European Review for Medical and Pharmacological Sciences 2018; 22: 6609-6615

MicroRNA-210 promotes spinal cord injury recovery by inhibiting inflammation via the JAK-STAT pathway

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Abstract. – OBJECTIVE: To investigate the effect of microRNA-210 on the spinal cord injury (SCI) and its underlying mechanism.

MATERIALS AND METHODS: The mouse SCI model was established. Mice were randomly assigned into 4 groups, namely the sham operation group (sham group), surgery group (SCI group), surgery+NC group (SCI+NC group) and surgery+microRNA-210 overexpression group (SCI+microRNA-210 mimics group). The mRNA levels of microRNA-210 and the key genes in the JAK-STAT pathway of the four groups were detected by Real-Time Polymerase Chain Reaction (RT-PCR) at different time points. Protein levels of JAK2 and STAT3 in mice of the four groups were detected by Western blot. To investigate the role of microRNA-210 in SCI recovery, changes in the motor function of mice were detected.

RESULTS: Grip strengths of right and left forelimbs in mice from the sham group were temporarily decreased at the early stage after surgery, which were gradually recovered to the preoperative levels on the 3rd postoperative day. However, mice in SCI group were unable to complete the grip strength determination at the early stage after surgery. Mice in SCI group were capable of grasping on the 7th postoperative day. Besides, grip strengths of mice in SCI group were remarkably lower than those of sham group until the end-point (on the 50th day). Furthermore, mRNA levels of microRNA-210 in mice of SCI group were decreased in a time-dependent manner ($p<0.05$). Higher grip strengths were observed in mice of SCI+microRNA-210 mimics group in comparison with those of SCI group and SCI+NC group ($p<0.05$). In addition, Western blot showed that protein levels of JAK2 and STAT3 in mice of SCI group were increased in a time-dependent manner ($p<0.05$). Moreover, protein levels of JAK2, STAT3, and MCP-1 in mice of SCI+NC group were remarkably higher than those in the sham group and SCI+microRNA-210 mimics group ($p<0.05$).

CONCLUSIONS: MicroRNA-210 is down-regulated in SCI mice. Grip strengths of SCI mice can be recovered after microRNA-210 overexpression via inhibiting inflammatory response by the JAK-STAT pathway.

Key Words:
Spinal cord injury, MicroRNA-210, JAK-STAT pathway, Inflammation.

Introduction

Spinal cord injury (SCI) is a damage to the spinal cord that has a change in its function, often caused by traffic accidents, sports injuries, falling, and violent injuries. In recent years, the incidence of SCI has been risen[1,2]. SCI can occur at any level of the spinal cord, leading to nerve dysfunction[3], loss of motor and sensory function, multiple organ damage, various complications, and even death. SCI not only results in tremendous physical and psychological trauma, but also brings a financial burden to patients and their families[4,5]. So far, there is no curable treatment for SCI. In-depth study of SCI mechanism is urgently needed for improving rehabilitation of SCI patients.

MicroRNAs (miRNAs) are a class of single-stranded molecules with 19-22 nucleotides in length, which are widely found in many species of plants and animals. Previous studies have shown that over 30% of human protein-coding genes are regulated by miRNAs[6]. MiRNAs are widespread in the nervous system[7], which are closely related to the development, differentiation, and proliferation of the nervous system[8-10]. Researches[11,12] have shown that some certain miRNAs could regulate occurrence and progression of degenerative diseases. So far, 97 differentially expressed miRNAs have been observed in SCI rats. Bioinformatics analysis showed that the target genes of these

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miRNAs may be greatly involved in the SCI pathophysiology[13].

Target genes of microRNA-210 are variously involved in angiogenesis, migration and adhesion, proliferation and differentiation, and tumor suppression[14-16]. They also participate in the development of multiple neurological diseases, including ischemia stroke[17], neurological tumors[18], and degenerative diseases[19]. This study aims to explore the role of microRNA-210 in SCI and its potential mechanism.

