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Research Article

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Observation of therapeutic effect of rats with spinal cord injury through HBO and BMSCs transplantation

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ABSTRACT

To *observe the therapeutic effect of rats with spinal cord injury through HBO and BMSCs transplantation. Jointly manufacture 60 SCI rat models, which shall be divided into the control group, BMSCs group and treatment group by adopting the random number table, 20 rats for each group. The HBO treatment shall be conducted to the rats in the treatment group for 2 times every day after molding; what's more, the transplantation treatment shall be conducted to the BMSCs group and treatment group after 1 week and 4 weeks respectively, and the equal amount of physiological saline shall be injected to the control group at the same time. BBB motor function scoring shall be adopted for assessment of the hind limb motor function of the rat weekly after molding; kill the rats in the 9th week after molding, and compare the expression situation of the nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) at the injured spinal cord of the rats in various groups. At different time points after molding, it is found that the hind limb motor function of the rats in the treatment group is remarkably better than the other 2 groups (P<0.05); in the 9th week after molding, it is found that the NGF and BDNF protein expressions of the treatment group are remarkably higher than that of BMSCs group and control group (P<0.05). HBO with BMSCs transplantation treatment can remarkably promote the recovery of the hind limb motor function of SCI rat. The treatment mechanism may be related with that HBO promotes BMSCs survival and improves the NGF and BDNF expression of the injured spinal cord.*

Keywords: HBO; BMSCs transplantation; spinal nerve; repair

INTRODUCTION

It has been confirmed through related fundamental research that after the spinal cord injury (SCI) of the mammal, there are few new nerve cells generated through the endogenous self-repair. It is difficult to initiate the functional axon regeneration[1]. The mesenchymal stem cell (MSC) is a kind of stem cell existing in the bone marrow and other tissues and having the multi-directional differentiation, which can differentiate into the tissue cell of multiple mesoderms under the suitable conditions; in recent years, the bone mesenchymal stem cells (BMSCs) transplantation has provided the new path for SCI treatment. However, the the repair function of the simple BMSCs transplantation on the injured spinal cord tissue is not ideal, and the therapeutic effect shall be further improved in combination with other measures.[2] The hyperbaric oxygen (HBO) is the common treatment means in the field of neural rehabilitation, and has the precise therapeutic effect on SCI. Based on the above-mentioned background, this research adopts the HBO and BMSCs transplantation to treat the SCI rat, and observe the changes in the hind limb motor function of the rat and the expressions of the nerve growth factor (NGF), and the brain-derived neurotrophic factor (BDNF) of the local injured spinal cord before and after the treatment.

EXPERIMENTAL SECTION

I. Main experimental materials

Select 75 Wistar rats as the stock object for experiment, male, weight of (160±14)g and 8 to 10 weeks; the flow cytometry (U.S. BD Company); CO_2 couveuse (Japan SANYO); DMEM culture medium (Dulbecco's Modified Eagle Medium, DMEM) (U.S. Gibco Company); micro-adding sample appliance; fluorescence microscope; fetal calf serum (purchased from Hangzhou Sijiqing); SP kit (pruchased from Beijing Zhongshan Jinqiao); BDNF and NGF antibodies (purchased from Santa Cruz company) and so on.

II. Separation, cultivation and mark

Take 1 Winstar healthy rat, male, weight of 165.8g and 8 weeks. Separate the bilateral femur of the rat under the sterile conditions, collect the bone marrow, separate the BMSCs with the adherence method, inoculate them to 25cm^{2}plastic bottle, add the culture medium (Dulbecco's Modified Eagle Medium, DMEM) including various amino acids and grape sugar and the fetal calf serum with the volume fraction of 10%. Cultivate them in the 37℃ incubator with the CO_2volume fraction of 5%. Change the solution after 24 hours, remove the non-adherent cells, change the solutions 1 time every 3 to 5 days afterwards, use the 2.5g/L trypsinase for digestion and passage when 80% of the cells are mixed. Change the solution for digestion and so forth, until the 7th generation of cells. Add 5-Bromodeoxyuridine (Brdu) in the culture solution for mark, and the final concentration is 15μg/ml.

