**Original** paper

Folia Neuropathologica

# Remote ischaemic perconditioning reduces the infarct volume and improves the neurological function of acute ischaemic stroke partially through the miR-153-5p/TLR4/p65/IκBα signalling pathway

**Hao Zha[1], Wei Miao[2], Wei Rong[2], Aimei Wang[2], Weiqi Jiang[3], Rui Liu[3], Lanqi Liu[3], Ying Wang[2]**

[1]Department of Reproductive and Genetics, The Second Affiliated Hospital of Kunming Medical University, China, [2]Department of Neurology, The Second Affiliated Hospital of Kunming Medical University, China, [3]The Second Clinical School of Kunming Medical University, Kunming, China

*Folia Neuropathol 2021; 59 (4): 335-349* *DOI: https://doi.org/10.5114/fn.2021.112127*

## Abstract

*Remote ischemic perconditioning (RIPerC) could improve neuronal damage and inhibit inflammation and apoptosis. We conducted an in-depth exploration of the protective mechanism of RIPerC in cerebral ischaemia injury. In this study, a middle cerebral artery occlusion (MCAO) mouse model was built. According to whether to undergo RIPerC treatment and the duration of cerebral infarction, mice were divided into 5 groups: Sham group, MCAO 3.0 h group, MCAO 4.5 h group, MCAO 3.0 h + RIPerC group, and MCAO 4.5 h + RIPerC group. Overexpressed or silenced miR-153-5p was transfected into the cells to analyse the effects of oxygen-glucose deprivation (OGD) treatment on Neuro-2a cell viability, apoptosis, and related gene expressions by performing quantitative real-time polymerase chain reaction (qRT-PCR), MTT assay, flow cytometry, and Western blot. Bioinformatics analysis, qRT-PCR, dual-luciferase experiment, and RNA immunoprecipitation (RIP) were used to screen and verify the miRNA and downstream mRNA-targeted Toll-like receptor 4 (TLR4). The rescue test further verified the effects of the above target genes and miR-153-5p on the apoptosis of OGD-injured cells, apoptosis-related proteins, and the p65/IκBα pathway. The plasma levels of miR-153-5p in 68 patients with ischaemic stroke were detected within 6 hours of onset, and the patients were followed up for 3 months. We found that, in in vivo studies, RIPerC treatment inhibits cerebral infarction volume and neurological damage, and promotes the expression of miR-153-5p in the MCAO animal model. The expression of miR-153-5p in OGD cells was inhibited, and its upregulation protected Neuro-2a cells. TLR4 was predicted to be the target gene of miR-153-5p and could offset the effect of miR-153-5p mimic on OGD cell protection after up-regulating TLR4. TLR4 overexpression promoted the activation of OGD on the p65/IκBα pathway. Compared with the high plasma miR-153-5p group, the 3-month overall survival rate of patients with ischaemic stroke in the low plasma miR-153-5p group was significantly lower ($\chi^2 = 5.095$, $p = 0.024$). In conclusion, RIPerC intervention inhibits the damage caused by cerebral ischaemia partially through the miR-153-5p/TLR4/p65/IκBα signalling pathway.*

***Key words:*** *remote ischaemic perconditioning, stroke, middle cerebral artery occlusion, TLR4.*

**Communicating author**

Ying Wang, PhD, Department of Neurology, The Second Affiliated Hospital of Kunming Medical University, China,
e-mail: wang_yy1983@yeah.net

## Introduction

Cerebrovascular disease, which is characterized by high disability, morbidity, and mortality, is currently one of the focuses of the prevention and treatment of diseases of the elderly [14]. Stroke is currently the most important cause of disability and death in China, among which ischaemic stroke accounts for about 87% of all strokes [9]. The only effective treatment for ischaemic stroke is recombinant tissue plasminogen activator (rt-PA) thrombolytic therapy. However, it is restricted by its strict time window, indications, and contraindications. Currently, less than 1% of patients in China qualify for rt-PA thrombolytic therapy [3,17]. Therefore, it is urgent to actively explore new, effective, and safe treatment methods to improve the prognosis of ischaemic stroke.

The American Stroke Association (ASA) has published research showing that before the occurrence of cerebral infarction, patients with repeated "small strokes" (i.e. transient ischemic attacks [TIA]) have lower infarct volume and better prognosis than patients without "small strokes" [20]. This may indicate that repeated and short-term ischaemia and hypoxia of brain tissue can enhance the ability of brain tissue to resist ischaemia. Remote ischaemic conditioning (RIC) is a new method of ischaemic adaptation, which protects target organs from continuous ischaemic damage through transient ischaemia-reperfusion of remote organs [10,26]. According to the implementation time of RIC, it can be divided into remote ischaemic preconditioning (RIPC), remote ischaemic perconditioning (RIPerC), and remote ischaemic postconditioning (RIPostC). RIPerC refers to the implementation of RIC after cerebral ischaemia and before reperfusion. It is worth noting that there have been many studies on the application of RIPerC to ischaemic animal models, and it was initially found that RIPerC can significantly reduce the infarct volume of the ischaemic animal model organs [5,27]. However, the mechanism by which RIPerC interferes with ischaemic stroke to reduce cerebral infarction volume and improve neurological function has not yet been reported.

MicroRNA (miRNA) is an endogenous gene that encodes a non-coding single-stranded RNA molecule with a length of approximately 20 nucleotides [6]. After being combined with the target mRNA, it can effectively inhibit the translation of target mRNA and induce the degradation process. With the development of whole-genome sequencing technology, miRNAs are receiving increasing attention. Studies have shown that miRNAs are involved in various biological functions, such as brain neurodevelopment [2], nerve cell apoptosis [16], and changes in synaptic plasticity [22]. Interestingly, when the brain is in a hypoxic and ischaemic environment, neurological impairment and neurodegenerative diseases will also have a close relationship with miRNA [4]. Several studies have reported that miR-153-5p has significant anti-tumour effects [7,23]. However, there is no report about the expression characteristic and potential value of miR-153-5p after ischaemic stroke.

In this study, a middle cerebral artery occlusion (MCAO) mouse model and oxygen-glucose deprivation (OGD) cell model were constructed *in vivo* and *in vitro*. In clinical terms, we further explored the relationship between the plasma miR-153-5p level within 6 hours of the onset of acute ischaemic stroke and the clinical characteristics and prognosis. The purpose is to clarify the mechanism of RIPerC in reducing the infarct volume and neurological damage of acute ischaemic stroke.

## Material and methods

### Collection of plasma samples from acute ischaemic stroke patients and healthy controls, and 3-month follow-up of patients

The study group comprised 68 patients with acute ischaemic stroke treated in our hospital from October 2018 to August 2019, including 42 males and 26 females. The National Institutes of Health Stroke Scale (NIHSS) score was assessed on admission. We collected plasma from patients with acute ischaemic stroke within 6 hours of onset and stored it at –80° for future use. Demographic and clinical information, including gender, age, hypertension, hypercholesterolaemia, diabetes mellitus, body mass index (BMI), alcoholism, and smoking were also collected. The cerebral infarct volume of patients was calculated by MRI (Vision Plus1.5T, Siemens, Germany). According to the time when the patients were included in the study, they were followed up for 3 months. During the same period, 68 healthy controls who underwent physical examinations from the Health Examination Centre were collected. Plasma

was acquired from the subject with written informed consent. Our research plans were approved by the Human Ethics Committee of our hospital, Kunming, China (no. 20180811H), according to the Declaration of Helsinki (as revised in 2013).

## Preparation of MCAO model and RIPerC

We purchased 50 C57BL/6J mice (male, 25 ±2 g) from the Experimental Animal Centre of Tianjin Medical University [SCXK (E) 2019-0115]. Experiments were performed under a project license (No. 20190408-3) granted by the Experimental Animal Centre of our hospital. Humane care was given during the experimental animal breeding and experimental procedures following the 3R principle of experimental animals. The mice were housed at 22°C, with humidity of 50%, and provided with an adequate diet. According to body weight, C57BL/6J mice were randomly divided into 5 groups (10 mice/group): Sham group, MCAO 3.0 h group, MCAO 4.5 h group, MCAO 3.0 h + RIPerC group, and MCAO 4.5 h + RIPerC group.

The MCAO was built based on a previous study [24]. The mice were anaesthetized with sodium pentobarbital at a dose of 30 mg/kg. A 6-0 surgical thread was inserted from the left external carotid artery of the mouse and extended into the internal carotid artery to block the middle cerebral artery and induce embolism. The blood flow of arterial embolism was detected by laser Doppler blood flow meter (Moor Instruments, UK). Ischaemia was defined when the blood flow dropped to 80% (compared to the baseline). Clinically, the best and latest time points for intravenous thrombolysis for patients with acute ischaemic stroke are within 3.0 h and 4.5 h of onset, respectively. Therefore, we chose the 2 time points of 3.0 h and 4.5 h.

