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Interaction between miRNA-155 targeting neuronal pacemaker ion channels and release of amino acid transmitters during cerebral ischemia

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Abstract. – OBJECTIVE: Cerebral hemorrhage can cause hemorrhagic stroke, leading to severe brain damage. MiRNA-155 is closely related to the development of stroke. However, the regulatory mechanism of miRNA-155 during cerebral ischemia has not yet been elucidated.

MATERIALS AND METHODS: SD rats were separated to sham operation group, model group, miRNA-155 inhibitor group and miRNA-155 agonist group followed by analysis of neural function, miRNA-155 and pacemaker ion channel hyperpolarization-activated, cyclic nucleotide-gated (HCN) expression by Real Time PCR. Meanwhile, Caspase 3 activity, glutamic acid (Glu) and γ-aminobutyric acid (GABA) content were also measured along with analysis of IL-6 and IL-1β secretion by ELISA as well as reactive oxygen species (ROS) content and superoxide dismutase (SOD) activity.

RESULTS: Compared to sham operation group, model group presented significantly elevated miRNA-155 level and Longa score, decreased HCN expression, elevated Caspase 3 activity and Glu content, and reduced GABA and SOD activity. Meanwhile, model group also had elevated IL-6 and IL-1β secretion, and ROS content ($p<0.05$). The miRNA-155 agonist group further exacerbated these changes ($p<0.01$). The miRNA-155 inhibitor group could inhibit miRNA-155 expression and significantly reverse the above pathological changes ($p<0.05$).

CONCLUSIONS: miRNA-155 is increased in cerebral ischemia. Regulation of miRNA-155 expression can target neuronal pacing ion channel HCN channel to regulate the release of amino acid transmitters, thereby alleviating the progress of cerebral ischemia.

Key Words:
Cerebral ischemia, MiRNA-155, Nerve cells, Pacemaker ion channels, Amino acid transmitters.

Introduction

Cerebral ischemia can cause ischemic stroke and bleeding, which is an acute cerebrovascular disease, characterized as accidental sudden rupture of cerebral blood vessels and hemorrhage in the brain parenchyma, leading to increased intracranial pressure and subsequent severe brain damage[1,2]. Cerebral ischemia accounts for more than 20% of stroke. It is more common in young and middle-aged people. The gender difference is not obvious. With the change of lifestyle and pace of life, the incidence is increasing year by year, and the age of onset is younger[3,4]. There are several causes of cerebral ischemia. Metabolic diseases including hyperlipidemia, diabetes mellitus, and cardiovascular diseases including hypertension and aging of blood vessels can cause cerebrovascular diseases. Smoking, drinking, and emotional excitement can also cause cerebral hemorrhage[5,6]. The acute mortality of cerebral ischemia is high and the disability rate of surviving patients with cerebral ischemia is high, which is often accompanied by sequelae such as dyskinesia, cognitive impairment, and speech dysphagia[7,8]. The mechanism of cerebral ischemia is mainly due to brain parenchymal

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damage. Metabolites produced after hematoma dissolution have strong cytotoxicity, which triggers oxidative stress, leading to inflammatory response and subsequent nerve cell excitement, pacing ion channel damage, and amino acid transmitter release which can worsen cerebral hemorrhage-based diseases[9,10].

MicroRNAs (miRNAs) can regulate the expression of several target genes[11,12]. More and more evidence indicates that microRNAs are involved in cell proliferation, differentiation, apoptosis, and cell migration[13,14]; In addition, miRNAs participate in several diseases, such as cardiovascular and cerebrovascular diseases[15]. miRNA-155 is a product of B-cell gene integration clusters and involves in inflammation, tumors, and cardiovascular diseases, and can participate in the occurrence and development of ischemic brain diseases[16-18]. However, the expression profile of miRNA-155 in cerebral hemorrhage and whether it can target nerve cell pacing ion channels and regulate the release of amino acid transmitters have not been reported. Our study aims to investigate miRNA-155's role in cerebral hemorrhage.

