Inhibition of miR-34a ameliorates cerebral ischemia/reperfusion injury by targeting brain-derived neurotrophic factor

Type

Research paper

Keywords

ischemia/reperfusion injury, BDNF, miR-34a, oxidative stress, neuronal apoptosis

Abstract

Introduction
Oxidative stress and neuronal apoptosis are strongly associated with the pathogenesis of ischemic stroke. In this study, we aimed to determine whether miR-34a was involved in ischemia/reperfusion (I/R) injury, oxidative stress, and neuronal apoptosis by targeting brain-derived neurotrophic factor (BDNF).

Material and methods
Rats received middle cerebral artery occlusion (MCAO) surgery to simulate I/R injury. At 24 h after MCAO surgery, neurological deficits and infarct volumes were evaluated according to Longa's scale and 2,3,5-triphenyltetrazolium (TTC) chloride staining. Neuronal apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), and the expression of miR-34a and associated proteins were detected by quantitative reverse-transcription polymerase chain reaction (qRT-PCR), and western blotting. Several markers of oxidative stress were detected using commercial kits, and the interaction between miR-34a and BDNF was measured by RNA immunoprecipitation (RIP).

Results
The results showed that miR-34a was upregulated ($p < 0.05$), whereas BDNF was downregulated ($p < 0.05$) in the MCAO rats, and this negative correlation was accompanied by clear oxidative stress and neuronal apoptosis. RIP demonstrated a clear interaction between miR-34a and BDNF. Furthermore, miR-34a was also found to inhibit oxidative stress and neuronal apoptosis, increase BDNF expression, and ameliorate neurological deficits and infarct volumes ($p < 0.05$) seen in the MCAO rats.

Conclusions
These data suggested that inhibition of miR-34a ameliorated cerebral ischemia/reperfusion injury by targeting BDNF. This mechanism represents a novel and promising target for the treatment of strokes.

Inhibition of miR-34a ameliorates cerebral ischemia/reperfusion injury by targeting brain-derived neurotrophic factor

Running title: miR-34a targets BDNF in strokes

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Highlights: 1. The putative interaction between miR-34a and BDNF was established by RIP.

2. Inhibition of miR-34a ameliorated I/R injury by targeting BDNF.

3. Inhibition of miR-34a alleviated oxidative stress and neuronal apoptosis.

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interaction between miR-34a and BDNF. Furthermore, miR-34a was also found to inhibit oxidative stress and neuronal apoptosis, increase BDNF expression, and ameliorate neurological deficits and infarct volumes ($p < 0.05$) seen in the MCAO rats.

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Introduction

Epidemiological evidence suggests that strokes represent the second largest cause of death worldwide, and in approximately 87% of victims, the cause is ischemia (ischemic stroke) due to thrombosis or embolism [1, 2]. The factors associated with ischemic strokes are principally divided into uncontrollable (such as old age, male, and ethnic minorities, mainly Afro-Caribbean) and controllable factors (such as hypertension, diabetes, and hypercholesterolemia). The controllable factors can be managed by removing the blockages within blood vessels. However, when this is performed, reperfusion and reoxygenation are also associated with damage [3]

(ischemia/reperfusion (I/R) injury), which is manifested as oxidative stress, damage to the blood-brain barrier (BBB), neurovascular dysfunction, and neuronal death, all representing significant obstacles to therapy [4]. Thus, inhibition of the I/R injury may represent a novel therapy and provide a better long-term prognosis for stroke patients.

Accumulating evidence has implicated oxidative stress in I/R injury [5], and a sudden burst of oxidative stress following I/R can promote neuronal apoptosis [6]. Although the underlying mechanisms of oxidative stress-induced neuronal apoptosis remain unknown, inhibition of oxidative stress in the early stages of a stroke may represent a promising strategy for suppressing neuronal apoptosis and alleviating injury. Recently, many researchers have found that brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, regulates neuron survival, differentiation, and apoptosis through different pathways, and is of great significance for maintaining the development and function of the nerves [7, 8]. More importantly, BDNF is closely associated with oxidative stress and neuronal apoptosis in cell and animal models of I/R [9].

MicroRNAs (miRNAs) are crucial post-transcriptional regulators of gene expression, and the expression of some, including miR-141, miR-429, miR-200, miR-182, miR-183, miR-33, miR-125a-5p, miR-155, miR-34a, and miR-96, are altered after

reperfusion [10]. Among these, miR-34a plays an important role in oxidative stress and apoptosis in human mesenchymal stromal/stem cells, human umbilical vein endothelial cells [11], and vascular endothelial cells [12]. Interestingly, miR-34a expression is decreased in patients with stroke [13] and rats exposed to middle cerebral artery occlusion (MCAO) [14]. Furthermore, *in vitro* evidence suggests that miR-34a triggers the breakdown of the BBB and abnormal oxidative phosphorylation in endothelial cells [15]. Moreover, previous studies have indicated that miR-34a is a potential target for neuropathology [16]. However, whether miR-34a is implicated in oxidative stress-induced neuronal apoptosis following I/R injury by targeting BDNF remains unknown.

