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miR-940 promotes spinal cord injury recovery by inhibiting TLR4/NF-κB pathway-mediated inflammation

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Abstract. – OBJECTIVE: The aim of this study was to investigate the effects of miR-940 and Toll-like receptor 4/Nuclear Factor κB (TLR4/NF-κB) pathways on inflammatory responses and spinal cord injury (SCI).

MATERIALS AND METHODS: This study first established a model of spinal cord injury in mice. The grip force measurement was used to detect the recovery of the forelimb, left forelimb and right forelimb of SCI mice. The quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to detect the expression of miR-940 and macrophage receptor TLR4 in SCI mice. In addition, the protein levels of TLR4 and inducible nitric oxide synthase (iNOS) in SCI mice were detected by Western blot. MiR-940 mimic was injected into the injured area of SCI mice to explore the effect of miR-940 overexpression on TLR4 and myeloperoxidase (MPO) expression as well as the protein levels of TLR4, P65 and iNOS. Furthermore, the grip strength of SCI mice with double forelimb, left forelimb and right forelimb was detected by the grip force test after miR-940 overexpression.

RESULTS: Compared with the sham-operated mice, the grip strength of the forelimb, left forelimb, and right forelimb of the SCI group showed significant obstacles. Meanwhile, the expression of miR-940 was remarkably decreased in SCI mice along with significant elevation of the inflammatory response-related factors including TRL4 and iNOS. Then we injected SCI mice with miR-940 mimics into the spinal cord injury area and found that miR-940 overexpression decreased the expression levels of TLR4 and MPO. At the same time, the overexpression of miR-940 markedly decreased the protein levels of TLR4, P65, and iNOS in SCI mice. In addition, miR-940 overexpression improved the grip strength of the left and right forepaws and the simultaneous grip strength of the two claws of the SCI mice than those of the simple injury group.

CONCLUSIONS: High expression of miR-940 can promote the recovery of spinal cord injury by downregulating the TLR4/NF-κB signaling pathway and inhibiting inflammation.

Key Words
miR-940, TLR4/NF-ÐB signaling pathway, Inflammatory response, Spinal cord injury.

Introduction

Spinal cord injury (SCI) refers to damage caused by external forces acting directly or indirectly on the spinal cord. The incidence of SCI has increased year by year, and statistics show that 11,000 SCI patients are added each year in the United States[1]. SCI patients generally have poor prognosis and often experience physical mobility disorders, which cause tremendous pain and heavy burden to patients and their families. According to the timing of the onset of the disease and the direct cause of the disease, SCI can be divided into primary spinal cord injury (PSCI) and secondary spinal cord injury (SSCI)[2]. More and more studies have found that the immune inflammatory response after SCI plays a key part in the recovery of SCI and post-injury. Researchers have confirmed the role of immune inflammatory response in SCI and its repair process through animal experiments[3-5], putting forward the idea of intervening in the inflammatory response process to promote SCI treatment.

Toll-like receptors (TLRs) are important receptors for macrophages involved in the inflammatory response and play a key role in immune defense. Currently, 13 TLRs have been found in mammalian organisms[6]. Among them, TLR4 is the first mammalian TLRs, which are mainly expressed on the surface of macrophages and dendritic cells. It can specifically recognize Lipopolysaccharide (LPS) derived from

