

## Participation of MiR-145-5p in Neuronal Ischemia/Reperfusion Injury by Targeting Fibroblast Growth Factor-5

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**ABSTRACT** The researchers made efforts in elucidating the mechanism for miR-145-5p participating in neuronal ischemia/reperfusion injury (I/R) via targeting fibroblast growth factor-5 (FGF5). OGD/R was performed to construct the neuronal injury model. After OGD/R, the oxidative stress injury of nerve cells was enhanced significantly, the levels of lactic dehydrogenase, reactive oxygen species and malondialdehyde rose, while the superoxide dismutase activity decreased. Both miR-145-5p and cell apoptosis rate increased. MiR-145-5p had a targeting relation to FGF5. After miR-145-5p inhibitor transfection of nerve cells, inhibition of oxidative stress together with apoptosis was observed. FGF5 siRNA could reverse the impact on oxidative stress, FGF5, and apoptosis exerted by miR-145-5p-inhibitor in the OGD/R model. Being a latent target of I/R treatment, miR-145-5p induces the oxidative stress injury as well as apoptosis of HT22 cells by targeting and binding FGF5.

### INTRODUCTION

As acute cerebrovascular diseases, ischemic cerebrovascular diseases have high incidence, disability and mortality rates (Fan et al. 2017; Wang et al. 2020), and ischemic stroke cases account for eighty-seven percent of all stroke cases. Thrombolytic therapy or thrombus displacement may cause ischemic site reperfusion, which in turn leads to I/R injury. Neurons have the highest sensitivity to ischemia and hypoxia, and I/R markedly damages neurons. The pathophysiological mechanisms of ischemic stroke include release of excitotoxic neurotransmitter, accumulation of intracellular  $Ca^{2+}$ , free radical injury, cell apoptosis, neuroinflammation and lipid decomposition (Huang et al. 2019). However, the pathogenesis of I/R is still not fully understood. Therefore, further study on the pathogenesis of ischemic stroke to find the potential therapeutic targets provides an important direction for improving the therapeutic efficacy on ischemic stroke, and reducing the mortality and disability rates of patients.

MiRNAs are short-stranded non-coding RNAs with regulatory functions in eukaryotes (Olena and Patton 2010), which contain 21-23 nucleotide sequences (Zhi et al. 2016). They can be paired with the target gene mRNA 3'UTR and play regulatory roles in the target gene. They have

regulatory effects on I/R injury. For example, the miR-429 expression can alleviate nerve cell injury resulting from OGD/R (Xiao et al. 2018), and miR-320 can regulate cerebral I/R injury by suppressing insulin-like growth factor-1 (Tian et al. 2018). Regulated by long-chain non-coding RNA SNHG14, miR-145-5p contributes to CI progression (Qi et al. 2017). Under ischemic conditions, changes in the miR-145-5p expression can affect the neuronal activity (Xie et al. 2017). Fibroblast growth factor-5 (FGF5) is an ectoderm marker of mouse embryoid that serves as an important player in embryonic and neurological development.

### Objectives

Mouse hippocampal neurons cultured *in vitro* were used to construct an OGD/R model, and the targeted correlation of miR-145-5p with FGF5 as well as its effect on oxidative stress were studied, aiming to provide an underlying target for cerebral I/R.

### MATERIAL AND METHODS

#### Experimental Cells and Materials

Mouse hippocampal neurons HT22 cells (Cell Bank of the Shanghai Institute of Cell Biology, CAS), DMEM, foetal bovine serum and trypsin (Gibco, USA) were used. MiR-145-5p inhibitor was synthesised by Guangzhou RiboBio Co. Ltd. (Chi-

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na). siRNA and siRNA targeting FGF5 (Shanghai GeneChem Co. Ltd., China), reactive oxygen species (ROS) detection kit, and RIPA lysis buffer and BCA kit (Beyotime Institute of Biotechnology, Shanghai, China) were employed, together with kits for MDA, SOD and LDH (Nanjing Jiancheng Bioengineering Institute, China).

### Cell Culture and OGD/R Modeling

After culture in a 5% CO<sub>2</sub> anaerobic petri dish at 37°C HT22 hippocampal neurons were induced and cultured by glucose-free DMEM. OGD was induced by adding one percent O<sub>2</sub>, ninety-four percent N<sub>2</sub> and five percent CO<sub>2</sub> at 37°C to simulate hypoxia-ischemia. Following 3 hours of OGD, glucose-free DMEM was replaced by glucose-containing medium, followed by reoxygenation under conventional conditions (5% CO<sub>2</sub>, 37°C) for 12 and 24 hours. The cells cultured at normal oxygen concentration were used as control group (Hu et al. 2020).

