INTRODUCTION

Spinal cord injury (SCI) is a serious disease of the central nervous system and an important cause of disability and death in patients. Its prevention, treatment and rehabilitation have always been the focus of attention and research by clinicians and related scholars (Lu et al., 2020; Kumar et al., 2019). Circular RNAs (circ RNAs) are a kind of non-coding RNAs with closed circular structure widely existing in eukaryotic cells. It is formed by reverse splicing and plays an important role in cell proliferation, apoptosis and inflammation through microRNA (miRNA) sponges, RNA-binding proteins, regulatory gene transcription and peptide translation. Some studies have pointed out that circRNAs with abnormal expression exist in the process of SCI, which may be a potential target for the treatment of SCI (Yao et al., 2020). Some scholars have found that circRNA plays a role in the pathogenesis and treatment of SCI based on circNA-miRNA-mrna network. The expression of circ_0003801, circ_0014620, circ_0013613 and circ_0007259 is up-regulated in rats after SCI (Peng et al., 2020). Based on previous studies, we analyzed serum samples and SCI cell models of patients with SCI. Among the four circRNAs, circ_0013613 was the most significantly up-regulated. At the same time, the mechanism of circ_0013613 in SCI recovery is not completely clear.

CASP1 is a member of the Caspase family. It is also the core executor of scorching cell death. It plays an important role in the process of cell death and inflammatory process. (Man et al., 2017; Sun & Scott, 2016). Some studies have pointed out that miR-370-3p is low expressed in SCI cell model in vitro and plays an important role in the process of neuronal apoptosis (Li et al., 2021). CASP1 activation mediated sorosis is closely related to SCI (Li et al., 2020). However, it is not clear whether miR-370-3p participates in the occurrence and development of SCI by regulating the CASP1-mediated sorosis pathway. Based on this, this study aims to explore the role of circ_0013613 in promoting SCI function recovery, and to observe whether the mechanism of action is related to the regulation of miR-370-3p/CASP1 pathway mediated neuronal pyroptosis.

MATERIALS AND METHODS

Experimental materials

This study was approved by the Ethics Committee of Ningxia Hui Autonomous Region People’s Hospital. A total of 40 SCI Patients and healthy subjects with similar age and gender distribution were enrolled in the spinal surgery department of our hospital from October 2020 to May 2021. The participants’ informed consent was obtained from all of the participants. The serum was collected by centrifugation at 3000 r/min for 10 min at 4°C and stored in the refrigerator at –80°C for detection. DMEM medium, fetal bovine serum and horse serum were purchased from Sigma Company in the United States (MA, USA). IL-1β (SP10180), IL-6 (SP10234) and TNF-α (SP10205) ELISA kits are purchased from Wuhan Saipei Biotechnology Co., Ltd (Wuhan, China).
an). The LDH test kit was purchased from Nanjing Jiancheng Bioengineering Research Institute (A020-2-2, Nanjing, China). Reverse transcription kits, Lipofectamine 2000 and TRIZol reagents were purchased from Invitrogen Company in the United States (MA, USA). SYBR Green Mix was purchased from TaKaRa Company in Japan (Tokyo, Japan). Biotin-labeled circ_0013613 probe was purchased from Shenggong Bioengineering (Shanghai) Co., Ltd (Shanghai, China). CircCCS WT, circCCS MUT, circCCS siRNA and siRNA construction were controlled by Shanghai Albus Biotechnology Co., Ltd (Shanghai, China). miR-370-3p mimic, miR-370-3p inhibitor and their corresponding negative controls were purchased from Guangzhou Ruibo Co., Ltd (Guangzhou, China). miR-370-3p mimic, miR-370-3p inhibitor and their corresponding negative controls were purchased from Guangzhou Ruibo Co., Ltd (Guangzhou, China). GSDMD-N (69469), NLRP3 (13158), ASC (67824) and GAPDH (5174) antibodies are purchased from Cell Signaling Technology Company in the United States (MA, USA). The double luciferase detection kit was purchased from Promega Company in the United States (CA, USA). BCA protein quantitative kit is purchased from Thermo Company in the United States (MA, USA).

