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MiR-543-5p inhibits inflammation and promotes nerve regeneration through inactivation of the NF-κB in rats after spinal cord injury

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Abstract. – OBJECTIVE: The aim is to determine the efficacy and specific mechanism of microRNA-543-5p (miR-543-5p) on spinal cord injury (SCI).

MATERIALS AND METHODS: The model of spinal cord injury was established in 6-week-old rats. Firstly, quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to detect the changes of miR-543-5p in the spinal cord of rats in each group after spinal cord injury. Next, we observed the alterations in nuclear factor-kappa B (NF-κB) RNA level after injection of miR-543-5p. In addition, the enzyme-linked immunosorbent assay (ELISA) was used to detect the expression of the inflammatory cytokines, and we used Western blotting to detect the protein associated with nerve regeneration at the protein levels. Finally, the Basso-Beattie-Bresnahan (BBB) rating scale was utilized to measure the recovery of hindlimbs function in rats.

RESULTS: After spinal cord injury, the RNA expression of miR-543-5p in the rat spinal cord was decreased, and the RNA level of NF-κB was found to be decreased after the artificial injection of miR-543-5p. In the inflammatory expression, we found that the expression of various inflammatory mediators was also downregulated. However, the expression of nerve regeneration related factors was significantly upregulated, and it was observed that the evaluated score of the miR-543-5p group was higher than that of the SCI group within 1 month.

CONCLUSIONS: MiR-543-5p inhibited NF-κB pathway and reduced the inflammatory factors, and ameliorated nerve regeneration, which ultimately promoted hindlimbs locomotor function.

Key Words:
Spinal cord injury, MiR-543-5p, NF-κB pathway, Nerve regeneration.

Introduction

Spinal cord injury (SCI) is a serious complication of spinal surgery, which leads to irreversible neurological damage, autonomic dysfunction, even impairment of sensory, and locomotor functions below the lesion[1-3]. Current studies[4,5] have demonstrated that the development of the biological cascades after SCI, including ischemic necrosis and activation of inflammatory responses, etc. Due to the destruction of the blood-spinal cord barrier (BSCB), tissue ischemia, and chemokine of inflammatory cells in the circulatory system induce the inflammation around the injured tissue, and result in necrosis and apoptosis of the surrounding tissue mediated by the inflammatory response[6,7], which are the main factors involved in the severity and expansion of the area of spinal cord injury. The nuclear factor-kappa B (NF-κB) is a transcription factor belonging to the Rel family, which is involved in the regulation of the gene transcription related to immunity, inflammatory response, and cell differentiation[8,9]. The regulation of NF-κB is beneficial to improve the recovery process at post-SCI[10]. Brambilla et al[11] found that the inhibition of NF-κB contribute to increased axonal sparing and sprouting following SCI, while Xu et al[12] demonstrated that modulating NF-κB in mice with SCI influenced neurogenesis status. Moreover, Han et al[13] drew the conclusion that targeting IKK/NF-κB pathway reduced the infiltration of the inflammatory cells and apoptosis after SCI in rats. Therefore, the recovery of the neurological function depends on the regulation of NF-κB after SCI. MicroRNA

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(miRNA) is a nucleic acid transcribed from a non-coding gene, with a length of about 22 nucleotides[14]. MiRNAs are involved in the regulation of various biological activities *in vivo*[15]. There are reports evidencing that the regulation of various miRNAs in the spinal cord changed abnormally after SCI, which might induce some pathological progression[16]. MiR-133b is reported to be an essential factor in nerve recovery following SCI[17], and miR-126 promotes angiogenesis and attenuates inflammation in SCI rats[18]. In the current study, we explored the relationship between miR-543-5p and NF-κB in SCI rats. We determined whether the increase of miR-543-5p could improve the locomotor function, and finally, we investigated the effect of miR-543-5p on SCI.