### Detection of Neuronal Apoptosis by Flow Cytometry

After the density was adjusted to  $2 \times 10^5$ /mL, HT22 cells were subjected to 6-well plate seeding. Later, they were treated with the above OGD/R method for 6, 12 and 24 hours. After harvest, the cells underwent centrifugation and twice rinsing in pre-cooled PBS, followed by adjustment of cell density to  $1 \times 10^6$ /mL. Subsequently, resuspension in binding buffer ( $1 \times 100 \mu\text{L}$ ), staining with Annexin V-FITC ( $5 \mu\text{L}$ ) and PI ( $10 \mu\text{L}$ ), and 5 min of room-temperature culture in the dark were performed for the cells. Finally, after even blending with  $1 \times$  binding buffer in a volume of  $400 \mu\text{L}$ , followed the cells were detected through flow cytometry and graphing was implemented using BD FACSuite software.

### Detection of Protein Expressions by Western Blotting

After 24 hours of reoxygenation, the cells were subjected to 30 minutes of ice lysis using RIPA lysis buffer supplemented by protease inhibitor and phosphatase inhibitor. A refrigerated centrifuge was utilized to centrifuge the supernatant at 12,500 rpm for 10 minutes, and the BCA kit was

applied to measure the protein concentration. After 100 minutes of SDS-PAGE was conducted by virtue of stacking gel (5%) mixed with separation gel (10%) at 80 V, the transfer of proteins onto PVDF membranes was carried out. After tris-buffered saline rinsing for 10 minutes, five percent blocking protein dry powder diluent was employed to block the membranes for 2 hours, and then the membranes were cultured with primary antibodies (overnight, 4°C) as well as relevant secondary antibodies (1-2 hours) the following day, followed by colour development by ECL. Finally, the grey values of protein bands were analysed using ImageJ2x analysis system, with GAPDH as the internal reference.

### Determination of Oxidative Stress Markers

By means of the MDA, SOD, ROS and LDH kits, MDA, SOD, ROS and LDH content was measured, respectively.

### Verification of Targeting Relationship by Luciferase Reporter Assay

TargetScan plus MiRDB was adopted for binding site prediction for miR-145-5p and FGF5. The synthesis and cloning of wild-type (WT) FGF5-5p-UTR carrying binding site of miR-145-5p into pMIR-control luciferase reporter plasmid were conducted, followed by inoculation into a 95-well plate. WT/mutant (Mut) pcDNA3.0-FGF5 reporter plasmid, together with miR-145-5p mimic, was co-transfected with Lipofectamine 3000. Following 48-hour transfection, dual-luciferase reporter assay was performed by (Fan et al. 2022).

### Cell Transfection

SiRNA targeting FGF5 and control siRNA were inserted into pcDNA3.0 plasmid for transfection. into A 24-well plate was used for HT22 cell inoculation ( $1 \times 10^4$ /mL/ well). After normal culture for 24 hours, HT22 cells were transfected with siFGF5 and its control using Lipofectamine 3000.

### Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

After being extracted by TRIzol, the total RNA underwent reverse transcription to obtain cDNA.

With cDNA as the template, the SYBR Green Real-Time PCR Master Mix was adopted to carry out qRT-PCR. The mRNA level of miR-145-5p was analysed *via* 7300 Fast Real-Time PCR System. The relative PBK/TOPK expression was measured by setting U6 as the internal reference. By virtue of the  $2^{-\Delta\Delta Ct}$  method, the relative mRNA expressions were determined (Table 1).

### Statistical Analysis

Statistical analysis was implemented through SPSS 22.0, and plotting was accomplished by means of GraphPad Prism 5.0. Comparison between two groups was made by *t*-test, while the among multiple groups was exerted using one-way ANOVA.  $P < 0.05$  indicated a difference of statistical significance.

## RESULTS

### Role of OGD/R in Affecting Oxidative Stress Response of Neurons

ROS and MDA in the nerve cells plus LDH in the supernatant increased, while the activity of SOD decreased with extended reoxygenation time through OGD/R treatment, showing significant differences from those in control group ( $P < 0.01$ ). Thus, ROS, MDA and LDH up-regulation, as well as the down-regulation of SOD, was induced by OGD/R (Fig. 1).

### Function of OGD/R for Impacting miR-145-5p Expression

Nerve cells were reoxygenated after OGD. In such cells, the miR-145-5p expression rose in con-

trast to that in control group at different times points of reoxygenation, and the miR-145-5p expression rose with prolonged reoxygenation time, suggesting that OGD/R facilitated miR-145-5p in nerve cells (Fig. 2A). After OGD for nerve cells subjected to miR-145-5p mimic transfection, compared with OGD/R group, miR-145-5p mimic group exhibited far higher miR-145-5p, and miR-145-5p inhibitor group had a far lower miR-145-5p expression ( $P < 0.01$ ). Accordingly, I/R injury was associated with a high miR-145-5p expression (Fig. 2B).

