miRNA-146a and miRNA-202-3p Attenuate Inflammatory Response by Inhibiting TLR4, IRAK1, and TRAF6 Expressions in Rats following Spinal Cord Injury

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Spinal cord injury (SCI) is a catastrophic disease that induces a complex cascade of cellular reactions at the local lesion area, including secondary cell death and inflammatory reactions. Accumulating evidence has showed pro- and anti-inflammatory roles of microRNAs (miRNAs), a class of small RNAs, in SCI. The present study is aimed at investigating the effects of two miRNAs, miRNA-146a and miRNA-202-3p, on inflammatory response after SCI. Initially, we found that the expression levels of miRNA-146a and miRNA-202-3p were increased in the plasma samples of 32 SCI patients at days 3 and 7 after admission and the rat spinal cord at days 3 and 7 after SCI modeling compared with healthy controls and sham-operated rats, respectively. The expression levels of TLR4, IRAK1, and TRAF6 were declined in the rat spinal cord at days 1, 3, and 7 after SCI modeling compared with sham-operated rats. Injection of miRNA-146a mimic or miRNA-202-3p mimic decreased TLR4, IRAK1, and TRAF6 expressions in the rat spinal cord at days 1, 3, and 7 after SCI modeling, while injection of miRNA-146a antagomir or miRNA-202-3p antagomir produced opposed results. Subsequent results showed that the expression levels of tumor necrosis factor-α (TNF-α), IL-1β, IL-6, and IL-8 were upregulated in the rat serum at days 1, 3, and 7 after SCI modeling compared with sham-operated rats. Injection of miRNA-146a mimic or miRNA-202-3p mimic decreased TNF-α, IL-1β, IL-6, and IL-8 expression levels in the rat serum at days 1, 3, and 7 after SCI modeling, while injection of miRNA-146a antagomir or miRNA-202-3p antagomir produced opposed results. The expression levels of TNF-α, IL-1β, IL-6, and IL-8 were higher in the supernatants of PC12 cells transfected with anti-miRNA-146a or anti-miRNA-202-3p than in those transfected with si-TLR4, si-IRAK1, or si-TRAF6. These findings support the notion that miRNA-146a/miRNA-202-3p exerts anti-inflammatory functions after SCI.

1. Introduction

Spinal cord injury (SCI) is a common neurodegenerative condition that remains a significant source of morbidity and cost to society [1]. Each year, more than 250,000 individuals suffer from SCI around the world, and it is estimated to be 2 to 3 million people worldwide living with functional deficits [2]. SCI may arise from traumatic causes such as high-energy mechanisms of injury and nontraumatic causes such as myelitis and spinal cord tumor [3]. Historically, clinical outcomes of patients after SCI have been frustrating. SCI typically leads to long-term or even lifelong disability, largely owing to primary and secondary white matter damage at the site of injury [4]. Therapeutic strategies for SCI involve surgical and no-surgical treatments, including early surgical decompression and fixation, utilization of vasopressor medications for mean arterial blood pressure augmentation to improve spinal cord perfusion, stem cell transplantation,
and corticosteroids [5]. However, the efficacy and timing of these adjuvant treatments remain controversial, and neuroprotective and regenerative approaches for treating SCI are still a challenge for clinical physicians.

SCI comprises a two-step process involving an initial mechanical injury followed by an inflammatory process and apoptosis [6]. Local inflammatory response in the spinal cord lesion by mechanical injury is one of the important contributors to secondary insult to neurons, glia, axons, and myelin, which is mediated by cells normally found in the central nervous system (CNS), infiltrating neutrophil and leukocytes [7]. Nevertheless, inflammatory response is required for clearance of tissue debris and promotes wound healing and tissue repair [8]. Imbalance of inflammatory response may indirectly or directly affect the prognosis of SCI.

