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Let-7 participates in the regulation of inflammatory response in spinal cord injury through PI3K/Akt signaling pathway

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Abstract. – OBJECTIVE: To study the potential mechanism of let-7 in participating in the regulation of inflammatory response in spinal cord injury (SCI).

MATERIALS AND METHODS: A total of 40 male Sprague-Dawley rats were randomly divided into four groups: group A (Sham, n=10), group B (SCI+NC, n=10), group C (SCI+antagomir, n=10), and group D (SCI+mimics, n=10). The SCI model was established *via* operation in all groups. After successful modeling, let-7-antagomir negative control (80 mg/kg) was intraperitoneally injected in SCI+NC group at 5 d, an equal amount of let-7-antagomir was intraperitoneally injected in SCI+antagomir group at 5 d, and an equal amount of let-7-mimics was intraperitoneally injected in SCI+mimics group at 5 d. The inflammatory cells in experimental groups and control group were observed *via* hematoxylin-eosin (HE) staining. At the same time, the expression of let-7 in the four groups was detected *via* Reverse Transcription-Polymerase Chain Reaction (RT-PCR), the expressions of phosphatidylinositol 3-hydroxy kinase (PI3K) and protein kinase B (Akt) in all groups were detected *via* Western blotting, and the inflammatory index levels in each group were detected *via* enzyme-linked immunosorbent assay (ELISA).

RESULTS: In Sham group, it was observed *via* HE staining that there were only a few bleeding or inflammatory cells. In SCI+NC group, bleeding and inflammatory cells basically tended to be stable. There were a large number of inflammatory cells in SCI+mimics group, while there were some inflammatory cells in SCI+antagomir group, but showing a decreasing trend compared with SCI+NC group. It was found in the RT-PCR detection of let-7 expression level in all groups that the expression of let-7 significantly declined in SCI+antangomir group compared with that in Sham group and SCI+NC group, and there were significant differences ($p<0.01$). The expression of let-7 was significantly increased in SCI+mimics group compared with that in Sham group and SCI+NC group, and there were significant differences ($p<0.01$). The results of Western blotting revealed that the PI3K and Akt protein expressions were significantly decreased in SCI+mimics group compared with those in SCI+antagomir group, SCI+NC group, and Sham group ($p<0.05$). The ELISA results showed that the levels of inflammatory factors in SCI+mimics group, SCI+antagomir group, and SCI+NC group were significantly higher than those in Sham group. In SCI+mimics group, the levels of inflammatory factors were abnormally high and reached extremely significant levels ($p<0.05$), indicating that let-7 promotes the inflammatory response after SCI.

CONCLUSIONS: Let-7 participates in the regulation of inflammatory response in SCI through the PI3K/Akt signaling pathway.

Key Words:
let-7, PI3K/Akt signaling pathway, Inflammatory response in SCI.

Introduction

Spinal cord injury (SCI) refers to the injury caused by external violence against the spinal cord directly or indirectly. SCI patients suffer great pain in exercise[1], significantly reducing the quality of life and bringing a heavy burden to family members and society[2]. Although greater progress has been made in the understanding of pathophysiological changes after SCI than before, the improvement in neurological function remains a challenge. Currently, the therapeutic methods for SCI patients mainly include drug therapy, operative treatment, and transplantation, the goal of which is to reduce secondary damage and promote nerve repair and regeneration after SCI[3-5]. Increasingly more studies have found that the immuno-inflammatory response after SCI plays an important role in SCI and recovery after injury. At present, some scholars[6-8] have proved the role of immuno-inflammatory response in SCI and its repair process through the animal experiments, and some studies have proposed the hypothesis about the intervention in the immuno-inflammatory response to promote SCI recovery.

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The phosphatidylinositol 3-hydroxy kinase/protein kinase B (PI3K/Akt) signaling pathway has a response to extracellular signals, growth factors, cellular energy state, and cell growth, proliferation, survival, and differentiation in SCI. This pathway plays a key role in neurophysiological and neuropathological processes[9]. For example, Liu et al[10] argued that thymoquinone can improve cardiovascular function and inhibit inflammation, oxidative stress, and apoptosis in diabetic rats through the PI3K/Akt pathway. Chen et al[11] proved that the activation of the PI3K/Akt signaling pathway is closely related to the anti-inflammatory effect on SCI patients.

