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Transplantation of bone marrow mesenchymal stem cells alleviates spinal cord injury *via* inhibiting Notch signaling

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Abstract. – OBJECTIVE: To analyze the mechanism of action by which the bone marrow mesenchymal stem cells (BMMSCs) repair the spinal cord injury (SCI) in rats via the Notch signaling pathway.

MATERIALS AND METHODS: A total of 75 male rats aged about 12 weeks old were equally divided into group A (sham operation group), group B (model group), and group C (model group + BMMSCs). The SCI model was established by Allen's method, and the differences in presenilin-1, Hes1 and Notch proteins among the three groups of rats were evaluated via immunohistochemical staining and Western blotting.

RESULTS: Group B exhibited a lower Basso, Beattie, and Bresnahan (BBB) score at each time point than group A and group C ($p<0.05$), and the BBB score in group C was lower than that in group A ($p<0.05$). According to the average optical density analysis results of the immunohistochemically stained proteins, the optical density of presenilin-1 protein in group A was lower than that in both group B and group C ($p<0.05$), and group C exhibited a lower optical density of presenilin-1 protein than group B. In group A, the protein expression of Hes1 in the bone marrow tissues of rats was not evident and weakly positive. Compared with that in group A, it was substantially raised ($p<0.05$), and the strongly positively expressed Hes1 proteins were yellow or dark brown in group B. Compared with that in group B, the color of Hes1 proteins was lighter ($p<0.05$), and the positive level of Hes1 proteins was lowered in group C. Group A showed inconspicuously positively expressed Notch proteins, group B brown active Notch proteins, while group C several brown Notch proteins. The optical density of Notch proteins in group A was overtly lower than that in group B and group C ($p<0.05$), and it was significantly lower in group C than that in group B ($p<0.05$). Additionally, group B had an evidently higher expression level of Notch proteins than the other two groups ($p<0.05$), and the expression level of Notch proteins in group C was a little higher than that in group A ($p<0.05$).

CONCLUSIONS: BMMSCs inhibit the Notch signals to promote the proliferation and differentiation of rat neurons, thereby repairing spinal neurons.

Key Words:
Bone marrow mesenchymal stem cells, Notch, Spinal cord injury.

Introduction

Spinal cord injury (SCI) is a damage-induced disease that is relatively severe clinically, but there are no rather effective treatments currently, and it is characterized by a high disability rate, so that the central nervous system injury has serious consequences[1]. This disease tends to involve tissue tearing and deformation due to external causes[2], and according to the analysis of numerous factual data, primary SCI is far milder than secondary SCI externally caused[3,4]. These injuries will change human spinal neurons, mainly in the forms of deterioration, necrosis, degeneration, and loss, throwing heavy burdens on the patients and society. Studies have manifested that the incidence rate of SCI in China exceeds the global level, making China a country with highly prevalent SCI. Therefore, it is a top priority in the medical field to explore the SCI mechanism and rehabilitation treatments now. Bone marrow mesenchymal stem cells (BMMSCs) are a kind of long diamond-like

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and fibrillar cells, and it has been found that they can adhere to plastic culture dishes when isolated through adherent culture[5]. Currently, these cells, as relatively ideal donor cells for SCI cellular transplantation, have a strong multiple-target regulatory effect on astrocyte activation. BMMSCs are considered as preferred seed cells in tissue engineering, since they have such features as low immunogenicity, easy access, and strong self-renewal ability[6,7]. Indeed, they have been widely applied to the SCI experiments[8]. The Notch signaling pathway is a highly conservative signaling pathway extensively present in cells and tissues. In the proliferation, differentiation, and apoptosis of the adjacent cells, it is able to transmit cell signals with them in neurons[9]. If nuclear Notch signaling is halted in adult zebrafishes, most cells will stop in the S phase of mitosis, increasing neurogenesis. In a zebrafish with SCI, the motor nerves, controlled by Notch signals, are very difficult to re-differentiate. It can be seen that once activated in the central nervous system, the Notch signaling pathway can damage neuronal axons to hinder their growth and make them retract in severe cases. In summary, the Notch pathway has an important influence on neural circuit reconstruction, but the specific regulatory mechanism of the Notch signals in nerve regeneration is still unknown and remains to be further investigated. Thus, in the present study, the model of SCI was established to analyze the mechanism of action of BMMSCs in rat SCI repair *via* the Notch signaling pathway.

