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Original Article

MiR-146a downregulation improves motor function of mice with spinal cord injury by activating IL-6/STAT3 signaling pathway

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Abstract: Objective: To investigate the mechanism of microRNA (miR)-146a inhibition in improving the motor function of mice with spinal cord injury. Methods: SPF-class C57B16 mice (n=40) were randomly divided into sham operation group (sham group), spinal cord injury group (SCI group), spinal cord injury + miR-146a inhibitor group (SCI + antagonist group), miR-146a inhibitor group (antagonist group), with 10 mice in each group. On the 1st, 3rd, and 6th day after modeling, the motor function scores (BBB locomotor scores) of mice were observed and the level of miR-146a and STAT3 expressions as well as IL-6 content in the spinal cord tissues were detected. Results: Compared with the sham group, the BBB locomotor scores of the SCI group and the SCI + antagonist group were significantly lower, but the expression levels of miR-146a and STAT-3 as well as IL-6 content were higher on the 1st, 3rd, and 6th day after modeling (P<0.05), while there was no obviously difference in these parameters between the SCI + antagonist group and the SCI group on the 1st and 3rd day after modeling (P>0.05). On the 6th day after modeling, the SCI + antagonist group had higher BBB locomotor score, and lower miR-146a and STAT-3 expression levels as well as IL-6 content than the SCI group (P<0.05). Conclusion: miR-146a downregulation can improve the motor function of mice with spinal cord injury via inhibiting IL-6/STAT3 signaling pathway.

Keywords: miR-146a inhibitor, IL-6/STAT3, spinal cord injury, motor function, antagonist

Introduction

Spinal cord injury is caused by external or internal factors, of which the common types include spinal cord concussion or shock, longitudinal spinal cord injury and transverse spinal cord injury [1, 2]. The function of the lower limbs of the affected side is severely impaired in patients with spinal cord injury, which affects their motor and physiological functions [3, 4]. In clinic, spinal cord injury is often treated with drugs, including corticosteroids, scopolamine, gangliosides, vitamin B12 and other neurotrophic drugs [5]. Krstačić A et al. found that corticosteroids have a better effect on acute spinal cord injury, but it is ineffective for chronic spinal cord injury. Moreover, corticosteroids have lots of side effects, which hinders its clinical use [6, 7].

MicroRNAs, as post-transcriptional regulatory elements, are involved in all aspects of biological transcription and regulate the development and evolution of organisms [8, 9]. Studies have found that the expression of miR-146a is positively correlated with the severity of spinal cord injury and is closely related to inflammatory response [10, 11]. In fibroblasts, miR-146a inhibits gene expression of interleukin-6 (IL-6) and interleukin-8, and decreases their secretion and release [12, 13]. IL-6 is a pleiotropic cytokine that regulates the proliferation and differentiation of a variety of cells, and regulates the expression of proteins in the acute phase of spinal cord injury. At present, studies have reported that IL-6 could promote neuronal cell proliferation and recovery of mice with spinal cord injury [14]. After spinal cord injury, IL-6 phosphorylates STAT3, then the phosphorylated STAT3 translocates into nucleus to affect the expression of relate genes related to inflammation, cell proliferation, and acute phase response [15]. The activated STAT3 generally occurs within 1-2 hours after recovery and lasts for 4-6 hours.

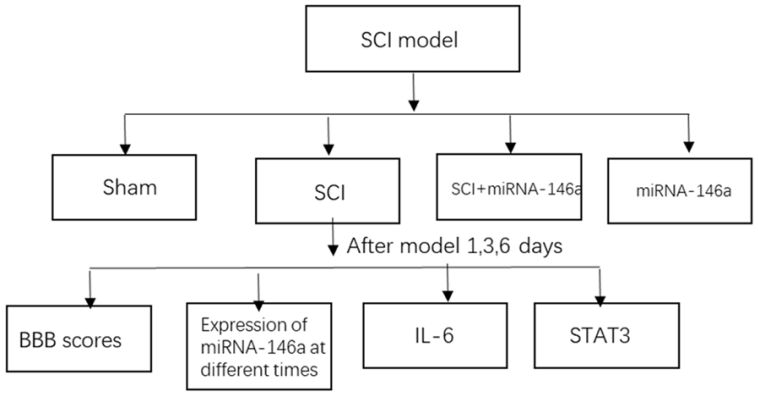

Figure 1. Experimental design flow chart. SCI + miRNA-146a, Spinal cord injury + miR-146a antagonist; miRNA-146a, miR-146a inhibitor group. Sham: sham operation group; SCI: spinal cord injury group; BBB score: Basso, Beattie & Bresnahan locomotor rating scale score; miRNA: microRNA; IL-6: interleukin-6; STAT3: signal transducing activator of transcription.

