ORIGINAL ARTICLES

Experimentation of acute injured spinal cord by transplanting the bone marrow stromal cells

Laixing Liu, Yude Zhu, Xiangyi Wang*, Jishen Li, Yixin Deng, Xin Sui

Department of Neurosurgery, Baogang Hospital, Bao Tou, Inner Mongolia, China

Received: August 16, 2015 **Accepted:** October 24, 2015 **Online Published:** December 10, 2015
DOI: 10.14725/dcc.v2n4p10 **URL:** http://dx.doi.org/10.14725/dcc.v2n4p10

Abstract

Objective: In this study, bone marrow stromal cells (BMSCs) were transplanted in situ after spinal cord injury (SCI) in rats. It was proved that the implanted BMSCs could differentiate into neuron-like cells in the injured spinal cord, and the long-term motor ability test was carried out to investigate the recovery of neurological dysfunction after transplantation.
Methods: (1) An improved Alien's SCI rat model (weight 10 g, height 30 mm) was made by Alien weight drop method. Methyl eosin (Haematoxyli - n/eosin, HE) was used to identify with SCI. (2) The BMSCs were identified by the method of bone marrow adherent culture in vitro. The morphology of the cells was observed by cell staining, and CD44 was detected by BMSCs. After three cultures, the cells were transfected with lentiviral vectors carrying Green Fluorescent Protein (GFP) gene. (3) Cell survival after passage and transplantation: the GFP labeled BMSCs in situ (injury zone) were implanted into the injury model. The spinal cord sections were sacrificed after 2 weeks (2w), 4 weeks (4w) and 6 weeks (6w) respectively, and the cells expressing GFP were observed under immunofluorescence microscope. (4) The modified Rivlin oblique plate test and spinal motor function blood brain barrir (BBB) score method were used to compare the changes of exercise ability in the simple injury group, 2w after transplantation group, 4w after transplantation group and 6w after transplantation group.
Results: (1) The Alien's SCI rat model was made by using Alien's heavy drop method, and the effect was reliable and stable. (2) The proliferation of BMSCs after 3 passages has stabilized. After culture and amplification in vitro, the cell morphology changed to be conical, protruding interwoven into a network. Therefore, it could be used as a source of cell transplantation after SCI. The morphology of the cells was observed by staining, and the positive CD44 was detected. (3) BMSCs were transfected into the SCI model by lentiviral vector carrying GFP gene, and the expression of GFP in 6w was observed. (4) The results of modified inclined plane test and Rivlin locomotor BBB score of rats in simple injury group were significantly worse than those in transplantation control group ($p < .05$). 2w, 4w and 6w after transplantation groups could improve long-term motor function. The recovery of neurological dysfunction after 6w was the best, and the difference was statistically significant ($p < .05$).
Conclusions: BMSCs transplantation could promote the recovery of neurological dysfunction after SCI, and the mechanism may be related to the differentiation of BMSCs into neurons and glial cells, and the BMSCs induced by transplantation could interact with peripheral nerve cells and produce some cytokines.

Key Words: Spinal cord injury, Bone marrow stromal cells, Transplantation

Spinal cord injury (SCI) is a common surgical emergency with high morbidity and mortality, which seriously threatens human health and quality of life.[1] Nerve cells are a kind of permanent cells, which would be permanently deletion once damaged. Furthermore, it could be only repaired by glial cells and fibers to form glial scar.[2] The discovery and transplantation of stem cells provide a new way to solve this problem, and is becoming the focus of research in recent years. At present, stem cell research has become one of the frontiers in the field of scientific research in twenty-

*Correspondence: Xiangyi Wang; E-mail: dcc54@ncspress.com; Address: Department of Neurosurgery, Baogang Hospital, Bao Tou, Inner Mongolia, China.

first century. More and more researchers pay attention to stem cell transplantation for the treatment of damaged cells and reconstruction of nerve function.[3,4] Embryonic stem cells, neural stem cells, umbilical cord blood stem cells and bone marrow stromal cells (BMSCs) are the most commonly used cells for the study of transplantation. BMSCs are non-hematopoietic stem cells widely found in bone marrow tissue, which are characterized by self-renewal, multidirectional differentiation potential and strong amplification ability. After the differentiation of BMSCs into neuron-like cells in vitro, the defect of SCI was filled to improve the function of the damaged nervous system. It has an incomparable advantage over other cells for the treatment of central nervous system diseases. Green fluorescent protein (GFP) can solve the problem of cell labeling effectively since it can express GFP in the experimental study of BMSCs in transfected rats. The purpose of this study was to culture and amplify the homologous BMSCs in vitro and to label the amplified cells with GFP. The labeled BMSCs were transplanted into the damaged model of SCI rats, and the cell differentiation of BMSCs in the injured spinal cord model was observed. The recovery of neurological deficits in SCI rats after BMSCs transplantation was investigated by measuring the exercise ability of rats at different stages. It provides more adequate theoretical and experimental basis for BMSCs transplantation in the treatment of SCI.

