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miR-590-3p protects against ischaemia/reperfusion injury in an oxygen-glucose deprivation and reoxygenation cellular model by regulating HMGB1/TLR4/MyD88/NF-κB signalling

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Summary. miR-590-3p has been reported to be reduced in myocardial ischaemia-reperfusion (I/R) injury, but its specific role in cerebral I/R injury is still uncertain. Thus, we explored the function and mechanism of miR-590-3p in cerebral I/R injury using a cellular model. miR-590-3p, high mobility group Box 1 (HMGB1), and signalling-related factor levels were assessed using qPCR or a western blot analysis. Cell apoptosis was measured by flow cytometry. Inflammatory factors were detected by ELISA. The target of miR-590-3p was confirmed by dual-luciferase reporter assay and western blot analysis. We found that miR-590-3p was decreased and HMGB1 was increased in the OGD/R model. Upregulation of miR-590-3p reduced cell apoptosis and inflammation in the OGD/R model, and the TLR4/MyD88/NF-κB signalling pathway was suppressed. However, inhibition of miR-590-3p showed the opposite effects. Moreover, HMGB1 was verified as a target gene of miR-590-3p. HMGB1 reversed the decrease in apoptosis and inflammation caused by overexpression of miR590-3p, and the TLR4/MyD88/NF-κB signalling pathway was activated. Our results suggest that miR-590-3p regulates the TLR4/MyD88/NF-κB pathway by interacting with HMGB1 to protect against OGD/R-induced I/R injury. Thus, miR-590-3p may serve as a potential therapeutic target in cerebral I/R repair.

Key words: HMGB1, Oxygen-glucose deprivation and reoxygenation, miR-590-3p, TLR4/MyD88/NF-κB pathway

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Introduction

Cerebral ischaemia/reperfusion (I/R) may cause neurovascular injury (Jung et al., 2010), resulting in hypoxia, apoptosis, inflammation, oxidative stress, neuronal damage, and aggravation of brain tissue injury. (Jung et al., 2010; Kraft et al., 2012; Lambertsen et al., 2012; Xue et al., 2020). However, to date, no effective prevention or therapy are available.

MicroRNAs (miRNAs) are a group of small noncoding RNAs of approximately 20-22 nucleotides that regulate various biological processes by binding to the 3'-untranslated regions (UTRs) of target mRNAs (Andersson et al., 2000; Park et al., 2003). Substantial evidence shows that miRNAs play a key role in regulating cerebral I/R injury (Treutiger et al., 2003; Messmer et al., 2004) and might represent a new promising strategy for treating cerebral I/R injury (Kim et al., 2006; Liu et al., 2007; Andrassy et al., 2008; Rossini et al., 2008; Youn et al., 2008). Recently, a considerable number of studies have demonstrated that miRNAs such as miR-125, miR-143, and miR-15a are relevant in ischaemic brain injury (Feng et al., 2017; Tiedt et al., 2017; Chen et al., 2018; Gao et al., 2019).

Several studies have also demonstrated the positive effects of microRNA-590-3p against ischaemic damage in cardiomyocytes (Gong et al., 2021; Huang et al., 2020; Zhao et al., 2018). miR-590-3p can regulate inflammation and apoptosis in rat H9c2 cardiomyocytes (Gong et al., 2021).

HMGB1 is an endogenous inflammatory mediator (Andersson et al., 2000; Park et al., 2003; Treutiger et al., 2003; Messmer et al., 2004; Rossini et al., 2008), as a key regulator of the inflammatory response, it acts as an important early inflammatory mediator in cerebral I/R injury (Kim et al., 2006; Liu et al., 2007; Andrassy et al., 2008). HMGB1 is an important protein associated with the Toll-like receptor 4 (TLR4)/MyD88/NF-κB pathway that triggers the expression of proinflammatory cytokines such as IL1 and TNFα (Bianchi, 2009; Zhang et al., 2017). In a rat model of cerebral I/R injury, inhibiting HMGB1 release relieved inflammatory damage in a cerebral I/R injury model with glycyrrhizic acid or berberine supplementation (Kim et al., 2006; Zhu et al., 2018). Another study reported that quercetin reduces myocardial I/R injury and limits the activity of HMGB1 protein. Under ischaemic conditions, it is released from many types of cells and promotes necrosis and an influx of damaging inflammatory cells (Hayakawa et al., 2010). The miRanda database was used to predict the target genes of HMGB1, and the results showed that miR-590-3p might bind to HMGB1.

I/R has a complex mechanism, and many studies have shown that cerebral I/R injury is related to the oxidative stress response and inflammatory response. miR-590-3p alleviates oxidative stress and the inflammatory response caused by myocardial I/R injury. However, the role and mechanism of miR-590-3p in cerebral I/R injury have not been reported. In addition, we hypothesized that miR-590-3p might repress the HMGB1/TLR4/MyD88/NF-κB pathway, reducing I/R injury in the cell model. We will provide evidence that miR-590-3p, as a gatekeeper that represses the target protein HMGB1, protects the cerebrum by inhibiting the TLR4/MyD88/NF-κB pathway.

Materials and methods

Cell culture and OGD/R cellular model construction

Mouse hippocampal neuron HT22 cells were obtained from Zolgene Biotechnology Co., Ltd. (Fuzhou, China). All cells were cultured in DMEM (Gibco, USA) with 10% foetal bovine serum (PAN Biotech, Germany), 1% streptomycin (GIBCO, USA), and 1% penicillin (GIBCO, USA). The cells were cultured in a water-saturated atmosphere under 5% CO_2 at 37°C.

