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Original Article

Downregulation of microRNA 132-3p protects neural stem cells (NSC) against injury of cerebral ischemia (CI) via HO-1/Nrf2 signaling pathway

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Abstract: It has been discovered that plasma concentration of miRNA-132-3p was remarkably elevated in terms of patients suffering from ischemic apoplexy (IA). Our study aimed to evaluate the influence of miRNA-132-3p on mice undergoing CI/cerebral reperfusion (CR). It was found that cortical miRNA-132-3p concentration was elevated subsequent to reperfusion. Preliminary supplement with miRNA-132-3p antagomir not only decreased the volume of cerebral infarctus and neuronal apoptosis but also increased neurological scores (NS). It was proved that miRNA-132-3p antagomir decreased the injury triggered by hydrogen peroxide. Furthermore, oxidative stress (OS) was depressed by miRNA-132-3p antagomir in CI/CR and cortical neurons supplemented with hydrogen peroxide. Additionally, it was demonstrated that decline of miRNA-132-3p enhanced expression of heme oxygenase-1 (HO-1) and nuclear factor erythroid 2-related factor (Nrf2). In the end, elimination of HO-1 or Nrf2 depressed protection of neurons triggered by miRNA-132-3p antagomir from OS. These findings indicated that CI injury could be relieved by miRNA-132-3p antagomir via HO-1/Nrf2 signaling pathway.

Keywords: miRNA-132-3p, neural stem cells, cerebral ischemia, heme oxygenase-1

Introduction

Apoplexy serves as the main contributor to mortality and paralysis in the long term of adult all around the world [1]. As the most prevalent kind of apoplexy, ischemic apoplexy (IA) accounts for almost 85% of all apoplexy [2]. Cerebral infarction (CI) tends to bring about irreversible damage to neurons in areas influenced by apoplexy in hours and days of apoplectic seizure. Numerous studies have been conducted on biochemistry and pathoph ysiology of apoplexy in recent years. However, the understanding of its mechanisms is still insufficient. It has been widely recognized that excessive generation of reactive oxygen species (ROS) subsequent to apoplexy, for example, H_2O_2, not only brings about oxidation of DNA, proteins, and lipids, but also promotes neuronal apoptosis [2].

Recently, more and more studies revealed that the expression level of miRNA was changed and play an important role in IA [3-5]. As a group of small noncoding RNAs, miRNAs modifies many fundamental cellular processes by negatively regulating expression levels of protein via binding to 3'-UTR of the target mRNAs. MiRNAs are indispensable in numerous reactions such as proliferation, metabolism, differentiation, development, and apoptosis due to its evolutionary conservation [6]. It has been widely reported that dysfunction of cerebral miRNA expression is related to pathogenesis of central nervous system (CNS) injuries [3, 5, 7]. For example, it has been discovered that miRNA-124 protect neurons from ischemic apoplexy [8]. Furthermore, in mice with middle cerebral artery occlusion (MCAO), miR-497 was up-regulated and its knockdown attenuated ischemic brain infraction [9]. Moreover, miR-210, a signature miRNA of hypoxia, has been shown to mediate apoptosis of neuroblastoma cells via targeting anti-apoptotic Bcl-2 expression [10]. However, research on influence of miRNAs on CNS pathogenesis is still at the ini-

tial stage. Consequently, throwing light upon the particular effect of miRNAs is crucial to reveal pathogenesis of CNS injuries and build innovative strategies to treat IA.

In our research, the effect of miRNA-132-3p on cerebral infarction/cerebral hemorrhage (CI/CU) was explored in neurons supplemented with hydrogen peroxide and in mice. It was proved that miRNA-132-3p could be crucial to modification of ischemic neurons and could affect OS by HO-1/Nrf2 signaling pathway.