### Role of OGD/R Treatment in Influencing Cell Apoptosis

As displayed in Figure 3, flow cytometry showed that in contrast with those in control group, the apoptotic cells and apoptotic rate increased after 6, 12 and 24 hours of treatment with OGD/R ( $P < 0.01$ ). Therefore, OGD/R triggered apoptosis, and the apoptosis rate increased with extended time.

### MiR-145-5p/FGF5 Targeting Relationip

As for the possible target genes of miR-145-5p, a total of 15,064, 890, 1,305 and 495 genes were found through miRTarBase, TargetScan, TargetMiner as well as miRDB, respectively. 100 potential target genes, including FGF5, were obtained finally (Fig. 4).

TargetScan was used for verification of the miR-145-5p/FGF5 targeting relation. Complementary sequences were found in the 3'-UTR of miR-145-5p and FGF5. The luciferase activity decreased greatly after WT FGF5 3'-UTR co-transfection with miR-145-5p mimic, but no significant

**Table 1: Primer sequences**

Gene	Primer sequence (5'-3')
miR-145-5P mimic	F: GUCCAGUUUCCCAGGAAUCCCU R: GG AUUCCUGGGAAAACUGGACUU
miR-145-5P	F: AACTCCAGCTGGGGTCCAGTTTTCCAGGA R: CTCAACTGGTTCGTGGAGTCGGCAATTCAGTTGAGAGGGATTC
FGF5 siRNA1	F: CAUAAGUUGUAUAGGCUAA R: CAACAAUAAGCCACGUCAA
FGF5 siRNA2	F: GCAAGUUCAGGGAGCGUUU R: GUAUUGAAGUCACGUCAUU
U6	F: CTCGCTTCGGCAGCACAA R: AACGCTTCACGAATTTGCGT

