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MiR-7a ameliorates spinal cord injury by inhibiting neuronal apoptosis and oxidative stress

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Abstract. – OBJECTIVE: The aim of this study was to investigate the therapeutic effect of microRNA-7a (miR-7a) on spinal cord injured rats and to explore its underlying mechanism *in vivo*.

MATERIALS AND METHODS: The spinal cord injury (SCI) model was first established in adult rats. The epicenter of the lesion was treated with miR-7a mimics via intrathecal injection. The Basso-Beattie-Bresnahan (BBB) locomotor rating scale was used to evaluate the functional recovery of hindlimbs in rats within 4 weeks following SCI. Western blotting and qPCR were utilized to detect the apoptosis and oxidative stress in rats treated with or without miR-7a. In addition, the neuron survival and neuro-filament amount were determined using immunofluorescence.

RESULTS: After SCI and miR-7a treatment, the locomotor recovery of treated rats was significantly improved when compared with rats without treatment. The mitochondrial disorder and cell death were significantly reduced in miR-7a treated rats. Meanwhile, the nuclear transcription factor-κB (NF-κB) pathway was significantly reduced as well. Contrarily, the expression of anti-apoptotic protein Bcl-2 and NF-κB inhibitor I-κB was remarkably elevated in miR-7a treated rats. In addition, up-regulation of miR-7a rescued neurons and maintained the neural structure.

CONCLUSIONS: The up-regulation of miR-7a alleviated the injury-induced oxidative stress and inhibited apoptosis by down-regulating NF-κB pathway in SCI rats.

Key Words:
MiR-7a, Spinal cord injury (SCI), Apoptosis, Oxidative stress.

Introduction

Spinal cord injury (SCI) is a common and grievous neural injury caused by external brute force. It engenders sensation, motor dysfunction and autonomic nervous disorder[1-3]. Injury-mediated tissue ischemia and hypoxia can induce oxidative stress in neural cells, which is one of the main pathological processes aggravating neuro-injury[4,5]. Under normal circumstances, the production and elimination of reactive oxygen species (ROS) are in a dynamic balance[6]. However, under stimulation of nerve tissue injury, excessive ROS production and reduced ROS elimination ability leads to an imbalance of ROS metabolism in the damaged area[7,8]. Excessive ROS attacks neural tissues and activate a series of cascade reactions. Meanwhile, it can lead to the apoptosis of nerve cells.

NF-κB is a multidirectional transcriptional regulatory protein commonly found in eukaryotic cells[9,10]. The activation or inactivation of NF-κB is involved in the regulation of various functional genes. The current study has found that it plays an important role in physiological and pathological processes, such as inflammation, immune response, oxidative stress, cell proliferation, and apoptosis[11-13]. Previous studies[14,15] have verified that ROS can deteriorate apoptosis by activating nuclear transcription factor kappa-B (NF-κB). This is one of the main mechanisms underlying ROS in aggravating cell damage. Therefore, the inhibition of the production of ROS and the activity of NF-κB pathway after nerve injury is crucial to the repair and survival of neural cells in later stages.

Recently, microRNAs (miRNAs) have been reported as effective targets for non-invasive repair of neural damage[16,17]. MiR-7a is one of the alterative miRNAs, which shows a continuous decreasing trend for 1 week after SCI[18]. Previous studies have demonstrated that miR-7a can improve the progression of diseases including ischemic brain damage and neuropathic pain[19,20].

In the current study, we hypothesized that declined miR-7a might be involved in cell damage and oxidative stress following SCI. Through miR-7a mimics administration, we investigated whether the repair of miR-7a could rescue neural survival and axonal structure, as well as inhibit oxidative

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stress and apoptosis. To determine the curative effect of miR-7a in SCI-induced secondary injury, we estimated the effects of intrathecal injection of miR-7a in SCI rats on cell damage, apoptosis-related indexes, and motor function recovery.

