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Effects of MiR-146a on repair and inflammation in rats with spinal cord injury through the TLR/NF-κB signaling pathway

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Abstract. – OBJECTIVE: This study aims to investigate the effects of micro ribonucleic acid-34a (miR-34a) on repair and inflammation of rats with spinal cord injury (SCI) through the toll-like receptor (TLR)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway.

MATERIALS AND METHODS: In this study, 12 healthy rats (control group (CG)) and 24 SCI rats (experimental group (EG-1)) were selected as subjects. A total of 12 experimental rats randomly selected from EG-1 were injected with 5 μL agomiR-146 as EG-2 group. Serum levels of miR-146a, TLR, NF-κB, interleukin-8 (IL-8) and IL-6 of rats in CG and EG-1 were detected by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Furthermore, the protein levels of miR-146a, TLR, NF-κB, IL-8 and IL-6 in rats of CG and EG were detected via Western blotting. Spinal cord tissue sections of SCI rats after treatment with agomiR-146 were observed by hematoxylin and eosin staining (H&E) staining.

RESULTS: The mRNA level of miR-146a in SCI rats was significantly lower than that in healthy rats, and the difference was statistically significant ($p < 0.05$). The mRNA levels of TLR, NF-κB, IL-8 and IL-6 in SCI rats were markedly higher than those in healthy rats, showing significant differences ($p < 0.05$). However, the relative mRNA level of miR-146a in EG-2 group was significantly higher than that in EG-1 group, with a significant difference ($p < 0.05$). Relative level of miR-146a was not significantly different between EG-2 group and CG group ($p > 0.05$). Meanwhile, the mRNA levels of TLR, NF-κB, IL-8 and IL-6 in EG-2 group were evidently lower than those in EG-1 group, displaying significant differences ($p < 0.05$).

CONCLUSIONS: MiR-146a can promote the repair of SCI and reduce inflammatory responses in rats through the TLR/NF-κB signaling pathway.

Key Words:
miR-146a, TLR/NF-κB, Repair of spinal cord injury, agomiR-146, Inflammation.

Introduction

As a kind of common diseases in spinal surgery, spinal cord injury (SCI) and other diseases can cause long-term cellular injury around the spinal cord and local inflammation in the later treatment[1-4]. SCI may even lead to injury of related nerve pathways, eventually causing numbness or even paralysis of the patient's limbs. Therefore, it is of great practical significance to strengthen the research. In recent years, with the continuous development of medical technology, non-surgical treatment methods have been widely applied in the treatment due to their unique advantages[5]. Secondary inflammatory response at post-SCI is one of the major reasons for aggravating the disease condition. Therefore, it is essential to explore how to prevent the occurrence of related inflammatory responses in the process of repair[6-8]. Genetic studies[9] have shown that messenger ribonucleic acids (mRNAs) carrying genetic information can be translated into proteins with certain specific functions to participate in different metabolic processes. In recent years, a large number of studies have found that the microRNA (miRNA), as a short-chain non-coding RNA, can inhibit or block the translation process of the target mRNA by binding to their 3'untranslated region (3'UTR). Finally, miRNAs downregulate or inhibit the expressions of the specific target proteins[10,11]. Studies[12] have revealed that miRNA-34a (miR-

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34a), as a member of miR-146a family, is capable of regulating downstream inflammatory genes by inhibiting the Toll-like receptor (TLR)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway. Nevertheless, the role of miR-146a in SCI is still unclear. In this study, we explored the role of miR-146a in the repair and inflammatory response at post-SCI, and its underlying mechanism.

Materials and Methods

General Materials

A total of 12 healthy rats (control group (CG)) and 24 SCI rats (experimental group, (EG-1)) with 12-14 weeks old and about 30 g in weight were selected. 12 experimental rats randomly selected from EG-1 group were injected with 5 μL agomiR-146 (Invitrogen, Carlsbad, CA, USA) as EG-2 group. All of them were purchased from Henan Provincial Laboratory Animal Research Center. This study was approved by the Animal Ethics Committee of Luoyang Orthopedic Hospital of Henan Province, Animal Center.