**Cultivate and identification of primary spinal cord neurons**

Spinal cord tissue isolated from pregnant mice was cut into small pieces according to references (Lan W et al., 2021) and digested using 0.125% trypsin for 25 min. The cells density was 6.0×10^5/mL and inoculated with 10% FBS on the petri dish of DMEM medium with 10% horse serum. After being cultured in a 5% CO₂ incubator at 37°C for 6 h, the medium was replaced with a serum-free medium containing 1% glut amylamine, 2% B27, 1% N₂ and 96% Neurobasal. After 7-9 days of culture, high purity primary spinal cord neurons were obtained by observing the number of tubulin β-positive cells under a microscope.

**Construction of SCI cell model, experimental grouping and cell transfection**

SCI cell model was constructed by stimulating primary spinal myeloid neurons with 100 ng/mL LPS according to the literature (Li et al., 2021). The experiment was divided into 1) Control group: no treatment was done; 2) LPS group: treated with 100 ng/mL LPS for 24 h; 3) NC inhibitor group: negative control transfected with miR-370-3p inhibitor; 4) NC mimic group: negative control was transfected with miR-370-3p mimic; 5) Inhibitor group: transfected miR-370-3p inhibitor; 6) Mimic group: transfected miR-370-3p mimic; 7) LPS+ Si-circ_0013613 + NC inhibitor group: 100 ng/mL LPS was added to si-circ_0013613 and miR-370-3p inhibitor negative irradiation after 24 h co-transfection for 24 h; 8) LPS+ Si-circ_0013613 + inhibitor group: After co-transfection of Si-circ_0013613 and miR-370-3p inhibitor negative control group, LPS treatment was added; 9) oe-nc group: transfection of empty plasmid; 10) oe-CASP1 group: transfection of CASP1 overexpression vector plasmid; 11) LPS+mimic+oe-nc group: co-transfection of miR-370-3p mimic and empty plasmid was given LPS treatment; 12) LPS+mimic+oe-CASP1 group: co-transfection of miR-370-3p mimic and CASP1 overexpression vector plasmid was co-transfected with LPS treatment. The transfection method was carried out according to the Lipofectamine 2000 instructions for 24 h.

**RT-qPCR detection**

After collecting the serum or cells to be detected, the total RNA was extracted by Trizol method. The purity of RNA was detected by ultraviolet spectrophotometer. After reverse transcription, cDNA was synthesized, and then PCR reaction was carried out with SYBR Green Premix Ex Taq. The 20 μL reaction system consisted of 2 μL cDNA, 10 μL TB Green Premix Ex Taq, 1 μL forward and backward primers and 6 μL ddH₂O. The 40 cyclic reaction conditions are: 94°C 30 s, 94°C 5 s, 60°C 30 s. The expression levels of miR-370-3p (with U6 as internal reference) and CASP1 (with GAPDH as internal reference). Primers for circ_0013613, miR-370-3p and CASP1 were provided by Shanghai Shenggong Biological Co., LTD.

**LDH activity detection**

The culture medium of the cells to be detected in each group was collected, and the LDH activity of the cells was detected using LDH detection kit according to the instructions.

**ELISA detection**

After collecting the cells to be detected in each group, the supernatant was collected by trypsin digestion and centrifugation. The contents of IL-18, IL-1β, IL-6 and TNF-α were detected with specific ELISA kits according to the instructions.

**Western blot detection**

After collecting the cells to be detected in each group, cell lysis solution was added and placed on ice for full lysis. Then, protein concentration was quantified by BCA method. The sample size of 50 μg per well was transferred onto membrane after SDS-PAGE electrophoresis. Next, the PVDF membrane was sealed with 5% skim milk powder for 2 h and incubated with the first antibody (GSDMD-N, 1:1000; NLRP3, 1:600; ASC, 1:800; GAPDH, 1:2500) overnight at 4°C. After being washed by TBST 3 times, the secondary antibody diluent (HRP labeled secondary antibody diluted according to 1/5 000) was incubated at room temperature for 2 h. ECL was used to develop color and Bio-Rad was used to take pictures. GAPDH was set as a reference to observe the grayscale of the target protein.

