

## Original Article

# Overexpressed miR-181b promotes nerve cell damage via inhibiting cell proliferation but inducing cell apoptosis during ischemic cerebral stroke

Jian Gong<sup>1</sup>, Jinling Ma<sup>2</sup>, Zhenying Liu<sup>1</sup>

<sup>1</sup>Department of Neurology, Linyi People's Hospital, Linyi, Shandong, China; <sup>2</sup>Department of Nephrology, Linyi People's Hospital, Linyi, Shandong, China

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**Abstract:** Increasing evidence has reported the pivotal roles of miRNAs involvement in the pathogen and progression of ischemic cerebral stroke (ICS). This study was aimed to investigate the potential role of miR-181b on protecting cerebral stroke under oxygen-glucose deprivation (OGD) condition. The miR-181b expression in the C57BL/6J rat isolated nerve cells was detected using the RT-PCR analysis. The effects of miR-181b expression on nerve cell viability and apoptosis were analyzed using the MTT assay and Annexin V-FITC respectively. Furthermore, the effects of miR-181b expression on the cell proliferation- or apoptosis-related protein expression were analyzed using the western blotting. Compared to the normal cells, miR-181b was significantly increased in nerve cells under OGD ( $P < 0.05$ ). The nerve cell viability was significantly increased by the knockdown miR-181b under OGD condition ( $P < 0.01$ ), also, the percentage of apoptotic cells under OGD treatment was significantly decreased by the knockdown miR-181b ( $P < 0.01$ ). The Atg5 level was highly increased while the cleaved caspase-9 level was decreased by the suppressed miR-181b in OGD-treated nerve cells. Taken together, our study revealed that the knockdown miR-181b might play certain protective role in resisting ICS via involving in the biological processes of proliferation and apoptosis by regulating Atg5 and caspase-9.

**Keywords:** Ischemic cerebral stroke, miR-181b, cell apoptosis, cell viability, Atg5

## Introduction

Ischemic cerebral stroke (ICS) remains to be one of the most threaten oxygen-glucose deprivation (OGD) diseases in worldwide, which is characterized by the high morbidity and mortality [1]. A series of pathological changes, which are caused by the persistent or temporary focal cerebral ischemia, often lead to the brain damage, and then result in the subsequent biological changes including dysfunctional energy metabolism, toxic effects of excitability amino acid, and inflammation [2-4]. Prevention or complication-regulation drugs including thrombolysis, anticoagulation, and brain protection are the major methods for ICS in clinical [5, 6], leading to the circumscribed treatment methods on ICS. Even though varieties of signal pathways are involved in the progression and development of ICS, but the pathogen mechanism for ICS still remain incompliantly illustrat-

ed. Hence, to develop several useful methods on ICS will be of great significance in clinical.

microRNAs (miRNAs) are some endogenous, highly conserved non-coding RNAs 20- to 22-nt in length that function in various biological processes at the transcriptional or post-transcriptional level via targeting the 3'UTR of genes [7]. Increasing evidence has reported the significant roles for miRNAs in the pathogen for ICS in recent years [8, 9]. For example, Sepramaniam et al report that miR-320a functions as a novel endogenous modulator for aquaporins 1 and 4 in cerebral ischemia [10]. Besides, Tan et al identified the characteristic patterns for miRNA in ischemic stroke by performing the miRNA profiling from peripheral blood of young stroke patients aged 18-49 years [11]. Additionally, miR-181b has been reported to be involved in a variety of diseases including cancer and neuron-related diseases [12, 13]. Moreover, Peng

et al proved that the down-regulated miR-181b may play neuroprotective roles in ICS by targeting HSPA5 protein [14], and Ouyang et al demonstrated that miR-181 play certain roles in brain cell death by regulating GRP78 during cerebral ischemia [15]. Although several studies have investigated the role and mechanism of miR-181 in ICS, few have reported that mechanism by which miR-181b regulates nerve cell viability and apoptosis via the cell apoptosis-related signal pathway and cell viability-related protein of autophagy related 5 (Atg5).

In the current study, we assessed the potential effects of miR-181b on C57BL/6J rats-isolated nerve cell viability and apoptosis under OGD treatment. Various kinds of experimental methods were used to analyze the possible molecular regulatory mechanism for miR-181b on ICS. The aim of this study was to reveal the possible effects of miR-181b on ICS under OGD condition and to identify its potential mechanism of action.