Materials and Methods

Cervical Contusion SCI

Experimental mice were obtained from SLAC Laboratory Animal Co, Ltd, (Shanghai, China). Procedures were approved by the Huaian First People's Hospital, Nanjing Medical University Ethics Committee. Briefly, mice were intraperitoneally anesthetized. The cervical dorsal skin was incised for exposure of C4-6, followed by unilateral laminectomy at C5. Mice in SCI group were subjected to the C5 spinal contusion injury using a tip. After surgical procedures, muscles and skin incisions were closed in layers.

Injection of MicroRNA-210 NC and MicroRNA-210 Mimics in the SCI Area

The animal model was conducted as previously described. After mice were subjected to spinal contusion injury, subdural injection of microRNA-210 NC and microRNA-210 mimics were subjected in mice from SCI+NC group and SCI+-microRNA-210 mimics group, respectively.

Griping Strength Meter (GSM)

Mice were gently held so that their tails were brought to the bar of GSM. Mice were then pulled back quickly in the horizontal direction when their paws grabbed in the bar. Forelimb griping strength was recorded when the grip was released. Grip strengths of left and right forelimbs were recorded, respectively. Four successful records, the average grip strength was calculated. The grip strength that mice could not grab in the bar was recorded as 0.

SCI Sample Collection

After mice were sacrificed, muscles and skin were cut open in layers, followed by unilateral laminectomy at C5. Spinal cord tissues were collected, extending 4 mm to the SCI area. Tissues were then placed in the 1.5 ml tube and preserved in a -80°C refrigerator for subsequent experiments.

RNA Extraction and Quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR)

50-100 g SCI tissues were selected for extraction of total RNA according to the instructions of the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was then performed based on the instructions of TaqMan MicroRNA kit (Thermo Fisher Scientific, Waltham, MA, USA). The relative concentration was calculated by the $2^{-\Delta\Delta CT}$ method using U6 as the loading control.

Western Blotting

The total protein was extracted by TRIzol reagent. Protein samples were then separated by 10% sodium dodecyl sulphate (SDS) protein electrophoresis after the concentration of each sample was adjusted to the same level. Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and routinely immunostained at 4°C overnight (diluted in 1:500). After washed 3 times with Tris-Buffered Saline-Tween (TBST), the membranes were incubated with the secondary antibody (l:1000) at room temperature for 1 h. All membranes were exposed by enhanced chemiluminescence (ECL) method.

Statistical Analysis

Statistical product and service solutions (SPSS16.0, SPSS Inc., Chicago, IL, USA) software was used for statistical analysis. Continuous variables were shown as mean ± standard deviation. The independent sample t-test was used to compare the data between two groups. $p<0.05$ indicated the difference was statistically significant.

Results

Behavior Features of SCI Mice

Grip strengths of right and left forelimbs in mice from sham group were temporarily decreased at the early stage after surgery, which were gradually recovered to the preoperative levels on the 3rd postoperative day. However, mice in SCI group were unable to complete the grip strength determination at the early stage after surgery. Mice in SCI group began to be capable of grasping on the 7th postoperative day. We observed decreased grip strengths of mice in SCI group

than those of sham group until the end-point (on the 50th day). The differences in grip strengths between the two groups were statistically significant (p<0.05, Figure 1A-C).

MicroRNA-210 Expression in Mice from SCI Group

The mRNA levels of microRNA-210, JAK2 and STAT3 in mice of SCI group and sham group were detected by RT-PCR. The results indicated that microRNA-210 expression in SCI group was remarkably decreased compared with that in sham group, which reached the lowest level on the 7th day (p<0.05, Figure 2A). However, expression levels of JAK2 and STAT3 were increased in a time-dependent manner, which reached peaks on the 10th day (Figure 2B, C). Our data suggested that the abnormally expressed microRNA-210, JAK2, and STAT3 may be involved in the SCI development. Previous studies have shown that JAK2-STAT3 is widespread in the central nervous system[20], which is capable of regulating astrocytic proliferation[21]. In this work, we detected protein expressions of JAK2 and STAT3 in mice of SCI group on the postoperative 1st, 5th, and 10th day by Western blot, respectively. The results showed that protein expressions of JAK2 and STAT3 were increased in a time-dependent manner (Figure 2D).