Materials and Methods

Animals and Grouping

A total of 108 male Sprague Dawley (SD) rats (aged 6-8 weeks, weighing 200-220 g) were used to establish the SCI model. The specific conditions for rats were as follows: 20-25°C, with 50-65% humidity and 12 h/12 h circadian cycle. All rats were given free access to normal food and water. Subsequently, the rats were randomly divided into three groups, including: the Sham group, the SCI group, and the miR-7a group. The rats in the Sham group only received laminectomy. The rats in the SCI group were subjected to SCI modeling and were injected with the same amount of normal saline in the injured area. Meanwhile, the rats in miR-7a group received SCI modeling and intrathecally injection of miR-34a mimics. This study was approved by the Animal Ethics Committee of Taizhou Central Hospital Animal Center.

Operative Procedure and Treatment

Briefly, the rats were anesthetized with 0.75 mL of 10% chloral hydrate. After limbs immobilization, the skin, anadesma, and muscles were cut to expose the lamina. Subsequently, we removed the lamina of the dorsal spinal cord at T10. The ALLEN bump equipment (10 g × 5 cm) was performed to collide the spinal cord. The spinal cord hemorrhage, delayed extension of hindlimbs, and tail swing indicated the successful establishment of the SCI model. Next, miR-7a mimics was intrathecally injected into the rats of the miR-7a group. After that, the incision skin was sutured and sterilized again. The supported urination was conducted twice a day until the recovery of the urination reflex.

Western Blotting

The total proteins in spinal cord samples were extracted on ice using a total protein extraction kit containing protease inhibitors and phosphatase inhibitors. After centrifugation (13000 g, 10 minutes) at 4°C, the purified proteins were obtained. The concentration of the proteins was determined by the Double Bicinchoninic Acid (BCA) method (Beyotime, Shanghai, China). Subsequently, the proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel and transferred into polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After that, the membranes were incubated with 5% of non-fatty milk in Tris-Buffered Saline with Tween 20 (TBST) to block nonspecific antigen for 1 h at room temperature. After washing 3 times with TBST, the membranes were incubated with primary antibodies (Drp-1, Abcam, Cambridge, MA, USA, Rabbit, 1:250; MFN-2, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; caspase-3, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; caspase-3, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; bcl-2, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; BDNF, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; NT-3, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; GAPDH, Proteintech, Rosemont, IL, USA, 1:10000) at 4°C overnight. After being washed for 3 times with TBST, the membranes were incubated with the corresponding secondary antibody (Goat Anti-Rabbit IgG, Yi Fei Xue Biotechnology, Nanjing, China, 1:5000) at room temperature for 1 h. The targeted proteins were visualized using the enhanced chemical luminescence (ECL; Pierce, Rockford, IL, USA) on an exposure machine.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was added to spinal cord tissue homogenate, and the nucleic acid-protein complex was completely separated after 5 minutes at room temperature. After being transferred into the EP tubes, chloroform was added. The tubes were then violently shaken for 15 seconds, followed by incubation at room temperature for 3 minutes. After centrifugation for 15 minutes (10000 RPM, 4°C), the upper water phase was removed. After re-adding pre-cooled alcohol, the mixture was vibrated and incubated at room temperature for 10 minutes. Subsequently, the mixture was centrifuged for 10 minutes (10000 RPM, 4°C). The supernatant was discarded, and the precipitation was further purified with 75% ethanol (10000 RPM, 4°C). Next, the precipitation was dissolved in 30μl RNase free water. The RNA concentrations were measured by Nano Drop at 260 nm, 230 nm, and 280 nm, respectively. A260/A280 between 1.8 and 2.0 was considered to be qualified. Quantitative analysis of mRNA was achieved using Prism 7300 Sequence Detection System. The relative expression level of genes was calculated by the $2^{-\Delta\Delta}$Ct method. The primer sequences used in this study were as follows: miR-7a,

F: 5'-GCCAGGGAAGCCGTCCGTACTC-3', R: 5'-GACTACGGAATGGGCAACAGCGA-3'; NF-κB, F: 5'-GCCTGTGTACATCAATCGAATG-3', R: 5'-AGGTTGATGTAGTGCCAGTCG-3'; I-κB, F: 5'-CCTTGTCCTATAGAAGCACAAC-3', R: 5'-GTCATTTCCACAGCCCTGTGA-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Immunofluorescence

The spinal samples were first immobilized with 4% paraformaldehyde for 24 h. Then, the spinal cords were dehydrated under different concentrations of ethyl alcohol solutions and embedded in paraffin. Subsequently, the samples were cut into 5 μm sections by a rotary microtome. After dewaxing and antigen repair, the bovine serum albumin (BSA) was utilized to block nonspecific combination at room temperature for 1 h. Subsequently, the sections were washed with Phosphate-Buffered Saline (PBS) and incubated with primary antibodies (NF 200, Cell Signaling Technology, Danvers, MA, USA, Rabbit, 1:200) at 4°C overnight. On the next day, fluorescent secondary antibodies were added to the sections, followed by incubation for 2 h in dark. Next, the nucleus was stained with 4',6-diamidino-2-phenylindole (DAPI) Fluoromount-G and sealed for 5 min. Finally, the sections were visualized using a fluorescence microscope.