1 Materials

1.1 Experimental animals and groups

Forty clean Wistar rats aged 6 weeks (supplied by the Animal Experimental Center of Inner Mongolia University), weighing 200 g $\pm$ 20 g, no matter male or female were selected. They were randomly divided into 4 groups: simple injury group (n = 10), 2w after transplantation group (n = 10), 4w after transplantation group (n = 10) and 6w after transplantation group (n = 10). SCI models were established in rats of simple injury group and transplantation group.

1.2 Reagents

Serum, F12 medium, DMEM medium, FBS reagent, chloral hydrate, lentiviral transfection of green fluorescent protein, eosin, hematoxylin, xylene, APES anti stripping tablets, absolute ethanol, 40 or 96 wells of polystyrene board, anti-GFAP antibody, anti-NSE antibody, goat anti-mouse IgG antibody.

1.3 Instruments

Electronic balance, super clean bench, centrifuge, inverted microscope, laser confocal microscope, LSM410, CO_2 incubator, ultra low temperature refrigerator, electric thermostatic drier, paraffin and frozen section machine, paraffin slicing machine, modified Alien'S device, modified Rivlin plate.

2 Methods

2.1 Establishment of SCI model in rats

An improved Alien's SCI rat model was made by using Alien's weight drop method (weight 10 g, height 30 mm).[5] Signs of successful SCI models: when the Alien's device hits the spinal cord, the body trembles, and the lower limbs rapidly retract and move. The tail tilts and falls rapidly, and it attacks the spinal cord rapidly. After the application of antibiotics, abdominal pressing method was employed for artificial urination, 3 times/d, until the recovery of the function of micturition in rats.

2.2 HE staining

Serial sections of the spinal cord were performed after transplantation of 2w, 4w and 6w, and the thickness of the slice was 20 μm. HE staining was measured to observe the distribution and migration of the transplanted cells under the microscope.

2.3 Culture of BMSCs in vitro

A clean Wistar rat weighing 200 g was killed by anesthesia and soaked with 75% alcohol for 15 min. The bilateral femur and tibia were removed. After removing the both ends of epiphysis, the DMEM/F12 complete medium was used to flush the bone marrow cavity. After repeated flushing 2 times, the density gradient centrifugation method was used. After repeated passages for 3 times, BMSCs were purified and amplified. The numbers, morphology and growth characteristics of cells were observed continuously and recorded every day.

2.4 Identification of BMSCs

BMSCs were identified by CD44 detection of cell surface markers. CD44 positive cells were observed by confocal laser scanning microscope.

2.5 Transfection of GFP gene into rat BMSCs lentivirus

The third generation of BMSCs was selected as the lentiviral vector for GFP gene transfection. The transfection rate of

GFP was determined by flow cytometry, and the uninfected BMSCs were used as controls. Fluorescence microscopy showed that the cells transfected with lentivirus GFP gene presented strong yellow green color. After 2-3 d, the number of yellow green cells increased, and the transfection expression reached peak value.

2.6 Situ cell transplantation in rat spinal cord

T10 was positioned by rib approach, and cell transplantation was performed at the T10 level as the transplantation site. BMSCs, which successfully transfected lentiviral GFP gene, were extracted from the 0.1 ml (10^5 cells) suspension with a 1 ml syringe at the site of injury, and were slowly injected into the dura in 5 min (without damage to spinal cord).

2.7 Modified Rivlin oblique plate test

Behavior and limb motor function were measured in rats. The recovery of hindlimb activity and muscle strength in rats was observed. The motor function of rats before and after transplantation was evaluated. The rats were placed on a rectangular wooden inclined plate and covered with a rubber pad. The inclined plate could be rotated to measure the angle of the inclined plate. The rat body axis was parallel to the longitudinal axis of the inclined plate, and the head of the rat was tilted toward the high end of the inclined plate. The high end oblique plate rose 5 degrees each time, and the maximum angle at which the rat could stay for 5 s was programmed as its function value. Each animal was measured 5 times, and the mean value was measured then.