Effect of Overexpressed MicroRNA-210 on the JAK-STAT Pathway

To investigate the effect of microRNA-210 on SCI, mice were injected with microRNA-210 NC or microRNA-210 mimics, respectively. As shown in Figure 3A, decreased microRNA-210 expressions were observed in mice of SCI group and SCI+NC group compared with those of sham group on the 7th day. MicroRNA-210 expressions in mice of SCI+microRNA-210 mimics group were increased within the first 7 days. However, mRNA expressions of JAK2 and STAT3 in mice of SCI+microRNA-210 mimics group were remarkably lower than those of sham group within the first 7 days, which were upregulated in SCI group and SCI+NC group on the 3rd day (Figure 3B, C). Western blot results showed that protein expressions of JAK2 and STAT3 in mice of sham group were remarkably lower than those in SCI+microRNA-210 mimic group and SCI+NC group (p<0.05). Scholars[22,23] have shown that monocyte chemoattractant protein-1 (MCP-1) is involved in the SCI-induced secondary inflammation by activating JAK2-STAT3 pathway. Our results demonstrated that lower MCP-1 expression was found in SCI+microRNA-210 mimics group in comparison with that of sham group and SCI+NC group, which was consistent with expression changes of JAK2 and STAT3 (Figure 3D).

Effect of MicroRNA-210 on Repair and Regeneration of SCI Mice

Improved grip strengths of right and left forelimbs were observed in mice of SCI+microRNA-210 mimics group than those of SCI group and SCI+NC group (p<0.05). However, there was no significance in improvement of grip strength between SCI group and SCI+NC group (p>0.05, Figure 4A-C), indicating that overexpressed microRNA-210 could promote SCI recovery.

Discussion

Spinal cord injury (SCI) can be divided into primary spinal cord injury (PSCI) and secondary spinal cord injury (SSCI). PSCI is an irreversible process that can directly lead to neuronal synaptic damage, dysfunctions of sensory, motor and autonomic nerves. Studies have found that SSCI can

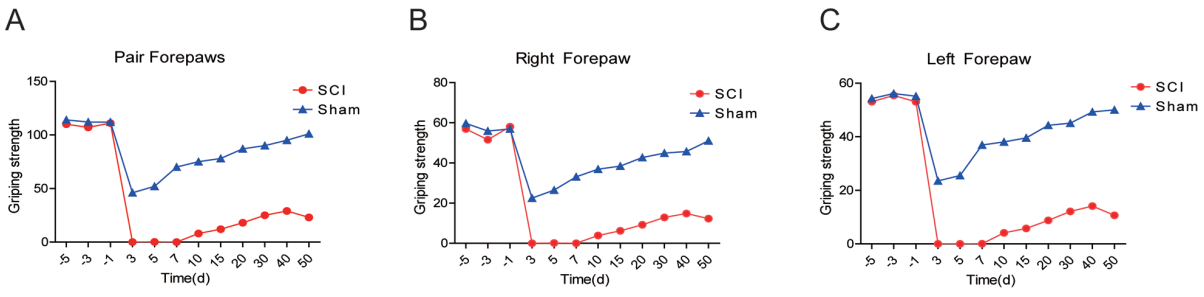

Figure 1. Behavior features of SCI mice. ***A-C***, Grip strength in each group (p<0.05).

