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Inhibition of miR-214-5p attenuates inflammatory chemotaxis and nerve regeneration obstruction after spinal cord injury in rats

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Abstract. – OBJECTIVE: To investigate the effect of miR-214-5p on spinal cord injury (SCI) and the possible mechanism in pathophysiological relevance, and to evaluate the therapeutic efficacy of the corresponding inhibitor.

MATERIALS AND METHODS: The SCI model was successfully established in 6-week-old rats. The levels of locomotor function recovery in rats of miR-214-5p inhibitor group and SCI group were detected one month later by Basso-Beattie-Bresnahan (BBB) locomotor rating scale, Western blotting, quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. Rat microglia cells were cultured *in vitro*. Furthermore, the effect of miR-214-5p and its inhibitor on inflammatory microglia was explored.

RESULTS: Compared with SCI group, rats in miR-214-5p inhibitor group showed a significant retardation of inflammatory diffusion in terms of reduced production of inflammatory factors and chemokines *in vivo*. MiR-214-5p inhibitor markedly attenuated antioxidant stress, inhibited apoptosis, and increased nerve fibers repair. Compared with lipopolysaccharide (LPS) group, tumor necrosis factor alpha (TNF-α) and interleukin 1 (IL-1) decreased significantly in microglia treated with miR-214-5p inhibitor *in vitro*. Furthermore, inhibition of miR-214-5p remarkably promoted locomotor function recovery in rats.

CONCLUSIONS: MiR-214-5p inhibitor retarded inflammatory diffusion by inhibiting inflammatory factors and chemokines after SCI. In addition, this might relieve nerve structure destruction, resist oxidative stress and inhibit apoptosis, eventually promoting function recovery.

Key Words:
Spinal cord injury (SCI), MiR-214-5p, Inflammation, Locomotor function recovery.

Introduction

Spinal cord injury (SCI) is one of the most serious complications after spinal trauma. Multiple severe neurological dysfunctions are caused by SCI[1], including loss of locomotor and sensory functions below the lesion and systemic autonomic disorders[2]. In the past few decades, great progress has been made in understanding the molecular and cellular events produced by SCI. These findings provide a theoretical basis for further exploring the key mechanisms of tissue damage and regeneration failure of injured neurons[3-5]. Current researches have confirmed that the formation of primary SCI triggers a series of biological chain reactions, which lead to the activation of inflammatory responses and the formation of chemokines[6]. This means that the primary injured area of the spinal cord is aggravated. Meanwhile, the injury area extends to the surrounding normal tissue. Effective control of inflammatory after injury and inhibition of apoptosis of injured neurons exerts a positive effect on the prognosis of the disease[7,8]. Reducing the accumulation of inflammatory reactants and weakening of chemokines can decrease the deterioration of immune inflammation, especially in the acute phase of injury. This has shown a predictive effect on the protection of neurons[9,10].

MiRNA is a type of non-coding single-stranded RNA molecule with about 22-24 nucleotides in length. It is known to all that miRNA is formed by the transcription of endogenous genes[11]. Some studies[12] have indicated that miRNA is mainly involved in the regulation of gene expression after transcription in organisms. It has been also re-

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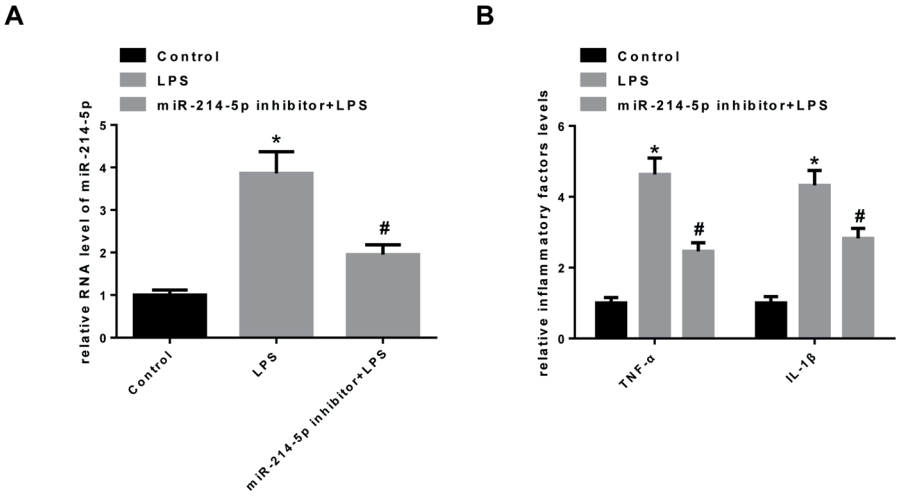