**Flow cytometry detection**

The cells were collected, washed twice with PBS solution, and then added to 100 μL Annexin V Binding Buffer suspension cells. Then, 5 μL Annexin V-FITC measurements.
and 5 μL PI were added. After being mixed, cells were incubated for 15 min in the dark. After the 400 μL Annexin V Binding Buffer was added, flow cytometry was performed within 60 min.

**Double luciferase reporter gene assay**

Circular RNA Interactome ([https://circinteractome.nia.nih.gov/index.html](https://circinteractome.nia.nih.gov/index.html)), TargetScan 7.2 ([http://www.targetscan.org/vert_72/](http://www.targetscan.org/vert_72/)) and miRcode data libraries were used to analyze the complementary binding sites between miR-370-3p and circ_0013613/CASP1. circ_0013613/CASP1 containing miR-370-3p binding sites were used to construct wild-type double luciferase reporter gene carrier, labeled WT. The mutated vector constructed by the sequence of circ_0013613 containing miR-370-3p binding site and CASP1 site-directed mutation was used as MUT. WT and MUT were co-transfected with miR-370-3p mimics (mimics) and negative control (NC mimics) for 48 h. To detect the luciferase activity of cells, refer to the double luciferase reporter gene detection kit for specific steps.

**RNA Pull down experiment**

Trizol reagent was added to 100 μg circ_0013613 or CASP1 overexpression cells to extract the total RNA. After washing the streptavidin magnetic beads, 200 pmol biotin labeled miR-370-3p mimic was added. After setting for 5 min at room temperature, shake it upside down. Next, it was added to the extracted total RNA and incubated for 30 min at room temperature. After the elution buffer was added, the pulled RNA complex was collected in the magnetic field. The expression of circ_0013613 or CASP1 was detected by RT-qPCR.

**RNase R and actinomycin D test**

After the total RNA of circ_0013613 overexpression cells was extracted, 5 mg and 3 U/mg RNase R were incubated at 37°C for 15 min. circ_0013613 expression was detected by RT-qPCR. Nerve cells were treated with 2 g/L actinomycin D for 0, 4, 8, 12, 24 h. The total RNA was extracted and the expression of circular and linear in circ_0013613 was detected by RT-qPCR.

**Statistical analysis**

SPSS26.0 software was used to analyze the experimental data. Data results were calculated as means ± standard deviation (x̄±s). Single factor variance is used to compare multi-group differences. T-test corrected by Bonferroni was used for pairwise comparison of data between groups. The difference was accepted as statistically significant at P<0.05.

**RESULTS**

**circ_0013613 expression increased in SCI**

RT-qPCR showed that compared with the control group, the expression of circ_003801, circ_0014620, and circ_0013613 were significantly up-regulated in LPS-induced neuronal cells, and circ_0013613 had the highest expression (P<0.001, Fig. 1A). Meanwhile, circ_0013613 was also found to be highly expressed in peripheral blood samples of 40 patients with spinal cord injury (Fig. 1B). The expression of circ_0013613 was not affected by RNase R digestion. The abundance of linear RNA decreased significantly. The results showed that the closed-loop structure circ_0013613 was not easily degraded by exonuclease (Fig. 1C). In addition, actinomycin D analysis showed that circ_0013613 was more stable than linear transcription in nerve cells (Fig. 1D).

