Original Article
Therapeutic effect of transplanting bone mesenchymal stem cells on the hind limbs’ motor function of rats with acute spinal cord injury

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Abstract: Purpose: To research the therapeutic effect of the allograft of bone mesenchymal stem cells (BMSCs) on hind limbs’ motor function of rats that underwent acute injury to their spinal nerve. Design: 40 Wistar rat samples with the acute injury to the spinal cord were established and divided into the transplantation group and the control group, 20 for each group; One week after injury, BMSCs were slowly injected into the center of the injured spinal cord of the rats, and the physiological saline was injected into the control group. Main Outcome Measures: The rehabilitation of the motor function of the rats’ hind limbs was observed; furthermore, eight weeks after the injury, the protein disparity of the nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) between the two groups of rats was noted. Results: The rehabilitation of the hind limbs’ motor function of the transplantation group was significantly better than that of the control group from the third week on after injury, and the difference was of significance (P<0.05). Conclusions: Transplanting BMSCs can boost the protein expression of NGF and BDNF in the rats which undergo acute injury to their spinal nerves. It can, therefore, significantly improve the rehabilitation of the motor function of their hind limbs. The improvement is associated with the transplantation of BMSCs which are beneficial for regeneration and repair of the rat’s spinal nerves.

Keywords: Mesenchymal stem cells, transplantation, spinal nerves, repair

Introduction

The acute injury to the spinal cord of the mammal much too often leads to limb paralysis and other motor function disorders, which are related to the injury to the spinal nerves. And basic research has proved that, after the spinal nerves injury, new nerve cells generated by endogenous self-repair are few, and it is difficult to start the functional axon regeneration [1]. As a result, it is difficult for late rehabilitation. In recent years, the transplantation of the bone mesenchymal stem cells (BMSCs) and the development of the nerve tissue engineering have opened up a new path to treat the spinal cord injury. The mesenchymal stem cells (MSC) are the stem cells which exist in the marrow and other tissues, and have multiple differentiation potentials. They have very strong plasticity and chemotaxis. They can be transplanted to the tissue, and differentiated into various tissue cells originated from mesodermers [2] under favorable conditions so as to induce cell regeneration and growth and repair the injured tissue. Owing to the rise of research in stem cells, BMSCs has provided a new direction for research in the treatment of many diseases and shown preliminary efficacy in the recent 20 years. With regard to transplanting BMSCs to intervene in the treatment of spinal nerve injury, Xu [3] pointed out in literature review that the neural precursor cells with particular differentiation potential could be transplanted to treat the injury to the central nervous system and various neurodegenerative diseases, thus promoting patients’ recovery of their neurological function. In this research, the researchers established sample rats with acute injury to their spinal nerves, allografted BMSCs for treatment, and observed the effect.
Materials and methods

Experimental materials

Healthy Wistar rats (purchased from the Animal Breeding Center of Medical School of Zhengzhou University), clean, male, (160±14) g in weight, 8 to 10 weeks old. Two groups of sample rats were uniformly fed in different cages and taken care of by the designated person (certificate number for the qualified animal room: YYD document No. 4104022). The indoor ventilation was good, and the temperature was controlled at about 24°C. The rats were cleaned and disinfected twice a day to avoid wound infection. The rats were fed every 8 hours, and the amount of food was decided mainly by the free intake of the rats, and aided by the fluid food injected into the oral cavity with a needle tube. There was no death in two groups of rats during the nurturing period; a flow cytometry (U.S. BD Company); a CO₂ couveuse (Japan SANYO); DMEM culture medium (U.S. Gibco Company); micro pipettes; a fluorescence microscope; fetal bovine serum (purchased from Hangzhou Sijiqing); SP kit (purchased from Beijing Zhongshan Jinqiao). Anti-nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) antibodies were purchased from Santa Cruz.