Materials and Methods

Animals and Grouping

A total of 81 male Sprague Dawley (SD) rats were used for the SCI model. The rats were aged from 6 to 8 week and weight of 200-220 g. The rats were bred in specific conditions (3 rats/cage) in a normal environment (conventional food and drinking water, in room temperature at 20-25°C, and 12 h/12 h artificial circadian cycle). All samples of the animal were randomly divided into three groups (Sham group, SCI group, miR-543-5p injection group). The Sham group was rats merely carried out the laminectomy. The rats in SCI group received injection with the same volume of normal saline intrathecally. The rats in the miR-543-5p group were treated with miR-543-5p intrathecally. This study was approved by the Animal Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong University Animal Center.

Operative Procedure of Modeling

Initially, the rats were anesthetized with 10% paraformaldehyde (4 mL/kg). Then, the skin preparation was accomplished, and the skin of the operation area was disinfected. The modeling process was generally described as follows: we located the spinous process of the T10 spine, and cut open the skin, separated the fascia and muscle tissues to expose the intact lamina structure. Laminectomy was performed to strip the upper lamina of the spinal cord and completely display the spinal cord. Spinal cord impingement (10 g, 5 cm) was performed to impact the spinal cord tissue. After successful modeling, the following reactions occurred in rats: spinal cord hemorrhage, the delayed extension of hindlimbs, and tail swing. Then, we performed an intrathecal injection, closed the incision, and sterilized it again. An assisted urination was carried out once a day following SCI until the micturition reflex was recovered.

Enzyme-Linked Immunosorbent Assay

The spinal cord tissues were extracted from rat bodies. After adding a proper volume of Phosphate-Buffered Saline (PBS) to the spinal cord tissues, the samples were homogenized and centrifuged for 10 minutes to gather supernatant. The standard product wells are set on the 96-well plate, and the standard products of different concentrations were added, respectively. The samples to be tested were added into the corresponding wells, and then, the plates were sealed with the sealing film and incubated at room temperature for 30 minutes. Then, we discarded the liquid and filled each well with washing solution for 30 seconds and repeated 5 times. Then, the enzyme standard reagent was added into each well, except blank wells. Then, the colorant was added into each well and we transferred it in a dark room for 15 minutes. The termination solution was added to terminate the reaction, and the absorbance (OD value) of each well was measured sequentially at the wavelength of 450 nm by zeroing in the blank hole. With the concentration of the standard product as the abscissa and OD value as the ordinate, the standard curve was drawn to calculate the sample concentration.

Western Blotting Technology

The spinal cord tissues in each group were transformed into proteins on ice using a total protein extraction kit with protease inhibitors and phosphatase inhibitors. The protein compounds were centrifuged in the high-speed centrifuge (13000 rpm, 15 minutes) and at 4°C for the aim of supernatant fluid of proteins. The concentration of the protein solution was determined by double Bicinchoninic Acid (BCA) method (Pierce, Rockford, IL, USA). The concentration of the proteins in different groups was balanced, the proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel, and then, we transferred the dispersed proteins to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) at low temperature. 5% non-fatty milk was prepared with Tris-buffered saline with Tween-20 (TBST). After being washed 3 times

with TBST, the membrane was incubated with primary antibodies (Caspase-3, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; bcl-2, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; cleaved PARP, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; NCAM1, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; NL1, Cell Signaling Technology, Danvers, MA, USA, Rabbit, 1:1000; glyceraldehyde 3-phosphate dehydrogenase GAPDH, Proteintech, Rosemont, IL, USA, 1:10000) at 4°C overnight. The membrane was separated from the primary antibody and washed 3 times. Specifically combined with the second antibody (Goat Anti-Rabbit IgG, Yi Fei Xue Biotechnology, Nanjing, China, 1:1000), it was incubated with the membrane at room temperature for 2 h and washed again for 3 times. Enhanced chemiluminescence (ECL) was used to display the targeted protein on an exposure machine.

