

MicroRNA-93 Downregulation Ameliorates Cerebral Ischemic Injury Through the Nrf2/HO-1 Defense Pathway

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Abstract The present study was designed to evaluate the potential role of miR-93 in cerebral ischemic/reperfusion (I/R) injury in mice. The stroke model was produced in C57BL/6 J mice via middle cerebral artery occlusion (MCAO) for 1 h followed by reperfusion. And miR-93 antagomir was transfected to down-regulate the miR-93 level. Our results showed that miR-93 levels in the cerebral cortex of mice increased at 24 and 48 h after reperfusion. Importantly, *in vivo* study demonstrated that treatment with miR-93 antagomir reduced cerebral infarction volume, neural apoptosis and restored the neurological scores. *In vitro* study demonstrated that miR-93 antagomir attenuated hydrogen peroxide (H₂O₂)-induced injury. Moreover, miR-93 antagomir suppressed oxidative stress in I/R brain and H₂O₂ treated cortical neurons. Furthermore, we founded that down-regulation of miR-93 increased the expression of nuclear factor erythroid 2-related factor (Nrf2) and heme oxygenase-1 (HO-1) and the luciferase reporter assay confirmed that miR-93 directly binds to the predicted 3'-UTR target sites of the *nrf2* gene. Finally, we found that knockdown of Nrf2 or HO-1 abolished miR-93 antagomir-induced neuroprotection against oxidative stress in H₂O₂ treated neuronal cultures. These results suggested

that miR-93 antagomir alleviates ischemic injury through the Nrf2/HO-1 antioxidant pathway.

Keywords MiR-93 · Nrf2 · HO-1 · Oxidative stress · Ischemic stroke

Introduction

It is known that stroke is a serious damage to human health worldwide, has high incidence, high morbidity and high mortality characteristics. The pathophysiological mechanisms of stroke-induced neuronal death are complex cellular biochemical events, involving oxidative stress-related pathways [1]. It is already well accepted that overproduction of reactive oxygen species (ROS) after stroke, such as hydrogen peroxide (H₂O₂), leads to the oxidation of proteins, lipids, and DNA and drive neuronal cell death [2]. Nuclear factor erythroid 2-related factor 2 (Nrf2) is important in the protection of cells against oxidative stress, that activates the transcription of antioxidant stress genes, including heme oxygenase-1 (HO-1) [3, 4]. The Nrf2/HO-1 pathway has been shown to play important neuroprotective role in brain injury after ischemic stroke [5–7].

MicroRNAs(miRs) are small noncoding single-stranded RNA molecules of 21–23 nucleotides that modulate gene expression by binding to the complementary seed sequences in the 3'-UTRs of mRNAs [8, 9]. MiRNAs are evolutionarily conserved, and play a critical role in a variety of normal biological processes including cell differentiation and development, metabolism, proliferation, and apoptotic cell death [10, 11]. Accumulating evidences have linked dysregulated miRs expression in brain to the pathological process of ischemic stroke [12–14]. The miR-106b-25 cluster, a paralog of the miR-17-92 cluster, which all resides in the

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thirteenth intron of the MCM7 gene, contains three miRs, miR-25, miR-93 and miR-106b. Until recently, only a few studies have assessed the effects of the miR-106b-25 cluster on ischemic stroke [15]. Notably, miR-93 was related to inflammation, oxidative stress, and cell apoptosis [16–18]. One previous expression analysis has revealed that miR-93 is upregulated in the postischemic brain, suggesting that miR-93 may act as a potential therapeutic target for acute ischemic stroke [12]. However, the functional significance and clear mechanism of miR-93 in cerebral ischemic injury have yet to be clarified.

In this study, we examined the role of miR-93 in cerebral ischemic/reperfusion (I/R) injury in mice and in H₂O₂-treated primary cortical neurons. We demonstrated that miR-93 might be a critical player in the regulation of neural ischemic injury and able to influence oxidative stress through the Nrf2/HO-1 defense pathway.

Methods

Animal Model

All procedures in this study were conducted according to the guidelines set by the Animal Care and Use Committee of Liaoning Medical University and are consistent with the NIH Guide for the care and use of laboratory animals. Adult male C57BL/6 J mice (weighing 22–25 g) purchased from Experimental Animal Center of Chinese Academy of Medical Sciences, PR China were maintained in temperature controlled rooms (20±2°C) with access to food and water ad libitum. The mouse model of ischemic stroke was prepared as described before [19, 20]. In brief, after anesthetized with pentobarbital sodium (60 mg/kg i.p.), the right common and the right external carotid artery were exposed and focal cerebral ischemia was produced by 1 h of middle cerebral artery occlusion (MCAO) with a 6-0 surgical nylon monofilament (0.23 mm in diameter) followed by 24 h of reperfusion. Sham-operated mice underwent an identical procedure, without inserting the suture.