Behavioral Assessment

Basso-Beattie-Bresnahan (BBB) locomotor rating scale was used to evaluate the recovery of hindlimb locomotor coordination function for four weeks after SCI. The rats were scored in a double-blind experiment on a scale of 0 to 21. The evaluation was conducted at 1, 7, 14, 21, and 28 days after SCI modeling, respectively.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 16.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. The measurement data were expressed as mean ± standard error. The *t*-test was used to compare the difference between the two groups. The One-way analysis of variance (ANOVA) was performed to compare the differences among different groups, followed by the post-hoc test (Least Significant Difference). The LSD test or Student-Newman-Keuls (SNK) test was used for pairwise comparison under the condition of homogeneity of variance. $p<0.05$ was considered statistically significant. All experiments were independently repeated for 3 times.

Results

Treatment of MiR-7a After SCI Improves Recovery of Locomotor Function

In this study, we first assessed the effect of miR-7a on SCI neural damage by comparing the locomotor recovery in SCI rats with or without miR-7a treatment. BBB rating scale was used to evaluate the locomotor performance of rats in an open field. Two researchers independently recorded the scores for 1 month. The results revealed that the scores of the locomotor function remained low for the first 3 days in both miR-7a treatment and SCI groups. However, the statistically significant difference in motor recovery was observed between two groups one week later. The rats in miR-7a treatment group showed a significantly delayed recovery when compared to the rats in the SCI group until 4 weeks later (Figure 1). Therefore, our data suggested that the administration of miR-7a significantly improved the recovery of the locomotor function in SCI rats.

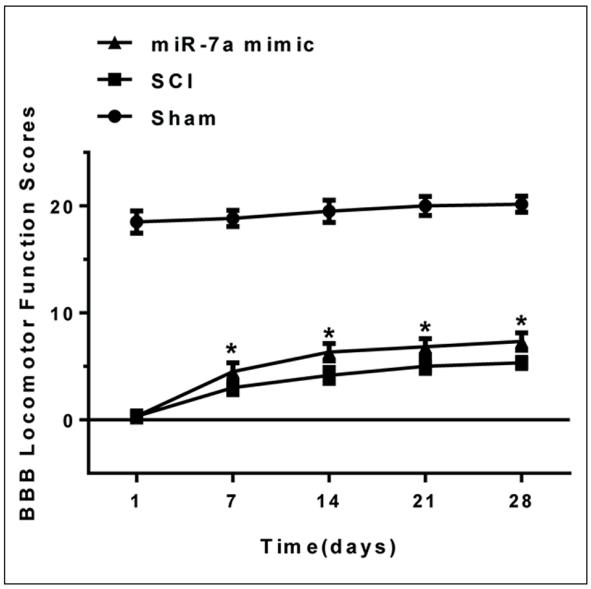

Figure 1. Treatment of miR-7a after SCI improves the recovery of locomotor function. Alterations trend of BBB rating scores in each group during 4 weeks after SCI.