Figure 1. MiR-214-5p increased significantly in the inflammatory microglia and the administration of miR-214-5p inhibitor ameliorated the expression of inflammatory cytokines. ***A,*** The mRNA expression of miR-214-5p in control group, LPS activation group and miR-214-5p inhibitor group. ***B,*** ELISA results of TNF-α and IL-1β in control group, LPS activation group and miR-214-5p inhibitor group.

ported[13-15] that miRNAs are expressed differently from normal in many diseases models. Similarly, an abnormal transcriptional regulation of miRNAs also exists in SCI. This may be involved in the regulation of various biological and pathological reactions after injury[16,17]. Currently, researches have demonstrated that abnormal changes of miR-21 after SCI play an important role in various pathological changes[18]. Meanwhile, miR-486 serves as a potential target of SCI therapy[19].

In the present research, we mainly explored the pathological changes caused by the abnormal expression of miR-214-5p in SCI. Moreover, the potential value of human intervention on the expression level of miR-214-5p in SCI therapy was investigated. The results showed that an increased expression of miR-214-5p after SCI significantly enhanced the activity of inflammatory reaction, induced the increase of chemokine aggregation, and inhibited the regeneration of nerve fibers. However, the application of its inhibitors impeded the regulatory effect of miR-214-5p, alleviated the levels of inflammation and chemotaxis, and assisted in the reconstruction of neural function.

Materials and Methods

Primary Microglia Isolation and Culture

Primary microglia were isolated from the cerebral cortex of 3-day-old newborn rats. After sacrificing the rats, brain tissues were removed from bodies, and tyrosine was used to dissolve the tissues. Subsequently, the cell mixture was purified to obtain microglia (95% purification rate). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% streptomycin and penicillin. When microglia grew to a satisfactory density, miR214-5p was transfected into cells. After that, LPS (100 ng/mL, 24 h) was used to activate the cells. Finally, cells in each group were collected for subsequent experiments.

Animals and Grouping

A total of 60 male, 6-week-old Sprague Dawley (SD) rats weighing 200-220g were used to construct the SCI model. Rats were fed in 20 cages (3 rats/cage) under normal environmental conditions (conventional food and drinking water, room temperature of 22-24°C, 12 h artificial circadian cycle). All experimental rats were randomly divided into three groups, including: Sham group, SCI group, and miR-214-5p inhibitor group. Rats in Sham group only underwent laminectomy. Rats in SCI group were injected with the same amount of normal saline intrathecally. Meanwhile, rats in miR-214-5p inhibitor group were those with suspended tails and injected with miR-214-5p inhibitor intrathecally. This study was approved by the Animal Ethics Committee of Jilin University Animal Center.

Operative Procedure and Treatment

First, rats were anesthetized with 10% paraformaldehyde at a dose of 4 mL/kg. Then the skin was prepared and disinfected for operation area. The modeling process was recorded as follows. After locating the spinous process of the 10th thoracic spine, the skin was cut open. The fascia muscle tissue was further separated to expose the intact lamina structure. Laminectomy was performed to strip the upper lamina of the spinal cord and completely expose it. Spinal cord impingement (10 g, 5 cm) was performed to hit spinal cord tissues. SCI modeling was successfully demonstrated in rats with spinal cord hemorrhage and delayed the extension of lower limbs and tail swing. After that, we performed an intrathecal injection, and closed and sterilized the incision. Manual urination was performed daily after modeling, until the micturition reflex's restoration.