Measurement of Neurological Deficit and Infarct Volume

Mice were tested for neurological deficits at 24 h after 1 h MCAO according to neurological disability status scale (NDSS) reported by Rodriguez et al. [21]. NDSS has ten progressive steps beyond zero (normal), extending to status ten (death). Briefly, the six major steps indicate: 0 represents no neurological dysfunction; 2 represents slight decrease in mobility and the presence of passivity; 4 represents moderate neurological dysfunction and including additional alterations, such as flattened posture, hunched back, lateralized

posture, ataxic gait, moderate hypomobility, decreased muscular strength and body tone and slight motor incoordination; 6 corresponding to more handicapped animals but still able to walk, with more tremor, jerks and/or convulsions, marked hypomobility, forelimb flexion, circling and moderate motor incoordination; 8 corresponding to respiratory distress, and total incapacity to move/coordinate. If the criteria for the accurate grade were not met, the nearest appropriate number was utilized: 1, 3, 5, 7 and 9.

Infarct volumes were determined by 2,3,5-triphenyltetrazolium chloride (TTC) staining. Mice were killed after 24 h reperfusion. The brain was quickly removed and cut into 1.0 mm thickness coronal sections. The sections were incubated for 15 min in a solution of 0.5% TTC (Sigma-Aldrich, St. Louis, MO, USA) at 37°C, and then the sections were scanned into a computer. The images of stained sections were analyzed using Image Pro Plus 1 6.0 (Media Cybernetics, Silver Spring, MD).

Intracerebroventricular Injection of miR-93 Antagomir

MiR-93 antagomir and nonsensical negative control were purchased from GenePharma (Shanghai, China). miR-93 antagomir (100 µm) or the control (100 µm) were mixed with Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA) and incubated at room temperature for 30 min; 7 µL of the mixture was injected into the right lateral ventricle and the needle was kept in the place for 10 min before the MCAO [22]. The injection position was at the point of 0.5 mm posterior to bregma, 1.0 mm lateral to bregma, and 3.5 mm below the skull surface.

Primary Neuronal Cell Culture and Treatment

Primary cortical neurons obtained from postnatal day 1 C57BL/6 J mice were cultured in neurobasal medium (Gibco Inc., Grand Island, USA) supplemented with 2% B27 (Gibco Inc., Grand Island, USA) and maintained at 37°C in a 5% CO₂/air environment. The miR-93 antagomir (GenePharma, Shanghai, PR China) and small interference RNA (siRNA) targeting to Nrf2 or HO-1 (GenePharma, Shanghai, PR China) were transfected into primary cortical neurons for 48 h using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen Tec., Carlsbad, CA, USA) after 6 days as manufacturer's instruction. To initiate oxidative stress, primary cortical neurons were stimulated by 200 µm H₂O₂ for 6 h. Then neurons and supernatant were collected for measurements.

Real-Time PCR

Total RNA including miRNAs was isolated from cerebral tissue with the NucleoSpin[®] miRNA kit (Macherey–Nagel,

Germany) according to the manufacturer's protocol. Reverse transcription were performed by using the miR-CURY LNATM Universal RT microRNA PCR (Exiqon A/S, Vedbaek, Denmark). The real-time PCR amplification was performed with an Mx3000PTM system (Agilent Technologies, CA 95051, USA) using Brilliant II SYBR[®] Green QPCR master mix (Agilent Technologies, CA 95051, USA) according to the manufacturer's protocol, with U6 used as an internal control.

Cell Viability Assessment

The viability of cortical neurons was evaluated by using thiazolyl blue tetrazolium bromide (MTT, 0.5 mg/mL; Applichem Inc., Omaha, NE, USA) and the CytoTox 96[®] Non-Radioactive Cytotoxicity Assay (lactate dehydrogenase; LDH; Promega Cor, Madison, WI, USA) following the manufacturer's instructions.

TUNEL Staining

Cell apoptosis was assessed by using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining kit (TMR Red in situ cell death detection kit; Roche Diagnostics, Indianapolis, IN), according to the manufacturer's instructions. Briefly, fix cell samples with a freshly prepared fixation solution for 1 h and then rinse slides with phosphate-buffered saline. Cells were incubated in permeabilization solution for 2 min on ice, and then incubated with TUNEL reaction mixture in a humidified atmosphere for 1 h at 37°C in the dark. MAP2 (1:500; RRID: AB_776174; Abcam, Cambridge, United Kingdom) and 4',6-diamidino-2-phenylindole (DAPI) were used to visualize the neurons and neural nuclei, respectively. Images were acquired by a fluorescence microscope system (DM4000B; Leica, Wetzlar, Germany).

Western Blot Analysis

The ipsilateral cortices were collected at 24 h after reperfusion and processed for Western blot as described previously [23, 24]. Antibodies in this study were rabbit anti-HO-1 (1:1000; Abcam, Cambridge, UK), anti-Nrf2 (1:1000; Abcam, Cambridge, UK), β -actin antibody (1:3000; Sigma-Aldrich Corp. St. Louis, MO 63103, USA), and corresponding secondary antibodies (Stressgen Biotechnologies Corporation, Victoria, BC, Canada). The Enhanced Chemiluminescence (ECL) kit (GE Healthcare, UK) was employed to detect the signals. Protein levels were quantified by densitometry and normalized to β -actin as a loading control.

