Original Article
Role of miRNA-185 in cerebral ischemia-reperfusion injury

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Abstract: Aims: This study is to investigate the role of miRNA-185 in cerebral ischemia-reperfusion injury. Methods: The cerebral ischemia-reperfusion rat model was established by clipping common carotid artery. HE staining was applied to observe the morphological changes of cerebral cortex cells in rats. qRT-PCR was applied to detect the changes of NOS2 mRNA and miRNA-185 expressions in rat cerebral cortex tissue specimens and blood samples. Immunohistochemical staining, Western blot and ELISA were also applied to detect the changes of NOS protein in these two specimens. Results: Compared with those in sham operation group, obvious pathological changes in cerebral cortex cells were shown by HE staining. The expression of *NOS2* mRNA and protein in cerebral cortex and blood were significantly up-regulated. The expression of miRNA-185 was down-regulated in model group. The difference was statistically significant (P < 0.05). Conclusions: The expression levels of *NOS* mRNA and protein were significantly up-regulated in cerebral cortex tissues and blood in cerebral ischemia-reperfusion rats, and these up-regulations may be related to the down-regulation of miRNA-185 expression. Our findings suggest that miRNA-185 may be involved in the pathogenesis of cerebral ischemia reperfusion injury through the regulation of *NOS2*.

Keywords: miRNA-185, Cerebral ischemia-reperfusion, NOS2

Introduction

Ischemic cerebrovascular disease, refers to the brain blood supply disorder due to cerebral vascular wall lesions or hemodynamic impairment, which further results in a corresponding cerebral tissue necrosis or softening due to ischemia and hypoxia [1, 2]. The current treatment method is to restore brain tissue blood supply [3]. However, there are many metabolic changes and injuries after ischemia when blood oxygen supply is restored, which is called reperfusion injury [4]. Many studies [5, 6] have shown that this is due to the generation of oxygen free radicals during reperfusion [7]. Once damage occurs, it is difficult to reverse [8-10]. Therefore, it is essential to clarify the pathogenesis of cerebral ischemia-reperfusion, to find new therapeutic gene targets, to improve the early diagnosis rate, and to implement individualized treatment, which is the key to improve the curative effect and prognosis.

Long term studies have found that a wide variety of mRNA and miRNA are involved in the pathogenesis of cerebral ischemia-reperfusion injury [11, 12], and many cytokines, such as TNF-α and NF-κB, are also involved in this process. Recent researches have focused on the roles [13] and signal transduction pathways [14] of these factors. However, the regulatory mechanism of cerebral ischemia-reperfusion is still not fully understood.

Nitric oxide synthase 2 (NOS2) is a key enzyme in the synthesis of endogenous NO in human body, which plays a vital role in the synthesis of NO in vivo. Studies [15, 16] have found that appropriate amount of NO can relax diastolic cerebral blood vessels in the early cerebral ischemia, thus playing a protective role for cerebral tissues in maintaining local cerebral blood flow [17]. However, with the extension of ischemic time, NOS2 will induce a large number of NO. NO acts as a chemically active oxygen free radical at this time, and will produce more toxic superoxide radicals to damage brain tissues [18].

In this study, qRT-PCR, Western Blot, HE staining, immunohistochemical staining, bioinformatics prediction, and ELISA were applied to

detect the expressions of *NOS2* mRNA and protein in cerebral tissues and blood of cerebral ischemia-reperfusion rat models. The relationship between *NOS2* expression and miRNA-185 expression was also predicted and verified, in order to explore the mechanism of miRNA-185 in cerebral ischemia-reperfusion injury.

Materials and methods

Experimental animal

Totally 60 male Sprague Dawley (SD) rats, weighing between 150-200 g, were purchased from Chongqing Tengxin Bill Experimental Animal sales Co. Ltd (Chongqing, China). All rats were adaptive fed for a week, with free access to food and drinking water. The 3R animal welfare principle was followed in this experiment. All animal experiments were conducted according to the ethical guidelines of Animal Protection Committee, Affiliated Hospital of North Sichuan Medical College.

