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Effect of exosomes derived from mir-126-modified mesenchymal stem cells on the repair process of spinal cord injury in rats

B. YUAN, S. PAN, Y.-Q. DONG, W.-W. ZHANG, X.-D. HE

Department of Spine Surgery, Xinchang Hospital Affiliated to Wenzhou Medical University, Shaoxing, China

Abstract. – OBJECTIVE: To investigate the effect of the micro ribonucleic acid (miR)-126-modified mesenchymal stem cell (MSC)-derived exosomes (MSC-exos) on the repair process of spinal cord injury (SCI) in rats.

MATERIALS AND METHODS: MiR-126-modified MSCs were cultured, the exosomes were extracted, and a rat model of SCI was established. The Quantitative Polymerase Chain Reaction (qPCR) was carried out to detect the expression of miR-126 in the injured spinal cord, the Western blotting (WB) was adopted to detect the expressions of the exosome-related molecules. Subsequently, the motor function recovery of rats was examined via the Basso, Beattie and Bresnahan (BBB) locomotor rating scale. The effects of miR-126 exosomes on the injury volume and NeuN retention were evaluated via immunohistochemistry.

RESULTS: MSCs were able to package miR-126 into exosomes secreted. After SCI, the recovery of the hindlimb motor function of rats was remarkably improved by miR-126-modified MSC-exos relative to the control group. Treatment with miR-126-modified MSC-exos remarkably decreased the injury volume, retained the neuronal cells, and triggered the axon regeneration following SCI. Besides, the expression of Ras homolog gene family member A (RhoA), identified as a downstream gene of miR-126, was downregulated in the group with miR-126-modified MSC-exos. Moreover, miR-126-modified MSC-exos activated the extracellular regulated protein kinases 1/2 (ERK1/2) pathway.

CONCLUSIONS: MiR-126-modified MSC-exos protect neurons of rats with SCI, stimulates axon regeneration, and improves the recovery of limb motor function after SCI.

Key Words:
MiR-126, Mesenchymal stem cell, Exosome, Spinal cord injury repair.

Introduction

Traumatic SCI is a severe damage to the central nervous system (CNS), often leads to irreversible pathological deficits. SCI involves complex pathophysiology as its direct primary mechanical injury is accompanied by a series of secondary processes including neuroinflammation and ischemia, aggravate SCI[1]. In spite of profound breakthroughs in preclinical studies on the protection and regeneration of neurons so far, the specific pathophysiological mechanism is still unclear. Therefore, basic science and clinical researchers are still facing a challenge to find an effective treatment method for SCI.

As an endogenous non-coding ribonucleic acid (RNA), a micro RNA (miRNA) can bind to the 3' untranslated region (3'-UTR) of its target gene messenger RNA (mRNA), so as to exert the post-transcriptional regulation[2]. MiRNAs have been revealed in recent studies to exert crucial effects in synaptic activity, regeneration, and neurogenesis of the CNS. Besides, it has been reported that several miRNAs are regarded as the underlying novel targets for treating SCI[3,4]. Yu et al[5] reported that miR-133b is critical for the function recovery after SCI in zebrafishes. Moreover, Gordon et al[6] have also indicated that miR-126 targets Ras homolog gene family member A (RhoA) to accelerate the neurite outgrowth *in vitro*.

Exosomes are a class of small membrane vesicles derived from the lumen of a multivesicular body (30-100 nm in diameter). Exosomes are considered as mediators of intercellular communication by transporting various RNAs, DNAs, and proteins between cells[7]. Many studies[8] have shown that, exosomes, as intercellular communicators, function both locally and systematically.