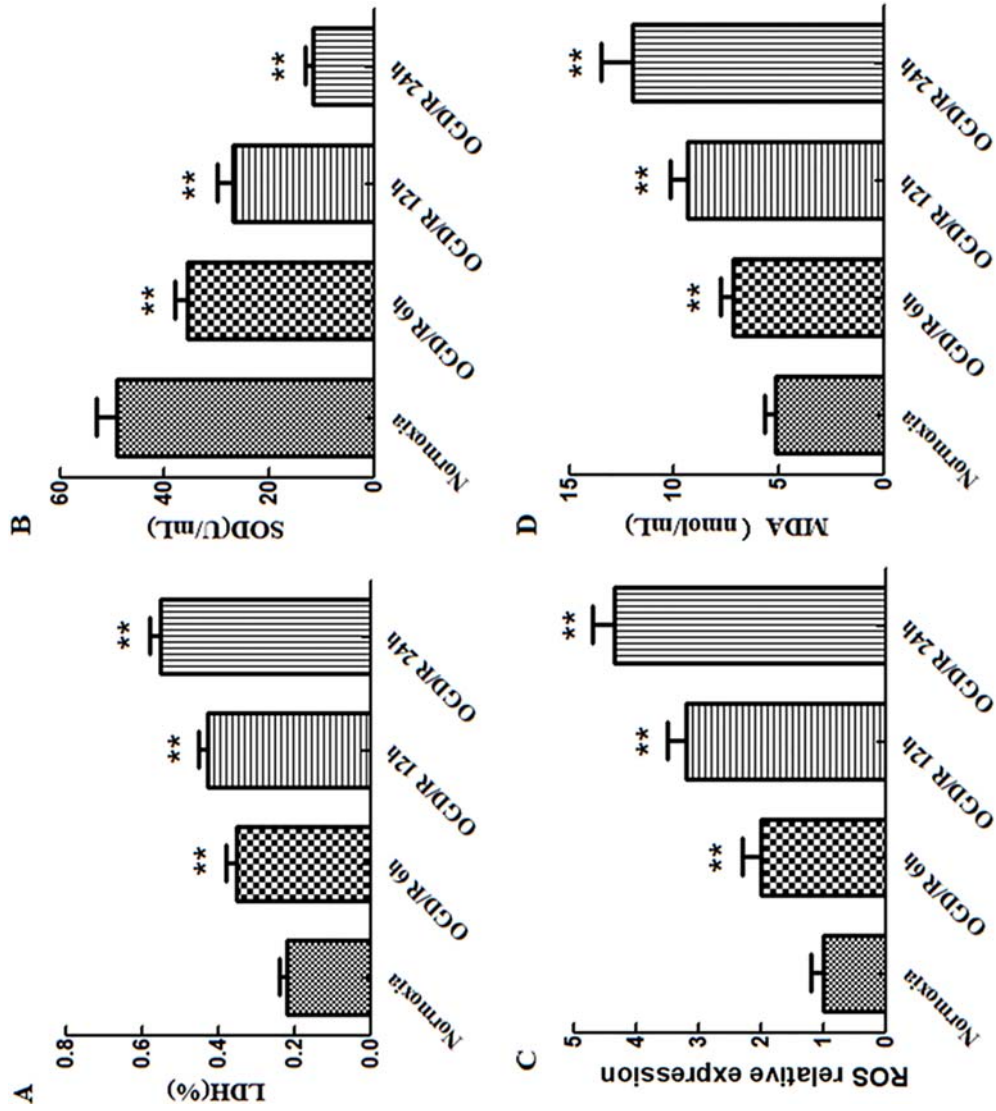


Fig. 1. Effect of OGD/R treatment on oxidative stress response of nerve cells. A: LDH content, B: SOD content, C: relative ROS level, D: MDA content. \*\*P<0.01 vs. control group

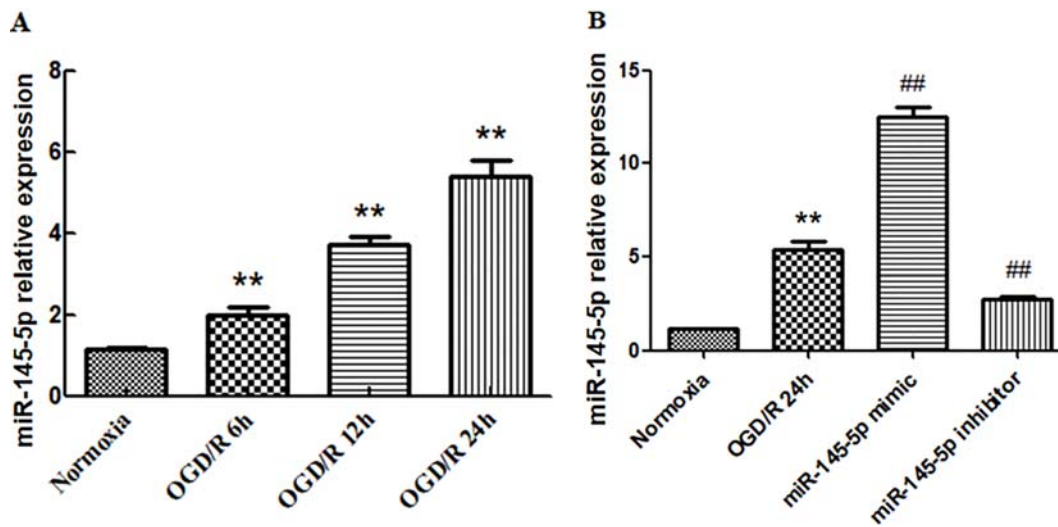


fig. 2. Effect of OGD/R treatment on expression of miR-145-5p. A, B: expression levels of miR-145-5p in HT22 neurons in different treatment groups, \*\*P<0.01 vs. control group, ##P<0.01 vs. OGD/R 24 h group

