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Original Article

The inhibition of microRNA-31 weakens acute spinal cord injury through nuclear factor-κB and TGF-β/Smad 2 in rat

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Abstract: Therefore, the aim of the present study is to evaluate that the therapeutic potential of microRNA-31 after spinal cord injury (SCI) in rats and to expound the potential neuroprotective mechanisms. In SCI model, microRNA-31 expression was up-regulated, compared with negative group. In vitro model, over-expression of microRNA-31 increases cell apoptosis and inflammation, compared with negative control group. Over-expression of microRNA-31 induced nuclear factor-κB (NF-κB), TGF-β and p-Smad 2 protein expression in vitro model of SCI, compared with negative control group. NF-κB inhibitor suppressed the effects of microRNA-31 on inflammation of vitro model of SCI. Meanwhile, TGF-β inhibitor suppressed the effects of microRNA-31 on apoptosis of in vitro model of SCI. The results clearly show that anti-microRNA-31 weakens inflammation and apoptosis by NF-κB and TGF-β/Smad 2 pathway in SCI.

Keywords: MicroRNA-31, spinal cord injury, inflammation, apoptosis, NF-κB, TGF-β/Smad 2

Introduction

Spinal cord injury (SCI) is a severe and disabling disease, of which the incidence is increasing year by year, but there are no effective measures to achieve clinically complete recovery of spinal cord function [1]. After SCI, ischemia and hypoxia, reperfusion injury, lipid peroxidation and expressions of and a variety of inhibiting molecules in local tissues lead to necrosis of nerve cells and axonal demyelination, forming glial scar, which seriously impede axonal regeneration and myelination and affect nerve function recovery [2].

SCI is one of the major causes for disability, of which the pathophysiological processes include primary and secondary trauma injuries. Secondary injury can cause a lot of initial recruitment of inflammatory cells around, resulting in a strong, to cause glial cell death [3]. This inflammatory response of secondary injury plays an important role in the late recovery of SCI. The ability to control and suppress inflammation affects the recovery of SCI. The oxidative stress after SCI plays an important role in the secondary injury mechanism, and the generation of various free radicals has been an indicator to determine the degree of injury [4]. Taking effective measures to prevent or weaken oxidative stress after damage become an effective intervention measure of SCI. Not only cellular oxidative stress response but also cellular necrosis and apoptosis will appear after SCI [5].

TGF-β/Smads signal pathway not only develops an important role on fibrosis of various tissues and scar growth. Particularly, it has a close relation with organs and tissues of renal fibrosis, hepatic fibrosis, pulmonary fibrosis, hypertrophic scar and peritoneum fibrosis, etc [6]. In recent years, overseas researchers have already studied therapeutic difficulties of glial scar after spinal cord injury and have acquired some performance [6, 7].

It is considered in research that miRNA plays an important role in regulating inflammatory response signal pathway and pathological

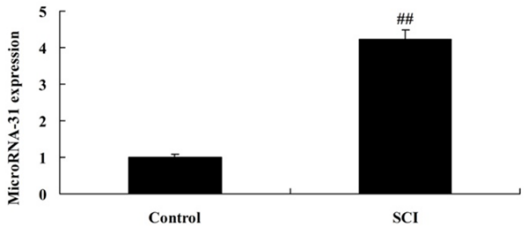

Figure 1. MicroRNA-31 expression of SCI model vitro. Control, control group; SCI, SCI model rat group. ##P < 0.01 compared with control group.

immune response [8]. Numerous miRNAs show changes in expression levels after spinal cord injury [9]. These changes are considered to be related to immune responses, such as immune cell invasion and inflammatory signal pathway regulation [8]. It is found in numerous studies that multiple miRNAs is related to cell apoptosis after spinal cord injury [10]. miRNA is also involved in regulating calcium ion signal and oxidative stress in cell after spinal cord injury [9]. The aim of this study was to investigate the effect of microRNA-31 weakens acute SCI and it might be involved in mechanism.

Materials and methods

Experimental animals and reagents

Healthy male Sprague-Dawley rats (200-250 g) were obtained from the Animal Center. This study was approved by the Scientific Review Committee and the Institutional Review board of Zhangjiagang First People's Hospital, Soochow University. All rats were maintained under a 12 h dark/light cycle (22-24°C, relative humidity 40-60%) and fed a standard laboratory diet and water ad libitum in the laboratory for 2 weeks.

Establishment of animal models

All rats were anesthetized with an intraperitoneal (i.p.) injection of 2.5% ketamine (20 mg/kg). All rats were fixed on the operating table for thoroughly disinfected. T8-T9 spinous processes and lamina were uncovered, and paraspinal muscles were stripped. After rat clamped with forceps, T8 and T9 spinous processes and lamina were removed in order to expose the dura mater. The right side of the spinal cord was sectioned. The right hind limb was indicated as successful SCI model rat. Meanwhile, rats were randomly divided into two groups: control and SCI model group.