Sample Collection and Main Reagents

6 mL blood was extracted from rats in CG, EG-1 and EG-2 group, centrifuged at 1000 rpm for 10 min to obtain the upper serum. Serum samples were stored at -80°C after the treatment with cryoprotectant at -80°C. The miR-146a, TLR, NF-κB, IL-8 and IL-6 antibodies used in this study were purchased from Roche (Basel, Switzerland), RNA extraction kits from Axygen (Tewksbury, MA, USA), and related molecular reagents from TaKaRa (Otsu, Shiga, Japan). Fluorescence quantitative polymerase chain reaction (qPCR) primers were synthesized by Shanghai Sangon Biotech Co., Ltd., (Shanghai, China). All PCR reagents used in this study were purchased from TaKaRa (Otsu, Shiga, Japan), agaroses from Shanghai Sangon Biotech Co., Ltd. (Shanghai, China), the DNA extraction kit from Axygen (Tewksbury, MA, USA), and the animal cell protein extraction kit from Roche (Basel, Switzerland).

Fluorescence qPCR

RNA Extraction

In this study, rats in CG, EG-1 and EG-2 group were taken as subjects to extract the RNA and determine the extraction quality[13].

Fluorescence qPCR

In order to study the difference in the expression level of the total mRNA in different samples, complementary DNAs (cDNAs) were obtained through RNA reverse transcription (RT) as a template to carry out fluorescence qPCR in this experiment. The primer sequences were shown in Table I.

Enzyme-Linked Immunosorbent Assay

The animal cell protein extraction kit (Roche, Basel, Switzerland) was used to extract the total protein. Enzyme-linked immunosorbent assay was carried out according to Connelly DJ experimental scheme[14].

Hematoxylin and Eosin (H&E) Staining

(1) Samples were selected from CG, EG-1 and EG-2 group, treated with 10% formaldehyde and then embedded in paraffin. In this experiment, sections were sliced into 4 μm in the thickness, fixed on a glass slide and baked at 70°C for about 1 h. (2) Samples were dewaxed with xylene prepared in advance and eluted with anhydrous alcohol. Remaining alcohol was washed off with ultrapure water, followed by washing with phosphate-buffered saline (PBS) (pH 7.2) for 5 times, with 5 min each time. The sterilization pot was heated at 121°C for 2 min and then placed in PBS solution for 30 min at room temperature after cooling. (3) The tissue slices prepared were placed in a solution containing 0.3% Tritonx-100 for 30 min of incubation, and then washed with 0.01 M PBS for 2-3 times, with 5 min each time. (4) After the slices were dried, they were dewaxed with xylene and treated with alcohol, followed by staining with hematoxylin aqueous solution and alcohol eosin dye solution. (5) The slices were observed under a microscope. The procedures were according to the Medvedev AE experimental scheme[15].

Statistical Analysis

Measurement data were expressed as ($\bar{x} \pm s$). All data were analyzed using Statistical Pro-

Table I. RT-PCR primer sequences of miR-146a, TLR, NF-κB and inflammation-related cytokines, IL-8 and IL-6.

Name	Sequence
MiR-146a	ATCGCTGTAGCTAGCTAGC
TLR	CTGATCGATAGCTGATCGATC
NF-κB	ATCGTCGCATCGACTGATCAGC
IL-8	CGTAGCGATCGAGAGCTAGCCA
IL-6	CTGATCGCGGATAGCTAGATCAG

duct and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA).

Results

Relative Levels of miR-146a, TLR, NF-κB in CG and EG-1 Group

The total RNA was extracted from serum samples of rats in CG and EG-1 for detecting mRNA levels of miR-146a, TLR and NF-κB by fluorescence qPCR. The results manifested that the mRNA level of miR-146a in EG-1 group was markedly downregulated compared with that in CG group, and the difference was significant (p<0.05). Expression levels of TLR and NF-κB were upregulated in EG-1 group relative to CG group (p<0.05) (Figure 1). The above results suggested that miR-146a was downregulated, whereas TLR and NF-κB were upregulated at post-SCI.