2.8 BBB scoring system for spinal motor function

The BBB score has been recognized as a scoring standard for evaluating the functional recovery of the hind limb after thoracic SCI in rats. Its basic tests include the number and range of joint movement, the degree of joint weight-bearing and the coordination of limbs, front and back claws and tail activity. The observation period was 4 min, so as to keep the animals in the center of the range of activity.

2.9 Survival and immunofluorescence staining of spinal cord cell transplantation

The rats in the 2w, 4w and 6w transplantation group were anesthetized and fixed by perfusion, and then stained with immunofluorescence staining. 5 fields of vision were selected randomly, the cells were counted and the distribution, differentiation and migration of the cells were observed under fluorescent microscope.

2.10 Statistical methods

Data were analyzed using by SPSS 13.0. The measurement data were expressed as mean ± standard deviation (x ± s), and multiple groups were analyzed by variance analysis with $p < .05$ indicating statistical significance.

3 Results

3.1 General performance of experimental animals after SCI

The rat body started shaking, and its lower limbs quickly bounced and retracted. Its tail tilted and fell rapidly when the modified Alien's device (parameters of weight 10 g, height 30 mm) hit the spinal cord. Less rats were died using this method, which was in line with the experimental requirements.

3.2 Pathological changes

3.2.1 Gross pathological changes

After the establishment of rat model, contusion and bleeding were seen on the surface of the spinal cord, but there was no bleeding in the parenchyma. No hematoma or injury occurred in the spinal cord. The gross pathological changes of the injured spinal cord showed that the extent and range of injury were basically the same, which met the requirements of the experiment.

3.2.2 Histological changes after hematoxylin eosin staining

Pathological observation showed that the range and degree of spinal cord tissue injury were basically consistent in each group after HE staining, which accorded with the experimental requirements. The gross pathological changes of the injured spinal cord showed swelling in the injured area, scattered spots in the cortex and medulla in the contusion area, and punctate hemorrhagic foci with different sizes. In the early stage of injury, the number of neurons in the injured area and its surrounding areas decreased significantly, and the swelling, necrosis, dissolution and degeneration of the nerve cells occurred. In the late stage of injury, the nucleus of nerve cells were shrunken and cracked, and the number of necrotic neurons increased significantly. The neural defects formed by necrotic neuronal lesions were replaced by glial scar formed by reactive proliferation of glial cells in the later stage.

3.3 CD44 staining of BMSCs

BMSCs were identified by cell surface marker CD44 detection. After BMSCs passage to the third generation, CD44 immunohistochemical staining showed that CD44 expression was positive, which was cytoplasm staining.

3.4 Lentiviral vector mediated GFP transfection in BMSCs

The third generation BMSCs was transfected with lentiviral vector carrying GFP gene, and the expression of GFP gene was observed under fluorescence microscope after transfection with BMSCs. It was observed that the cells appeared polygonal and protruding out of the antenna, mostly appeared at 12 h. After replacement of the culture medium, the morphology of the cells returned to normal after 24 h. Two days later, green fluorescence could be observed stronger in BMSCs with GFP than 24 hours before. It was suggested that BMSCs have been successfully transfected with lentiviral vector carrying GFP gene and stably and highly expressed in cells.

3.5 Survival of transplanted cells in situ transplantation of SCI

BMSCs with GFP gene were transplanted in situ of SCI. The rats in the transplantation group were sacrificed, and the spinal cord slices were taken in 2w, 4w and 6w after transplantation. Under the fluorescence microscope, the GFP labeled cells in the survival site of transplanted spinal cord showed green fluorescence, and the transplanted cells were scattered in the lesion.

3.6 Changes of motor function of spinal cord

3.6.1 BBB scoring of motor function of hindlimb of rats

After 3 weeks of experiment, the scores of hindlimb motor function in each group were shown in Table 1. The motor ability of the treatment group gradually recovered, and the recovery of motor ability in the simple injury group was not obvious with the treated days. The results of the simple injury group were significantly worse than those of the transplantation control group ($p < .05$).