Increasing studies have discovery that miR-146a plays a role in the regulation of genes involved in the inflammatory pathway and is therefore closely related to the body's inflammatory response. However, the role of miR-146a in the regulation of spinal cord injury has not been determined. Therefore, our study aimed to investigate the effect of miR-146a on the function of mice with spinal cord injury and its possible mechanisms, with the hope to provide a reliable basis for the treatment of patients with spinal cord injury.

Materials and methods

Animals and grouping

SPF-class C57BL6 mice (7 weeks, weight between 18-21 g) were used to establish the spinal cord injury model and 20 successfully modeled mice were randomly divided into spinal cord injury group (SCI group), spinal cord injury + miR-146a inhibitor group (SCI + antagonist group), with 10 mice in each group. In addition, another 10 SPF-class C57Bl6 underwent sham operation (sham group) and 10 healthy mice were selected as miR-146a inhibitor group (antagonist group). Before modeling, the SCI + antagonist group and the antagonist group intrathecally administrated with miR-146a inhibitor (purchased from Beijing Ruibo Biotechnology Co., Ltd.), and the SCI group and sham group intrathecally received the same dose of normal saline [16]. The present study was approved by the Renmin Hospital of Wuhan University.

The motor function scores (Basso, Beattie & Bresnahan locomotor rating scale, BBB locomotor score) of the four groups of mice were observed on 1st, 3rd, and 6th day after modeling. C57BL6 mice were anesthetized by intraperitoneal injection of 1% pentobarbital sodium (50 mg/kg), and eyeballs were picked to take blood for the detection of IL-6 by ELISA. After the mice died of bleeding, the spinal cord tissues were collected from 10 mice from the four groups, respectively, to detect miR-146a level by qPCR, STAT3 protein expression level by Western Blot. The experiment design is shown in **Figure 1**.

Spinal cord injury modeling

C57BL6 mice were intraperitoneally injected with 1% sodium pentobarbital (50 mg/kg) for anesthesia, and no peritonitis was found. After the anesthesia was completed, the mice were placed at prone position on the operating table, and the back hair of the mice was shaved. After disinfection with alcohol, the skin was incised and the spinous process and lamina on T8 segment were separated to expose the spinal cord. Subsequently, the spinal cord was clamped with tweezers for 20 seconds. The spinal cord of mice in the sham operation group wasn't clamped. Later, muscle and skin were sutured layer by layer and mice were placed in a 37°C environment to keep warm until their vital signs recovered to the normal level. Finally, the mice were placed back to the squirrel cage [5].

Criteria for successful modeling are as follows: When knocking on the exposed spinal cord by a hammer, the bilateral hind limbs of mice twitched and the tail of mice also twitched around and then the hind limbs completely relaxed after a few seconds. It can be seen that there are congestion and swelling in the spinal cord, which indicates a successful model. The success rate of modeling is 80%.

Motor function evaluation

The motor function of mice after the modeling was evaluated by Basso, Beattie & Bresnahan

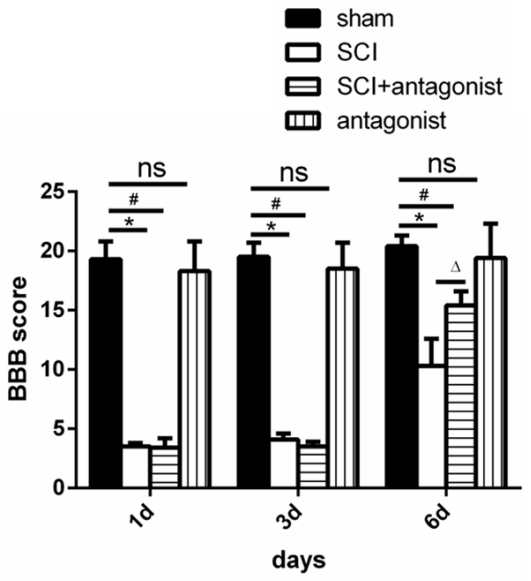

Figure 2. Motor function evaluation. SCI + antagonist, Spinal cord injury + miR-146a antagonist; antagonist, miR-146a inhibitor group. *P<0.05, sham vs. SCI; #P<0.05, sham vs. SCI + antagonist; ΔP<0.05, SCI vs. SCI + antagonist. Sham: sham operation group; SCI: spinal cord injury group; BBB score: Basso, Beattie & Bresnahan locomotor rating scale score; ns: no statistical difference; Days: days after modeling; miRNA: microRNA.

locomotor rating scale (BBB locomotor score) to determine whether the animal's hip, knee, ankle joint and trunk movements are coordinated, which was proposed by Basso et al. in 1995. 0 points represent no visible hindlimb movement; 21 points represent motor coordination, and the trunk is stable. Low BBB locomotor score indicates severe the spinal cord injury [17].