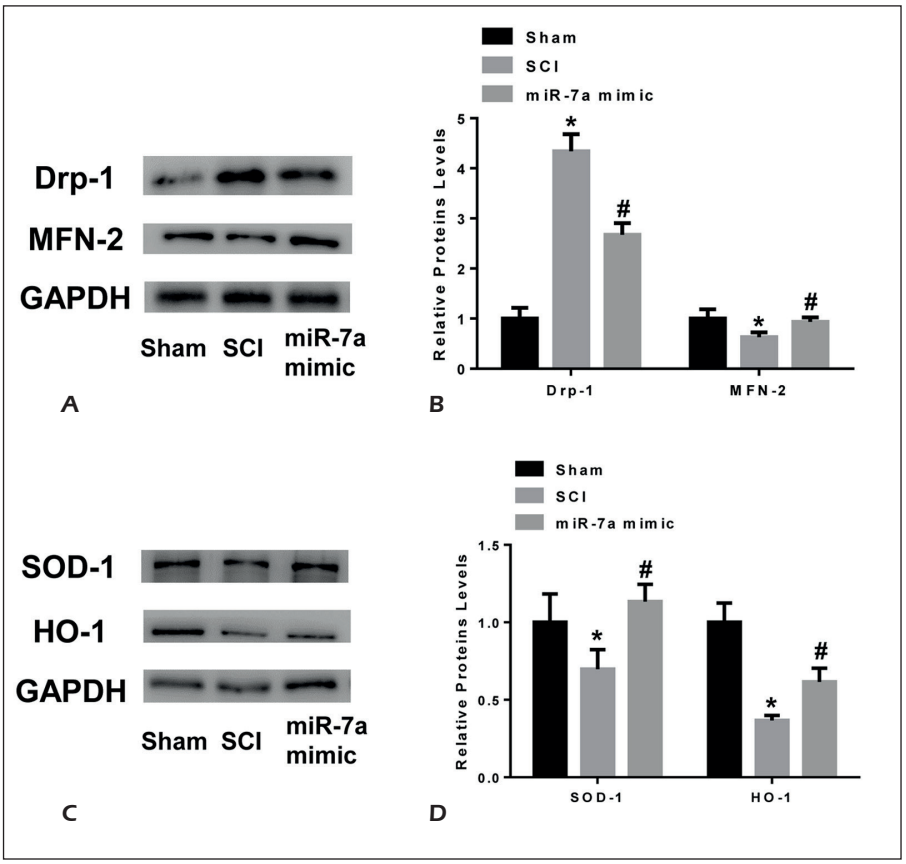

Figure 2. Administration of miR-7a inhibits oxidative stress reaction and mitochondrial dysfunction following SCI. **A**, The protein expressions of Drp-1 and MFN-2 at 3 days following SCI in rats. **B**, Statistically significant differences were observed among all groups. **C**, The protein levels of SOD-1 and HO-1 at 3 days in rats of the three groups. **D**, There were statistically significant differences in the protein expressions among the three groups.

Administration of MiR-7a Inhibits Oxidative Stress Reaction and Mitochondrial Dysfunction Following SCI

Due to the recovery of locomotor function, we continued to explore the underlying mechanism of improvement of the injured spinal cord. Currently, an excessive ROS production derived from SCI can cause a series of damaging effects, such as lipid peroxidation, protein oxidation, and DNA damage. Besides, mitochondrial dysfunction results in a decrease in molecular oxygen metabolism. Dynamin-related protein-1 (Drp-1) and mitofusin-2 (MFN-2) have been reported to regulate mitochondrial division and fusion. Meanwhile, the abnormal expression of both Drp-1 and MFN-2 contributes to the abundance of ROS. Hence, we detected the protein expression of the two molecules by Western blotting at 3 days following SCI. The results showed that the mitochondrial function was remarkably declined in SCI rats without miR-7a treatment. However, the ectopic expression of miR-7a effectively rescued mitochondrial activity by down-regulating Drp-1 and up-regulating MFN-2 (Figures 2A, 2B). Furthermore, the levels of antioxidant enzymes in the injured spinal cord were measured as well. Both Superoxide dismutase-1 (SOD-1) and Heme oxygenase 1 (OH-1) were markedly up-regulated in the rats of miR-7a group (Figures 2C, 2D). All the above results indicated that miR-7a up-regulation inhibited the progression of oxidative stress by regulating mitochondrial function and enhancing the expression of antioxidase.