Material and methods

Blood samples of patients with acute stroke

Ten patients with acute CI and healthy participants were recruited in Puai Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology from March to June 2016. The inclusion criteria for IA patients were listed as follows: male aged from 55 to 65, first diagnosis of IA depending on the combination of clinical manifestations and MRI, subject appeared in seventy-two hours subsequent to apoplectic seizure, apoplexy TOAST subtype of large-artery atherosclerosis, National Institutes of Health Stroke Scale score of four to fifteen, and fully informed written consent. 12 healthy male participants that matched in age were recruited as control group. Blood was drawn from all participants for evaluation.

Animal model of cerebral ischemia

Every experiment in our research was performed in conformity with guidelines of Animal Care and Use Committee of Puai Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology in terms of use and care of lab animals. C57BL/6 J male mice in adulthood (body weight, 22-25 g) were purchased from Experimental Animal Center of Chinese Academy of Medical Sciences. Mice with ischemic apoplexy were obtained as previously mentioned [11]. In a word, subsequent to intraperitoneal anaesthesia with pentobarbital sodium at the concentration of 60 mg/kg, 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). Subsequently, reperfusion was conducted for twenty-four hours. Mice received sham operation in the same way, except suture inserting. We purchased miRNA-132-3p antagomir and its corresponding negative control from GenePharma (Shanghai, P.R.C.). Either 100 um miRNA-132-3p antagomir or 100 um control was mingled Lipofectamine RNAiMAX Transfection Reagent (LRTR) (Invitrogen, CA) and underwent 30-minute-incubation under circumstances of room temperature. Right lateral ventricle was injected with 7 uL mixture. The needle remained in the ventricle for ten minutes prior to MCAO [12]. Subsequently, mice underwent examination on neurological damage at 24th hour in conformity with neurological disability status scale (NDSS) [13]. 2,3,5-triphenyltetrazolium chloride (TTC) staining was utilized to detect the volume of infarct. Image Pro Plus1 6.0 (Media Cybernetics, Silver Spring, MD) was applied to analysis of the staining image. The infarct area was calculated as previous described [14].

Primary neuron culture and subsequent treatment

On day 1 after birth, primary neurons of mice were acquired and subsequently underwent culture in neurobasal medium (Gibco Inc., United States) added 2% B27 (Gibco Inc., United States) under circumstances of 5% CO_2 at 37°C. miRNA-132-3p and siRNA aimed at HO-1 or Nrf2 underwent transfection into primary neurons with the help of LRTR for forty-eight hours subsequent to six days. In order to generate OS, neurons were supplemented with 200 um hydrogen peroxide for six hours. The supernatant and sediment were obtained for further examination.

Real-time PCR

Total RNA was obtained from the brain utilizing NucleoSpin® miRNA kit (Macherey-Nagel, Germany). miRCURY LNATM Universal RT microRNA PCR (Exiqon A/S, Vedbaek, Denmark) was applied to reverse transcription. For cDNA synthesis, 1 μg of RNA was mixed with Oligo(dT) primers, dNTP mix, 5× first-strand buffer, Ditiotreitol, RNAseOUT and SuperScript II RT, all according to manufacturer's protocol (Invitrogen). Mx3000PTM system (Agilent Technologies, United States) with Brilliant II SYBR® Green QPCR master mix (Agilent Technologies, United States) was required for amplification. The primers for specific miRNA and U6, the internal control, are from manufacturer (Exiqon,

Denmark). The primers for HO-1, Nfr2, and control GAPDH are as follows: HO-1, GGTGATGGCTTCCTTGTACC (forward) and AGTGAGGCCCATACCAGAAG (reverse); Nrf2, TCTCCTCGCTGGAAAAAGAA (forward) and AATGTGCTGGCTGTGCTTTA (reverse); GAPDH, ATGACATCAAGAAGGTGGTG (forward) and CATACCAGGAAATGAGCTTG (reverse). Thermocycling conditions were 10 min of initial denaturation at 94°C, followed by 40 cycles of denaturation at 94°C for 10 s, annealing at 58°C for 30 s and extension at 70°C for 30 s with a single fluorescence acquisition step at the end of extension, followed by final cooling at 4°C for 30 s.

