Abstract. – OBJECTIVE: To explore the role of microRNA (miR) 137 in spinal cord injury and its mechanism.

MATERIALS AND METHODS: The model of spinal cord injury in mice was established to detect the recovery differences of grip strength in upper and lower limbs of mice. The expressions of miR-137 and neuronal differentiation 4 (NEUROD4) were detected at the same time. The inflammation level and the oxidative stress response after spinal cord injury were subsequently detected after overexpression of miR-137. Target genes of miR-137 were identified by bioinformatics. Finally, dual-luciferase reporter gene assay was used to identify the target genes of miR-137.

RESULTS: By establishing the model of spinal cord injury in mice, the strength of upper and lower limbs recovered after 7 days of injury in mice. The expression of miR-137 in spinal cord injury was found to decrease in a time-dependent manner by quantitative Real-time polymerase chain reaction (qRT-PCR), while the expression of NEUROD4 gradually increased. Inflammation indicators and oxidative stress level were found to be significantly higher after spinal cord injury. However, the inflammation level and oxidative stress were significantly reduced after transfection of miR-137. Finally, we predicted the target gene of miR-137 through bioinformatics website and found that NEUROD4 was a potential target gene of miR-137. Using dual luciferase reporter assays, we found that NEUROD4 bound to miR-137. After overexpression of miR-137, the expression of NEUROD4 was significantly reduced. Overexpression of NEUROD4 could promote spinal cord injury inflammation and oxidative stress. After intracellular transfection of NEUROD4 and miR-137 at the same time, the inflammation level and oxidative stress of spinal cord injury decreased significantly.

CONCLUSIONS: These results suggested that miR-137 promoted the recovery of spinal cord injury by degrading NEUROD4 to relieve the spinal cord inflammation and the progression of oxidative stress, thus promoting the recovery of spinal cord injury.

Key Words: Spinal cord injury, miR-137, NEUROD4, Oxidative stress.

Introduction

Spinal cord injury is a common spine surgical injury that often causes great physical and psychological trauma. It brings financial burden to patients and their families due to impaired sensory motor function, which is difficult to recover. The current incidence of spinal cord injury has been high and increasing year by year. Current literature showed that the annual incidence of new diseases in Asian countries and regions was about 19.5-56.1 cases/million. In the United States, about 11,000 new cases were reported each year, with a morbidity of about 54 cases/million. The current prevalence was about 1,275,000 cases. Injury mechanism of the traumatic spinal cord injury can be divided into blunt, pulling, cutting, tearing, etc., among which the most common form was blunt compression injury caused by violent impact. The cervical spine injury was the most common segment, accounting for more than half of all injuries. For patients with cervical spinal cord injury, sensory dyskinesia can affect limbs, seriously affecting patients’ self-care ability and life quality. In particular, the recovery of upper limb function in patients with cervical spinal cord injury was even more important.
MicroRNA (miRNA) is a single-stranded, non-coding RNA over 20 nucleotides in length and has a wide range of functions in regulating protein expression. Under RISC mediation, its “seed sequence” binds to the target mRNA untranslated region, thereby inhibiting its translation process, or affecting its stability. In the spinal cord and other central nervous system of mammals, researchers detected a variety of miRNAs that were highly expressed with tissue specificity. MicroRNA plays an important role in all aspects of the central nervous system. It not only participates in the occurrence and development of nerve cells, but also in the synaptic connections. It is also associated with glial differentiation and myelination. In addition to maintaining the normal function of the central nervous system, a large number of studies also confirmed that the microRNA imbalance was related to a variety of central nervous system diseases. However, the imbalance of microRNA after spinal cord injury may be involved in all aspects of inflammation, apoptosis, glial scar formation and regeneration inhibition in the process of injury.

Materials and Methods

Experimental Animals
Thirty 8-10 week-old female C57bl/6J mice weighing 20-25 g were utilized, fed in a normal light cycle with normal diet. Experimental animals were purchased from the Jackson Lab (Bar Harbor, MI, USA). Grip training and gripping strength meter (GSM) baseline data measurements were adjusted according to previous studies. Grip strength of left and right single forelimb was recorded for 4 times, and the average value was calculated. This study was approved by the Animal Ethics Committee of Huai’an First People’s Hospital Animal Center.

Establishment of Sham Operation Group and C5 Spinal Cord Contusion Model
Mice in the injury group were incised longitudinally in the neck, the skin and fascia dissected, and the C4-6 spinous processes were revealed. Next, the C5 lamina was excised to expose the dural sac, and the C5 spinal cord was injured. After the successful injury, bleeding was stopped, and muscles, fascia and skin incision were sutured. Dural sac was exposed by the same way. Protection was exerted in order not to damage the dural sac and spinal cord.