Corresponding Author: Bo Yuan, BM; e-mail: 627582584@qq.com

Additionally, some investigations have evidenced that exosomes contribute to the transportation of therapeutic miRNAs to cells producing exosomes. There are numerous cell types that could produce exosomes, and MSCs are one of the most common types. MSCs have been confirmed in multiple investigations to be the ideal candidate cells for cell therapy of SCI. It is reported that MSCs could promote tissue repair by inhibiting the inflammatory response and activating endogenous repair mechanisms. It has been verified in a previous study that MSCs decreased the secretion of multiple inflammatory cytokines [tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and IL-6] and blocked the activation of the nuclear factor kappa-B (NF-κB)[9]. Furthermore, MSCs are able to guide the transformation of the stimulated macrophages from a proinflammatory M1 phenotype to an anti-inflammatory M2 phenotype. It has been revealed in a recent study that the therapeutic effect of MSCs may be mediated by exosomes[10]. According to reports, MSC-derived exosomes (MSC-exos) can mimic most of the biological functions of MSCs, and also attenuate inflammatory response and apoptosis, reduce the level of proinflammatory cytokines, and inhibit the activation of astro and macrophages[11]. Similar to that of MSC, th fusion of MSC-exos is capable of suppressing activation of NF-κB. Besides, the ther tic effe of the cell-free exosomes is equi nt that o the intact MSCs after brain in Acco g to a previous study, the applicati n SC SCI model has manifested t a sy c admins tration can lower the le f the pro- mmatory cytokines and imp th covery o nction. Nevertheless, th xact bei l mechanism to SCI is still un .

On the b s of r ed studies reported, it is speculated t -126b modified MSC-exos admini tio imp d the functional re- co e inj l cord by transferring -126b the les .

Materials and Methods

Animals

Male adult Sprague-Dawley rats (80-100 g) purchased from the Animal Center of the Chinese Academy of Sciences in Shanghai, China, were used in this study. All of the experimental procedures were approved by the Animal Care and Use Committee of our hospital and the rats were raised in line with the guidelines for the care and use of laboratory animals approved by the National Institutes of Health.

Preparation of MiR-126-Modified MSC-Exos

The primary MSCs were isolated from the bone marrow of male rats. Briefly, femur and tibia bone marrow were separated and rinsed with Phosphate-Buffered Saline (PBS). C trifuged precipitates were suspended and ther- mal inactivation in Dulbecco' odified e's Medium (DMEM; Life Tech ies, Gai s- burg, MD, USA) contain 10% l bovi se- rum (FBS; Life Techn gies, Gaith MD, USA) and 1% penic l-stre ycin. en, they were maintained in tor (5% CO_2, 37°C).

MSCs wer hen fected ith miR-126 mimics or tive cont g Lipofectamine 3000 (In ogen Carlsbad, A, USA). The exosomes were harve from the supernatant using the uick-TC k ystem Biosciences, Palo A , CA, USA) at 72 h. Subsequently, the exosome p cipitates w resuspended in PBS at 10 μg/μL.

Co of the SCI Model

All rats were anesthetized with 400 mg/kg ral hydrate before the operation. After the pa rtebral muscle was dissected, T9-T11 laminectomy was conducted. After that, the muscle was kept with the aneurysm clamp at T10 level with a closing force of 35 g for 60 s, and finally the incision was sutured with the silk thread. The rats in the sham operation group only received laminectomy. All rats were administrated with penicillin and analgesics for 3 days after the operation and urinated manually three times a day. At 24 h after trauma, the rats were injected miR-126 exosomes (100 μg exosomes in 0.5 mL PBS) and miR-con exosomes were treated with PBS (0.5 mL) in the tail vein.

Animal Grouping

The rats were randomly allocated into 4 groups by a blind method: the sham operation group (sham operation); the control group (SCI and PBS treatment); the miR-con group (SCI and miR-con exosomes treatment); and the miR-126 group (SCI and miR-126 exosomes treatment).

Immunohistochemistry

The rats were finally anesthetized with isoflurane on the 4th day after injury. The spinal cord was embedded into compounds with the optimal cutting temperature. Then, T9-T11 segments of the

spinal cord near the lesion center were collected for histological evaluation. Hematoxylin and eosin (H&E) staining was conducted to quantify the lesion degree by Imagepro-Plus software. T9-T11 segments of the spinal cord were also dissected in 6 rats in each group, and 8 sections of each rat were selected to calculate cystic cavity size.

Subsequently, the sections were deparaffinized in xylene, dehydrated in gradient ethanol, and then boiled twice in citrate buffer (pH 6.0) for 5 min. Thereafter, the cooled sections were incubated with 3% H_2O_2 at room temperature for 15 min to inactivate the endogenous peroxidase. Then, the glass slides were sealed with 10% FBS for 10 min before incubation with the corresponding primary antibody at 4°C overnight. After TBS washing, the slides were incubated with the fluorescent-labeled secondary antibody (Abcam, Cambridge, MA, USA). Ultimately, the sections were stained with 4',6-diamidino-2-phenylindole (DAPI) and visualized under a confocal laser scanning microscope (Olympus LSM-GB200, Tokyo, Japan).