Luciferase Assays

The 3'-UTR of the Nrf2 was amplified by PCR using primers (forward: 5'-atttaggagattgacc-3'; reverse: 5'-ttttgccagagc-taaacaattt-3') and cloned into the pmiR-RB-REPORTTM luciferase reporter vector (RiboBio, Guangzhou, People's Republic of China). The mutant 3'-UTR of the Nrf2 gene was constructed with QuikChange II Site-Directed Mutagenesis (Stratagene, USA). Mouse N2A cells were plated at 0.5×10^5 cells per well in 24-well plates. The following day, cells were co-transfected pmiR-RB-ReportTM vector (RIBOBIO, Guangzhou, China), including the 3'-UTR of Nrf2 (with either wild-type or mutant miR-93 binding sites), and miR-93 agomir or miR control plasmid using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen Tec., Carlsbad, CA, USA). Luciferase assays were performed 48 h after transfection by using Dual-Luciferase[®] Reporter Assay System (Promega Cor, Madison, WI, USA) according to the manufacturer's protocols.

Determination of Oxidative Stress

Brain homogenates and primary cortical neurons were prepared with cold phosphate-buffered saline. The superoxide dismutase (SOD) activity, malondialdehyde (MDA) and ROS levels were assessed using SOD, MDA and ROS detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively, following the manufacturer's instructions.

Statistical Analysis

We performed quantitative analysis of Western blot by using GelDoc-2000 Imagine System. For protein expression level, the protein ratio (band density of protein/band density of β -actin) in the control group was expressed as one, and then the other groups were expressed as percentage of that from control group. Statistical analysis was performed with the *t* test and by one-way analysis of variance (ANOVA) followed by all pairwise multiple comparison procedures using Bonferroni's test. Data are expressed as mean \pm SEM, and differences were considered significant at $P < 0.05$.

Results

The Expressions of miR-93 were Upregulated in MCAO-Induced Ischemic Stroked Mice

To determine the expression of miR-93 in MCAO-induced ischemic stroke mice, we examined miR-93 levels using quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) in the mouse cerebral cortex. Our

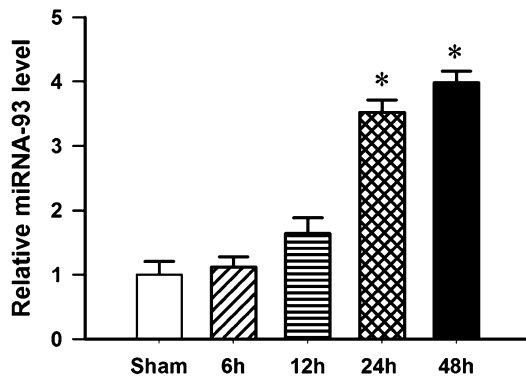


Fig. 1 The expression of miR-93 in MCAO-induced ischemic stroke mice. MiR-93 was detected by RT-PCR in mice that treated with MCAO for 1 h and reperfusion for 6–48 h. Compared with the sham group, miR-93 levels were increased at 24 and 48 h in the cerebral cortex of mice after 1 h MCAO-induced ischemic stroke values present as mean \pm SEM (n = 8 per group). * P < 0.05 vs. sham group

results showed that miR-93 levels on the ipsilateral side of the brain significantly increased at the 24 and 48 h reperfusion time point after 1 h MCAO compared with the sham group (Fig. 1). MiR-93 expression was not significantly changed at 6 and 12 h.

MiR-93 Antagomir Ameliorated Ischemic Brain Damage

To evaluate the role of miR-93 in ischemic brain injury in vivo, the miR-93 antagomir was administered via intracerebroventricular injection. The down-regulated miRNA-93 levels in ischemic cortex of MCAO mice were confirmed by using quantitative RT-PCR (Fig. 2a). TTC staining showed that miR-93 antagomir effectively attenuated cerebral infarction, as compared with those in the I/R group (Fig. 2b). In addition, the western blotting results showed