Reagents and instruments

Chloral hydrate was purchased from Qingdao Yulong seaweed Co. Ltd (Qingdao, China). miRcute miRNA Isolation Kit, miRcute miRNA First-strand cDNA Synthesis Kit, miRcute miRNA qPCR Detection Kit, SuperReal PreMix (SYBR Green) and TIANScript II First-strandcDNA Synthesis Kit were all purchased from TIANGEN Biotech (Beijing) Co, Ltd (Beijing, China). Rabbit anti-rat NOS2 primary antibody (ab15323), rabbit anti-rat β-actin primary antibody (ab-129348) and goat anti-rabbit secondary antibody were all purchased from Abcam Inc, (MA, USA). Trizol was purchased from Yeasen Co, Ltd (Shanghai, China). BCA Protein Assay Kit was purchased from Real-Times (Beijing) Biotechnology Co. Ltd (Beijing, China). miR-Neasy Serum/Plasma Kit was purchased from Guangzhou Jianlun Biological Technology Co. Ltd (Guangzhou, China). NOS2 ELISA kit was purchased from Shanghai Xin Yu Biotechnology Co., Ltd (Shanghai, China), and HE staining kit was purchased from Beyotime Biotechnology Co., Ltd (Shanghai, China). iQ5 real-time PCR detection systems were purchased from Bio-Rad Corporation (Hercules, CA, USA). Nano-Drop ND-1000 UV-Vis Spectrophotometer purchased from Thermo Fisher Scientific Inc. (Wilmington, USA). Automatic tissue embed-ding machine Leica EG1150 was purchased from Leica Biosystems (Wetzlar, Germany). Image lab3.0 software was purchased from Bio-Rad Corporation (Hercules, CA, USA).

Construction of cerebral ischemia-reperfusion rat model

Rats were fasted 24 hours before surgery with free drinking water. 30 rats were anesthetized by intraperitoneal injection with 10 percent of chloral hydrate at the dose of (weight * 3 + 0.1) ml. Then, the common carotid arteries on both sides were exposed and separated by a glass probe. The common carotid arteries on both sides were clamped to induce cerebral ischemia, thus establishing the cerebral ischemia rat model. After 2 hours, the clamp was released to make total bilateral carotid artery recanalization, and the common carotid arteries were reperfused for 24 hours to form the cerebral ischemia-reperfusion rat model. Rats in the sham operation group received the same procedure without clamping the common carotid artery. Rectal temperature of rats was maintained at 37.0 ± 0.5°C in the process of operation, and 4 rats with coma, epilepsy, seizures and other complications were abandoned.

Sample collection

Blood was collected from abdominal aorta with puncture. And, serum was separated and stored at -80°C. The rats were decapitated after anesthesia and the cerebral cortex tissues were collected. After washing with pre-cold 0.9% saline, one part of the cerebral cortex tissue was stored at -80°C, and the other part of the cerebral cortex tissue was fixed with 10% formalin and paraffin-embedded sectioned for HE and immunohistochemical staining.

HE staining

HE staining was performed with HE staining kit. Briefly, tissues were fixed, embedded in paraffin with Leica EG1150 and cut into tissue sections. Then tissue sections were dewaxed in xylene and rehydrated in graded alcohols. After washing with running water and distilled water, sections were stained with hematoxylin for 3-5 min. After washing again with running water, sections were differentiated with 1% HCl in 70% alcohol. Then sections were stained with eosin for 1-4 min after washing with running

water. After dehydration and differentiation in alcohol, sections were mounted and observed under ECLIPSE TS100-F microscopy (Nikon Instruments (Shanghai) Inc, Shanghai, China).

qRT-PCR

Total RNA was extracted by Trizol according to the manufacturer's protocol. Then RNA was reverse transcribed into cDNA. The cDNA was used as a template for PCR to detect the expression of *NOS2* and miRNA-185. The expression of β-Actin and U6 were set as internal controls to *NOS2* and miRNA-185, respectively. The primer sequences were as follows:

NOS2: Sense: 5'-ATCCCGAAACGCTACACTT-3'; Anti-sense: 5'-GGTCT GGCGAAGAACAATC-3'.

β-actin: Sense: 5'-CGTGCG TGACATTAAAGAG-3'; Anti-sense: 5'-CTGGAAGGTGGACAGTGAG-3'.