Materials and Methods

Experimental Animals

Forty female SD rats, 2 months of age, SPF grade, body weight (250 ± 20) g, were bought from our animal center and fed in SPF conditions including maintaining the temperature at (21 ± 1)°C and relative humidity (50-70%) under constant temperature and humidity conditions, with a guarantee of 12/12 h day/night cycle. Animal experiments were conducted by experienced and skilled technicians to minimize animal suffering. This study was approved by the Ethical Committee of Qingdao Hospital of Traditional Chinese Medicine.

Reagents and Instruments

Sodium pentobarbital was purchased from Shanghai Zhaohui Pharmaceutical Co., Ltd (Shanghai, China). The miRNA-155 inhibitor group and miRNA-155 agonist were designed and synthesized by Shanghai Gima Gene Co., Ltd (Shanghai, China). The Caspase 3 activity detection kit was from Cell Signaling Technology (Danvers, MA, USA). RNA extraction and reverse transcription kit were from American ABI (Waltham, MA, USA). The IL-6 and IL-1β ELISA kits, as well as glutamic acid (Glu) and γ-aminobutyric acid (GABA) content detection kits, were purchased from R&D (Minneapolis, MN, USA).

Grouping and Processing of Experimental Animals

SD rats were randomly and equally divided into sham operation group; a model group of cerebral ischemia; a miRNA-155 inhibitor group and a miRNA-155 agonist group, in which miRNA-155 inhibitors or agonist were injected intraventricularly on the basis of the model group.

Preparation and Management of Rat Cerebral Ischemia Model

The rat cerebral ischemia model was established according to the literature[19]. After intraperitoneal anesthesia with 30 mg/kg sodium pentobarbital, the rat stereotactic apparatus was used to fix the rats in the prone position and the anterior and posterior condyles were adjusted at the same horizontal position. The head was skinned and sterilized with alcohol, and a 1.5 cm incision was made longitudinally in the middle of the skull. The surrounding soft tissue was removed, the skull was exposed, and the right middle cerebral artery of the rat was modified with a modified thread embolization method to create a cerebral ischemia model. An incision in the middle of the rat's neck was made, which was separated layer by layer to expose carotid artery and ligate the external carotid artery to make a V-shaped incision. After the plug was coated with polylysine, it was gently inserted. When the middle arterial artery was 1.8 cm away from the common carotid bifurcation and there was a sense of resistance, the plug was stopped, and the thread was ligated and fixed plug to the common carotid artery. In the sham operation group, only the blood vessels were separated, the artery was not ligated without insertion of nylon thread plug. 100 pmol/L miRNA-155 inhibitor or agonist was injected into the lateral ventricle of rats.

Rat Neurological Scores in Each Group

After treatment of each group of rats, the rats had to be fully awake before scoring the nerve function, referring to the Longa score method. The scoring criteria were as follows: 0 indicated normal function of the limbs; 1 indicated the rat's tail lifted, the limb was paralyzed, and one side of the forelimb couldn't be straightened; 2 indicated the paralysis of the limb of the body

while the rat was walking; 3 indicated that when the rat was standing, it will fall to the paralyzed side; 4 indicated that rat cannot stand and walk at all, accompanied by a disturbance of consciousness.

Caspase 3 Activity Test

The changes in Caspase3 activity in each group of cells were examined according to the kit instructions. Trypsin digested cells were centrifuged at 600 g at 4°C for 5 min and cell lysate was lysed followed by addition of 2 mM Ac-DEVD-pNA to detect the OD value change at 405 nm to calculate Caspase3 activity changes.

Real-Time PCR

TRIzol reagent was used to extract mRNA from spinal cord tissue and oligodendrocytes followed by DNA reverse transcription synthesis. The primers were designed by Primer Premier 6.0 based on each gene sequence and synthesized by Shanghai Yingjun Biotechnology Co., Ltd. (Table I). Real-time PCR was conducted with reaction conditions: 92°C 30 s, 58°C 45 s, 72°C 35 s, and a total of 35 cycles were performed. Data was collected using the PCR reactor software and GAPDH was used as a reference. According to the fluorescence quantification, the starting cycle number (CT) of all samples and standards was calculated. Based on the standard CT value, a standard curve was drawn, and then the semi-quantitative analysis was carried out using $2^{-\Delta Ct}$ method.