**circ_0013613 down-regulation promotes SCI recovery by alleviating pyroptosis of nerve cells**

RT-QPCR results showed that compared with Si-NC, the expression of circ_0013613 was down-regulated after transfection with Si-circ_0013613-1 and Si-circ_0013613-2 (P<0.05), and si-circ_0013613-1 has a more significant effect (P<0.01, Fig. 2A). The results of MTT assay showed that the down-regulation of circ_0013613 significantly weakened the inhibitory effect of LPS on the viability of nerve cells (Fig. 3B). Additionally, the LDH activity of nerve cells induced by LPS was significantly higher than that of control (P<0.01). However, the down-regulation of circ_0013613 could attenuate the effect of LPS on the activity of LDH in nerve cells (P<0.01, Fig. 3C). The contents of IL-18, IL-1β, IL-6 and TNF-α in the supernatant of nerve cells treated with LPS were significantly higher than those of control. However, the above-mentioned effects of LPS on nerve cells were significantly weakened after transfection with Si-circ_0013613 (P<0.01, Fig. 3D–G). The protein expression of pyroptosis biomarkers (such as GSDMD-N, NLRP3, and ASC) was decreased by circ_0013613 knockdown (Fig. 3H). The pyroptosis rates of neuronal cells were significantly decreased after transfection of Si-circ_0013613 (P<0.01, Fig. 3H–I).
miR-370-3p is the target gene of circ_0013613 and has low expression after SCI

It was predicted by Circular RNA Interactome that there were complementary binding sites between circ_0013613 and miR-370-3p (Fig. 3A). Dual-luciferase reporter gene assay confirmed that miR-370-3p mimics reduced luciferase activity of circ_0013613-WT (Fig. 3B). circ_0013613 was highly enriched in the biotin-miR-370-3p group compared with the Biotin-NC group (P<0.01, Fig. 3C). In addition, transfection of Si-circ_0013613 significantly induced the upregulation of miR-370-3p (Fig. 3D). Meanwhile, miR-370-3p expression was significantly decreased in SCI cell model and patient plasma (Fig. 3E~F).

miR-370-3p low expression can reverse the promoting effect of circ_0013613 downregulation on SCI recovery

Compared with the corresponding control, nc inhibitor and NC mimic, transfection with miR-370-3p inhibitor and mimic successively induced low expression and overexpression of miR-370-3p in nerve cells (Fig. 4A). We further verified whether circ_0013613 plays a role in promoting SCI recovery by directly regulating miR-370-3p expression. Compared with LPS+ Si-circ_0013613 + NC inhibitor, cell viability of LPS+ Si-circ_0013613 + inhibitor group was decreased. The contents of IL-18, IL-1β, IL-6 and TNF-α in cell supernatant, as well as the gray level of protein expression of GSDMD-N, NLRP3 and ASC, LDH activity and pyroptosis rates were significantly increased (P<0.01, Fig. 4B–I).

CASP1 is the target gene of miR-370-3p

It was predicted by TargetScan that there were complementary binding sites between CASP1 and miR-370-3p (P<0.01, Fig. 5A). Dual luciferase reporter gene assay, RNA pull-down assay and RT-QPCR detection were found. miR-370-3p mimics reduced luciferase activity and CASP1 mRNA expression of CASP1-WT, and CASP1 was highly enriched in the biotin-miR-370-3p group (P<0.01, Fig. 5B and C). Moreover, miR-370-3p overexpression significantly decreased the expression of CASP1 (P<0.01, Fig. 5D). In addition, RT-QPCR detection showed that miR-
circ_0013613 was highly expressed in SCI cell model and SCI patients (P<0.01, Fig. 5E–F).

Up-regulation of CASP1 induced the degradation of neuronal cells

As shown in Fig. 6A, the expression of CASP1 was significantly increased in CASP1 OE group (P<0.01), suggesting that cells were successfully transfected. Over-expression of CASP1 suppressed the cell viability of neuronal cells (P<0.01, Fig. 6B), and promoted cytotoxicity (P<0.01, Fig. 6C), inflammatory response (P<0.01, Fig. 6D–G) and pyroptosis (P<0.01, Fig. 6H and I).

DISCUSSION

Previous studies have shown that there are abnormal circRNAs in SCI. Restoration of its expression can inhibit secondary injury and promote functional recovery after SCI, which is expected to be a potential molecular marker for the diagnosis and treatment of SCI (Dai et al., 2019). In this study, the expression of circ_0013613 was up-regulated, especially that of circ_0013613. Scorched cell death is one of the important ways of programmed cell death. Including the classic CASP1-mediated inflammasome pathway and/or the non-classical caspase-11/4/5 pathway, inhibition of pyroptosis may be a promising treatment for SCI (Chalfouh et al., 2020; Dai et al., 2019).