U6: Sense: 5'-CTCGCTTCGGCAGCACA-3'; Anti-sense: 5'-AACGCTTCACGAATTTGCGT-3'.

miR-185: Sense: 5'-ACACTCCAGCTGGG TGGA-GAGAAAGGCAGT-3'; Anti-sense: 5'-TGGTGTCG-TGGAGTCG-3'.

Quantitative PCR for *NOS2* and β-actin were performed with the following procedure: 95°C for 10 min, followed by 35 cycles of 95°C for 45 s, 52°C for 45 s and 72°C for 45 s. And quantitative PCR for miR-185 and U6 were performed with the following procedure: 95°C for 3 min, followed by 40 cycles of 95°C for 12 s, 62°C for 40 s and 72°C for 20 s. The relative expression was calculated by $2^{-\Delta\Delta CT}$ method.

Immunohistochemical staining

Immunohistochemical staining was performed as follows. Paraffin embedded tissues were dewaxed and rehydrated in graded alcohols. Antigen retrieval was achieved by incubating with Citric acid sodium antigen retrieval solution P0081 (Beyotime Biotechnology Co., Ltd, Shanghai, China). And heating at 97°C for 12 min. After washing, sections were incubated with 0.3% hydrogen peroxide at room temperature for 10 min to inactivate endogenous peroxidase. After blocking, anti-NOS2 antibody (1:50) was added and incubated overnight at 4°C. After washing, the secondary antibody was added and incubated at room temperature for 1 h. Then sections were developed with DAB

chromogenic reagent. Finally, sections were counterstained with haematoxylin. Sections were mounted with neutral gum. The NOS2 expression positive cerebral cells were observed under ECLIPSE TS100-F microscopy (Nikon Instruments (Shanghai) Inc, Shanghai, China).

The staining positive cells were brown or tan, and NOS2 positive expression was mainly localized in the cell membrane, or cytoplasm. Five fields under high optical magnification (10×40) were randomly selected in each section. Positive cells were counted, and the mean number of positive cells was calculated.

Western blot

The total protein was extracted and the protein concentration was determined by BCA protein assay kit. After boiling in loading buffer for 5 min, 20 μg samples were subjected to 10% SDS-PAGE for Western Blot analysis. The primary antibodies, including anti-NOS2 antibody (1:800) and anti-β-actin antibody (1:5000) were added and incubated overnight at 4°C. Then, secondary goat anti-rabbit antibodies (1:3000) was added and incubated at room temperature for 1 h. Membrane was placed in ECL solution for color development. And, image was obtained by gel imaging system and analyzed by image lab3.0 software. The relative content of NOS2 protein was calculated as the ratio of NOS2 gray value to β-actin gray value.

ELISA assay

Serum was separated from blood sample by centrifugation at 3000 rpm for 10 min. Then, 50 μg samples (1:4 dilution) or standard reference solutions were added to the corresponding well. HRP-conjugated detection antibody (100 μl) was added to each well, sealed and incubated in constant temperature incubator for 1 h. After washing for 5 times, 50 μl substrate A and B was added to each well. After incubation at 37°C for 15 min, 50 μl termination solutions were added, and OD value of each well at 450 nm wavelength were determined within 15 minutes.