Table 1: BBB scoring of rats in each group ($\bar{x} \pm$ s)

Groups	1d	3d	1w	2w	3w
Simple injury group	0.51 ±0.54	0.68 ±0.81	3.34 ±1.02	4.84 ±1.32	6.34 ±1.74
transplantation control group	0.66 ±0.83	0.82 ±0.99	4.32 ±1.38	6.82 ±1.18	12.32 ±1.64*

Note. * Compared with simple injury group, $p < .05$

3.6.2 Modified Rivlin oblique plate experiment in rats

In the experiment, the average angle was $60° \pm 1.8°$ degree before SCI model establishment. After SCI, there was no significant difference in the angle of oblique pulling test between the simple treatment group and the transplantation group ($p > .05$) at the first day of the experiment. In the experimental group, the angle of the inclined plate was increased at the beginning of the 5^{th} day, and increased to $39.4° \pm 2.191°$ in the transplantation group 7 days after transplantation. Compared with the previous week, the difference was statistically significant ($p < .05$). The difference was still statistically significant when compared with the simple injury group ($p < .05$). After 3 weeks, the angle of the inclined plate did not increase significantly ($p > .05$). After 3 weeks of observation, the angle of the plate in the simple injury group recovered slowly, which was significantly worse than that in the transplantation group, and the difference was statistically significant ($p < .05$), as shown in Table 2.

3.7 Differentiation of BMSCs after implantation in vivo

BMSCs with GFP gene were transplanted in situ of SCI. The rats in the transplantation group were sacrificed and the spinal cord slices were taken at 2w, 4w and 6w after transplantation. Under the fluorescence microscope, the survival of transplanted spinal cord cells was marked by GFP labeled cells dry green fluorescence, and the transplanted cells were scattered in the lesion. At the 2^{nd} week, the transplanted BMSCs were observed to migrate to the injured spinal cord under fluorescent microscope. At the 4^{th} week, BMSCs began to focus around the injured cortex. After 6 weeks, the number of cells expressing GFP began to decrease. Immunofluorescence staining showed the expression of glial cells specific protein (glial fibrillary acidic protein, GFAP), microtubule associated protein and neuronal marker protein (neuron specific enolase, NSE) of transplanted cells.

Table 2: Comparison of oblique plate experiment in each group

Post-surgery	Groups	Angle	*SD*	*t*	*p*
1d	Simple injury group	24.800	1.799	0.390	.717
	transplantation group	25.200	2.960		
2d	Simple injury group	30.200	2.208	0.279	.790
	transplantation group	30.800	2.260		
7d	Simple injury group	36.500	2.618	2.498	.037
	transplantation group	39.500	2.191		
2w	Simple injury group	41.200	2.740	3.725	.006
	transplantation group	46.800	1.948		
3w	Simple injury group	41.800	2.281	4.619	.002
	transplantation group	49.200	2.645		

4 Discussion

Acute SCI is a common surgical emergency in clinic. Because of its high disability rate and expensive treatment cost, it brings heavy burden to the family and society, so it receives wide attention from clinicians. To date, there is no effective treatment method for SCI. The most common causes of SCI are spinal trauma, vertebral degeneration and ischemic lesions of the spinal cord, among which spinal trauma is the most common one. A large number of experiments have shown that BMSCs can differentiate into neurons and glial cells through the germinal layer, so it can be used as seed cells for repairing nerve injury.[6, 7] BMSCs transplantation provides new hope for the recovery of neurological function after SCI. At present, the methods of SCI cell transplantation mainly focus on direct transplantation, intravenous transplantation, and abdominal transplantation. Abdominal cavity transplantation of rats with immune response has been proved to induce abdominal lymph node enlargement in many experiments and the effect is weak. Vein transplantation is superior to peritoneal transplantation, but it is characterized by more immune reaction. Direct transplantation is easy to operate, effective, safe and light in immune response, so we use the method for our experiment. Schoch et al.[2] showed that destruction of BBB structure might result from many damage factors, such as trauma, hypoxia, ischemia and so on. The mechanism may be related to the disintegration of the basement membrane under the action of metalloproteinases, the close connection of endothelial cells and the necrosis and apoptosis of astrocytes. The permeability of BBB is greatly enhanced, which makes it easy for the transplanted cells to pass through the blood-brain barrier and reach the injured site. Fluorescent stem cell transplantation was also performed in normal rats in China,[8] and no fluorescent stem cells were found in the spinal cord and brain. In this study, we observed that BMSCs had the ability of directional migration and site-specific differentiation to injured tissues. The increased permeability of BBB and the directional migration of BMSCs in injured sites were consistent with the injury area and the accumulation of transplanted cells in our study. The results of this experiment have fully demonstrated the role of BMSCs in the repair of nerve injury and promoted the recovery of nerve function through the proliferation, differentiation, migration and the fusion of nerve tissue structure.