Measurement of Forelimb Grip Strength
4 successful measurements of grip strength were recorded, and the average value was recorded. If the mouse was unable to grasp the crossbar due to severe forelimb movement disorder, grip score was marked as 0. For the measurement of forelimb locomotor scale (FLS), mice were placed in an open field of 90 × 120 cm for video recording and observation for 4 min. The FLS score was recorded separately by two experimenters and divided into 0-17 points according to the severity.

Cell Culture
Mouse spinal cord tissue was removed and cultured in a mixed medium with cells for 2 days. Next, the medium was replaced by Dulbecco’s Modified Eagle Medium (DMEM, KenGen, Nanjing, China) containing 20% fetal bovine serum (FBS, Gibco, Rockville, MD, USA). After 10 days, microglia cells were separated.

Total RNA Extraction From Spinal Cord Tissue
Quantitative Real-time polymerase chain reaction (qRT-PCR) was used to detect the mRNA level from tissues of mouse spinal cord and the cells transfected with miR-NC and miR-137 mimic, respectively. Total RNA from tissues or cultured cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). qRT-PCR analysis was performed using a PrimeScript reverse transcription kit and SYBR Premix (TaKaRa, Otsu, Shiga, Japan), and the results were normalized with the expression of phosphoglycerate dehydrogenase (GAPDH).

Western Blotting
Cells transfected for 48 h and injured tissues were collected and lysed by radioimmunoprecipitation assay (RIPA) to extract the total protein. After sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and membrane transformation, antibodies of neuronal differentiation 4 (NEUROD4), inducible nitric oxide synthase (iNOS), and endothelial nitric oxide synthase (eROS) (Invitrogen, Carlsbad, CA, USA) were added for incubation at 4°C overnight, followed by horseradish peroxidase (HRP)-rabbit IgG (Invitrogen, Carlsbad, CA, USA) incubation. Chromogenic agent was used for protein band exposure and Gene Tools software was used to analyze protein expressions.
Dual Luciferase Reporter Assay

The 3'-UTR of NEUROD4 was inserted into the vector, transformed and extracted into the plasmid. 150 ng of the plasmids were mixed with 3 pmol of miR-137 mimics and mimics control, and added into 25 μL of opti-MEM medium containing 1 μL of Lipofectamine for 30 min incubation at room temperature. After incubation, the mixture was added in corresponding wells containing glial cells, and incubated for 24 h. Dual luciferase assay was then performed according to protocol from the American Gene Copoeia (Rockville, MD, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

The production of TNF-α and IL-10 in cell supernatants were measured using ELISA kits (Boster, Wuhan, China) according to the manufacturer’s instructions. The OD value at 450 nm wavelength was recorded, and the concentration was calculated based on standard curve.

Statistical Analysis

All data were expressed as the average ± standard deviation. Statistical product and service solutions 17.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Comparison between groups was done using One-way ANOVA test followed by Least Significant Difference (LSD). $p < 0.05$ indicated statistically significant difference between groups.

Results

Recovery of Grip Strength in Spinal Cord Injury Group is Significantly Lower Than That in Sham Operation Group

Mice in the sham operation group were unable to complete the full stretch at the early stage when we carried out the measurement of grip force, resulting in a temporary drop in the measurement of grip strength. 1 week after the operation, the grip strength gradually recovered to preoperative level. After surgery, mice in the injury group could not use the forepaw for any action within the first week, and they could not resist the stretch from 7 d-14 d to complete the measurement (Figure 1A, 1B and 1C). After 21 d-56 d, grip strength of single or both forepaws gradually improved slightly over time (Figure 1A-C). At each time point after operation, the grip strength of mice in the injury group was significantly lower than that of the sham operation group ($p < 0.05$).

Expression of miR-137 in Spinal Cord Injury Group Decreases and the Expression of NEUROD4 increases

The dysregulated microRNAs in spinal cord injury have been published in previous studies (Figure 2A). We found that the expression level of miR-137 gradually decreased in the first 7 days after injury. To confirm this finding, we evaluated the expression level of miR-137 and NEUROD4. The expression of miR-137 in the spinal cord of mice from the sham operation group on the first day after operation was taken as the control.

Figure 1. Significant recovery dysfunction of the grip strength in the upper and lower limbs after spinal cord injury. A, Recovery of grip strength in mice forelimbs from spinal cord injury group was significantly less than those from the sham operation group. B, Recovery of grip strength in right forelimb from spinal cord injury group was significantly less than those from the sham operation group. C, Recovery of grip strength in left forelimb from spinal cord injury group was significantly less than those from the sham operation group. Grip strength of mice in the injured group was lower than that in the sham operation group at each time point after operation.
QRT-PCR result demonstrated that the expression of miR-137 in the injured group gradually decreased (Figure 2B). At the same time, NEUROD4 expression significantly increased in a time-dependent manner (Figure 2C-D). These results showed that miR-137 played a protective role in the process of spinal cord injury, while NEUROD4 mainly inhibited the repair process during the repair of spinal cord injury.