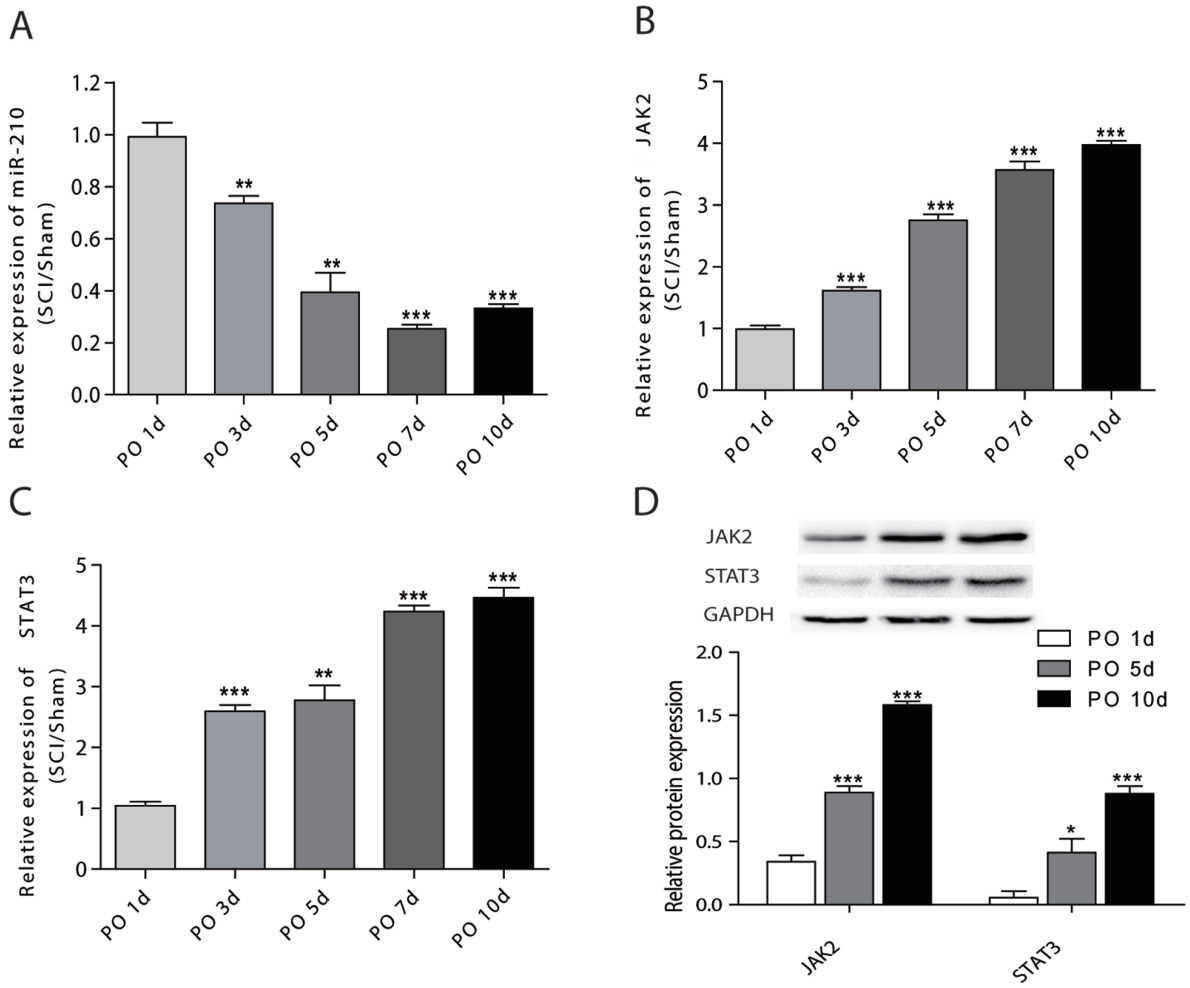

Figure 2. Expressions of microRNA-210 and JAK-STAT pathway in SCI group. ***A,*** MicroRNA-210 expression in SCI group. ***B,*** and ***C,*** The mRNA levels of JAK2 and STAT3 in SCI group were increased in a time-dependent manner. ***D,*** The protein levels of JAK2 and STAT3 in SCI group were increased in a time-dependent manner.