RNA Extraction and q-PCR

The tissues and cells were lysed using the TRIzol kit (Invitrogen, Carlsbad, CA, USA) followed by the measurement of RNA concentration using an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan). The complementary Deoxyribonucleic Acid (cDNA) was synthesized following the protocols of the PrimeScript RT MasterMix kit (Invitrogen, Carlsbad, CA, USA). Thermal cycle protocols were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s for a total of 40 cycles. The target gene level was calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in this study were as follows: microRNA-126, F: 5'-GCTTCGTCATAACTCGCCTG-3', R: 5'-AGGTTTGTGCAGACAG-3'; U6: F: 5'-GCTTCGGCAGCATATACTAAAAT-3', R: TCATGCGTGTCAT-3'.

Western Blotting (WB)

Total proteins were isolated with radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After the proteins were successfully transferred, the membrane was sealed with 5% skim milk at 37°C for 2 h and reacted with the primary antibodies (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. The membranes were then washed with TBST (Tris-buffered Saline with Tween 20) and followed by the incubation of the secondary antibodies conjugated with horseradish peroxidase at 37°C for 1 h. The exposed bands were observed with enhanced chemiluminescence (ECL) detection kit.

Statistical Analysis

The data were analyzed by Statistical Product and Service Solutions (SPSS) 20.0 (IBM Corp., Armonk, NY, USA). All the data collected from at least three independent experiments were expressed as mean ± standard deviation. $p<0.05$ signified significant difference determined at $p<0.05$.

Results

MiR-126 Expression Was Reduced in Rats

MiR-126 level in the SCI rats was measured via qRT-PCR at 12 h, 24 h, 2 days, 3 days, 4 days, 5 days, and 7 days following acute SCI. It was found that miR-126 was evidently down-regulated since 24 h following SCI ($p<0.05$) (Figure 1).

miR-126 Level in MSC-Exos

MSCs manifested as cluster of differentiation 73+ ($CD73^+$), $CD90^+$, $CD105^+$ $CD34^-$, and $CD45^-$ were cultured as described above. The transfection efficiency of miR-126 mimic reached about 90%. The exosomes were isolated from MSC supernatant 72 h after transfection. Subsequently, WB analysis was carried out and the com-

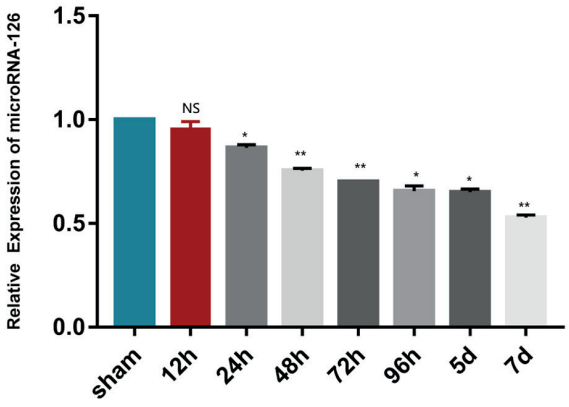

Figure 1. Expression level of miR-126 in the injured spinal cord measured by qRT-PCR compared with those in control group at 12 h, 24 h, 2 day, 3 day, 4 day, 5 day, and 7 day after acute SCI (NS: $p>0.05$, *$p<0.05$, **$p<0.01$).