MSCs separation, cultivation and labeling

Take 1 healthy Wistar rat, male, 168.9 g in weight, 9 weeks old. Separate the bilateral femur of the rat under sterile conditions, collect the bone marrow, separate the BMSCs using the adherence method, inoculate BMSCs to an 25 cm² plastic bottle, add L-DMEM and the fetal bovine serum with the volume fraction of 10%, cultivate BMSCs in the 37°C incubator with the CO₂ volume fraction of 5%. Change the solution after 24 hours, remove the non-adherent cells, change the solutions every 3 to 5 days afterwards, and use the 2.5 g/L trypsin for absorption and regeneration when 80% of the cells are mixed. Change the solution repeatedly, until the 7th generation of cells surfaced. Take the seventh generation of cells, add BrdU in the culture solution for mark, and the final concentration is 15 μg/ml. Digest the marked BMSCs with the trypsogen (7°C and 5 min), to free the cells, add them to the fresh culture medium including serum to suspend the trypsogen function, gently blow the cells with the elbow straw, put the cell suspension into the centrifugal tube for centrifugation of 1500 r/min and for 5 minutes. Then pull the supernatant, add the non-complete medium of 0.5-Iml, re-suspend the cells, take 30 μL to count the trypan blue cells, and the cell population is about 5×10⁴/μL.

Establishment of the samples with spinal cord injury

Take 50 Wistar rats as experimental subjects, clean, male, and mark the weight and week of each rat. Use the special vertebral lamina clamp to remove the T8 and T9 processus spinosus and vertebral lamina of the sampled rats so as to expose the putamen. Use the aneurysm clamp to directly clamp the T9 segment spinal cord for about 0.5 s (operation method: use the improved aneurysm clamp to remove the T9 segment processus spinosus and vertebral lamina, with the calibrated force of 35 g, use the clamp holder to open the aneurysm clamp, and then suddenly release the aneurysm clamp, to hit the spinal cord all of a sudden. The hitting time shall be about 0.5 s, and the position of the aneurysm clamp shall be accurate during the operation. It is pointed out in the related research that: [4] the method can maintain the integrity of the spinal dura mater, and the changes in the anatomical structure and the nerve function after the spinal cord injury is quite similar to the contusion-type spinal cord injury. Meanwhile, it is found that when the force is changed from 2 g to 98 g, if the clamping force is higher, the remaining axons in the injured area are less, and the functional recovery is less ideal. Therefore, the nominal force in this research is 35 g for rapid extrusion, causing the acute complete injury to the spinal cord, and establish the sample of acute injury to the spinal nerve. The spastic swing of the rats’ tail and paralysis of the lower limbs shall be taken as the standard injury. After the completion of establishing samples, select 40 of them as experimental animals, and divide them into the control group (n=20) and BMSCs transplantation group (n=20) at random. There is no significant difference between the weights, weeks, lower limb paralysis and other basic indicators of the two groups of rats (P>0.05), and they are, therefore, of comparable value.
BMSCs transplantation

BMSCs transplantation group: perform the 2nd and 3rd surgical operations on the 7th day and 28th day of spinal cord injury. Expose the injured spinal cord, use the micro-injector to slowly inject the culture solution of 5 μL including BMSCs (about 5×10^4/μL) into the spinal cord injury center, which shall be completed within 3 min. Retain the needle for 5 min, and use the medical biogum to seal the pinhole, to prevent the cell suspension from overflow, and suture wounds layer by layer. The same amount of physiological saline shall be injected to the control group in accordance with the method used with the model group.

Assessment of the hind limb motor function

This research adopts the BBB motor function scoring system [5] to assess the recovery of the hind limb motor function of the rat. The same experimenter shall conduct the single-blind method to the hind limb motor function of the rat for scoring continuously in the 1st, 2nd, 3rd, 4th, 6th and 8th weeks after the 2nd surgical operation respectively in accordance with the detailed rules of BBB scoring method. The BBB scoring standard is a new neurological function rating method formally proposed by the research personnel of U.S. Ohio University in 1995-BBB scoring method. It divides the hind limb movement of the rat into 22 grades. The full paralysis of the hind limb is 0 point, and the completely normal is 22 points. Refer to the literature for specific contents and methods [5].

NGF and BDNF examination

After 8 weeks, the rats in the control group and the transplantation group were sacrificed; then open the chest, pour the physiological saline of 200 mL 4°C through the heart to flush away the blood in the blood vessel, and then pour 500 mL of 4% 4°C paraformaldehyde phosphate buffer solution (pH 7.2-7.4). Pour the solution and fix it for 30 minutes, and put the T8 and T9 segment spinal cord of the injury area into the 4% paraformaldehyde phosphate buffer solution for over 24 hours. Cut 1 cm at the position nearest to the crossing the night before slicing, and put it into the 20% sucrose solution 4°C to stay over. Embed it with the paraffin wax; slice with the cryostat continuously, and the slice thickness is 25 μm. Immunohistochemical staining of the tissue were conducted in strict accordance with the operation instructions on the kit, and PBS shall substitute the primary antibody as the negative control. 5 slices shall be taken for each rat at random, the image processing system consisting of the image analysis card (produced by Beijing Tiandi Company), IBM586 and Olympus Bx60 microscope shall be applied for observation, and SPSS l3.0 software shall be used to calculate the amount of positive reaction cells. Calculate the average value of the NGF and BDNF positive cells of each group.