aggravate neuronal degeneration, necrosis, and apoptosis process. Although SSCI can expand the injury scope and damage the remaining neurons, it is a reversible and controllable process[24,25]. Inflammation may be a major mechanism of SSCI[26]. The activation of the local glial cells, infiltration of monocytes/macrophages and recruitment of chemotactic cytokines to peripheral blood cells all participate in the inflammatory response of SSCI.

Some works have shown that MCP-1 is a chemokine involved in the secondary inflammatory response by activating macrophages[22]. JAK2-STAT3, an important signaling pathway of JAK-STAT family, can participate in multiple biological processes under activation of inflammatory responses[23,27-29]. Previous studies have reported that cerebral ischemia can activate JAK-STAT pathway. Subsequently, phosphorylated STAT3 is transferred into the nucleus, thus regulating a large number of gene expressions and directly participating in neuronal apoptosis[30]. JAK2-STAT3 pathway also mediates apoptosis of cerebral cortical neurons[31]. Moreover, JAK2-STAT3 pathway is activated in many solid tumors and hematological malignancies[32]. STAT3 exerts a crucial role in regulating astrocyte reactivity during the motor function recovery and tissue repair after SCI[33-36].

In this report, we constructed the mouse SCI model to analyze the motor dysfunction induced by SCI. Our data suggested that grip strengths of right and left forelimbs in mice from sham group were temporarily decreased at the early stage after surgery, which were gradually recovered to the preoperative levels on the 3rd day after surgery.