Cell viability evaluation

Neuron viability was examined with thiazolyl blue tetrazolium bromide (Applichem Inc., United States; MTT, 0.5 mg/mL). Briefly, 1×10^4 cells/well of neurons were seeded in 96-well plates. After 24 h, the cells were transfected with Nrf2 or Ho-1 siRNA, and treated with 200 μm H_2O_2 for 6 h. Subsequently, the cell culture medium was replaced with MTT solution and incubated at 37°C for 3 h. A microplate reader (Thermo Fisher Scientific, USA) was used to detect the absorbance at 590 nm.

Apoptosis assay

Annexin V-FITC/PI apoptosis detection kit (4 A Biotech, Beijing, China) was applied to apoptotic detection. In short, 200 μL binding buffer including 5 μL PI and 10 μL Annexin V-FITC was used to suspend the cells again. Subsequent to 30-minute-incubation in dark under circumstances of room temperature, flow cytometry (FC) (Becton Dickinson, Mountainview, CA) were used to identify PI-negative and FITC-positive cells.

Western blot (WB) analysis

The cortex at the same side was obtained twenty-four hours subsequent to repurfusion and treated for WB as mentioned before [15]. Briefly, a total of 40 μg/lane protein lysates were loaded onto 10% SDS-PAGE gels and then transferred to 0.22 μm polyvinylidene difluoride membranes. The membranes were blocked with 5% fat-free milk in 0.1% Tween-20 (PBST) and incubated with primary antibodies overnight at 4°C. Following washes with PBST for 3 times, the membranes were incubated with secondary antibodies for 1 h at room temperature. Following washing with PBST, specific antibody binding was detected using ECL kit detection system, according to the manufacturer's protocol. Antibodies utilized in our research included rabbit anti-caspase-3, anti-Nrf2 (Abcam, Great Britain; 1:1000), anti-β-actin (1:3000; Sigma-Aldrich Corp. St. Louis, MO 63103, USA), and anti-HO-1 (Abcam, Great Britain; 1:1000) antibody and their relative secondary antibodies (Stressgen Biotechnologies Corporation, Canada). Signals were measured with ECL kit (GE Healthcare, Great Britain). Densitometry was applied to determine proteins. Proteins were normalized to β-actin to serve to be a loading control.

OS examination

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

Measurement Data were presented in the form of mean ± standard error; and the comparison between two groups was conducted using *t* test while the multiple groups comparison was conducted using ANOVA followed with bonfferroni's test. The categorical data was expressed in percent, and the comparison was conducted using chi-square test. Differences were considered significant at $P<0.05$.

Results

MiRNA-132-3p increases in patients with IA and mice with MCAO

In order to explore the influence of miRNA on CI/CR apoplexy, 6 miRNAs were chosen, such as miRNA-125a, miRNA-15a, miRNA-132-3p, miRNA-93, miRNA-324, and miRNA-132-3p. Their expression was determined by RT-PCR. It was discovered that miRNA132-3p and miRNA-93 were remarkably enhanced in plasma of acute IA patients in comparison to normal controls (**Figure 1**).

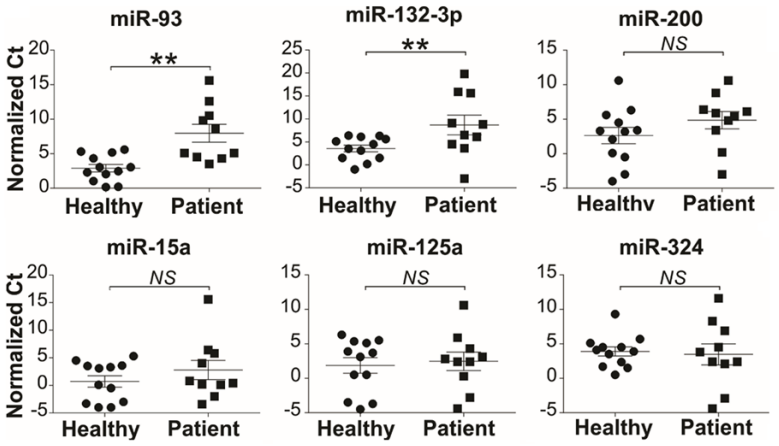

Figure 1. Analysis of error distribution presented by normalized ΔCt of the chosen six miRNAs in healthy control samples (N=12) and the patients with apoplexy (N=10). NS, P>0.05; **, P<0.01.