RIPerC treatment of MCAO mice: After the successful establishment of the MCAO model, the mice were subjected to cerebral ischaemia for 2.0 h and 3.5 h, respectively, without removing the threaded plug. Then, a non-invasive tourniquet was used to tighten the right hind limb of the mouse for the first time for 10 min and loosen it for 10 min. For the second time, the right hind limb of the mouse was tightened for 10 min and loosened for 10 min. For the third time, the right hind limb of the mouse was tightened for 10 min and loosened for 10 min. After the above 3 cycles, the threaded plug was removed. Then, the blood supply was restored and blood flow after reperfusion was maintained at higher than 70%. Finally, the neurological function (Menzies score, Belayev score, and Garcia score) of each group of mice was scored. All the mice were sacrificed 24 hours after reperfusion.

## Cell and model establishment

Mouse Neuro-2A neuroblastoma (Neuro-2a) cells were established by R. J. Klebe and F. H. Ruddle with spontaneous tumours of strain A mice, and most of them were neuron-like with axon-like structures. Therefore, we purchased Neuro-2a cells (CL-0168) from Wuhan Procell for *in vitro* research. Neuro-2a cells were inoculated in MEM complete medium (PM170410B, Procell, China) mixed with foetal bovine serum. When the serum-free medium was needed in subsequent experiments, the medium was replaced with a pure MEM medium (PM170409B, Procell, China). The conventional cell culture was uniformly completed in a Heracell™ VIOS 250i $CO_2$ Incubator (Thermo Scientific™) at 37°C with 5% $CO_2$.

An OGD cell model was constructed as described before [19]. Neuro-2a cells were adjusted to $1 \times 10^5$ cells/ml and then uniformly inoculated in a pure MEM medium. Neuro-2a cells in the control group were inoculated in MEM complete medium. The cells in the OGD group were transferred to a 37°C Heal Force 3-gas incubator (HF100) containing 1% $O_2$, 5% $CO_2$, and 94% $N_2$. The subsequent OGD treatment was conducted with the continuous filling of an anoxic mixture for 6 hours. During this period, the cells in the control group were cultured routinely.

## Cell transfection

miR-153-5p mimic, small interfering RNA (siRNA)-miR-153-5p, and Toll-like receptor 4 (TLR4) overexpression plasmid, as well as their negative controls (NC), were synthesized by Shanghai HANBIO Company. The sequence of siRNAs was provided in Table I. After completing the plasmid preparation, we used the Lipofectamine 3000 liposome transfection reagent (L3000009, ThermoFisher) to transfect the overexpression plasmid into the Neuro-2a cells. The transfection efficiency was assessed 48 hours after transfection by quantitative real-time polymerase chain reaction (qRT-PCR).

**Table I.** Sequence of primers and siRNAs

| | Gene | Forward primer (5'-3') | Reverse primer (5'-3') |
|---|---|---|---|
| Primers used for qRT-PCR | miR-153-5p | GGCTGTAATTGGGCGCGTCG | GTGCGTATAGTCCAGAATTGTGC |
| | TLR4 | GTGAGACCTGAAACTGCGTCA | TGTCGTGGTGAGACTTATTAC |
| | HGF | ACCACTTCGGGGTGATCGG | CGCACCACTTCGAATTCGCAGA |
| | GDAP1 | TAACCACCACTTCACACTACGGA | GAATTAACCACGCACCGCTCTTT |
| | TBX22 | ACCTCCTAACCGAGGAGACC | TCTCTTGATCGTTCACGTCTGG |
| | HNMT | CACTTGAGACCTCATCGTGAACC | GGAGCCCACTTTCATCACTTAGAG |
| | FREM2 | CATAACCTTGGAAGCCAGGAA | GTTTCAAGGATCAGATGGAGCAT |
| | U6 | CTCGCTTCGGCAGCACA | AACGCTTCACGAATTTGCGT |
| | GAPDH | TGTGGGCATCAATGGATTTGG | ACACCATGTATTCCGGGTCAAT |
| siRNAs | siNC | UUCGCCAGACGUUGCGCGUdTdT | ACGUGACACGUUCGGAGAAdTdT |
| | si-miR-153-5p | CGCGGCACUGCGACAATCdTdT | ACCGCUGGGACTGCATTCGdTdT |

## QRT-PCR

Total RNAs in Neuro-2a cells, mouse brain tissues, and stroke patients' plasma were separated by TRIzol (15576425, Invitrogen, USA), and then cDNA was further synthesized by PrimeScriptTM RT reagent Kit (RR039A, TaKaRa, Japan). Pre-synthesized gene primers (Sangon, China), Roche SYBR Green Master (05629017212), and DEPC water were added to the cDNA and mixed and tested in the detection instrument (thermal cycler T100, Bio-Rad, USA), according to the following settings: pre-denaturation at 95°C for 10 min, denaturation at 95°C for 15 s, and annealing at 58°C for 1 min, for a total of 40 cycles. For calculation of RNA levels, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 were used as internal references. The gene RNA level was determined by the qRT-PCR detection and calculated by $2^{-\Delta\Delta CT}$. The amplification efficiencies of miR-153-5p, TLR4, HGF, GDAP1, TBX22, HNMT, FREM2, U6, and GAPDH were 98.7%, 101.5%, 99.4%, 96.5%, 104.7%, 99.6%, 97.2%, 103.3%, and 98.9%, respectively. The test was repeated 3 times for each sample. The detailed primer sequences are listed in Table I.

## Apoptosis test

Neuro-2a cells were trypsinized and washed with phosphate-buffered saline (PBS). $1 \times 10^5$ Neuro-2a cells were resuspended in 100 µl of 1 × Binding Buffer (from Annexin V-FITC/PI Apoptosis Detection Kit; 39211ES60, YEASEN, China), then stained by Annexin V-FITC (5 µl) and PI Staining Solution (5 µl) in dark condition. Next, 400 µl of 1 × Binding Buffer was added to the Neuro-2a cell mixture, and the apoptosis changes were analysed by BD FACSVerse flow cytometer (USA).

## Western blot

The RIPA (P0012K, Beyotime, China) and the BCA detection kit (P0010S, Beyotime, China) were applied in the extraction and quantification of proteins from Neuro-2a cells or brain tissues. The proteins were transferred to the NC membrane (N8633, Millipore, USA) with a pore size of 0.22 µm by the SDS-PAGE method. After treating the NC membrane with the blocking solution at room temperature for 2 hours, corresponding primary antibodies were used to fully cover the protein-laden NC membrane (at 4°C). After 24 hours, the primary antibody was washed off and replaced with the corresponding secondary antibody for further incubation. The NC membrane was developed with Beyotime ECL Luminescent Solution (P0018S) after 1 h. The fluorescent signal generated by the protein on the membrane was collected by the BIO-RAD Gel Doc™ XR+ instrument and processed into a corresponding greyscale band image. GAPDH was an internal reference. The primary and secondary antibodies used in the experiment were purchased from Abcam and CST in the United States as follows: Bcl-2 (1 : 2000, ab196495, 26 kDa, Abcam), Bax (1 : 2000, ab53154, 21 kDa, Abcam), cleaved Caspase-3 (1 : 5000, ab52072, 17 kDa, Abcam), phosphorylated-p65 (p-p65; 1 : 1000, ab76311, 60 kDa, Abcam), p65 (1 : 1000, ab19870, 65 kDa, Abcam), TLR4 (1 : 1000, ab22048, 96 kDa, Abcam), GAPDH (1 : 10000, ab8245, 36 kDa, Abcam), Rabbit Anti-Mouse antibody (1 : 5000, ab46540, Abcam), Goat Anti-Rabbit antibody (1 : 5000, ab97051, Abcam),

p-inhibitor a of NF-κB (p-IκBα; 1 : 1000, #9246, 40 kDa, CST), and IκBα (1 : 1000, #4814, 39 kDa, CST).

### Dual-luciferase reporter experiment

Based on the binding sequence provided by the starBase database, we constructed TLR4-wt and TLR4-mut reporter plasmid by pmirGLO vector (E1330, Promega, USA). The reporter plasmid and miR-153-5p mimic or mimic control were co-transfected into Neuro-2a cells (48 h). Neuro-2a cells treated with Dual-Luciferase® Reporter Assay kit (E1910, Promega, USA) were then transferred to the GloMax 20/20 Luminometer (Promega) to analyse luciferase activity.

### RNA immunoprecipitation (RIP)

We collected Neuro-2a cells with transfection (TLR4 + miR-153-5p mimic or TLR4 + mimic control), and the Neuro-2a cells were lysed with Cell lysis buffer in the RIP kit (KT100-01, GZSCBio, China). Cells in the RNase-free EP tubes were labelled as the IgG + mimic-NC group, anti-Argonaute-2 (Ago2) + mimic-NC group, IgG + miR-153-5p group, Ago2 + miR-153-5p group, Input-mimic-NC group, and Input-miR-153-5p group, respectively. The antibodies used were the Ago2 antibody (1 : 50, ab156870, Abcam, USA) and the NC antibody IgG provided in the kit. In RNA binding protein immunoprecipitation, the magnetic bead-antibody mixture was mixed with cell lysate for incubation overnight (at 4°C). The Input-mimic-NC group and the Input-miR-153-5p group were not given the magnetic bead-antibody mixture. On the second day, the purified RNA was analysed for the expression of TLR4 by qRT-PCR, and the enrichment level was calculated.