MicroRNAs (miRNAs), a group of small RNA with length ranging from 18 to 24 nucleotides, have been underlined as gene governors by binding to their target messenger RNAs and thus mediate the process of physiology and pathology [8]. Accumulating evidence shows that numerous miRNAs are expressed in the CNS, and some of them have been confirmed to be involved in traumatic CNS injury and neurodegenerative diseases [9]. The underlying mechanisms of miRNA in CNS injuries are reviewed including inflammation, apoptosis, oxidative stress, blood-brain barrier protection, neurogenesis, and angiogenesis [10]. miRNA-146a has been reported to modulate innate immune response via the toll-like receptor (TLR) and inflammatory pathways in neurodegenerative diseases [11, 12]. miRNA-202-3p is located in a chromosomal fragile site in 10q26.3 and recently has been investigated in several human diseases including colo-retal cancer [13], gastric cancers [14], sclerodera fibrosis [15], and lymphomagenesis [16]. However, there is currently limited information with regard to the roles of miRNA-146a/miRNA-202-3p and its mechanism of action in the context of SCI. To fill this gap, the present study determined the expressions of miRNA-146a and miRNA-202-3p in blood samples collected from patients at an indicated day of admission post-SCI and rat spinal cord lesion following weight-drop injury and the effects of two of them on inflammatory response in SCI.

2. Methods and Materials

2.1. Clinical Sample Collection. A total of 32 patients who were diagnosed with SCI by neurological examination and magnetic resonance imaging at the First Affiliated Hospital of Harbin Medical University between January 2018 and December 2019 were recruited into the study. These patients, aged from 18 to 70 years, had a hospitalization of less than 7 days, and among them, none had autoimmune diseases, neurodegenerative diseases, infectious symptoms, severe cardiovascular diseases, or malignant tumors. Additionally, 32 age-matched healthy controls (18 to 70 years) who received physical examinations at the same period were recruited as controls, and none had neurodegenerative diseases, autoimmune diseases, neurodegenerative diseases, infectious symptoms, severe cardiovascular diseases, or malignant tumors. The study was approved by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University. Informed consent was obtained from each participant. Fasting peripheral venous blood (5 mL) was collected from SCI patients at their initial diagnosis and healthy controls and placed into EDTA-containing test tubes for supernatant collection after centrifugation (3000 r/min, 10 min, 4°C). The supernatants were frozen at -80°C.

2.2. Animals. A total of 54 clean Sprague-Dawley male rats, aged 6-8 weeks and weighing 240 ± 10 g, were purchased from Beijing Huafukang Bioscience Co., Inc. (license no., SCXK [jing] 2009-0004) and housed in a humidity-controlled clean environment (40-60%) under a 12 h light/12 h dark cycle at a controlled temperature of 20-25°C in the laboratory of the First Affiliated Hospital of Harbin Medical University. These rats had free access to both food (sterilized pelleted feeds) and water. The animal experiments were approved by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University and performed following the National Institutes of Health’s Guidelines for Laboratory Animal Care and Use.

2.3. Establishment of the SCI Rat Model. Rats were anesthetized with 2% sodium pentobarbital (i.p. 0.25 mL/100 g). After dissection of the paraspinal muscles, a laminectomy from the lower thoracic spinal cord (T10) was performed. SCI at the T10 was induced in rats using a 10 g weight drop from a height of 50 mm [17]. Briefly, the rats were anesthetized with pentobarbital (40 mg/kg, i.p.), and then, the spine was exposed, followed by laminectomy at the T10 level. The appliance (# WH160162, Convergence Technology Co., Ltd., Shenzhen, China) (10 g) was dropped from a height of 50 mm on the exposed spinal cord. After standing for 20 seconds, the correcotr was then withdrawn to produce a moderate contusion. Finally, the incision was closed in layers. The rats’ hind legs began to twitch unconsciously and tails started to twist, which means the successful establishment of the SCI models. Rats in the sham group were only given a T10 laminectomy in the absence of the weight-drop injury. After surgery, all rats received erythromycin ointment for 3 consecutive days to avoid infection, and the bladders were manually voided three times daily.

2.4. Alternation of miRNA-146a and miRNA-202-3p In Vivo. SCI rats (n = 45) were assigned into five groups at a random manner and given tail vein injections of normal saline, miRNA-146a mimics, miRNA-146a antagonir, miRNA-202-3p mimics, and miRNA-202-3p antagonir, respectively. These plasmids were purchased from RiboBio Inc. (Guangzhou, China).

2.5. Behavioral Tests. The Basso, Beattie, and Bresnahan (BBB) locomotion rating scale [18] was employed 6th, 24th, and 48th hours after weight-drop injury or sham operation. The BBB score evaluated hindlimb locomotor function on a scale from 0 to 21, with 0 indicating no observable movement and 21 indicating normal movement.