In this study, we attempted to determine the relationship between miR-34a and BDNF in the MCAO rat model. Furthermore, an miR-34a inhibitor was administered to determine the potential role of miR-34a as a mediator of oxidative stress and oxidative stress-induced neuronal apoptosis. RNA immunoprecipitation (RIP) was also performed to establish putative interactions between miR-34a and BDNF.

Materials and Methods

Animals and study groups

A total of 60 male Sprague-Dawley rats (250–300 g) were obtained from the Animal

Experiment Center at the Institute of Radiation Medicine of the Chinese Academy of Medical Sciences. The rats were fed with standard chow, and drinking water was freely available. Animals were kept in a 12:12 h light/dark cycle, a humidity of 40%, and a temperature of 22 ± 2 °C. Rats were randomly divided into the sham group (n = 15), MCAO group (n = 15), miR-34a inhibitor negative control group (reflected by miR-34a inhibitor NC in the figures; n = 15), and miR-34a inhibitor group (n = 15). All rats except for those in the sham group underwent MCAO surgery. In addition, rats in the negative control group and inhibitor group received treatment with a miR-34a inhibitor negative control or a miR-34a inhibitor by intracerebroventricular injection (ICV injection). 2% pentobarbital sodium (30 mg/kg) was used to induce euthanasia. The use of experimental animals in the present study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (USA) [17]. All experimental protocols were approved by the Animal Ethics Committee of the Institute of Radiation Medicine of the Chinese Academy of Medical Sciences. The experiments were conducted at The Institute of Radiation Medicine of the Chinese Academy of Medical Sciences.

MCAO surgery

Surgery was performed according to previous studies [18]. Briefly, the rats were

anesthetized using 30 mg/kg pentobarbital sodium (2% solution, intraperitoneal injection). The right common carotid artery, internal carotid artery, and external carotid artery were exposed, and the body temperatures were continuously monitored and maintained at 36.5–37.5 °C with a thermostatic blanket. Following 1 h of transient occlusion, the cerebral blood flow was restored by removing the suture for 24 h. Subsequently, the right common carotid artery, external carotid artery, and internal carotid artery were exposed via a midline cervical incision. A piece of 4/0 monofilament nylon suture with a heat-induced rounded tip was inserted through the right internal carotid artery to the base of the middle cerebral artery, which occluded the blood flow to the cortex and striatum. For the sham surgery, all arteries were exposed during the surgical period, but the filament was not inserted into the MCA. Following surgery, the rats were housed individually and closely monitored for changes in behavior and vital signs. The MCAO model was considered successfully established when the following observations were indicated: i) Horner syndrome occurred in the ipsilateral (left side) when the rat displayed wakefulness after surgery; ii) the forelimbs did not completely stretch; and iii) contralateral circling occurred when walking. Simultaneously, the Zea-Longa neurological deficit scores were calculated. Scores of 2 and 3 were included in the MCAO model. The neurological scores were blindly assessed independently by two pretrained technicians when the rats awoke after MCAO surgery according to the Zea-

Longa neurological deficit scores [19], The Zea-Longa assessment criteria were as follows: Score 0, normal, no neurological sign; score 1, cannot completely stretch contralateral forelimbs; score 2, contralateral circling when walking; score 3, contralateral fall over when walking; and score 4, cannot walk and lowered consciousness.

Intracerebroventricular injection

The miR-34a inhibitor negative control and miR-34a inhibitor were purchased from RiboBio (Guangzhou, China). Five minutes after MCAO surgery, the rats in the miR-34a negative control group were given miR-34a inhibitor negative control (5 mg/ml) according to the manufacturer's protocol (RiboBio, Guangzhou, China) and the rats in the miR-34a inhibitor group were given miRNA-34a inhibitor (5 mg/ml). These were administered by ICV injection through a skull hole into the left lateral cerebral ventricles (coordinates: 0.9 mm caudal, 1.4 mm lateral, and 4.6 mm deep with respect to Bregma) no more than 5 min after MCAO [20].

Scoring of neurological deficits

Scoring was performed 24 h after MCAO according to Longa's scale [21]. Briefly, this scale was as follows: score 0, no deficits; score 1, difficulty in extending the

147 contralateral forelimb; score 2, mild circling to the contralateral side; score 3, severe
148 circling to the contralateral side; and score 4, no spontaneous motor activity.