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

The moderate TRIzol (Invitrogen, Carlsbad, CA, USA) was added to spinal cord tissue and homogenized after shearing. The nucleic acid-protein complex was completely separated after 5 minutes at room temperature. 0.2 mL chloroform was added into each 1 mL TRIzol, and the tubes were violently shaken for 15 seconds and then left at room temperature for 3 minutes. The mixture was centrifuged for 15 minutes (10000 RPM, 4°C) and then, the upper water phase was sucked out to join the isopropyl alcohol. The mixture was vibrated and placed at room temperature for 10 minutes. After the mixture has been centrifuged for 10 minutes (10000 RPM, 4°C), we get the RNA precipitation and discard the supernatant. After washing RNA precipitation with 75% ethanol, the mixture was centrifuged (10000 RPM, 4°C) for 5 minutes. The supernatant was discarded and then, we add 30 μL RNase free water to dissolve it. RNA concentrations were measured on NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) to determine the absorbance at 260 nm, 230 nm, and 280 nm. If A260/A280 were between 1.8 and 2.0, the RNA quality was considered to be standard and could be used in subsequent experiments.

The quantitative analysis of mRNA is achieved using Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), 25 μL reaction System is used including SYBR Green (12.5 μL), 10 Mm of primers (0.5 mL each from the stock), 10.5 μL of water, and 0.5 μL of template. The PCR conditions were as follows: 10 min denaturation at 95°C, 40 cycles of denaturation at 95°C for 15 s, 60°C annealing for 30 s, and 72°C extension for 30 s. The data are analyzed by SDS software, and the results were output to EXCEL for further analyses. Endogenous GAPDH was used to standardize the data. The comparative threshold cycle (Ct) method, that is, the $2^{-\Delta\Delta Ct}$ method was used to calculate the fold amplification.

Behavioral Assessment

The Basso-Beattie-Bresnahan (BBB) locomotor rating scale was utilized to evaluate the recovery of the locomotor coordination function of hindlimbs within one month after spinal cord injury in rats. Two different investigators scored the samples on a range of 0 to 21 as they analyzed their movements in an open field. The evaluation was conducted at 1, 3, 7, 14, 21, and 28 days after modeling, respectively.

Statistical Analysis

The data were analyzed with Statistical Product and Service Solutions (SPSS 18.0) software (SPSS Inc., Chicago, IL, USA). All data were expressed as mean±SEM ($\bar{x} \pm s$). The *t*-test was used for comparisons between the two groups. The single-factor analysis of variance (ANOVA) was used for the comparison between groups with different concentrations. The Least Significant Difference (LSD) test or the 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 repeated 3 times.

Result

MiR-543-5p Was Downregulated in Spinal Cord Injury and Inhibited NF-κB Elevation After Injection of MiR-543-5p

Firstly, we used qt-PCR to measure miR-543-5p and NF-κB RNA levels in different treated groups within one week. The results showed that the expression of miR-543-5p showed an increasing trend within one week in the injured spinal cord, reaching the highest level at 7 days (Figure 1A). However, the level of NF-κB rose and at the top after 7 days. This indicates that the decrease of miR-543-5p may be negatively correlated with the increase of NF-κB. For further confirmation,

