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Effect of exosomes derived from MiR-133b-modified ADSCs on the recovery of neurological function after SCI

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Abstract. – OBJECTIVE: To investigate the recovery effect of exosomes derived from micro ribonucleic acid (miR)-133b-modified adipose-derived stem cells (ADSCs) on neurological function after spinal cord injury (SCI) and its mechanism.

MATERIALS AND METHODS: The SCI model of rats was used and divided into the following 5 groups: sham-operation group, 4 d SCI group, phosphate-buffered saline (PBS) group, miR-control group and miR-133b group. At 96 h after operation, rats were euthanatized, and spinal tissues were removed. Next, the level of miR-133b was detected via reverse transcription-polymerase chain reaction (RT-PCR), and the expression of RhoA protein was measured via Western blotting. Moreover, expressions of proteins associated with the axon regeneration pathway, including phosphorylated-cAMP-response element binding protein (p-CREB), CREB, phosphorylated-signal transducer and activator of transcription 3 (p-STAT3) and STAT3, along with expressions of neurofilament (NF), growth associated protein 43 (GAP43), glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP), were tested by Western blotting and immunofluorescence staining.

RESULTS: The miR-133b mimics significantly upregulated the expression of miR-133b in adipose-derived stem cells (ADSCs), compared to blank group ($p<0.05$). The expression of miR-133b was significantly decreased in 4 d SCI group compared with that in sham-operation group ($p<0.001$). The RhoA expression was statistically increased in 4 d SCI group compared with that in sham-operation group ($p<0.001$), and it was partially impaired by using miR-133b compared with that in 4 d SCI group ($p<0.001$). Expressions of NF, GAP43, GFAP and MBP were remarkably higher in 4 d SCI group than those in sham-operation group ($p<0.01$), and they were also significantly increased in miR-133b group than those in 4 d SCI group ($p<0.01$). Besides, our data showed a significant increase of p-CREB/CREB, p-STAT3/STAT3, NF, GAP-43, GFAP and MBP in miR-133b group compared to those in 4 d SCI group, with statistical reduction of RhoA ($p<0.05$).

CONCLUSIONS: We showed that exosomes derived from miR-133b-modified ADSCs can significantly promote the recovery of neurological function of SCI animals through affecting the signaling pathway related to axon regeneration and expressions of NF, GAP-43, GFAP and MBP.

Key Words:
Spinal cord injury, Mesenchymal stem cells, Exosomes, miR-133b.

Introduction

Spinal cord injury (SCI) is a kind of severe disease causing nervous system injury and damage in neurological function of patients. The incidence rate, disability rate and treatment expenses pose heavy burdens for patients and their families[1]. The surgical intervention, drug and high pressure oxygen are major measures in the clinical treatment of SCI[2], but clinical effects remain unsatisfactory. In recent years, stem cell technology has emerged, but only a small number of stem cells can differentiate and integrate with damaged tissues after transplantation[3]. According to recent studies, the paracrine of stem cells is one of the main effects in the treatment of tissue and organ damage with stem cell transplantation, in which exosomes play an extremely important role[4,5]. The exosome is a kind of vesicle with 50-100 nm in size, which is generally released outside cells in the pathological process[6]. Scholars[7] have found that a large number of exosomes can be secreted by adipose-derived stem cells (ADSCs), and the protective effect of transplantation of exosomes secreted by stem cells alone on tissue damage is similar to that of

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transplantation of stem cells. It is speculated that exosomes may be one of the major substances in the treatment of damage with stem cells. Moreover, exosomes can transmit genetic materials, such as messenger ribonucleic acid (mRNA) and micro RNA (miRNA), to recipient cells, and effectively regulate a variety of physiological functions of recipient cells. For example, exosomes secreted by ADSCs can transmit miR-122 to primary liver cancer, regulate the expression of a variety of cytokines and increase the sensitivity of cancer cells[8]. In this study, we sought to determine the protective role of exosomes derived from mir-133b-modified ADSCS against from SCI.

Materials and Methods

Experimental Animals

A total of 42 healthy male adult Sprague-Dawley (SD) rats weighing (300±50) g were purchased from Shanghai Bioray Laboratories Inc. This research was approved by Affiliated Concord Hospital of Tongji Medical College, Huazhong University of Science And Technology Ethics Committee.