Enzyme-linked immunosorbent assay (ELISA)

The collected 1 ml venous blood was centrifuged (3,000 rpm/min, 10 min) to obtain serum, and the level of inflammatory factors IL-6 in each group of samples was examined according to the instructions of the kit which was purchased from Xitang Biotechnology Co., Ltd.

Western Blot

The protein was extracted from the spinal cord tissue by lysis after trituration. The protein concentration was determined by BCA method. Protein sample with a volume of 40 μg was loaded and separated by SDS-PAGE electrophoresis, and then transferred to nitrocellulose membrane and blocked by skimmed milk at room temperature for 1 h. Primary antibodies were added (STAT-3, 1:600, Abcam; β-Actin, 1:1,000, Abcam) onto the membrane followed by incubation at 4°C overnight. Subsequently, the membranes were washed by TBST for 3 times and then the membranes were incubated with goat anti-rabbit secondary for 1 h at room temperature followed by washing with TBST for 3 times. Finally, the membrane were developed by Ultra-sensitive ECL luminescent solution and X-ray. Image J software was used to analyze the gray scale of each strip.

Statistical analysis

All data were analyzed using SPSS 19.0 software and GraphPad Prism 6.01 software. Measurement data were expressed as mean ± sd and all measurement data were in normal distribution and with homogeneity of variance, and one-way ANOVA followed by post hoc LSD tests was used for comparison between groups. P<0.05 was considered to be statistically significant.

Results

Motor function evaluation

Compared with the sham group, the BBB locomotor scores of the SCI group and the SCI + antagonist group were significantly lower on the 1st, 3rd, 6th day after modeling (P<0.05). There was no significant difference in the BBB locomotor scores between the SCI + antagonist group and the SCI group on the 1st and 3rd day after modeling (P>0.05). On the 6th day after modeling, the SCI + antagonist group had higher BBB locomotor score than the SCI group (P<0.05). There was no difference in BBB locomotor score between the antagonistic group and the sham group at any time points (P>0.05). See **Figure 2**.

Evaluation of miRNA-146a expression level in the four groups of mice

Compared with sham group, the expression of miR-146a in SCI group and SCI + antagonist group was significantly higher in the SCI group (P<0.05). There was no significant difference in miR-146a expression level between the SCI + antagonist group and the SCI group on 1st and

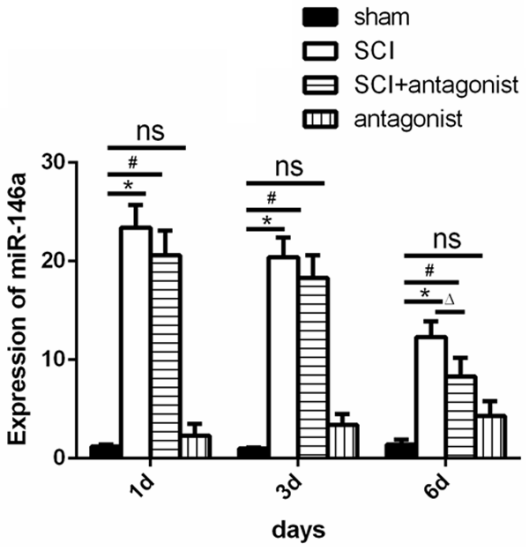

Figure 3. Evaluation of miRNA-146a expression level in the four groups of mice. SCI + antagonist, Spinal cord injury + miR-146a antagonist; antagonist, miR-146a inhibitor group. *P<0.05, sham vs. SCI; #P<0.05, sham vs. SCI + antagonist; ΔP<0.05, SCI vs. SCI + antagonist. Sham: sham operation group; SCI: spinal cord injury group; ns: no statistical difference; Days: days after modeling; miRNA: microRNA.