2.6. Histological Examination. SCI rats and sham-operated rats were sacrificed using 2% sodium pentobarbital (i.p.
0.25 mL/100 g), with 3 cm spinal cord segments containing the spinal cord lesion excised. Rat spinal cord tissues were fixed with 4% paraformaldehyde at 4°C and paraffin-embedded for hematoxylin-eosin (HE) staining. In brief, paraffin-embedded tissues were longitudinally sectioned (4 μm thick) and mounted on slides. After deparaffinization, the slides were immersed in xylene I and xylene II (each for 15 min), in 100% twice, 95%, 80%, and 70% ethanol (each for 5 min) and water (1 min) in order. Subsequently, the slides were stained with hematoxylin for 1-5 min and eosin 20 s-5 min and finally observed under the optical microscope.

2.7. RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). In order to determine plasma miRNA-146a and miRNA-202-3p expression, total RNA was extracted from human plasma samples using the kit (Life Technologies, USA) following the manuals provided by the manufacturer. The complementary DNA (cDNA) was generated using EzOmics™ One-Step qPCR Kit (BK2100, Biomics Biotechnologies Co., Ltd.) according to the manufacturer’s instructions. To determine miRNA-146a and miRNA-202-3p expression in rat spinal cord tissues, total RNA was extracted from tissues using TRIzol reagents (Invitrogen, Carlsbad, CA, USA) following the manuals provided by the manufacturer. Synthesis of cDNA was performed using GoScript™ Reverse Transcriptase System (Promega, Madison, WI, USA) according to the manufacturer’s instructions. The qPCR was performed using GoTaq® qPCR Master Mix (Promega) with SYBR Green (Promega) on the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The primer information is listed in Table 1. The miR-144 level was standardized as U6 and the target mRNA level as GAPDH. Results were calculated by using the 2−ΔΔCT method.

2.8. Enzyme-Linked Immunosorbent Assay (ELISA). The expression levels of inflammatory cytokines, TNF-α, IL-1β, IL-6, and IL-8, in serum collected from SCI rats and sham-operated rats were determined using commercial ELISA kits following the manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA).

2.9. Cell Harvest and Transfection. The rat pheochromocytoma cell line PC12 (ATCC, USA) was maintained in DMEM (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS) in a 5% CO2 incubator with saturated humidity (95%) at 37°C. PC12 cells were seeded in 6-well plates (5 × 104 cells per mL) and then transfected with anti-miRNA-146a, anti-miRNA-202-3p, si-TLR4 (5′-GGCGCUAAAGACTCAGGATTTCCAGTCA CACCCAA-3′), si-IRAK1 (5′-GGGACAGAAGACTGGTCGTTACCTCA AGGTTTTGG-3′), si-TRAF6 (5′-GACUGAUUUGAGCGUGAAGUGCU ATT-3′), and si-NC (5′-UUCUCAGGUGUCAGCATT-3′) (Thermo Fisher Scientific, Waltham, MA, USA) using Lipofectamine 2000 reagents (Invitrogen) following the manuals provided by the manufacturer. After 48 h transfection, lipopolysaccharide (LPS) stimulation (100 ng/mL) was performed for 4 h.

Table 1: Primer sequences used for qRT-PCR.

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR4</td>
<td>F: 5′-GGCACAGGTGAAATGATGTT-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-GGTTGTTGATTCTCTCAGGAATGTT-3′</td>
</tr>
<tr>
<td>TRAF6</td>
<td>F: 5′-GCTTTCTCGTGGCAGAACCTAC-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-GACAGAGACGCTGGAAGGCT-3′</td>
</tr>
<tr>
<td>IRAK1</td>
<td>F: 5′-TACCTGCGCGGAGTCCACATCAA-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-GACGTGGAAGGATAGAAGA-3′</td>
</tr>
<tr>
<td>NF-xB</td>
<td>F: 5′-GATCGCCACCGGATTGAAGA-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-GACGGGAAGCAGCGAGA-3′</td>
</tr>
<tr>
<td>miRNA-146a</td>
<td>F: 5′-CTCCATGCTGGCTTCATGTT-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-GACGTGGAAGGATAGAAGA-3′</td>
</tr>
<tr>
<td>miRNA-202-3p</td>
<td>F: 5′-AGGGAGAACACCAGCATTACC-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-AGTGGCAGGTTCCAGGATT-3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: 5′-GCTTCGCGACGACATATACCT-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-GCAATTTGGTGTCATCCT-3′</td>
</tr>
</tbody>
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F: forward; R: reverse.