Neural Tissue Apoptosis is Alleviated Through Inhibition of NF-κB Pathway Caused by MiR-7a

ROS accumulation aggravates injured tissues and initiates cell apoptosis through the activation of a series of specific cascades. NF-κB pathway regulates various cellular biological events, including inflammation, tumorigenesis, and apoptosis. As a classical signaling pathway inducing cell apoptosis, NF-κB can eventually stimulate the caspase system via modulating the expression of various pro-apoptotic proteins and genes. In this study, the expression level of miR-7a within 1 week was detected by qRT-PCR (Figure 3A). To explore whether miR-7a exerted an anti-apoptosis function, we measured the mRNA levels of

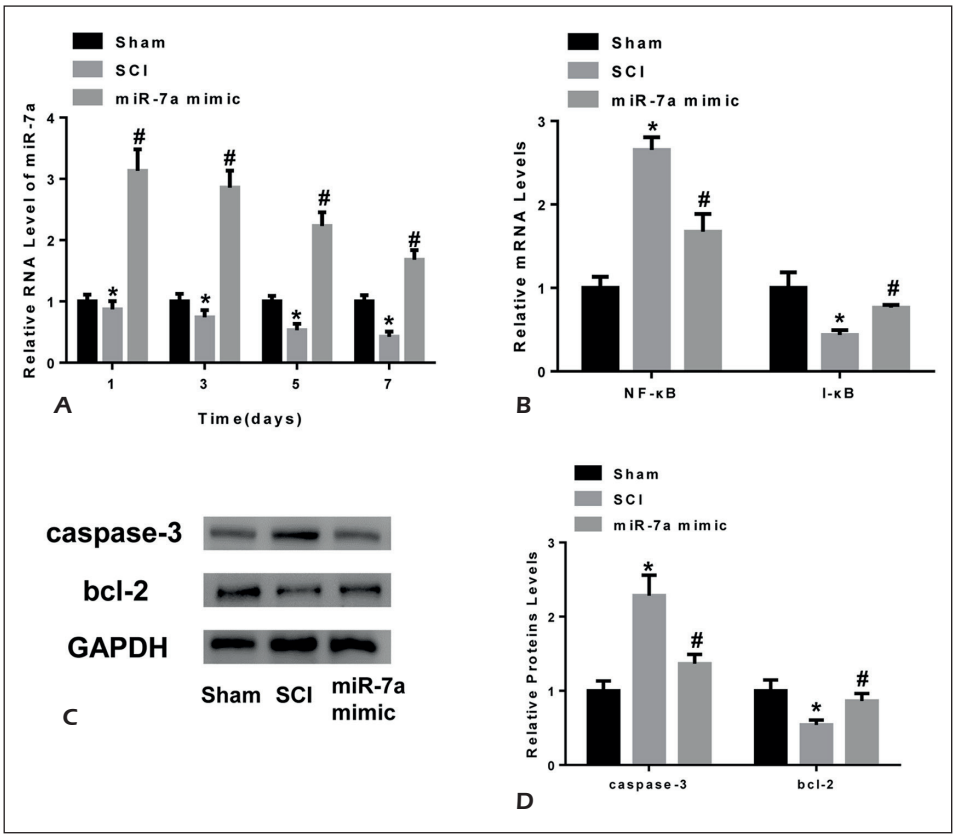

Figure 3. Neural tissue apoptosis is alleviated through the inhibition of NF-κB pathway caused by miR-7a. **A**, MiR-7a level within 1 week after SCI in different groups. **B**, The mRNA levels of NF-κB and I-κB at 7 days after SCI. **C**, The proteins levels of caspase-3 and bcl-2 in each group at 7 days after SCI. **D**, There were statistically significant differences in protein levels among the three groups.

NF-κB and I-κB at 7 days after SCI. The results demonstrated that NF-κB was significantly inhibited due to the increase of I-κB induced by miR-7a treatment (Figure 3B). Moreover, the protein expressions of caspase-3 and bcl-2 were also detected to evaluate cell apoptosis. The results indicated that caspase-3 was significantly elevated, while bcl-2 was significantly declined after SCI. Furthermore, miR-7a mimics could depress the expression of caspase-3 and enhance the expression of bcl-2 (Figures 3C, 3D). These results revealed that miR-7a treatment attenuated apoptosis via suppressing NF-κB pathway.