Western Blotting Technology

Spinal cord tissues or microglia treated differently were transformed into proteins on ice using a total protein extraction kit containing protease inhibitors and phosphatase inhibitors. After centrifugation at 13000 rpm for 15 minutes at low temperature (4°C), the supernatant fluid was collected. The concentration of protein samples was determined by the double bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Subsequently, protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA,

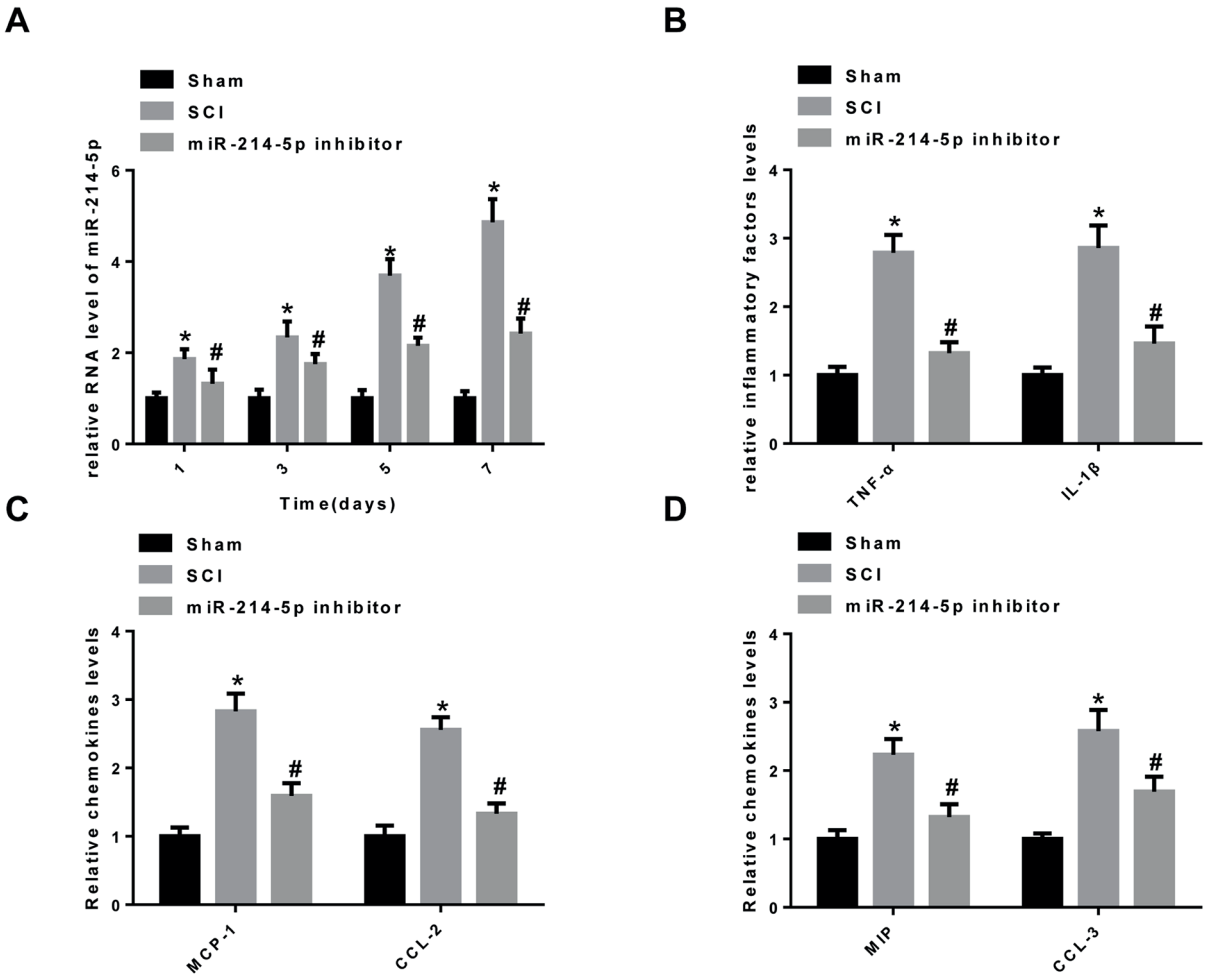

Figure 2. Decrease of miR-214-5p expression down-regulated inflammatory response and impeded the expression of chemokines in SCI rats. ***A,*** The mRNA expression of miR-214-5p within one week following SCI. ***B,*** ELISA results of TNF-α and IL-1β at 7 days after SCI. ***C,*** ELISA results of monocyte chemotactic protein 1 (MCP-1) and chemokine (C-C motif) ligand 2 (CCL-2) at 7 days after SCI. ***D,*** ELISA results of macrophage inflammatory protein (MIP) and chemokine (C-C motif) ligand 3 (CCL-3) at 7 days following SCI.