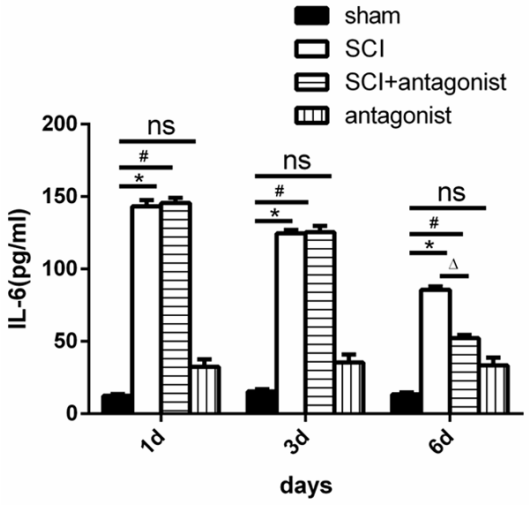

Figure 4. Evaluation of IL-6 expression level in the four groups of mice. SCI + antagonist, Spinal cord injury + miR-146a antagonist; antagonist, miR-146a inhibitor group. *P<0.05, sham vs. SCI; #P<0.05, sham vs. SCI + antagonist; ΔP<0.05, SCI vs. SCI + antagonist. Sham: sham operation group; SCI: spinal cord injury group; ns: no statistical difference; Days: days after modeling; miRNA: microRNA; IL-6: interleukin-6.

3rd day after modeling (P>0.05), while on the 6th day after modeling, the SCI + antagonist group had lower miR-146a expression level than the SCI group (P<0.05). There was no difference in miR-146a expression level between the antagonistic group and the sham group at any time points (P>0.05). See **Figure 3**.

Evaluation of IL-6 expression level in the four groups of mice

Compared with sham group, the content of IL-6 in SCI group and SCI + antagonist group was significantly higher in the SCI group (P<0.05). There was no significant difference in IL-6 content between the SCI + antagonist group and the SCI group on 1st and 3rd day after modeling (P>0.05), while on the 6th day after modeling, the SCI + antagonist group had lower IL-6 content than the SCI group (P<0.05). There was no difference in IL-6 content between the antagonistic group and the sham group at any time points (P>0.05). See **Figure 4**.

Evaluation of STAT-3 protein level in the four groups of mice

Compared with sham group, the expression of STAT-3 protein in SCI group and SCI + antagonist group was significantly higher after modeling (P<0.05). There was no significant difference in STAT-3 protein level between the SCI + antagonist group and the SCI group on 1st and 3rd day after modeling (P>0.05), while on the 6th day after modeling, the SCI + antagonist group had lower STAT-3 protein level than the SCI group (P<0.05). There was no difference in STAT-3 protein level between the antagonistic group and the sham group at any time points (P>0.05). See **Figure 5**.

Discussion

MiR-146a, as a promoter of spinal cord injury, aggravates motor disorders and inflammatory responses after damage [18]. miR-146a inhibition attenuates the pro-inflammatory and injurious effects of miR-146a [19]. Xi Y et al. have confirmed that miR-146a downregulation plays a critical role in the recovery of intervertebral disc degeneration and spinal cord injury [20]. Our study found that compared with the mice administrated with normal saline, the motor function was evidently improved in mice treated with the miR-146a inhibitor. The possible reason might be that the miR-146a inhibitor binds to its receptor and competitively inhibits the miR-146a expression, thereby the