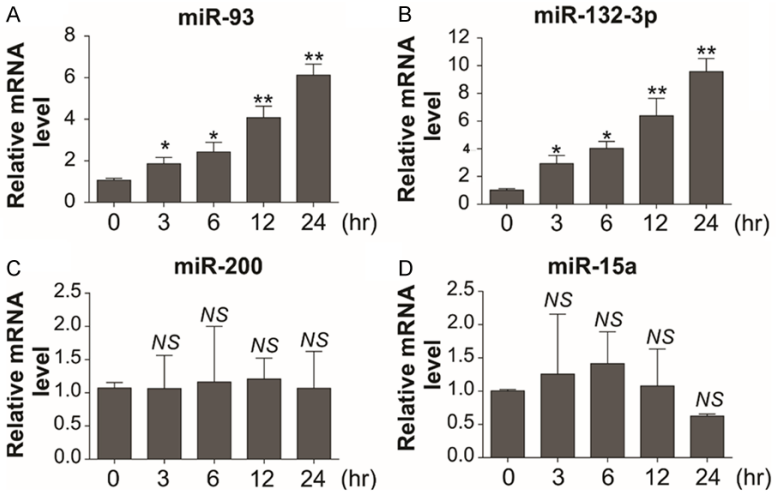

Figure 2. miRNA expression in mice with MCAO. Expression of miRNA-93 (A), miRNA132-3p (B), miRNA200 (C), and miRNA15a (D) was examined with RT-PCR in mice with MCAO for one hour as well as reperfusion for three to twenty-four hours. Each group in the time points from 3-24 hour was compared with the control group in the time point 0 hour. NS, P>0.05; *, P<0.05.

Subsequently, mice with MCAO were obtained to verify expression of miRNA-200, miRNA-93, miRNA-15a, and miRNA-132-3p. Our results showed that miR-93 and miR-132-3p levels on the ipsilateral side of the brain significantly increased in a time-dependent manner after reperfusion compared with the sham group (**Figure 2A** and **2B**). However, the other two miRNAs did not significantly change after reperfusion (**Figure 2C** and **2D**). Our findings indicated that upregulation of miRNA-93 and miRNA-132-3p is correlated to CI/CR injury in human and mice. As miRNA-93 was reported to contribute to mice I/R injury [3], we next focused on influence of miRNA-132-3p on CI/CR injury.

Suppression of MiRNA-132-3p by its antagomir reduced ischemic brain damage

In order to investigate the influence of miRNA-132-3p on CI damage *in vivo*, a miRNA-132-3p antagomir was designed and injected to mice to test whether it has protective effect in mice with MCAO. As predicted, the miRNA-132-3p antagomir suppressed miR-132-3p expression in ischemic regions of mice with MCAO (**Figure 3A**). Accordingly, miR-132-3p antagomir effectively reduced cerebral infarction in comparison to CI/CR group (**Figure 3B**). As CI/CR damage is majorly caused by apoptosis, caspase-3 activation was examined by WB. Our results showed the caspase-3 was activated upon CI/CR damage in brains of mice, but compromised in the miR-132-3p antagomir pre-treatment groups (**Figure 3C**). Furthermore, NS test suggested that neuronal damage of CI/CR mice might decrease due to miRNA-132-3p antagomir (**Figure 3D**). Findings of our research collectively indicated that suppression of miR-132-3p expression was able to protect mice from CI/CR damage.