Micro-ribonucleic acids (miRNAs) are endogenous single-stranded non-coding RNAs consisting of 21-24 bases, whose main role is to participate in the regulation of post-transcriptional gene expression, thereby affecting cell proliferation, differentiation, and apoptosis. Let-7, one of the small RNAs first found in *Caenorhabditis elegans*, exerts a variety of biological effects and displays a strong evolutionary protective function from nematodes to humans. Latest studies[12,13] have found that the let-7 cluster plays a key role in regulating such inflammatory responses as the production of B cell antibody and macrophage reaction. The role of let-7 cluster in the regulation of the immune system has been proved by increasingly more evidence, so studying how let-7 participates in the regulation of inflammatory response in SCI and further clarifying the important role of this miRNA in immuno-inflammatory response in SCI will help guide the future pathophysiological research, thereby providing better therapeutic methods and ideas for clinical treatment of SCI patients.

Materials and Methods

Animals and Treatment

A total of 40 healthy adult female Sprague-Dawley rats aged 8 weeks old and weighing 180-220 g were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China), and fed under the room temperature of (24±1)°C, appropriate humidity and 12/12 h light/dark cycle. All rats were randomly divided into four groups: group A (Sham), group B (SCI+NC), group C (SCI+antagomir), and group D (SCI+mimics). To avoid confusion, the rats in each group were fed in separate cages and had free access to water and food. After successful modeling, let-7-antagomir negative control (80 mg/kg) was intraperitoneally injected in SCI+NC group at 5 d, an equal amount of let-7-antagomir was intraperitoneally injected in SCI+antagomir group at 5 d, and an equal amount of let-7-mimics was intraperitoneally injected in SCI+mimics group at 5 d. All rats were executed at 7 d after modeling. This study was approved by the Animal Ethics Committee of Wuhan University Animal Center.

Establishment of Rat Model of SCI

In group A, the vertebral plate was resected without damaging the spinal cord. In group B, C and D, all rats were injected with amobarbital sodium (300 mg/kg) (Xinyu Biological Technology Co., Ltd., Shanghai, China) for anesthesia, and placed on a sterile operating table, followed by skin preparation and draping. The dorsal skin was cut along the spine, and the spinous process and vertebral plate of the T10 segment were dissociated and exposed. Under a microscope, the spinous process and vertebral plate of the T10 segment were removed using rongeur forceps, and the spinal cord was fully exposed and clamped using 0.3 mm-wide forceps for 15 s. After SCI, muscles, and skin were sutured layer by layer, the wound was disinfected and bandaged, and the urine was artificially discharged twice a day after the operation. The above establishment method was based on that described by Plemel et al[14].

HE Staining

All rats were executed at 7 d after modeling. The spinal cord tissues of the T10 segment were taken, fixed with 10% formaldehyde (Sembaiga Biological Technology Co., Ltd., Nanjing, China), decalcified with 9% formic acid and embedded in paraffin. After standardized treatment, the tissues were sliced into 5 μm-thick sections, deparaffinized and stained with Harris hematoxylin and 0.5% eosin (Sembaiga Biological Technology Co., Ltd., Nanjing, China) for HE staining, and the staining results were observed under the microscope. Whether there were significant differences in inflammatory cells under the microscope was compared among groups.

Real Time-Polymerase Chain Reaction (RT-PCR) Analysis

At 3 d and 7 d after modeling, 3 rats were randomly executed in each group, and the total RNA was extracted from the tissues. Whether there was differential expression of let-7 among groups was detected *via* RT-PCR. The total RNA

was reversely transcribed by TRIzol (Sangon Biotech, Shanghai, China) using the NanoDrop 2000 device (Thermo Fisher Scientific, Waltham, MA, USA), and the complementary deoxyribose nucleic acid (cDNA) samples were obtained using the TaKaRa RNA PCR kit (TaKaRa, Dalian, China) and Oligo dT primers (Invitrogen, Carlsbad, CA, USA). The let-7 expression level was detected using the SYBR mixture (TaKaRa, Dalian, China) on the LightCycler 480 device (Roche, Basel, Switzerland). Each sample was measured for 3 times. The reaction conditions are as follows: at 95°C for 5 min for 1 cycle, a total of 40 cycles (95°C for 30 s, 60°C for 30 s, and 72°C for 30 s). The primer design and synthesis are shown in Table I. The let-7 expression level was normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and data were analyzed using the $2^{-\Delta\Delta CT}$ method.