Quantitative RT-PCR detection

TRIZOL reagent (Invitrogen, USA) was used to extract total RNA from tissues or cell. A large capacity complementary DNA (cDNA) library kit (Applied Biosystems) was used to synthesize cDNA. Quantitative RT-PCR was quantitated using Power SYBR Green PCR (MasterMix; Applied Biosystems).

Cell lines and transfection

The astrocyte cell lines CTX TNA2 cell was cultured in Dulbecco's Modified Eagle's Medium (DMEM; HyClone, Logan, UT, USA) with 10% fetal bovine serum (FBS; Gibco, Mulgrave Victoria, Australia) at 37°C and 5% CO_2. MicroRNA-31, anti-microRNA-31 and control negative mimics were purchased from by Guangzhou Ribo Bio Co., Ltd. (Guangzhou, China). These mimics were transfected into cell using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.).

Cell proliferation

Cell after transfection for 24, 48 and 72 h, and stained with MTT assay (120 μL, 5 mg/ml) for 4 h at 37°C. Then, DMSO assay was added into cell after removing old Medium for 20 min at 37°C. The absorbance at 490 nm was measured in a spectrophotometer (Bio-Rad Laboratories, California, USA).

Apoptotic cell proportions

Cell after transfection for 48 h was washed with PBS, and stained with annexin-V-fluorescein isothiocyanate/propidium iodide (Annexin-V-FITC/PI, BD Biosciences, San Jose, CA, USA) for 15 min at darkness. Apoptosis was measured using cytometric analysis (BD FACS Calibur™, BD Biosciences, San Jose, CA, USA).

Measurement of inflammation factor

The supernatant of cell after transfection for 48 h was used to assess activities of TNF-α, IL-6, IL-1β and IL-18 following the manufacturer's protocol (Jiancheng Bioengineering Institute, Nanjing, China).

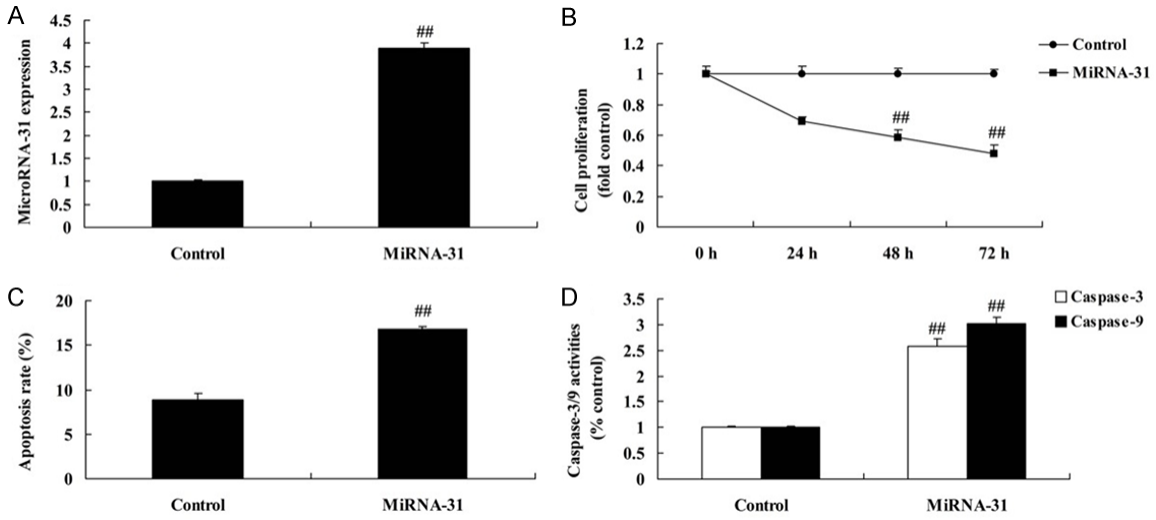

Figure 2. Over-expression of microRNA-31 increases cell apoptosis. MicroRNA-31 expression (A), cell proliferation (B), cell apoptosis rate (C) and caspase-3/9 activity (D). Control, control negative group; MiRNA-31, Over-expression of microRNA-31 group. ##P < 0.01 compared with control group.

Western blot analysis

Cell after transfection was split using lysis buffer (10 mM NaCl, 0.1 mM EDTA, 20 mM Tris-acetate, 1 mg/mL aprotinin, pH 7.4) and homogenized. The samples were centrifuged at 12,000 g for 10 minutes at 4°C. The protein concentration was performed using BCA assay kit (Beyotime Bioengineering Institute, Nanjing, China). Equal protein was resolved on 10% (w/v) SDS-PAGE gel and transferred onto polyvinylidene difluoride (PVDF) membranes. The membrane was blocked with 5% non-fat milk to block nonspecific binding sites for 2 h at room temperature. Then, membrane was incubated with the following primary antibodies overnight at 4°C: anti-NF-κb (1:1000, Santa Cruz Biotechnology, Inc, Calif, USA), anti-TGF-β (1:1000, Santa Cruz Biotechnology, Inc, Calif, USA), anti-p-Smad 2 (1:1000, Santa Cruz Biotechnology, Inc, Calif, USA), and anti-GAPDH (1:500, Santa Cruz Biotechnology, Inc, Calif, USA). After washing, the membrane was incubated for 2 h at room temperature with secondary antibodies. The analysis of the bands was resolved by a gel image analysis system (Media Cybernetics, Rockville, MD, USA).