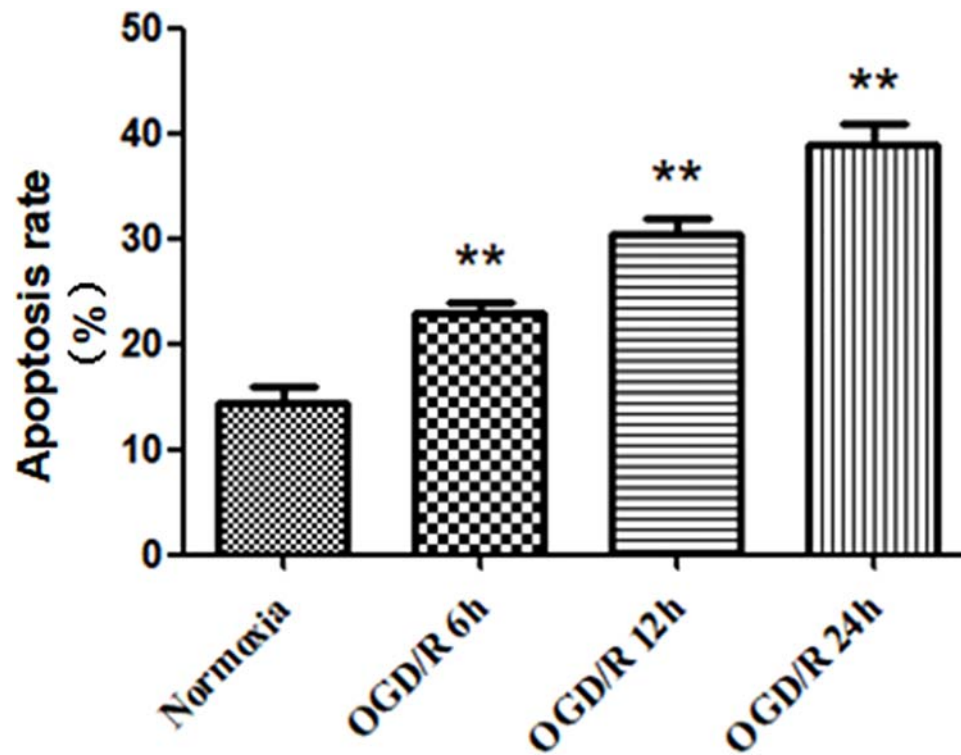


Fig. 3. Effect of OGD/R treatment on apoptosis of HT22 neurons

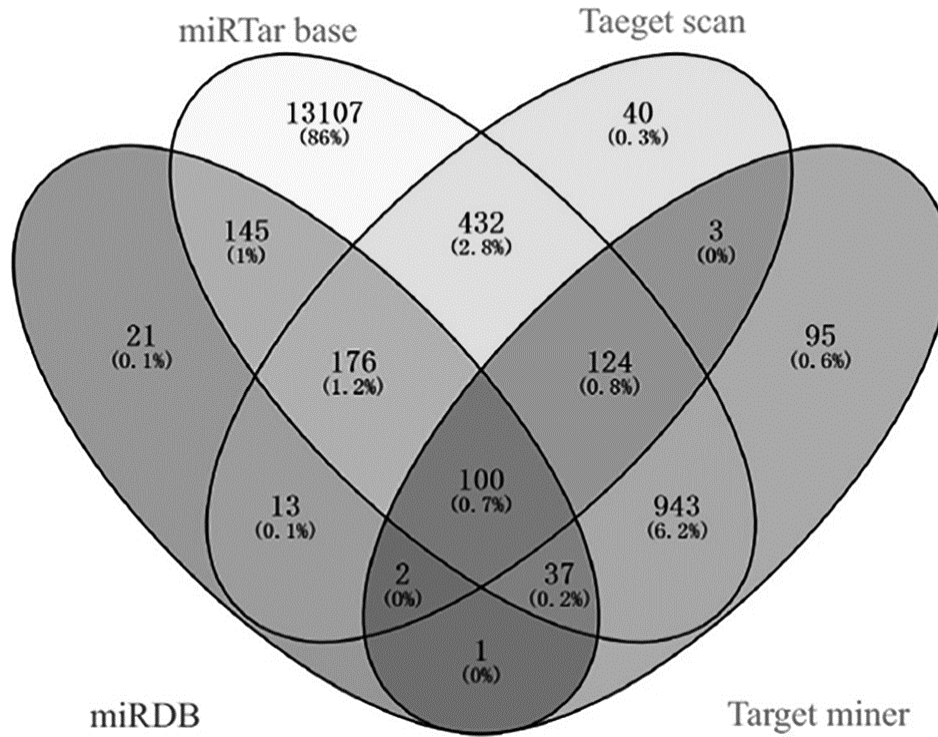
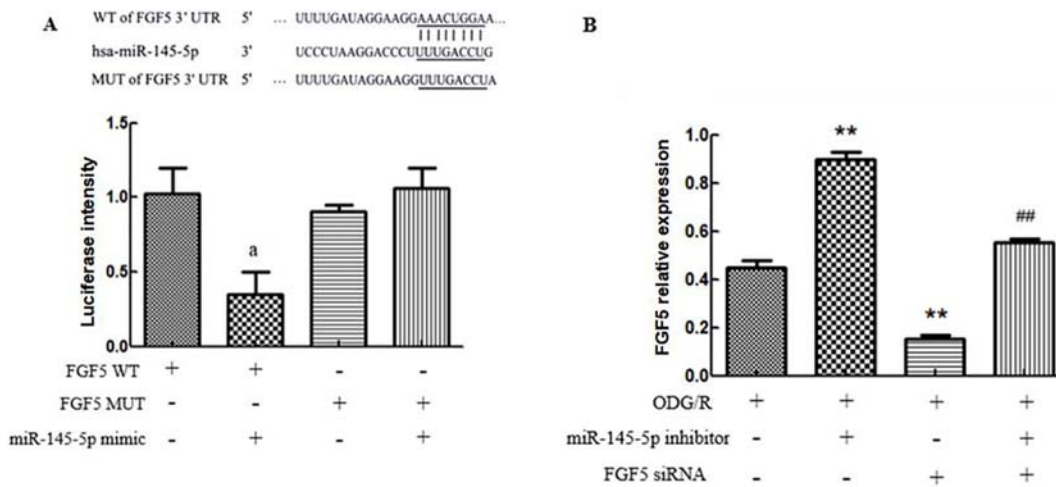