Materials and Methods

Laboratory Animals

The present experiment was conducted in the Experimental Center of Sichuan University using 75 healthy male rats aged 10 weeks old and weighing 200-275 g (provided by the Experimental Center of Nanjing Medical University). This study was approved by the Animal Ethics Committee of Sichuan University Animal Center.

Animal Grouping

A total of 75 healthy rats were randomly divided into 3 groups, namely group A (sham operation group, n=25), group B (model group, n=25), and group C (model group + BMMSCs, n=25), and the spinal cord tissues were taken from each group of rats to detect the presenilin-1 and Hes1 proteins therein. The rats in group A only underwent laminectomy without SCI, those in group B were given a certain volume of normal saline after the spinal cord was damaged by Allen's combat method, and those in group C were directly injected with BMMSCs daily (6 h/time) for 28 d in a row after spinal cord was injured by Allen's combat method.

Main Instruments and Reagents

The main instruments and reagents used in this experiment are as follows: HI-0400 Spinal Cord Impactor (Thermo Fisher Scientific, Waltham, MA, USA), Olympus IX70 Inverted Microscope (Olympus, Tokyo, Japan), H-7500 Transmission Electron Microscope (Hitachi, Tokyo, Japan), staining solution (Solarbio, Shanghai, China) and kits (SBI, San Francisco, CA, USA).

Establishment of SCI Model by Allen's Method

The model was established according to the following operations. With T_{10} as the center, the rats were disinfected and anesthetized, and a small about 2 cm-long incision was made along the upper spine to separate and expose the spine. Then, the T_{10} vertebral plate was excised, while the spinal dura was retained. Acute SCI was caused at T_{10}-T_{11} segment using Allen's impactor at the height of 6 cm and injury magnitude of 60 g/cm. The sign indicating that the model was successful were both lower limbs and the body of rats retracted and fluttered, with tail swinging reflex, and the incomplete paralysis occurred in both lower limbs.

Collection of Specimens and Culture of BMMSCs

The three groups of rats were persistently fed with drugs for intervention, and 5 weeks later, they were anesthetized *via* intraperitoneal injection of 1.5% pentobarbital sodium at a dose of 0.15 g/kg (weight). Then, the rats were sacrificed, and the bilateral femurs were taken out under aseptic conditions and cryopreserved at -80°C for later detection of the signaling pathway-related proteins presenilin-1 and Hes1 in the femurs. The blood was extracted from the carotid artery of the rats to determine the content of alkaline phosphatase (ALP), phosphorus, and calcium in rats, and the BMMSCs rat were cultured. After the femurs were rinsed using Phosphate-Buffered Saline (PBS), the bone marrow cells were harvested, centrifuged for 10 min at 350 g at

normal temperature, and cultured in the DMEM/F12 complete medium containing 100 g/L fetal bovine serum (FBS, Hyclone, South Logan, UT, USA), with the medium replaced once every 3-4 d. In the present experiment, the BMMSCs rats sub-cultured into the 4-6th generations were used for immunohistochemical staining.

Image Acquisition

A total of 3 images (×400) were selected from each group and analyzed using the Image-Pro Plus 6.0 (Silver Springs, MD, USA) analysis software, and the accumulated optical density value was measured based on the effective area selected.

Determination of Rat Basso, Beattie, and Bresnahan (BBB) Score

The behaviors and hind limb motor function of rats in the three groups were evaluated before SCI and at 7, 14, 21, or 28 d after SCI using the SCI motor function scoring method. Before scoring, all the rats were examined for the filling degree of the bladder so as to reduce the experimental errors.