### MTT assay

According to the recommended inoculation requirements, the cell density of Neuro-2a was adjusted to $1 \times 10^4$ cells/ml and 100 µl of it was transferred to a sterilized 96-well plate (6 wells per treatment group). After 48 hours of routine culture, 10 µl of APE × BIO MTT solution (B7567) was added to Neuro-2a cells in the control group and other treatment groups. After 4 hours, the Neuro-2a cells were detected by the Molecular Devices microplate reader (SpectraMax iD5) for absorbance (OD) at 490 nm.

### Triphenyl tetrazolium chloride staining

The brain tissues of the sacrificed mice were quickly removed and transferred to an environment of –20°C for freezing for 30 min. The frozen brain tissues were incised according to the coronal position and made into brain slices with a thickness of 2 mm. The brain tissues were then reacted with triphenyl tetrazolium chloride (TTC) Stain Kit (D025-1-1, Nanjing Jiancheng Bioengineering Institute, China) for 30 min. The brain tissues were fully stained in a water bath (37°C). Image information was collected immediately after the removed brain tissues were washed with PBS. The white part of the result was the infarcted tissues. The proportion of the infarct area in the total area was measured and calculated.

### Immunohistochemical analysis

Immunohistochemical analysis was constructed as described before [21]. Primary antibody TLR4 (1 : 1000, ab22048, 96 kDa, Abcam) was added to the brain tissues, and the biotinylated Goat Anti-Rabbit secondary antibody (1 : 5000, ab97051, Abcam) was incubated at 4°C. Finally, the sections were incubated with DAB substrate for 5 min.

### Statistical analysis

All experiments were performed in triplicate unless specified. Experiments were performed at least 3 times. The results are represented as the mean ± standard error of the mean (SEM) or median (interquartile range – IQR). The differences between normally distributed numeric variables were evaluated by Student's *t*-test, whereas non-normally distributed variables were analysed by Mann-Whitney *U*-test. One-way ANOVA was used for the comparison among multiple groups if the variance was homogeneous, while non-normally distributed variables were evaluated by Kruskal-Wallis variance analysis. Multiple comparisons between the groups were performed using the S-N-K method. Correlations were analysed using the Spearmen method. The survival curve was analysed by the Kaplan-Meier method. $P < 0.05$ was considered significant.

## Results

### RIPerC treatment reduces cerebral infarction volume and neurological damage and promotes the expression of miR-153-5p in the MCAO animal models

In this study, 5 groups (10 mice/group) of mice were analysed, namely the Sham group, MCAO 3.0 h

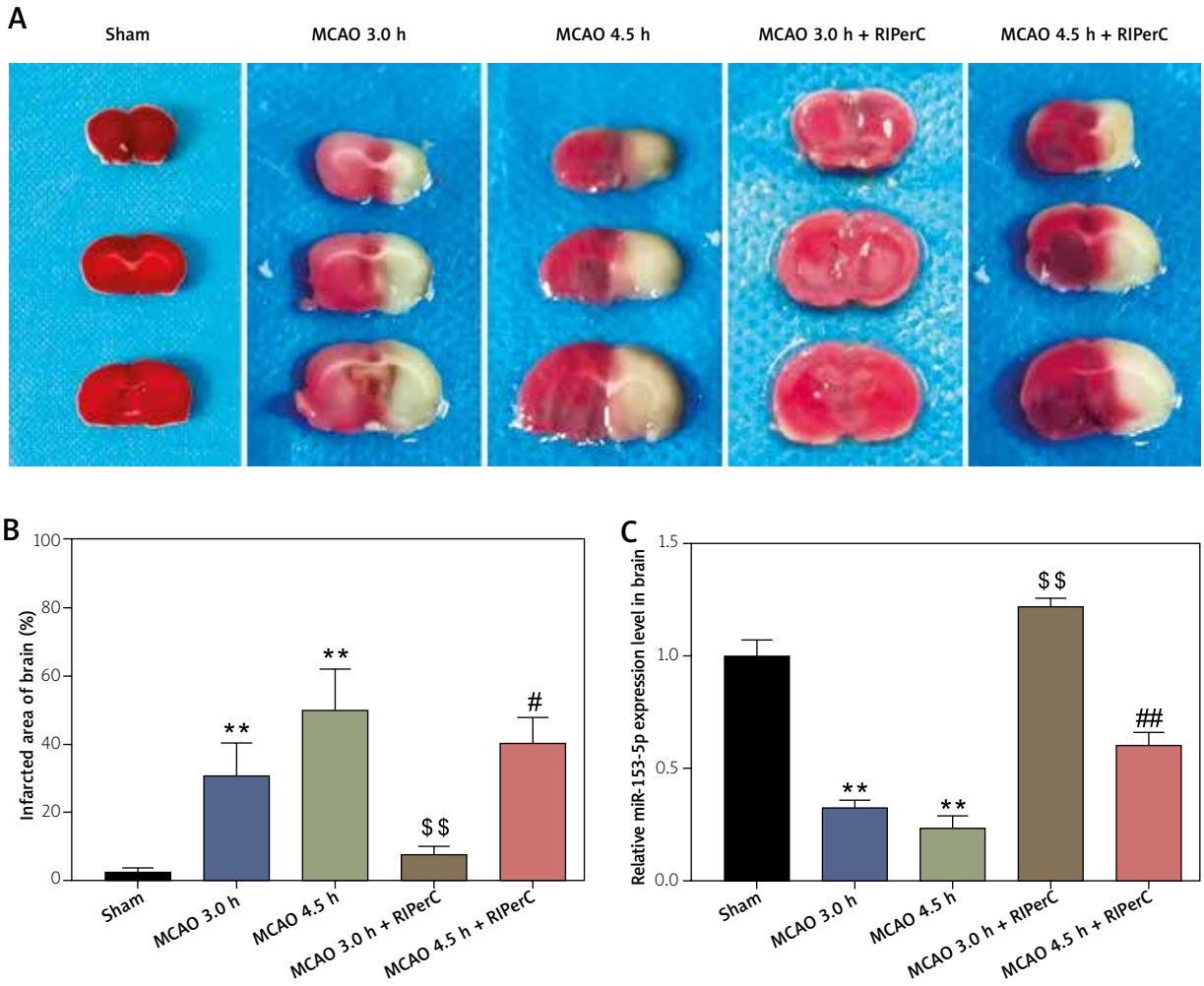


**Fig. 1.** RIPerC treatment reduced brain tissue infarction and promoted the miR-153-5p level caused by MCAO. **A, B**) The effect of RIPerC on the infarct area of MCAO model brain tissues was observed and analyzed by TTC staining. **C**) The effect of RIPerC on the miR-153-5p expression of MCAO model brain tissues was analyzed by qRT-PCR. The test was repeated three times for each sample. $^{**}p < 0.01$ vs. Sham group; $^{\$\$}p < 0.01$ vs. MCAO 3.0 h group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. MCAO 4.5 h group. RIPerC – remote ischemic perconditioning, MCAO – middle cerebral artery occlusion, TTC – triphenyltetrazolium chloride.

**Table II.** Comparison of neurological function scores of rats in each group (median [IQR])

| Variables | Sham group | MCAO 3.0 h group | MCAO 4.5 h group | MCAO 3.0 h + RIPerC group | MCAO 4.5 h + RIPerC group | Z | *P* |
|---|---|---|---|---|---|---|---|
| Number of rats | 10 | 10 | 10 | 10 | 10 | | |
| Menzies score | 0 (0-0) | 2 (1-3)[a] | 4 (3-4)[a] | 1 (0-1)[b] | 2 (1-4)[c] | 6.284 | 0.004 |
| Belayev score | 0 (0-0) | 8 (7-10)[a] | 11 (9-12)[a] | 4 (3-5)[b] | 7 (6-10)[c] | 7.694 | 0.001 |
| Garcia score | 18 (17-18) | 11 (9-14)[a] | 7 (5-10)[a] | 16 (14-17)[b] | 12 (10-14)[c] | 4.356 | 0.013 |

*MCAO – middle cerebral artery occlusion; RIPerC – remote ischemic perconditioning; IQR – interquartile range. [a] Compared with the Sham group, p < 0.05; [b] Compared with the MCAO 3.0 h group, p < 0.05; [c] Compared with the MCAO 4.5 h group, p < 0.05*

group, MCAO 4.5 h group, MCAO 3.0 h + RIPerC group, and MCAO 4.5 h + RIPerC group. As shown in Figure 1A, B, and Table II, compared with MCAO mice not treated with RIPerC, the proportion of infarcts in the entire brain tissues and neurological damage (Menzies score, Belayev score, and Garcia