2.10. Immunoblotting Analysis. Total protein was extracted from rat spinal cord tissues and PC12 cells using RIPA lysis buffer (Solarbio, Beijing, China). The protein sample was separated using freshly prepared SDS-PAGE (Beyotime Biotechnology, Jiangsu, China), electrotransferred onto PVDF membranes (IPVH0001, Millipore, MA, USA), and probed with primary antibodies to TLR4 (ab13556), IRAK1 (ab180747), TRAF6 (ab33915), or GAPDH (ab9485, Abcam, Cambridge, UK). Immunoreactive bands were visualized with goat anti-rabbit IgG (1:10000, ab205718) and enhanced chemiluminescence detection reagents (Billerica, MA, USA). The blots were developed, followed by quantitative analysis using ImageJ software (National Institutes of Health, USA). The quantitative level of the protein was determined based on the gray ratio of each protein to the gray ratio of GAPDH.

2.11. Statistical Analysis. All data (mean ± standard deviation) were representative of three independent experiments (each in triplicate), and the statistical analysis was performed with SPSS 21.0 software (IBM, Armonk, NY, USA), with two-tailed P < 0.05 as a level of statistical significance. For statistical comparisons, unpaired Student’s t-test and a one-way analysis of variance (ANOVA) with Tukey’s test were performed as appropriate.

3. Results

3.1. miRNA-146a and miRNA-202-3p Were Upregulated in Patients with SCI. To study the clinical significance of miRNA-146a and miRNA-202-3p in SCI, we collected blood samples from healthy controls and SCI patients at days 1, 3, and 7 after admission to examine the plasma expression.
levels of miRNA-146a and miRNA-202-3p. Results of qRT-PCR showed that miRNA-146a and miRNA-202-3p expression levels did not differ between healthy controls and SCI patients at day 1 after admission \( (P > 0.05) \). At days 3 and 7 after admission, miRNA-146a and miRNA-202-3p were upregulated in the plasma of SCI patients, which were remarkably higher than those in the plasma of healthy controls \( (P < 0.05, \text{Figures 1(a) and 1(b)}) \).

3.2. Behavioral and Histological Characterization of Rats following SCI. As shown in Figure 2(a), the BBB scores of the rats at the 6th, 24th, and 48th hours after weight-drop injury were lower than those of sham-operated rats \( (P < 0.05) \). Next, SCI rats and sham-operated rats were sacrificed, and spinal cord segments containing the spinal cord lesion were excised for histological examination. After fixation with paraformaldehyde, evident weight-drop injury was observed in the spinal cord segments of SCI rats (Figure 2(b)). Results of HE staining (Figure 2(c)) showed accumulation of erythrocytes, inflammatory infiltration, and hemorrhage; however, no evident inflammatory infiltration and hemorrhage were found in the spinal cord segments of sham-operated rats.

3.3. Expression of miRNA-146a and miRNA-202-3p in Rats following SCI. In order to ascertain the effects of miRNA-146a and miRNA-202-3p on SCI, SCI rats were injected with normal saline, miRNA-146a mimics, miRNA-146a antagonim, miRNA-202-3p mimics, and miRNA-202-3p antagonim, respectively. It revealed that the expression levels of miRNA-146a did not significantly differ in the spinal cord tissues among sham, SCI, miRNA-146a mimic, and miRNA-146a antagonim groups at day 1 after weight-drop injury \( (P > 0.05) \). The expression level of miRNA-146a was increased in rats at days 3 and 7 after weight-drop injury compared with sham-operated rats. At day 3 after weight-drop injury, injection of miRNA-146a mimics significantly increased the signal of miRNA-146a expression in SCI rats \( (P < 0.05) \), and injection of miRNA-146a antagonim signifi-

\[ \text{Relative miRNA-146a expression} \]

\[ \text{Relative miRNA-202-3p expression} \]

