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Original Article

Effect of hyperbaric oxygen on bone mesenchymal stem cells transplant in spinal cord injury rats

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Abstract: Purpose: To observe the effect of bone mesenchymal stem cells (BMSCs) transplant on spinal cord injury (SCI) rats with the intervention of hyperbaric oxygen (HBO). Method: 60 SCI rats are divided into an Experimental Group, a BMSCs Group and a Control Group, with each group consisting of 20 rats. After the model is set up, the Experimental Group are given HBO treatment for 2 times a day; BMSCs are transplanted into the BMSCs Group and the Experiment Group on the 7th days and the 28th day after the model, while the same amount of normal saline is injected into the Control Group on the same dates. After the model is set up, the motor functions of the rear limbs of all the rats will be assessed applying BBB motor function evaluation. On the 57th day after the model is built, all the rats are killed to compare the nerve growth factor (NGF) in the injured spinal cord of the rats and the expression of brain-derived neurotrophic factor (BDNF). Result: At different points after the model is built up, the motor function of the Experimental Group is better than the other two groups (P<0.05); on the 57th day, the expression of NGF and BDNF protein of the Experimental Group is obviously higher than that of the BMSCs Group and the Control Group (P<0.05). Conclusion: Combination of HBO with BMSCs can evidently promote the recovery of the motor functions of SCI rats probably because HBO can promote the survival of BMSCs and enhance the expression of NGF and BDNF in the injured spinal cord.

Keywords: HBO, BMSCs transplant, spinal nerves, repair

Introduction

The relevant fundamental researches have confirmed that, after the mammals suffer spinal cord injury, the new nerve cells created by endogenous repair are very few and unable to start the regeneration of functional axons, therefore, their recovery is difficult [1, 2]. Mesenchymal stem cells (MSC) which exist in many organs like spinal cord and can differentiate cells with multiple mesoderm sources in an agreeable situation; in recent years, BMSCs transplant has provided a new way for SCI treatment, however, the SCI repair is not satisfactory only by means of BMDCs transplant, so, other treatment methods shall be combined

Materials and methods

Experiment materials

70 Wistar healthy rats (male, clean, 8-10 weeks old with a weight of (164±12) g); CO_2 incubator (Japanese Sanyo); Dulbecco's Modified Eagle medium (DMEM) (made by American Gibco Company); pipettes; fluorescence microscope; Fetal Bovine Serum (FBS) (bought from Hangzhou Sijiqing company); SP kit (bought from Beijing ZSGB-BIO company); BDNF and NGF antibody (bought from American Santa Cruz company).

Separation, culture and mark of MSCs

Take one healthy Wistar rat (male, 9 weeks old with a weight of 165.8 g). Separate the femurs of the rat and gather marrow under the aseptic condition. Adopt absorption separation to separate BMSCs and inoculate them into a plastic bottle with an area of 25 cm^2; then put various amino acid and glucose DMEM and FBS with a volume fraction of 10% into the bottle and culture it in the CO_2 incubator with a temperature of 37°C and a volume fraction of 5%. Change the liquid after 24 hours and rid off the non-

adherent cells. Afterwards, change the liquid once for every 3~5 days, and when 80% of the cells are fused, use 2.5 g/L trypsin for absorption and passage. Repeat the said process until the 7th generation of the cells, then put in 10 mg/L 5-Bromodeoxyuridine and mark it.

Building and grouping of SCI models

Take the rest 69 Wistar rats (male and clean) and record their age (in terms of weeks) and weight. Use special laminectomy rongeur to bite T_8 and T_9 spinal process and vertebral plate to reveal the dura mater. Use a Microscopic tweezer to directly clip the T_9 spinal cord (about 0.5 s). The spastic wag of the tails and paralysis of the rear limbs of the rats is deemed as the successful building of the models. Then, choose 60 rats for which the models are successfully build and randomly divide them into Control Group (n=20), BMSCs Group (n=20) and Experimental Group (n=20). There are no obvious differences among the basic indicators of the three groups of rats like their weight, age and the extent of paralysis of their rear limbs (P>0.05), therefore, they are comparable.

Therapeutic intervention

On the 7th and the 28th day after the models of BMSCs rats are built, conduct operation on them to expose their spinal cord and slowly inject 5 μL BMSCs (about 5×10^4 pieces/μL) DMEM into the center of the injured spinal cord within 3 minutes, retain the needle for 5 minutes, then use the medical biogum to seal the outflow of DMEM and suture the wound layer by layer. The same operation is done on the Control Group except that what is injected into the rats is the same quantity of normal saline. While for the Experimental Group, the rats are placed into the hyperbaric oxygen chamber one hour after their models are built. The chamber is washed with pure oxygen for 10 minutes, then increase its pressure to 0.2 MPa at the constant rate of 0.01 MPa/min, keep the pressure stable for 30 minutes, during which time, put in pure oxygen at intervals to keep the volume fraction of oxygen over 70%, then reduce the pressure of the chamber at a constant rate to a normal pressure for 10 minutes and take out the rats. Treat the same rats 2 times a day in the hyperbaric oxygen chamber for a consecutive period of 28 days; during this period, transplant BMSCs into such rats in the same method and for the same duration as those of BMSCs Group.