The cells (1×10^5 cells/well) were seeded into a 24-well plate and cultured in glucose-free DMEM. The cells were then placed in a hypoxic incubation chamber filled with a gas mixture (1% O_2, 94% N_2, and 5% CO_2) for 4h at 37°C. After incubation for 4h, the culture medium was exchanged for DMEM containing 4.5 g/L glucose, and the cells were incubated at 37°C with 95% O_2 and 5% CO_2 for reoxygenation for 24h. Cells cultured under normal conditions were used as controls.

Transfection

The miR-590-3p mimic, miR-590-3p inhibitor, mimic NC, inhibitor NC, pcDNA, and pcDNA-HMGB1 were obtained from Zolgene Biotechnology Co., Ltd. (Fuzhou, China). Transfection was performed using Lipofectamine 2000 (Invitrogen, USA) following the manufacturer's instructions.

Cell apoptosis assay

Cell apoptosis was observed by flow cytometry. HT22 cells were inoculated in six-well plates one day in advance. HT22 cells were transfected when the confluency of HT22 cells reached 80-90%. After transfection, the cells were incubated at 37°C and 5% CO_2 for 24h. The OGD/R treatment was then performed for 24h, and trypsin without EDTA was used for the digestion and collection of HT22 cells. Cell apoptosis was detected using an Annexin V Alexa Fluor 647/PI Apoptosis Detection Kit (CA1050, Solarbio, Beijing, China) according to the manufacturer's instructions. Finally, flow cytometry was conducted to calculate the rate of cell apoptosis in each group.

Inflammatory cytokine detection

The inflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α were detected by ELISA according to the manufacturer's instructions.

Luciferase reporter assays

The wild-type 3'UTR (HMGB1-WT) and mutant 3'UTR of HMGB1 (HMGB1-Mut) were ligated into the psiCHECK-2 luciferase reporter vector (Promega, USA). The 293T cells were cotransfected with miR-590-3p mimic or mimic NC and the HMGB1-WT or HMGB1-Mut reporter plasmid. After 48h of transfection, the luciferase activity was analysed with a dual reporter assay (Promega Corporation, USA).

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was isolated from the cells using NucleoZol (Gene Company Ltd., China). Two micrograms of prepared RNA was immediately reverse transcribed to generate cDNA using a qPCR mix reverse transcription kit (Promega, USA). qPCR was then performed using SYBR Green qPCR Master Mix (TaKaRa, China). Relative gene expression was assayed with an ABI 7500 PCR system (ABI, USA) and calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). miR-590-3p expression was normalized to the U6

level, and HMGB1 expression was normalized to the internal control β-actin level.

All primers are shown in Table 1.

Western blot analysis

Total protein was isolated from cells using RIPA buffer. The protein concentration of the sample was detected with a BCA Protein Assay Kit PC0020-500 (Solarbio, Beijing, China). Based on a previous study, SDS-PAGE was performed to separate proteins. After separation, the blots were blocked and transferred onto NC membranes. The membranes were blocked with 5% BSA for 1h at room temperature. After washing with TBS, the membranes were incubated with anti-TLR4, anti-NF-κB, anti-NF-κB p65 (phospho S276), anti-anti-IκBα (phospho S36), anti-IκBα, and anti-HMGB1 antibodies overnight at 4°C. The anti-mouse HRP-conjugated secondary antibody was used and incubated for 2h at room temperature. All antibodies were purchased from Abcam (Beijing, China). Anti-β-actin acted as a loading control. The proteins were then detected with an ECL assay (34080, Thermo) and captured with the Versa Doc TM imaging system (Peiqing Technology Co. LTD, Shanghai, China).

Table 1. Primer sequences.

Gene	Direction	Primer sequences
HMGB1	FORWARD	AGGAGTGGCTTTTGTCCCTC
	REVERSE	CATCAAACTTTGCTCGGGCG
GAPDH	FORWARD	CCCTTAAGAGGGATGCTGCC
	REVERSE	TACGGCCAAATCCGTTCACA
miR-590-3p	FORWARD	GCGCGCGTAATTTTATGTATAA
	REVERSE	AGTGCAGGGTCCGAGGTATT
	RT Primer	GTCGTATCCAGTGCAGGGTCCGAG GTATTCGCACTGGATACGACACTAGC
U6	FORWARD	CTCGCTTCGGCAGCACATATACT
	REVERSE	ACGCTTCACGAATTTGCGTGTC
	RT Primer	AAAATATGGAACGCTTCACGAATTTG

Statistical analysis

In this study, all data are expressed as the mean ± SD. The results were analysed with SPSS 22.0 statistical software using a Student's t-test or one-way ANOVA. $P<0.05$ (marked with an asterisk) indicates a significant difference.

Results

miR-590-3p and HMGB1 levels in the OGD/R cell model

To explore the role of miR-590-3p in cerebral ischaemia injury, an OGD/R HT22 cell model was constructed to mimic cerebral I/R injury *in vitro*. The qPCR results showed that the miR-590-3p level (Fig. 1A) declined and HMGB1 (Fig. 1B) was elevated in the OGD/R cell model group compared with those in the control group.