Bioinformatics prediction

The miRanda, TargetSean, PieTar, MiRanda, BibiServ and other target gene prediction software were applied for predicting *NOS2*
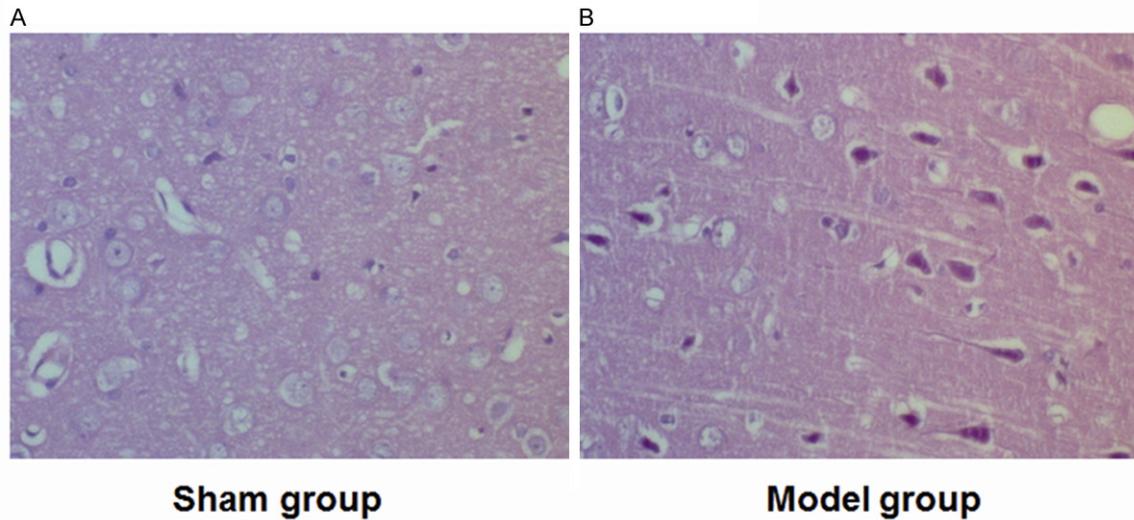
Figure 1. Morphological changes of cerebral cortex tissues in model group rats (B) and sham group rats (A).
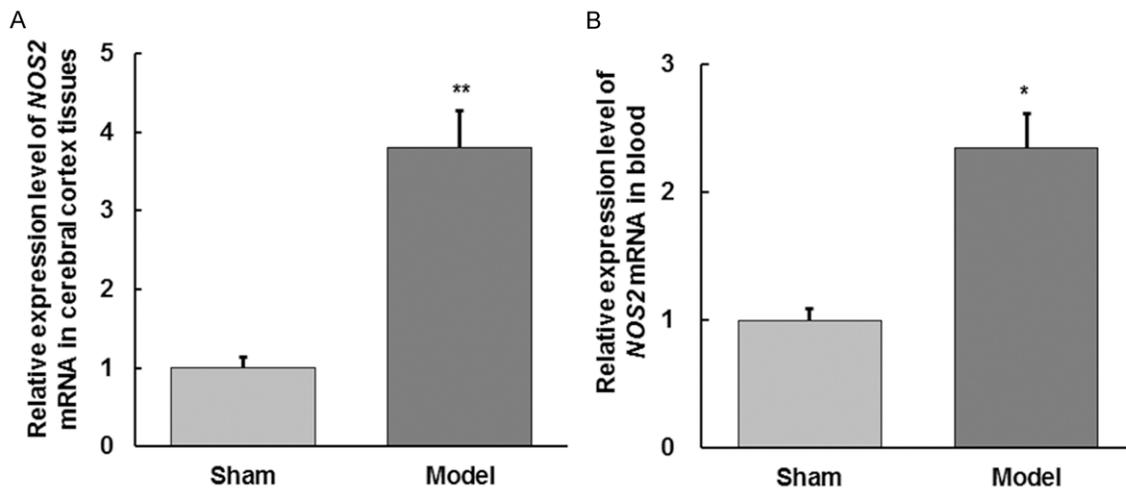
Figure 2. Relative expression levels of *NOS2* mRNA in cerebral cortex tissues (A) and blood (B) in rats. Compared with sham group, *Represents P < 0.05, **Represents P < 0.01.

upstream regulatory miRNAs, and the possible regulatory sites were also predicted.

Statistical analysis

All statistical analyses were performed by using the Statistical Package for Social Sciences software (SPSS, Windows version release 18.0; SPSS Inc.; Chicago, IL, USA). Data were presented as mean ± standard deviation. All data were analyzed with normality test. One-way ANOVA was applied for multiple sets of measurement data analysis. LSD and SNK method were applied when there was homogeneity of

variance, and Tamhane's T2 or Dunnett's T3 method was applied when there was not homogeneity of variance. A *P* value < 0.05 was considered statistically significant, and P < 0.01 difference was considered extremely statistically significant.