Measurement of caspase-3/8/9 activity

The activity of caspase-3 was measured using a colorimetric assay kit following the manufacturer's protocol (Jiancheng Bioengineering Institute, Nanjing, China). Homogenates were incubated with 100 μL tissue lysis buffer for 30 min on ice. 2 μL of caspase-3/8/9 activity kits was added into equal protein solution and incubated at 37°C for 4 h in the dark. Then the activity of caspase-3 was measured at an absorbance of 405 nm.

Statistical analysis

The data are expressed as the mean ± SEM and statistical analysis was performed using SPSS 16.0 using one-way ANOVA followed by Dunnett's test. A value of $P < 0.05$ was considered statistically significant.

Results

MicroRNA-31 expression of SCI model in vitro

To explore that the neuroprotective effect of microRNA-31 influence on development and progression of SCI, we analyzed the change of microRNA-31 in normal rat or SCI rat. In SCI model, microRNA-31 expression was up-regulated, comparison with control group (**Figure 1**).

Over-expression of microRNA-31 increases cell apoptosis

To discovery that the effect of microRNA-31 on SCI, cell apoptosis was measured after over-expression of microRNA-31. The expression of microRNA-31 level in microRNA-31 group was

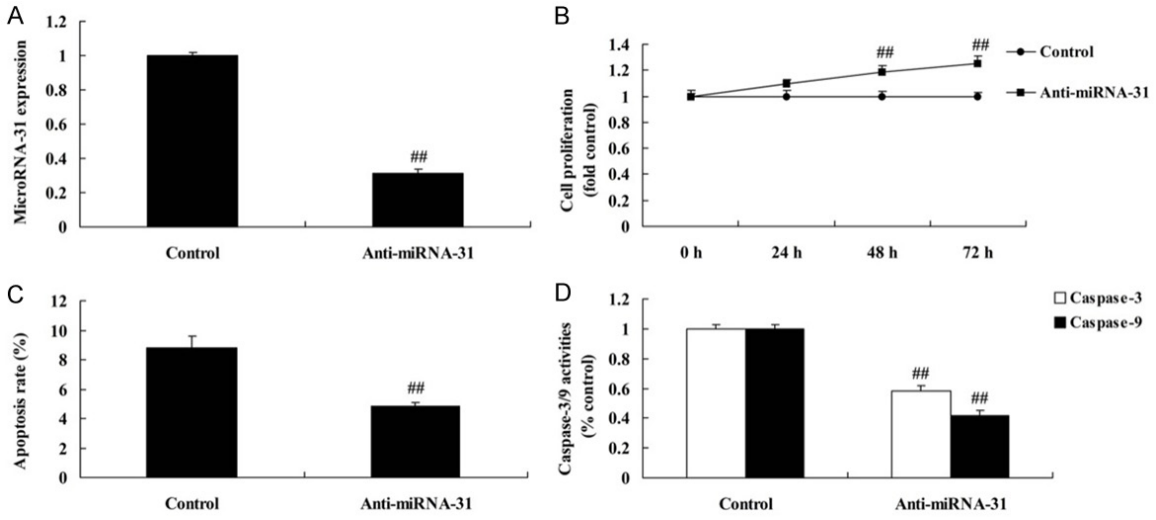

Figure 3. Down-expression of microRNA-31 decreases cell apoptosis. MicroRNA-31 expression (A), cell proliferation (B), cell apoptosis rate (C) and caspase-3/9 activity (D). Control, control negative group; Anti-miRNA-31, down-expression of microRNA-31 group. ##P < 0.01 compared with control group.