MiR-7a Up-regulation Promotes the Release of Neurotrophic Factors and Neurostructural Repair

In the late stage of SCI, the expression of neurotrophic factors in the lesion of the spinal cord dominates neural repair. Axon regeneration and re-establishment of the synaptic structure depend on the release of multiple neurotrophic factors. Therefore, we detected the expressions of brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) in SCI rats at 4 weeks. The results demonstrated that miR-7a administration could effectively stimulate the expressions of BDNF and NT-3 in the lesion of the spinal cord (Figure 4A). Besides, the neuro-filament regeneration was imaged using immunofluorescence technique. The results manifested that treatment of miR-7a remarkably facilitated the growth of axons and enhanced the number of neuro-filaments in injured rats (Figure 4B). The above findings illustrated that the up-regulation of miR-7a in the SCI models contributed to the increase of neurotrophic factors, thereby promoting neuro-structural repair.

Discussion

ROS production and aggregation in the injured neural tissues cause continuous and escalating damage[21]. The disruption of the blood-spinal cord barrier and microangiopathy lead to edema and ischemia, eventually causing oxidative death in spinal cord neurons[22,23]. MiRNAs are a class of non-coding RNAs, with capacities

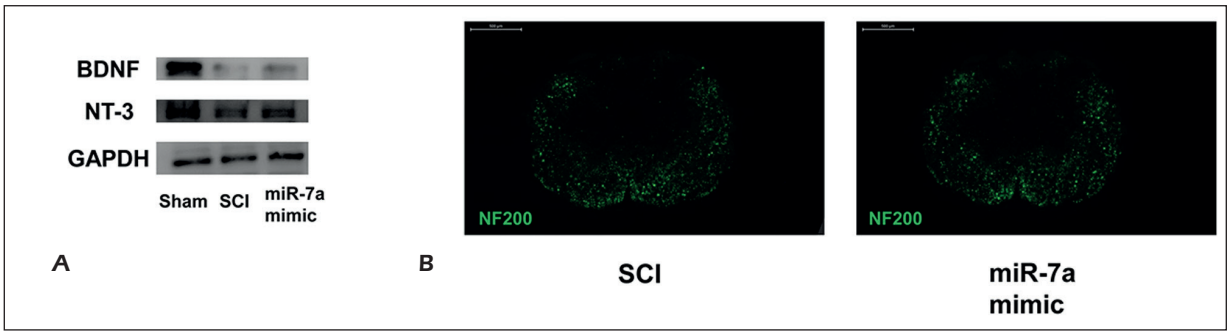

Figure 4. MiR-7a up-regulation promotes the release of neurotrophic factors and neuro-structural repair. **A**, The protein levels of BDNF and NT-3 at 28 days in rats with injured spinal cord. **B**, Immunofluorescence staining of NF-200 in the epicenter of damage at 28 days following SCI (magnification × 40).

of transcriptional regulation[24]. Liu et al[25] have verified that miRNAs modulate the pathogenesis of SCI at multiple levels. Astrocyte proliferation and glial inflammation have been reported to be regulated by various miRNAs[26,27]. In this study, we found that decreased miR-7a was associated with ROS production and apoptosis of neural tissues in rats with SCI. Moreover, the up-regulation of miR-7a *in vivo* significantly accelerated ROS catabolism by improving mitochondrial function and maintaining antioxidant enzymes. Besides, SCI-induced neuronal apoptosis is a fatal pathological event. This may ultimately lead to irreversible neurons loss and neuro-logical deficits. The effective control of apoptosis after injury can change the prognosis of nerve injury. In this study, we inhibited the activation of NF-κB pathway to induce apoptosis and alleviate caspase function. It was noteworthy that the administration of miR-7a promoted the release of neurotrophic factors and the elongation of axons. The above findings suggested that miR-7a enhanced the neuro-structural re-establishment and neurologic improvement in SCI rats. Endanger factors of ROS always play an important role in SCI. However, the mechanism of ROS generation following SCI has not been fully elucidated. Few studies have focused on the regulation and control of ROS production in SCI. In this study, we elucidated the interaction between miRNA and ROS in SCI treatment. The results improved the treatment of neurotraumatic diseases by miRNAs and developed an emerging therapeutic approach. Meanwhile, this study also provided fundamental basis for other ROS researches after SCI. However, specific targets of miR-7a for ROS regulation have not been identified and need further exploration.

Conclusions

This study demonstrated that increased miR-7a suppressed ROS accumulation and cell apoptosis by inhibiting NF-κB pathway. In addition, this might improve the release of neurotrophic factors to ameliorate locomotor function recovery after SCI.

Conflict of Interests

The Authors declared that they have no conflict of interests.

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