149 **Sample collection**

150 After scoring the neurological deficits for 24 h, the animals were euthanatized with 30
151 mg/kg pentobarbital sodium. The brains from a portion of the rats (n = 5 per group)
152 were rapidly removed, sliced into five coronal sections, and used for 2,3,5-
153 triphenyltetrazolium chloride (TTC) staining. A further portion of the rats (n = 5 per
154 group) was sacrificed after their cortices had been collected under anesthesia. The
155 cortices were stored at −80 °C for quantitative reverse-transcription polymerase chain
156 reaction (qRT-PCR), western blot, RIP, and the detection of oxidative stress-related
157 markers. A portion of rats (n = 5 per group) received intracardial perfusion first with
158 saline and then with 4% paraformaldehyde in PBS. The brains from these animals were
159 removed, and 10-μm frozen sections were prepared for terminal deoxynucleotidyl
160 transferase-mediated dUTP nick-end labeling (TUNEL) staining.

161 **Measurement of infarct area**

162 Sections were incubated in 2% TTC solution (Sigma-Aldrich, St. Louis, MO, USA) at
163 37 °C for 30 min and incubated in fixative (4% formaldehyde) for 24 h. From these

sections, images were captured using a digital camera, and Image-Pro Plus 6.0 software was used to measure the infarct volume according to a previous study [22].

RT-qPCR

Hippocampus tissue from three rats from each group was taken for total RNA extraction using the TRIzolRNAiso Plus kit (TaKaRa, Dalian, China). The Prime Script RT reagent kit with gDNA Eraser (TaKaRa) was used for reverse transcription of total RNA according to the manufacturer's instructions. Next, RT-qPCR was performed with SYBR Premix Ex TaqII (TaKaRa) using a CFX96 detection system (Bio-Rad, Hercules, CA, USA). The primers used were as follows [23]: miRNA-34a forward 5'-CATGGCAGTGTCTTAGCTGGTT-3'; reverse 5'-CAGTGCAGGGTCCGAGGTAT-3', and U6 forward 5'-CTCGCTTCGGCAGCACA-3'; reverse 5'-AACGCTTCACGAATTTGCGT-3'. U6 snRNA was used to standardize the expression levels of miRNA-34a.

Western blot

Hippocampal tissues from three rats in each group were taken and washed twice with PBS. Then, they were lysed in lysis buffer (Boster, Wuhan, China), vortexed, and centrifuged at 12,000 × g for 30 min at 4 °C. The supernatant was removed, and the

total protein concentration was measured using a BCA kit (Beyotime). Total protein was separated using 10% sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE). Then, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Billerica, MA, USA). After blocking with 5% skimmed milk in Tris-buffered saline/Tween-20 (TBST) for 1 h at room temperature, the membranes were incubated with primary antibodies overnight at 4 °C. The following primary antibodies were used: rabbit anti-BDNF (1:1,000; Abcam, Cambridge, MA, USA), rabbit anti-cleaved caspase-3 (1:1,000; Abcam), rabbit anti-Bax (1:1,000; Abcam), rabbit anti-Bcl-2 (1:1,000; Abcam), and rabbit anti-GAPDH (1:3,000; Abcam). After washing three times in TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:3,000; Abcam) for 1 h at room temperature. Finally, images were visualized using chemiluminescence (Boster) and analyzed by Quantity One software (Bio-Rad, Hercules, CA, USA).

RIP

RIP analysis was performed to detect a possible interaction between miR-34a and BDNF using the Magna RIP RNA-binding protein immunoprecipitation kit (Millipore Corporation) in accordance with the manufacturer's instructions. Co-precipitated RNA was detected by RT-qPCR. Here, the anti-IgG represented the negative control, and the

input represented the cell lysates.

Detection of oxidative stress-related markers

The levels of ROS, glutathione (GSH), glutathione peroxidase (GSH-Px), and glutathione reductase (GR) were detected using their appropriate kits (Beyotime, Shanghai, China) and according to the manufacturer's instructions.

TUNEL staining

Brain tissues were taken from 15 rats from each group to detect the rate of apoptosis in the CA1 area of the hippocampus. Paraffin-embedded sections from the hippocampus were placed onto poly-lysine coated slides. Neuronal apoptosis was determined by a one-step TUNEL apoptosis detection assay kit (Beyotime) according to the manufacturer's instructions. Mouse anti-NeuN (1:100; Millipore Corporation) was used to label neuronal nuclei, and DAPI (Beyotime) was used to counterstain the nuclei. Images were captured using a fluorescence microscope (Olympus, Tokyo, Japan). Neuronal apoptosis in the penumbral region was assessed by overlapping ratios between NeuN and TUNEL.

Statistical analysis

215 Data were expressed as mean ± standard deviation and analyzed by one-way analysis of
216 variance (ANOVA) followed by post hoc Tukey's test. Differences at the $p<0.05$ level
217 were deemed to be statistically significant.