MiRNA-132-3p antagomir decreased OS in mice with transient MCAO

Reperfusion of oxygenated blood into ischemic tissue usually results in production of ROS, which could regulate almost all molecules of cells, consequently triggering malfunction of cells [16]. We therefore investigate the eff-

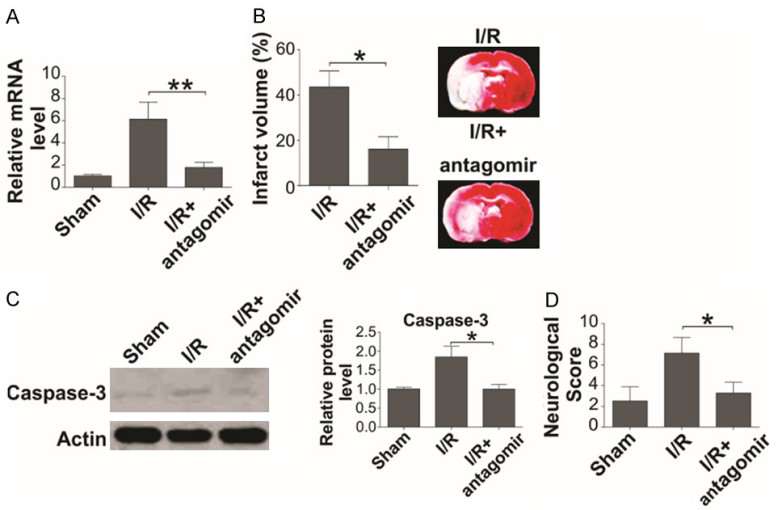

Figure 3. miR-132-3p antagomir protects mice from CI/CR injury. (A). The suppression effect of miR-132-3p antagomir on its mRNA expression was tested by RT-PCR. (B). Volume of brain infarct assessed by coronal sections stained by TTC. The representative picture of brain infarct was shown in the right panel. (C). Expression of caspase-3 on the same side was examined by WB. The quantification of caspase-3 was shown on the right panel. (D). NS results. Data were displayed in the form of mean ± standard error (n=5 per group). *P<0.05, **P<0.01.

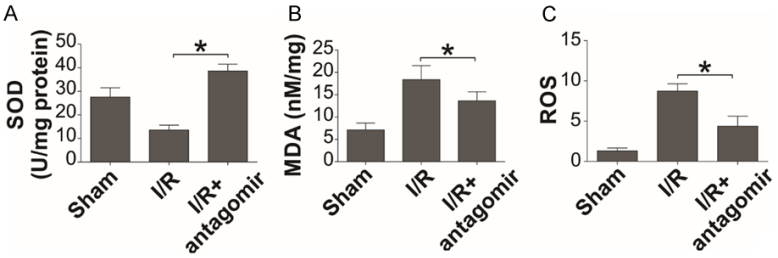

Figure 4. Influence of miRNA-132-3p antagomir on OS triggered by CI/CR of mice. A. Biochemical kit was applied to examination of SOD activity. B. Biochemical kit was applied to examination of MDA concentration. C. Biochemical kit was applied to examination of ROS concentration. Data were displayed in the form of mean ± standard error (n=5 per group). *P<0.05.

ect of miRNA-132-3p on OS triggered by CI/CR *in vivo* by examination of concentration of ROS and MDA and activity of SOD. Our results indicated that SOD activity dropped in ischemic region of CI/CR mice but rebounded upon miR-93 antagomir treatment in comparison to CI/CR group (**Figure 4A**). MDA concentration was promoted in CI/CR group in comparison to mice that underwent sham operation. However, MDA decreased subsequent to supplementation of miRNA-132-3p antagomir (**Figure 4B**). Similarly, CI/CR promoted generation of ROS of the cortex, which was suppressed by miR-132-3p antagomir supplementation (**Figure 4C**). Therefore, effect of miRNA-132-3p on CI/CR damage might be attributable to ROS generation.