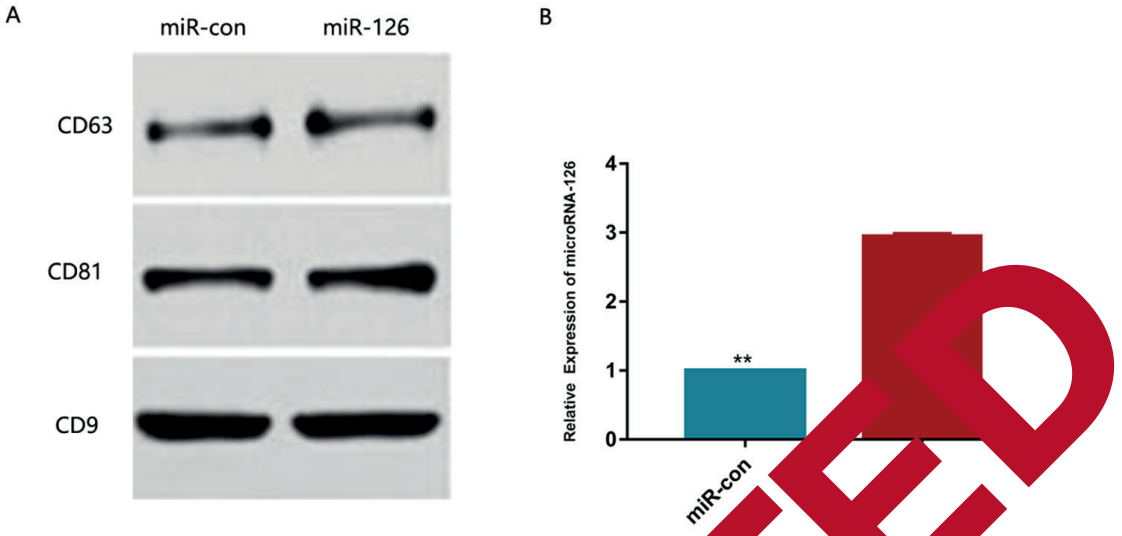

Figure 2. Expressions of CD63, CD81 and CD9 in MSC-exos transfected wi[illegible]126 or mi[illegible]aluated by WB and qRT-PCR.

mon exosome markers, including CD9, CD63, and CD81 were detected (Figure 2A). QRT-PCR manifested that miR-126 level was about 3.1 times higher in MSC-exos overexpressing miR-126 than that of the controls ($p<0.01$) (Figure 2B). The above results proved that miR-126 was effectively packaged into secreted exosomes from MSCs.

MiR-126-Modified Exosome[illegible]ibuted to Function Recovery and Injury Volume Decline

First, qRT-PCR was a[illegible]ed to explore *in vivo* the miR-126 level in [illegible]pinal cord. Compared with the control group, [illegible]-126-mod[illegible]ed exosome markedly increased the miR-126 level on the 4th day after SCI (Figure 3A).

In order to investigate whether miR-126-modified exosome[illegible]nhanced the motor function recovery following acute SCI, the BBB locomotor scale was performed at different time points to assess our SCI model. After SCI, 0-1 point of BBB score indicated the successful modeling of SCI. As mentioned earlier (after SCI), we observed a spontaneous functional recovery in all groups. After 5 days, a significant difference was observed in the functional recovery between the rats injected with miR-126 exosomes and those injected with miR-con exosomes ($p<0.01$, on the 9th day: $p<0.001$, and on the 14th day: $p<0.01$), indicating that miR-126-mediated triggered the recovery of the hind limb motor function after SCI (Figure 3B).

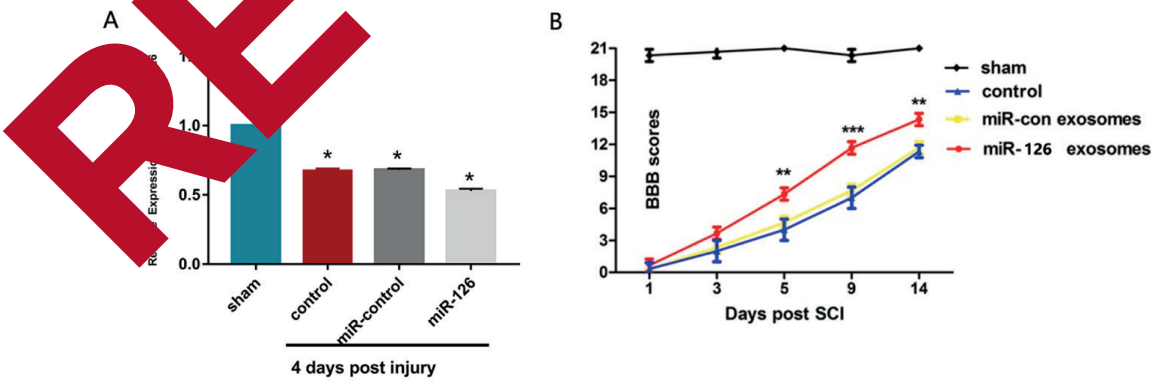

Figure 3. Injection of miR-126 exosomes improved the recovery after SCI. **A,** After SCI, the relative expression of miR-126 in the lesion site compared with that in control group is detected by qRT-PCR, and the expression of miR-126 is notably increased by tail vein injection of miR-126 exosomes (*$p<0.05$). **B,** The recovery of function (BBB score) is monitored on the 1st, 3rd, 5th, 9th, and 14th day after lower limb SCI (***$p<0.001$, **$p<0.01$, *$p<0.05$, n=4).