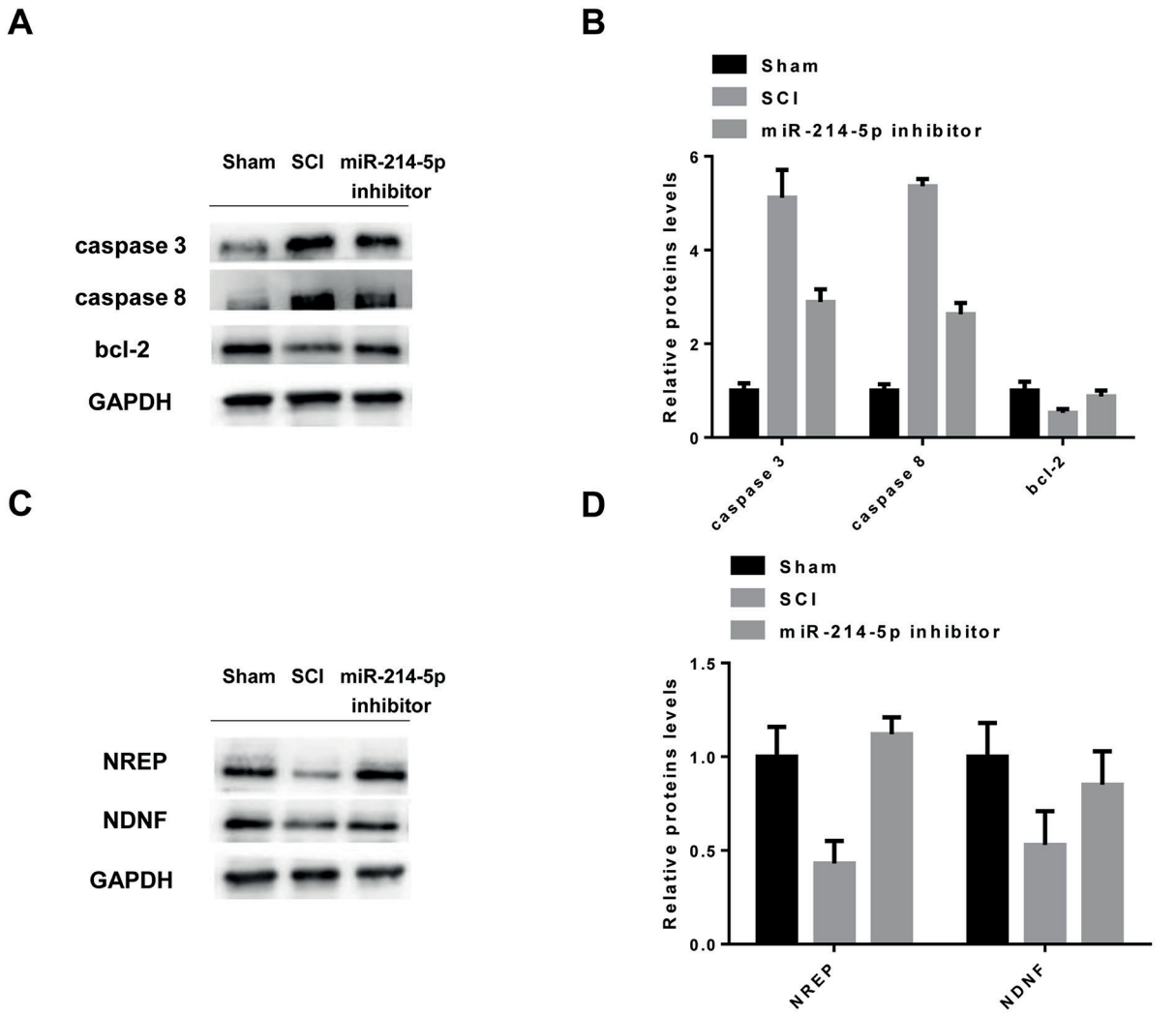

Figure 3. Inhibition of miR-214-5p alleviated tissue apoptosis and contributed to neurostructural remodeling. ***A,*** Western blotting results of the expressions of apoptosis-related proteins at 14 days following SCI. ***B,*** Gray value analysis of protein bands showed that the differences of each component were statistically significant. ***C,*** Western blotting results of the expressions of neurofilament regeneration proteins at 14 days following SCI. ***D,*** Statistically significant differences were found in the expressions of proteins among groups.