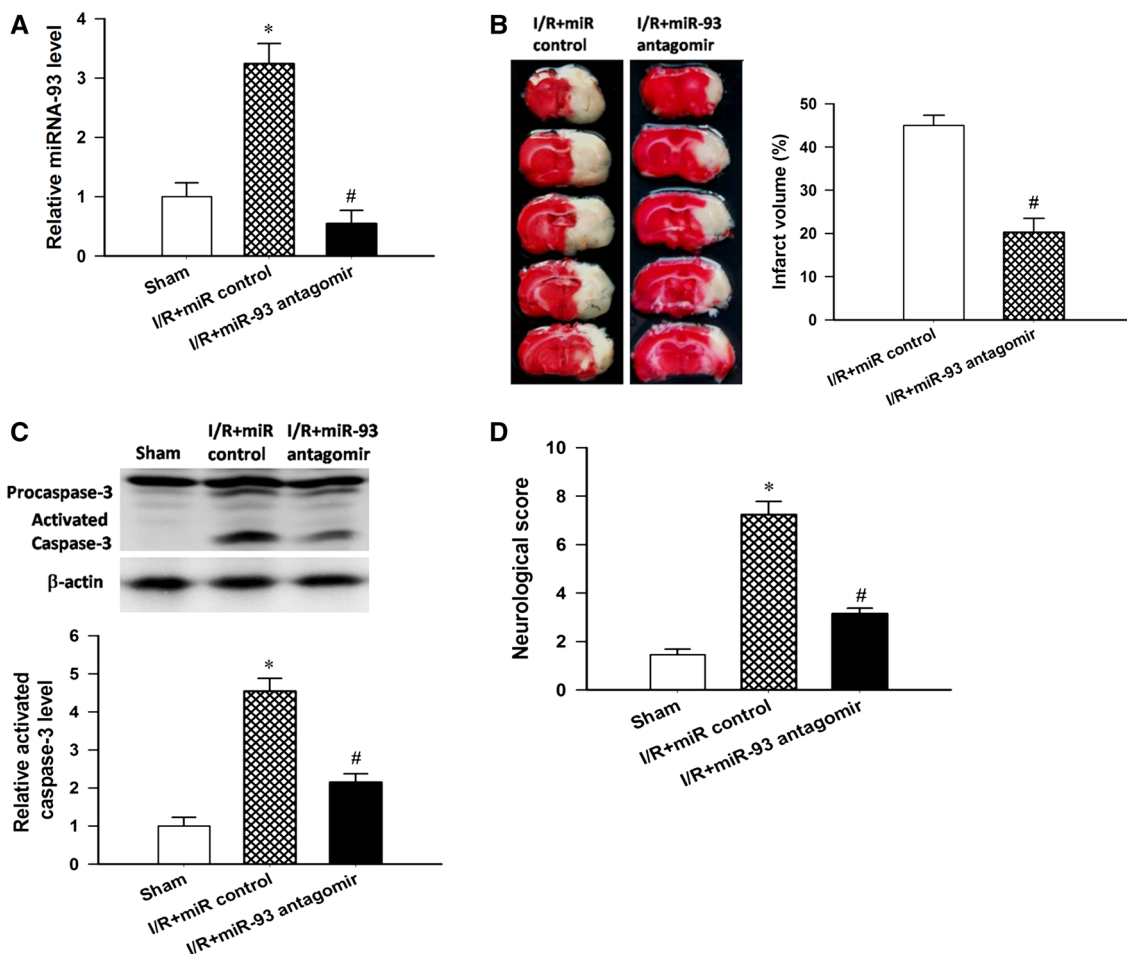


Fig. 2 MiR-93 antagomir protects against cerebral I/R injury in mice. **a** The RT-PCR results showed that intracerebroventricular injection of miR-93 antagomir could effectively down-regulated miRNA-93 expression level in ischemic cortex of 1 h MCAO/24 h reperfusion treated mice. **b** Cerebral infarct volume evaluated by TTC-stained

cerebral coronal sections. **c** The apoptosis in the ipsilateral cortex as detected by the activated caspase-3 levels using western blotting. **d** Results of the neurological score. Values present as mean \pm SEM (n = 10 per group). * P < 0.05 vs. sham group; # P < 0.05 vs. I/R+miR control group

the miR-93 antagomir significantly reduced the activated caspase-3 levels in the cortex of mice relative to the I/R group (Fig. 2c). Results of the neurological score indicated that miR-93 antagomir could improve the neurological deficit of mice with ischemic stroke (Fig. 2d). These results suggested that down-regulation of miR-93 could attenuate cerebral I/R injury in mice.

MiR-93 Antagomir Attenuated Oxidative Stress in Transient MCAO Mouse Model

To investigate the influence of miR-93 on cerebral I/R-induced oxidative stress in vivo, SOD activity and the levels of MDA and ROS in the cortex were evaluated. It was shown that SOD activity which was decreased in the ischemic brain of I/R mice compared with the sham group, was significantly upregulated by miR-93 antagomir treatment compared with the I/R group (Fig. 3a). MDA levels increased in the I/R group compared with the sham group, but were down-regulated by miR-93 antagomir treatment (Fig. 3b). I/R similarly increased ROS production in the cortex relative to the sham group, but the level was suppressed by miR-93 antagomir treatment (Fig. 3c).