Effects of MiR-126 Exosomes on the Injury Volume and Neuron Retention After SCI

Next, the effects of miR-126-mediated exosomes on the injury volume and NeuN retention after SCI were detected via immunohistochemistry. HE staining demonstrated that, compared with that in miR-con or control group, miR-126 exosomes markedly reduced the lesion cavity area (Figures 4A and 4B). On the 4th day after SCI, NeuN, a specific marker of mature neurons, was stained to visualize the number of neurons in the injured spinal cord. Relative to rats receiving miR-con exosomes, the number of mature neurons in the injured rats receiving miR-126 exosomes was significantly increased ($p<0.01$) (Figures 5A and 5B).

MiR-126 Exosomes Promoted Axon Growth Following SCI

To study the underlying mechanisms of miR-126 exosomes on axon growth, WB was carried out for growth-associated protein 43 (GAP43) and NF. GAP43 level in miR-126 exosome group was raised compared with that in miR-con or control group (Figure 6A). Besides, the treatment with miR-126 exosomes also elevated NF expre on the 4th day after SCI (Figure 6B).

Effects of MiR-126 Exosomes on the Phosphorylation of cAMP R… Element-Binding Protein (CREB) and the Activation of Signal Tra… Activator of Transcri… 3 (STAT3) Pathways

RhoA is the dir… tar… of miR-1… as previously indicate… In addit… RhoA has been reported to participate in the neuronal cell death in the spinal cord. Compared with the miR-con exosomes group, RhoA level in the miR-126 exosomes group was notably reduced ($p<0.05$) (Figure 7A). Furthermore, the phosphorylation of ERK1/2 was pronounced in the SCI rats treated with miR-126 exosomes (Figure 7B).

Discussion

SCI has been showing a g… impact o… patients and society. The f… tion … very f… wing SCI is usually slig… and there … ctive treatment method c… ally a… ied so … Many studies have ind… th… volve… ent of miRNAs in the p… gene… SCI … r example, it has also be… oved that … reduces neuronal cell … th … promotes … unctional recovery after SCI. Long e… [13] have shown that miR-126 ex… ucial effe… neuronal differentiation, a… ptosis, and neurite outgrowth in the CNS[13]. H… eover, fu… r research has revealed that miR-12… verexpr… on is able to improve the functio… in rats with stroke. The exosome is a new … pe of intercellular communication de… which has been applied as a biological carrier … local or systemic miRNA delivery and treatment for stroke and Parkinson's disease[14]. In this paper, the role of exosome-mediated miR-126 transfer in SCI therapy was explored. After SCI, evident differences in the functional recovery were identified in each group. Niu et al[15] manifested that there were a variety of treatment methods improving the functional recovery 3 days after SCI. It was observed in this study that at 5 days