MiRNA-132-3p antagomir promoted HO-1 expression by Nrf2

In terms of OS, it is usually involved with dysregulation of Nrf2 pathway, we therefore investigate the regulation of Nrf2 and downstream target, HO-1 [17]. As shown in (**Figure 5A**), CI/CR damage suppressed the modification of Nrf2 and HO-1. However, miRNA-132-3p antagomir supplementation remarkably promoted Nrf2 expression in CI/CR mice (**Figure 5A**). Furethermore, the RT-PCR also confirmed that the miRNA-132-3p antagomir could significantly rescue the decrease of HO-1 and Nrf2 by CI/CR damage (**Figure 5B** and **5C**). Therefore, these results indicated that miRNA-132-3p might exert its function by suppress the expression of NrF2 and HO-1 in oxidative stress caused by CI/CR damage.

HO-1/Nrf2 modulates the protective effects of miR-132-3p antagomir in primary neurons

In order to confirm the role of HO-1/Nrf2 signaling pathway in miRNA-132-3p regulated CI/CR damage, we utilized the H_2O_2-induced cell death in vitro model. As demonstrated in (**Figure 6A**), treatment with miR-132-3p antagomir effectively attenuated H_2O_2-triggered apoptosis in comparison with control. In contrast, elimination of either HO-1 or Nrf2 by siRNA abandoned protection by miRNA-132-3p antagomir. The findings in our research suggested that inhibition of HO-1 or Nrf2 could eliminate protection by miRNA-132-3p antagomir under circumstances of H_2O_2 stress. As to the mechanism of cell death, we found that

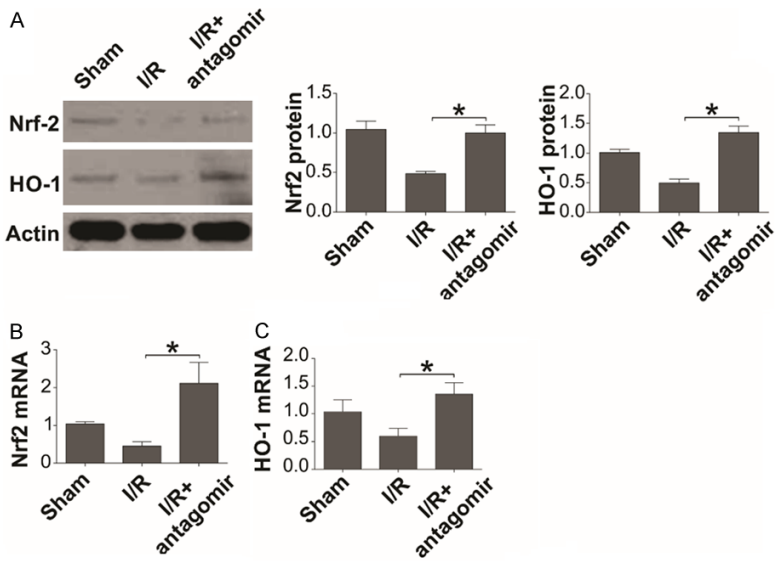

Figure 5. Influence of miRNA-132-3p antagomir on HO-1/Nrf2 concentration. A. Influence of miRNA-132-3p antagomir on cortical HO-1 or Nrf2 concentration, which was examined by WB (n=5 per group). The quantification of Nrf2 and HO-1 was shown on the right panel. B. RT-PCR was applied to examination of Nrf2 mRNA concentration (n=5 per group). C. RT-PCR was applied to examination of HO-1 mRNA concentration (n=5 per group). *P<0.05.

H_2O_2 induced cell death by apoptosis (**Figure 6B** and **6C**), which could be inhibited by miR-132-3p antagomir. However, elimination of either HO-1 or Nrf2 could re-sensitize the primary cortical neurons to H_2O_2 induced apoptosis (**Figure 6B** and **6C**). Additionally, the elevated ROS concentration in neurons was suppressed by miRNA-132-3p antagomir, which was abandoned by depletion of HO-1 or Nrf2 (**Figure 6D**). The findings of our research proved that miRNA-132-3p is crucial to regulation of OS tolerance in neurons under circumstances of H_2O_2 stimulation.