Reagents and Instruments

Rabbit anti-human neurofilament (NF), growth associated protein 43 (GAP43), glial fibrillary acidic protein (GFAP), myelin basic protein (MBP), extracellular signal-regulated kinase 1/2 (ERK1/2), phosphorylated-ERK1/2 (p-ERK1/2), signal transducer and activator of transcription 3 (STAT3), p-STAT3, RhoA, cluster of differentiation 9 (CD9), CD63, CD81, cAMP-response element binding protein (CREB) and p-CREB monoclonal antibodies and corresponding secondary antibodies were obtained from Beijing Ding Guo Chang Sheng Biotechnology Co., Ltd. (Beijing, China). Diaminobenzidine (DAB) color developing agent was bought from Shanghai Xinyu Biotechnology Co., Ltd. (Shanghai, China). Citrate buffer powder was obtained from Shanghai Xinyu Biotechnology Co., Ltd. (Shanghai, China). Real-time fluorescence quantitative polymerase chain reaction (PCR) kit was collected from Guangzhou Vipotion Biotechnology Co., Ltd. (Guangzhou, China). TRIGene reagent was acquired from Beijing Kangrun Chengye Biotechnology Co., Ltd. (Beijing, China). Reverse transcription kit was originated from Shanghai Yuduo Biotechnology Co., Ltd., (Shanghai, China). SBI Exo Quick-TC kit was purchased from Shanghai Mito Biological Technology Co., Ltd., (Shanghai, China). Real-time fluorescence quantitative PCR instrument was made by Applied Biosystems, ABI 7500 (Thermo Fisher Scientific, Waltham, MA, USA). Paraffin-embedding machine was provided by SPCC-6D, Dongguan Pubiao Laboratory Equipment Co., Ltd., (Dongguan, Guangdong, China). Inverted optical microscope was from Olympus BX40 (Shinjuku, Tokyo, Japan). Aneurysm clip was from Aesculap, FT280T (Center Valley, PA, USA). Agarose horizontal electrophoresis apparatus was from DYCP-31BN, Beijing Liuyi Instrument Co., Ltd., (Beijing, China)

Cell Transfection with miR-133b

MiR-133b-3p mimics and miR-133b-3p negative control were synthesized by Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA), added with diethylpyrocarbonate (DEPC)-treated water according to instructions and stored in a dark place at low temperature. ADSCs were purchased from Cyagen Biosciences Inc., (Guangzhou, Guangdong, China) and were inoculated into a 6-well plate. In blank group, miR-133b-3p negative control group and miR-133b-3p mimics group, 5 repeated wells, were set till the cell density was up to 80%. The mixed solution of 5 μL RNA mimics or 5 μL negative control was added into the serum-free culture solution containing 1 μL Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA). After standing for 0.5 h, the cell solution in the plate was removed. The plate was washed, added with 2 mL serum-free cell solution, slowly added with the oligonucleotide-Lipofectamine 3000 polymer and mixed evenly, followed by incubation for 5 h. The transfection solution was replaced with the complete culture solution for culture. Cells were collected after stable transfection for 72 h.

Extraction of Exosomes

Exosomes were extracted from the culture solution strictly according to the procedure of the Exo Quick-TC kit. ADSC culture solution was collected and centrifuged to collect the supernatant. Exo Quick-TC Exosome precipitation solution was added, mixed evenly and placed in a refrigerator at 4°C overnight. After centrifugation for 0.5 h, the supernatant was transferred and centrifuged for 5 min, the residual liquid in the test tube was sucked dry, and an appropriate amount of phosphate-buffered saline (PBS) was added for re-suspension of precipitate.

Establishment of SCI Model of Rat and Grouping

The SCI model of rat was established according to the method reported by Xin et al[9]. All SD rats were fasted for solids at 12 h before modeling and liquids at 4 h before modeling, and they were fixed on the fixing plate after abdominal anesthesia. The boundary between lowermost rib and soft tissue was disinfected, and skin and subcutaneous fascia were cut longitudinally to expose the muscle. The supraspinous ligament and interspinous ligament were cut off under observation by naked eye, T9 and T11 spinous processes were removed using a mosquito clamp, the *ligamentum flavum* was pried, and T10 posterior wall of spinal canal was cut off using ophthalmic scissors to expose the spinal cord. After the T10 spinal cord was clamped using the aneurysm clip for 10 s, it was observed that both hind limbs of SD rats straightened out and jerked, and muscular tension was lost in both hind limbs and tail, suggesting the successful modeling. Next, the aneurysm clip was released, and there were congestion and swelling in the spinal cord with bruise focus. Rats were fed separately after resuscitation. A total of 30 experimental animals were taken and divided into the following 5 groups: sham-operation group (n=6), 4 d SCI group (n=6), PBS group (n=6), miR-control group (n=6) and miR-133b group (n=6).