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Gram-negative bacteria. LPS first binds to the LPS binding protein in serum and then is transported to the LPS receptor CD14 on the surface of macrophages, thereby releasing LBP so that TLR4 and LPS jointly activate the activation signal of the macrophages[7]. At the same time, TLR4 activates the Toll-like receptor 4/Nuclear Factor κB (TLR4/NF-κB) signaling pathway. NF-κB is a protein complex widely expressed in eukaryotic cells, which is closely related to pathological and physiological processes such as cell proliferation, apoptosis, immune response and inflammatory response[7]. At rest, it binds to the inhibitory protein IκB as a trimer that is concealed in the cytoplasm and is inactive. When it is activated by stress and pro-inflammatory factors such as tumor necrosis factor (TNF), interleukin 1 (IL-1), etc., NF-κB can be transferred into the nucleus to induce the production of pro-inflammatory cytokines, such as IL-1, IL-6, TNF-α and to induce inflammation reaction. These inflammatory factors can also activate NF-κB cyclically, leading to an enlarged inflammatory response[8]. After spinal cord injury, TLR4/NF-κB signaling pathway is activated; meanwhile, upregulation of multiple cytokine expressions can also negatively activate NF-κB, which causes an inflammatory response in the injured area and induce cascade effect, which is involved in the secondary spinal cord injury.

MicroRNA (miRNA) is a kind of non-coding small RNA of about 20 nucleotides in length, which can be involved in many pathophysiological processes such as growth and development, apoptosis, inflammation and tumor[9]. Yang et al[10] have shown that the expression of multiple miRNAs may be dysregulated after acute SCI, suggesting that miRNA may be involved in the regulation of SCI. miR-128 downregulation can activate the P38 signaling pathway, which helps to alleviate neuropathic pain after SCI. miRNA-21 regulates the glial response after SCI[11]. Besides, miR-133b promotes functional recovery of adult zebrafish after SCI[12]. miRNA-124 can reduce the activation of microglia in SCI model rats[13].

However, the current relationship between the expression of miR-940 and SCI has not been clearly reported. Moreover, there is no definitive study to confirm its role in the prognosis of SCI. This investigation demonstrated that high expression of miR-940 can down-regulate TLR4/NF-κB-mediated inflammatory response and promote SCI recovery, providing a viable reference for the diagnosis and treatment of SCI.

Materials and Methods

Establishment of Mice SCI Model

48 mice were randomly divided into 4 groups. After all the mice were anesthetized, they were placed on the thermostatic pad of the operating table and the neck was raised. The spinous process of the neck 5 was recognized as the center and then a longitudinal incision was made, followed by incision of the skin and fascia. The C4-6 spinous process was exposed and then the C5 laminar was cut off by ophthalmology scissors to reveal the dural sac. Then the position of the spinal cord hitter against the hammer was adjusted to combat the dural sac successfully, while the muscles and fascia were sutured in four lines, and the skin incision was sutured with sutures. Mice were resuscitated on a 37°C pad. This study was approved by the Animal Ethics Committee of Nanjing Medical University Animal Center.

Notes: 1) The simple injury group only received cervical blunt contusion; 2) the injury group + miR-940 mimic group: mice were injected with exogenous miR-940 mimic in the injury area after injury; 3) the injury group + negative control group: mice were injected with of miR-negative control. 4) The sham operation group only used the ophthalmic scissors to remove the C5 lamina, revealed the dural sac, and sutured the muscle fascia and skin incision after hemostasis. After successful modeling, the mice were sacrificed at the indicated time points to collect spinal cord specimens.

Mice Grip Measurement

All mice were subjected to acute grip training 1 week prior to the start of the experiment to stimulate the grip reflection to ensure a stable grip. Mice were lightly lifted by the tail, and when the two forelimbs were close to the crossbar, they could be induced to actively grasp the claw to grasp the crossbar. After the mouse was able to complete the double front paw grip, the unilateral forelimb grip was trained by wrapping one side of the front paw with a small piece of paper tape and using the above method to induce it to grip the crossbar with the other front paw. Each mouse was subjected to 5-10 minutes of grip training daily for 3-5 days until it was able to complete a qualified forefoot grip and left and right forelimb grip. After the mouse was able to perform a satisfactory grip, the grip strength baseline data was measured. Mice were lightly lifted by the tail for them to grasp the crossbar. Then we pulled straight back in the horizontal direction and pulled it off

the crossbar. At this time, the grip force measuring device showed the greatest double forelimb grip strength for resisting pulling. The front paw of one side of the mouse was wrapped with a tape, and the forearm grip strength of the left and right sides was measured by the above method.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) Detection