Results

Morphological changes of rat cerebral cortex tissues

Changes of rat cerebral cortex cells in cell morphology were preliminary detected by HE stain-
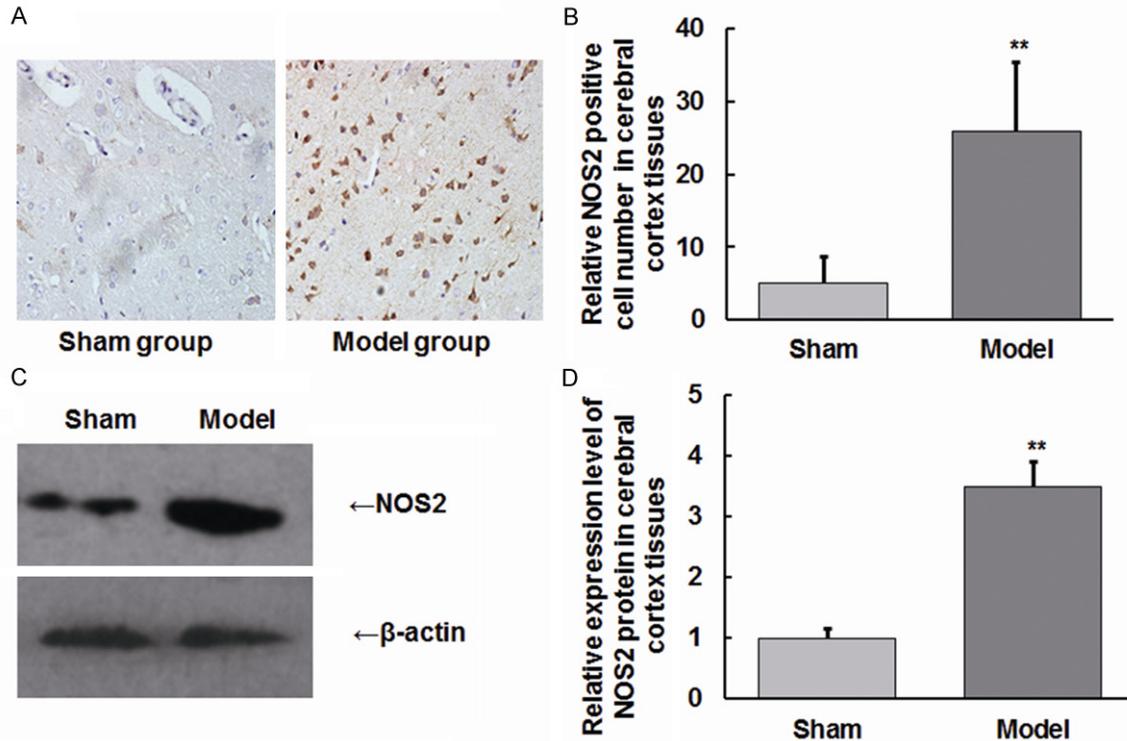
Figure 3. Relative NOS2 expression positive cell numbers and corresponding expression levels in rat cerebral cortex tissues. A. Relative NOS2 expression positive cells in rat cerebral cortex tissues. B. Relative NOS2 expression positive cell numbers in rat cerebral cortex tissues. Compared with sham group, **Represents P < 0.01. C. Relative NOS2 expression in rat cerebral cortex tissues. D. Relative NOS2 expression levels in rat cerebral cortex tissues. Compared with sham group, **Represents P < 0.01.

ing. As shown in **Figure 1**, cell size was decreased, cytoplasm had a tendency to concentrate, and nuclear chromatin condensed in the model group. In the sham group, cell morphology was more complete, nucleus was in high visibility, and cytoplasm was in normal color. This result indicates that compared with that in sham group, a series of morphological changes occurred in brain cells in model group, which may be an early sign of apoptosis or necrosis.

Changes of NOS2 mRNA expression in cerebral cortex tissues and blood samples

The qRT-PCR was applied to detect the expression of *NOS2* mRNA in different samples. As shown in **Figure 2**, compared with that in the sham group, *NOS2* mRNA expressions in cerebral cortex tissues and blood samples were significantly increased, and these differences were statistically significant (P < 0.05). This result indicates that NOS2 may play a certain role in the regulation of ischemia and reperfusion.

Expression changes and distribution of NOS2 protein in cerebral cortex tissues

Immunohistochemical staining and Western Blot was applied to detect the expression and distribution of NOS2protein in cerebral cortex tissues. The number of NOS2 positive cells in cerebral cortex tissues in model group was significantly higher than that in sham group (**Figure 3A** and **3B**), and the difference was statistically significant (P < 0.05). And compared with that in sham group, the expression level of NOS2 protein in cerebral cortex tissues in model group was significantly higher than that in sham group (**Figure 3C** and **3D**), and the difference was statistically significant (P < 0.05). This result indicates that consistent with the trend of mRNA, the expression of NOS2 protein in cerebral cortex tissues was also up-regulated.