Fig. 4. Venn diagram

Fig. 5. MiR-145-5p targeted FGF5 and negatively regulated its expression. <sup>a</sup>P<0.01 vs. WT FGF5, <sup>\*\*</sup>P<0.01 vs. ODG/R group, <sup>##</sup>P<0.01 vs. ODG/R + siRNA group

changes occurred after Mut FGF5 3'-UTR co-transfection with miR-145-5p mimic, according to the results of Luciferase reporter assay. In contrast to those in OGD/R group, miR-145-5p inhibitor greatly up-regulated whereas FGF5 siRNA down-regulated the expression of FGF5 ( $P < 0.01$ ) (Fig. 5).

#### MiR-145-5p Inhibitor Repressed HT22 Cells Apoptosis Induced by OGD/R

As compared to OGD/R group, miR-145-5p inhibitor group manifested a greatly decreased cell apoptosis rate ( $P < 0.01$ ), whereas it increased greatly in FGF5 siRNA group ( $P < 0.01$ ). Moreover, compared with FGF5 siRNA group, miR-145-5p inhibitor group and FGF5 siRNA group had a far lower cell apoptosis rate ( $P < 0.01$ ) (Fig. 6).

#### MiR-145-5p Inhibitor Alleviated OGD/R-induced Oxidative Stress Damage

In comparison with OGD/R group, miR-145-5p inhibitor group exhibited greatly lowered

MDA, ROS and LDH, as well as greatly enhanced activity of SOD ( $P < 0.01$ ). In FGF5 siRNA group, MDA, ROS plus LDH greatly rose, while the activity of SOD greatly declined compared with those in OGD/R group ( $P < 0.01$ ). In contrast to FGF5 siRNA group, ROS, MDA, and LDH were lower in FGF5 siRNA group and miR-145-5p inhibitor group, while the SOD activity increased significantly ( $P < 0.01$ ) (Fig. 7).

#### DISCUSSION

I/R is a complex physio-pathological process involving neuronal death, glial cell activation and inflammatory response. MiRNAs play crucial roles in brain function, including neurogenesis, neurodevelopment and cellular responses, thus altering the synaptic plasticity. MiR-145-5p remarkably increases during the pathological process of vascular endothelial cell injury, as well as cardiomyocyte survival and  $H_2O_2$ -induced neuronal injury (Saugstad 2010; Chen et al. 2015). It is significantly up-regulated in the cerebral cortex 2 hours after microglia OGD/R and 12 hours