Effect of miR-590-3p on cell survival and inflammation in a cell model of OGD/R

To explore the effect of miR-590-3p on cell survival and inflammation during cerebral I/R injury, miR-590-3p mimic or miR-590-3p inhibitor was transfected into HT22 cells. We found that the miR-590-3p level was increased by the miR-590-3p mimic and reduced by the miR-590-3p inhibitor (Fig. 2A). Flow cytometry (Fig. 2B) and ELISA (Fig. 2C-F) showed that the miR-590-3p mimic markedly attenuated OGD/R-induced apoptosis and the inflammatory response, whereas the miR-590-3p inhibitor aggravated OGD/R-induced apoptosis and the inflammatory response. These data suggest that

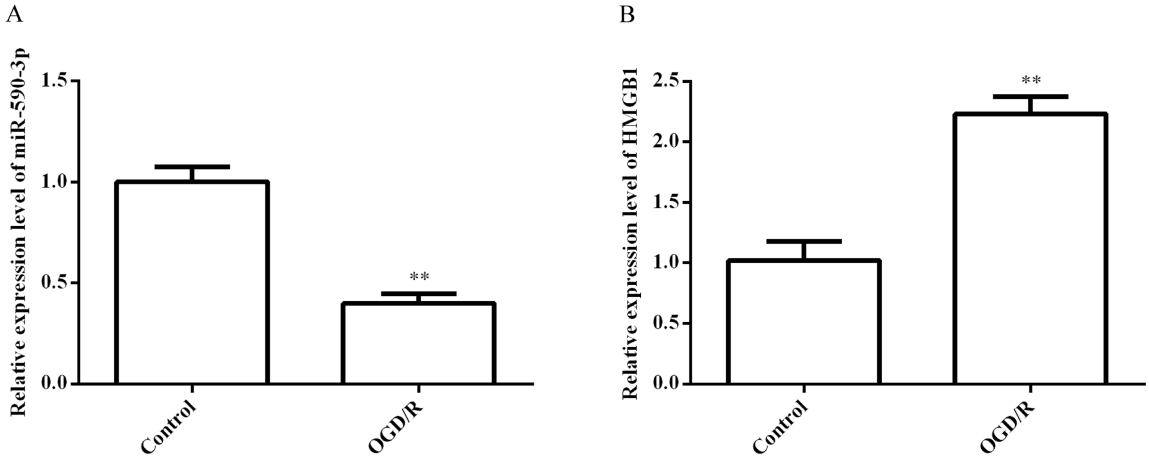

Fig. 1. MiR-590-3p and HMGB1 levels in cell model of OGD/R. Levels of miR-590-3p **(A)** and HMGB1 **(B)** in the OGD/R cell model. **P<0.01.

