3)]	194.42±19.17[a(1)(2)(3)]	281.31±35.26[ab(1)(2)(3)]

[a]: compared with the 3 d after treatment, there are differences, [b]: compared with the 5 d after treatment, there are differences; [(1)]: compared with control group, there are differences, [(2)]: compared with BMSCs group, there are differences, [(3)]: compared with EPO group, there are differences.

recovery degree of spinal cord function. MBP is the marker protein of neuron and axon structure remyelination, and in the function reconstruction process of injured spinal cord, regenerated axon structures can only complete remyelination before they can realize rapid and effective transmission of nerve impulse. In the research, analysis of marker molecule contents in spinal cord structure showed that both BMSCs and EPO treatment could increase mRNA contents of NF-200 and MBP, and after BMSCs combined with EPO treatment, increase of mRNA contents of NF-200 and MBP was more significant, which further proved that BMSCs combined with EPO therapy could promote spinal cord repair.

BMSCs implantation therapy can not only repair injured spinal cord through differentiating into neurons, but also differentiate into gliacytes to secrete a variety of neurotrophic molecules to improve the micro-environment of spinal cord injury. Meanwhile, auxiliary EPO treatment can reduce the inhibiting effect of local injury factors on BMSCs cells, and guarantee that BMSCs can exert corresponding treatment effect. Currently known neurotrophic factors synthesized and secreted by gliacytes include NGF and BDNF, the role of the former is to promote and maintain neuron growth, survival and differentiation as well as save injured neurons[12], and the role of the latter is to maintain normal physiological function of neurons, induce directed growth of neurites as well as nourish motor and sensory nerves[13]. In the research, analysis of neurotrophic factor contents in spinal cord tissue showed that both BMSCs and EPO treatment could increase protein contents of NGF and BDNF, and after BMSCs combined with EPO treatment, increase of protein contents of NGF and BDNF was more significant, which indicated that BMSCs combined with EPO therapy could improve nutritional status of local injured spinal cord.

In pathological process of spinal cord injury, expression of a variety of apoptotic molecules and signal molecules will be abnormal. Caspase-3 is an important member of Caspase family and executive molecule of apoptosis, exogenous trauma factors will cause increase of Caspase-3 expression and induce apoptosis of neurons[14]. c-fos is a proto-oncogene in nerve cells and rather sensitive to sunlight, mechanical damage and other stimuli; noxious stimulation on neurons will promote c-fos expression[15]. In order to make clear the intervention and reverse effect of BMSCs combined with EPO therapy on pathological process after spinal cord injury, mRNA contents of Caspase-3 and c-fos in spinal cord tissue were analyzed, and results showed that both BMSCs and EPO treatment could decrease mRNA contents of Caspase-3 and c-fos, and after BMSCs combined with EPO treatment, decrease of mRNA contents of Caspase-3 and c-fos was more significant.

Based on above analysis, it is concluded as follows: bone marrow mesenchymal stem cells combined with erythropoietin therapy can inhibit cell apoptosis in the injured spinal cord tissue, increase neurotrophic factor levels and inhibit apoptosis and injury molecule expression; it has protective effect on spinal cord injury.

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