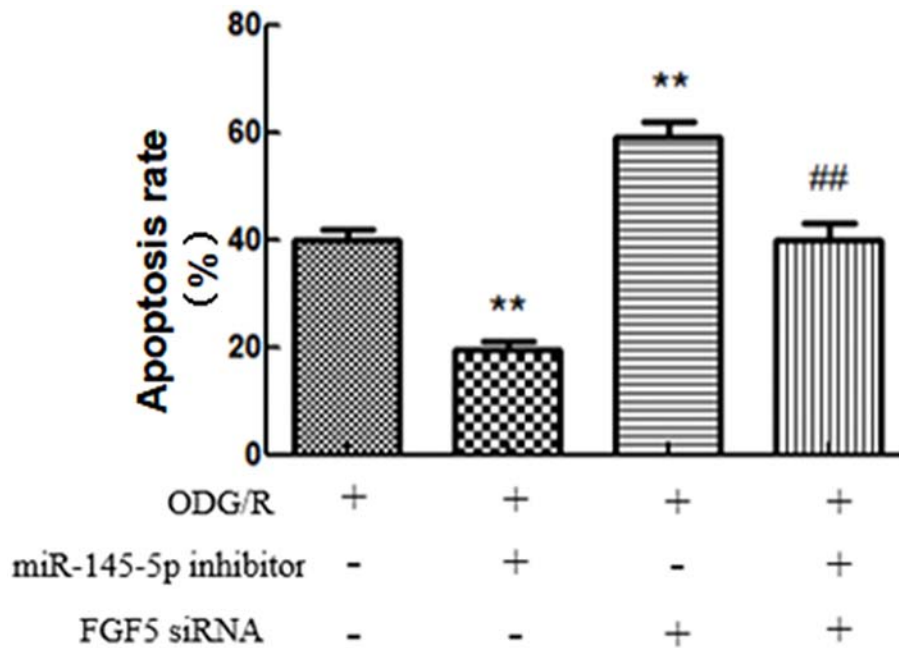


Fig. 6. MiR-145-5p inhibitor suppressed OGD/R-induced apoptosis of HT22 cells

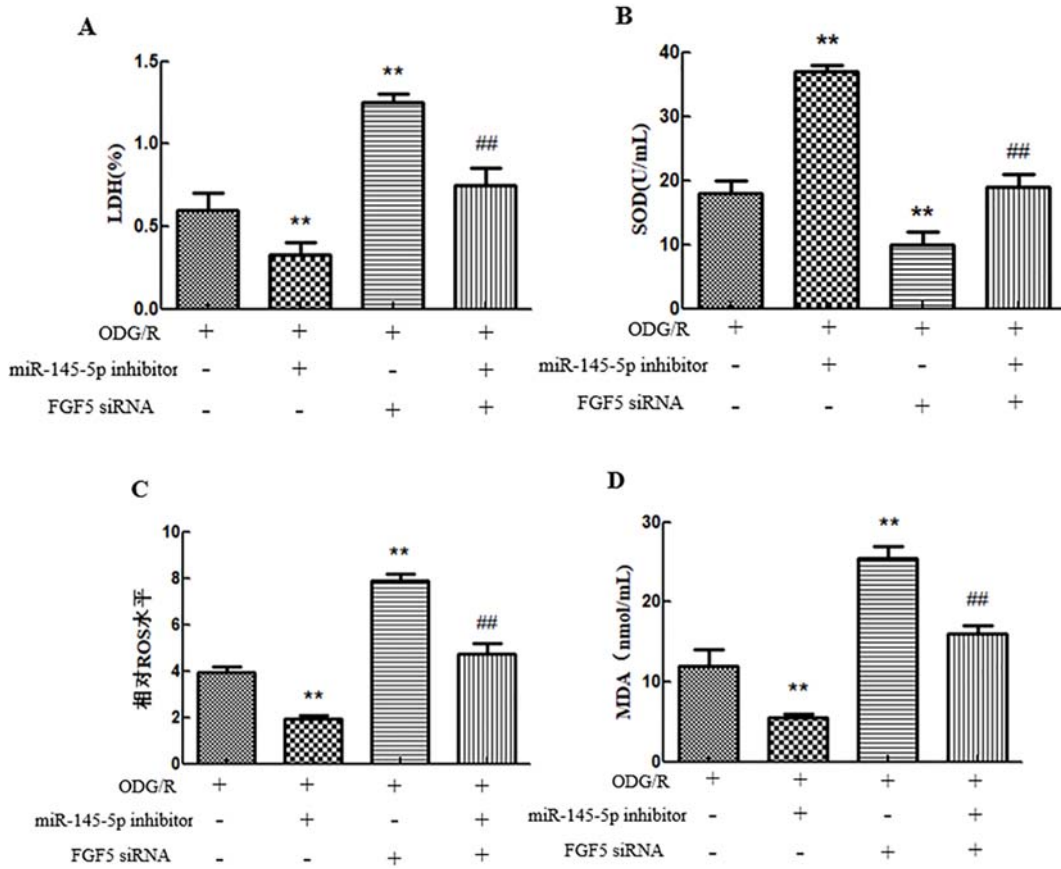


Fig. 7. MiR-145-5p inhibitor alleviated OGD/R-induced oxidative stress injury. A: LDH content, B: SOD content, C: relative ROS level, D: MDA content. \*\* $P < 0.01$  vs. OGD/R group, ## $P < 0.01$  vs. FGF5 siRNA group

after MCAO. Accordingly, miR-145-5p may damage neurons during OGD/R. In this study, after OGD/R for different time periods, ROS, MDA and LDH in HT22 cells increased significantly, while the activity of SOD decreased, indicating successful modelling. The oxidation/antioxidation balance is broken after I/R, which leads to oxidative stress reaction and production of massive oxygen free radicals. Therefore, I/R can be effectively treated by eliminating free radicals and suppressing oxidative stress. The studies on miR-34a in I/R injury focus on organs such as the myocardium, liver and intestine, while there is little knowledge about the influence on brain I/R injury. By virtue of the SIRT1 pathway, myocardial I/R injury can be prevented through inhibit-

ing miR-34a expression (Fu et al. 2017). Shao et al. (2017) argued that miR-34a inhibited autophagy by targeting TNF- $\alpha$ , thereby mitigating myocardial I/R injury. Through the miR-34a/SIRT1 pathway, carbon monoxide exerted a protective effect on hepatic I/R injury (Kim et al. 2015). Moreover, the report of Huang et al. (2014) indicated that miR-34a modulated the Nrf-2 pathway in young and aged rats to play different roles in H<sub>2</sub>S-induced liver I/R injury. LncRNAMEG3 prevents I/R injury of liver via downward modulation of miR-34a expression, while suppressing miR-34a-5p is capable of relieving intestinal I/R injury through ROS accumulation plus apoptosis induced by SIRT1 signalling activation (Wang et al. 2016). Studies in recent years have uncovered that miR-



34a-5p has high expression in rat models and patients with acute ischemic stroke (Liang and Lou 2016), but in the case of brain I/R injury, miR-34a-5p or its mechanism needs to be clarified.