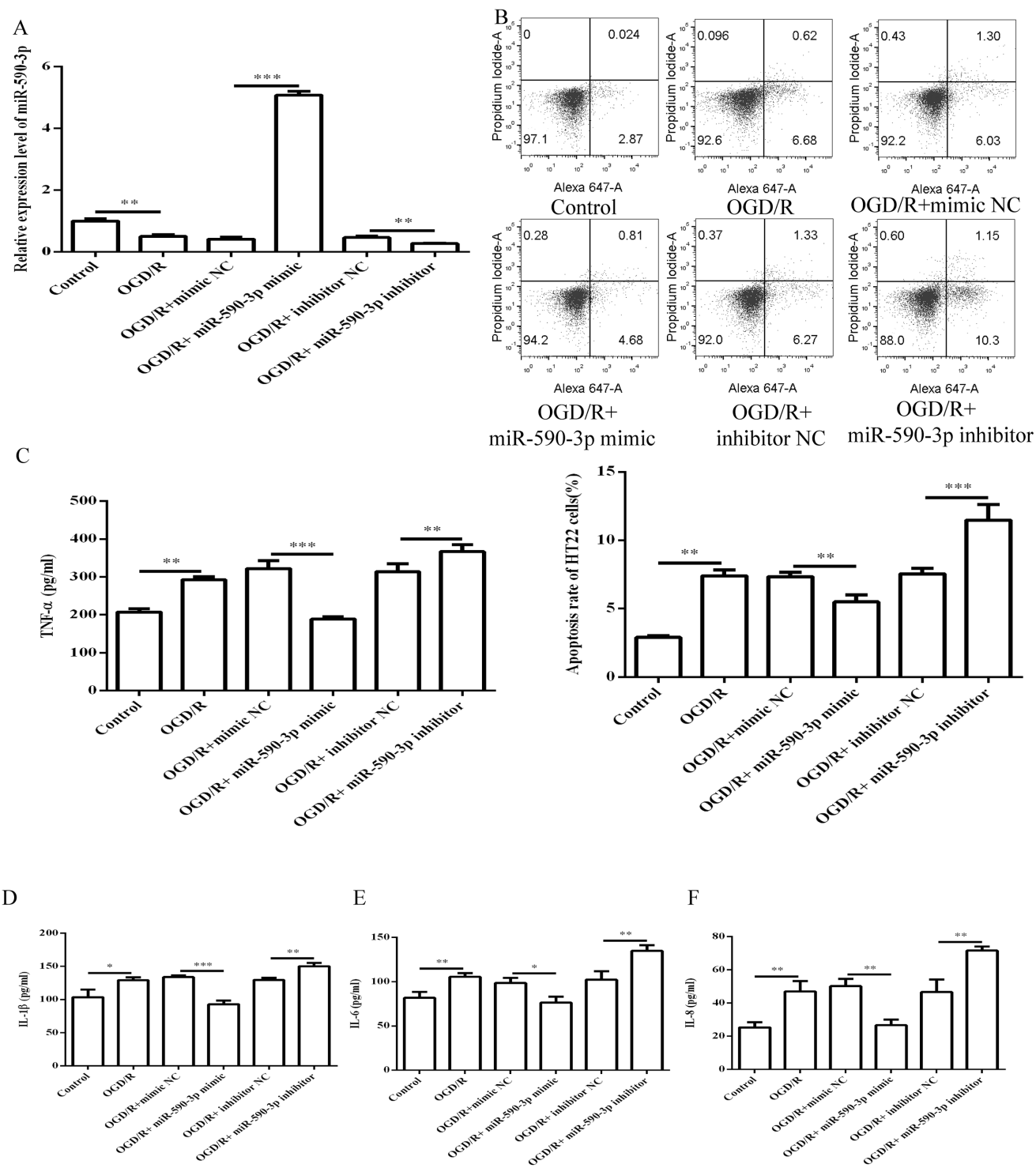

Fig. 2. Effect of miR-590-3p on cell survival and inflammation in cell model of OGD/R. HT22 cells were transfected with miR-590-3p mimic or miR-590-3p inhibitor for 24h prior to OGD/R treatment. **A.** MiR-590-3p expression levels in OGD/R-treated HT22 cells transfected with the mimic or inhibitor and measured by qPCR. **B.** Cell apoptosis detected by flow cytometry. **C-F.** Inflammatory cytokine level detected by Elisa assay. *P<0.05, **P<0.01, ***P<0.001.

overexpression of miR-590-3p has a protective effect on OGD/R-induced HT22 cell injury by decreasing apoptosis.

HMGB1 is a direct target of miR-590-3p

To explore the mechanism by which miR-590-3p mediates OGD/R-induced injury, the target genes of miR-590-3p were predicted, and HMGB1 was selected. The binding site of miR-590-3p and HMGB1 is shown in Fig. 3A. The luciferase assay results showed that the miR-590-3p mimic reduced the luciferase activity of HMGB1-WT. However, it did not change the activity of HMGB1-Mut (Fig. 3B). A western blot analysis further confirmed that the miR-590-3p mimic suppressed HMGB1 and that the miR-590-3p inhibitor promoted HMGB1 in the OGD/R cell model (Fig. 3C). These data indicate that miR-590-3p targets the 3′-UTR of HMGB1.

Effect of miR-590-3p on the TLR4/MyD88/NF-κB pathway in a cell model of OGD/R

To further delineate whether miR-590-3p is involved in the regulation of the TLR4/MyD88/NF-κB signalling pathway during the progression of cerebral I/R, we explored the role of miR-590-3p in the TLR4/MyD88/NF-κB pathway. As shown in Fig. 4, the miR-590-3p mimic repressed TLR4, MyD88, p-NF-κB, and p-IκBα protein levels after OGD/R treatment, whereas the miR-590-3p inhibitor showed the opposite effects. These results suggest that miR-590-3p inactivated the TLR4/MyD88/NF-κB signalling pathway.

miR-590-3p targets HMGB1 to regulate cell survival and inflammation via the TLR4/MyD88/NF-κB pathway in a cell model of OGD/R

Previous studies have reported that HMGB1 acts as an important inflammatory mediator in cerebral I/R injury. The reverse experiments were conducted to explore whether HMGB1 was involved in the biological roles of miR-590-3p in the cell OGD/R model. We initially transfected an HMGB1 overexpression vector (pcDNA-HMGB1) into OGD/R-treated HT22 cells and found that pcDNA-HMGB1 transfection elevated HMGB1 expression (Fig. 5A,B). Moreover, HMGB1 overexpression reversed the inhibitory effects of the miR-590-3p mimic on cell apoptosis (Fig. 5C) and inflammation (Fig. 5D) in the cell model of OGD/R. Moreover, as shown in Fig. 6, the miR-590-3p mimic