218

Results

Inhibition of miR-34a ameliorates ischemic infarction and neurological deficits.

To determine the effects of miR-34a on I/R injury, MCAO rats received a treatment with the miR-34a inhibitor negative control or the miR-34a inhibitor. We observed that the expression of miR-34a was increased in the MCAO group compared with the sham group (Fig. 1D, $p < 0.05$). There was no significant change in the expression of miR-34a between the negative control group and the MCAO group (Fig. 1D, $p > 0.05$). However, compared with the MCAO group, the expression of miR-34a was consistently and significantly decreased in the inhibitor group (Fig. 1D, $p < 0.05$). Next, we determined the infarct volumes and neurological deficit scores in these groups. Our results showed that there was a larger infarct volume (Fig. 1A and 1B, $p < 0.05$) and a more serious neurological deficit score (Fig. 1C, $p < 0.05$) in the MCAO group than the sham group. However, these differences did not reach statistical significance between the negative control group and the MCAO group (Fig. 1A and 1B, $p > 0.05$, Fig. 1C, $p > 0.05$). Notably, however, we found a lower infarct volume (Fig. 1A and 1B, $p < 0.05$) and lower neurological deficit scores (Fig. 1C, $p < 0.05$) in the miR-34a inhibitor group than the MCAO group.

Inhibition of miR-34a upregulates BDNF expression.

237 It has been reported that miR-34a can inhibit the expression of BDNF, as assessed by
238 luciferase reporter assays [24]. In agreement with this, we found a strong interaction
239 between miR-34a and BDNF using RIP analysis (Fig. 2A). In addition, western blotting
240 revealed that BDNF expression was decreased in the MCAO group compared with the
241 sham group (Fig. 2B and 2C, $p < 0.05$). However, there was no statistically significant
242 difference in the expression of BDNF between the miR-34a negative control group and
243 the MCAO group (Fig. 2B and 2C, $p > 0.05$). However, BDNF expression was
244 upregulated following the addition of the miR-34a inhibitor compared with the MCAO
245 group (Fig. 2B and 2C, $p < 0.05$).

246 **Inhibition of miR-34a alleviates oxidative stress following MCAO.**

247 To detect the effects of miR-34a on oxidative stress following MCAO, we monitored
248 the levels of oxidative stress markers, such as ROS, GSH, GSH-Px, and GR. We found
249 that ROS levels (Fig. 3A, $p < 0.05$) were increased, but GSH (Fig. 3B, $p < 0.05$), GSH-
250 Px (Fig. 3C, $p < 0.05$), and GR (Fig. 3D, $p < 0.05$) were decreased significantly in the
251 MCAO group compared with the sham group. However, there were no significant
252 differences in ROS, GSH, GSH-Px, or GR levels between the miR-34a negative control
253 group and MCAO group (Fig. 3A-3D, $p > 0.05$). However, the miR-34a inhibitor group
254 displayed decreased ROS (Fig. 3A, $p < 0.05$) and increased GSH (Fig. 3B, $p < 0.05$),

GSH-Px (Fig. 3C, $p < 0.05$), and GR (Fig. 3D, $p < 0.05$) compared with the MCAO group.

Inhibition of miR-34a reduces neuronal apoptosis following MCAO.

It is well known that oxidative stress can induce neuronal apoptosis. Therefore, we measured the extent of neuronal apoptosis by double-label immunofluorescence staining, TUNEL, NeuN, and western blot. The MCAO group showed greater neuronal apoptosis than the sham group (Fig. 4A and 4B, $p < 0.05$). While there was no significant difference in neuronal apoptosis between the miR-34a negative control group and MCAO group, decreased neuronal apoptosis was clearly seen in the miR-34a inhibitor group compared with the MCAO group (Fig. 4A and 4B, $p < 0.05$). In addition, cleaved-caspase-3 and Bax expressions were increased, whereas Bcl-2 expression was decreased in the MCAO group compared with the sham group (Fig. 4C and 4D, $p < 0.05$). The addition of the miR-34a inhibitor reversed this trend (Fig. 4C and 4D, $p <0.05$).

Discussion

It has been reported that after MCAO intervention, the level of ROS increases significantly, which activates different signaling pathways to generate oxidative stress

and cause neuronal apoptosis [25, 26]. Furthermore, BDNF regulates the metabolism of free radicals and increases the content of SOD and GSH-Px in neurons, thus reducing the accumulation of free radicals [27], and protecting neurons after cerebral ischemia [28]. In our present study, we have shown high expression levels of miR-34a and low expression of BDNF 48 h after MCAO, which was paralleled by clear oxidative stress and neuronal apoptosis. However, the inhibition of miR-34a could increase the expression of BDNF and suppress oxidative stress and neuronal apoptosis in MCAO rats, resulting in reduced ischemic infarction and neurological deficits. These results suggested that inhibition of miR-34a expression could ameliorate the injury caused by I/R through the inhibition of BDNF expression. Therefore, miR-34a may be a novel and promising target for the suppression of injuries associated with I/R.