Preparation of Spinal Tissue Specimen

All SD rats were subjected to abdominal anesthesia at the corresponding time points after operation. The spinal cord was exposed along the modeling incision, the T9-T11 segment was taken out using scissors, the spinal cavity was opened using ophthalmic scissors to remove the spinal cord, and the injury center was marked. The specimen was taken and stored in a refrigerator at -80°C for standby application.

Immunofluorescence Assay

Spinal cord tissues were embedded into paraffin, sliced, dewaxed, hydrated, washed with PBS and sealed for 15 min. The non-specific site was blocked using 10% serum at room temperature for 15 min, and the primary antibody (diluted at 1:300) was added and placed in the refrigerator at 4°C overnight. Then sections were taken, washed with PBS, and added with the target protein and NeuN secondary antibody (diluted at 1:800), followed by incubation at room temperature for 1 h. The sections were washed again with PBS, added with 4',6-diamidino-2-phenylindole (DAPI) and observed (488: blue green, 555: green red, DAPI: ultraviolet blue).

Detection of miR-133b Expression via Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The total RNA in spinal cord tissues and exosome precipitate after centrifugation was extracted according to instructions of the kit. The concentration and purity of total RNA were determined using a spectrophotometer, and the absorbance $(A)_{260}/A_{280}$ value was 1.8-2.0. The primer sequences were synthesized by Shanghai Jiran Biotechnology Co., Ltd. (Shanghai, China) according to instructions of the Reverted Fist Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription was performed to obtain cDNA using the reaction system with a total volume of 20 μL on the RT-PCR instrument (Bio-Rad, Hercules, CA, USA). Reaction was performed using 25 μL reaction system according to instructions of the Real-time fluorescence quantitative PCR kit. Reaction conditions are as follows: 95°C for 10 min, 95°C for 30 s, 59.4°C for 30 s, 40 cycles, 95°C for 15 s, then cooling to 65°C. The fluorescence value was recorded, and the relative expression level of miR-133b was calculated automatically using the RT-PCR instrument with β-actin as an internal reference.

Rno-miR-133b UUUGGUCCCCUUCAACCAG CUA
Rno-miR-133bRT GCGCGTGAGCAGGCTGGA GAAATTAACCACGCGCTAGCTG
Rno-miR-133bF CCTTTGGTCCCCTTC
Rno-miR-133bP FAM+AACCACGCGCTAGCT-G+MGB
Reverse primer R GAGCAGGCTGGGAA
Internal reference gene
Rat β-actin F TGGAGTCTACTGGCGTCTT
Rat β-actin R TGTCATATTTCTCGTGGTTCA
Rat β-actin probe 5'-FAM+CTGAAGGGTGGG-GCCAAAAG+BHQ1-3'

Detection of Protein Expression via Western blotting

The total protein was extracted from spinal cord tissues according to instructions of the total protein extraction kit; the concentration of protein extracted was determined, and the protein was stored at -70°C for standby application. 10% separation gel was prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the gel site for protein was selected based on the marker

band, and the protein was transferred onto a membrane for 35 min. Then, the protein was blocked with 5% skim milk powder at 37°C for 90 min, incubated with primary antibody at 4°C overnight, and then washed with Tris-buffered saline and Tween-20 (TBST) for 3 times (15 min/time). The protein was incubated with secondary antibody at 37°C for 1 h and then washed with Tris-buffered saline with Tween-20 (TBST) for 3 times (15 min/time). Enhanced chemiluminescence (ECL) solution was added in a dark room for exposure, followed by color development and fixation. Finally, the protein was scanned using the ChemiDocTM-MP imaging system, the image was analyzed using the Image J professional image analysis software, and the gray value of target band was recorded.

Statistical Analysis

Statistical Product and Service Solutions (IBM, Armonk, NY, USA) 19.0 was used for data analysis in this experiment. Continuous data are presented as means ± standard deviation (SD), and were analyzed by using one-way ANOVA, with the Tukey's post-hoc test. $p<0.05$ was set as the test statistical standard.

Results

Detection of miR-133b Expression

The expression level of miR-133b in mimics group was significantly higher than those in blank group and negative control group ($p<0.05$). There was no statistical difference of the miR-133b expression between blank group and negative control group ($p>0.05$) (Figure 1).