![Figure 1: Relative expression levels of miRNA-146a (a) and miRNA-202-3p (b) in the plasma of SCI patients and healthy controls were determined by qRT-PCR. \( ** P < 0.01 \) by Student’s t-test.}](image)

3.4. The mRNA and Protein Expressions of TLR4, IRAK1, and TRAF6 in Rats following SCI. Results of qRT-PCR showed that the mRNA expressions of TLR4, IRAK1, and TRAF6 were declined in SCI rats compared with sham-operated rats at days 1, 3, and 7 after surgery \( (P < 0.05, \text{Figure 4(a)}) \). At days 1, 3, and 7 after weight-drop injury, injection of miRNA-146a antagonim remarkably elevated TLR4, IRAK1, and TRAF6 mRNA expressions in SCI rats \( (P < 0.05) \), and injection of miRNA-146a mimics yielded opposed results \( (P < 0.05, \text{Figure 4(b)}) \). As shown in Figure 4(c), injection of miRNA-202-3p antagonim also remarkably increased TLR4, IRAK1, and TRAF6 mRNA expressions in SCI rats \( (P < 0.05) \), and injection of miRNA-146a mimics yielded opposed results \( (P < 0.05, \text{Figure 4(e)}) \). Injection of miRNA-202-3p antagonim also remarkably increased TLR4, IRAK1, and TRAF6 protein expressions in SCI rats.
3.5. The Levels of Inflammatory Cytokines in Rats following SCI.

Subsequently, ELISA was performed to detect the levels of inflammatory cytokines, TNF-α, IL-1β, IL-6, and IL-8, in rats after weight-drop injury or sham operation. The levels of TNF-α, IL-1β, IL-6, and IL-8 were elevated in SCI rats compared with sham-operated rats at days 1, 3, and 7 after surgery (P < 0.05). Injection of miRNA-146a mimic or miRNA-202-3p mimic remarkably reduced the release of inflammatory cytokines in SCI rats at days 1, 3, and 7 after surgery (P < 0.05), and injection of miRNA-146a antagonir or miRNA-202-3p antagonir yielded opposed results (P < 0.05, Figures 5(b) and 5(c)).

3.6. miRNA-146a and miRNA-202-3p Inhibited the Release of Inflammatory Cytokines in LPS-Stimulated PC12 Cells by Regulating TLR4, IRAK1, and TRAF6.

In order to confirm whether the anti-inflammatory roles of miRNA-146a and miRNA-202-3p in SCI is achieved by affecting TLR4, IRAK1, and TRAF6 expressions, PC12 cells were transfected with anti-miRNA-146a, anti-miRNA-202-3p, si-TLR4, si-IRAK1, si-TRAF6, and si-NC. As shown in Figure 6(a), the protein expressions of TLR4, IRAK1, and TRAF6 were declined in PC12 cells transfected with si-TLR4 compared with PC12 cells transfected with si-NC (P < 0.05). PC12 cells transfected with anti-miRNA-146a or anti-miRNA-202-3p presented higher protein expressions of TLR4, IRAK1, and TRAF6 than PC12 cells transfected with si-TLR4 (P < 0.05). The levels of TNF-α, IL-1β, IL-6, and IL-8 were declined in PC12 cells after transfection with anti-miRNA-146a or anti-miRNA-202-3p mimics (P < 0.05, Figures 5(b) and 5(c)).
transfected with si-TRAF6 compared with PC12 cells transfected with si-NC (P < 0.05). PC12 cells transfected with anti-miRNA-146a or anti-miRNA-202-3p exhibited higher levels of inflammatory cytokines than PC12 cells transfected with si-TLR4 (P < 0.05, Figure 6(b)). As shown in Figure 6(c), the protein expressions of IRAK1 and TRAF6 were declined in PC12 cells transfected with si-IRAK1 compared with PC12 cells transfected with si-NC (P < 0.05). PC12 cells transfected with anti-miRNA-146a or anti-miRNA-202-3p presented higher protein expressions of IRAK1 and TRAF6 than PC12 cells transfected with si-IRAK1 (P < 0.05). The levels of TNF-α, IL-1β, IL-6, and IL-8 were declined in PC12 cells transfected with si-IRAK1 compared with PC12 cells transfected with si-NC (P < 0.05). PC12 cells transfected with anti-miRNA-146a or anti-miRNA-202-3p exhibited higher levels of inflammatory cytokines than PC12 cells transfected with si-IRAK1 (P < 0.05, Figure 6(d)). Finally, it was found that PC12 cells transfected with si-TRA6 showed declined protein expression of TRAF6 compared with PC12 cells transfected with si-NC (P < 0.05). PC12 cells transfected with anti-miRNA-146a or anti-miRNA-202-3p presented higher protein expression of TRAF6 than PC12 cells transfected with si-TRA6 (P < 0.05, Figure 6(e)). The levels of TNF-α, IL-1β, IL-6, and IL-8 were declined in PC12 cells transfected with si-TRA6 compared with PC12 cells transfected with si-NC (P < 0.05). PC12 cells transfected with anti-miRNA-146a or anti-miRNA-202-3p exhibited higher levels of inflammatory cytokines than PC12 cells transfected with si-TRA6 (P < 0.05, Figure 6(f)).