USA) at 4°C for 2 h. 5% non-fatty milk was prepared with Tris-Buffered Saline and Tween-20 (TBST) to block non-specific antigen binding for 1 h. After washing 3 times with TBST, the membranes were incubated with primary antibodies (Caspase3, Cell Signaling Technology, Danvers, MA, USA, Rabbit, 1:1000; Caspase 8, Cell Signaling Technology, Danvers, MA, USA, Rabbit, 1:1000; bcl-2, Abcam, Cambridge, MA, USA, Rabbit, 1:2000; NREP, Abcam, Cambridge, MA, USA, Rabbit, 1:1000, NDNF, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; GAPDH, Proteintech, Rosemont, IL, USA, 1:10000) at 4°C overnight. On the next day, the membranes were washed with TBST for 3 times and incubated with the corresponding secondary antibody (Goat Anti-Rabbit IgG, YiFeiXue Biotechnology, Nanjing, China. 1:1000) at room temperature for 2 h. Enhanced chemiluminescence (ECL) was used to display target proteins on the exposure machine.

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

1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) was added to spinal cord tissues and homogenized after shearing. The nucleic acid protein complex was completely separated after 5 min of incubation at room temperature. For cells, 0.5 mL

of TRIzol was added to six-well plates, followed by shaking on ice for 10 min. 0.2 mL of chloroform was added into every 1 mL of TRIzol. The tubes were violently shaken for 15 s and incubated at room temperature for 3 min. After centrifugation for 15 min (10000 RPM, 4°C), the upper water phase was collected and isopropyl alcohol was added. Subsequently, the mixture was vibrated and placed at room temperature for 10 min. After centrifugation for 10 min (10000 RPM, 4°C), the supernatant was discarded and RNA precipitation was obtained. After washing RNA precipitation with 75% of ethanol, the mixture was centrifuged at 10000 RPM, 4°C for 5 min. After discarding the supernatant, 30 μl RNase free water was added to dissolve the RNA precipitation. The concentration of extracted RNA was measured by NanoDrop, followed by determination of absorbance at 260 nm, 230 nm and 280 nm. RNA samples with A260/A280 between 1.8 and 2.0 were considered of good quality, which could be used for subsequent experiments.

Quantitative analysis of mRNA was achieved using Prism 7300 Sequence Detection System. 25 μL reaction system was used, including SYBR Green (12.5 μL), 10 Mm of primers (0.5 mL each from the stock), water (10.5 μL) and template (0.5 μL). Specific PCR conditions were as follows: denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 s; annealing at 60°C for 30 s, and extension at 72°C for 30 s. Experimental data was analyzed by SDS software, and the results were output to EXCEL for further analysis. GAPDH was used as an internal reference. Comparative threshold cycle (Ct) method, namely the $2^{-\Delta\Delta Ct}$ method, was used to calculate fold amplification. The primer sequences used in this study were as follows: miR-214-5p, F: 5'-GCAGAAGAACCTCGGCTTACTC-3', R: 5'-GCCTAGGGATAAGTCCGAAGGA-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

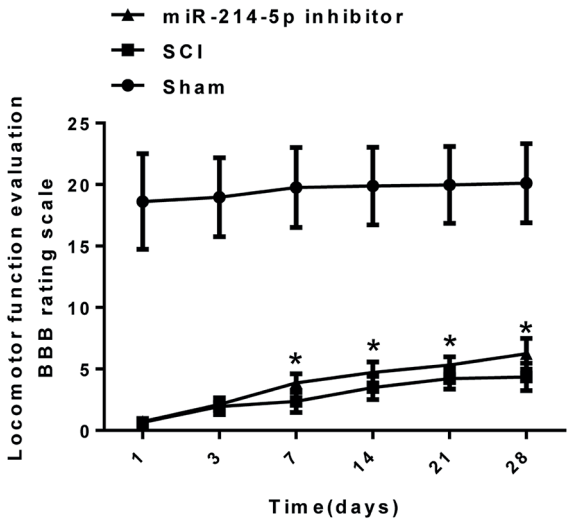

Figure 4. Restrain of miR-214-5p improved the recovery of locomotor function after SCI. Locomotor function recovery score of each time period within 4 weeks after SCI.