### Materials and methods

#### *Cerebral cell isolation and cell culture*

All the experimental procedures were approved by the relevant local research animal ethics committee. The male C57BL/6J rats (The Animal Center of the Capital University of Medical Sciences, Beijing, China) was disinfected using the 75% ethyl alcohol (Sigma, USA) to obtain the cerebral tissue. After being cut into pieces, cerebral tissues were digested in trypsin (Sigma) at 37°C in the Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) for the nerve cell suspension preparation.

#### *OGD model*

The digested nerve cells were exposed to OGD as previously described [16]. In brief, cells were washed once with the Dulbecco's Phosphate Buffered Saline buffer (D-PBS; France) and then cultured in serum-free OGD medium, DMEM medium without glucose and L-glutamine (Sigma). The OGD medium was saturated for 1 h at 37°C in a humidified hypoxic chamber with a gas mixture containing 5% CO<sub>2</sub>. Then the nerve cells were placed into the OGD medium for 90 min cultivation. After deprivation, cells were washed with D-PBS buffer and then incu-

bated under normoxic condition for another 72 h. The control cells were maintained under a normoxic atmosphere in culture medium for 90 min at the same time.

#### *Cell transfection*

The mature miR-181b and the anti-miR-181b (purchased from Sangon Biotech, Shanghai, China) were transfected into the OGD-treated nerve cells using the Lipofectamine 2000 according to manufacturer's protocol. Non-transfected cells and cells transfected with the scramble RNA vector were used as controls.

#### *Cell viability assay*

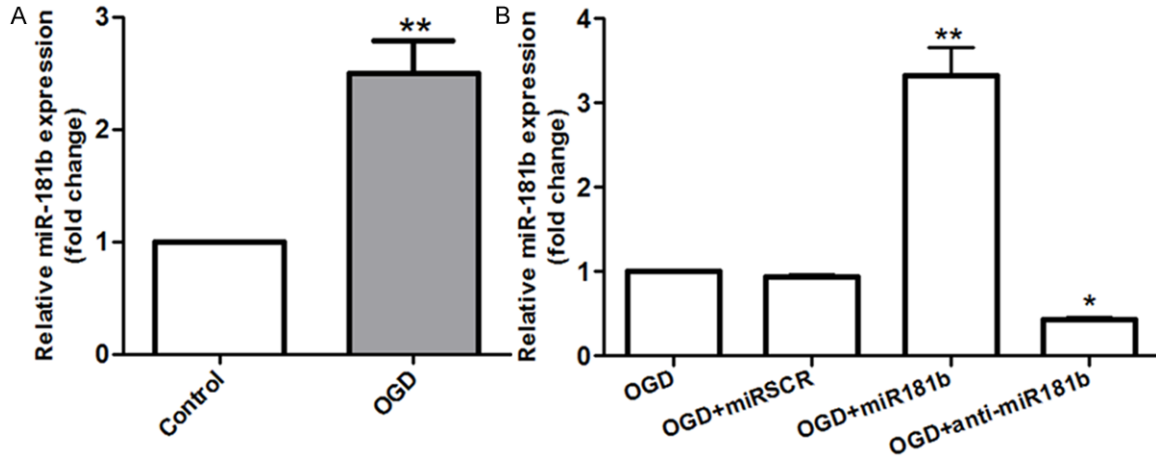
MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-tetrazolium bromide) assay was used to analyze the nerve cell viability as previously described [17]. In brief, after transfection for 24 h, cells (5×10<sup>3</sup> cells) were transferred onto the 96-well plates for another 24 h incubation. Then cells were centrifuged at 12,000 rpm to remove the supernatant. Then 20 µL MTT was added into the cultured cells for 4 h. Finally, 150 µL dimethylsulfoxide (DMSO) was used to mix with the cells for 10 min. Absorbance for cells was observed at 570 nm under an absorption spectrophotometer (Olympus, Japan).