**MiR-137 Relieves the Inflammation Level and Reduces Oxidative Stress**

To test the changes of cytokines induced by miR-137 during spinal cord injury, we performed ELISA assay. We found that the expressions of inflammatory cytokines, including TNF-α and IL-6 in spinal cord injury group were significantly increased, whereas their expressions significantly decreased after transfection of miR-137 (Figure 3A). To investigate the role of miR-137 in oxidative stress induced by spinal cord injury, we examined the expressions of SEPN1 and GPX1 (Figure 3B). We found that the expressions of SEPN1 and GPX1 significantly decreased after miR-137 transfection. In addition, overexpression of miR-137 significantly decreased the inducible nitric oxide synthase and eROS (endothelial nitric oxide synthase) induced by spinal cord injury (Figure 3C and 3D). These results showed that miR-137 could inhibit the inflammatory response and reduce oxidative stress, which significantly relieved spinal cord injury.

**MiR-137 Targets the Degradation of NEUROD4**

Through bioinformatics analysis, we found that NEUROD4 was a potential target gene of miR-137 (Figure 4A). To confirm whether there was a binding site of miR-137 with NEUROD4, we performed dual luciferase reporter assay. The results suggested that miR-137 significantly reduced the luciferase activity of wild-type NEUROD4, but did not reduce the activity of mutation type (Figure 4B). In order to validate the above results, we found that the mRNA and protein expression of NEUROD4 was significantly reduced after overexpression of miR-137 (Figure 4C-D). These results illustrated that NEUROD4 was the downstream target of miR-137. Therefore, it suggested that miR-137 promoted the recovery of spinal cord injury by targeting the degradation of NEUROD4 to relieve the inflammatory level and oxidative stress in spinal cord injury.
Figure 3. MiR-137 reduces levels of inflammatory cytokines and oxidative stress. **A**, MiR-137 significantly reduced the level of inflammatory cytokines. **B**, **C**, and **D**, MiR-137 significantly reduced the level of oxidative stress.

Figure 4. NEUROD4 is the target of miR-137. **A**, The binding sites of NEUROD4 and miR-137. **B**, The luciferase activity of NEUROD4 down regulated by miR-137. **C**, The mRNA level of NEUROD4 significantly decreases in miR-137 mimic group. **D**, The protein level of NEUROD4 significantly reduces in miR-137 mimic group.
Discussion

The animal model used in this experiment was a modified model of C5 spinal cord contusion, using the Infinite Horizon spinal cord striker to accurately quantify the spinal cord injury. The advantage of this model was the precise and controllable dose of striking. In this study, the method used for the animal model establishment was similar to spinal cord injury in humans. MicroRNAs play a regulatory role in gene expression under both normal physiological and pathological conditions. Recent studies have identified that there were many differentially expressed miRNAs in the central nervous system, such as in the mouse spinal cord. MiRNAs are involved in the regulation of various transcription factors that play important roles in the development and function establishment of the spinal cord, such as neuronal cell differentiation and cell type maintenance. It has been reported that inflammation induced by IL-7 could be effectively reduced by down-regulating miR-136-5p through NF-kB/A20. MiR-99b-5p modulated neural spinal cord injury in mice via Mtor pathway. In addition, miR-214-3p and miR-211 played essential role in the repair of spinal cord injury. Therefore, studying the role of microRNA in spinal cord injury can provide a basis for future treatment.

We first established a spinal cord injury model and then examined the grip strength recovery of the upper and lower limbs of mice. It was found that the strength of upper and lower limbs began to recover in mice 7 days after injury. Next, by searching the literature, we found that the expression of miR-137 in spinal cord injury decreased with time prolonged. At the same time, we found that the expression of NEUROD4 increased gradually with the increase of time. Subsequently, we examined the inflammatory and oxidative stress levels after spinal cord injury, and found that the expressions of TNF-α and IL-6 and other inflammation markers were significantly increased. Inflammation level and oxidative stress were significantly decreased after miR-137 transfection. Finally, we predicted the target gene of miR-137 through bioinformatics website and found that NEUROD4 was a potential target gene of miR-137. Using dual luciferase reporter assays, it was found that NEUROD4 bound to miR-137. After overexpressing miR-137, the expression of NEUROD4 was significantly reduced. These results suggested that miR-137 promoted the recovery of spinal cord injury by targeting the degradation of NEUROD4 to relieve inflammation and oxidative stress in spinal cord injury.

Conclusions

MiR-137 promoted the recovery of spinal cord injury by degrading NEUROD4 to relieve the inflammatory reaction and oxidative stress of spinal cord injury.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References