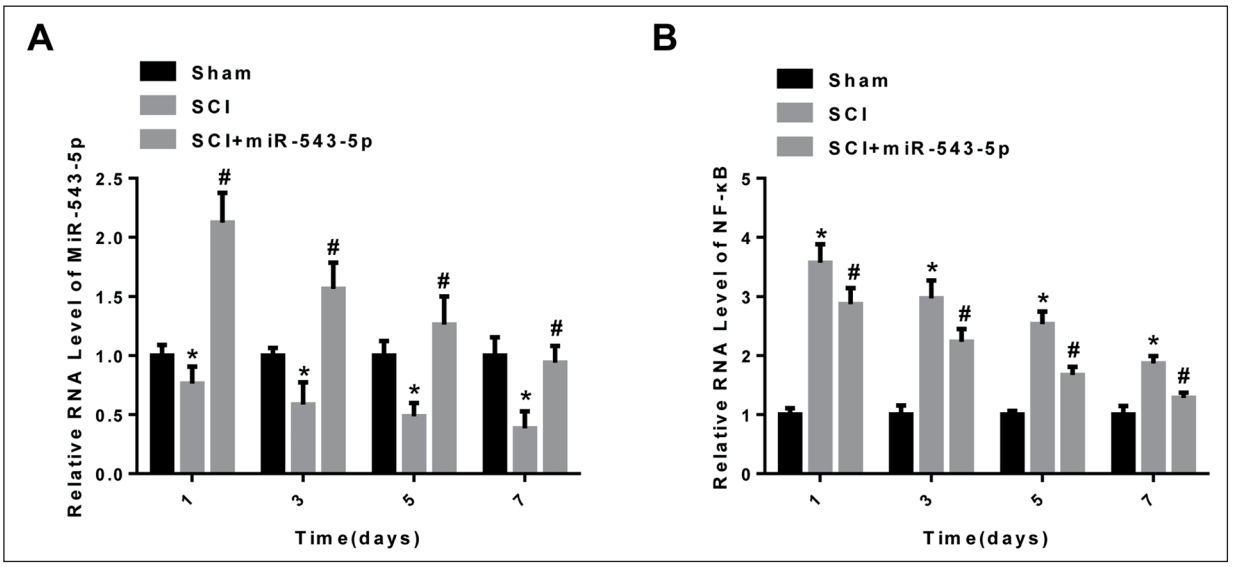

Figure 1. MiR-543-5p was down-regulated in spinal cord injury and inhibited NF-κB elevation after injection of miR-543-5p. ***A,*** the RNA levels of miR-543-5p within one week after SCI in the three groups. ***B,*** The NF-κB transcription levels during one week at post-SCI among groups.

we compared the expression levels of miR-543-5p and NF-κB in the SCI group and the injection group. It was found that the level of NF-κB was inhibited with the increase of the dose of miR-543-5p (Figure 1B). Therefore, we concluded that miR-543-5p was reduced following SCI, and its upregulated expression inhibited the level of NF-κB.

MiR-543-5p Induced Reduction of Inflammation and Inhibition of Apoptosis

Furthermore, we measured the inflammatory factors like interleukin 1 beta (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor-alpha (TNF-α) with ELISA assay. Because of the inhibition effect of miR-543-5p to NF-κB, we found that the results of the ELISA analysis of IL-1β, IL-6, and TNF-α in the miR-543-5p injection group were relatively inferior to those in the SCI group at 3 days following SCI (Figure 2A-2C). On the other hand, the key factors of the apoptosis system were detected by Western blotting technology to reflect the degree of apoptosis in the spinal cord at 7 days post-injury. Then, we obtained the results that the protein level of caspase-3 and cleaved PARP were significantly decreased after the administration of miR-543-5p, while the content of bcl-2 was significantly increased (Figure 2D), indicating that the inhibition of NF-κB by miR-543-5p reduced apoptosis.

Administration of MiR-543-5p Facilitated Nerve Remodeling and Axonal Regeneration Following SCI

Inducing neurogenesis, promoting neuronal differentiation, and protecting nerve cells play a decisive role in the recovery of nerve function after SCI. The level of nerve repair factor in the later stage of injury promotes the structural remodeling of nerve to a certain extent. The neural cell adhesion molecule (NCAM) can promote the regeneration and repair after nerve injury, such as SCI and the traumatic brain injury[19]. Neuroligin 1 (NL1) is the most clearly studied member of the neuroligins family. Pan et al[20] have shown that NL1 is involved in the formation of excitatory post-synaptic structures, as well as the formation of synaptogenesis and N-methyl-D-aspartate (NMDA) receptors. NL1 is not only a structural protein that forms synapses, but also a functional protein that affects the synaptic formation and information transmission. After injection of miR-543-5p, we detected the proteins of NCAM1 and NL1 in rat spinal cords among groups at 14 days post-SCI (Figures 3A and 3B). The results exhibited the hypo-expression of NCAM1 and NL1 in the SCI group. Treatment with miR-543-5p significantly attenuated the SCI-induced hypo-expression of nerve regulated factors. Consistent with the other findings in this work, the miR-543-5p treatment prevented the decline in NCAM1 and NL1.