Immunohistochemical Staining

After being deparaffinized, the spinal cord tissue sections were soaked in 3% methanol-H_2O_2 for 20 min and washed using PBS for more than 3 times (10 min/time). Then, they were dropwise added with goat serum blocking solution and placed at room temperature for 15-30 min, and with the excess liquid discarded, they were incubated with 100 μL of primary antibody at 35°C overnight and warmed at 37°C for 35 min. After being washed with PBS for 3 times (5 min/time), the sections were added with 100 μL of secondary antibody, let stand at room temperature for 1 h, and washed using PBS for 3 times (5 min/time), followed by diaminobenzidine (DAB) color development for 2 min and observation of the staining degree under the microscope. The resulting sections were washed with PBS for another 3 times (once every 2 min), counterstained with hematoxylin for 10-15 min, washed using tap water for 5-10 min, and finally sealed.

Detection of Presenilin-1, Hes1, and Notch Proteins Via Western Blotting

A 6-well plate was added with 120 μg of trypsin extract and then with 3 mL of medium to terminate the digestion. Subsequently, the cell extract was placed in an Eppendorf (EP) tube, mixed with the trypsin extract at 1:100, and frozen in a refrigerator for 10 min. Once the cells were completely divided into E solution, the EP tube was added with spinal tissues and 3 mL of trypsin extract at 1:100 to fully split the cells into F solution. Then, the E and F solution was mixed at the volume ratio of 80:1, shaken evenly, and prepared into the working solution. After that, the working solution was stored in an incubator at 37°C for 20-30 min. Finally, the concentration of proteins in the cooled solution was calculated.

Detection of Presenilin-1, Hes1, and Notch Proteins in Rat Brain Tissues Via Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

The neurons of brain tissues were digested using trypsin. After being rinsed using PBS, they were dropwise added with 0.9% sodium chloride solution and then added with 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) reagent and 2 mL of ribonucleic acid (RNA) extraction reagent to extract RNAs. Subsequently, 20 mg of neurons of brain tissues were placed into an EP tube, and the RNAs extracted were reversely transcribed into complementary deoxyribonucleic acids (cDNAs) using the A3500 RT kit. The cDNAs were stained with the nucleic acid gel dye to detect the expression of target genes using the CFX-96 qRT-PCR instrument. The related primers used were designed using the NCBI Primer-BLAST, and the reaction conditions are as follows: 98°C for 6 min, 98°C for 28 s, 75°C for 30 s, and 80°C for 4 min for 55 cycles in total. The denaturalization was effective in PCR cycles. The gene primer sequences are shown in Table I.

Table I. Primer sequence.

Gene	Primer sequence
Notch	Forward: 5'GACTCCAAGATGAAGAAGATGTG3' Reverse: 5'GAGCATTCGCAGGTVCAAGCC3'
GAPDH	Forward: 5'CAACGGGAAAGCCATCACCA3' Reverse: 5'ACGCCAGTAGACTCCACGACAT3'

Statistical Analysis

In the current analysis, the Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp, Armonk, NY, USA) software was used for statistical analysis. The *t*-test was performed for the comparison of the correlation among the three groups. The experimental results were expressed as mean ± standard deviation (`χ±s). The comparison between multiple groups was done using One-way ANOVA test followed by the post-hoc test (Least Significant Difference). $p<0.05$ denoted that the difference was statistically significant.

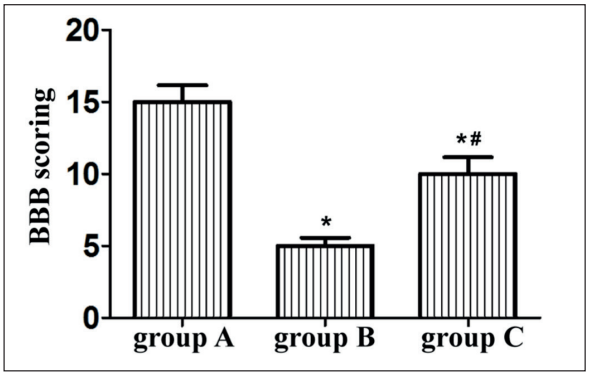

Figure 1. BBB scoring results. Note: $^{*}p<0.05$, *vs.* group A and $^{\#}p<0.05$, *vs.* group B

Results

Comparison of BBB Score at Each Time Point Among the Three Groups

The BBB score at each time point in group B was lower than that in both group A and group C ($p<0.05$), and group C showed a lower BBB score than group A ($p<0.05$) (Figure 1).