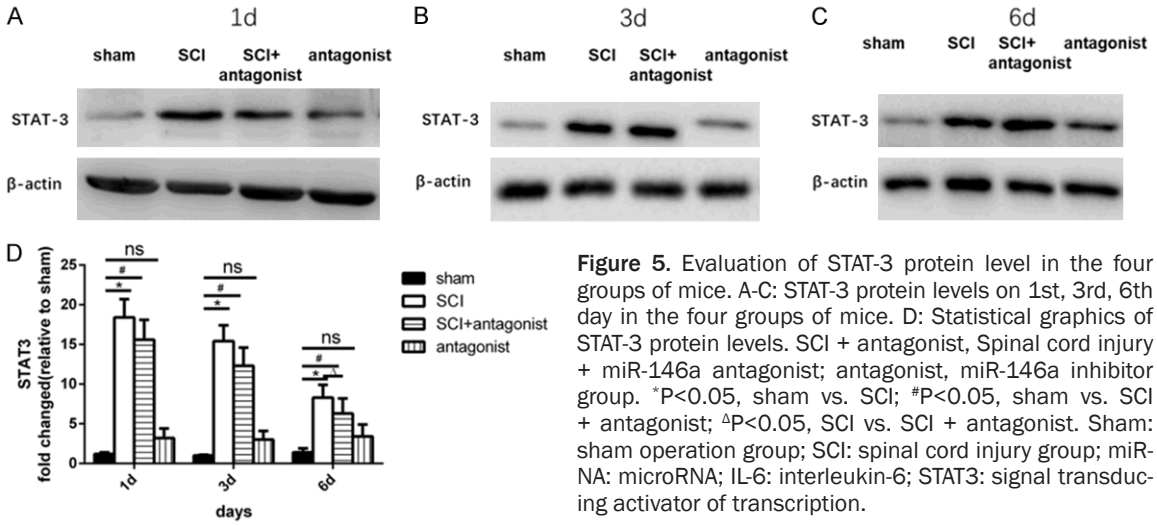

Figure 5. Evaluation of STAT-3 protein level in the four groups of mice. A-C: STAT-3 protein levels on 1st, 3rd, 6th day in the four groups of mice. D: Statistical graphics of STAT-3 protein levels. SCI + antagonist, Spinal cord injury + miR-146a antagonist; antagonist, miR-146a inhibitor group. *P<0.05, sham vs. SCI; #P<0.05, sham vs. SCI + antagonist; ΔP<0.05, SCI vs. SCI + antagonist. Sham: sham operation group; SCI: spinal cord injury group; miRNA: microRNA; IL-6: interleukin-6; STAT3: signal transducing activator of transcription.

vicious circle produced after spinal cord injury is inhibited.

He Y et al. reported that miR-146a had a time-regulated expression after spinal cord injury: miR-146a was significantly up-regulated on day 1 after spinal cord injury, and its expression remains at high level on day 3 until day 7 [21]. Our study also found that after spinal cord injury, miR-146a expression levels of mice kept increasing on the 1st, 3rd, and 6th day after spinal cord injury compared with sham group. However, the expression of miR-146a in mice treated with miR-146a inhibitor decreased on day 6 after spinal cord injury, which is consistent with the reports of previous studies, indicting the successful establishment of spinal cord injury model.

The immune response that occurs after spinal cord injury involves various signal pathways and signal molecules [22, 23]. Jaerve et al. found that after spinal cord injury, a variety of pro-inflammatory and chemokines were generated, which ultimately led to destructive damages such as nerve fiber demyelination, and necrosis and apoptosis of nerve cells [24]. The results of our study showed that IL-6 secretion increased obviously after spinal cord injury, and on the 6th after modeling, the IL-6 secretion was significantly lower in mice treated with miR-146a inhibitor than that in mice treated with normal saline, indicating that the inhibition of miR-146a may restore motor function and reducing the secretion and release of inflammatory factors.

STAT3 is involved in early embryonic development, bone marrow cell differentiation as well as the regulation of cell differentiation, proliferation, invasion, etc. [25]. The report from Park et al. proved that JAK2/STAT3 signaling pathway plays a role in the regulation of apoptosis in acute spinal cord injury in rats [26]. Our study found that the expression of STAT3 in mice with spinal cord injury was higher compared to the controls, possibly due to that the damage of spinal cord cells promotes the gene transcription of STAT-3, which induces the differentiation of spinal cord cells into spinal glial cells and parenchyma cells to protect organisms. Moreover, the administration of the miR-146a inhibitor could decrease the STAT3 transcription, so as to protect the spinal cord parenchyma cells and injured spinal cord.

The IL-6/STAT3 signaling pathway is participated in various physiological processes related to cancer, inflammation, and cell differentiation, etc. [27, 28], which are also involved in the process of spinal cord injury. In our study, mice with spinal cord injury were pre-administered with miR-146a inhibitor, which significantly inhibited the secretion of IL-6 and expression of STAT3. Therefore, miR-146a inhibitors may be involved in spinal cord injury recovery via the IL-6/STAT3 signaling pathway.

However, this experiment only investigated the effect of miR-146a inhibitor on spinal cord injury recovery by inhibiting the expression of miR-146a by its inhibitor, rather than from the genetic level. Therefore, we will knock out the

miR-146a gene in the future to observe the changes of IL-6 secretion and STAT3 expression. Meanwhile, we will study that whether acute spinal cord injury could be reversed by using miR-146a, IL-6 or STAT3 as specific target, or not, which may provide an important reference for clinical intervention of acute spinal cord injury.

In conclusion, miR-146a inhibitor may improve the motor function in mice with spinal cord injury by regulating IL-6/STAT3 signaling pathway, which provides a reliable basis for clinical treatment of spinal cord injury.

Disclosure of conflict of interest

None.

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