After the mice were sacrificed by CO_2 asphyxiation, the spinal cord tissue containing the upper and lower 4 mm was cut out from the lesion area and stored in a 1.5 mL centrifuge tube. During the experiment, the spinal cord tissue was taken out into a pre-cooled glass mortar and triturated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA was extracted by sequentially adding chloroform, isopropanol and ethanol, and the RNA concentration was measured using Nanodrop. Total RNA was reverse transcribed into complementary Deoxyribose Nucleic Acid (cDNA) using reverse transcription kit. qPCR was performed with the SYBR mix (TaKaRa, Otsu, Shiga, Japan). The relative expression levels of miR-940, TLR4 and MPO were calculated by $2^{-\Delta\Delta CT}$ method. The primer sequences were as follows: GAPDH (forward): 5'-CGGAGTCAACGGATTTGGTCGTAT-3'; GAPDH (reverse): 5'-AGCCTTCTCCATGGT GGTGAAGAC-3'; miR-940 (forward): 5'-CCTGTCTTACTTTTCCGAAGGAC-3'; miR-940 (reverse): 5'-TTGCTGTATTGTTGCCCATGT-3'; TLR4 (forward): 5'-AAGGCATGGCATGGCTTACAC-3'; TLR4 (reverse): 5'-GGCCAATTTTGTCTCCACAGC-3'; MPO (forward): 5'-GGCCAGCCCTATGGAACT-3'; MPO (reverse): 5'-GCCACTGCCATTGACCTT-3';

Western Blot

Radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) cell lysate containing the protease inhibitor was added to lyse the spinal cord tissue of the mice on ice to extract the total proteins. The protein concentration was measured by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). The sample was electrophoresed in a 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel for about 3 h and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was blocked for 1 h in Tris-Buffered Saline and Tween 20 (TBST) containing 5% skim milk. Then the membranes were incubated with TRL4 antibody (1:500), inducible nitric oxide synthase (iNOS) antibody (1:500), P65 antibody (1:500) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:2000) at 4°C overnight. After being washed 3 times in TBST, horseradish peroxidase-labeled antibody (1:1000) was added for 30 min at room temperature. After being washed 3 times in TBST, protein bands were detected by enhanced chemiluminescence (ECL) method (Thermo Fisher Scientific, Waltham, MA, USA).

Statistics Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 18.0 software (SPSS Inc., Chicago, IL, USA). Data were expressed in terms of mean ± standard deviation ($\overline{x}\pm s$). Differences between the two groups were tested by *t*-test. $p<0.05$ was considered statistically significant.

Results

The Recovery of Forelimb Grip Strength in SCI Group Was Significantly Worse than that in Sham-Operated Group

In the sham operation group, when the grip force was measured at the early stage, the wound was painful due to the surgical wound, and the whole process was not pulled, so that the grip force measurement value temporarily decreased. One week after the operation, the wound gradually recovered and the grip strength gradually returned to the preoperative level. Similarly, in the first week of the SCI group, the motor function of the limbs was severely damaged, while the forepaw could not be used for any action. After 7 days, there was a slight improvement over time. Although mice were able to complete the measurement, the reading number was extremely low, indicating that SCI mice had severe motor dysfunction. At each time point after surgery, SCI mice's forelimb grip strength (Figure 1A), right forelimb grip strength (Figure 1B), and left forelimb grip strength were markedly lower than those in the sham group (Figure 1C). These results suggested that the forearm grip strength of the SCI group was markedly worse than that of the sham-operated group.