III. SCI modeling and grouping

Take the rest 74 Wistar rats for backup, clean grade, male, and mark the weight and weeks of each rat. Adopt the special vertebral lamina clamp to remove the T_8 and T_9 processus spinosus and vertebral lamina of the rat, to expose the putamen. Use the microforceps to clamp the spinal cord in the T_9 segment for 0.5 s, causing the spinal cord injury. The spastic swing of the rat tail and paralysis of the lower limbs shall be taken as the standard of successful molding. Successfully select the model, and let in 60 experimental animals, which shall be divided into the control group (n=20), BMSCs transplantation group (n=20) and the treatment group by adopting the random number table. There is no significant difference between the weights, weeks and lower limb paralysis degrees and other basic indicators of 3 groups of rats (P>0.05), with comparability.

IV. Treatment intervention

Perform the surgical operations on the 7th day and 28th day of spinal cord injury. Expose the injured spinal cord, use the microinjector to slowly inject the culture solution of 5 μlincluding BMSCs (about 5×10^4 /μl) into the spinal cord injury center, which shall be completed within 3min. Retain the needle for 5min, and use the medical biogum to seal the pinhole, to prevent the cell suspension from overflow, and suture wounds layer by layer. The surgical operation of the control group is the same with that of BMSCs group. However, the same amount of physiological saline shall be injected during the period. Put the rats in the treatment group into the hyperbaric oxygen chamber 1h after molding. First of all, use the pure oxygen to clean the chamber for 10min, pressure to 0.2Mpa at the constant speed of 0.01 MPa/min and stablize the pressure for 30min. During the period, supply the pure oxygen discontinuously, to maintain the volume fraction of the oxygen above 70%. Afterwards, reduce the pressure at constant speed for 10 min to the ordinary pressure, and get out of the chamber. Perform the HBO treatments 2 times every day for 4 weeks continuously; meanwhile, the group of rats shall be subject to the HBO treatment, aided with BMSCs transplantation treatment. The specific operation and the operation time are same with that of BMSCs group.

V. Assessment of the hind limb motor function of the rat

The hind limb motor function of the rats of various groups shall be assessed by adopting the BBB (Basso-Beatlie-Bresnahan) motor function scoring[3] in the 2nd, 3rd, 4th, 5th, 7th and 9th week after molding respectively. If the hind limb has no visual movement, it shall be counted as 0 point. If the hind limb motor function is normal, it shall be counted as 21 points. Each rat shall be assessed for 3 times in a day, and the average value shall be included for analysis.

VI. NGF and BDNF protein inspection

Adopt the cervical vertebra dislocation method (press the rat head with a hand, and press the rat tail with the other hand to broke the spinal cord at the cervical vertebra, and the rat is dead immediately) to uniformly kill the rats of various groups in the 9th week after molding, and inspect the NGF and BDNF protein expressions. Under the western blot sterile operation, cut 15mg of spinal cord tissues from two groups of objects with T9 segment as the center, and put them into the liquid nitrogen for storage. During the test, take the specimen in cryopreservation to extract the protein and measure the concentration, and freeze it in the -70℃environment for backup. Make the separation gel and stacking gel of sodium dodecyl sulfate (SDS) - polyacrylamide, add Tris-glycine electrophoretic buffer solution, and pluck the comb to add sample. Add 20μL sample for each hole, and add the pre-stained molecular weight protein with the low molecular weight standards in the first electrophoresis course. The initial

voltage is 8V / cm, and shall be increased to 15V / cm after the sample enters the separation gel. Transfer the protein on the nitrocellulose membrane after the electrophoresis is completed. After the closing membrane of the skimmed milk powder, add the primary antibody (1:500) Tris (hydroxymethyl) aminomethane buffer saline (TBS) of NGF and BDNF, and stay overnight at 4℃ environment for storage. Wash the membrane, and then add the second antibody respectively for reaction for 1h. Add DAB coloration after membrane washing. For the protein inspection, PBS shall be the negative control, and the internal reference (β-actin) shall be the positive control. Adopt the gel imaging and analysis system, compare the strip gray value of each sample with that of the corresponding sample of β-actin, and obtain the ratio.