During the ischemic phase of a stroke, interruption of blood flow causes a severe reduction in oxygen and nutrients at the ischemic area. This causes abnormal oxidative phosphorylation and the accumulation of metabolites, which cause an imbalance between oxidative stress and antioxidant mechanisms [29]. At reperfusion however, sudden blood flow is restored causing an acute increase in ROS to the ischemic area, representing the most important trigger for oxidative stress [5]. Next, anabatic oxidative stress induces neuronal injuries, including apoptosis [30]. In this study, we found that increased ROS but decreased GSH, GSH-Px, and GR were accompanied by obvious

neuronal apoptosis 48 h after MCAO. Oxidative stress and oxidative stress-induced neuronal apoptosis are closely related to the pathophysiology of strokes [31, 32], and many studies suggest that miRNAs have a regulatory role in oxidative stress. For example, miR-23a-3p can increase the production of manganese SOD and decrease the production of peroxidative nitric oxide and 3-nitrotyrosine in MCAO mice and H_2O_2-treated neuro-2a cells, resulting in the downregulation of cleaved caspase-3 [33]. In addition, the inhibition of miR-106b-5p downregulates the malondialdehyde content and Bax expression but upregulates Bcl-2 expression and SOD activity in MCAO rats and glutamate-treated PC12 cells, thereby inhibiting oxidative damage and neuronal apoptosis [34]. In this study, we found that oxidative stress and neuronal apoptosis were positively related to high levels of miR-34a expression in the MCAO rats. Moreover, oxidative stress, neuronal apoptosis, ischemic infarction, and neurological deficits were dramatically decreased in MCAO rats when an miR-34a inhibitor was used. These data indicated that miR-34a was involved in oxidative stress and neuronal apoptosis, which is consistent with previous studies [11, 35, 36].

As early as 1993, Mattson et al. found that neurotrophic factors contribute to calcium homeostasis and the suppression of ROS production [37]. Since then, research has confirmed a role for neurotrophic factors in oxidative stress and oxidative stress-associated cell injuries, such as apoptosis. Here, we found that the expression of the

neurotrophic factor BDNF was negatively correlated with oxidative stress and neuronal apoptosis in the MCAO rats. Furthermore, previous studies have found a potential protective mechanism for BDNF against mitochondrial dysfunction-related neurodegenerative disorders [38]. Therefore, enhancing the secretion of BDNF following I/R may represent an effective strategy for blocking the progression of strokes in experimental models. Previous studies, for example, found anti-oxidative, anti-apoptotic, and anti-inflammatory effects of bone marrow mononuclear cells by increasing BDNF expression in the MCAO rats [39]. Similar to this, in our study, miR-34a induced the upregulation of BDNF and demonstrated a neuroprotective effect by inhibiting oxidative stress and neuronal apoptosis, which ameliorated the ischemic infarction and neurological deficits. Moreover, luciferase reporter assays detected BDNF as a target of miR-34a, which is consistent with our results from RIP assays [24]. Our findings revealed that upregulated miR-34a expression following I/R may inhibit BDNF expression, resulting in oxidative stress and neuronal apoptosis. Thus, the inhibition of miR-34a can upregulate BDNF expression and might be able to suppress I/R injury.

Conclusions

In summary, our study demonstrated that inhibition of miR-34a blocked I/R induced

injury by promoting the expression of BDNF, which may prove to be a potential and promising new therapeutic target for the treatment of ischemic stroke. However, other biomarkers involved in this neuronal injury that are associated with the miR-34a/BDNF axis need to be further clarified for subsequent diagnosis and treatment.

Acknowledgments

Not applicable.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The Authors declare that there is no conflict of interest.