MiR-93 Antagomir Induced HO-1 Expression Through Nrf2

Using bioinformatics analysis, we found a binding site for miR-93 at the 3'-UTR of Nrf2 mRNA with a high possibility ranking, implying that Nrf2 may be one of the possible target genes of miR-93. Nrf2, a member of the transcription factor family, functions in HO-1 induction. As shown in Fig. 4a, miR-93 antagomir treatment effectively increased the expression of Nrf2 relative to the I/R group. In addition, miR-93 antagomir treatment also induced an upregulation in HO-1 expression in the cerebral cortex of I/R mice (Fig. 4b).

To test whether miRNA-93 directly targeted Nrf2 by binding to the 3'-UTR sequence, we cloned a luciferase reporter vector in which T7 driven-luciferase cDNA was followed by a fragment of the Nrf2 3'-UTR or the miR-93 binding site mutated Nrf2 3'-UTR (Fig. 4c). The luciferase activity assay indicated that miR-93 agomir significantly decreased luciferase activity of the reporter vector containing miR-93 binding sequences of Nrf2 3'-UTR but not the mutant 3'-UTR vector (Fig. 4d). These results suggested that miRNA-93 could directly target the 3'-UTR of Nrf2 to negatively regulate Nrf2-protein levels in cortical neurons.

MiR-93 Antagomir Alleviated H₂O₂-Induced Oxidative Stress Damage in Primary Cortical Neurons Through the Nrf2/HO-1 Pathway

To confirm that miR-93 regulates H₂O₂-induced cell death in vitro, we transfected primary cortical neurons with miR-93 antagomir. H₂O₂-induced cell death was simultaneously assayed by MTT, LDH and TUNEL methods. As demonstrated in Fig. 5a–c, treatment with miR-93 antagomir effectively reduces H₂O₂-induced cell death compared to control groups. To demonstrate that antioxidants contribute to the effects of miR-93 in H₂O₂-induced cell death, we co-transfected Nrf2 or HO-1 siRNA with miR-93 antagomir in primary cortical neurons, and then detected the cell death rate. The results indicated that suppressing Nrf2 or HO-1 had the ability to block the protective effect of miR-93 antagomir in response to H₂O₂ stress.

To further confirm whether silencing of miR-93 increased anti-oxidative ability of neurons, SOD activity and MDA content in primary cortical neurons were detected. It was shown that SOD activity which was reduced in primary cortical neurons upon H₂O₂ exposure, was elevated by miR-93 antagomir treatment (Fig. 6a). The effect of miR-93 antagomir on SOD activity was blocked by Nrf2 or HO-1 knockdown treatment (Fig. 6a). Additionally, MDA

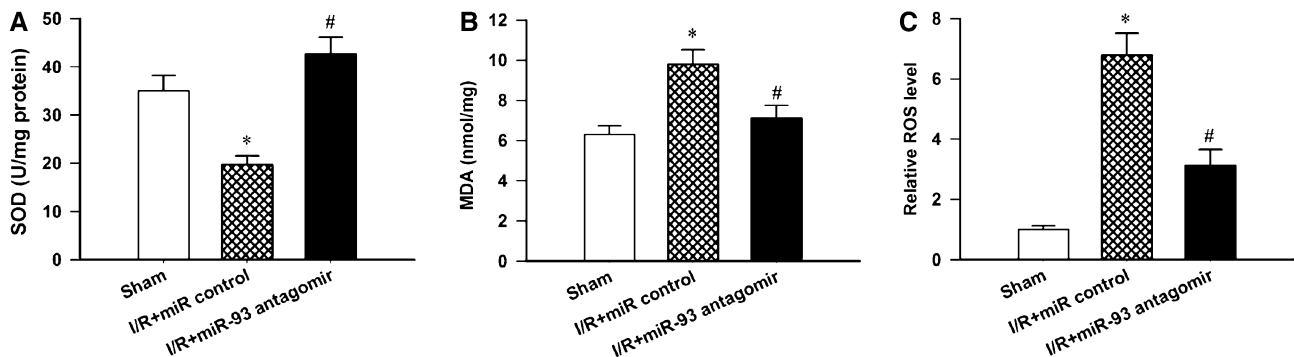
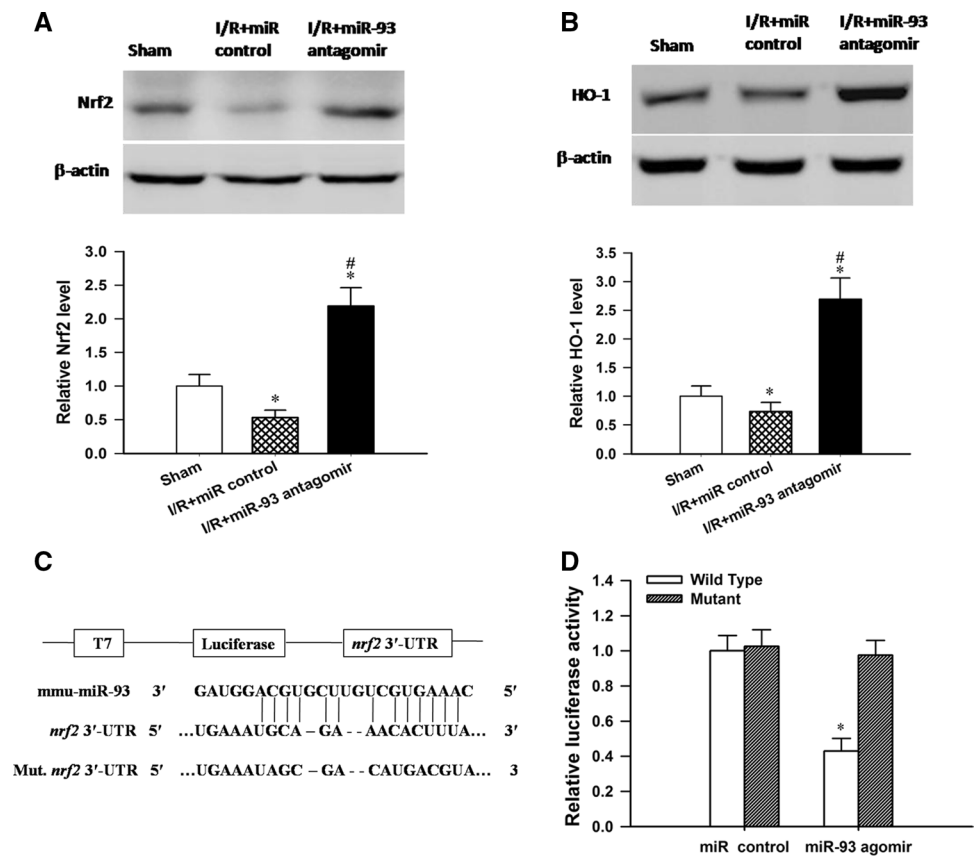