In this study, it was found that circ_0013613 had good stability in the nerve cells. Knocking down the expression of circ_0013613 can improve the activity of nerve cells by inhibiting the expression of IL-18, IL-1β, IL-6, TNF-α and related proteins GSDMD-N, NLRP3, ASC and scorched death to reduce SCI damage and promote SCI recovery. The results showed that circ_0013613 down-regulation may promote SCI recovery by alleviating pyroptosis of nerve cells. The results suggest that circ_0013613 may be a potential therapeutic target for SCI.
As is known to all, circRNAs are rich in specific binding sites with miRNAs. CircRNA is a new type of regulatory molecule that regulates the function of miRNA. The role of competitive endogenous RNA can be exerted by targeted adsorption of miRNA to relieve its inhibitory effect on target genes (Chen et al., 2021).

In this study, we used bioinformatics software to screen the circ_0013613 target gene. MiR-370-3p is a miRNA closely related to neurosystemic diseases and tissue damage. It has been reported that it can inhibit the occurrence and development of glioma by targeting β-catenin to regulate cell proliferation and cycle. It can reduce neuronal damage in rats with depression and play an important role in the protective effect of paclitaxel on acute renal injury induced by LPS (Mamun et al., 2020; Shi et al., 2021). In addition, in LPS-induced acute kidney injury, MiR-370-3p can be targeted by LNC-MALAT1 to play a positive role in the protection of paclitaxel against renal tissue injury (Du & Andrea, 2013). We found that MiR-370-3p is the target gene of circ_0013613, and its low expression can promote the recovery of nerve cell scoria inhibition SCI. This is because there are complementary binding sites between circ_0013613 and MiR-370-3p. Double luciferase reporter gene assay and RNA Pull down assay confirmed that down-regulation of circ_0013613 could increase the expression of miR-370-3p. In addition, low expression of miR-370-3p could reverse the down-regulated effect of circ_0013613 on the recovery of SCI. This suggests that circ_0013613 may regulate cell pyroptosis by regulating MiR-370-3p and thus affect SCI recovery. Our results may provide new insights into the molecular regulation of circ_0013613 on the circRNA/miRNA network, thus regulating the progression of SCI.

In order to further explore the mechanism of MiR-370-3p in regulating SCI injury, we used bioinformatics software to predict its potential target genes. CASP1 is a cysteine aspartate specific proteolytic enzyme that cleaves the N-terminal and C-terminal domains of the executive protein GSDMD. The activation of N-domain can promote the release of IL-1β and IL-18, which play an important role in the process of inflammation and cell death (Peng et al., 2016). The decrease in Caspase-1 activity causes a decrease in the amount of IL-1β because caspase-1 activates pro-IL-1β to IL-1β (Malik & Kaneganti, 2017). Some studies have pointed out that inhibition of Caspase-1 can reduce the expression of IL-1β and IL-18 after SCI, reduce the secondary injury after SCI, and may play a positive role in the repair of SCI (Nadaradjane et al., 2018). We found that CASP1 is the target gene of MiR-370-3p. Its overexpression can promote the scorched death of nerve cells and inhibit the recovery of SCI. This is because we found that there are complementary binding sites between CASP1 and miR-370-3p. At the same time, double luciferase reporter gene assay and RT-qPCR confirmed that MiR-370-3p could target the regulation of CASP1. In addition, up-regulation of CASP1 expression could reverse the promoting effect of MiR-370-3p overexpression on SCI recovery. The results suggest that miR-370-3p can promote the recovery of SCI through targeted regulation of CASP1-mediated neuronal necrotic death.

In conclusion, circ_0013613 expression was increased in SCI. circ_0013613 sponged miR-370-3p promoted degradation of neurons by promoting CASP1-mediated inflammatory response and inflammation-induced pyroptosis. circ_0013613/miR-370-3p/CASP1 axis may be a therapeutic target for SCI.
REFERENCE