The Expression of miR-940 in SCI Group Was Significantly Decreased

After spinal cord injury, the expression of miR-940 was maintained at a stable level in the sham-operated mice at each time point. However, miR-940 expression in the SCI group was remarkably lower than that in the sham-operated group,

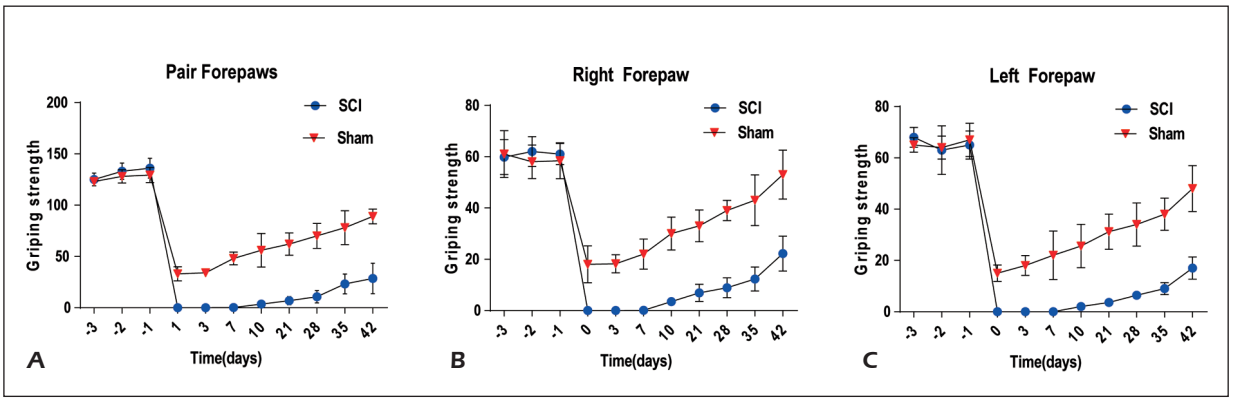

Figure 1. The recovery of forelimb grip strength in the spinal cord injury group (SCI group) was significantly worse than that in the sham-operated group (Sham group). **A**, The recovery of the forelimb grip strength of the mice in the SCI group was significantly smaller than that in the Sham group. **B**, The recovery of right forearm grip strength in SCI group was significantly less than that in Sham group. **C**, The recovery of the left forelimb grip strength of the SCI group was significantly smaller than that of the Sham group.

which decreased as the damage time increased (Figure 2A). At the same time, TLR4 mRNA levels in the SCI group increased with injury time, which was remarkably higher than that of sham operation group (Figure 2B). Furthermore, we detected the protein levels by Western blot and found that the protein expression of TLR4, as well as iNOS in the SCI group, increased with injury time (Figure 2C, 2D). These results indicated that miR-940 was involved in the spinal cord injury and might be related to inflammatory responses.

miR-940 Inhibited Inflammatory Response by Inhibiting TLR4-NF-κB Pathway

To further explore the effect of miR-940 on the TLR4 pathway, we injected the miR-940 mimic sequence into the spinal cord lesion of SCI mice to achieve miR-940 overexpression (Figure 3A). At the same time, we observed that the expression of TLR4 was markedly decreased over time in the miR-940 mimic group (Figure 3B). After overexpression of miR-940, the expression of myeloperoxidase (MPO) was markedly decreased than that of the SCI group (Figure 3C). Similarly, TLR4, P65 (NF-κB family members) and iNOS protein expression levels were also remarkably decreased after miR-940 overexpression (Figure 3D). These results suggested that miR-940 overexpression could inhibit the TLR4-NF-κB pathway, thereby inhibiting the inflammatory response.

miR-940 can Promote the Recovery of Spinal Cord Injury

After the operation, the mice in the SCI group suffered from severe motor function damage in the first week, and could not use the forepaw to perform any movement. When the grip force was measured from 7 d to 10 d, the mouse tried to grasp the measuring rod but could not complete grasp succesfully. After 21 days, the left and right front paws alone and the simultaneous grip strength of the two jaws gradually improved slightly with time. Although the measurement was completed, the reading was significantly lower than that before surgery, indicating that there was a serious movement disorder.