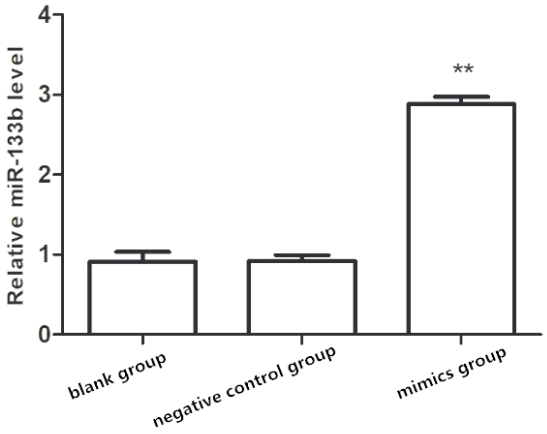

Figure 1. MiR-133b expression in the three groups.

MiR-133b Expressions in Spinal Cord Tissues of Rats in Different Groups

The expression of miR-133b was significantly decreased in 4 d SCI group compared with that in sham-operation group ($p<0.001$), while there were no statistically significant differences among 4 d SCI group, PBS group and miR-control group ($p>0.05$) MiR-133b mimics significantly up regulated the miR-133b level in the SCI model compared with that of miR-control group ($p<0.001$) (Figure 2).

RhoA Expressions in Spinal Cord Tissues of Rats in Different Groups

The RhoA expression was significantly increased in 4 d SCI group compared with that in sham-operation group ($p<0.001$), whereas the over expression of miR-133b significantly reduced the RhoA level, compared with that in miR-control group ($p<0.001$) (Figure 3).

Expressions of NF, GAP43, GFAP and MBP in Spinal Cord Tissues of Rats in Different Groups

Expressions of NF, GAP43, GFAP and MBP were remarkably higher in 4 d SCI group than those in sham-operation group ($p<0.01$). No statistically significant differences were found in expressions of NF, GAP43, GFAP and MBP among 4 d SCI group, PBS group and miR-control group ($p>0.05$). The overexpression of miR-133b significantly elevated the levels of NF, GAP43, GFAP and MBP compared to that in miR-control group ($p<0.001$) (Figure 4, 5).

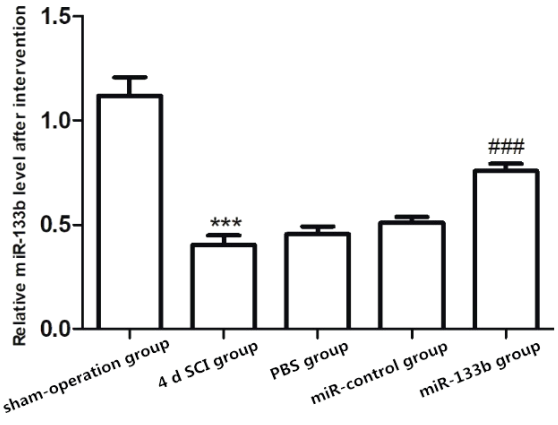

Figure 2. MiR-133b expressions in spinal cord tissues of rats in different groups. Note: $p<0.001$ in the comparison between 4 d SCI group and sham-operation group, $p<0.001$ in the comparison between miR-control group and miR-133b group.

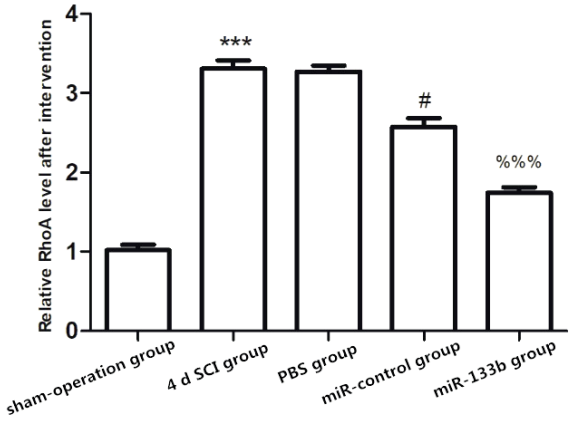

Figure 3. RhoA expressions in spinal cord tissue of rats in different groups. Note: $p<0.001$ in the comparison between 4 d SCI group and sham-operation group, $p<0.05$ in the comparison between PBS group and miR-control group, $p<0.001$ in the comparison between miR-control group and miR-133b group.