Fig. 3 Effect of miR-93 antagomir on oxidative stress induced by I/R in mice. **a** SOD activity in the cortex was detected by biochemical kit. **b** MDA content in the cortex was detected by biochemical kit. **c** ROS

level in the cortex was detected by biochemical kit. Values present as mean \pm SEM ($n=10$ per group). * $P<0.05$ vs. sham group; # $P<0.05$ vs. I/R + miR control group

Fig. 4 Effect of miR-93 antagomir on Nrf2/HO-1 levels. **a** Effect of miR-93 antagomir of Nrf2 protein level in cerebral cortex, as determined by western blotting (n=10 per group). **b** HO-1 level in cerebral cortex as determined by western blotting (n=10 per group). **c** Showed the design of a miR-93 reporter vector containing a T7-driven-luciferase cDNA fused to Nrf2 3'-UTR or mutated Nrf2 3'-UTR. The putative binding position between miR-93 and the 3'-UTR of Nrf2 mRNA was also shown. **d** Luciferase reporter assay was performed by cotransfection of luciferase reporter containing Nrf2 3'-UTR or mutated Nrf2 3'-UTR with miR-93 agomir or its control into N2A cells. (n=6 per group). Values present as mean \pm SEM. * P <0.05 vs. sham group or miR control; # P <0.05 vs. I/R+miR control group



level which was increased in primary cortical neurons was blocked by miR-93 antagomir treatment; this effect was inhibited by both Nrf2 depletion and HO-1 depletion (Fig. 6b). These results demonstrate that miR-93 plays an important role in the modulation of oxidative stress tolerance in primary cortical neurons upon H₂O₂ stimulation.

Discussion

In this study, we investigated the role of miR-93 in mice with transient focal I/R injury and in primary cortical neurons subjected to oxidative stress induced by H₂O₂. MiR-93 levels increased in a time-dependent manner in the cerebral cortex following ischemia and reperfusion in mice. Importantly, intracerebroventricular injection of miR-93 antagomir reduced cerebral infarct volume, neural cell apoptosis, and oxidative stress after transient MCAO. Meanwhile, miR-93 antagomir increased HO-1 and Nrf2 expression levels. Further studies revealed that the mechanisms of miR-93 in ischemic cerebral injury may involve the Nrf2/HO-1 defense pathway by directly targeting Nrf2. Taken together, our data demonstrated that miR-93 down-regulation protects against cerebral I/R injury by stimulating antioxidant responses in neurons, providing a new therapeutic target in acute ischemic stroke.

Until now, studies of miR-93 have been focused mainly on its involvement in many types of human cancers, such as glioblastoma, gastric cancer, breast carcinoma, and others. miR-93 is overexpressed in a number of types of human cancers and functions as an oncogene by targeting important cancer-related genes [25–29], whereas, in some types of cancers miR-93 was down-regulated and repressed proliferation paradoxically [30, 31]. However, the present study is the first to investigate its role in ischemia-induced cerebral injury. Our results showed that miR-93 was up-regulated at 24 h and persistently increased until 48 h in the cerebral cortex of mice after 1 h MCAO-induced ischemic stroke *in vivo*, which is comparable to the results reported by Dharap et al. [12]. These data suggest that miR-93 might be a critical role in the pathogenesis of ischemic stroke, proposing the potential of miR-93 down-regulation as a neuroprotective strategy for cerebral ischemia injury. Follow-up *in vivo* studies indicated that treatment of C57BL/6 J mice with intracerebroventricular injection of miR-93 antagomir reduced infarct volume, neural apoptosis, and oxidative stress in the brain, and improved behavioral outcome of mice with ischemic stroke. Taken together, these results demonstrate that downregulation of miR-93 protects the brain from focal cerebral ischemia injury.