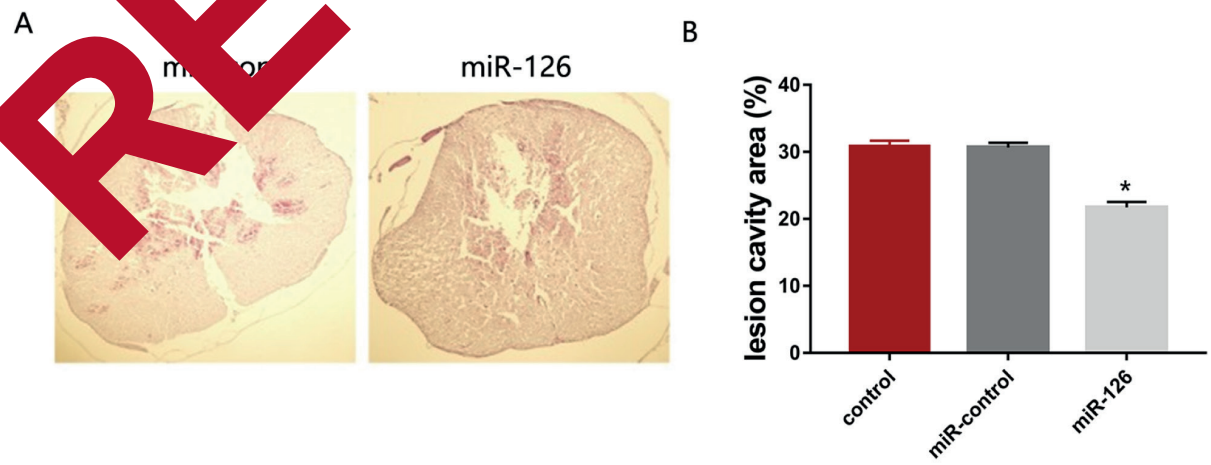

Figure 4. Injection of miR-126 exosomes reduces the lesion volume and retains NeuN after SCI (magnification × 10) (*$p<0.05$).

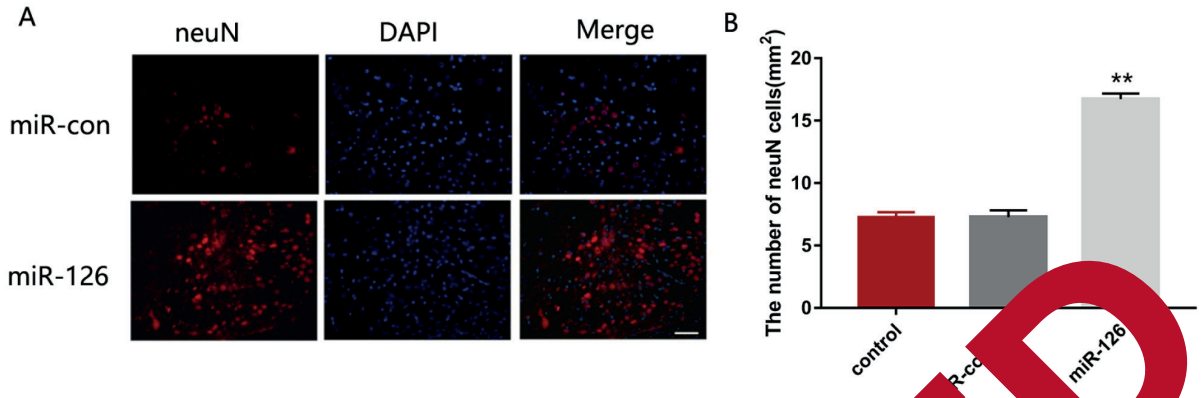

Figure 5. Effect of miR-126 exosomes on NeuN evaluated by calculating the cell num through immu scence staining (magnification × 40) (**p<0.01).

after SCI, the BBB score of the rats injected with miR-126 exosomes was remarkably higher than that of the rats injected with miR-con exosomes. Lentiviral delivery of miR-126b promotes the recovery in the SCI mice, which was consistent with our results[16].

Several mRNAs, such as RhoA and MST2, have been identified as the target genes of miR-126[17]. Here, we found that the RhoA level e ited a significant decrease in SCI rats rece g miR-126 exosomes. Together with the result previous study *in vitro*, our data s sted th RhoA is a target of miR-126[18]. R ember of the Rho family, is up-regul in the CI rats and act on the downstream el r, R ated protein kinase (RO). Be Umezu et al[19] indicated that Rh ROCK pa ay exerts a vital effect on th eu death in I mice. Thus, whether t survival neurons after the injection of r -126 exosom enhanced *in vivo* was f er in igated. Within 24 hours, the neuron d e to mechanical forces and secondary i es follow . In this study, miR-126 osc group s wed a notably increased number o ture neurons on the 4th day aft . Our resu evealed that the silence o hoA evidently reduced cell death following s . Besides, treatment of miR-126 exosomes n edly enh ced the ERK1/2 phosphorylation leve ured site of the spinal cord. MiR-126 activated ERK1/2 phosphorylation *via* RhoA. results also demonstrated a neuroprotective of miR-126 exosomes attributing to RhoA downregulation and ERK1/2 activation.