#### *Cell apoptosis assay*

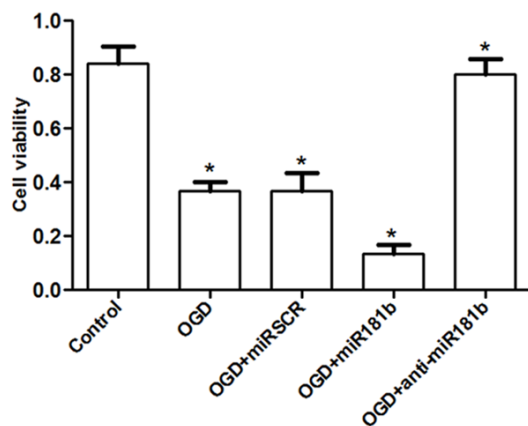
Cell apoptosis was analyzed using Annexin V-FITC cell apoptosis kit (Invitrogen, USA) according to manufacturer's protocol [18]. Briefly, after transfection for 24 h, cells were cultured in serum-free DMEM medium for another 24 h. Total cells were harvested and then washed using PBS buffer (PH 7.4) for 3 times, and then resuspended in the staining buffer. After that, 5 µL of annexin-V-FITC and 5 µL of propidium iodide (PI) were mixed with the cells. After being cultivated at room temperature for 10 min, mixtures were analyzed using the FACScan flow cytometry. Annexin V-positive and propidium iodide-negative cells were considered to be apoptotic cells.

#### *Real-time PCR*

Total RNA was isolated from the nerve cells using TRIzol Reagent (Invitrogen) as previously described [19], and was treated with Rnase-free DNase I (Promega Biotech, USA). Consequently, concentration and purity of isolated



**Figure 1.** Expression of miR-181b in nerve cells during ICS. A: RT-PCR analysis showed that miR-181b was highly expressed in nerve cell under oxygen glucose deprivation (OGD) condition than that in normal cells; B: After cells were treated with OGD, the miR-181b expression was significantly increased by mature miR-181b compared to the control cells (no transfected vectors) and the positive (transfected with scramble miRNA) or negative controls (transfected with anti-miR-181b). \*\*:  $P < 0.01$ , compared to the control cells (cells treated without OGD).



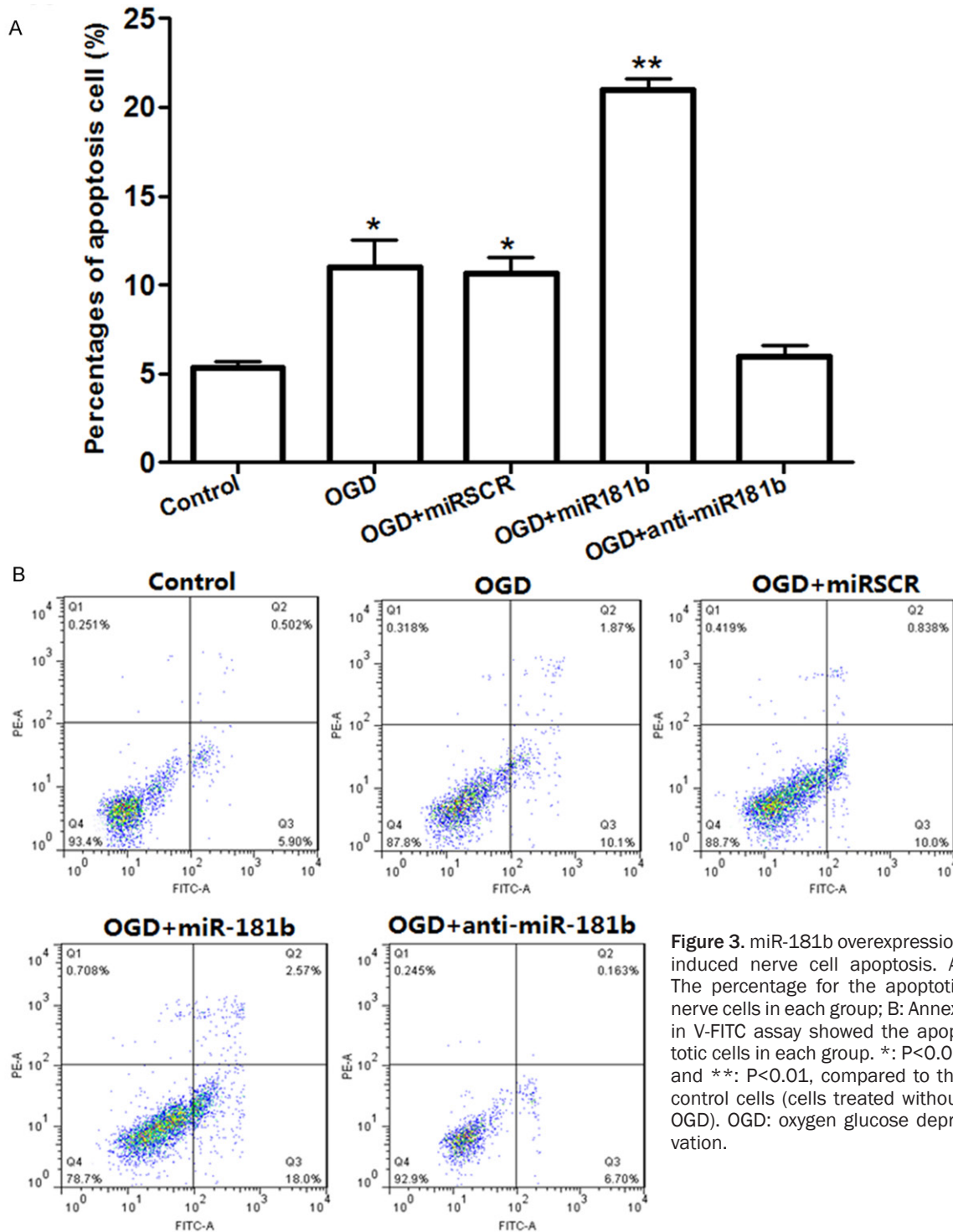
**Figure 2.** Effects of miR-181b expression on nerve cell viability. Cell viability was suppressed by the oxygen glucose deprivation (OGD) treatment, but was drastically inhibited by the overexpressed miR-181b. \*:  $P < 0.05$  and \*\*:  $P < 0.01$ , compared to the control cells (cells treated without OGD).