VII. Statistical analysis

The data obtained in this research shall be represented in ($\bar{x} \pm s$), SPSS 13.0 is adopted for data processing. The comparison among groups shall adopt the single factor variance analysis for inspection. The counting data shall be inspected with χ^2, and α=0.05 shall be the inspection level.

RESULTS

I. Comparison of the BBB motor function scores of rats in various groups before and after molding

The BBB motor function scores of rats in various groups are 21 points before molding; the rats in various groups on the next day after molding are completely paralyzed, and the BBB score is 0 point; in the 5th day after molding, the pinprick the hind legs of the rats of various groups, and there is the retraction reaction, but there is no difference significance among various groups (P>0.05); the slight movement appears in the rats of various groups in the 2nd week after molding, the obvious hind limb activities appears after 3 weeks, and the hind limb activities are coordinated after 6 weeks. Through comparison of the hind limb BBB scores at different time points of the rats in various groups, it is found that the BBB motor function scores of the BMSCs group are obviously better than those of the control group since 4th week after molding (P<0.05); the BBB motor function scores of the treatment group are obviously better than those of the control group since 2nd week after molding (P<0.05), which indicates that the hind limb motor function of the rats in the treatment group is obviously better than that of the control group and BMSCs group. See Table 1 for the details of the specific data.

Table I Comparison of the BBB motor function scores of the hind limbs of rats in various groups before and after molding (points, $\bar{x} \pm s$)

Group	n	2nd week after molding	3rd week after molding	4th week after molding	5th week after molding	7th week after molding	9th week after molding
Control group	20	1.9±0.2	3.6±1.0	4.5±1.1	6.2±1.2	8.6±1.4	9.4±1.3
BMSCs group	20	2.1±0.3	4.8±1.2	8.6±1.0[a]	11.1±1.8[a]	15.5±1.7[a]	17.2±1.6[a]
Treatment group	20	2.7±0.3[ab]	5.9±1.3[ab]	10.1±1.3[ab]	12.5±1.7[ab]	16.8±1.8[ab]	18.7±1.7[ab]

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

II. Comparison of NGF and BDNF protein expressions of rats of various groups

See Table 2 for the NGF and BDGF protein expressions at the local injured spinal cords of rats of various groups in the 9th week after molding. The data in the table shows that the NGF and BDNF protein expression amounts of BMSCs group are remarkably higher than those of the control group (P<0.05); the increase extent of the treatment group is respectively obvious, and the difference between the BMSCs group and control group is of statistical significance (P<0.05).

Table 2 Ratio of the positive cells of NGF and BDNF protein expressions of rats with injured spinal cord in various group in the 9th week after molding (%, $\bar{x} \pm s$)

Group	n	NGF	BDNF
Control group	20	0.21±0.04	0.36±0.06
BMSCs group	20	0.53±0.05[a]	0.61±0.05[a]
Treatment group	20	0.73±0.07[ab]	0.82±0.07[ab]