Relative Levels of miR-146a, TLR, NF-κB in EG-1 and EG-2 Group

Rats in EG-2 group were randomly selected in EG-1 group, and they were administrated with the agomiR-146, a miR-146a enhancer. The mRNA level of miR-146a was higher in EG-2 group than that of EG-1 group (p<0.05), indicating the successful efficacy of agomiR-146. Besides, mRNA levels of TLR and NF-κB were lower in EG-2 group relative to EG-1 group (p<0.05, Figure 2). The above results demonstrated that upregulated miR-146a could downregulate TLR and NF-κB in SCI rats.

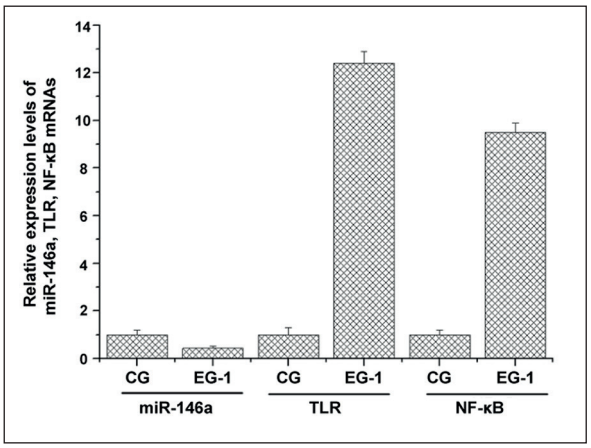

Figure 1. Relative levels of miR-146a, TLR and NF-κB in CG and EG-1 group detected by PCR. p<0.05.

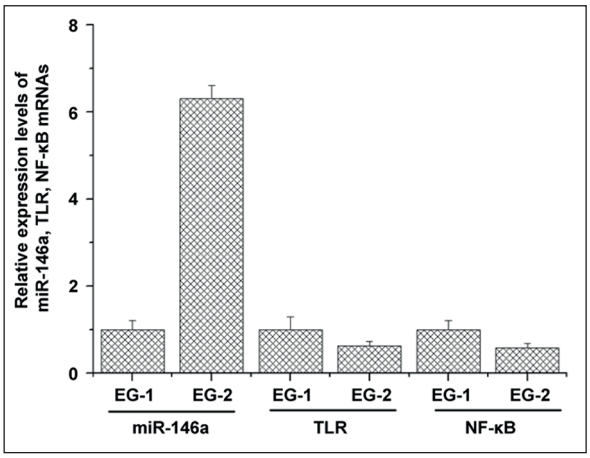

Figure 2. Relative levels of miR-146a, TLR and NF-κB in EG-1 and EG-2 group detected by PCR. p<0.05.

Protein Levels of TLR and NF-κB in CG and EG-1 Group

Protein levels of TLR and NF-κB in rats of CG and EG-1 group were measured. According to the results, protein levels of TLR and NF-κB in rat serum samples of EG-1 group ((8.1±0.42) μg/L and (9.2±0.45) μg/L, p<0.05) were evidently higher than those of CG group (3.6±0.34) μg/L and 3.8±0.41) μg/L, p<0.05), which were consistent with their mRNA levels (Figure 3).

Protein Levels of TLR and NF-κB in EG-1 and EG-2 Group

Protein levels of TLR and NF-κB were markedly downregulated in EG-2 group relative to

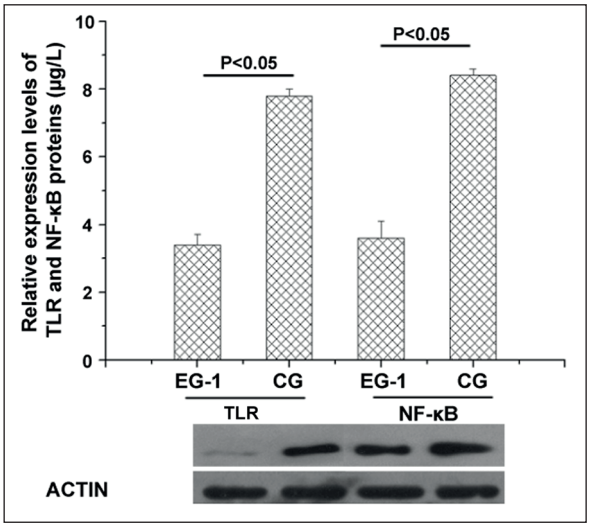

Figure 3. Protein levels of TLR and NF-κB in CG and EG-1 group detected by Western blot. p<0.05.