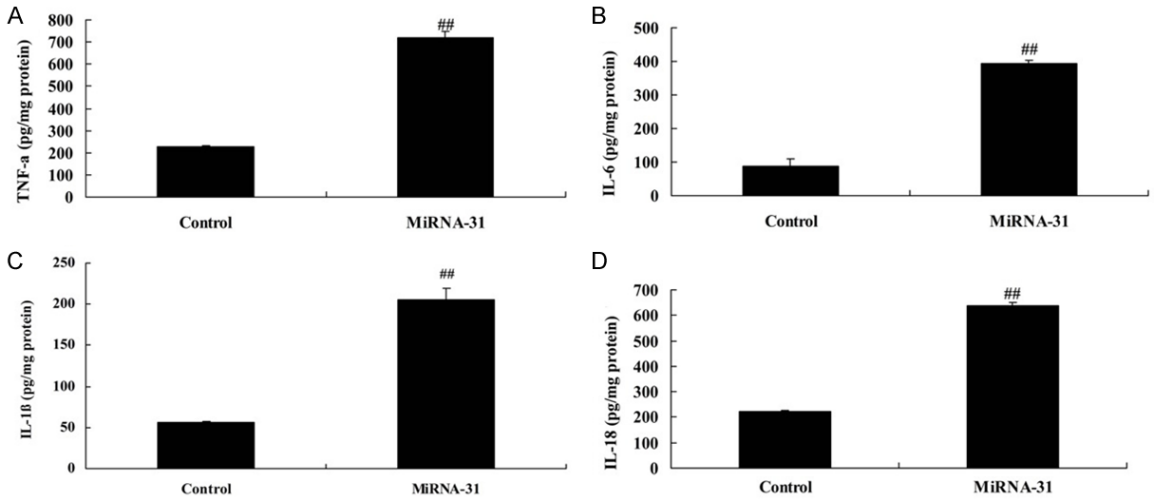

Figure 4. Over-expression of microRNA-31 increases inflammation. TNF-α (A), IL-6 (B), IL-1β (C) and IL-18 (D) levels. Control, control negative group; MiRNA-31, Over-expression of microRNA-31 group. ##P < 0.01 compared with control group.

higher than that of control group (**Figure 2A**). However, over-expression of microRNA-31 inhibited cell proliferation, and increased apoptosis rate and caspase-3/9 activity in vitro model of SCI, comparison with control group (**Figure 2B-D**).

Down-expression of microRNA-31 decreases cell apoptosis

Next, the expression of microRNA-31 level in microRNA-31 group was lower than that of control group (**Figure 3A**). Down-expression of microRNA-31 increased cell proliferation, and decreases cell apoptosis rate and caspase-3/9 activity in vitro model of SCI, comparison with control group (**Figure 3B-D**).

Over-expression of microRNA-31 increases inflammation

To explain that the effects of microRNA-31 on inflammation of SCI, the TNF-α, IL-6, IL-1β and IL-18 levels were analyzed with colorimetric

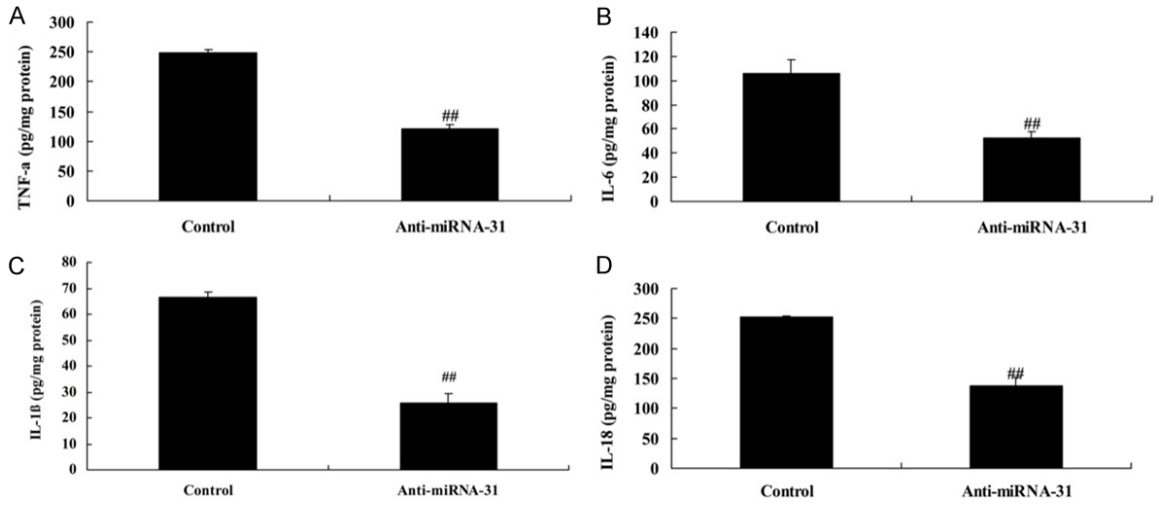

Figure 5. Down-expression of microRNA-31 decreases inflammation. TNF-α (A), IL-6 (B), IL-1β (C) and IL-18 (D) levels. Control, control negative group; Anti-miRNA-31, down-expression of microRNA-31 group. ##P < 0.01 compared with control group.