RNA were measured with SMA 400 UVVIS (Merinton, Shanghai, China). Purified RNA at density of  $0.5 \mu\text{g}/\mu\text{L}$  with nuclease-free water was used for cDNA synthesis with the PrimerScript 1<sup>st</sup> Strand cDNA Synthesis Kit (Invitrogen). Expressions of targets in OVCAR-3 cells were detected in an Eppendorf Mastercycler (Brinkman Instruments, Westbury, NY) using the SYBR ExScript RT-qPCR Kit (Takara, China). Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one product was ampli-

fied and detected. Phosphoglyceraldehyde dehydrogenase (GAPDH) was chosen as the internal control. Primers used for targets amplification were as follows: caspase-9 sense, 5'-GCTCTCCTTTGTTTCATCTCC-3' and caspase-9 anti-sense, 5'-CATCTGGCTCGGGGTTACTGC-3'; Atg5 sense, 5'-TTTGCATCACCTCTGCTTTC-3' and Atg5 antisense, 5'-TAGGCCAAAGGTTTCA-GCTT-3', and GAPDH sense, 5'-TATGATGATCAAGAGGGTAGT-3', and GAPDH anti-sense, 5'-TGTATCCAAACTCATTGTCATAC-3'.

#### Western blotting

Protein samples ( $50 \mu\text{g}$  per lane) were separated on the 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transferred onto a Polyvinylidene difluoride (PVDF) membrane (Mip-pore). Then the PVDF membranes were blocked in Tris-Buffered Saline Tween (TBST) containing 5% non-fat milk for 1 h at room temperature. Consequently, the membranes were incubated with rabbit anti-human antibodies (Atg5, caspase-9, and cleaved caspase-9, 1:100 dilution, Invitrogen, USA) and overnight at  $4^\circ\text{C}$ . Then membrane was incubated with horseradish peroxidase labeled goat anti-rat secondary antibody (1:1000 dilution) at room temperature for 1 h. Finally, the PVDF membranes were washed 3 times with  $1\times$  TBST buffer for 10 min each. The signals were detected after incubation with a chromogenic substrate using the enhanced