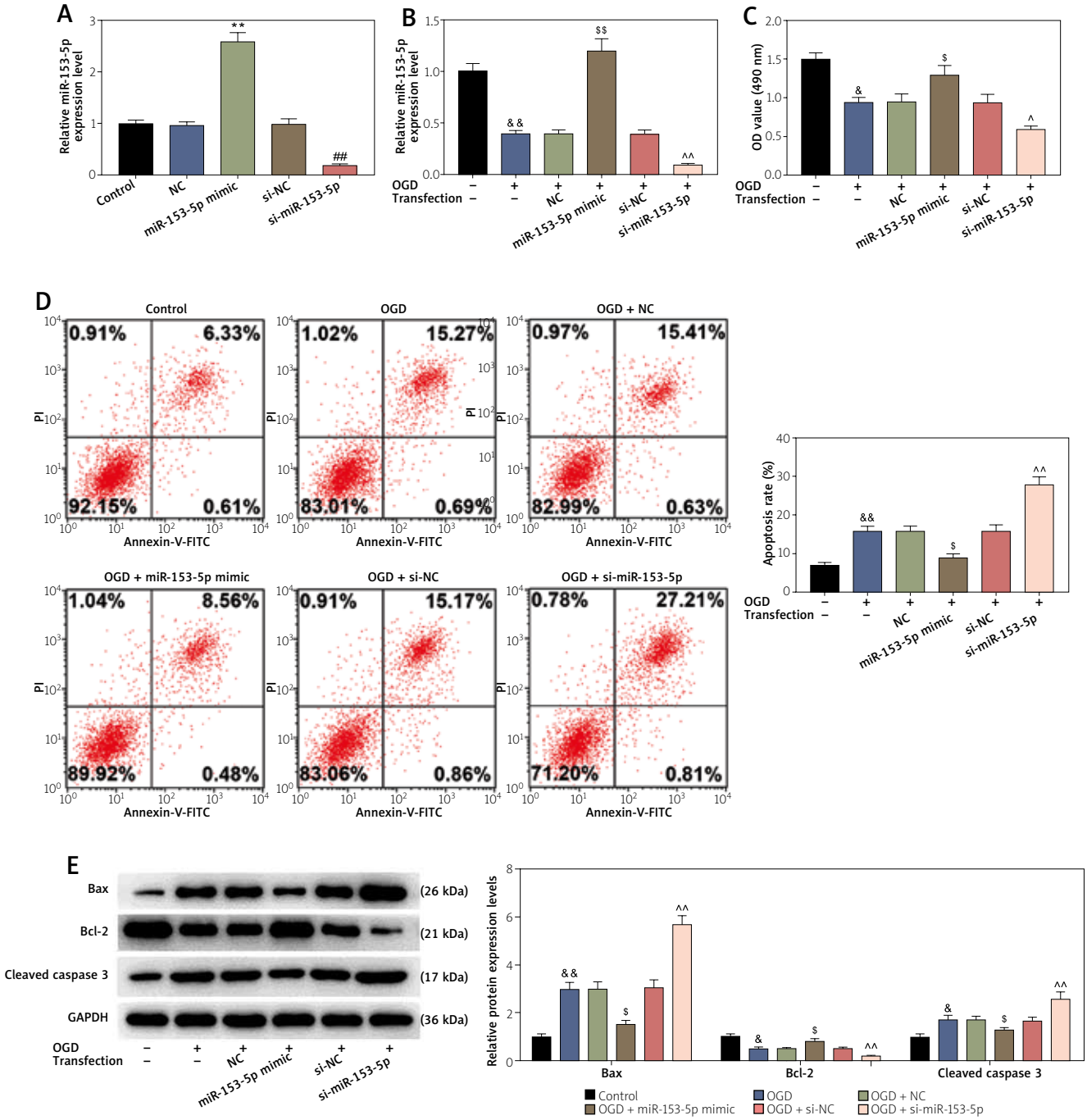


**Fig. 2.** Up-regulated miR-153-5p enhanced the viability of OGD cells and reduced apoptosis by regulating apoptosis-related proteins, while si-miR-153-5p had the opposite effect. **A**) The transfection efficiency of overexpressed/silenced miR-153-5p was determined by qRT-PCR. The test was repeated 3 times for each sample. **B**) The effects of overexpressed/silenced miR-153-5p and OGD treatment on miR-153-5p expression in Neuro-2a cells were detected by qRT-PCR. U6 was an internal reference. The test was repeated 3 times for each sample. **C**) The effects of overexpressed/silenced miR-153-5p and OGD treatment on Neuro-2a cell viability were determined by the MTT experiment (OD = 490 nm). The experiment was performed 3 times. **D**) The effects of overexpressed/silenced miR-153-5p and OGD treatment on Neuro-2a cell apoptosis were measured by flow cytometry. The experiment was performed 3 times. **E**) The effects of overexpressed/silenced miR-153-5p and OGD treatment on apoptosis-related proteins in Neuro-2a cells were detected by Western blot. GAPDH was an internal reference. The test was repeated 3 times for each sample. $^{**}p < 0.01$ vs. NC; $^{\#\#}p < 0.01$ vs. si-NC; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ vs. Control; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ vs. OGD + NC; $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$ vs. OGD + si-NC. OGD – oxygen-glucose deprivation; qRT-PCR – quantitative real-time polymerase chain reaction; GAPDH – glyceraldehyde-3-phosphate dehydrogenase.

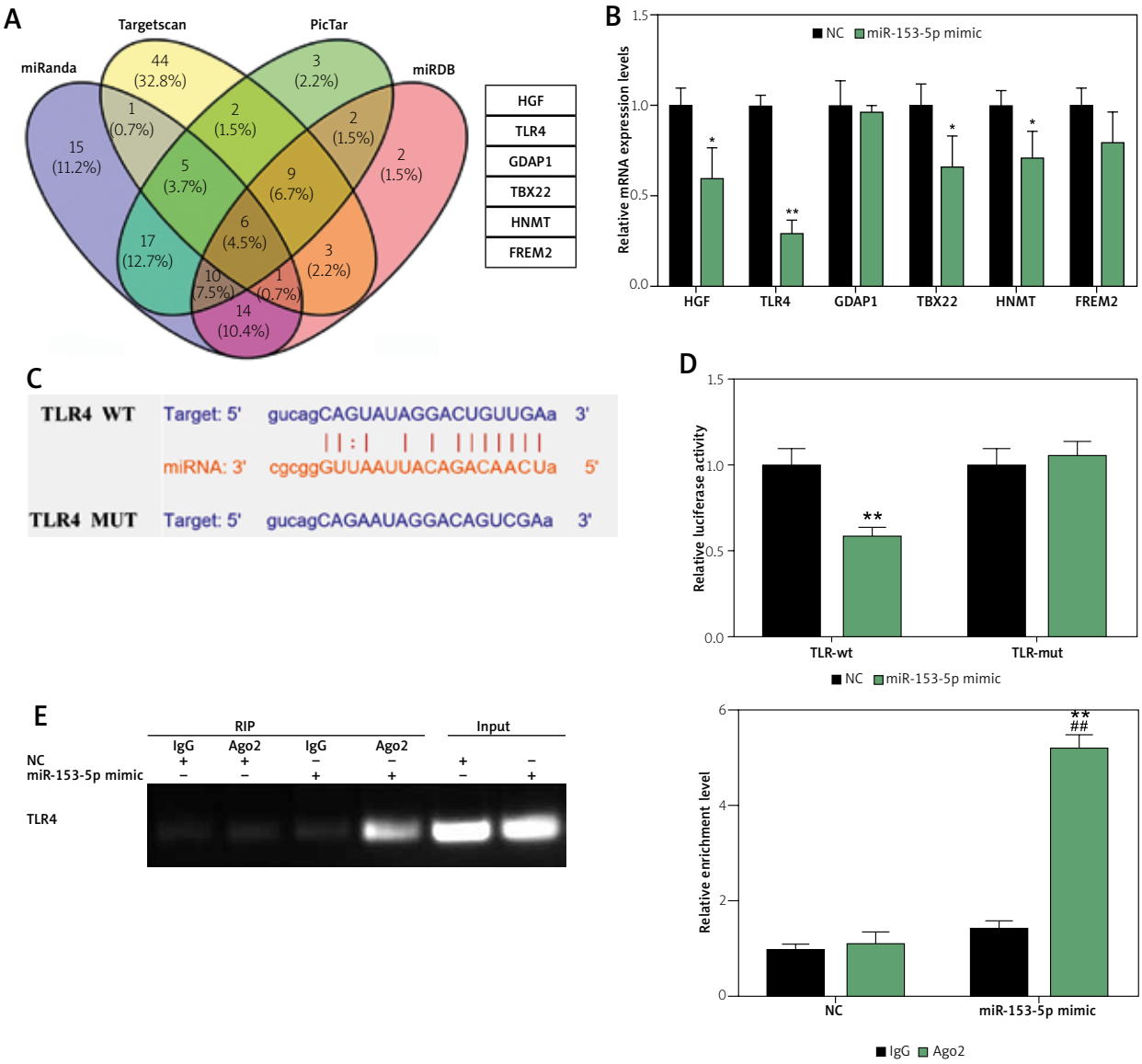


**Fig. 3.** TLR4 was a downstream target gene of miR-153-5p. **A**) The intersection mRNAs of the target genes of miR-153-5p predicted by PicTar (https://pictar.mdc-berlin.de/), miRanda (http://www.microrna.org/), miRDB (http://www.mirdb.org/), and Targetscan (http://www.targetscan.org/) were obtained through the Venn diagram. **B**) The regulatory relationship between the intersection of mRNAs and miR-153-5p mimic was further verified by qRT-PCR. GAPDH was an internal reference. The test was repeated 3 times for each sample. **C**) The miRDB database showed the binding sequence of miR-153-5p and TLR4. **D**) The dual-luciferase experiment verified the binding sequence of miR-153-5p and TLR4. The experiment was performed 3 times. **E**) The association of miR-153-5p and TLR4 was verified by an RNA immunoprecipitation experiment. The experiment was performed 3 times. *$p < 0.05$, **$p < 0.01$ vs. NC; $^{\#\#}p < 0.01$ vs. IgG + miR-153-5p mimic. qRT-PCR – quantitative real-time polymerase chain reaction; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; TLR4 – Toll-like receptor 4.

score) of MCAO mice treated with RIPerC were significantly reduced ($p < 0.05$). Moreover, compared with MCAO mice not treated with RIPerC, the relative miR-153-5p expression level in the brain of MCAO mice treated with RIPerC was significantly increased (Fig. 1C, $p < 0.05$).