Western Blotting Analysis

The spinal cord tissue extracts were lysed homogeneously using lysis buffer (Beyotime, Shanghai, China) *via* radioimmunoprecipitation at 4°C for 30 min, followed by centrifugation at 12000 rpm and 4°C for 5 min. The protein content was detected using the protein assay kit (Bio-Rad Hercules, CA, USA). The protein (50 μg/sample) was added into 12% polyacrylamide gel, separated *via* Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), and transferred from the gel to the nitrocellulose membrane. The membrane was sealed with 1% bovine serum albumin (BSA) dissolved in Tris Buffered Saline-Tween (TBST) at room temperature for 1 h and incubated with antibodies, including PI3K antibody (Cat. No.: SC-7175, diluted at 1:1000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Akt antibody (Cat. No.: SC-8312, diluted at 1:500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and GAPDH antibody (Cat. No.: SC-25778, diluted at 1:2000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), at room temperature for 1 h or at 4°C overnight. After the membrane was washed with TBST for 3 times, it was incubated again with the HRP-labeled goat anti-mouse IgG (Cat. No.: SC-2005, diluted at 1:1000, Santa Cruz, Santa Cruz, CA, USA), followed by detection using the EasyBlot ECL kit (Cat. No.: C506668, Sangon Biotech, Shanghai, China) and visualization using the enhanced chemiluminescence system Image Lab 3.0 (Bio-Rad, Hercules, CA, USA). The experiment was repeated for 3 times.

Detection of Inflammatory Factors Via Enzyme-Linked Immunosorbent Assay (ELISA)

Interleukin-1β, IL-6, and tumor necrosis factor-α (TNF-α) were detected using ELISA kits (Bonyi Biotechnology Co., Ltd., Shanghai, China). The coating antigen was diluted with the coating buffer CBS till 2×10^8 CFU, added into each well of the plate (50 μL/well) and placed in a wet box at room temperature or 4°C or 37°C overnight. Then, the coating buffer was patted dry, and the plate was fully washed with 1×PBST (Phosphate-Buffered Saline and Tween) for 3 times (3-5 min/time) and sealed with 250 μL 1% BSA at 37°C for 2 h. After the plate was washed again with 1×PBST for 3 times (3-5 min/time), the serum to be detected was diluted (1:100) with 1×PBST (50 μL/well), followed by incubation at 37°C for 1.5 h. The negative control was set up. After the plate was washed as above, the horseradish peroxidase (HRP)-labeled IgG secondary antibody (diluted at 1:500 with 1×PBST, the dilution ratio was different according to the product and could be based on the instructions) was added into each well for incubation at 37°C for 1.5 h. After the plate was washed as above, 50 μL OPD was added into each well, and the color was developed in a dark box at room temperature for 10 min. After that, 50 μL stop buffer (2M H_2SO_4) was added into each well to terminate the reaction. The optical density (OD) value was read at 492 nm using a microplate reader.

Table I. Primer sequences used for the RT-PCR.

Gene	Primer sequence
let-7-F	ACACTCCAGCTGGGAGGCGGGGCGCCGCGGGA
let-7-R	CTCAACTGGTGTCGTGGA
U6-F	CTCGCTTCGGCAGCACA
U6-R	AACGCTTCACGAATTTGCGT

Statistical Analysis

All data in this experiment were expressed as mean ± standard error of mean (Mean ± SEM), and the experimental results were statistically analyzed using Statistical Product and Service Solutions (SPSS) 21.0 software (IBM, Armonk, NY, USA). The *t*-test was used for the mean comparison between the two groups, and one-way analysis of variance was used for the sample comparison among groups. $p<0.05$ suggested that the difference was statistically significant.