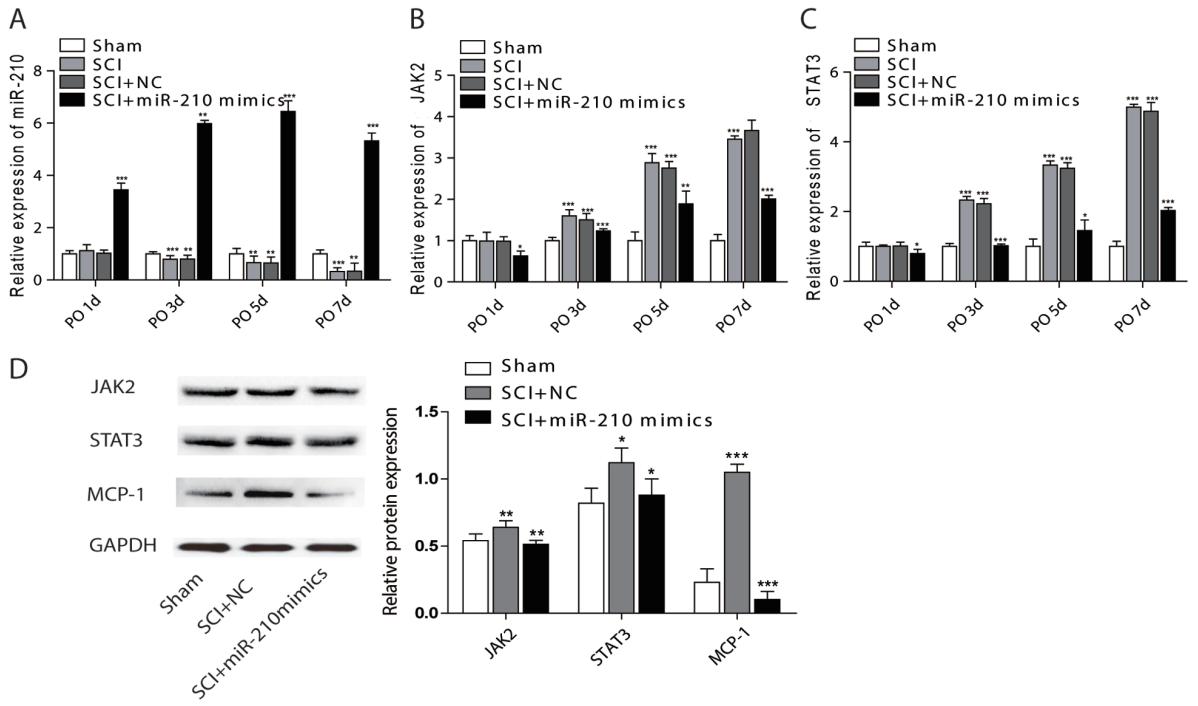

Figure 3. Effect of overexpressed microRNA-210on JAK-STAT pathway. ***A,*** MicroRNA-210 expressions in SCI area. ***B,*** and ***C,*** Effects of overexpressed microRNA-210 in SCI area on mRNA levels of JAK2 and STAT3. ***D,*** Effects of overexpressed microRNA-210 in SCI area on protein levels of JAK2, STAT3 and MCP-1.

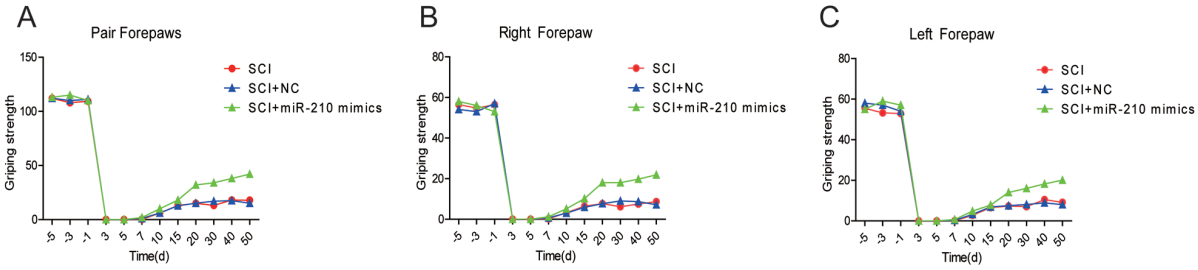

Figure 4. Effect of microRNA-210 on repair and regeneration of SCI. ***A-C***, Grip strength after overexpression of microRNA-210.

However, mice in SCI group were unable to complete the grip strength determination at the early stage after surgery. Mice in SCI group began to grasp the bar on the 7th postoperative day. The grip strengths of mice in SCI group were always lower than those of sham group until the endpoint. A large number of researches have pointed out that microRNA-210 is involved in many neuronal diseases. In the present work, lower microRNA-210 expression was found in SCI group than that of sham group, indicating that microRNA-210 could exert a certain effect on SCI. Therefore, we then randomly assigned mice into SCI group, SCI+NC group and SCI+microRNA-210 mimics group. Our results elucidated that mouse grip strength in SCI+microRNA-210 mimics group was remarkably improved than that of SCI group and SCI+NC group ($p<0.05$). However, no significant difference in grip strength was found between SCI group and SCI+NC group ($p>0.05$), indicating that overexpressed microRNA-210 could protect SCI. Subsequently, the specific role of microRNA-210 in protecting SCI-induced motor dysfunction was analyzed by detecting key genes in JAK-STAT pathway. We found that expressions of JAK2 and STAT3 in SCI group were increased in a time-dependent manner. Moreover, lower expressions of JAK2, STAT3, and MCP-1

were found in SCI+microRNA-210 mimics group than those of sham group and SCI+NC group. It is suggested that microRNA-210 promotes SCI recovery by inhibiting inflammatory response via the JAK-STAT pathway.

Conclusions

We found that overexpressed microRNA-210 can promote SCI recovery via inhibiting inflammatory response by the JAK-STAT pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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