## Up-regulated miR-153-5p enhanced the viability of OGD cells and reduced apoptosis by regulating apoptosis-related proteins, while si-miR-153-5p had the opposite effect

The cell transfection experiment fully confirmed that the transfection study was successfully constructed. The miR-153-5p expression level was up-regulated in the miR-153-5p mimic group but down-regulated in the si-miR-153-5p group (Fig. 2A, $p < 0.01$). *In vivo*, as expected, the expression of miR-153-5p was suppressed by the OGD treatment (Fig. 2B, $p < 0.01$). In the following loss or gain cell function experiments, OGD treatment produced greater effects on inhibiting cell viability and increasing apoptosis of Neuro-2a cells (Fig. 2C, D, $p < 0.05$). More importantly, OGD treatment up-regulated Bax and cleaved caspase 3 proteins but prevented the activation of Bcl-2 (Fig. 2E, $p < 0.05$). Exogenous up-regulation or silencing of miR-153-5p also produced reversal or enhanced effects: miR-153-5p overexpression reduced cell damage and protein regulation caused by OGD treatment, whereas silenced miR-153-5p further increased OGD's damage effects on Neuro-2a cells (Fig. 2C-E, $p < 0.05$).

## TLR4 was a downstream target gene of miR-153-5p

In this study, HGF, TLR4, GDAP1, TBX22, HNMT, and FREM2 with MCAO differences were screened (Fig. 3A). We further determined the expression changes of the above 6 target genes under miR-153-5p mimic intervention. The results showed that TLR4 was the most inhibited (Fig. 3B, $p < 0.01$). Therefore, TLR4 was determined as the downstream target gene of miR-153-5p for further analysis. We first verified the association between TLR4 and miR-153-5p based on the binding sequence predicted by the miRDB database (Fig. 3C). As shown in Fig. 3D, the co-transfection of TLR4-wt with miR-153-5p mimic significantly inhibited the luciferase activity of the cells ($p < 0.01$). Further RIP testing also confirmed the binding between miR-153-5p and TLR4 (Fig. 3E, $p < 0.01$).

## Overexpressed TLR4 neutralized the anti-apoptosis and gene regulation effects of miR-153-5p mimic in OGD cells

The transfection of overexpressed TLR4 into Neuro-2a cells significantly upregulated the mRNA level of TLR4 (Fig. 4A, $p < 0.05$). In the following Western blot detection, miR-153-5p mimic further inhibited the TLR4 expression overexpressed by OGD, while overexpressed TLR4 neutralized the above regulatory effect of miR-153-5p mimic (Fig. 4B, $p < 0.05$). Overexpressed TLR4 also showed the same neutralization effect in the subsequent apoptosis test (Fig. 4C, $p < 0.05$). We also analysed the regulation of TLR4 on the p65/IκBα pathway. The results showed that TLR4 overexpression promoted the activation of OGD on the p65/IκBα pathway (and the ratio of p-p65/p65 and p-IκBα/IκBα) (Fig. 4D, $p < 0.05$). More importantly, overexpression of TLR4 neutralized the OGD attenuation effect of the miR-153-5p mimic (Fig. 4D, $p < 0.05$).

## The MCAO animal model confirmed the results of the cell experiment

Furthermore, we validated the results of the cell experiment through *in vivo* study. The results of immunohistochemistry and Western blot detection showed that the expression of TLR4 protein in the brain tissue of MCAO mice treated with RIPerC was significantly lower than that of the group without RIPerC treatment (Fig. 5A-C, $p < 0.05$). Interestingly, the ratios of p-p65/p65 and p-IκBα/IκBα also decreased significantly after treatment with RIPerC (Fig. 5B, D, E, $p < 0.05$). The above results suggest that RIPerC treatment may inhibit the infarction and neurological damage of MCAO mice by regulating the miR-153-5p/TLR4/p65/IκBα signalling pathway.

## Plasma miR-153-5p levels in ischaemic stroke patients are negatively correlated with infarct volume and NIHSS, and low levels of miR-153-5p are associated with poor prognosis

Plasma was collected within 6 hours after the onset of ischaemic stroke, and the plasma miR-153-5p