Immunohistochemical Staining Results of Presenilin-1 Protein Expression in All Groups of Rats

As shown in the staining images, the immunopositive expression signals of presenilin-1 protein were dark brown and mainly present in the anterior angle and posterior angle and near the central canal in the rat spinal cord tissues. Group A had a lower expression level of presenilin-1 protein in the rat spinal cord tissue cells than group B and group C, and the expression level of presenilin-1 protein was the highest in group B (Figure 2). The average optical density analysis results of the immunohistochemical staining showed that the optical density of presenilin-1 protein in group A was lower than that in group B and group C, and that it was lower in group C than that in group B (Figure 3).

Immunohistochemical Staining Results of Hes1 Proteins in All Groups of Rats

In the staining figures, the positively expressed Hes1 proteins were brownish yellow or brownish gray. Group A exhibited inconspicuously and weakly positively expressed Hes1 proteins in the rat spinal cord tissues. Compared with that in group A, the expression of Hes1 proteins was substantially enhanced ($p<0.05$), and the Hes1 proteins were strongly positive and yellow or dark brown in group B. Compared with group B, group C showed a lower staining intensity in Hes1 proteins ($p<0.05$) and a decreased positive level of Hes1 proteins (Figures 4 and 5).

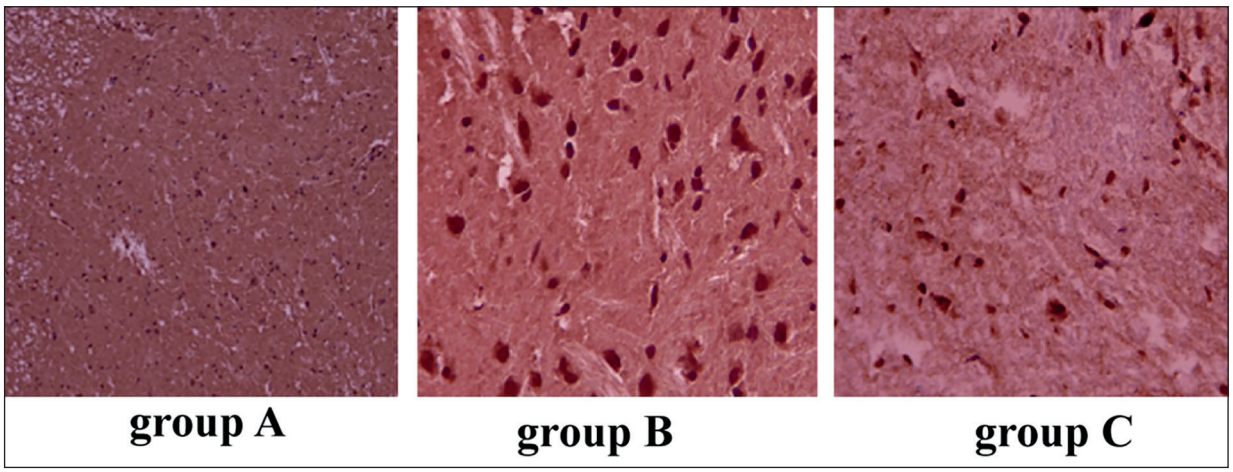

Figure 2. Immunohistochemical staining results of presenilin-1 protein in all the groups (×200).

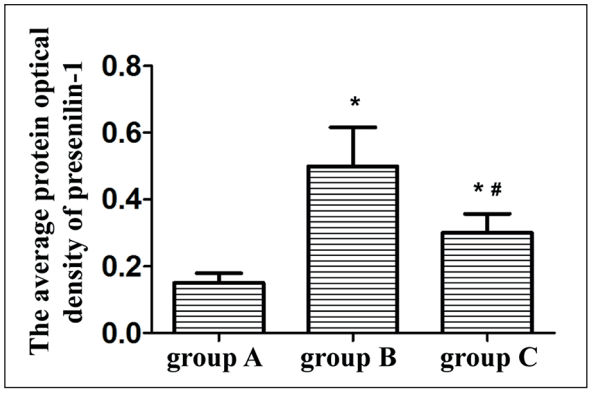

Figure 3. Comparison of the average optical density of presenilin-1 protein expressed among all groups of rats ($\bar{x} \pm s$, n=12). Note: $^{*}p<0.05$, *vs.* group A and $^{\#}p<0.05$, *vs.* group B.