Enzyme-Linked Immunosorbent Assay

Spinal cord samples were first taken from rats. An appropriate amount of phosphate-buffered saline (PBS) was added to spinal cord tissues. Then, the mixture was homogenized and centrifuged for 10 min to collect the supernatant. Standard product wells were set on 96-well plates, and standard products of different concentrations were added successively. After that, the samples were added into corresponding wells. After sealing with the sealing film, the plates were incubated at room temperature for 30 min. Then, the liquid was discarded, and each well was filled with washing solution for 5 times, with 30 s for each time. Enzyme standard reagent was added into each well, except blank ones. Next, the colorant was added into each well and incubated in the dark for 15 min. The final solution was added to terminate the reaction. The absorbance (OD value) of each well at the wavelength of 450 nm was measured sequentially by zeroing in the blank hole. The concentration of standard product and OD value were used as the abscissa and the ordinate, respectively. Finally, the standard curve was drawn to calculate the sample concentration.

Behavioral Assessment

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

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 18.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Measurement data were expressed as $\chi \pm s$; the *t*-test was used to compare the difference between the two groups. One-way ANOVA test was used to

compare the differences among different groups, followed by Post-Hoc Test (Least Significant Difference). The Least Significant Difference (LSD) test or the Student-Newman-Keuls (SNK) test was used for pairwise comparison under homogeneity of variance. $p<0.05$ was considered statistically significant. All experiments were repeated for 3 times.

Results

MiR-214-5p Increased Significantly in Inflammatory Microglia and Administration of MiR-214-5p Inhibitor Ameliorated Expression of Inflammatory Cytokines

An excessive activation of microglia after SCI mediates the deterioration of inflammatory response. Uncontrolled microglia can cause neurotoxicity because they are important sources of pro-inflammatory factors and oxidative stress, such as TNF-α, nitric oxide, and IL-1β. The mRNA expression of miR-214-5p after the activation of microglia cells was detected. The results found that the expression of miR-214-5p increased significantly in inflammatory microglia cells. However, the mRNA level of miR-214-5p in cells was obviously inhibited after treatment with inhibitor (Figure 1A). Meanwhile, ELISA results showed that the expressions of TNF-α and IL-1β were remarkably down-regulated (Figure 1B). The results demonstrated that the elevation of miR-214-5p after activation of microglia provoked inflammation. Meanwhile, inhibition of miR-214-5p ameliorated the release of inflammatory factors.

Decreased MiR-214-5p Expression Down-regulated Inflammatory Response and Impeded the Expression of Chemokines in SCI Rats

To verify the inflammatory regulatory effect of miR-214-5p *in vivo*, the expression of miR-214-5p in fresh spinal cord extracted from rats within one week after SCI modeling was determined by qRT-PCR. Results indicated that the mRNA level of miR-214-5p was significantly up-regulated one week after injury. QRT-PCR also showed that the mRNA expression of miR-214-5p in the inhibitor group was significantly down-regulated when compared with that in SCI group (Figure 2A). At the level of inflammation, ELISA results demonstrated that the expressions of inflammatory factors (Figure 2B) and chemokines (Figure 2C and 2D) in inhibitor group were obviously down-regulated at 7 days after injury. All the results indicated that the expression of miR-214-5p increased in SCI rats and was positive in inflammatory response and chemotaxis process.