Expressions of Related Proteins in Spinal Cord Tissues of Rats After SCI

We showed that there were significant differences of RhoA protein expression ($p<0.05$), levels of p-CREB/CREB ($p<0.01$), p-STAT3/STAT3 ($p<0.05$), NF, GAP-43, GFAP and MBP levels ($p<0.05$) between miR-control group and miR-

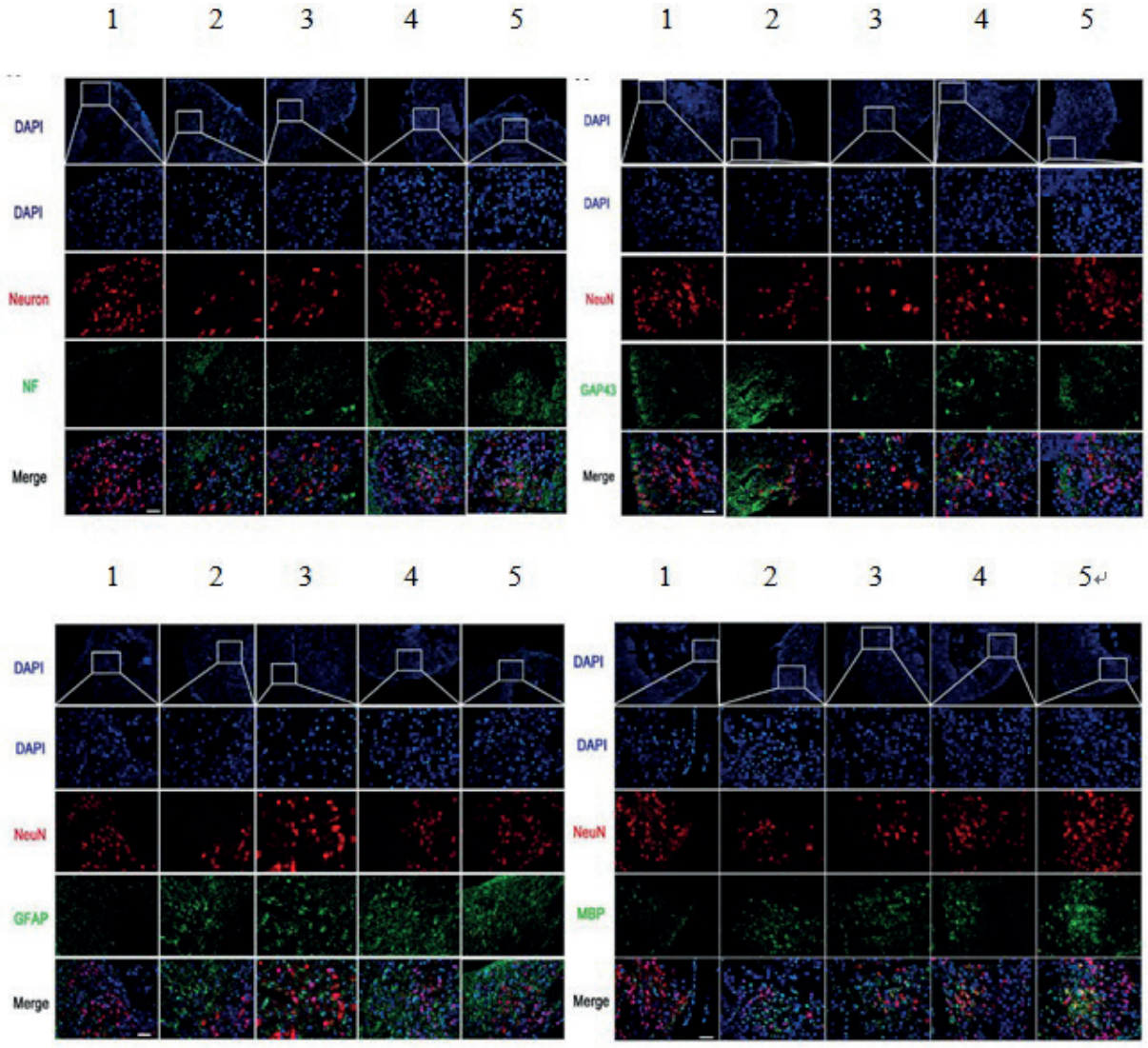

Figure 4. Immunofluorescence staining of NF, GAP43, GFAP and MBP in spinal cord tissues of rats in different groups. Note: 1) Sham-operation group, 2) 4 d SCI group, 3) PBS group, 4) miR-control group, 5) miR-133b group. NF, GAP43, GFAP and MBP (green): target proteins, NeuN (red): neuronal marker protein, DAPI (blue): nucleus, and Merge: combined location of the first three (Bar=60 μm).