Discussion

In our research, the effect of miRNA-132-3p on ischemic cerebral damage was explored. The findings of our research demonstrated that miRNA-132-3p concentration was elevated in patients with ischemic apoplexy and mice with MCAO in twenty-four hours. Furthermore, intraventricular injection of miRNA-132-3p antagomir remarkably protected from ischemic brain injury. Further studies revealed that the protection by miRNA-132-3p antagomir was closely linked with HO-1/Nrf2 signaling pathway. In a word, findings of our research proved that suppression of miR-132-3p reduces CI/CR damage by triggering antioxidant reactions of neurons, proposing an innovative target to treat acute IA.

MicroRNAs are crucial not only to brain development but also to generation of its functional connectivity, which was proved by the changed pattern of spine, declined dendritic complexity, and cognition shortcomings arising from malfunction of miRNA processing procedures [1, 18]. In our research, it was discovered that miR-132-3p is upregulated in IA patients and mice with MCAO. miR-132 was found involved in generation and development of vessels in various animal research or in vitro experiment [19, 20], down-regulating p120RasGAP expression [20]. The miRNA mentioned above is abundant in human brains and closely linked to a quantity of neurologic reactions [18, 21]. Here, our results further identify that miR-132-3p up-regulation plays a crucial part in modulated OS in the brain IA. The findings of our research indicated that miRNA-132-3p could serve as a promising predictor for IA. Further research is required to verify the conformity to another research applying standard curves to qPCR absolute measurement.

As to effects of miRNA-132-3p on the IA, we found that the up-regulation of miRNA-132-3p is correlated to the ROS generation in the brain. OS is a crucial contributor to CI/CR damage leading to dysregulation of neurons and apoptosis [22, 23]. It has been proved that a swift promotion of ROS generation immediately subsequent to acute IA could counteract antioxidant defences, bringing about further damage to tissues through apoptosis, necrosis, and autophagy. Furthermore, the swift recovery of blood supply promotes oxygenation of tissues and is responsible for a second wave of ROS production, bringing about reperfusion damage. A number of miRNAs are found related to OS response through antioxidant defending system [12]. MiRNA-708 and miRNA-135b were