Statistical analysis

Apply SPSS 13.0 software for statistical treatment. The measurement data shall be expressed in (x±s), the comparison among groups adopt the t inspection, and P<0.05 indicates that the discrepancy has statistical significance.

Results

BBB scoring results

The BBB score of each group before injury is 21 points. The rats of each group after injury are fully paralyzed, and the score is 0 point. Pinprick the hind leg 5 days later, and there is the retraction reaction, but there is no significant differences between two groups. Conduct the comparison therapy of both groups after transplantation 1 week later (on the 7th day of damage), the hind limb activities appear after 2 weeks, the obvious hind limb activities appears after 3 weeks, and the hind limb activities are coordinated after 6 weeks. The scoring difference between 2 groups after 3 to 8 weeks of the injury has the statistical significance (P<0.05). See Table 1.

NGF and BDNF protein examination result

After BMSCs transplantation treatment, check the influence of BMSCs transplantation on the expression of NGF and BDNF by comparison of the expression of NGF and BDNF in the transplantation group and control group. After 8 weeks, kill the rats in the control group and transplantation group, and measure the NGF and BDNF protein expression. It is found that the NGF and BDNF protein amount in the transplantation group is significantly higher than that of the control group (P<0.05) (Table 2).
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Discussion

For a long time, it is considered in the medical circle that the nerve cell is a kind of permanent cell, which lacks the regeneration capacity. Therefore, traditional view holds that if the central nervous system is injured and causes loss of a great amount of neurones, the injured function is difficult to recover because the new neurones cannot be generated and the new synaptic connection cannot be established [6]. Therefore, the disease of the central nervous system has always been a worldwide difficulty in clinical treatment.

Along with the improvement of the medical treatment, people find the huge potential of the stem cell in treatment of the central nervous system injury. The discovery of the stem cell challenges the knowledge that the neurone of the nervus centralis of the adult mammals cannot be regenerated [7]. For example, Park et al [8] put MSC, the genetically modified bone marrow, into healthy female rats, and observe the protective function of the bone marrow MSC to the substantia nigra cells by establishing the animal model with Parkinson’s disease. It is found that the positive substantia nigra neurons of immunological competence tyrosine hydroxylase in the treatment group is obviously higher than that of the control group, which indicates that MSC transplantation can improve the neurological symptoms of the Parkinson’s patients, and realize the treatment effect. Harvey et al [9] think that BMSCs can be induced and divided into the neurons and neuroglial cells, and move to the diseased region, generating many kinds of neurotrophic factors and acceptors, which can promote the repair of the injured spinal cord neuron, and inhibit the nerve regeneration cicatrix. Han et al [10] point out that BMSCs have the function to regenerate and repair the spinal cord injury. In the research, by transplanting the bFGF genetically modified BMSCs into the injured spinal cord, they find that it can greatly improve the expression level of bFGF, and can effectively promote the recovery of motor function after spinal cord injury of the rats Many researches show that the neural stem cell can connect the broken end of the spinal cord injury, establish the new synaptic connection, and meanwhile secrete the neurotrophic factor at the injured place, so as to improve the microenvironment of the spinal cord injury, promote the marrow sheath regeneration, and recover the nerve conduction. Therefore, the stem cell transplantation has become the hot issue in the current international medical circle, and brings hope to the treatment of the nerve diseases of human beings [11].

Through BMSCs transplantation to the rat with acute injury to the spinal cord in this research, we observe the influence on the treatment of the spinal nerve injury of the rat. It is found that after the treatment, the rehabilitation efficacy of the hind limb motor function of the rat in the transplantation group is obviously higher than that in the control group, and the NGF and BDNF protein expressions of the rats in the transplantation group is obviously higher than that in the control group. NGF is a special protein which has proven to be able to promote and maintain the nerve growth, survival and executive function, and save the injured neurones [12]. Through gene transfer NGF