In the present study, upregulation of miR-145-5p expression was detected in I/R cell model, and the oxidative stress response was alleviated after miR-145-5p was inhibited. Oxygen free radicals can trigger massive production of cytokines, thus facilitating the expression of cerebrovascular endothelial cell adhesion factor, triggering massive inflammatory response induced by reperfusion, and finally leading to apoptosis and necrosis of nerve cells (Zhu et al. 2018). ROS is a small molecule with high oxygen content and activity, and effective clearance of excess ROS can improve the treatment outcome of ischemic stroke. As a protease that eliminates oxygen free radicals, SOD can reduce cell apoptosis caused by ischemic brain injury through phosphorylating AKT (Dong et al. 2020). Herein, nerve cells were reoxygenated after OGD. ROS and MDA in the cells and LDH in the supernatant increased, while the activity of SOD decreased with prolonged reoxygenation time.

Through complementary binding to target mRNA 3'UTR, mature miRNAs regulate gene expression, resulting in target mRNA degradation or protein translational inhibition, resulting in cell function changes (Men et al. 2020). In HT22 cells in this paper, the miR-145-5p expression rose, while FGF5 expression declined. It was confirmed by bioinformatics analysis and luciferase assay that miR-145-5p and FGF5 had a strong targeting relation. Oxidative stress, inflammatory response and cell apoptosis were significantly attenuated after transfection of HT22 cells with miR-497-5p inhibitor and OGD/R was conducted. However, oxidative stress and cell apoptosis were enhanced after FGF5 siRNA transfection into HT22 cells. Collectively, miR-145-5p targeted FGF5, thus regulating the cell apoptosis and oxidative stress injury induced by I/R.

As a FGF family member, FGF5 is detected in the CNS and in the formation of gastrula, muscle and motor neuron of mouse embryos and adult mice (Reuss et al. 2000). The FGF5 expression in adult mice is slightly higher in the hippocampus, spinal cord and cerebral cortex than that in other ganglia, cerebellum, midbrain and hindbrain, and FGF5 can be considered as a neurotrophic factor continuously released by neurons or glia (Tian et

al. 2019). FGF5 has a variety of biological functions. Allerstorfer et al. (2008) found that the signals mediated by FGF5 and its main receptor FGFR1 were therapeutic targets for human glioblastoma multiforme. Additionally, as reported by Fang et al. (2015), miR-188-5p suppressed hepatocellular carcinoma by targeting FGF5 gene to restrain HCC cell proliferation as well as metastasis. Besides, FGF5 plays key roles in hypertensive diseases. The inner or nearby region of FGF5 gene is prone to essential hypertension. The T/C mutation of peripheral blood FGF5 gene of essential hypertension patients is positively associated with diastolic and systolic blood pressures, suggesting that the abnormal expressions of FGF5 mRNA and protein may have correlation with hypertension in terms of onset and progression (Huang et al. 2018; Ren et al. 2018). In this study, the expression of FGF5 decreased in HT22 cells, and FGF5 was verified to be the downstream target of miR-145-5p. Following HT22 cell transfection using miR-145-5p inhibitor, the FGF5 protein increased, and the cell apoptosis and oxidative stress injury were alleviated. However, after transfection of HT22 cells with FGF5 siRNA, FGF5 protein decreased and the oxidative stress response was enhanced. To sum up, the OGD/R-induced injury and apoptosis of HT22 cells were increased by miR-145-5p through restraining FGF5 expression.

## CONCLUSION

In summary, miR-145-5p can induce the apoptosis and oxidative stress injury of HT22 cells by targeting and binding FGF5. MiR-145-5p is an underlying therapeutic target for I/R. However, the related mechanism was only explored through cell culture *in vitro*, so it is necessary to further explore the role of miR-145-5p in affecting I/R injury together with its mechanism.

## RECOMMENDATIONS

Further studies using human neuronal cells are ongoing in the group. Besides, FGF5 is also involved in human diseases, so this study paves the way for human genetic studies.

## CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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