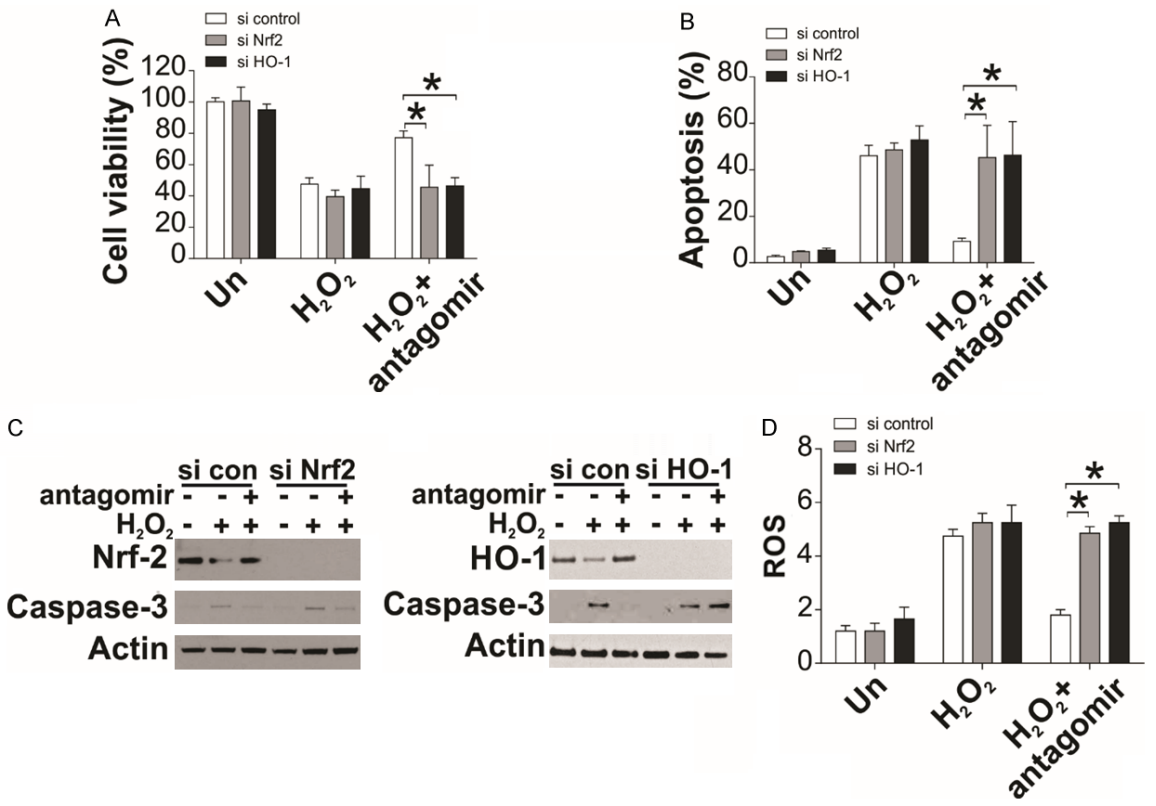

Figure 6. Protective effect of miRNA-132-3p antagomir is via HO-1/Nrf2 pathway. (A) Neurons that underwent transfection with HO-1 or Nrf2 siRNA were treated with H_2O_2 for twenty-four hours. Neuron viability was examined with MTT assay. (B) Neurons were treated as in (A), the apoptosis was analyzed by FC. (C) Neurons were treated as in (A), caspase-3 expression was analyzed by WB. The quantification of caspaspe-3 was shown on the bottom panels. (D) Neurons were treated as in (A), Biochemical kit was applied to examination of cortical ROS concentration. *P<0.05.

remarkably enhanced upon H_2O_2 stimulation, and their targets were related with DNA recombination, protein autophosphorylation, protein ubiquitination, and neurons development [24]. Also, miRNA-424 reduced OS in the cortex and protected against transient CI/CR damage [12]. It has been discovered in our research that MDA and ROS concentrations decrease due to miRNA-132-3p antagomir in ischemic region of CI/CR mice. Nrf2 and HO-1 expression and activity of SOD are promoted by miRNA-132-3p antagomir, which meets our expectations.

Nrf2 is a short-lived protein that acts as a transcription factor, driving expression of numerous cytoprotective genes related to xenobiotic metabolism, antioxidant responses, and anti-inflammatory responses [17]. Proteins upregulated by Nrf2 signalling include haeme oxygenase-1 (HO-1), superoxide dismutase (SOD1), catalase, and enzymes related to glutathione metabolism, such as glutathione cysteine ligase modifier subunit, glutathione S-transferase, and glutathione cysteine ligase catalytic subunit [25]. Dysregulation of the Nrf2 pathway is implicated in various diseases, including ovarian, prostate, and breast cancers; and in inflammatory conditions, including hepatitis, atherosclerosis, and neurodegenerative disease [17]. Our research demonstrates that depletion of HO-1 or Nrf2 by siRNA attenuates the protective effects of miRNA-132-3p antagomir against H_2O_2-triggered neuronal damage and OS. Our findings indicate that miRNA-132-3p antagomir was able to promote HO-1/Nrf2 expression, thus reinforcing defending system via anti-oxidative signaling pathway.

In a word, our research proved that miRNA-132-3p regulated neuronal ischemic damage by stimulating HO-1/Nrf2 signaling pathway, proposing an innovative strategy to consider miRNA-132-3p as the target to treat IA. Nevertheless, there are several limitations of

our research, such as delivery specific to cells or tissues in vivo, target specificity, and degeneration averting. Furthermore, miRNA delivery that can effectively get across blood brain barrier and aim at brain tissues is the focus of future research.

Disclosure of conflict of interest

None.

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