Evaluation of motor functions of rear limbs

Use the Basso-Beatlie-Bresnahan (BBB) System for Motor Functions Evaluation to evaluate the motor functions of the rear limbs of the two groups of rats respectively on the 7th, 14th, 28th, 42nd and 56th day after the models are built, with zero mark representing total paralysis and 21 marks representing total recovery of the motor functions of the rear limbs. Each rat is evaluated for 3 times and the average of the 3 results of evaluation is adopted for analysis.

Inspection of NGF and BDNF protein

On the 57th day after the models after built, use cervical dislocation method (press the head of the rats on one hand and the tail with the other hand to cause disconnection of the spinal cord at the cervical vertebra) to kill the rats of all the groups and inspect the expression of NGF and BDNF protein. In the western blot aseptic operation, take 15 mg spinal cord with T_9 as the core from the rats of the groups and preserve it in liquid nitrogen. When inspection is conducted, freeze the specimen and extract its protein for measurement of its concentration, then cryopreserve the same under -70°C for future use. Then make a casting of sodium dodecyl sulfate (SDS)-polyacrylamide separation gel and stacking gel, and add Tris-Glycine electrophoresis buffer to it, then pull off the comb to add 20 μ the specimen to each hole. Then add pre-dyed Molecular low weight markers proteins to the first electrophoresis. The initial voltage which is 8 V/cm is increased to 15 V/cm after the specimen enters the separation gel. After the electrophoresis, transfer the protein to nitrocellulose membranes and seal the membranes with skim milk powder before TBS with NGF and BDNF primary antibodies (1:500) are added. Keep the membrane in a surrounding at the temperature of 4°C, then wash it before adding secondary antibodies for reaction for 1 hour. After the membrane is washed, add DAB for color development. In the inspection of protein, PBS is used for negative control, while β-actin is used for positive control. The gel imaging and analysis system is adopted to compare the gray value of the electrophoretic band of each specimen with the β-actin band gray value and find their ratio.

Table 1. Comparison of BBB ratings of motor functions of the three groups of rats ($\bar{x}$±s)

Groups	n	7th day	14th day	21st day	28th day	42nd day	56th day
Control Group	20	1.93±0.21	3.45±1.16	4.39±1.20	6.17±1.44	8.55±1.53	9.40±1.51
BMSCs Group	20	1.90±0.26	4.87±1.22	7.58±1.31[a]	10.10±1.71[a]	14.13±1.85[a]	16.22±2.31[a]
Experimental Group	20	2.11±0.25	5.69±1.28[a,b]	9.84±1.37[a,b]	12.36±1.80[a,b]	16.32±1.89[a,b]	18.37±2.29[a,b]

Note: compared with the Control Group, [a]P<0.05; compared with BMSCs Group, [b]P<0.05.

Table 2. Ratio of positive cells of NGF and BDNF protein expression of the injured spinal cord of the three groups of rats on the 57th day (%, $\bar{x}$ ±s)

Group	n	NGF	BDNF
Control Group	20	0.24±0.04	0.35±0.06
BMSCs Group	20	0.51±0.05[a]	0.57±0.07[a]
Experimental Group	20	0.72±0.07[a,b]	0.80±0.09[a,b]

Note: compared with the Control Group, [a]P<0.05; compared with BMSCs Group, [b]P<0.05.

Statistical analysis

Conduct statistical treatment with SPssl3.0 software and the measurement data shall be expressed with ($\bar{x}$±s). Variance analysis is used to make inter-group comparison of the relevant data, if the difference showed by such comparison has statistical difference, then make pairwise comparison (also called multiple comparison) of the average data of the samples. This article employs the Dunnett-t test for pairwise comparison between a number of control group and one experimental group and P<0.05 demonstrates the statistical significance of the difference.

Result

Comparison of BBB ratings of motor functions of the three groups of rats

Before the models are built, all the rats score 20 in BBB ratings of their motor functions, and after the models are built, they are paralyzed and score zero in BBB rating; on the 7th days after the models are built, the rear legs of all the rats show retraction reaction to acupuncture, but the inter-group difference has no statistical significance (P>0.05); 14 days after the models are built, the rats in all the three groups begin slight movement which becomes obvious 21 days after the models are built; 42 days after the models are built, the motions of their rear legs appear harmonious. The comparison of their scores in BBB rating at different times shows that, from the 28th day of the model, the score of BBB rating of the BMSCs Group is evidently higher than that of the Control Group (P<0.05); while, from the 28th day of the model, the score of BBB rating of the Experimental Group is evidently higher than those of both the Control Group and the BMSCs Group (P<0.05), implying that the motor function of the Experimental Group is better than that of the Control Group and the BMSCs Group See **Table 1**.