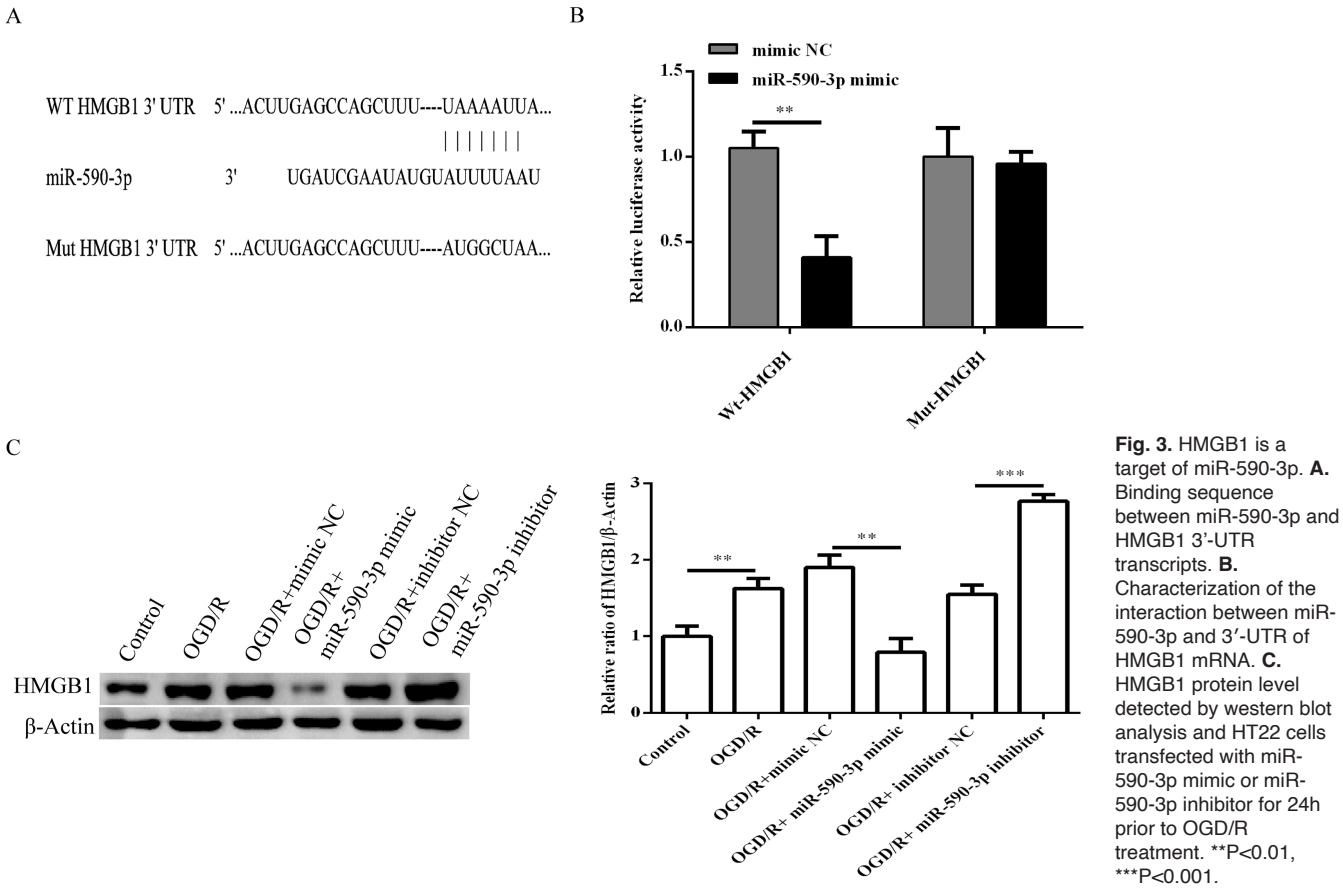

Fig. 3. HMGB1 is a target of miR-590-3p. **A.** Binding sequence between miR-590-3p and HMGB1 3'-UTR transcripts. **B.** Characterization of the interaction between miR-590-3p and 3′-UTR of HMGB1 mRNA. **C.** HMGB1 protein level detected by western blot analysis and HT22 cells transfected with miR-590-3p mimic or miR-590-3p inhibitor for 24h prior to OGD/R treatment. **P<0.01, ***P<0.001.

repressed TLR4 and MyD88 protein expression levels, and the protein phosphorylation ratio of NF-κB and IκBα was induced by OGD/R treatment. However, these changes were reversed by transfection with the pcDNA-HMGB1 mimic. Collectively, these data indicate that miR-590-3p protects against OGD/R-induced cell apoptosis and inflammation in OGD/R-treated HT22 cells by regulating the TLR4/MyD88/NF-κB pathway.

Discussion

According to statistics, more than 80% of all reported strokes are ischaemic strokes (Liu et al., 2020). A previous study indicated that inhibiting the apoptosis rate and suppressing inflammation were important protective approaches in cerebral I/R (Hua et al., 2015), and specific microRNAs, as immunomodulatory factors, play an essential role in regulating I/R injury (Zhao et al., 2019). However, the role of miR-590-3p in the cerebral I/R process remains unexplored. Our findings revealed that 1) the miR-590-3p level is markedly decreased and HMGB1 was increased in the OGD/R cell model, 2) overexpression of miR-590-3p has a protective effect on OGD/R-induced HT22 cell injury by decreasing apoptosis, 3) HMGB1 is a direct target of miR-590-3p, 4) miR-590-3p inactivates the TLR4/MyD88/NF-κB pathway by inhibiting HMGB1 in the cell OGD/R model, and 5) miR-590-3p targets HMGB1 to regulate cell survival and inflammation in the OGD/R cell model via the TLR4/MyD88/NF-κB pathway.

In this study, an *in vitro* model of cerebral I/R injury was established using OGD/R. We found that downregulation of miR-590-3p in OGD/R-induced brain neural cells promoted inflammatory cytokine secretion, cell apoptosis and activation of the TLR4/MyD88/NF-κB pathway. Previous studies have shown that cytokine secretion can be changed during the injury process by regulating miRNA and the NF-κB pathway, particularly IL-6, IL-11, IL-8, and TNF-α, which are important mediators involved in injury (Heinrich et al., 2003; Alsaleh et al., 2009; Yamamoto et al., 2014; Li et al., 2019). Furthermore, miR-590-3p mimic transfection was found to reduce the levels of IL-1β, IL-6, IL-8, and TNF-α in OGD/R-simulated neural cells in the vitro