The mechanism of SCI treatment may be stated as follows:[9, 10] There have been some achievements in the treatment of SCI with BMSCs, but there are still many doubts about the mechanism of BMSCs in repairing SCI. At present, two kinds of view regarding the mechanism of the disease are generally accepted in the scientific community. One is to transplant the induced BMSCs in the injured spinal cord. These cells repair the damaged spinal cord in structure and accumulate in the damaged area. Firstly, the structure breaks up the interruption of the upper and lower spinal cord, so that the interrupted nerve pathway can be re-connected. Furthermore, BMSCs differentiated neural-like cells, which are extensively connected through the dendrites and peripheral nerve cells, compensate for the loss of signal transduction function. Another mechanism is that BMSCs induced by transplantation interact with peripheral nerve cells and produce some cytokines. These cytokines not only inhibit glial scar formation, but also protect cell survival. In the microenvironment of these cytokines, neural cells are regenerated, survived, neovascularization, and local vascular remodeling. It creates favorable conditions for the growth of damaged spinal nerve and the functional repair of the injured spinal cord.

The neuronal marker protein NSE, glial specific protein GFAP and microtubule associated protein 2 expressed by BMSCs were observed in immunofluorescence staining. It was proved that BMSCs differentiated into neurons and glial cells in the spinal cord after transplantation injury. In this study, we evaluated the recovery of neurological deficits after SCI by monitoring the motor function in rats within 3 weeks. The experimental results showed that the results of BBB and oblique plate test were significantly different between the simple injury group and the transplantation control group, and the difference was statistically significant ($p < .05$). 1w, 2w and 3w after transplantation groups could improve long-term behavioral outcome, and the recovery of neurological dysfunction after 3w was the best, the difference was statistically significant ($p < .05$). These findings

suggest that BMSCs can differentiate into neurons and glial cells after SCI, and promote the recovery of neurological deficits caused by traumatic SCI.

In conclusion, BMSCs transplantation can promote the recovery of neurological deficits after SCI, and improve long-term behavioral outcomes. The mechanism may be related to the differentiation of BMSCs into neurons and glial cells, and the induction of BMSCs after transplantation, which may interact with the neural cells of the descendants and produce some cytokines. This study provides a new experimental and theoretical basis for BMSCs transplantation in the treatment of SCI.

Conflicts of Interest Disclosure

The authors have no conflicts of interest related to this article.

References

[1] Fang B, Ma H. Research progress of bone marrow stromal stem cell transplantation for spinal cord injury. Chinese Journal of Rehabilitation Medicine. 2011; 26(10): 985-989.

[2] Wang SY. Transplantation of bone marrow stromal stem cells for the treatment of spinal cord injury. Journal of Kunming Medical University. 2007(2B): 86-90.

[3] Kim HJ, Lee JH, Kim SH, et al. Therapeutic effects of human mesenchymal stem cells on traumatic brain injury in rats: secretion of neurotrophie factors and inhibition of apoptosis. Neurotrauma. 2010; 27(1): 131-138. PMid: 19508155. https://doi.org/10.1089/neu.2008.0818

[4] Mahmood A, Lu D, Qu C, et al. Treatment of traumatic brain injury with a combination therapy of marrow stromal cells and atorvastatin in rats. Neurosurgery. 2007; 60(3): 546-553. PMid: 17327800. https://doi.org/10.1227/01.NEU.0000255346.25959.99

[5] Xiong CX, Zong SH, Zeng F. Establishment and evaluation of rat Allens spinal cord injury model. Journal of Guangxi Medical University. 2011; 28(2): 215-217.

[6] Englund U, Bjorklund A, Wietorin K, et al. Grafted neural Stem cells develop into functional Pyralnidal neurons and Integrate into hest cortical circuitry. Proc Natl Acad Sci USA. 2002; 99(26): 17089-17094. PMid: 12471158. https://doi.org/10.1073/pnas.252589099

[7] Cunullings BJ, Tamaki SJ, Tamaki SJ. Human neural stern cells differentiate and Promote locomotorrecovery in spinal cord-injured mice. Proc Natl Acad Sci USA. 2005; 102(39): 14069-14074. PMid: 16172374. https://doi.org/10.1073/pnas.0507063102

[8] Yang XH, Li M, Li J, et al. Bone marrow mesenchymal stem cell transplantation for the treatment of spinal cord injury in different experimental study. Journal of Kunming Medical University. 2006; 6(2): 30-33.

[9] Zompa EA, Cain LD, Everhart AW, et al. Transplant therapy: recovery of function after spinal cord injury. J Neurotrauma. 1997; 14: 479-506. PMid: 9300561. https://doi.org/10.1089/neu.1997.14.479

[10] Murray M, Fischer I, Smeraski C, et al. Towards a definition of recovery of function. J Neurotrauma. 2004; 21: 405-413. PMid: 15115590. https://doi.org/10.1089/089771504323004557