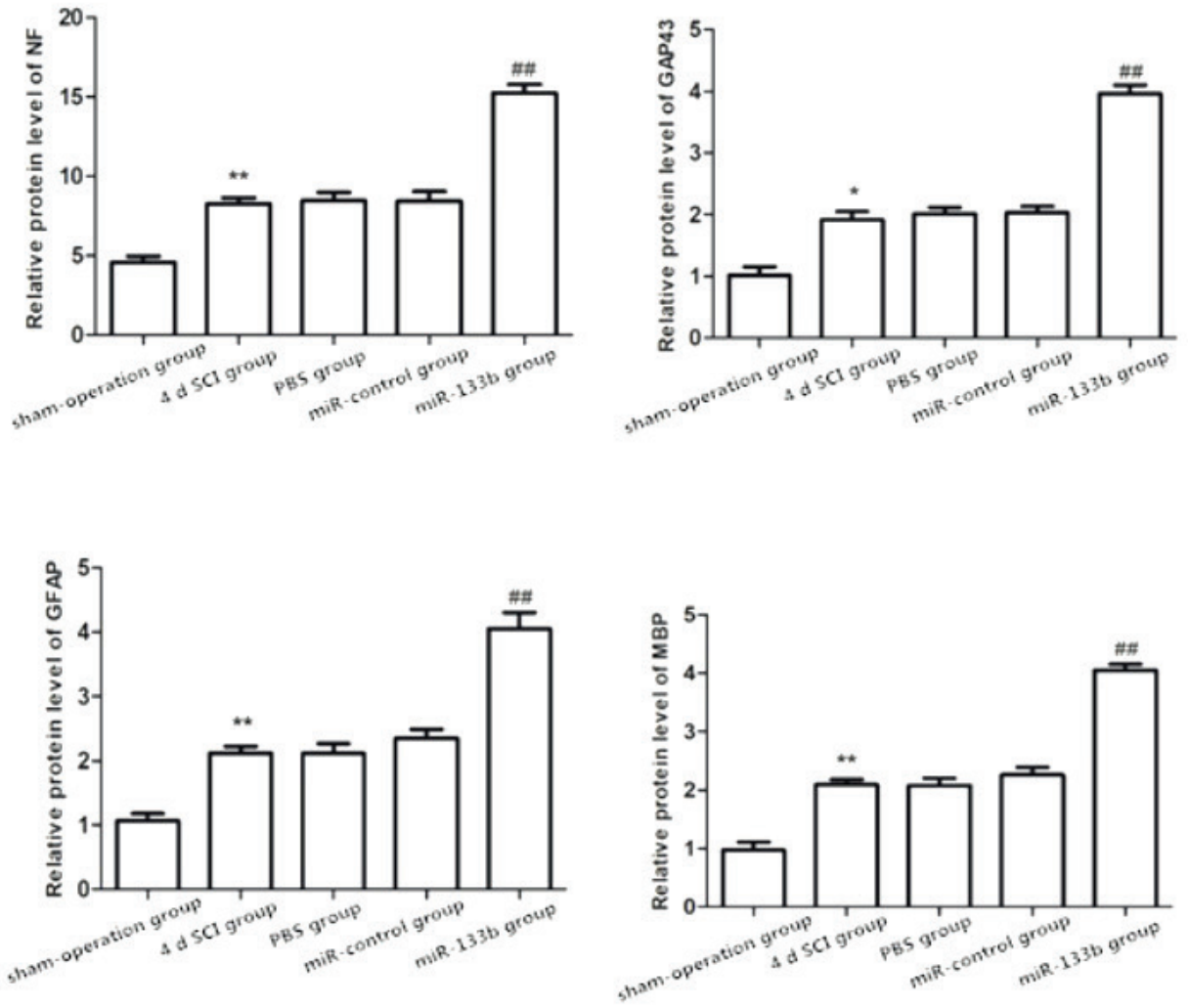

Figure 5. Bar graph of NF, GAP43, GFAP and MBP expressions in spinal cord tissues of rats in different groups. Note: $p<0.001$ in the comparison between 4 d SCI group and sham-operation group, $p<0.001$ in the comparison between miR-control group and miR-133b group.

133b group. Expressions of GAP-43, GFAP and MBP were also significantly different between 4 d SCI group and miR-control group ($p<0.05$) (Figures 6,7).