Comparison of NGF and BDNF protein expressions of the three groups on the 57th day

See **Table 2** for NGF and BDNF protein expressions of part of the injured spinal cord of the three groups of rats on the 57th day, which shows that, the NGF and BDNF protein expressions of the Experimental Group and the BMSCs Group is obviously higher than that of the Control Group (P<0.05); and the increase of the Experimental Group is most obvious, providing a statistical significance to the difference between the Experimental Group and the BMSCs Group and the Control Group (P<0.05).

Discussion

According to the traditional view, after the nerve system is injured, new nerve cells are hard to generate to build new synaptic connection, thus making it hard for the injured nerves to recover their function, so, how to prompt the recovery of the central nervous system disease has always been a globally knotty problem [6, 7]. With the improvement of medical technology, some academicians found in recent years that, stem cells have the great potential for treatment of the central nervous system. For example, Brazelton [8] has proved that, MSC of the mouse can be differentiated in to nerve cells in its brain. Some academicians, by studying the cerebral ischemic animal model, found that, the transplanted MSC can survive in the

brain and move to the ischemic part of the brain, and that the recovery of the nervous system of that group is obviously better than that of the control group [9]. Another example is Park [10] who injected MSC into the healthy female rat before building it into a Parkinson disease (PD) model to observe the protective function of MSC for nigral cells. The result was that the number of nerve cells with tyrosine hydroxylase positive immune activity is obviously larger than that of the control group. As for the work mechanism of MSC transplant, most researchers believe that, the stem cells can build new synaptic connection by connecting the injured part of the spinal cord while generating GDNF at the injured part to improve the micro-condition of the injured spinal cord so that the generation of myelin sheath is accelerated to prompt the recovery of the nerve conduction [11-13]. At present, transplant of stem cells has become the focus of the international medical circle and brings hope to cure the disease of nervous system of human beings.

A lot of fundamental and clinical studies show that HBO has the confirmed treatment effect on SCI and has become one of the most common therapies in nerve rehabilitation. HBO can speed up the recovery of the functions of the injured spinal nerves by increasing the content of oxygen and the distance of oxygen diffusion and improving the aerobic metabolism and microcirculation [14, 15]. Other researches show that, HBO can promote the survival of endogenous and exogenous BMSCs, increase the expression of various GDNF and re-generation-related genes in the spinal cord, speed up the differentiation of endogenous and exogenous stem cells into nerve cells and induce them to move the injured part of the spinal cord [16]. The experiment conducted by Dayan shows that [17], after SCI occurs to the rat, the reaction of the endogenous stem cells proliferates which is further promoted and induced by HBO treatment to the injured part of the spinal cord, thereby, accelerating the recovery of the injured spinal cord.

The present study shows that, NGF is a special protein that can promote and maintain the growth, survival, differentiation and function execution of the nerves and protect the injured nerve cells [18]; the relevant study on animals proves that, NGF induce the esthesioneure and the noradrenergic neuron of SCI rats to extend to prompt the sprouting of the fibers of the nerve cells of the focus; the injection of NGF into the focus of cross-sectional SCI rat increases the concentration of Spinal cord axon [19, 20]. NDGF is an important member of GDNF family and has 50% of homology with NGF. It not only plays an important role in maintaining the normal physiological functions of the nerve cells, but induces the oriented growth of nervous process and adjusts the growth direction of sensory and sympathetic nerve fibers. Meanwhile, BDNF can provide nutrition to the nerve fibers and protect the injured nerve cells. As the study of Jakeman et al. [21] pointed out, BDNF can promote the development, differentiation and re-generation of various nerve cells; prevent the death of motor neuron in case of trans-section of sciatic nerves, and save the red nucleus in case of Brown-Sequard. The results of the said studies and researches show that, the increase of NGF and BDNF protein expression at the focus is essential to the recovery of functions of the injured nerves in case of SCI.

This study, after treatment of SCI rats by combining BMSCs cells transplant with HBO, finds that, after treatment for 56 days, the motor functions of the rear legs of the BMSCs Group and the Experimental Group of rats are obviously better than that of the Control Group, and the NGF and BDNF expression of the motor functions and injured spinal cord of the Experimental Group is evidently better than that of the BMSCs Group. This study implies that the combination of BMSCs transplant with HBO has a synergistic treatment effect which can further increase the motor functions of the rear limbs of the rats. This may be linked to the fact that HBO can promote the survival of exogenous BMSC and increase the NGF and BDNF protein expression. However, the definitive treatment mechanism still calls for further exploration.

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Disclosure of conflict of interest

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