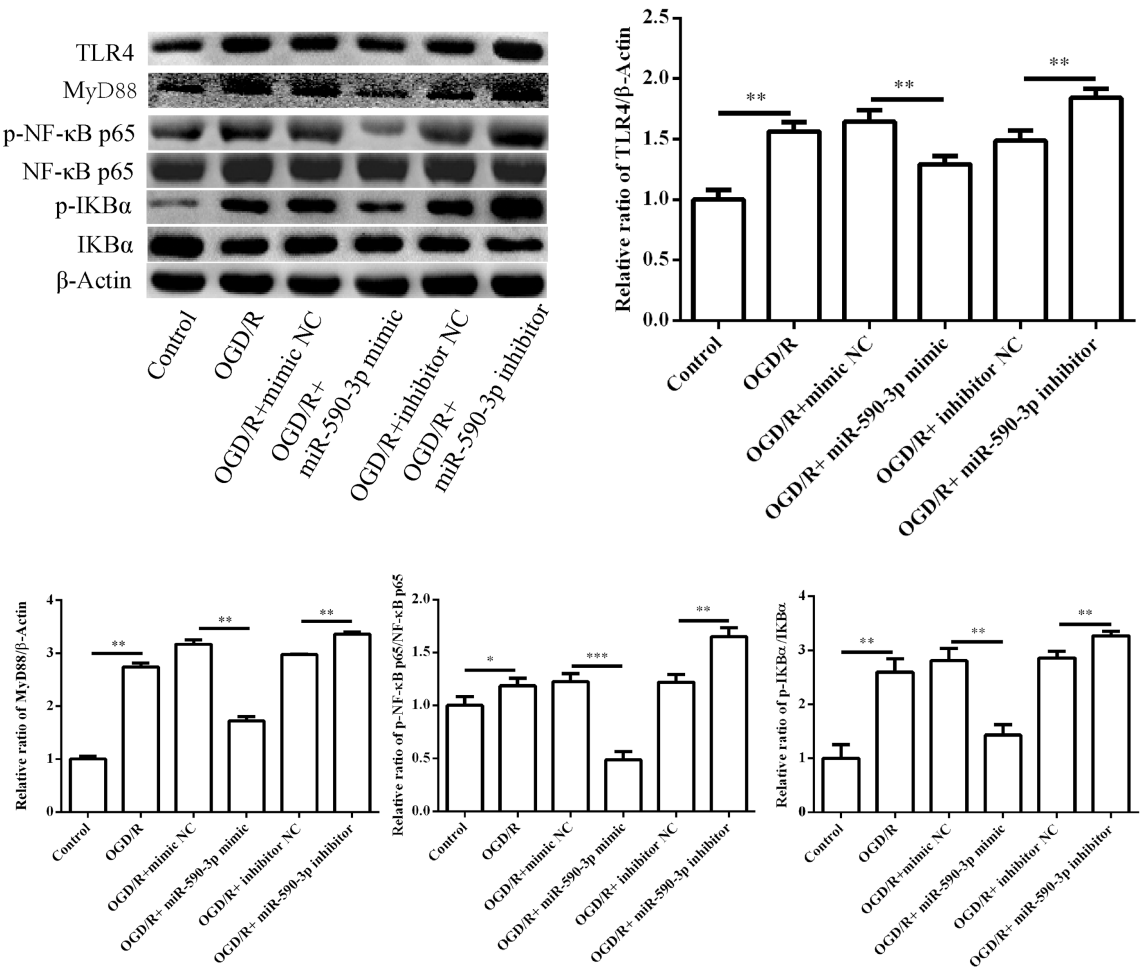

Fig. 4. Effect of miR-590-3p on TLR4/MyD88/NF-κB pathway in the OGD/R cell model detected by western blot analysis. HT22 cells transfected with miR-590-3p mimic or miR-590-3p inhibitor for 24h prior to OGD/R treatment. *P<0.05, **P<0.01, ***P<0.001.