4. Discussion

Inflammatory response begins within minutes to hours following SCI and peaks approximately 1 day later, which was characterized by very early production of cytokines, chemokines, and the infiltration of neutrophils into the site of injury [19]. The resolution of inflammation is a highly controlled and coordinated process that involves the suppression of proinflammatory gene expression and inhibition of leukocyte migration and activation, followed by clearance of inflammatory cells by apoptosis and phagocytosis [20]. miRNAs have been well studied in the SCI model. For example, miR-9, miR-219, and miR-384-5p increased in the serum of mice 12 h following SCI-like surgery [21]. miR-10b, miR-100, miR-130a, miR-133a-5p, miR-133b, miR-208b, miR-365-3p, miR-378, miR-378b-3p, and miR-885-5p were changed in serum at 1 and 3 days in pig models of SCI, and these miRNAs were related to outcome measures at 12 weeks following SCI [22]. In this work, we demonstrated anti-inflammatory action of miRNA-146a and miRNA-202-3p in SCI and their mechanism of action involving three target genes, TLR4, IRAK1, and TRAF6.

Initially, we collected blood samples from healthy controls and SCI patients at days 1, 3, and 7 after admission to examine the plasma expression levels of miRNA-146a and miRNA-202-3p. It revealed that miRNA-146a and miRNA-202-3p were upregulated in the plasma of SCI patients at days 3 and 7 after admission, which was further confirmed by results from an animal SCI model after weight-drop injury. The inflammatory response post-SCI consists of two phases, early and late. The early inflammatory response may be neurotoxic in nature by releasing proinflammatory cytokines including TNF-α, IL-1β, IL-6, and IL-8 [23], which is detrimental to functional recovery. The late inflammatory response may be essential for wound repair and recovery of function [8]. miRNA-146a and miRNA-202-3p are released to regulate inflammatory response after SCI through a feedback mechanism and prevent further damage to the body, thus promoting the recovery. In a previous study reported by Tan et al. [24], they showed that miR-146a expression was declined in the SCI rat model, which is different from our results. However, their final conclusion was that miR-146a reduces inflammation in an SCI model. Similar to our results, Wei et al. determined an upregulation of...
Figure 4: Continued.
miR-146a in the animal model of SCI except on the first day after surgery [25]. No experiment based on a human sample was performed in these two previous studies. Fortunately, our study collected blood samples from healthy controls and SCI patients at days 1, 3, and 7 after admission and demonstrated upregulated miR-146a following SCI. Few previous data reported an anti-inflammatory role of miR-202-3p. Most of the studies focus on its function as a tumor suppressor in human cancer. Since inflammation plays an important role in cancerogenesis, coupled with our results, there is possibility that miR-202-3p inhibits cancer progression potentially by exerting anti-inflammatory effects.