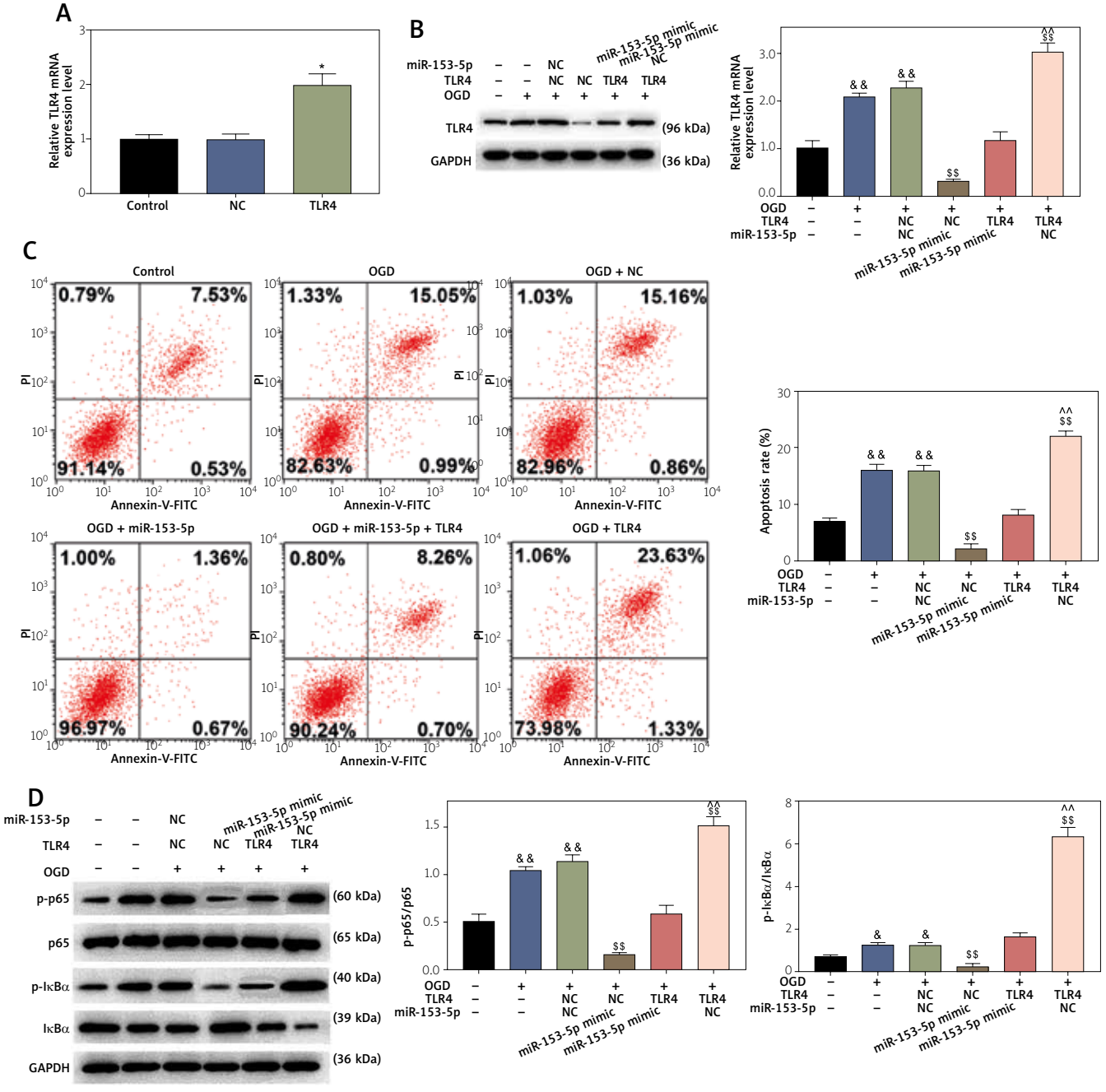


**Fig. 4.** Overexpressed TLR4 neutralized the anti-apoptosis and gene regulation effects of miR-153-5p mimic in OGD cells. **A**) The transfection efficiency of TLR4 was tested by qRT-PCR. The test was repeated 3 times for each sample. **B**) The effects of overexpressed TLR4 and miR-153-5p mimic on TLR4 protein expression in OGD model cells were analysed by Western blot. GAPDH was an internal reference. The test was repeated 3 times for each sample. **C**) The effects of overexpressed TLR4 and miR-153-5p mimic on OGD cell apoptosis were analysed by flow cytometry. The experiment was performed 3 times. **D**) The effects of overexpressed TLR4 and miR-153-5p mimic on the p65/IκBα pathway in OGD model cells were detected by Western blot. GAPDH was an internal reference. The test was repeated 3 times for each sample. $^{*}p < 0.05$ vs. NC; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ vs. Control; $^{\$\$}p < 0.01$ vs. OGD + NC; $^{\wedge\wedge}p < 0.001$ vs. OGD + miR-153-5p mimic + TLR4. TLR4 – Toll-like receptor 4; OGD – oxygen-glucose deprivation; qRT-PCR – quantitative real-time polymerase chain reaction; GAPDH – glyceraldehyde-3-phosphate dehydrogenase.

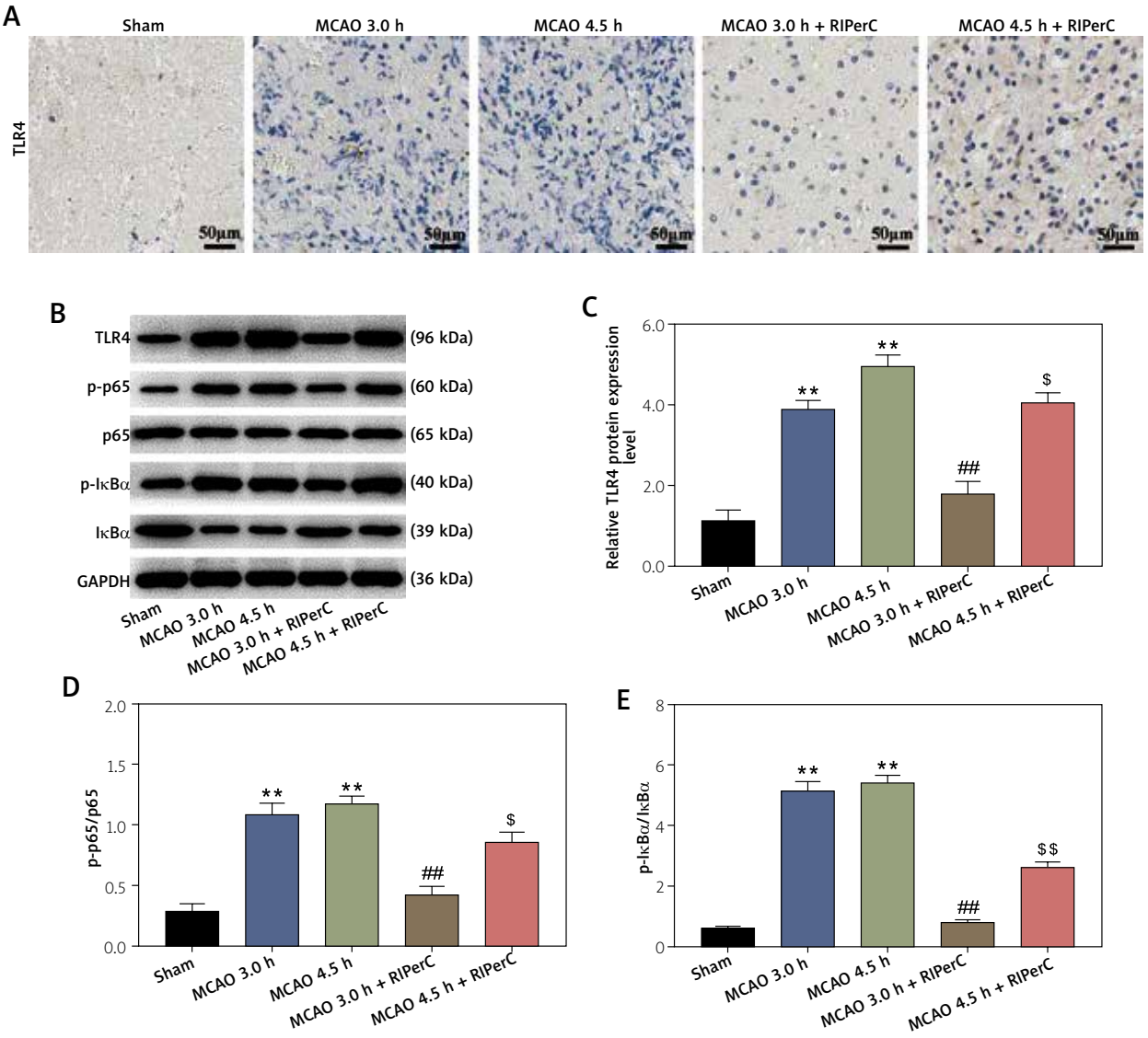


**Fig. 5.** RIPerC treatment reduced the TLR4 expression and the ratios of p-p65/p65 and p-IκBα/IκBα in brain tissue of MCAO mouse model. **A**) The levels of TLR4 in the brain tissue of mice in each group were detected by immunohistochemistry. The experiment was performed 3 times. **B-E**) The levels of TLR4 and p65/IκBα pathway proteins in the brain tissues of mice in each group were detected by Western blot. GAPDH was an internal reference. The test was repeated 3 times for each sample. $^{**}P < 0.01$ vs. Sham group; $^{\#\#}p < 0.01$ vs. MCAO 3.0 h group; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ vs. MCAO 4.5 h group. TLR4 – Toll-like receptor 4; RIPerC – remote ischemic perconditioning; MCAO – middle cerebral artery occlusion; GAPDH – glyceraldehyde-3-phosphate dehydrogenase.

level was detected by qRT-PCR. As shown in Figure 6A, plasma miR-153-5p levels in patients with ischaemic stroke were significantly lower than healthy controls. Spearman correlation analysis showed that plasma miR-153-5p levels in patients with ischaemic stroke were significantly negatively correlated with cerebral infarction volume (Fig. 6B, $r = –0.596$, $p < 0.001$) and NIHSS score (Fig. 6C, $r = –0.571$, $p < 0.001$). Then, the patients were divided into 2 groups based on the median of their plasma miR-153-5p levels. Survival analysis showed that, compared with the high miR-153-5p group, the 3-month overall survival rate of patients in the low miR-153-5p group was significantly lower (Fig. 6D, $\chi^2 = 5.095$, $p = 0.024$). Cox regression analysis is shown in Table III. Hypertension and infarct volume are independent risk factors for poor progno-