After overexpression of miR-940 in SCI mice, the mice also suffered from severe motor impairment after spinal cord injury. However, after the 7th day of injury, some mice had a preliminary recovery of the forepaw function and that their grip strength gradually improved over time. From 28 d to 42 d after injury, miR-940 overexpression mice had significant grip strength and double paw grip strength compared with the simple injury group (Figure 4A, 4B, 4C). These indicated that the overexpression of miR-940 can promote the recovery of the spinal cord injury in SCI mice.

Discussion

miRNA is a single-stranded, non-coding RNA with a length of more than 20 nucleotides and has a broad regulatory function for protein expression. miRNA binds to the untranslated region of the target mRNA to inhibit its translation process or affect its stability[14]. In the central nervous system such as the spinal cord of mammals, researchers have found that various miRNAs are specifically overexpressed[15,16]. miRNAs play important parts in all aspects of the central nervous system. They

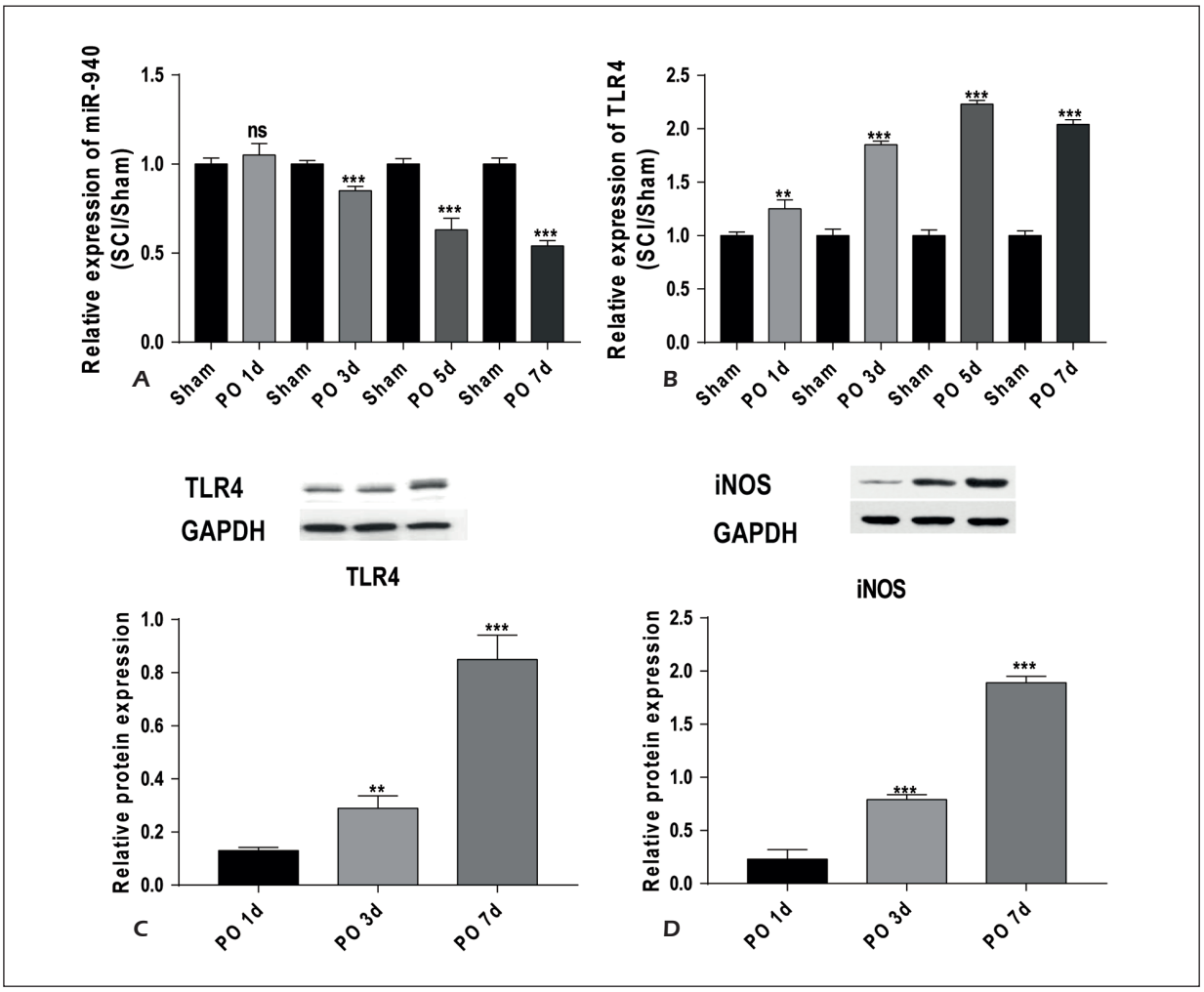

Figure 2. Reduced expression of miR-940 in mice with spinal cord injury (SCI group). **A**, The expression level of miR-940 decreased as the time of injury increased. **B**, After spinal cord injury, the mRNA expression level of TLR4 increases with the increase of injury time. **C**, After spinal cord injury, the protein expression of TLR4 increases with increasing injury time. **D**, After spinal cord injury, the expression level of iNOS protein level increased significantly with time.