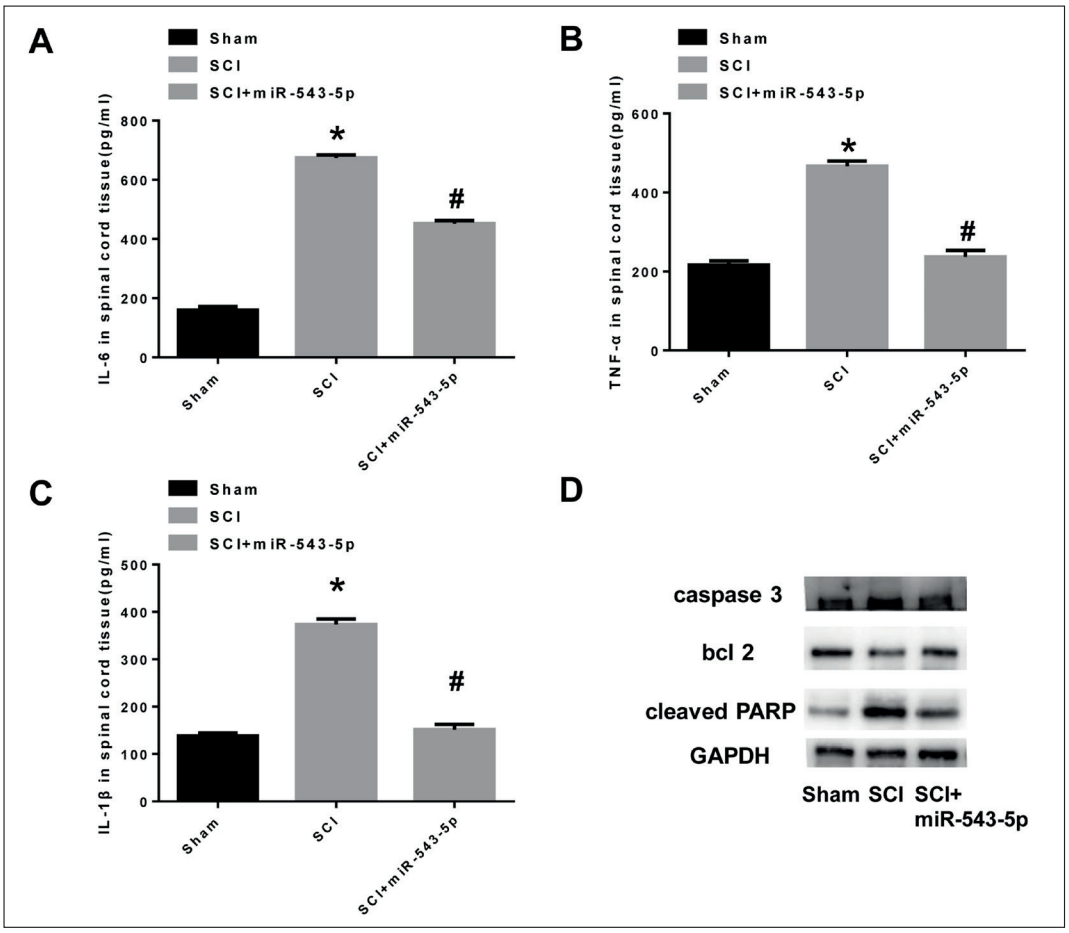

Figure 2. MiR-543-5p induced reduction of inflammation and inhibition of apoptosis. ***A,*** The ELISA results analysis of IL-6 at 3 days following SCI in the three groups. ***B,*** Concentration determination of TNF-α at 3 days after SCI among the three groups. ***C,*** Concentration determination of IL-1β with ELISA at 3 days following SCI. ***D,*** Protein levels of apoptotic factor, apoptotic product, and anti-apoptotic factor at 7 days after SCI.