Subsequent experiments were performed to confirm whether TLR4, IRAK1, and TRAF6 were involved in the anti-inflammatory action of miRNA-146a and miRNA-202-3p in SCI. Toll-like receptors (TLRs) provide critical signals to induce innate immune responses, and TLR4 is the receptor for lipopolysaccharide. TLR4 stimulation activates pro-inflammatory pathways and induces secretion of cytokines in various cell types [26]. IRAK1 and TRAF6 are known to be part of the common signaling pathway of the toll-like receptor/interleukin 1 receptor (TIR) superfamily. Several studies have reported that TLR4, IRAK1, and TRAF6 expressions were inhibited following miRNA-146a overexpression [27–29]. In our study, we found that upregulation of miRNA-146a could inhibit expressions of TLR4, IRAK1, and TRAF6 at mRNA and protein levels. Consistent with our results, Liu et al. demonstrated that miR-146 could attenuate microglial inflammatory response through TLR4/IRAK1/TRAF6-related pathways [30]. Additionally, the TLR4/IRAK1/TRAF6 axis was revealed in our work, as
Figure 5: The levels of inflammatory cytokines in rats following SCI. (a) ELISA detection of TNF-α, IL-1β, IL-6, and IL-8 in the serum of sham-operated and SCI rats at days 1, 3, and 7 after surgery. (b) ELISA detection of TNF-α, IL-1β, IL-6, and IL-8 in rat serum among SCI, miRNA-146a mimic, and miRNA-146a antagonir groups at days 1, 3, and 7 after surgery. (c) ELISA detection of TNF-α, IL-1β, IL-6, and IL-8 in rat serum among SCI, miRNA-202-3p mimic, and miRNA-202-3p antagonir groups at days 1, 3, and 7 after surgery. **P < 0.01 by Student’s t-test in (a); **P < 0.01 by one-way ANOVA with Tukey’s test in (b) and (c).
Figure 6: Continued.
Figure 6: miRNA-146a and miRNA-202-3p inhibited the release of inflammatory cytokines in LPS-stimulated PC12 cells by regulating TLR4, IRAK1, and TRAF6. (a) Immunoblotting analysis of TLR4, IRAK1, and TRAF6 proteins in PC12 cells transfected with si-TLR4, anti-miRNA-146a, anti-miRNA-202-3p, and si-NC. (b) ELISA detection of TNF-α, IL-1β, IL-6, and IL-8 levels in PC12 cells transfected with si-TLR4, anti-miRNA-146a, anti-miRNA-202-3p, and si-NC. (c) Immunoblotting analysis of IRAK1 and TRAF6 proteins in PC12 cells transfected with si-IRAK1, anti-miRNA-146a, anti-miRNA-202-3p, and si-NC. (d) ELISA detection of TNF-α, IL-1β, IL-6, and IL-8 levels in PC12 cells transfected with si-IRAK1, anti-miRNA-146a, anti-miRNA-202-3p, and si-NC. (e) Immunoblotting analysis of TRAF6 protein in PC12 cells transfected with si-TRAF6, anti-miRNA-146a, anti-miRNA-202-3p, and si-NC. (f) ELISA detection of TNF-α, IL-1β, IL-6, and IL-8 levels in PC12 cells transfected with si-TRAF6, anti-miRNA-146a, anti-miRNA-202-3p, and si-NC. **P < 0.01 by one-way ANOVA with Tukey’s test.
evidenced by results of IRAK1 and TRAF6 inhibition after TLR4 knockdown and TRAF6 inhibition after IRAK1 knockdown, concurring with other studies [31, 32]. Similar to the anti-inflammatory action and mechanism of miRNA-146a in SCI, miRNA-202-3p was observed to ameliorate inflammation following SCI. It is the first time to report the anti-inflammatory role of miRNA-202-3p and its targeted relationship with TLR4, IRAK1, and TRAF6 in the context of SCI, required for further investigations.

In conclusion, our study provides evidence of anti-inflammatory action and mechanism of miRNA-146a and miRNA-202-3p following SCI. Once SCI occurs, miRNA-146a and miRNA-202-3p were secreted to inhibit TLR4, IRAK1, and TRAF6, three crucial parts of inflammatory pathways, protecting the body from excessive inflammation-induced secondary damage. However, our study is a preliminary study. Further in vivo investigations about miRNA-146a and miRNA-202-3p interaction with TLR4, IRAK1, and TRAF6 and inflammation-related signal pathways responsible for the anti-inflammatory functions of miRNA-146a and miRNA-202-3p in SCI were required.

**Data Availability**

The data used to support the findings of this study are included within the article.

**Conflicts of Interest**

All authors declare that they have no conflict of interest.

**References**