Results

HE Staining

The spinal cord samples of rats were taken for HE staining (Figure 1A). In Sham group, it was observed *via* HE staining that there were only a few bleeding or inflammatory cells. In SCI+NC group, bleeding and inflammatory cells basically tended to be stable and recovered. There were a large number of inflammatory cells in SCI+mimics group, indicating that the inflammatory response may be stronger. There were some inflammatory cells in SCI+antagomir group, but showing a decreasing trend compared with SCI+NC group (Figure 1B).

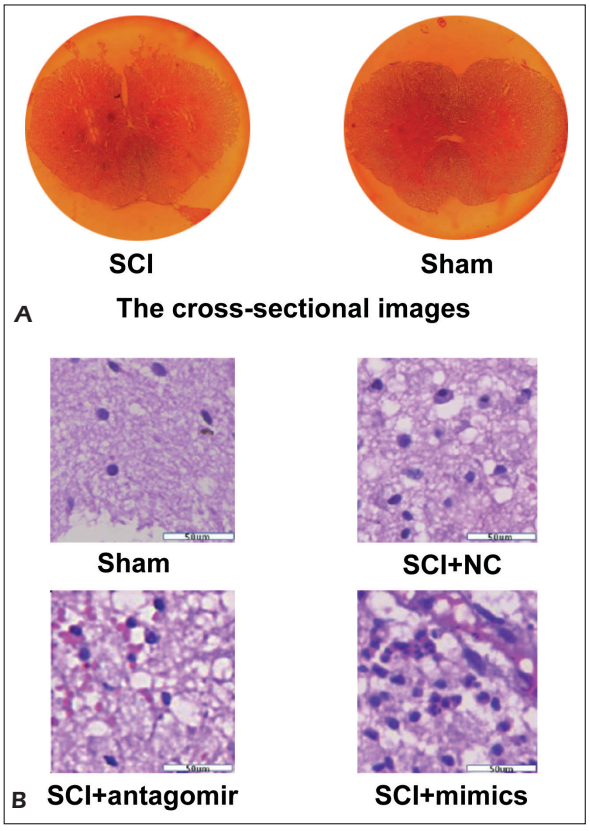

Figure 1. HE staining for the spinal cord samples: ***A***, gross view of the cross-sectional images of the spinal cords; ***B***, HE staining results of spinal cord tissues (400×).

Expression Level of let-7 i n Each Group

As shown in Figure 2, the expression of let-7 significantly declined in SCI+antangomir group compared with that in Sham group and SCI+NC group, and there were significant differences ($p<0.01$). The expression of let-7 was significantly increased in SCI+mimics group compared with that in Sham group and SCI+NC group, and there were significant differences ($p<0.01$), suggesting that the let-7 mimics (SCI+mimics) has evident efficacy and can significantly increase the let-7 level, while the let-7 inhibitor (SCI+antagomir) also has evident efficacy and can significantly reduce the let-7 level.

Western Blotting Results

The PI3K and Akt protein levels in spinal cord tissues in the four groups were detected *via* Western blotting. As shown in Figure 3A and 3B, the PI3K and Akt protein expressions were signifi-

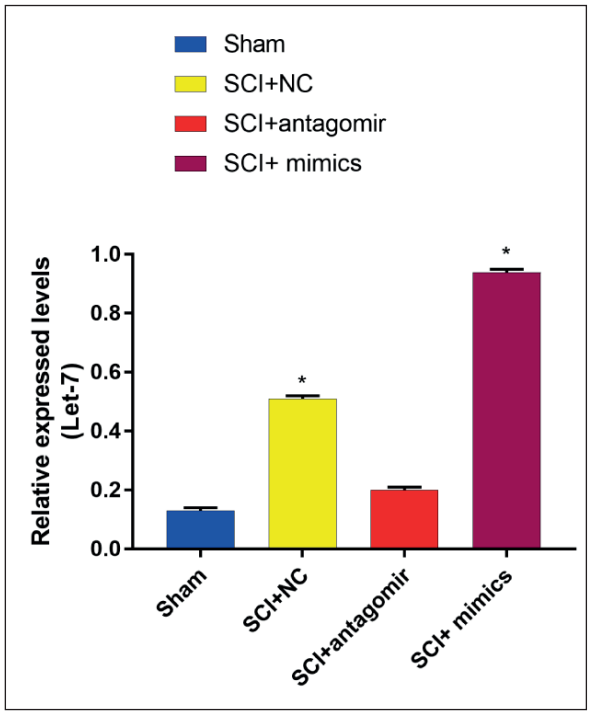

Figure 2. RT-PCR detection results of let-7 in the four groups. Expression in SCI+mimics group, SCI+antagomir group, SCI+NC group, and Sham group at 7 d (*$p<0.01$).