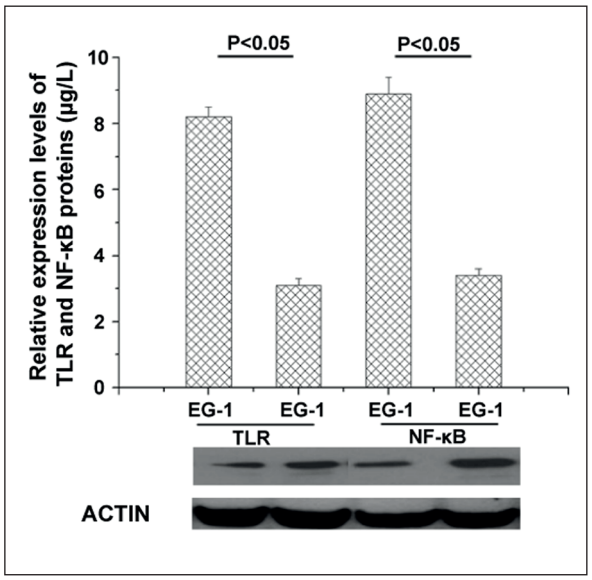

Figure 4. Protein levels of TLR and NF-κB in EG-1 and EG-2 group detected by Western blot. $p<0.05$.

EG-1, indicating that upregulated miR-146a greatly inhibited inflammatory response at post-SCI ($p<0.05$, Figure 4).

Relative Levels of IL-6 and IL-8 in EG-1 and EG-2 Group

To further clarify the anti-inflammatory role of miR-146a in SCI rats, we determined relative levels of IL-6 and IL-8. Here, serum levels of IL-6 and IL-8 were remarkably lower in CG and EG-2 group compared with those of EG-1 group, indicating that miR-146a could reduce the levels of inflammatory cytokines in SCI rats ($p<0.05$, Figure 5).

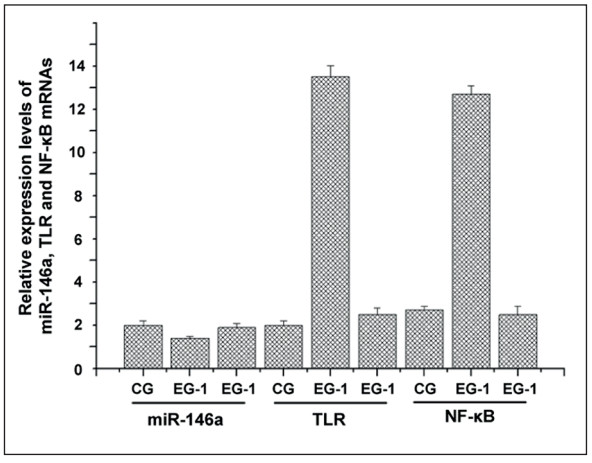

Figure 5. Relative levels of miR-146a, TLR and NF-κB in EG-1 and EG-2 group detected by PCR. $p<0.05$.

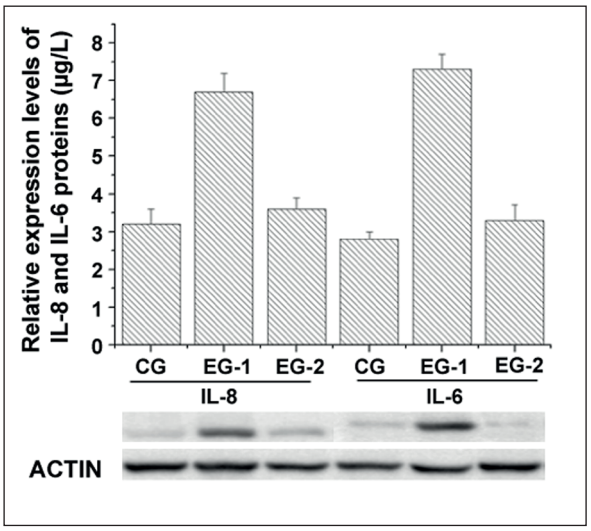

Figure 6. Protein levels of IL-8 and IL-6 in EG-1 and EG-2 group detected by Western blot. $p<0.05$.