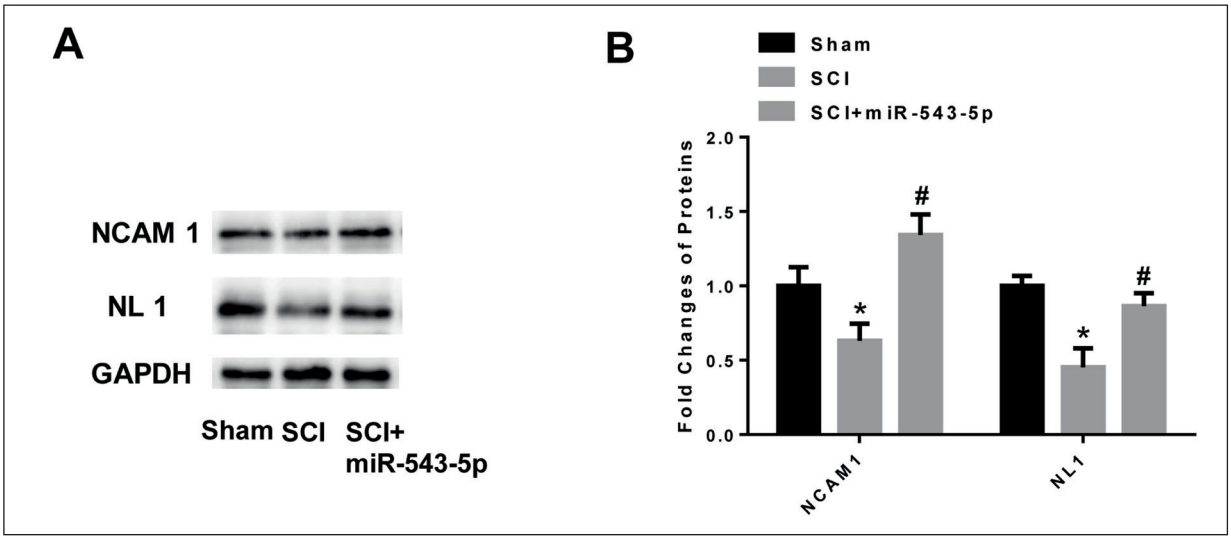

Figure 3. Administration of miR-543-5p facilitated nerve remodeling and axonal regeneration following SCI. ***A,*** Protein expressions of NCAM 1 and NL 1 at two weeks following injury. ***B,*** According to the analysis results of IMAGE J software, the protein expressions were statistically significant.

Inhibition of MiR-543-5p to NF-κB Ameliorated Locomotor Function Recovery in SCI Rats

Finally, we performed behavioral tests at 1, 7, 14, 21, and 28 days following SCI on untreated rats and rats treated with miR-543-5p. The performance of the Sham rats remained unchanged throughout the testing period. Functional recovery was also assessed in an open-field testing using the 21-point BBB locomotor rating scale. Animals underwent paraplegia after SCI and displayed a low BBB score after 1 day. However, with the development of continued recovery, the early improvement of motor function was showed after 7 days. Untreated samples were revealed a modest recovery while rats injected with miR-543-5p displayed a significantly higher improvement within 4 weeks after SCI (Figure 4). So, we drew the conclusion that the inhibition of miR-543-5p to NF-κB promoted the function recovery after SCI in rats.