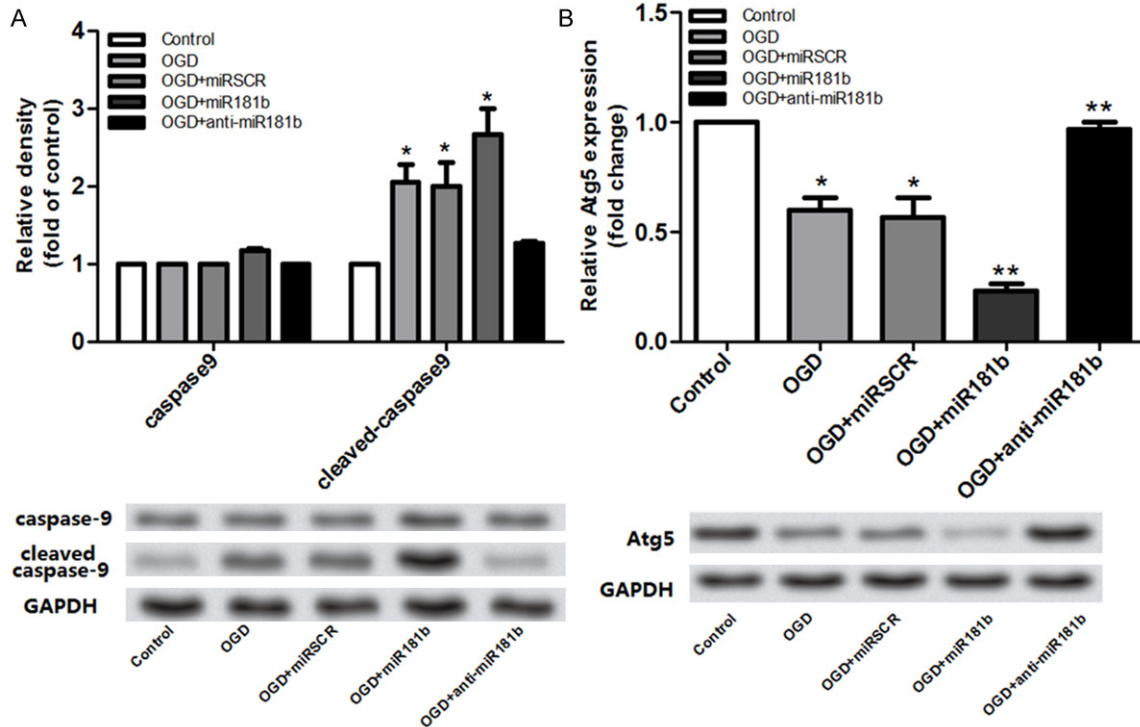
**Figure 3.** miR-181b overexpression induced nerve cell apoptosis. **A:** The percentage for the apoptotic nerve cells in each group; **B:** Annexin V-FITC assay showed the apoptotic cells in each group. \*: P<0.05 and \*\*: P<0.01, compared to the control cells (cells treated without OGD). OGD: oxygen glucose deprivation.

chemiluminescence (ECL) method. Additionally, GAPDH served as the internal control.

*Statistical analysis*

All experiments were independently repeated 3 times. The data are expressed as the mean ±

standard deviation (SD). Statistical analyses were performed using the Graph Prism 5.0 software. Significance for data between two groups was calculated using a one-way analysis of variance (ANOVA). The P<0.05 was considered as statistically significant.



**Figure 4.** Effects of miR-181b expression on ICS-related protein expression in nerve cells. A: No significant difference for the mRNA and protein levels for caspase-9 was observed in each group cells, but the cleaved caspase-9 level was significantly increased by the overexpressed miR-181b expression in nerve cells; B: The overexpressed miR-181b drastically decreased the Atg5 protein level in nerve cells. \*: P<0.05 and \*\*: P<0.01, compared to the control cells (cells treated without OGD). OGD: oxygen glucose deprivation.

**Results**

*Expression of miR-181b in nerve cells*

The results showed that the mRNA level for miR-181b in brain nerve cells was significantly increased compared to the control normal cells (P<0.01; **Figure 1A**). Subsequently, we further analyzed the miR-181b expression in cells at the different condition of OGD treatment. The results showed that the mRNA level for miR-181b was significantly decreased by the knock-down miR-181b than that in the negative or in positive control cells (P<0.01; **Figure 1B**).

*miR-181b suppression promotes nerve cell viability*

The results showed that cell viability was significantly inhibited by OGD treatment than that in control cells, similar results was observed when cells were treated with OGD and miR-181b scramble transfection (P<0.05; **Figure 2**). However, when cells were treated with the knockdown miR-181b, the cell viability was

drastically increased compared to the controls, which was opposite to the cells treated with the overexpressed miR-181b (P<0.01; **Figure 2**).

*miR-181b knockdown suppresses nerve cell apoptosis*

The percentage of apoptotic nerve cells was significantly increased by the OGD or OGD and miR-181b scramble co-treatment. However, the apoptotic cells were drastically decreased when cells were treated with the anti-miR-181b than that in the OGD group cells (**Figure 3**), which was accordant with the results showed in **Figure 3B**. These data suggested that miR-181b suppression may block the nerve cell apoptosis.