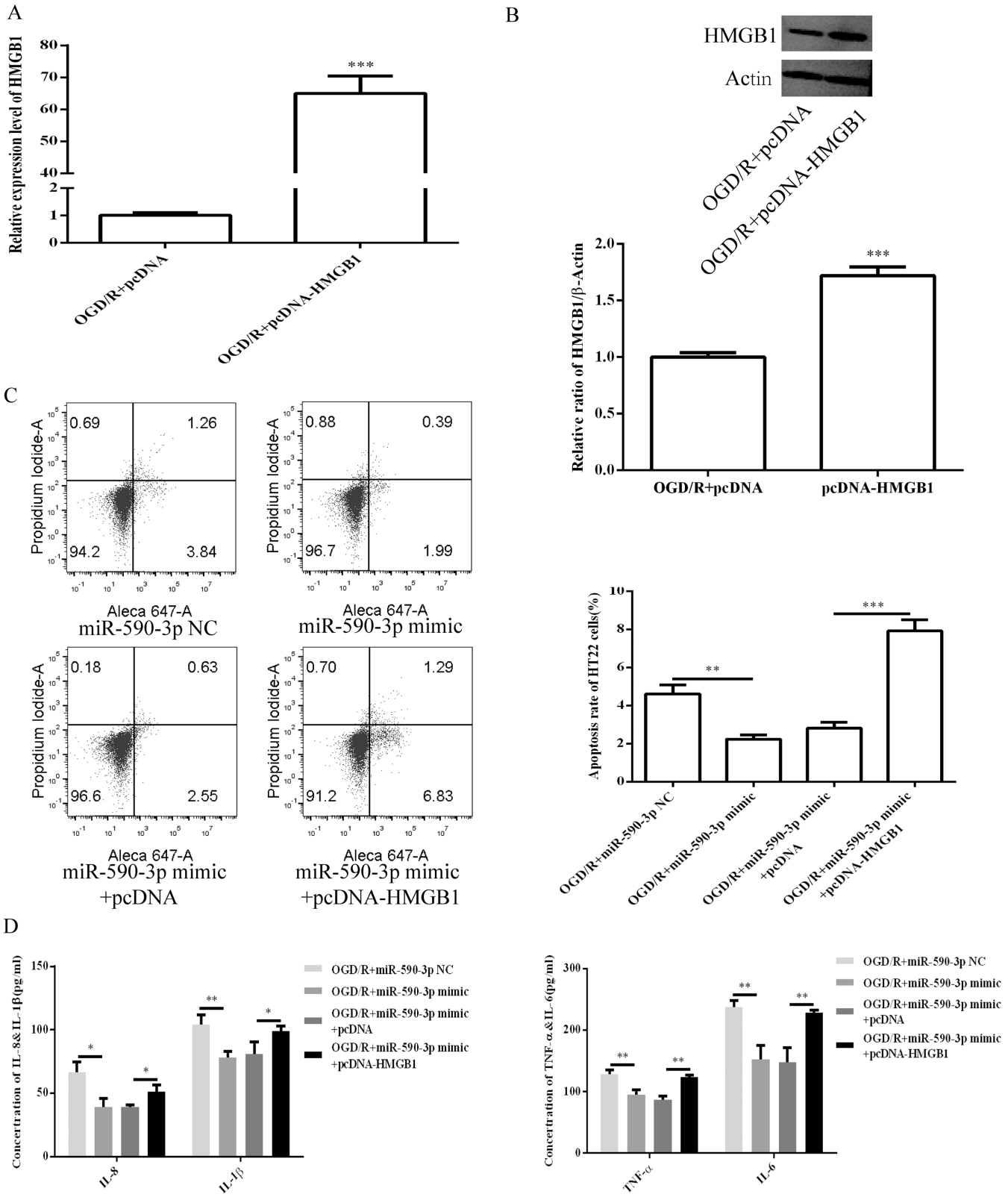

Fig. 5. miR-590-3p targets HMGB1 to regulate cell survival and inflammation in the OGD/R cell model. HT22 cells cotransfected with miR-590-3p mimic and pcDNA-HMGB1 or the control vector pcDNA for 24h, then treated by OGD/R. **A.** Relative expression level of HMGB1 by qPCR. **B.** HMGB1 protein expression assessed by western blot analysis. **C.** Cell apoptosis detected by flow cytometry. **D.** Inflammatory cytokine level detected by Elisa assay. *P<0.05, **P<0.01, ***P<0.001.

model, which was considered in previous studies of IL-1β and TNF-α production (Guijarro-Munoz et al., 2014). Moreover, miR-590-3p was able to interact with HMGB1 and inhibit HMGB1 expression, thereby significantly downregulating the protein levels of TLR4 and MyD88 and the protein phosphorylation levels of NF-κB and IκB. These results suggest that miR-590-3p can target HMGB1, which inhibits TLR4/MyD88/NF-κB signalling.

In cancer research, miR-590-3p promotes apoptosis in breast cancer cells (Rohini et al., 2018), intrahepatic cholangiocarcinoma (Zu et al., 2017), and head and neck cancer cells (Di Agostino et al., 2018). These results of miR-590-3p were inconsistent with our results, but no evidence has confirmed the role of miR-590-3p in I/R. Some previous studies suggested that overexpression of miR-496 (Yao et al., 2019), miR-34b (Huang et al., 2019) or miR-484 (Liu et al., 2021) was able to decrease cerebral I/R injury, which indicates that these microRNAs inhibit apoptosis in I/R, and these outcomes were consistent with our results, which demonstrated that the miR-590-3p mimic reduced the apoptosis rate of HT22 cells (Fig. 2B). However, some cancer studies reported that miR-484, miR-496 and miR-34b overexpression significantly inhibited cell proliferation in gastric cancer (Liu et al., 2020; Su et al., 2021) and colon cancer (Ye et al., 2019). The mode of action of miRNA in the pathophysiological processes of I/R injuries appears to differ from that in cancer cells.

In a series of studies, TLR4 expression and IL-1β production increased in rat lung pericytes with LPS-induced inflammation (Edelman et al., 2006, 2007a,b),