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Figure legends

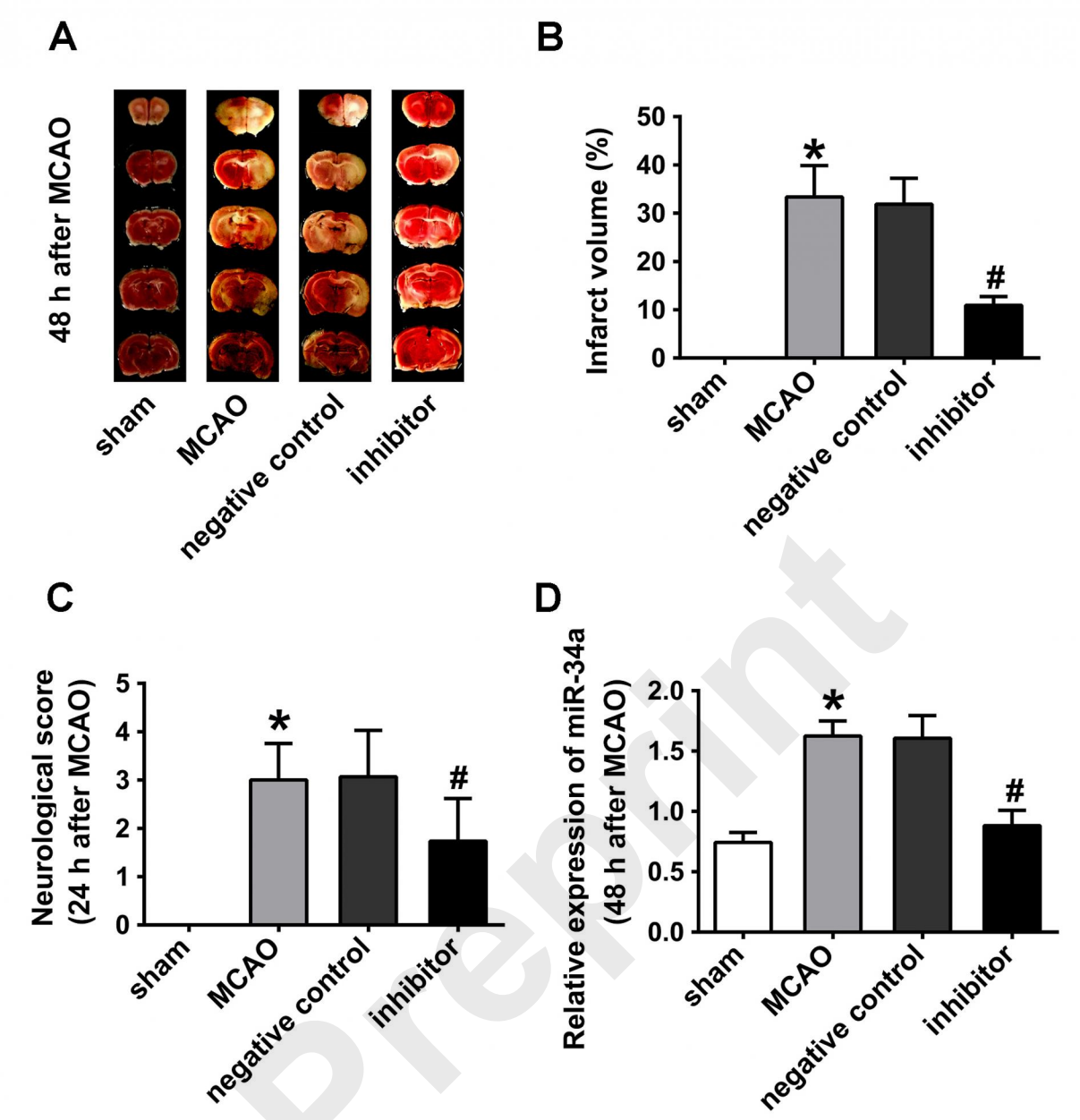
Figure 1. Inhibition of miR-34a ameliorates ischemic infarction and neurological deficits. (A) Representative images of TTC staining. **(B)** Histogram representing infarct volume (n = 5 per group). **(C)** Histogram representing neurological deficit scoring (n = 15 per group). **(D)** Relative expression of miR-34a in the various groups (n = 5 per group). *$p < 0.05$ compared with the sham group. #$p < 0.05$ compared with the MCAO group.

Figure 2. Inhibition of miR-34a upregulates BDNF expression. (A) Histogram representing RIP analysis (n = 5 per group). **(B)** Representative images from western blots for BDNF. **(C)** Histogram representing western blots for BDNF (n = 5 per group). $^{*}p < 0.05$ compared with the sham group. $^{\#}p < 0.05$ compared with the MCAO group.

Figure 3. Inhibition of miR-34a alleviates oxidative stress following MCAO. (A) ROS concentration (arbitrary units), **(B)** GSH, **(C)** GSH-Px, and **(D)** GR concentrations in the various groups (arbitrary units) (n = 5 per group). $^{*}p < 0.05$ compared with the sham group. $^{\#}p < 0.05$ compared with the MCAO group.

Figure 4. Inhibition of miR-34a reduces neuronal apoptosis following MCAO. (A) Representative images of TUNEL staining, scale bar: 50 μm. **(B)** Histogram

representing neuronal apoptosis (n = 5 per group). **(C)** Representative images from western blots for cleaved-caspase-3, Bax, and Bcl-2. **(D)** Histogram representing western blots for cleaved-caspase-3, Bax, and Bcl-2 (n = 5 per group). $^{*}p < 0.05$ compared with the sham group. $^{\#}p < 0.05$ compared with the MCAO group.