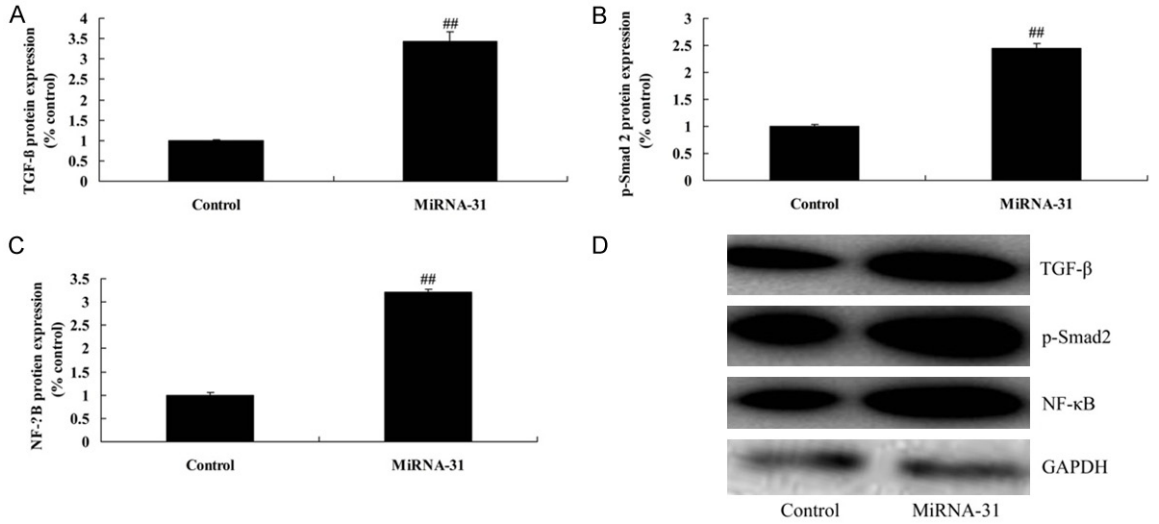

Figure 6. Over-expression of microRNA-31 induced NF-κB, TGF-β and p-Smad 2 protein expression. NF-κB, TGF-β and p-Smad 2 protein expression by statistical analysis (A-C), and western blotting assays (D). Control, control negative group; MiRNA-31, Over-expression of microRNA-31 group. ##P < 0.01 compared with control group.

assay kits in this study. Over-expression of microRNA-31 increased TNF-α, IL-6, IL-1β and IL-18 levels in vitro model of SCI, comparison with control group (**Figure 4**).

Down-expression of microRNA-31 decreases inflammation

Down-expression of microRNA-31 decreases TNF-α, IL-6, IL-1β and IL-18 levels in vitro model of SCI, comparison with control group (**Figure 5**).

Over-expression of microRNA-31 induced NF-κB, TGF-β and p-Smad 2 protein expression

To discovery that the mechanism effect of microRNA-31 on SCI, the anti-inflammatory and anti-apoptosis activity of microRNA-31 was analyzed in this study. As showed in **Figure 6**, over-expression of microRNA-31 induced NF-κB, TGF-β and p-Smad 2 protein expression in vitro model of SCI, comparison with control group.

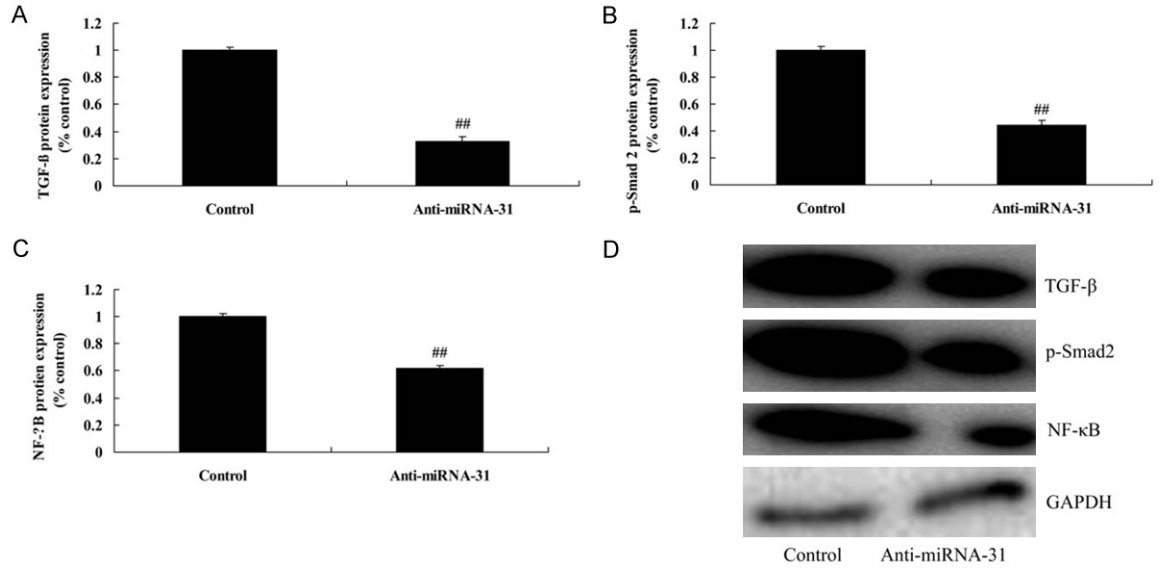

Figure 7. Down-expression of microRNA-31 suppressed NF-κB, TGF-β and p-Smad 2 protein expression. NF-κB, TGF-β and p-Smad 2 protein expression by statistical analysis (A, B and C), and western blotting assays (D). Control, control negative group; Anti-miRNA-31, down-expression of microRNA-31 group. ##P < 0.01 compared with control group.