Expression changes of NOS2 protein in blood samples

ELISA was applied to detect the expression of NOS2 protein in blood samples. As shown in
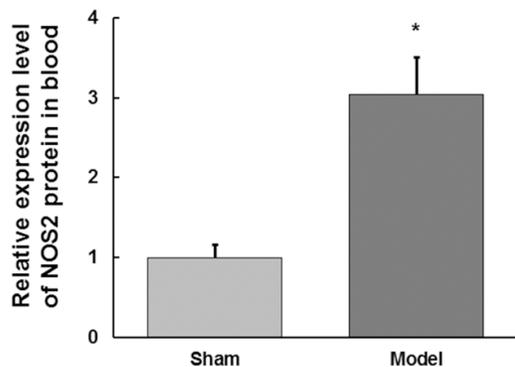
Figure 4. Relative expression levels of NOS2 protein in rat blood. Compared with sham group,*Represents P < 0.05.

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3' aguCCU—UGACGGAAAGAGAGGu 5'  rno-miR-185
         |||  ||||  |  ||||||||
133:5' uguGGACUACUGAAUCUCUCUCCu 3'  Nos2
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Figure 5. The predicted specific regulatory binding sequences of miR-185 to *NOS2*.

Figure 4, compared with that in sham group, the expression level of NOS2 protein in blood sample in model group was significantly higher than that in sham group, and the difference was statistically significant (P < 0.05). This result indicates that consistent with the trend of mRNA, the expression of NOS2 protein in blood was also up-regulated.

Prediction miR-185 targeted regulation of NOS2

The *NOS2* upstream regulatory miRNAs were predicted with target gene prediction software. Results showed there may have targeted regulatory relationship between miR-185 and *NOS2*, and the specific regulatory binding sequences as shown in **Figure 5**.

Expression changes of miRNA-185 in cerebral cortex tissues and blood samples

The qRT-PCR was applied to detect the expression of miRNA-185 in cerebral cortex tissues and blood samples. As shown in **Figure 6**, the expression level of miRNA-185 in cerebral cortex tissues and blood samples in model group were significantly lower than those in sham group, and these differences were statistically significant (P < 0.05). This result indicates that changes in *NOS2* mRNA and protein expression

may be caused by the down-regulation of miRNA-185.

Discussion

In this study, we observed the morphological changes of cerebral cells in cerebral ischemia-reperfusion injury rat model, detected the expression levels of *NOS2* mRNA and protein and miRNA-185 in cerebral cortex tissues and blood, and preliminary explored the molecular mechanisms of miRNA-185 on cerebral ischemia-reperfusion injury through regulating *NOS2*.

Cerebral ischemic stroke accounts for about 75% of all strokes, and the most common cause of cerebrovascular diseases is cerebral atherosclerosis, followed by cerebral arthritis [19]. Blood supply re-perfusion to cerebral ischemic areas is the primary treatment to avoid ischemic injury, but reperfusion injury may occur [20]. Experiments show that many pathological processes, including oxidative stress, blood-brain barrier damage, excitatory amino acid toxicity, infiltration of inflammatory cells, medium infiltration and so on, can result in ischemia-reperfusion injury [21-23]. It has very important theoretical and practical values to investigate the pathogenesis of cerebral ischemia-reperfusion injury, and to research development drugs for the protection of ischemic brain damage [24].