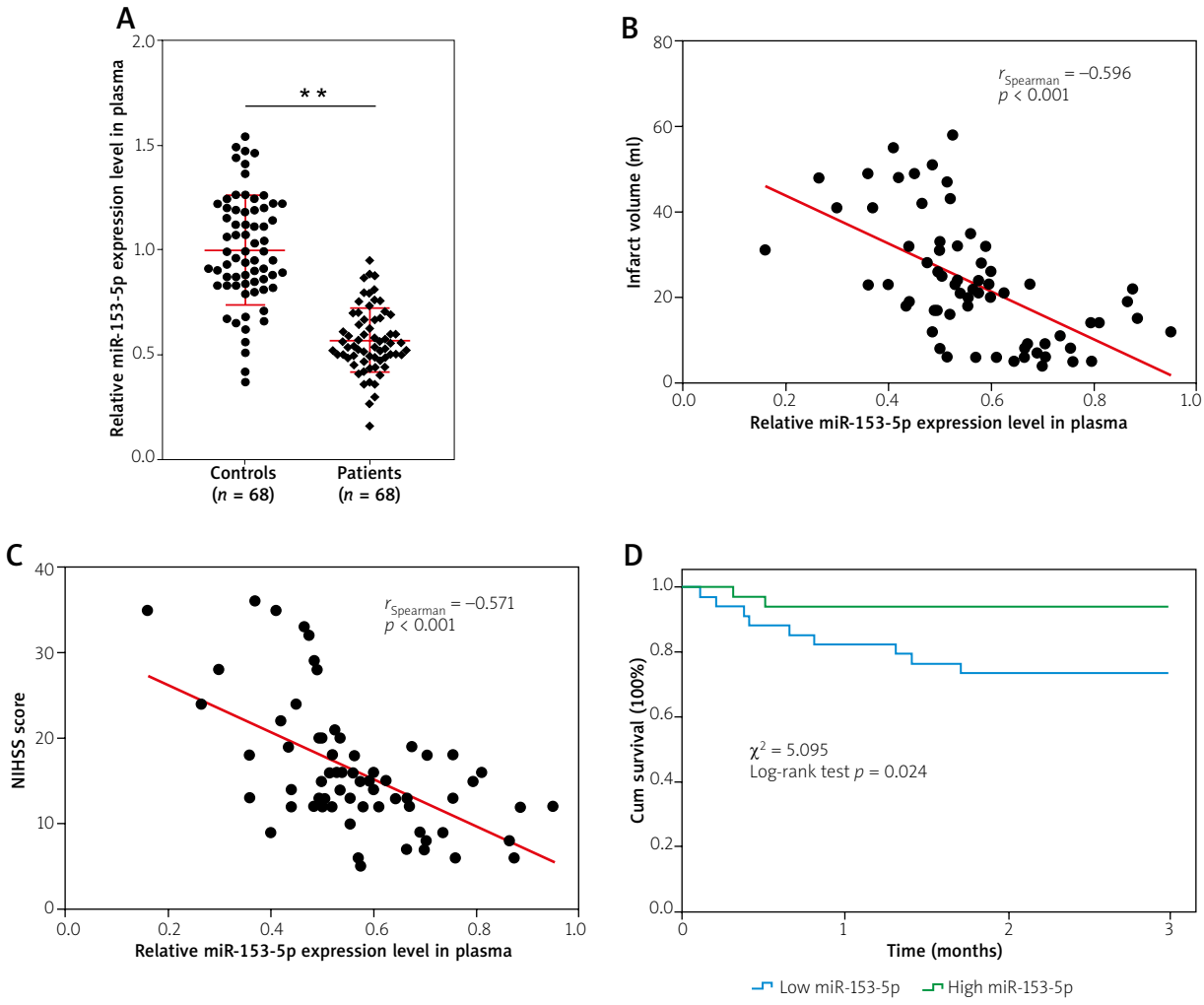


**Fig. 6.** Plasma miR-153-5p levels in ischaemic stroke patients are negatively correlated with infarct volume and NIHSS, and low levels of miR-153-5p are associated with poor prognosis. **A**) The plasma miR-153-5p levels in patients with ischaemic stroke and healthy controls were tested by qRT-PCR. The test was repeated 3 times for each sample. **B**) Spearman correlation analysis between plasma miR-153-5p levels and cerebral infarction volume. **C**) Spearman correlation analysis between plasma miR-153-5p levels and NIHSS score. **D**) Survival analysis showed that compared with the high miR-153-5p group, the 3-month overall survival rate of patients in the low miR-153-5p group was significantly lower. $^{**}p < 0.01$ vs. Controls. qRT-PCR – quantitative real-time polymerase chain reaction.

sis in patients with ischaemic stroke, while miR-153-5p is an independent protective factor.

## Discussion

Brain tissue ischaemia could cause local cerebral ischaemic injury. RIPerC refers to repetitive and intermittent blood flow blocking stimulation of the limbs, through nerve conduction, body fluids, and systemic inflammatory responses, to modulate the endogenous protective mechanism of motivation [5,10,26]. Thereby, it induces the tolerance of the heart, brain, liver, kidney, lung, and other organs to ischaemia and hypoxia, and improves the ability of remote vital organs to resist ischaemia and hypoxia damage. A study has shown that RIPerC cannot only protect ischaemic brain tissue, but also can further improve the effect of thrombolytic therapy for myocardial infarction [15]. To better improve the clinical treatment effect of RIPerC, the improvement effect of miR-153-5p on cerebral ischaemia injury was examined. The up-regulation of miR-153-5p effectively improved the cell damage caused by OGD and

**Table III.** Analysis of univariate and multivariate Cox models

| Variables | Univariate Cox model | | | | | | Multivariate Cox model | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | β | SE | $\chi^2$ | Wald | RR | *P* | β | Standardized β | $\chi^2$ | Wald | RR | *P* |
| Age | –0.172 | 0.117 | 2.148 | 2.143 | 0.842 | 0.143 | | | | | | |
| Male | 0.023 | 0.120 | 0.037 | 0.037 | 1.023 | 0.847 | | | | | | |
| Hypertension | 0.263 | 0.128 | 4.288 | 4.264 | 1.268 | 0.039 | 0.232 | 0.132 | 3.948 | 3.942 | 1.193 | 0.046 |
| Hypercholesterolaemia | –0.081 | 0.118 | 0.474 | 0.472 | 0.922 | 0.491 | | | | | | |
| Diabetes mellitus | –0.025 | 0.125 | 0.040 | 0.039 | 0.975 | 0.842 | | | | | | |
| BMI | 0.217 | 0.110 | 3.924 | 3.919 | 1.243 | 0.048 | 0.176 | 0.132 | 2.331 | 2.327 | 1.027 | 0.537 |
| Alcoholism | 0.016 | 0.105 | 0.875 | 0.876 | 0.932 | 0.337 | | | | | | |
| Smoking | 0.031 | 0.114 | 1.102 | 1.100 | 1.015 | 0.548 | | | | | | |
| Infarct volume | 0.534 | 0.098 | 30.356 | 29.728 | 1.706 | 0.000 | 0.346 | 0.277 | 6.324 | 8.589 | 1.413 | 0.012 |
| miR-153-5p | –0.288 | 0.106 | 7.451 | 7.417 | 0.134 | 0.006 | –0.403 | –0.160 | 6.576 | 7.308 | 0.297 | 0.010 |

β – *regression coefficient; SE – standard error; RR – risk ratio; BMI – body mass index*

reduced the brain tissue infarction induced by MCAO in mice. At the molecular level, overexpression of miR-153-5p prevented the excessive activation of pro-apoptotic genes (Bax and cleaved caspase 3), and the p65/IκBα inflammatory pathway, while promoted the expression of apoptosis inhibitor Bcl-2. These data suggested that miR-153-5p is a potential marker of cerebral ischaemia injury. Thus, the pathway in which miR-153-5p exerted its effects was further analysed.

Further screening of mRNAs and verification demonstrated that among the mRNAs screened, TLR4 changed the most obviously after being regulated by miR-153-5p. The TLR4 gene is an important member of the interferon regulatory factor (IRF) family and a specific transcription factor that can bind to the A/T-rich DNA sequence to regulate the expressions of related genes [11]. Studies have shown that TLR4 is the core transcription factor for heart development [12,25]. Abnormal expression of TLR4 induces abnormal development of the heart and blood vessels, leading to the death of mice in the embryo [25]. TLR4 also has a wide range of regulatory effects on cell apoptosis and inflammation [1,13]. These reports confirmed the results of this study that TLR4 expression in OGD cells was up-regulated by MCAO but down-regulated by miR-153-5p. More importantly, TLR4 aggravated the cell damage caused by OGD and neutralized the regulatory effect of miR-153-5p. Our experimental results revealed that by regulating the miR-153-5p/TLR4 axis, RIPerC alleviated the damage to tissues and cells caused by cerebral ischaemia.

In addition, according to our results, miR-153-5p regulated the apoptosis and inflammation of tissues and cells through the miR-153-5p/TLR4 axis. Consistent with previous reports, up-regulation of Bcl-2, which inhibited apoptosis, blocked the expression of pro-apoptotic genes (Bax and Cleaved caspase 3), thereby reducing the apoptosis of Neuro-2a cells [18]. It also blocked the p65/IκBα pathway and alleviated the excessive activation of the inflammatory response. Similar results have also been reported in the research of Hsieh *et al*. [8].

Based on the MCAO mouse model and the OGD cell experiments, we further verified the relationship between the expression of plasma miR-153-5p level and cerebral infarction volume, neurological damage, and prognosis in clinical patients. We found that low levels of plasma miR-153-5p are closely related to poor prognosis of patients with ischaemic cerebral infarction, and the follow-up results further confirmed our conclusion. Combined with the above research results, it seems that RIPerC can inhibit the damage caused by cerebral ischaemia by regulating the miR-153-5p/TLR4/p65/IκBα pathway. However, this study has some shortcomings: 1. We only applied RIPerC to the MCAO mouse model, and the clinical application effect of RIPerC in patients with ischaemic stroke needs to be further verified. 2. The way through which RIPerC treatment affects the expression of miR-153-5p remains to be explored. 3. In this study, we found for the first time that RIPerC intervention can inhibit ischaemic brain damage by regulating the expression of miR-153-5p in patients with ischaemic stroke. Then, the expres-

sion regulation of miR-153-5p in the ischaemia of the myocardium, liver, kidney, and other important organs needs to be further explored.

In conclusion, this study confirmed that RIPerC intervention reduces apoptosis and inflammatory response in cerebral ischaemia injury by regulating the miR-153-5p/TLR4/p65/IκBα pathway, suggesting that RIPerC may be a potential therapeutic intervention to inhibit cerebral ischaemic injury. Our research only elucidated the protective effect of RIPerC/miR-153-5p at a basic level; therefore, in-depth research is also required to further confirm the current findings.

## Acknowledgments

Thanks for the support of the Emergency Department of our hospital in this study.