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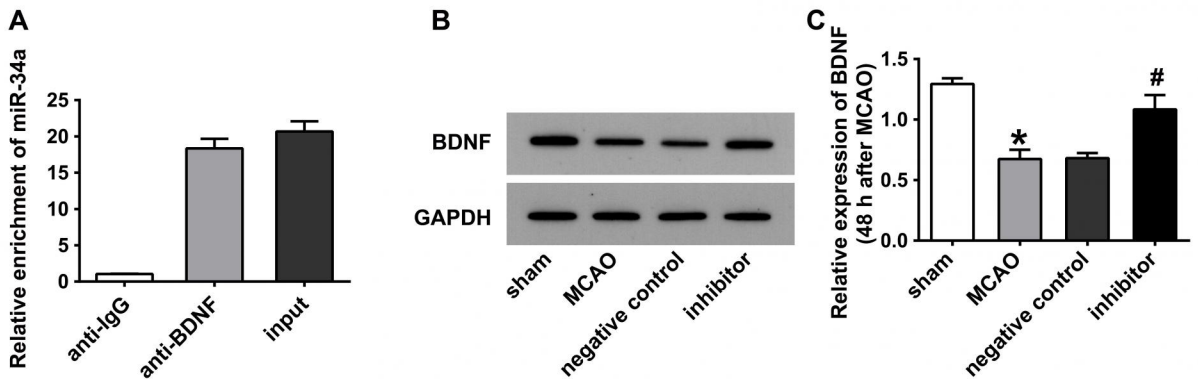

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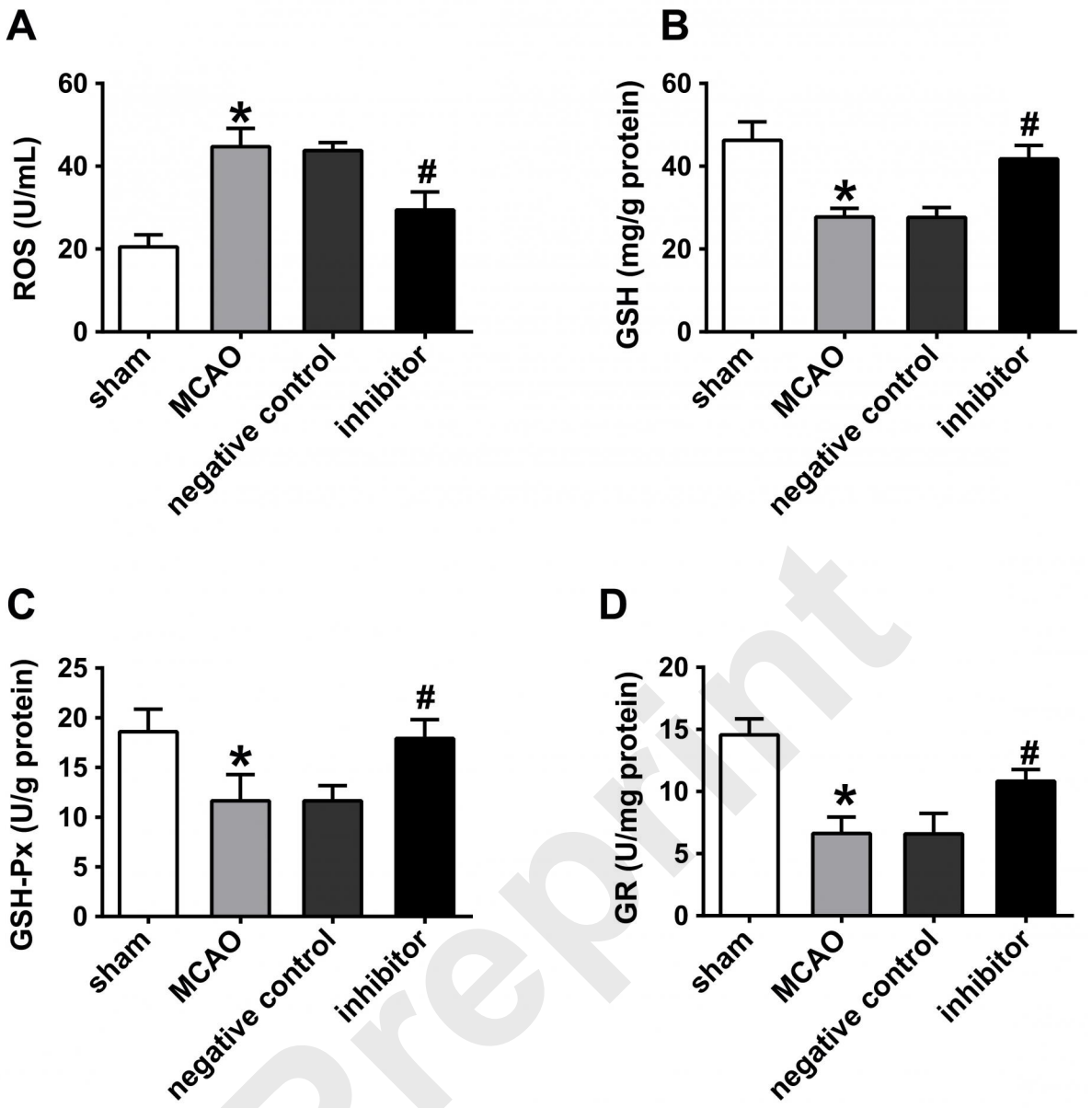