Notch Proteins After SCI According to the Immunohistochemical Observation Results

At the distal SCI site, the brown positively expressed Notch proteins were mainly present in the white matter. In group A, the positively expressed Notch proteins were not evident, the brown active Notch proteins were seen in group B, and several brown Notch proteins were found in group C (Figure 6). The optical density of Notch proteins in group A was evidently lower than that in both group B and group C ($p<0.05$), and compared with that in group B, it was significantly lowered in group C (Figure 7).

Protein Expression of Notch in the Three Groups of Rats

Group B had a significantly higher expression level of Notch proteins than group A and group C ($p<0.05$), and the expression level of Notch proteins in group C was slightly higher than that in group A ($p<0.05$) (Figure 8).

Discussion

SCI is a secondary injury mainly caused by an external force, in which neurons are impaired. If neurons are not effective, resulting in neuron apoptosis, SCI may further cause permanent neuronal damage, ultimately leading to irreparable central nervous injury. Therefore, how to reduce the disability rate of SCI is the current hotspot in clinical research[10,11]. Now, the key of studies is how to alleviate the secondary injuries to a maximum extent to rescue the injured spinal cord neurons, and meanwhile, to promote the repair of spinal nerves after injury, thereby rebuilding the impaired spinal cord function[12]. The specific mechanisms in SCI involve cell apoptosis, inflammatory response, and mitochondrial dysfunction, and they can induce more inflammatory factors, thus causing spinal neuron necrosis and apoptosis.

According to the results of the present investigation, the BBB score at each time point in group B and group C was notably lower than that in group A ($p<0.05$), and group C had substantially higher BBB scores than group B from the 28th d ($p<0.05$). BBB scores are classified into 0-21 grades, and the lower the grade, the higher the severity. Experimental results have proven that the rats scoring 0-5 grades experience different degrees of paralysis in hind limbs. In the present study, the rat spinal cord in group B scored the

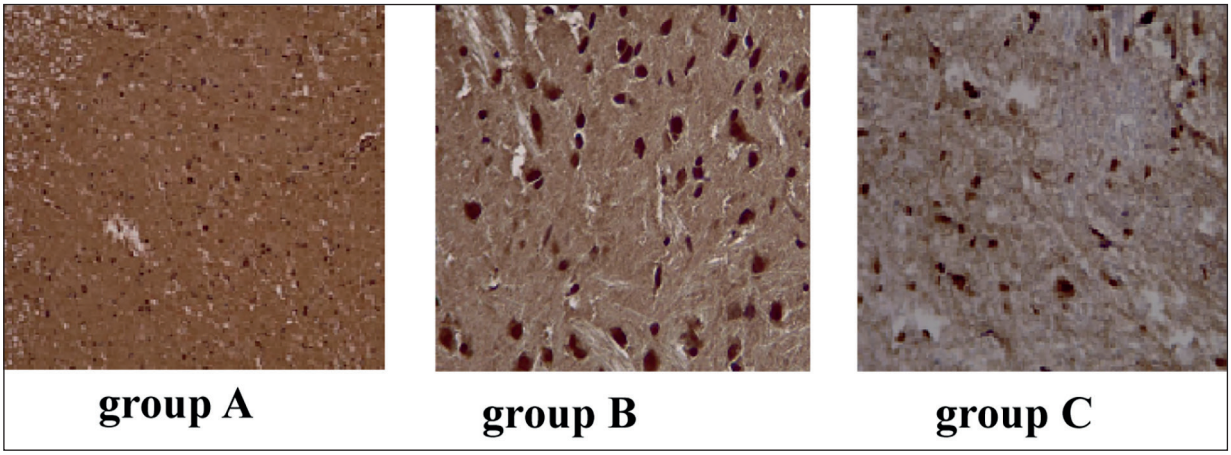

Figure 4. Immunohistochemical staining results of Hes1 proteins in all the groups (×200).