To further elucidate the mechanism of miR-93 on alleviating MCAO-induced cerebral ischemic injury, the

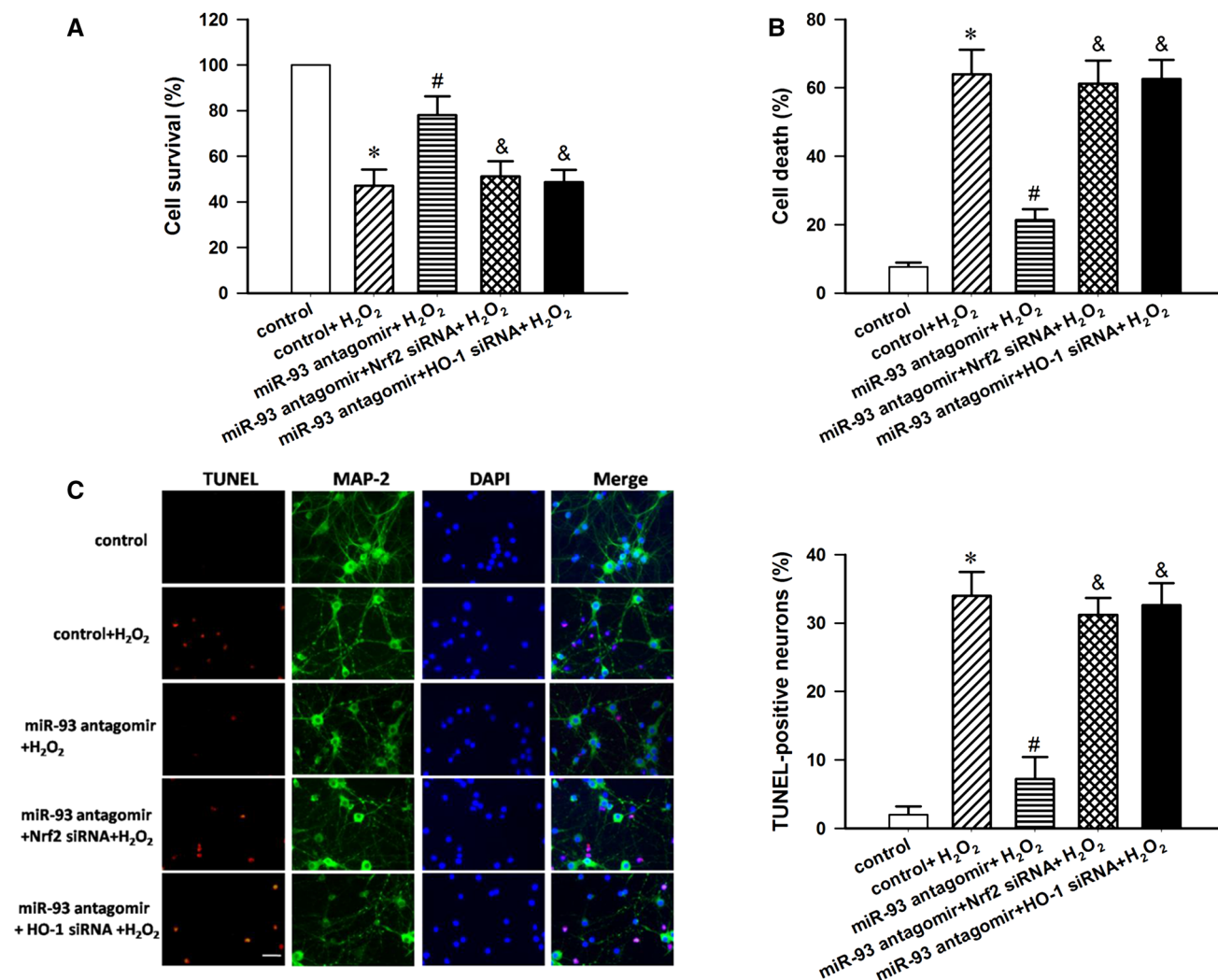


Fig. 5 MiR-93 antagonist inhibits primary cortical neurons death after H₂O₂ treatment. **a** Neurons viability was detected by MTT. **b** LDH assay in cultured cortical neurons was conducted. **c** Representative microscopic image of the neuronal apoptosis detected

by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and DAPI double staining (scale bar 100 μm). Values present as mean ± SEM (n=8 per group). *P<0.05 vs. control; #P<0.05 vs. control+H₂O₂, &P<0.05 vs. miR-93 antagonist+H₂O₂

present study investigated the effects of miR-93 on the levels of oxidative stress markers and antioxidants. Oxidative stress is a core pathological component of brain ischemia-reperfusion injury causing neuronal malfunction and cell death [32–34]. Several miRNAs have been reported to be involved in the cellular response to oxidative stress via the antioxidant defense system [22, 24]. As expected, we found that treatment with miR-93 antagonist reduced ROS and MDA levels in the ischemic brain of I/R mice, and increased the SOD activity as well as the expression of HO-1 and Nrf2.