## Funding sources

This work was supported by the applied basic research foundation of Yunnan Province (202101AT070229, 2019FB090), the Innovation and Entrepreneurship Training Program for College Students in Yunnan Province (202110678069), and the in-hospital science and technology project of the Second Affiliated Hospital of Kunming Medical University (2020yk008).

## Disclosure

The authors report no conflict of interest.

### References

1. Bai XJ, Hao L, Guo YE, Shi XB, Wu WP. Bone marrow stromal cells reverse the microglia type from pro-inflammatory tumour necrosis factor a microglia to anti-inflammatory CD206 microglia of middle cerebral artery occlusion rats through triggering secretion of CX3CL1. Folia Neuropathol 2021; 59: 20-31.
2. Channakkar AS, Singh T, Pattnaik B, Gupta K, Seth P, Adlakha YK. MiRNA-137-mediated modulation of mitochondrial dynamics regulates human neural stem cell fate. Stem Cells 2020; 38: 683-697.
3. Che R, Zhao W, Ma Q, Jiang F, Wu L, Yu Z, Zhang Q, Dong K, Song H, Huang X, Ji X. rt-PA with remote ischemic postconditioning for acute ischemic stroke. Ann Clin Transl Neurol 2019; 6: 364-372.
4. Eyileten C, Sharif L, Wicik Z, Jakubik D, Jarosz-Popek J, Soplinska A, Postula M, Czlonkowska A, Kaplon-Cieslicka A, Mirowska-Guzel D. The relation of the brain-derived neurotrophic factor with microRNAs in neurodegenerative diseases and ischemic stroke. Mol Neurobiol 2021; 58: 329-347.
5. Gholampour F, Bagheri A, Barati A, Masoudi R, Owji SM. Remote ischemic perconditioning modulates apelin expression after renal ischemia-reperfusion injury. J Surg Res 2020; 247: 429-437.
6. Giordano M, Trotta MC, Ciarambino T, D'Amico M, Galdiero M, Schettini F, Paternosto D, Salzillo M, Alfano R, Andreone V, Malatino LS, Biolo G, Paolisso G, Adinolfi LE. Circulating MiRNA-195-5p and -451a in diabetic patients with transient and acute ischemic stroke in the emergency department. Int J Mol Sci 2020; 21: 7615.
7. He Y, Zhang L, Tan F, Wang LF, Liu DH, Wang RJ, Yin XZ. MiR-153-5p promotes sensibility of colorectal cancer cells to oxaliplatin via targeting Bcl-2-mediated autophagy pathway. Biosci Biotechnol Biochem 2020; 84: 1645-1651.
8. Hsieh CH, Jeng SF, Hsieh MW, Chen YC, Rau CS, Lu TH, Chen SS. Statin-induced heme oxygenase-1 increases NF-kappaB activation and oxygen radical production in cultured neuronal cells exposed to lipopolysaccharide. Toxicol Sci 2008; 102: 150-159.
9. Kanai M, Noguchi M, Kubo H, Nozoe M, Kitano T, Izawa KP, Mase K, Shimada S. Pre-Stroke Frailty and Stroke Severity in Elderly Patients with Acute Stroke. J Stroke Cerebrovasc Dis 2020; 29: 105346.
10. Kloner RA, Shi J, Dai W, Carreno J, Zhao L. Remote ischemic conditioning in acute myocardial infarction and shock states. J Cardiovasc Pharmacol Ther 2020; 25: 103-109.
11. Kumari M, Wang X, Lantier L, Lyubetskaya A, Eguchi J, Kang S, Tenen D, Roh HC, Kong X, Kazak L, Ahmad R, Rosen ED. IRF3 promotes adipose inflammation and insulin resistance and represses browning. J Clin Invest 2016; 126: 2839-2854.
12. Li W, Feng G, Gauthier JM, Lokshina I, Higashikubo R, Evans S, Liu X, Hassan A, Tanaka S, Cicka M, Hsiao HM, Ruiz-Perez D, Bredemeyer A, Gross RW, Mann DL, Tyurina YY, Gelman AE, Kagan VE, Linkermann A, Lavine KJ, Kreisel D. Ferroptotic cell death and TLR4/Trif signaling initiate neutrophil recruitment after heart transplantation. J Clin Invest 2019; 129: 2293-2304.
13. Luo M, Yan D, Sun Q, Tao J, Xu L, Sun H, Zhao H. Ginsenoside Rg1 attenuates cardiomyocyte apoptosis and inflammation via the TLR4/NF-kB/NLRP3 pathway. J Cell Biochem 2020; 121: 2994-3004.
14. Matsumoto K, Jin Z, Homma S, Elkind MSV, Rundek T, Mannina C, Lee TC, Yoshita M, DeCarli C, Wright CB, Sacco RL, Di Tullio MR. Association between central blood pressure and subclinical cerebrovascular disease in older adults. Hypertension 2020; 75: 580-587.
15. Pilz PM, Hamza O, Gidlöf O, Gonçalves IF, Tretter EV, Trojanek S, Abraham D, Heber S, Haller PM, Podesser BK, Kiss A. Remote ischemic perconditioning attenuates adverse cardiac remodeling and preserves left ventricular function in a rat model of reperfused myocardial infarction. Int J Cardiol 2019; 285: 72-79.
16. Song B, Xu J, Zhong P, Fang L. MiR-125a-5p silencing inhibits cerebral ischemia-induced injury through targeting IGFBP3. Folia Neuropathol 2021; 59: 121-130.
17. Yang P, Zhang Y, Zhang L, Zhang Y, Treurniet KM, Chen W, Peng Y, Han H, Wang J, Wang S, Yin C, Liu S, Wang P, Fang Q, Shi H, Yang J, Wen C, Li C, Jiang C, Sun J, Yue X, Lou M, Zhang M, Shu H, Sun D, Liang H, Li T, Guo F, Ke K, Yuan H, Wang G, Yang W, Shi H, Li T, Li Z, Xing P, Zhang P, Zhou Y, Wang H, Xu Y, Huang Q, Wu T, Zhao R, Li Q, Fang Y, Wang L, Lu J, Li Y, Fu J, Zhong X, Wang Y, Wang L, Goyal M, Dippel DWJ, Hong B, Deng B, Roos YBWEM, Majoie CBLM, Liu J;

DIRECT-MT Investigators. Endovascular Thrombectomy with or without Intravenous Alteplase in Acute Stroke. N Engl J Med 2020; 382: 1981-1993.
18. Wang K, Zhu Y. Dexmedetomidine protects against oxygen-glucose deprivation/reoxygenation injury-induced apoptosis via the p38 MAPK/ERK signalling pathway. J Int Med Res 2018; 46: 675-686.
19. Xie W, Zhu T, Zhou P, Xu H, Meng X, Ding T, Nan F, Sun G, Sun X. Notoginseng leaf triterpenes ameliorates OGD/R-induced neuronal injury via SIRT1/2/3-Foxo3a-MnSOD/PGC-1α signaling pathways mediated by the NAMPT-NAD pathway. Oxid Med Cell Longev 2020; 2020: 7308386.
20. Yaghi S, Willey JZ, Cucchiara B, Goldstein JN, Gonzales NR, Khatri P, Kim LJ, Mayer SA, Sheth KN, Schwamm LH. Treatment and outcome of hemorrhagic transformation after intravenous alteplase in acute ischemic stroke: a scientific statement for healthcare professionals from the American Heart Association/American Stroke Association. Stroke 2017; 48: e343-e361.
21. Yan Q, Sun SY, Yuan S, Wang XQ, Zhang ZC. Inhibition of microRNA-9-5p and microRNA-128-3p can inhibit ischemic stroke-related cell death in vitro and in vivo. IUBMB Life 2020; 72: 2382-2390.
22. Yoshino Y, Roy B, Dwivedi Y. Differential and unique patterns of synaptic miRNA expression in dorsolateral prefrontal cortex of depressed subjects. Neuropsychopharmacology 2021; 46: 900-910.
23. Zhang W, Liu K, Pei Y, Tan J, Ma J, Zhao J. Long Noncoding RNA HIF1A-AS2 promotes non-small cell lung cancer progression by the miR-153-5p/S100A14 axis. Onco Targets Ther 2020; 13: 8715-8722.
24. Zhang Y, Li M, Li X, Zhang H, Wang L, Wu X, Zhang H, Luo Y. Catalytically inactive RIP1 and RIP3 deficiency protect against acute ischemic stroke by inhibiting necroptosis and neuroinflammation. Cell Death Dis 2020; 11: 565.
25. Zhao J, Li X, Hu J, Chen F, Qiao S, Sun X, Gao L, Xie J, Xu B. Mesenchymal stromal cell-derived exosomes attenuate myocardial ischaemia-reperfusion injury through miR-182-regulated macrophage polarization. Cardiovasc Res 2019; 115: 1205-1216.
26. Zhao W, Li S, Ren C, Meng R, Jin K, Ji X. Remote ischemic conditioning for stroke: clinical data, challenges, and future directions. Ann Clin Transl Neurol 2018; 6: 186-196.
27. Zhao W, Ren C, Ji X. Remote ischemic perconditioning for the treatment of acute ischemic stroke. JAMA Neurol 2020; 77: 1451-1452.