### Table 1. BBB Scoring of Hind Limb Motor Function of Rats of 2 Groups (n=20, x±s)

<table>
<thead>
<tr>
<th>Group</th>
<th>1 week</th>
<th>2 weeks</th>
<th>3 weeks</th>
<th>4 weeks</th>
<th>6 weeks</th>
<th>8 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>1.93±0.22</td>
<td>3.59±1.03</td>
<td>4.51±1.07</td>
<td>6.24±1.20</td>
<td>8.64±1.39</td>
<td>9.35±1.33</td>
</tr>
<tr>
<td>Transplantation group</td>
<td>2.14±0.31</td>
<td>4.77±1.18</td>
<td>7.58±1.03*</td>
<td>9.06±1.81*</td>
<td>15.45±1.66*</td>
<td>17.17±1.62*</td>
</tr>
<tr>
<td>T</td>
<td>0.314</td>
<td>1.746</td>
<td>3.175</td>
<td>3.842</td>
<td>5.215</td>
<td>5.376</td>
</tr>
<tr>
<td>P</td>
<td>0.717</td>
<td>0.285</td>
<td>0.026</td>
<td>0.021</td>
<td>0.007</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Note: *P<0.05.

### Table 2. Counting of the positive motor nerves of chemical staining of the NGF and BDNF immune tissue of the control group and the transplantation group (piece, n=20, x±s)

<table>
<thead>
<tr>
<th>Group</th>
<th>NGF</th>
<th>BDNF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>37.56±4.82</td>
<td>124.31±8.63</td>
</tr>
<tr>
<td>Transplantation group</td>
<td>95.36±6.51</td>
<td>256.42±9.15</td>
</tr>
<tr>
<td>T</td>
<td>6.485</td>
<td>5.016</td>
</tr>
<tr>
<td>P</td>
<td>0.004</td>
<td>0.012</td>
</tr>
</tbody>
</table>

Note: Comparison between the transplantation group and the control group, P<0.05.
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research, it is proven that after spinal cord injury of the adult rat, NGF can induce the axon elongation of the sensory neuron and the noradrenephrine neuron, so that the local motion neurofibillary in the nidus can sprout [13]. The exogenous NGF is injected into the part of the cross-sectional spinal cord injury, which can remarkably increase the density of the axon of corticospinal tract [14]. BDNF is the important member in the neurotrophic factor family, and it has 50% homology with NGF [15]. It not only plays an important role in maintaining the normal physiological functions of the neurones during the developmental process of the central nervous system, but can also induce the orientated growth of the neurite, and determine the growth direction of the sensory and sympathetic nerve fiber. Meanwhile, it has the nutrition activity of the motor nerve fiber, and protects the spinal motor neuron from death after spinal cord injury. For example, Schanker et al [16] point out that BDNF can maintain and promote the development, differentiation, growth and regeneration of multiple neurones, can prevent numerous motor neurones from death after transverse cutting of the sciatic nerve, and can save the red nucleus neurons after semi-cutting of the spinal cord. It can be seen that NGF and BDNF are the important substances in the mechanism intervening in the neural repair, and have important influence on the nerve growth and repair effect. That is to say, the expression of NGF and BDNF substance can directly influence the neural repair effect. Therefore, after BMSCs transplantation of the rat with spinal cord injury, the NGF and BDNF protein expression is remarkably improved, this can promote the repair of the injured spinal cord.

This experiment successfully establishes the in vitro culture system of the allograft BMSCs of rats. After BMSCs transplantation experiment, the reailitation efficacy of the hind limb motor function of the rats of 2 groups shall be inspected. The rehabilitation efficacy of the transplantation group is remarkably better than that of the control group, which is due to that BMSCs transplantation can induce the NGF and BDNF expression. Because the leg motor function of the rat model with spinal nerve injury is related with the spinal nerve injury, the spinal nerve repair directly influences the motor function of the leg. However, NGF and BDNF are the important substances intervening in the neural repair mechanism. Their full expression can promote the survival and regeneration of the neurone of the rats with spinal nerve injury and regeneration and repair of the spinal nerve, and has an important influence and restrictive function in good growth and repair of the nerves. Through comparison of the NGF and BDNF protein expression levels of rats of 2 groups, it is found that the transplantation group is obviously better than the control group, which indicates that BMSCs transplantation can promote the NGF and BDNF protein expression. However, the significant expression of the nerve factor can promote the regeneration, growth and repair of the injured spinal nerve tissue cells of rats. Therefore, the rehabilitation of the leg motor function of the rat in the transplantation group benefits more from the BMSCs’ induction on NGF and BDNF, to promote the repair of the spinal nerve function.

Disclosure of conflict of interest

None.

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