ELISA

The ELISA kit was used to analyze IL-6 and IL-1β secretion. According to the instructions, a standard curve was prepared. Each sample had 3 duplicate wells. Sample was added to 96-well plate and then enzyme labeling reagent was added followed by addition of developer A and subsequent addition of developer B. After that, the reaction was stopped by adding of stop solution. The blank value was set to zero. The absorbance value (OD) of each well was measured with a microplate reader at 450 nm. The linear regression equation of the standard curve was calculated based on the concentration of the standard and the corresponding OD value, and then calculate the corresponding sample concentration on the regression equation based on the OD value of the sample.

Detection of the Neurotransmitters Glutamic Acid (Glu) and γ-Aminobutyric Acid (GABA)

HI-TACHI83550 amino acid automatic analyzer and Glu and GABA determination kit were used to determine the content of Glu and GABA.

Detection of ROS Content in Serum

Changes in ROS levels in the serum of each group were detected. The treated cells were subjected to a 95°C water bath, and after 40 minutes, they were taken out, washed with cold water and incubated with 2',7'-dichlorofluorescein diacetate (DCF-DA) for 15 min at 37°C followed by centrifugation and resuspending the pellet in sterile PBS phosphate buffer. After incubation at 37°C for 60 min, ROS production was measured by a spectrophotometer and the percentage of ROS generation was compared and analyzed.

SOD Activity Detection

The changes of SOD activity in the serum of each group of rats were detected following the kit instructions for specific tests. Tissue protein was extracted and placed in a 95°C water bath. After 40 minutes, it was removed and rinsed with cold water. After cooling and subsequent centrifugation at 4000 rpm for 10 minutes, the ethanol-chloroform mixture (5: 3, v / v volume ratio 5: 3) was used to extract the ethanol phase in the tissue homogenate for detection of total SOD activity.

Statistical Analysis

SPSS 19.0 software (IBM Armonk, NY, USA) was adopted for processing data which were displayed as mean ± standard deviation (SD) and assessed by single factor analysis of variance. $p<0.05$ was considered as statistically significant.

Table I. Primer sequences.

Gene	Forward 5'-3'	Reverse 5'-3'
GAPDH	ACCAGGTATCTTGGTTG	TAACCATGTCAGCGTGGT
MiR-155	TCTCCCAGACTCACAGT	GCCGGCTGGTCATTAATATT
HCN	ACGCTCCCGTGGTCT	ACCTGCCCATGTGCTGTCA

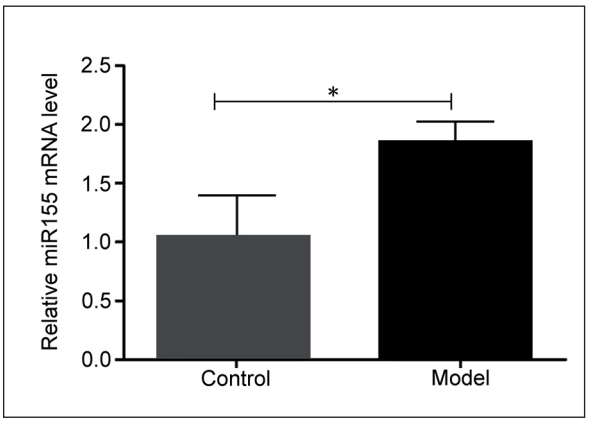

Figure 1. Analysis of miRNA-155 expression in cerebral ischemia. Compared with sham operation group, *p<0.05.

Results

MiRNA-155 Expression in Cerebral Ischemic Brain Tissue

miRNA-155 expression in cerebral ischemic brain tissue was significantly increased compared to sham operation (p<0.05) (Figure 1).

Effect of Regulating MiRNA-155 on MiRNA-155 Expression in Cerebral Ischemia

Transfection of miRNA-155 agonist in cerebral ischemia rat model significantly increased miRNA-155 expression (p<0.05); transfection of miRNA-155 inhibitor significantly reduced miRNA-155 expression (p<0.05) (Figure 2).

Effect of Regulating MiRNA-155 on Longa Score

miRNA-155 level was elevated in cerebral ischemia and the Longa score was significantly increased (p<0.05). The miRNA-155 agonist group further aggravated the above changes, and the Longa score was further significantly increased (p<0.01); the miRNA-155 inhibitor group could inhibit miRNA-155 expression and significantly decrease Longa score (p<0.05) (Figure 3).