are involved not only in the development of nerve cells and the formation of synaptic connections, but also in the differentiation of glial cells and myelination[17,18]. In addition to its extremely important role in maintaining the normal function of the central nervous system, a large number of studies have confirmed the association of miRNA dysregulation with a variety of central nervous system lesions[16]. The miRNA dysregulation after SCI may be involved in the inflammatory reaction, apoptosis, glial scar formation and regeneration inhibition during the injury process. Researchers[19-21] have shown that miR-940 is involved in the migration, metastasis and proliferation of cancers such as gastric and pancreatic cancer, and can be applied to target genes including Wnt/β-catenin, ZNF24, GSK3β and MIEN1. However, there were few investigations about changes in the expression of miR-940 in SCI mice. We found that miR-940 expression was significantly downregulated in SCI mice, suggesting that miR-940 may play a crucial role in SCI.

NF-κB is a nuclear protein found in mammalian mature B cell nuclear extracts and is named for its specific binding to the kappa B site in the immunoprotein kappa light chain gene enhancer. It is now believed that NF-κB is involved in the transcriptional regulation of all genes involved in immune and inflammatory responses. The NF-κB family members include 5 p65, p50, p52, RelB, and c-Rel. Typically, NF-κB is present in the cytoplasm in a homologous or heterodimeric