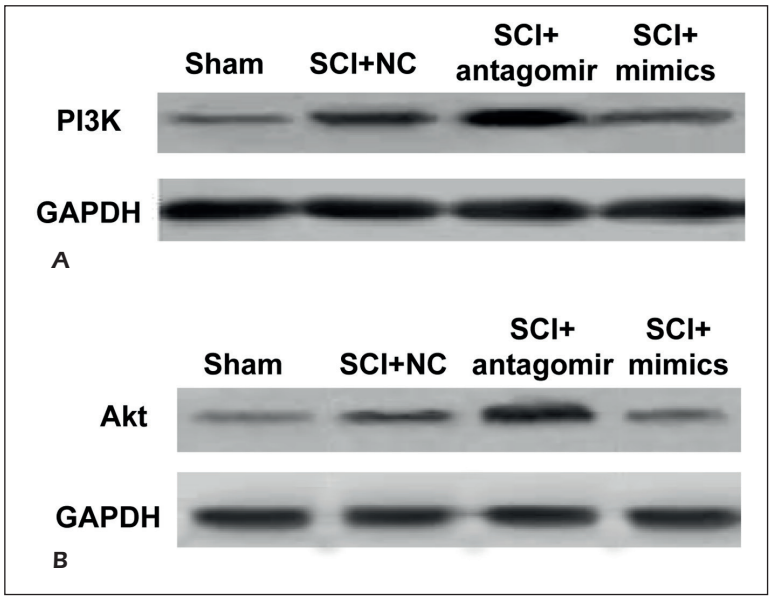

Figure 3. Protein levels of PI3K and Akt in spinal cord tissues in different groups. ***A***, The PI3K protein expressions are significantly decreased in SCI+mimics group compared with those in SCI+antagomir group, SCI+NC group, and Sham group ($p<0.05$); ***B***, The Akt protein expressions are significantly decreased in SCI + mimics group compared with those in SCI + antagomir group, SCI+NC group, and Sham group ($p<0.05$).

cantly decreased in SCI+mimics group compared with those in SCI+antagomir group, SCI+NC group, and Sham group ($p<0.05$).

Inflammatory Factor Levels Detected Via ELISA

The levels of inflammatory factors (IL-1β, IL-6, and TNF-α) in each group were detected *via* ELISA. It was found that the levels of inflammatory factors in SCI+mimics group, SCI+antagomir group, and SCI+NC group were significantly higher than those in Sham group. In SCI+mimics group, the levels of inflammatory factors were abnormally high and reached extremely significant levels (Figure 4), suggesting that SCI+mimics can significantly increase the level of inflammatory response in animal models, and let-7 promotes the inflammatory response after SCI.

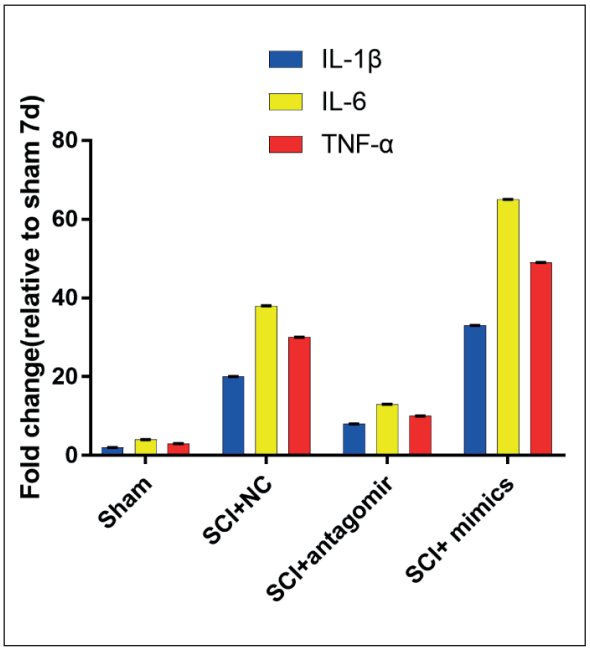

Figure 4. Inflammatory factor levels in SCI animal models in different groups.