The Nrf2/HO-1 pathway is an important cellular defense mechanism against I/R-induced oxidative stress [35, 36]. To further directly determine the role of the Nrf2 antioxidant pathway in miR-93 antagonist-mediated neuroprotection, the present study utilized H₂O₂ treatment of primary

neurons to stimulate oxidative stress in vitro. The results showed that miR-93 significantly regulated Nrf2/HO-1 levels in ischemic model. Furthermore, luciferase reporter assay also confirmed that Nrf2 is a direct target of miR-93. In primary cortical cultures, miR-93 antagonist treatment alleviated H₂O₂-induced injury, as evidenced by increased cell viability and SOD activity, and decreased lactate dehydrogenase leakage and MDA production, which is one of the classic oxidative stress markers to directly reflect the rate and extent of lipid peroxidation. The current study also shows that knockdown of Nrf2 or HO-1 can block miR-93 antagonist-mediated neuroprotection against H₂O₂-induced cortical neuron injury and oxidative stress. These results suggest that miR-93 antagonist could upregulate Nrf2/HO-1 expression levels, thereby enhancing the protective defense mechanisms through anti-oxidative pathway.

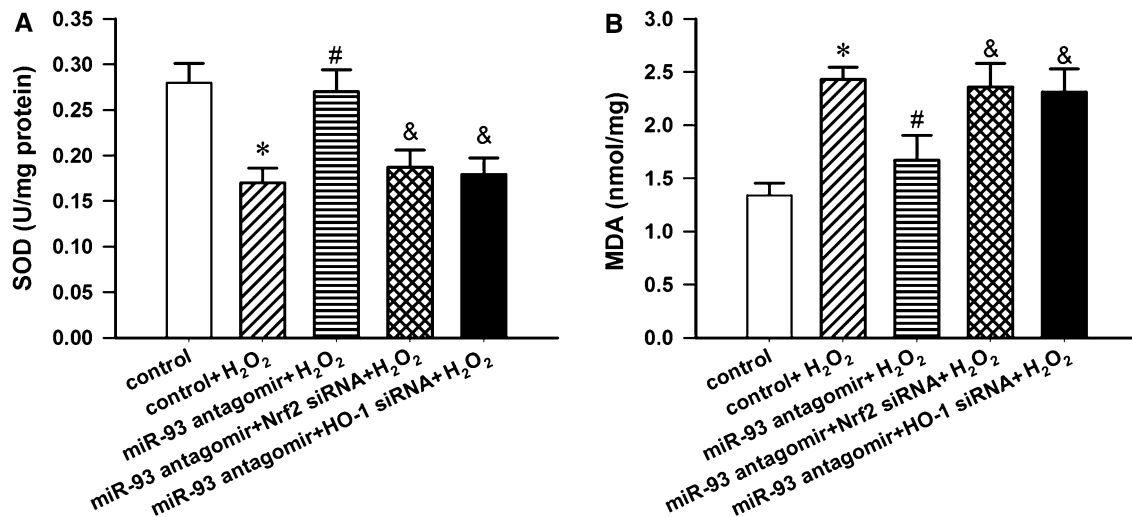


Fig. 6 MiR-93 antagomir attenuates oxidative stress damage in primary cortical neurons. Effect of miR-93 antagomir on SOD activity (a) and MDA content (b) in primary cortical neurons were

evaluated by biochemical kit. Values present as mean \pm SEM ($n=8$ per group). * $P<0.05$ vs. control; # $P<0.05$ vs. control+H₂O₂ and & $P<0.05$ vs. miR-93 antagomir+H₂O₂

In summary, the present study demonstrated that the down-regulation of miR-93 could alleviate neural ischemic injury through activating Nrf2/HO-1 antioxidant pathway, raising an interesting prospect for using miR-93 as a therapeutic target for ischemic stroke. However, it is far away from the clinical application. There are some severe problems waiting to solve, including tissue and cell-specific delivery in vivo, degradation avoidance, and target specificity. How to bring the miRNA into the brain is an important problem. Obviously, intracerebroventricular injection would be limited in the patients for the injury caused by the surgery. Delivery systems for miRNA to efficiently cross the blood brain barrier and target brain tissue will be the subject of future investigations.

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Compliance with Ethical Standards

Conflict of Interest The authors confirm that there are no conflicts.

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