Discussion

SCI leads to the dysfunction in central nervous system. Drug and operative treatments are major therapeutic methods for SCI currently, but the clinical therapeutic effect is still to be further improved. Studies[10] have found that in the treatment of SCI animal model with stem cell transplantation, exosomes function in stem cells and play a role in nerve injury. Exosomes widely exist in human body fluid and contribute to regulatory role in a variety of pathological processes by the release outside cells. Eissa et al[11] studied and found that exosomes, as a novel carrier of cell communication, can bind to receptors for the potential therapy of diabetic nephropathy. Huang et al[12] found that exosomes increase the expression level of miR-21 in bronchial epithelial cells and activate the expressions of STAT3 and vascular endothe-

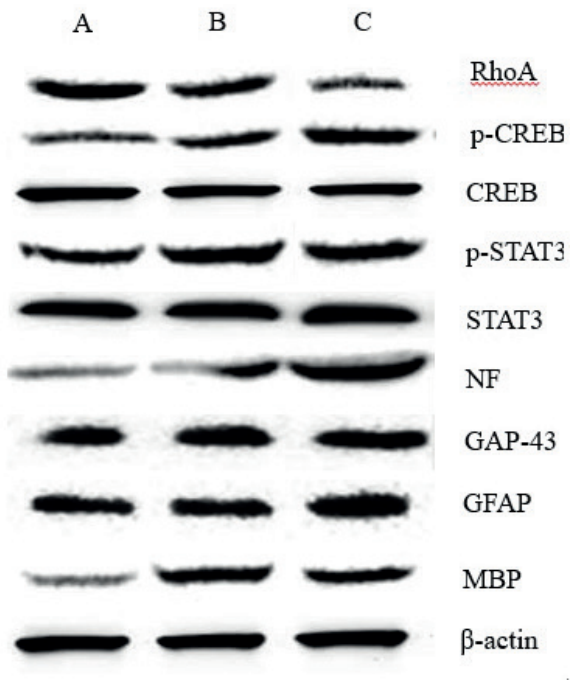

Figure 6. Western blotting graph of expressions of related proteins in spinal cord tissues of rats after SCI. A) 4 d SCI group, B) miR-control group, C) miR-133b group.

lial growth factor (VEGF), ultimately promoting angiogenesis. Moreover, Sun et al[13] injected exosomes derived from bone mesenchymal stem cells into the animal model with primary brain tumor, and found that exosomes can transmit the genetic material miR-146b to glioma and significantly inhibited the growth of glioma, indicating that exosomes may also exert effects as a drug carrier. In this study, the recovery effect of exosomes derived from miR-133b-modified ADSCs on neurological function of SCI animal model was investigated, and it was found that the expression level of miR-133b in mimics group was significantly higher than those in blank group and negative control group, indicating that a large number of miR-133b is expressed in exosomes secreted by ADSCs after transfection. Notably, the expression of miR-133b was significantly decreased in 4 d SCI group compared with that in sham-operation group, with the increase of RhoA expression. However, the level of RhoA was significantly decreased in miR-133b group compared with that in miR-control group, which were similar to the findings of Tofiño-Vian et al[14]. Baglio et al[15] found that, when the expression of miR-133b in neuronal cells is significantly increased, the axon growth can be promoted through regulating the expression of RhoA, but its concrete mechanism remains unclear. Huang et al[16] demonstrated that exosomes can increase the expressions of related factors to nerve growth through transmitting genetic materials to recipient cells, which may play a role in SCI repair. Besides, Xin et al[17] indicated that exosomes can effectively relieve the inflammatory response via transmitting small-interfering RNA (siRNA) to target cells. According to Chang et al[18], ischemic preconditioning (IPC) can lead to the increase in VEGF expression in plasma, and effectively protect the body from the ischemic state after SCI, which is realized through down-regulating expressions of various miRNAs by exosomes. Results of this study also revealed that expressions of NF, GAP43, GFAP and MBP were remarkably higher in 4 d SCI group than those in sham-operation group, but they were also remarkably higher in miR-133b group than those in miR-control group. De RiveroVaccari et al[19] revealed that the phosphorylation levels of CREB and STAT3 had a close correlation with the repair state of neurological function after SCI. Hayashi et al[20] further presented that the phosphorylation levels of CREB and STAT3 were elevated in the case of neurological repair and further induced levels of related factors to nerve repair (NF, GAP-43, GFAP and MBP), which are consistent with conclusions in this experiment. Further investigation is still required for the development of miRNAs-based therapy according to previously findings[21,22].