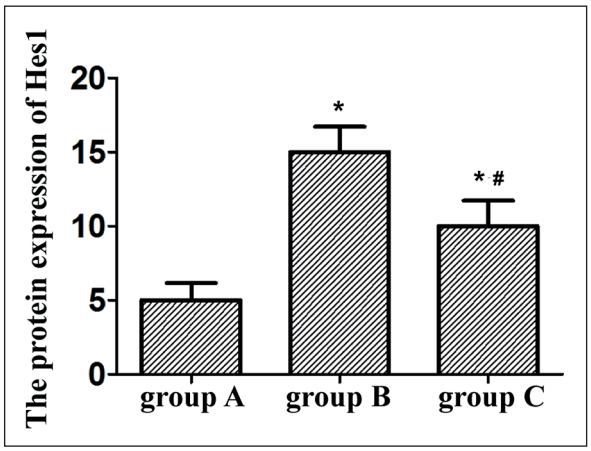

Figure 5. Comparison of Hes1 protein expression among the three groups of rats. *Note:* $^{*}p<0.05$, *vs.* group A and $^{\#}p<0.05$, *vs.* group B.

lowest level, suggesting that SCI was severe. On the contrary, the higher were the scores, the milder was the SCI.

As shown in the staining images, group A had a lower expression level of presenilin-1 protein in the rat spinal cord tissue cells than group B and group C, and the expression level of presenilin-1 protein was the highest in group B (Figure 2). According to the average optical density analysis results of immuniohistochemical staining images, the optical density of presenilin-1 protein in group A was lower than that in group B and group C, and it was lower in group C than that in group B (Figure 3). Group A exhibited inconspicuous and weakly positive Hes1 protein expression in the rat spinal cord tissues. Compared with that in group A, the expression of Hes1 proteins was substantially enhanced ($p<0.05$), and the Hes1 proteins were strongly positive and yellow or dark brown in group B. Besides, compared with group B, group C showed lighter stained Hes1 proteins ($p<0.05$) and a decreased positive level of Hes1 proteins. Presenilin gene was firstly found in the elderly patients with cerebellar atrophy[13], and both presenilin-1 gene and PSI gene chromosomes have been found in them[14,15]. The inhibition of the presenilin-1 signaling pathway can accelerate the differentiation of the embryonic stem cells into motor neurons. In the growth and development of neurons, a low level of presenilin-1 signals can promote the growth and extension of neurites, while the up-regulated presenilin-1 signals can suppress the neurite extension and even result in neurite retraction. It suggests that the activation of presenilin-1 can worsen SCI and stimulate the inflammatory factors in SCI. Hes1 is a protein factor that can promote the proliferation of neural stem cells when lowly expressed. The decrease in Hes1 expression can accelerate the differentiation of neural stem cells into neurons, so the Hes1 in the proliferation and differentiation of neurons can be repressed to protect against SCI. In other words, Hes1 protein protects from SCI *via* inhibiting inflammatory factors. Presenilin-1 and Hes1 factors play the same roles in SCI, which are positively correlated with each other.

At the distal SCI site, the Notch proteins are positively expressed as brown. In group A, the positively expressed Notch proteins were not evident, brown active Notch proteins were seen in group B, and several brown Notch proteins were found in group C (Figure 6). The optical