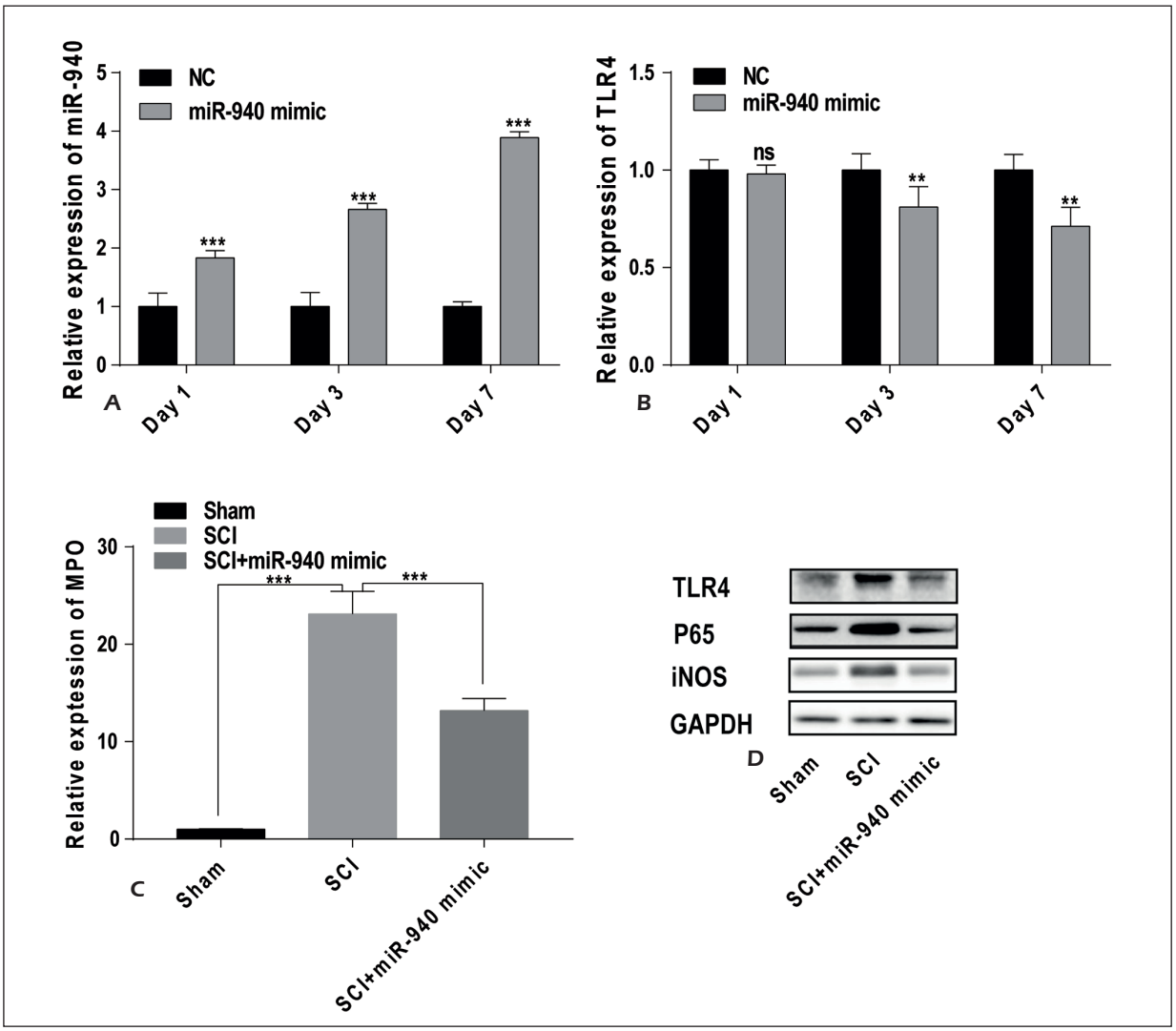

Figure 3. miR-940 inhibits the inflammatory response by inhibiting the TLR4/NF-kB pathway. **A**, The expression of miR-940 in the miR-940 mimic group increased significantly with time after the injection of the interference sequence into the spinal cord injury area of SCI mice. **B**, After the injection of the interference sequence into the spinal cord injury zone of SCI mice, the expression of TLR4 in the miR-940 mimic group decreased with time. After **C**, Overexpression of miR-940, MPO expression decreased compared with SCI group. **D**, After overexpression of miR-940, the expression levels of TLR4, P65 and iNOS protein levels were significantly decreased.

situation, with heterodimers consisting of p65 and p50 being the most typical combination. NF-κB is a cytokine produced by the activation of the classical pathway. It not only mediates the infiltration of inflammatory cells in the lesion, but also aggravates the inflammatory response. In addition, it can promote secondary damage such as apoptosis and glial scar formation. As one of the causes of SCI, NF-κB is found in almost all cells of spinal cord tissue, the most important function of which is to regulate the transcription of inflammation-related genes and the activation of NF-κB signaling pathway, thereby leading to post-SCI inflammatory response and secondary neurological damage. Yin et al[22] found that after SCI, NF-κB levels along with iNOS, caspase 3, TNF-α, IL-1β, and astrocytes were remarkably increased. The inhibition of the NF-κB activity in astrocytes can reduce the expression of inflammatory factors and promote the recovery of the spinal cord function after injury[23]. NF-κB is abnormally highly expressed after SCI, while hyperbaric oxygen therapy reduces inflammation after SCI by downregulating the expression of high mobility family protein B1/NF-κB[24]. Downregulation of the NF-κB signaling pathway by IKK inhibitors can reduce the expression of intercellular cell adhesion molecule-1 (ICMA-1) and reduce the infiltration