As shown in Figure 4, the expression levels of IL-1β, IL-6, and TNF-α in Sham group, SCI+NC group, SCI+antagomir group, and SCI+mimics group are displayed as different colors from left to right. The expression levels of inflammatory factors in Sham group at 7 d after SCI were used as the reference standards. The levels of all inflammatory factors in all groups were up-regulated in different degrees at 7 d after SCI. The levels were increased very significantly in SCI+mimics group at 7 d after SCI, indicating the strong inflammatory response. On the contrary, the levels declined significantly in SCI+antagomir group at 7 d after SCI and were even lower than that in SCI+NC group, indicating the inhibited inflammatory response ($p<0.05$).

Discussion

SCI is a kind of severe nervous system injury, which can lead to motor dysfunction and severe disability, bringing heavy economic burden to individuals, families, and society[15]. Therefore, it is of important significance to treat acute SCI and alleviate nerve injury. Currently, acute SCI is divided into primary and secondary SCI[16], the latter

of which is reversible and controllable. Therefore, secondary SCI determines the final outcome of patients[17]. At present, the treatment of secondary SCI is a key in the treatment of acute SCI. The inflammatory response is a major component of secondary SCI[15]. Therefore, the regulation of inflammatory response in SCI has been studied widely in recent years. MiRNAs play important roles in the complex process of SCI. To apply miRNAs in clinical treatment, a specific miRNA involved in the regulation of inflammatory response, let-7, was studied in this paper.

We found that there were only a few bleeding or inflammatory cells in Sham group, and a large number of inflammatory cells in SCI+mimics group, indicating the stronger inflammatory response. There were some inflammatory cells in SCI+antagomir group, but showing a decreasing trend compared with SCI+NC group. It was found in RT-PCR that the expression of let-7 significantly declined in SCI+antangomir group compared with that in Sham group and SCI+NC group, and there were significant differences ($p<0.01$). The expression of let-7 was significantly increased in SCI+mimics group compared with that in Sham group and SCI+NC group, and there were significant differences ($p<0.01$). It can be seen from the above experiments that the overexpression and inhibition of let-7 will lead to significant difference in the inflammatory response after SCI, so it is speculated that let-7 is involved in the regulation of the inflammatory response in SCI. Such regulation is also further confirmed by the ELISA detection of inflammatory factor levels. The ELISA results showed that the levels of all inflammatory factors in all groups were up-regulated in different degrees at 7 d after SCI. The levels were increased very significantly in SCI+mimics group at 7 d after SCI, indicating the strong inflammatory response. On the contrary, the levels declined significantly in SCI+antagomir group at 7 d after SCI and were even lower than that in SCI+NC group, indicating the inhibited inflammatory response. The above results suggest that let-7 promotes the inflammatory response. Chen et al[18] reported that let-7 plays an important role in maintaining the innate immune response. Studies have also demonstrated that, in the gastric mucosa infected with *Helicobacter pylori*, the expression of let-7b is related to the neutrophil infiltration process in acute inflammation, and also related to the acute and chronic mononuclear inflammatory infiltration[19]. The results of this research were consistent with the reports. The results of Western blotting revealed that the PI3K and Akt protein expressions were significantly decreased in SCI+mimics group compared with those in SCI+antagomir group, SCI+NC group, and Sham group, indicating that the PI3K/Akt signaling pathway is inhibited in the regulation of inflammatory response in SCI, so it is speculated that the activation of PI3K/Akt signaling may inhibit inflammatory response, which is also consistent with the research results of Chen Y. et al[11].

Conclusions

The miRNAs closely related to SCI were screened using the SCI animal model in this study, and it was found that the changes in the expression level of let-7 in the SCI animal model were closely related to the expression levels of inflammatory factors. This work provides a living model for the further study on the mechanism of inflammatory response in SCI and lays a foundation for the in-depth study on the inflammatory response in SCI. Further investigations are needed in the future to explore the mechanism of inflammatory response in SCI deeply.

Conflict of Interests

The authors declare that they have no conflict of interest.

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