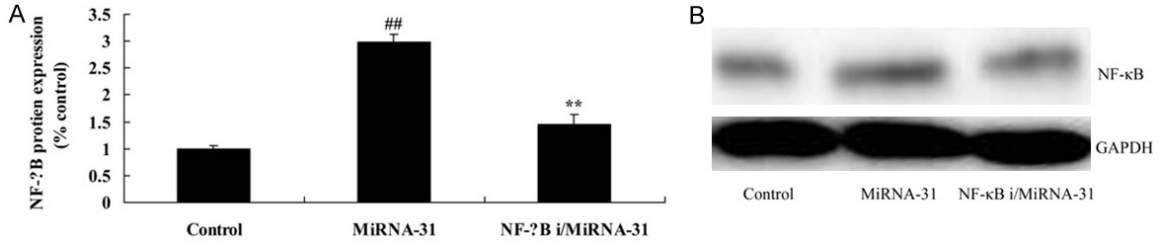

Figure 8. The inhibition of NF-κB reduced the effects of microRNA-31 on NF-κB protein expression of SCI. NF-κB protein expression by statistical analysis (A), and western blotting assays (B). Control, control negative group; MiRNA-31, Over-expression of microRNA-31 group; NF-κB i/MiRNA-31, Over-expression of microRNA-31 group and NF-κB inhibitor. ##P < 0.01 compared with control group, **P < 0.01 compared with Over-expression of microRNA-31 group.

Down-expression of microRNA-31 suppressed NF-κB, TGF-β and p-Smad 2 protein expression

Then, we found that down-expression of microRNA-31 suppressed NF-κB, TGF-β and p-Smad 2 protein expression in vitro model of SCI, comparison with control group (**Figure 7**).

The inhibition of NF-κB reduced the effects of microRNA-31 on inflammation of SCI

We further check into whether NF-κB pathway played a protection role in protective effect of anti-microRNA-31 in inflammation of SCI. NF-κB inhibitor, Curcumin, inhibited the microRNA-31-induced NF-κB protein expression in vitro model of SCI, comparison with microRNA-31 group (**Figure 8**). Meanwhile, the inhibition of NF-κB reduced the effects of microRNA-31 on TNF-α, IL-6, IL-1β and IL-18 levels in vitro model of SCI, comparison with microRNA-31 group (**Figure 9**).

The inhibition of TGF-β reduced the effects of microRNA-31 on cell apoptosis of SCI

To check into whether TGF-β protein expression played a protection role in protective effect of anti-microRNA-31 in SCI, TGF-β and p-Smad 2 protein expressions were measured using Western blot analysis. TGF-β inhibitor, SIS3 HCl, suppressed the microRNA-31-induced TGF-β and p-Smad 2 protein expressions in vitro model of SCI, comparison with microRNA-31 group