It is determined that the presence of the inhibitory molecules, including oligodendrocyte myelin glycoproteins and myelin-associated glycoproteins, inhibits axonal regeneration in the CNS. To reveal the effect of miR-126 exosomes on neurite outgrowth, the immunohistochemical staining was performed to analyze the NF expression in the spinal cord. The NF expression was observed to be increased in miR-126 exosome group, which is consistent with the results of the WB. GAP43

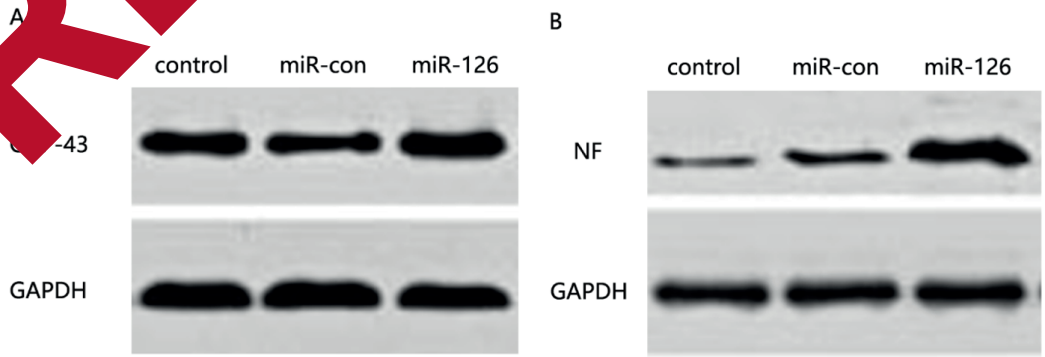

Figure 6. WB results show that miR-126 exosomes can increase the expressions of GAP43 **(A)** and NF **(B)**.

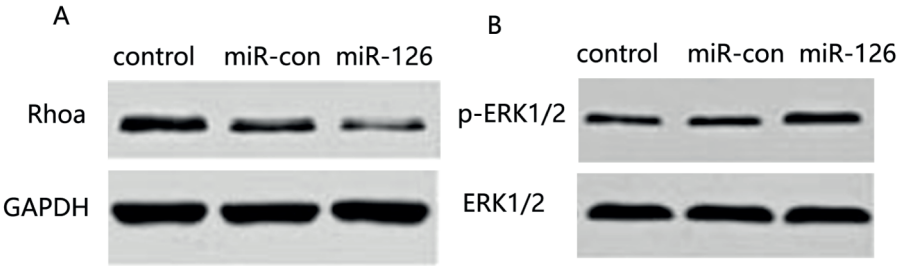

Figure 7. WB results indicate that miR-126 exosomes are capable of lowering the expression of RhoA **(A)** [illegible] the phosphorylation of ERK1/2 **(B)**.

is a regeneration-related gene that is up-regulated in regenerative neurons[20]. We found that the GAP43 expression in SCI rats injected with miR-126 exosomes was remarkably raised on the 4th day following SCI, indicating that miR-126 exosomes promote axon regeneration in the injured spinal cord.

Zhang et al[21] have indicated that the transcription factor CREB played a vital role in axon regeneration. The activation of CREB has been shown to counteract myelin inhibitors and trigger the regeneration of spinal axons. In this asp[illegible] was discovered that miR-126 exosomes activ[illegible] CREB expression in SCI rats. In addition, ph[illegible] phorylated STAT3 (involved in spina[illegible]on regeneration) in miR-126 exosome-tre[illegible]ed rats markedly increased. It can be [illegible]n that [illegible]R-126 exosomes enhanced the ax[illegible] SCI by enhancing the [illegible] levels of CREB and STAT3.

Conclusion

The syst[illegible]ction of miR-126 exosomes could [illegible] the mi[illegible]26 level on the lesion spi[illegible] and [illegible] the functional recov[illegible] follow[illegible] SCI. [illegible]ditionally, it was also ob[illegible]ed t[illegible]-126 exosomes retained residential [illegible]onal cells and increased axon regeneration, [illegible]h partly resulted from the activation of ERK1/2, [illegible]AT3, and CREB and the inhibition of the RhoA expression. The above findings imply that miRNA-modified MSC-exos can be used as a new treatment method for SCI.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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