Conclusions

We indicate that exosomes derived from miR-133b-modified ADSCs can significantly promote the recovery of neurological function of SCI animals possibly through affecting the signaling pathway related to axon regeneration, NF, GAP-43, GFAP and MBP, providing leads for the further treatment of spinal cord injury.

Conflict of Interest

The Authors declare that they have no conflict of interest.

Acknowledgments

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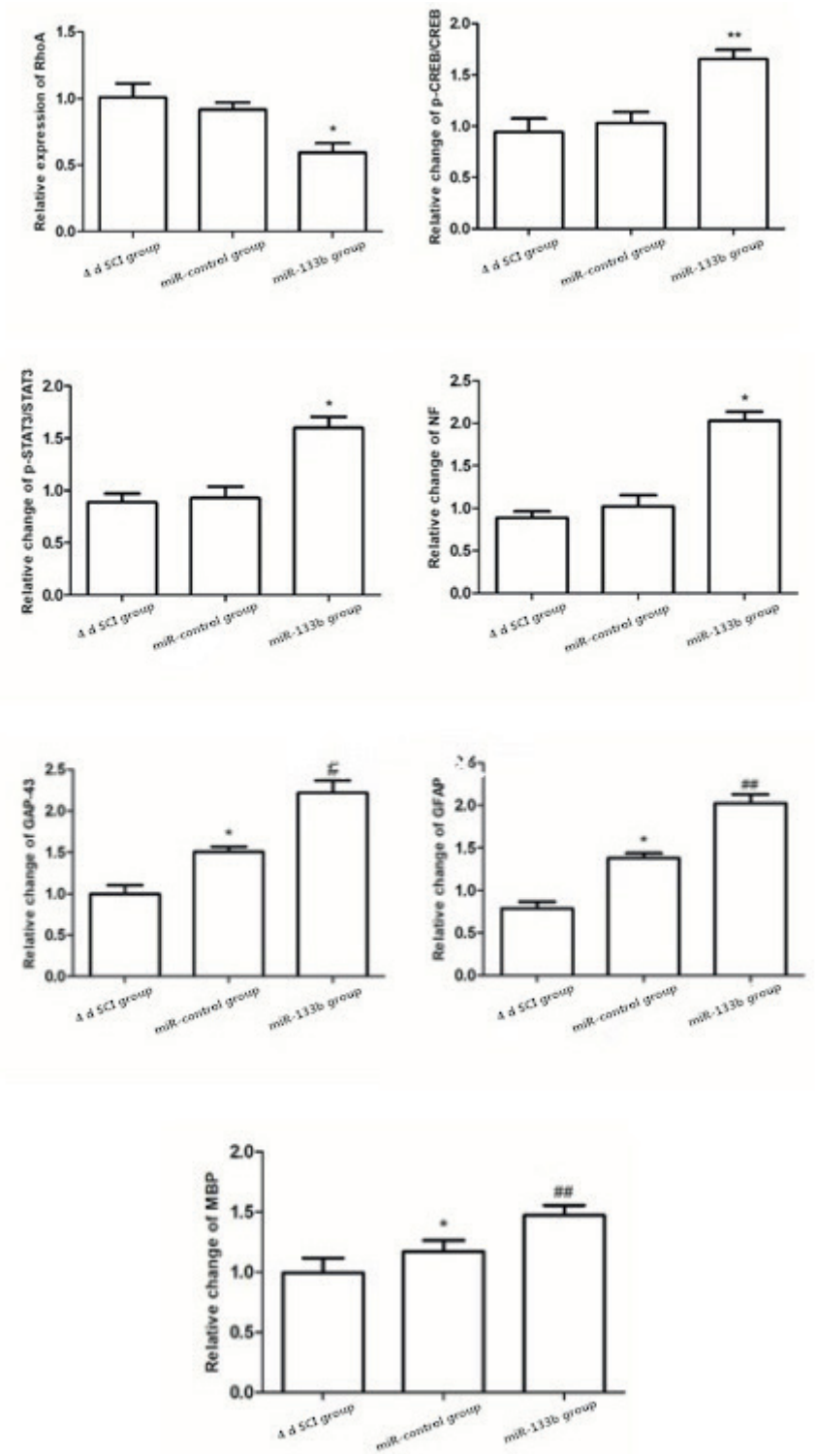

Figure 7. Bar graph of expressions of related proteins in spinal cord tissues of rats after SCI.

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