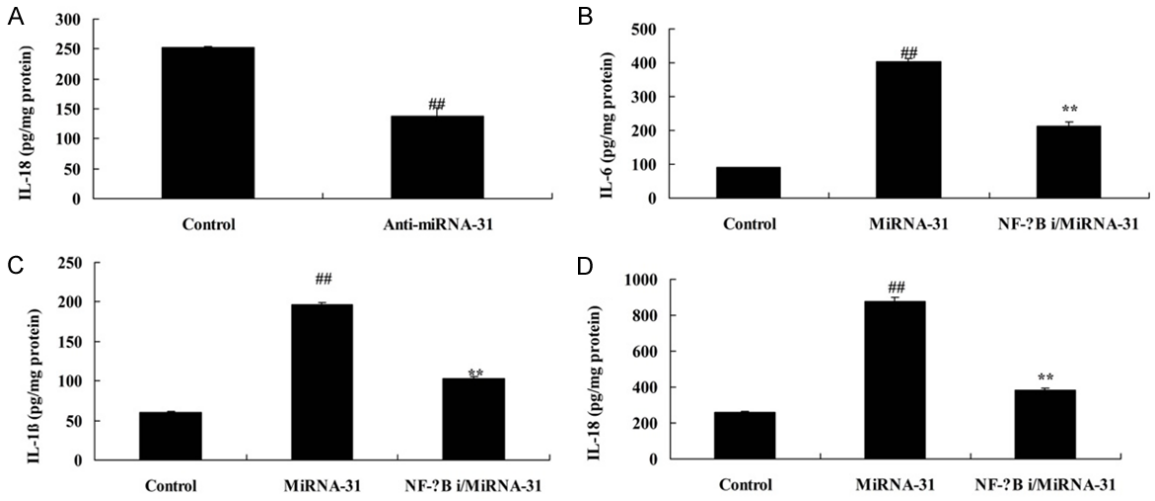

Figure 9. The inhibition of NF-κB reduced the effects of microRNA-31 on inflammation of SCI. TNF-α (A), IL-6 (B), IL-1β (C) and IL-18 (D) levels. Control, control negative group; MiRNA-31, Over-expression of microRNA-31 group; NF-κB i/MiRNA-31, Over-expression of microRNA-31 group and NF-κB inhibitor. ##P < 0.01 compared with control group, **P < 0.01 compared with Over-expression of microRNA-31 group.

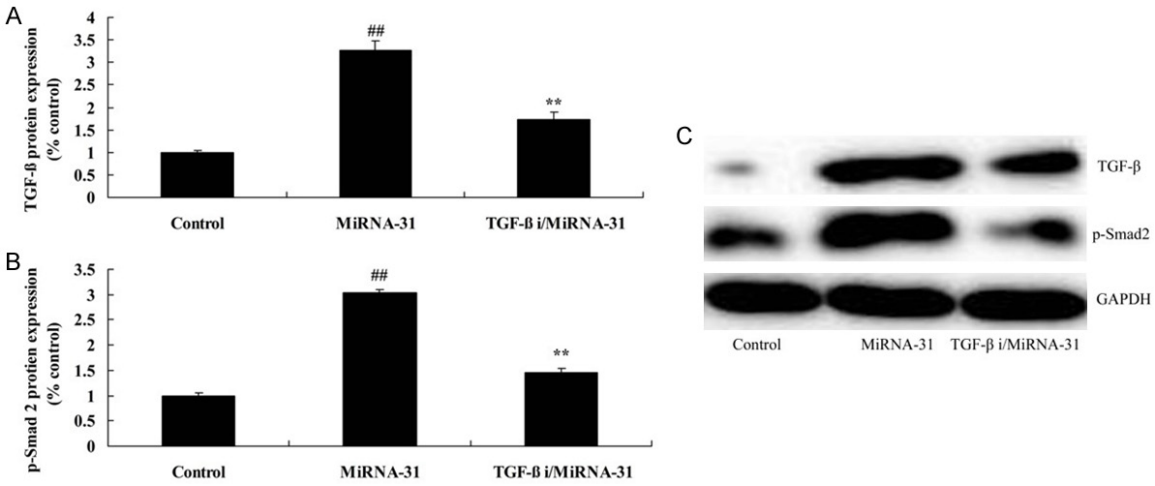

Figure 10. The inhibition of TGF-β reduced the effects of microRNA-31 on TGF-β and p-Smad 2 protein expression of SCI. TGF-β and p-Smad 2 protein expression by statistical analysis (A and B), and western blotting assays (C). Control, control negative group; MiRNA-31, Over-expression of microRNA-31 group; TGF-β i/MiRNA-31, Over-expression of microRNA-31 group and TGF-β inhibitor. ##P < 0.01 compared with control group, **P < 0.01 compared with Over-expression of microRNA-31 group.

(**Figure 10**). The inhibition of TGF-β reduced the effects of microRNA-31 on the inhibition of cell proliferation and activation of cell apoptosis and caspase-3/9 activity of SCI, comparison with microRNA-31 group (**Figure 11**).

Discussion

In secondary injury of SCI, the view that cell apoptosis occurs in neuronal injury site has been recognized, while there are different views about the pathological changes after SCI on the corresponding motor cortex neurons [11]. If retrograde secondary pathological changes (such as apoptosis, death, etc.) appear in the motor cortex neurons after SCI, even though the damaged axons in spinal cord can be regenerated or the structure and function can be rebuilt, motor function below the surface of injury cannot be completely recovered [12]. The study showed that In SCI model, microRNA-31 expression was up-regulated,