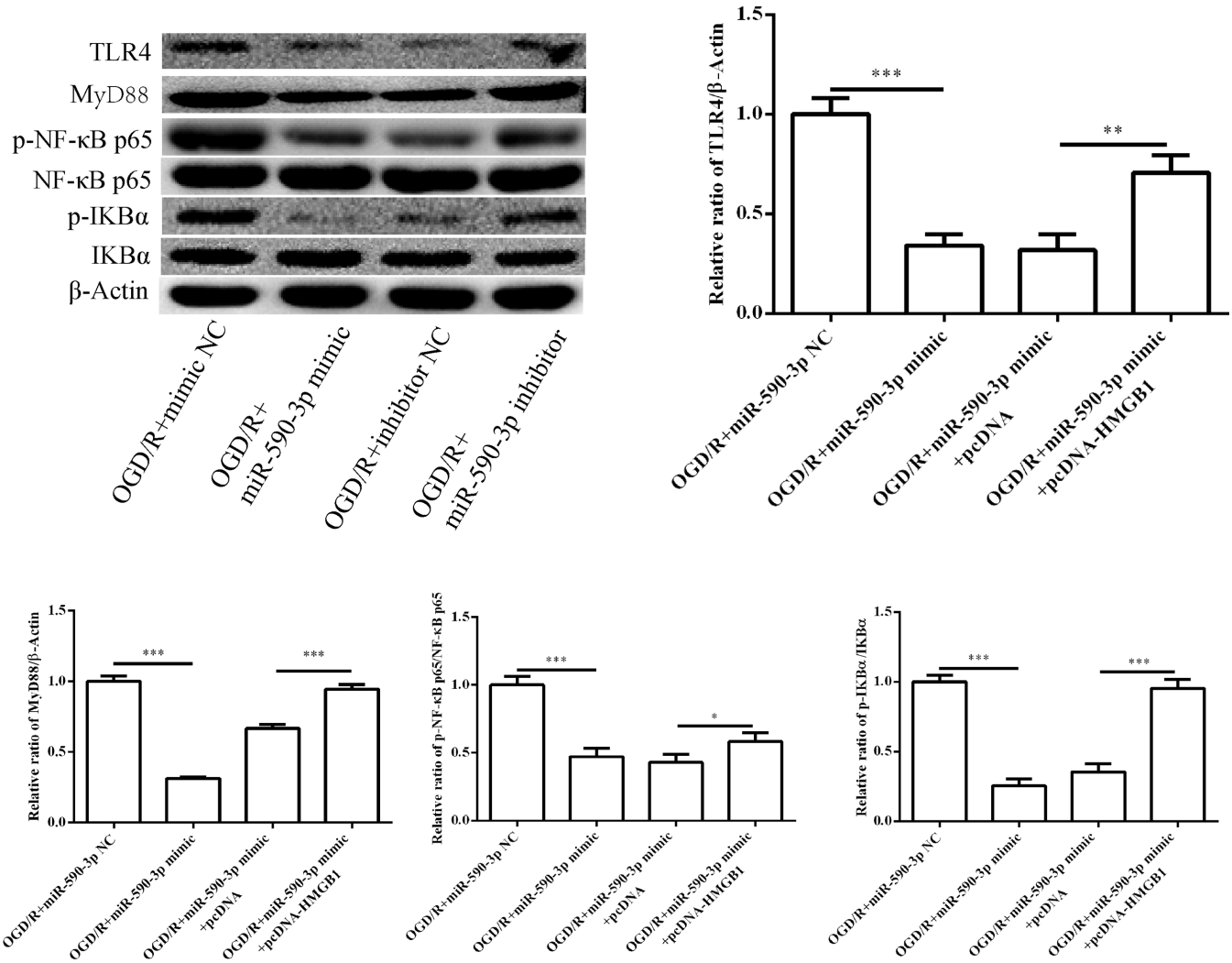

Fig. 6. Effect of miR-590-3p and HMGB1 on TLR4/MyD88/NF-κB pathway in the OGD/R cell model was detected by western blot. HT22 cells cotransfected with miR-590-3p mimic and pcDNA-HMGB1 or the control vector pcDNA for 24h, and then treated by OGD/R. *P<0.05, **P<0.01, ***P<0.001.

suggesting that they have an active role in the inflammatory response. TLR4 is an important receptor complex that binds the endogenous ligands of the proinflammatory cytokine-like protein HMGB1 (Treutiger et al., 2003; Yang et al., 2016). In addition, HMGB1 has been described to have a proinflammatory effect on monocytes, endothelial cells, neutrophils, fibroblasts, and dendritic cells (Andersson et al., 2000; Park et al., 2003; Treutiger et al., 2003; Messmer et al., 2004; Rossini et al., 2008). Importantly, HMGB1 has proinflammatory activity based on the combination of HMGB1 and LPS, which triggers the expression of proinflammatory cytokines (Youn et al., 2008; Jin et al., 2010; Khoshnam et al., 2017; Zhang et al., 2017). Increased release of HMGB1 leads to TLR4/MyD88/NF-κB activation, which exacerbates inflammation (Zhu et al., 2018; Yan et al., 2019). In our research, the expression of HMGB1 was significantly increased, and the concentrations of IL-1β, IL-6, IL-8, and TNF-α were also evidently increased in OGD/R-induced brain neural cells. miR-590-3p targets HMGB1 and negatively regulates HMGB1 expression. In the recovery experiment, after the overexpression of HMGB1, the inhibitory effect of miR-590-3p on cell apoptosis was abolished. HMGB1 overexpression significantly increased the cell apoptosis rate and the inflammatory cytokine concentrations, but these parameters were reduced with miR-590-3p mimic transfection. These results confirmed previous results of HMGB1 as a proinflammatory factor (Youn et al., 2008). Furthermore, miR-590-3p reduced the TLR4 protein level and the protein phosphorylation ratio of NFκB and IκBα. These results suggest that HMGB1, as a target of miR-590-3p, negatively regulates HT22 cell apoptosis by activating the TLR4/MyB88/NFκB pathway in OGD/R-induced brain neural cells, thereby regulating the I/R process.

In summary, this study demonstrated that overexpression of miR-590-3p attenuated OGD/R-induced injury in HT22 cells by targeting HMGB1 through the TLR4/MyD88/NF-κB pathway, implying a potential role of miR-590-3p in cerebral I/R injury. Targeting of HMGB1/TLR4/MyD88/NF-κB signalling by miR-590-3p may provide a promising approach for I/R injury therapy.

However, the precise function and mechanism of miR-590-3p in I/R injury *in vivo* remain to be fully elucidated using an animal model. These results would be more convincing if the target gene knockout model was applied to controlled studies in animal models of the mechanism and function of miR-590-3p.

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Competing Interest statement. The authors declare no competing interests.

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