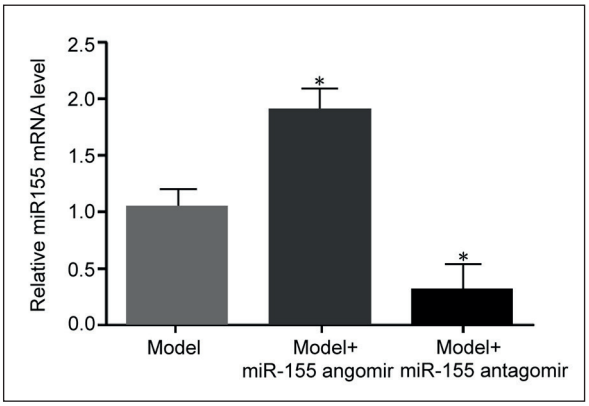

Figure 2. Regulation of miRNA-155 on miRNA-155 expression in cerebral ischemic brain tissue. Compared with the model group, *p<0.05.

Effect of MiRNA-155 on the Expression of HCN Channels

The expression of miRNA-155 was increased and the expression of HCN channels was significantly decreased in cerebral ischemia (p<0.05). The miRNA-155 agonist further aggravated the above changes and the expression of HCN channels was further significantly reduced (p<0.01); the miRNA-155 inhibitor could inhibit miRNA-155 expression and significantly promote HCN channel expression (p<0.05) (Figure 4).

Effect of MiRNA-155 on Caspase 3 Activity

miRNA-155 was increased and the activity of Caspase 3 was significantly increased in cerebral ischemia (p<0.05). miRNA-155 agonist further aggravated the above changes and Caspase 3 activity was significantly increased (p<0.01); miRNA-155 inhibitor could significantly inhibit miRNA-155 expression and Caspase 3 activity (p<0.05) (Figure 5).

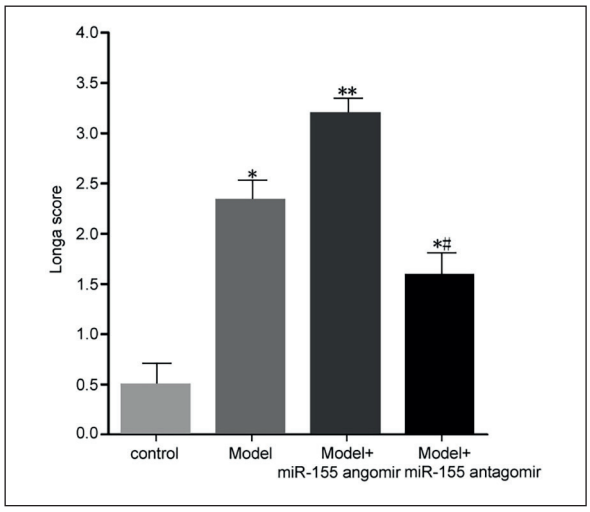

Figure 3. Effect of regulating miRNA-155 on Longa score in rats with cerebral ischemia. Compared with the sham operation group, *p<0.05, **p<0.05; compared with the model group, $^{\#}p$<0.05.

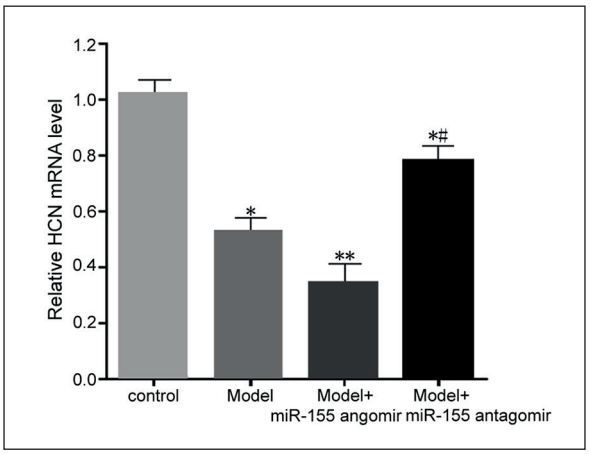

Figure 4. Regulation of miRNA-155 on HCN channel expression in brain tissue of rats with cerebral ischemia. Compared with the sham operation group, *p<0.05, **p<0.05; compared with the model group, $^{\#}p$<0.05.