Inhibition of MiR-214-5p Alleviated Tissue Apoptosis and Contributed to Neurostructural Remodeling

The remodeling of nerve structure is closely related to the degree of neuronal apoptosis and the regeneration of nerve fibers. The expression levels of Caspase 3, Caspase 8, and Bcl-2 (involving in the process of apoptosis) were detected by Western blotting at 14 days after SCI. The results showed that the expression levels of Caspase 3 and Caspase 8 in rats of SCI group were significantly higher than those of rats in Sham group. However, Bcl-2, an anti-apoptotic cytokine, exhibited the opposite results. After the administration of miR-214-5p inhibitor, the apoptosis was significantly prevented (Figure 3A and 3B). Neuronal regeneration related protein (NREP) and neuron-derived neurotrophic factor (NDNF) are key factors in the nerve fiber regeneration. The expressions of NREP and NDNF were determined by Western blotting as well. The results revealed that after SCI, the expression levels of nutritive factors related to nerve regeneration were markedly suppressed. However, after the miR-214-5p inhibitor treatment, the expression levels of functional proteins in nerve regeneration were significantly ameliorated (Figure 3C and 3D), which exerted potential positive effects on nerve structure remodeling.

Restrain of MiR-214-5p Improved the Recovery of Locomotor Function after SCI

Locomotor function of rats in each group was assessed and analyzed by BBB rating scale within one month after modeling. The results showed that no evident abnormal locomotor function was observed in the Sham group. However, the rats in the other two groups developed severe hindlimbs dysfunction after modeling. Until one week after injury, the recovery of hindlimbs movement of rats injected with miR-214-5p inhibitor was significantly improved compared with the SCI group. Meanwhile, the difference was most pronounced at 28 days (Figure 4). BBB rating scale demonstrated that inhibition of miR-214-5p activity could improve the locomotor function of hindlimbs in rats after SCI.

Discussion

Inflammatory chemotaxis after SCI leads to a large number of inflammatory activation of spinal cord microglia[20]. Currently, microglia are constantly scavenging damaged neurons in the central nervous system[21]. However, an excessive activation or loss of control of microglia may cause neurotoxicity[22]. On the one hand, microglia release a large number of pro-inflammatory factors to aggravate the apoptosis of nerve tissues[23]. Activated microglia mediates the proliferation of astrocytes which release inhibitory nerve regeneration factors. This may eventually form a glial scar at a specified future date[24].

Multiple miRNAs are important regulators of SCI, which have also been proved to be related to the expression level of post-transcriptional genes[25,26]. However, the regulatory targets and mechanisms of miR-214-5p after SCI have not been elucidated. Previous studies have reported that miR-214 is highly expressed in many diseases, such as gastric cancer, ovarian cancer and even osteoporosis. The above findings indicate that miR-214 participates in apoptosis, metastasis and progression. However, no report has fully elucidated the role of miR-214 in the regulation of nerve injury function yet. In the present work, we found that increased levels of miR-214 were associated with an increased expression of inflammatory and apoptotic markers and decreased levels of nerve growth factors in SCI rats. The above findings provided another insight into the role of miRNA in the pathophysiological regulation of SCI throughout the process from inflammation to inhibition of nerve repair. Rats injected with miR-214-5p showed a significant recovery of the locomotor function. Meanwhile, only a slight recovery of locomotor ability was observed in rats with SCI. Therefore, down-regulation of inflammatory factors and chemokines might lead to the reduction of nerve structure damage. However, the increase of nerve growth factors and the decrease of apoptosis resulted in the improvement of hindlimbs locomotor function.

Functional and structural repair of central nervous system injury is an increasingly serious public health problem. The major requirement for the treatment of SCI is to identify effective strategies to save neuron survival and to reconstruct neural pathway connections. These treatments have been widely used to compensate for locomotor sensory impairments caused by loss of neurons. We confirmed that rat SCI was accompanied by an increased expression of miR-214-5p and an improved recovery of nerve tissue by blocking the effect of inhibitors. However, due to the limitations of this study, it was difficult to attribute the high expression of miR-214 in rat SCI only to specific targets. Nevertheless, we provided a new discovery, that miRNA in microglia could regulate inflammatory responses *in vitro* and *in vivo*. Furthermore, specific miRNA inhibitors could regulate the remodeling effect of nerve injury in the pathological environment after SCI.

Conclusions

We showed that the inhibition of miR-214-5p alleviated inflammatory chemotaxis and progression, inhibited apoptosis and upregulated nerve regeneration. Furthermore, it ultimately improved the locomotor function recovery in rats after SCI.

Conflict of interest

The authors declare no conflicts of interest.

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