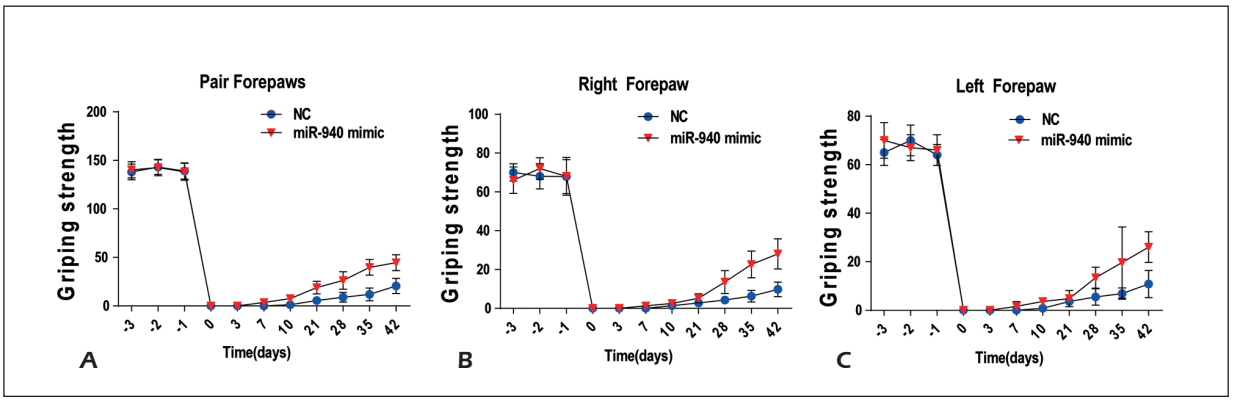

Figure 4. miR-940 can promote spinal cord injury recovery. **A-C**, After overexpression of miR-940, the grip strength of the mice's forelimb, left forelimb and right forelimb was significantly improved.

of inflammatory cells after injury[25]. The inhibition of TLR4/NF-κB/IL-1β pathway by drugs such as minocycline can effectively reduce neuroinflammation and apoptosis after spinal cord ischemia-reperfusion injury[26].

In this research, we found that SCI mice showed significant obstacles in the recovery of grip strength in the forelimbs, left forelimb, and right forelimb when compared to sham operation mice. Meanwhile, the expression of miR-940 was remarkably decreased in SCI mice along with significant elevation of inflammatory response-related factors including TRL4 and iNOS. Then we injected SCI mice with miR-940 mimics into the spinal cord injury area and verified that miR-940 overexpression decreased the expression levels of TLR4 and MPO. At the same time, the overexpression of miR-940 markedly decreased the protein levels of TLR4, P65, and iNOS in SCI mice. In addition, miR-940 overexpression improved the grip strength of the left and right forepaws and the simultaneous grip strength of the two claws of the SCI mice than those of the simple injury group.

Our study demonstrated that high expression of miR-940 could downregulate TLR4/NF-κB-mediated inflammatory responses and promote SCI recovery, providing a viable reference for the diagnosis and treatment of SCI.

Conclusions

We showed that the overexpression of miR-940 can improve the recovery of the spinal cord injury by downregulating the TLR4/NF-κB signaling pathway and inhibiting inflammation and it can be used as a target molecule for treating spinal cord injury.

Conflict of Interest

The authors declare that they have no conflict of interest.

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