Protein Levels of IL-6 and IL-8 in EG-1 and EG-2 Group

Identical to the mRNA levels, protein levels of IL-6 and IL-8 were markedly higher in EG-1 group compared with those of CG and EG-2 group ($p<0.05$, Figure 6). It is believed that miR-146a could suppress the inflammation at post-SCI through downregulating inflammatory cytokines.

H&E Staining Results of SCI in CG, EG-1 and EG-2

To investigate the role of miR-146a in the repair of SCI in rats, the repair status of SCI in rats in CG, EG-1 and EG-2 were detected. According to the results in Figure 7, the percentage of spinal cord vacuoles in EG-1 group markedly increased compared with that in CG group, which was reduced in EG-2 group. It is indicated that miR-146a contributed to the repair of SCI in rats.

Discussion

SCI can be generally divided into two different types according to its different pathological changes, namely, acute SCI and chronic SCI[16]. Currently, the mostly applied treatment includes drug treatment, surgical treatment and reconstruction after SCI[17,18]. At present, although some progresses have been made in the treatment, there are few reports on the repair mechanism of SCI and related complications such as inflammation

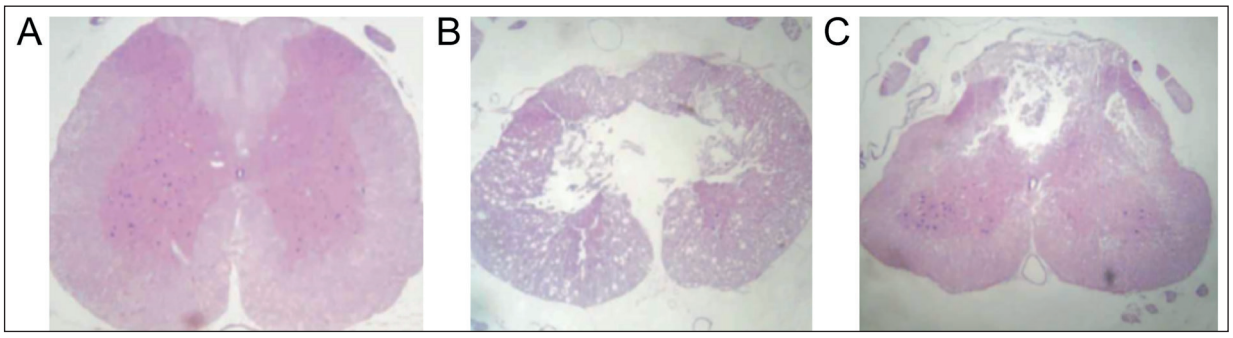

Figure 7. H&E staining results of SCI in CG, EG-1 and EG-2 group. ***A,*** CG: healthy rats, ***B,*** EG-1: SCI rats, and ***C,*** EG-2: SCI rats treated with agomiR-146.

in the repair process[19,20]. As an important complication after SCI repair, the inflammatory response aggravates the disease condition. Therefore, it is of great practical and theoretical significance to explore the repair mechanism and strengthen the research on related inflammatory responses. This study for the first time explored the role of miR-146a in mediating repair and inflammation in SCI rats by regulating the TLR/NF-κB signaling pathway. MiR-146 was lowly expressed in SCI rats than those of controls, whereas relative genes in TLR/NF-κB signaling pathway and inflammatory cytokines were upregulated. We therefore speculated that miR-146 may participate in the repair and inflammation at post-SCI. Subsequently, SCI rats were administrated with agomiR-146, the miR-146 enhancer. Interestingly, inflammatory genes in TLR/NF-κB signaling pathway and cytokines (IL-6 and IL-8) were markedly downregulated in rats administrated with agomiR-146. Moreover, agomiR-146 administration reduced the percentage of spinal cord vacuoles, suggesting the improved repair at post-SCI. In conclusion, miR-146 participated in the repair and inflammation regulation at post-SCI through the TLR/NF-κB signaling pathway.

Conclusions

We observed that MiR-146a can promote the repair of SCI and reduce inflammatory responses in SCI rats through the TLR/NF-κB signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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