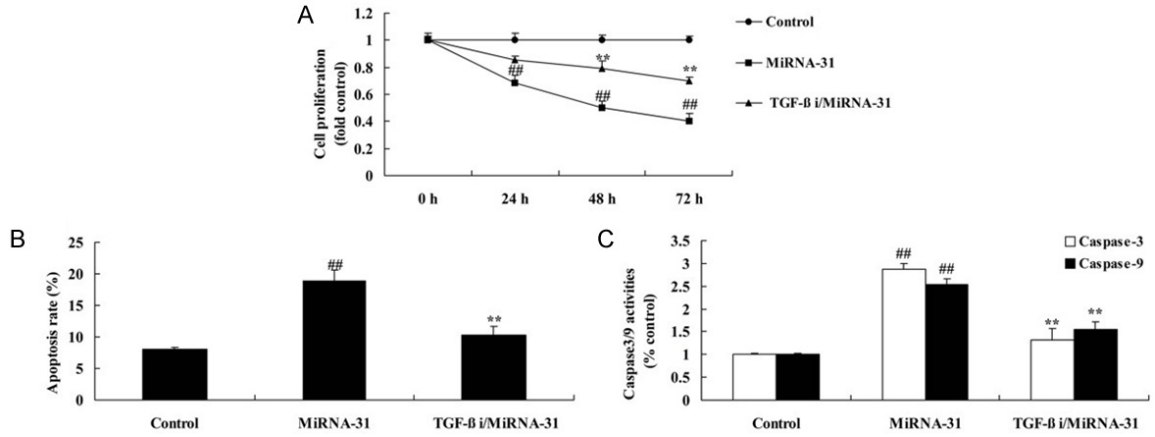

Figure 11. The inhibition of TGF-β reduced the effects of microRNA-31 on cell apoptosis of SCI. Cell proliferation (A), cell apoptosis rate (B) and caspase-3/9 activity (C). Control, control negative group; MiRNA-31, Over-expression of microRNA-31 group; TGF-β i/MiRNA-31, Over-expression of microRNA-31 group and TGF-β inhibitor. ##P < 0.01 compared with control group, **P < 0.01 compared with Over-expression of microRNA-31 group.

comparison with control group. Therefore, microRNA-31 might be one potential factor for SCI.

Inflammatory cytokine is a major neurotransmitter in regulating the immune reaction, which is a bridge to connect the immune cells with immune cells, immune cells and other cells [13]. IL-1β and IL-6 are mainly secreted by activated macrophages/microglia, and can also be secreted in neurons and glial cells in early SCI [14]. As neurotransmitters of intercellular signaling, inflammatory IL-1β and IL-6 regulate the inflammation of central nervous system, autoimmune reactions, trauma and other defense reactions. IL-1β can promote the infiltration polymorphonuclear cells and macrophage in blood into inflammation sites, and IL-6 can promote activation of astrocyte, resulting in the formation of glial scar, which is an important factor in leading to permanent loss of nerve function [15]. In this study, over-expression of microRNA-31 increases cell apoptosis and inflammation, compared with negative control group. Olaru et al. identified microRNA-31 expression levels increase inflammation in inflammatory bowel disease patients.

NF-κB is extensively participated in stress reaction like inflammatory response and radioactive damage [16]. It has known that NF-κB is involved in signal transduction during nerve cell apoptosis, its express level may indicate whether nerve cells are alive or not, and it is the emergency response of cells for different injures. It can be seen from the process of neuron and glial cells apoptosis after spinal cord injury that increase of NF-κB express shows changes of signal transduction pathway may exist after spinal cord injury [17]. In this study, we demonstrated that over-expression of microRNA-31 induced NF-κB protein expression in vitro model of SCI, comparison with control group. Yan et al. identify that microRNA-31 promotes epidermal hyperplasia by repressing protein phosphatase 6 in psoriasis through NF-κB expression [18].

It has an important role on studying the astrocyte and high TGF-β expression after spinal cord injury of rats and reminds astrocyte and TGF-β in secondary lesion of spinal cord injury. Hamada et al. shows that locomotor function is recovered obviously within 5 d after giving TGF-β to rats' spinal cord injury, but afterwards, the results show that growth of glial scar is more obvious than the saline treatment group [19]. In the model of TFG-β curing spinal cord injury that after spinal cord injury, astrocyte, TGF-β is tumor stimulating factors and mononuclear macrophage increase expression [6]. After giving external TGF-β, proliferation of astrocyte is more obvious. Buss et al. studies the relation between TFG-β and TGF-Bβ2 after spinal cord injury and shows that early increase of TGF-β1 is related to the inflammatory response and formation of glial scar. TGF-β2 is related to the maintenance of glial scar, as well as TGF-β/Smads signal pathway, etc. [20]. The current studies indicate that genesis and devel-

opment of fibrosis in multiple organs and tissues, such as renal fibrosis, hepatic fibrosis, pulmonary fibrosis, hypetrophic scar fibrosis and peritoneum fibrosis have a close relation with TGF-β/Smads signal pathway [6]. In this study, we found that over-expression of microRNA-31 induced TGF-β and p-Smad 2 protein expression in vitro model of SCI, comparison withcontrolgroup.Katsuraetal.showedthatmicroRNA-31 modulates endothelial-mesenchymal transition and associated secretory phenotype induced through TGF-β [21].

In summary, we demonstrated that pretreatment with anti-microRNA-31 protects against SCI., these protective effects were partially dependent on suppression of inflammation and apoptosis, through NF-κB and TGF-β/Smad 2 pathway in SCI rat. Our results imply that the neuroprotective effect of anti-microRNA-31 may be useful in the therapy of SCI.

Disclosure of conflict of interest

None.

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