Discussion

The problem of functional recovery after SCI has always been urgent to be solved. The regeneration of nerve filaments after injury and the remodeling of synaptic structure contribute to the recovery of nerve function[21]. However, the re-formation of the neural structure is a multi-factor complex process with many limiting factors. Considering the weak regeneration ability of nerve cells and the activation of a large number of glial cells after injury, resulting in neuroinflammatory reaction and glial scar hyperplasia, the remodeling of the nervous system after SCI is greatly restricted[22,23]. Due to the destruction of the blood spinal cord barrier, the inflammatory chemokines entering the spinal cord tissue affect nerve regeneration through the receptor binding pathway, and the glial cell population also produces blocking factors that hinder nerve regeneration[24,25]. In addition, the intensification of inflammation can induce the cells to initiate the apoptosis process, and thus, more surviving neurons will self-destruct under the induction of inflammation[26]. NCAM, belonging to the immunoglobulin superfamily cell adhesion points, is mainly expressed in the surface of neurons and glial cells[27,28]. Abnormal expression of NCAM will lead to the hindrance of the growth of newborn neurons, block the connection between nerve cells, and affect the development of the nervous system[28]. The lack of NCAM can promote the apoptosis of nerve cells at the injured site, while the presence of NCAM can induce neural differentiation, neuroprotection, and anti-inflammatory effects[29]. Similarly, NL1 is a post-synaptic transmembrane protein synthesized by neurons, which is bound to post-synaptic density protein 95 (psd-95) on excitatory post-synaptic membranes in the cell through the C-terminal PDZ domain-binding sequence, and is bound to the the gephyrin through the consistency sequence in the middle of the intracellular domain[30]. Meanwhile, NL1 relates to presynaptic axon-1 through the extracellular domain[31]. NL1 and axon albumen-1 play important roles in synaptogenesis and maturation. Burton et al[32] have shown that the overexpression of NL1 in neurons can promote the synapse formation. When NL1 is silenced, the number of synapses decreases[33]. However, in the present report, we confirmed the abnormal downregulation of miR-543-5p after SCI, and the reduction of NF-κB expression mediated the inflammatory suppression by miR-543-5p treatment. Moreover, miR-543-5p decreased the level of apoptosis and promoted the release of nerve repair factors. This analysis identified a new therapeutic strategy for SCI and opened-up a pathway for the application of miRNA in the SCI disease models, but the mechanism of miR-543-5p-specific targets in SCI still needs to be further explored.

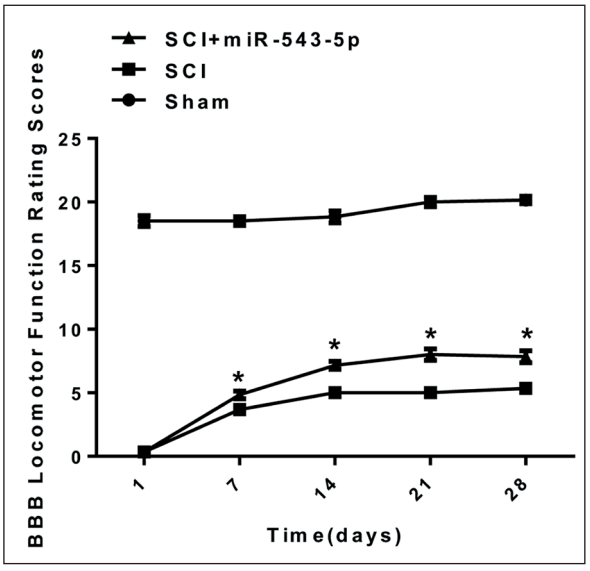

Figure 4. Inhibition of miR-543-5p to NF-κB ameliorated the locomotor function recovery in SCI rats. According to the statistics, there was a statistically significant difference in locomotor function recovery of rats after one week of post-SCI.

Conclusions

We demonstrated that miR-543-5p inhibited NF-κB to reduce glial inflammation and cell apoptosis, promoting NCAM and NL1expression, and improving the function recovery.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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