*Possible regulatory mechanism for miR-181b in OGD nerve cells*

The former results revealed that potential effects of miR-181b expression on nerve cell viability and apoptosis, we consequently analyzed the possible mechanism at the protein

level (**Figure 4**). The results showed that there was no significant difference for caspase-9 expression among each group, but mRNA and protein levels for cleaved caspase-9 were significantly increased when cells were on the OGD condition, while its level was significantly decreased by the knockdown miR-181b (**Figure 4A**). Besides, the mRNA and protein level for Atg5 was significantly increased by the knockdown miR-181b in OGD-treated nerve cells (**Figure 4B**).

### Discussion

ICS remains to be one of the most threatened OGD-related brain diseases, and is characterized with the high morbidity and mortality in worldwide [1, 20]. Increasing evidence reveals that miRNAs are involved in the biological or pathogen for ICS [21, 22]. To date, miR-181b alterations have been reported to be involved in various diseases such as cancers and neuron-related disease development [12, 15]. In this study, we analyzed the potential effects of miR-181b on nerve cell viability and apoptosis under OGD treatment during ICS. In accordance with the previous data [14], we found that miR-181b was highly expressed in OGD nerve cells. Moreover, the knockdown miR-181b promoted the nerve cell viability but suppressed cell apoptosis under OGD condition. In addition, the knockdown miR-181b increased Atg5 level but decreased the cleaved caspase-9 expression in nerve cells under OGD condition.

Cell necrosis and apoptosis are the major symbols for nerve cell damage during ICS [23, 24], implying that cell viability and apoptosis are the indexes for measuring ICS. Peng et al have demonstrated that the percentage of cell necrosis in nerve cells was increased by the down-regulated miR-181b [14]. Besides, Sun and his colleagues proved that the glioma cell viability was suppressed by the overexpressed miR-181b [25]. In this study, the cell viability was significantly increased by the knockdown miR-181b in nerve cells under OGD condition (**Figure 2**), indicating that the down-regulated miR-181b could enhance the nerve cell viability. On the other side, our results revealed that nerve cell apoptosis was suppressed by the knockdown miR-181b in cells under OGD condition (**Figure 3**), indicating the important correlation between miR-181b and nerve cell apoptosis. Hutchison et al said that miR-181b was

involved in astrocytes apoptosis in neuroinflammatory responses [26], while Sun et al proved that glioma cell apoptosis was induced by the overexpressed miR-181b [25]. Similar results for miR-181b on neuron cell apoptosis were also observed in the study performed by Peng et al [14]. Based on our results, we speculated that the knockdown miR-181b might be a protector for ICS via involving in the biological processes including cell viability and apoptosis.

Meanwhile, our results showed that the knockdown miR-181b significantly increased the Atg5 expression but decreased the cleaved caspase-9 expression in nerve cells under OGD condition (**Figure 4**). Atg5 is a salmonella infection pathway-related protein that involved in autophagic vesicle formation, and also plays a role in translation or maintenance of axon morphology and membrane structures and cell proliferation [27]. Fimia et al said that Atg5 deficient was correlated to the nerve cell proliferation and survival in nervous system [28]. Also, the release of caspase-9 from mitochondria promotes cell apoptosis during neuronal apoptosis and cerebral ischemia [29]. Taken together, our results revealed that the knockdown miR-181b may protect nerve cells from necrosis and apoptosis by increasing Atg5 protein and decreasing the caspase-9 expression.

In conclusion, the data presented in our study suggests that the knockdown miR-181b functions as a protector on nerve cells in OGD-induced ICS by affecting the cell viability and apoptosis. The down-regulated miR-181b promotes nerve cell viability but suppresses apoptosis under OGD condition, as well as increases Atg5 while decreasing the cleaved caspase-9 level. Our study may provide a theoretical basis for the possible application of miR-181b in ICS treatment and in illustrating its complicated mechanism. However, further experimental studies are still needed to develop the deep molecular mechanism for miR-181b in ICS.

### Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Zhenying Liu, Department of Neurology, Linyi People's Hospital, No. 27, Jiefang Road, Linyi 276003, Shandong, China. E-mail: liuzhenying5812@126.com

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