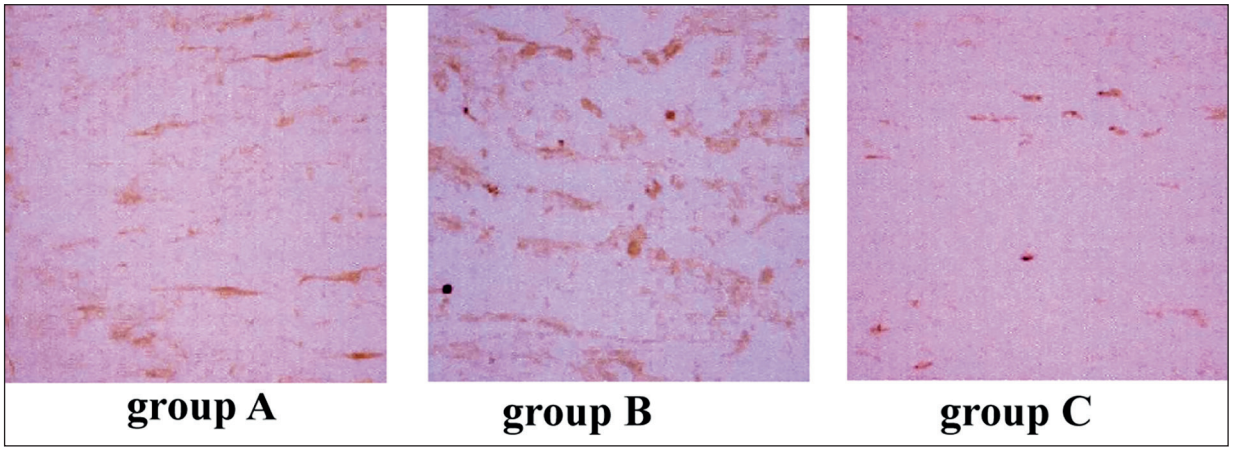

Figure 6. Immunohistochemical staining results of Notch protein (×400, 50 μm).

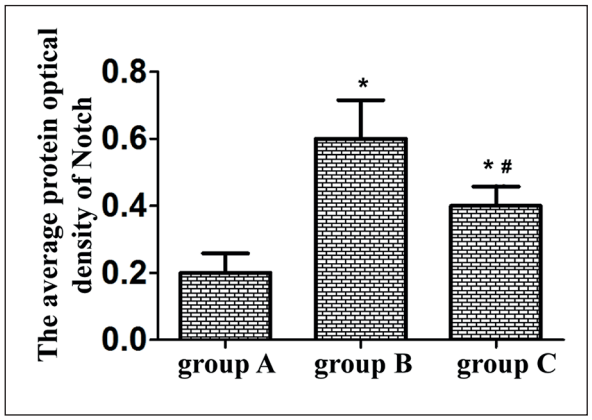

Figure 7. Protein expression of Notch in the three groups of rats. Note: $^{*}p<0.05$, *vs.* group A and $^{\#}p<0.05$, *vs.* group B.

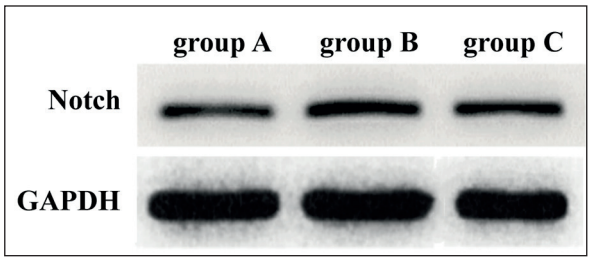

Figure 8. Protein expression of Notch in the three groups of rats.

density of the Notch proteins in group A was evidently lower than that in both group B and group C ($p<0.05$), and compared with that in group B, it was remarkably lowered in group C ($p<0.05$). Notch signal refers to a kind of cellular communication and can recognize the cells in cell development[16,17]. Human diseases develop based on the aberration of the components in the Notch signaling pathway. Greenwald et al[18] showed that the Notch signaling pathway plays an important role in regulating the self-maintenance, proliferation, and differentiation of neural stem cells. The Notch signaling pathway is distributed in different regions and can be expressed in both pluripotent stem cells of nervous tissues and non-nervous tissues. After binding to the ligands, its extracellular domain conformation allows the presence of tumor necrosis factors and promotes the activation and reproduction of these factors, while inducing the apoptosis of other cells[19-21]. Therefore, when Notch is upregulated, SCI is severe. It is speculated that the suppression of the Notch factors can relieve SCI.

Conclusions

We found that BMMSCs inhibit the Notch signals to promote the proliferation and differentiation of rat neurons, thereby repairing spinal neurons.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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