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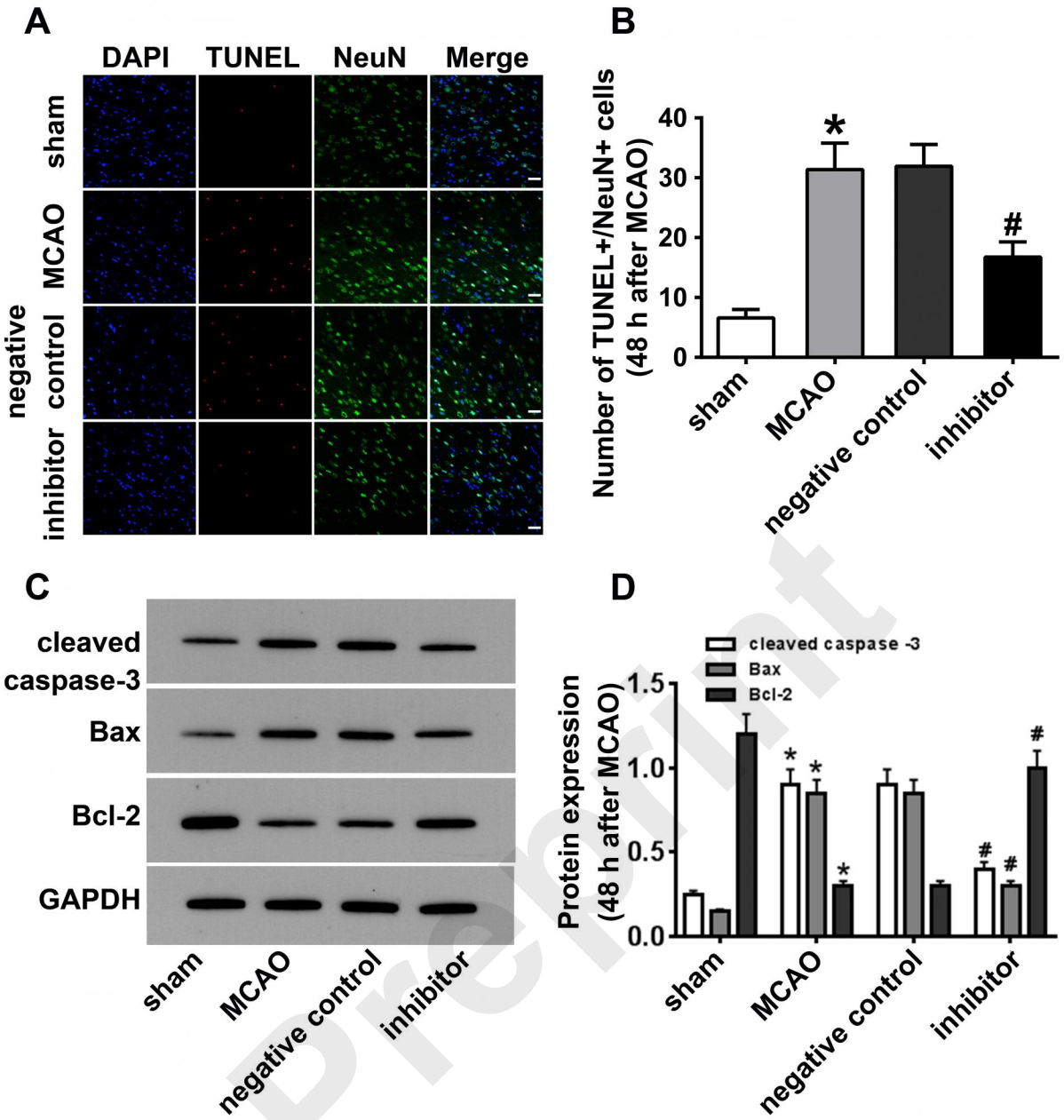

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