As early as 1980, Furchgoll *et al* found that under the action of acetylcholine, a substance named endothelium-derived relaxing factor (EDRF) was produced in blood vessels, which can cause vascular smooth muscle to relax [25]. This substance was then confirmed as NO. NO plays a double role in cerebral ischemia-reperfusion injury, which can be synthesized by the NOS2 catalysis. It is considered that NOS2 is not expressed in physiological state, and is induced expression after stimulation by bacterial lipopolysaccharide, γ-interferon, tumor necrosis factor, interleukin-1β and some other cytokines [26]. In this study, we have observed that NOS2 was abnormality highly expressed in cerebral cortex tissues and blood in cerebral ischemia-reperfusion injury rat model. And, a certain pathological changes of cell morphology occurred in NOS2 high-expressed cerebral cortex tissues, which suggest that NOS2 plays an important biologi-
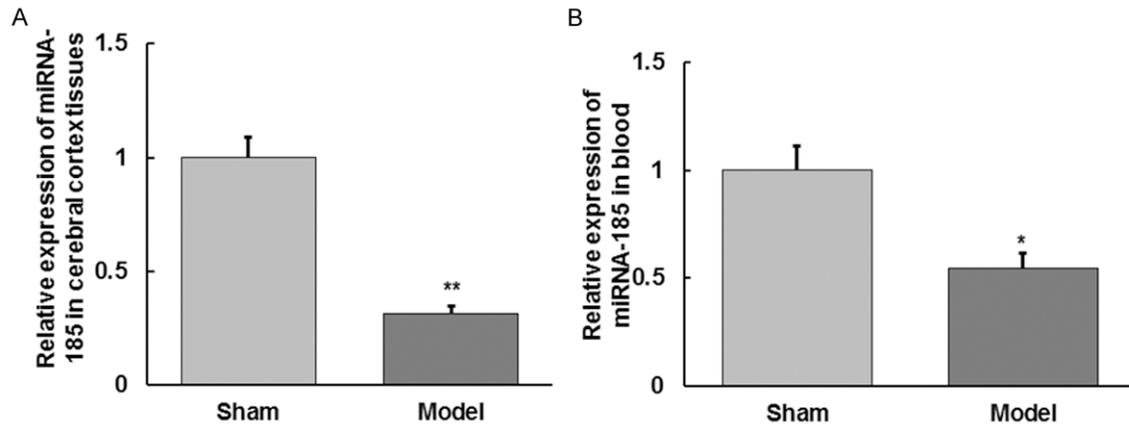
Figure 6. Relative expression levels of mRNA-185 in cerebral cortex tissues (A) and blood (B) in rats. Compared with sham group, *Represents P < 0.05, **Represents P < 0.01.

cal role in cerebral ischemia-reperfusion injury, and abnormal expression of NOS2 may be a key regulator of cerebral ischemia-reperfusion injury. In view of the current study, a consensus is that NOS2 has a close relationship with vascular diseases. Some vascular diseases can cause abnormal blood flow and then leads to a variety of pathological conditions including cerebral ischemia, anoxia, and metabolite accumulation [27]. Therefore, it is even more important to study the regulation mechanism of NOS2 in cerebral ischemia-reperfusion injury.

After prediction, we found that NOS2 may be one of the targets of miRNA-185. miRNA-185 genes were differentially expressed in a variety of diseases. For example, Kim et al showed that miRNA-185 played a role in reversal of cardiac hypertrophy through a variety of signaling pathways [28]. Ma et al showed that miRNA-185 inhibited the proliferation of clear cell, renal cell and carcinoma cell through the targeted gene VEGFA, and also induced apoptosis of these cells [29]. Bao et al showed that miRNA-185 inhibited the functional disorder of β cells caused by diabetes through targeted on SOCS3 [30]. Fu et al showed that miRNA-185 inhibited proliferation and metastasis of human breast cancer cells through targeting c-met [31]. Wang et al also found that miRNA-185 had a similar effect in breast cancer, but the target gene was VEGFA [32]. All these results suggest that miRNA-185 is closely related to human diseases. In this study, we have observed that miRNA-185 expression was significantly decreased in cerebral cortex tissues and blood in cerebral ischemia-reperfusion injury rat model. Considered that NOS2 was highly expressed in cerebral cortex tissues and blood in cerebral ischemia-reperfusion injury rat model, we speculate that the down-regulation of miRNA-185 is one of the reasons leading to the increase of NOS2. Considered that HE staining of brain cells in rats, we find that three are regulatory relationships in miRNA-185-NOS2-morphological changes of rat cerebral cells. Changes of miRNA-185, especially changes in blood, may be used as a strong indicator of cerebral ischemia-reperfusion injury.

In conclusion, one of the mechanisms of cerebral ischemia-reperfusion injury may be that reduced miRNA-185 expression in cerebral cortex tissues and blood resulted in up-regulated expression of NOS2 mRNA and protein, and finally caused a series of vascular lesions.

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