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

DISCUSSION

For a long time, it is considered in the traditional view that after the central nervous system is injured, the neurone cannot be regenerated and the new synaptic connection cannot be established. It is difficult to recover the injured

neurological function. Therefore, how to promote the rehabilitation of central nervous system diseases has always been one of the worldwide difficulties.[4] Along with the improvement of the medical treatment, some scholars have found the enormous potential of the stem cell in treatment of the central nervous system injury in recent years. For example, Brazelton and so on [5] confirm that the mouse MSC can be differentiated into the nerve cells in the brain; through research of the animal model with cerebral ischemia, some scholars find that the marrow MSC transplanted can survive in the brain, and move to the focal zone of ischemia, and the recovery degree of the neurological function of the group of animals is also obviously better than that of the control group[6]; for another example, Park and so on[7] inject the bone marrow MSC into the body of healthy female rat, make it the Parkinson's disease animal model, and observe the protective effect of the bone marrow MSC on the substantia nigra cells. It is found that the quantity of the positive substantia nigra neurons of tyrosine hydroxylase in the treatment group is obvious more than that of the control group. As for the treatment mechanism of MSC transplantation, it is currently considered by many researchers that the neural stem cells can establish the new synaptic connection by connecting the broken end of the spinal cord injury, secrete the neurotrophic factor at the injured position, improve the microenvironment of the injured spinal cord, accelerate the remyelination, and promote the recovery of the nerve conduction function[8]. At present, the stem cell transplantation has become the attention focus of attention in the international medical community, which brings hope to the treatment of the nervous system diseases of human beings.

It is indicated through a large number of basic and clinical researches that HBO has the definite therapeutic effect on the SCI, and has become one of the common treatment means in the field of neurological rehabilitation. HBO can accelerate the recovery of the neurological function of the injured spinal cord by improving the oxygen content in the tissue, increasing the oxygen diffusion distance, promoting the aerobic metabolism, improving the microcirculation and other ways[9]. What's more, studies also have shown that HBO can promote the survival of the endogenous and exogenous BMSCs, strengthen the expression of multiple neurotrophic factors and regeneration-related expression in the spinal cord tissue, accelerate the endogenous and exogenous stem cells to neuron differentiation, and induce them to move to the area with spinal cord injury[10]. For example, it is indicated through experiment and research by Liu Hai and so on[11] that the endogenous neural stem cells are proliferated after SCI of the rat. The HBO treatment can further promote the proliferation of the endogenous neural stem cells, and induce them to move to the injured region of the spinal cord, so as to accelerate the recovery process of the injured spinal cord.

At present, it is found through the research that NGF is a kind of special protein which can promote and maintain the growth, survival, differentiation and executive function of the nerve, and can protect the injured neurons[12]; it is confirmed through the related animal experiments that NGF can induce the axon elongation of the sensory neuron and the norepinephrine neurone, so that the local motion neurofibrillary in the nidus can sprout[13]; inject the exogenous NGF into the transverse SCI rat infection focus, which can increase the axon density of the corticospinal tract of the rat[14]. BDNF is one of the important members of the neurotrophic factor family, and has the 50% homology with NGF. It can not only play an important role in maintaining the normal physiological function of the neurone, but also induce the orientated growth of the neurite, control the growth direction of the sensory and sympathetic fibers. Meanwhile, BDNF also has the function to provide nutrition to the nerve fiber, and protect the injured neurons. For example, Jakeman and so on[15] point out that BDNF can promote the development, differentiation, regeneration and so on of various neurones of the organism, can prevent death of a large number of motor neurones after transection of the sciatic nerve, and can save the red nucleus neurons after semi-cutting of the spinal cord. It is indicated in the above-mentioned research results that how to improve the NGF and BDNF protein expression at the infection focus is of great significance in promoting the functional recovery after SCI.

Through BMSCs transplantation and hyperbaric oxygen treatment of SIC rat in this research, it is found that after 9-week treatment, the hind limb motor functions of the BMSCs group and treatment group are obviously better than that of the control group, and the hind limb motor function and the NGF and BDNF expressions of the injured spinal cord of the treatment group are better than those of the BMSCs group, which indicates that the BMSCs transplantation and hyperbaric oxygen can have a synergistic effect on the SCI treatment, and can further improve the hind limb motor function of the rat. The therapeutic mechanism may be related with that HBO promotes the exogenous BMSC survival and improve the NGF and BDNF protein expression. The more definite therapeutic mechanism is to be further researched.

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