Effect of MiRNA-155 on Release of Amino Acid Transmitters in Brain Tissue of Rats with Cerebral Ischemia

In cerebral ischemia, miRNA-155 expression was increased, Glu content was significantly increased, and GABA was significantly decreased (p<0.05); miRNA-155 agonist further aggravated the above changes and further significantly increased Glu and decreased GABA (p<0.01); miRNA-155 inhibitor could inhibit the expression of miRNA-155, significantly decrease Glu content and increase GABA (p<0.05) (Figure 6).

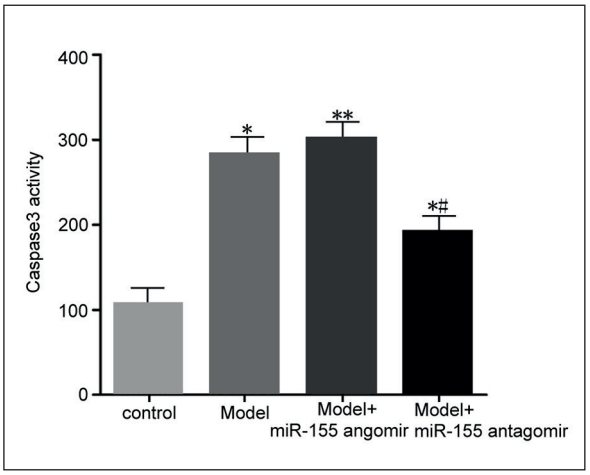

Figure 5. Effect of regulating miRNA-155 on the activity of Caspase 3 in the brain tissue of rats with cerebral ischemia. Compared with the sham operation group, *p<0.05, **p<0.05; compared with the model group, $^{\#}p$<0.05.

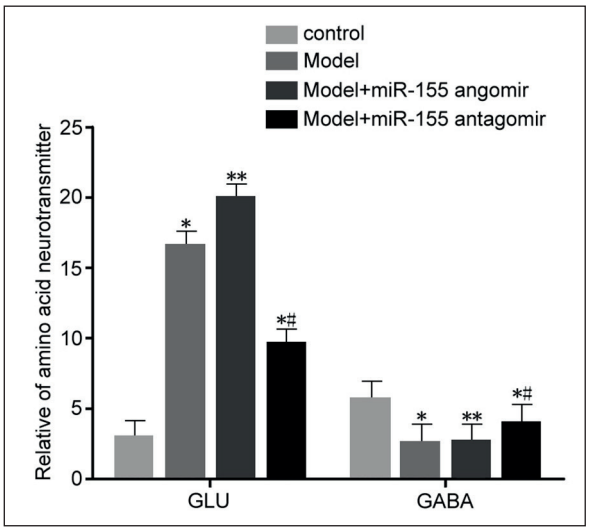

Figure 6. Effect of regulating miRNA-155 on the release of amino acid transmitters in the brain tissue of rats with cerebral ischemia. Compared with the sham operation group, *p<0.05, **p<0.05; compared with the model group, $^{\#}p$<0.05.

Effect of MiRNA-155 on the Secretion of Inflammatory Factors

The expression of miRNA-155 was increased and IL-6 and IL-1β secretion was significantly increased in cerebral ischemia (p<0.05); miRNA-155 agonist further aggravated the above changes and further significantly promoted the increase of IL-6 and IL-1β (p<0.01); miRNA-155 inhibitor can inhibit miRNA-155 expression and significantly inhibit IL-6 and IL-1β secretion (p<0.05) (Figure 7).

Effect of MiRNA-155 on Oxidative Stress in Brain Tissue of Rats with Cerebral Ischemia

The content of ROS was significantly increased, and the activity of SOD was significantly decreased in cerebral ischemia brain tissue (p<0.05). miRNA-155 agonist further aggravated the above changes and further significantly promoted the increase in ROS content and decrease in the SOD activity (p<0.01). miRNA-155 inhibitor can inhibit miRNA-155 expression, significantly reduce ROS content and increase SOD activity (p<0.05) (Figure 8).

Discussion

Cerebral ischemia ranks first in cerebrovascular diseases, mainly in middle-aged and elderly

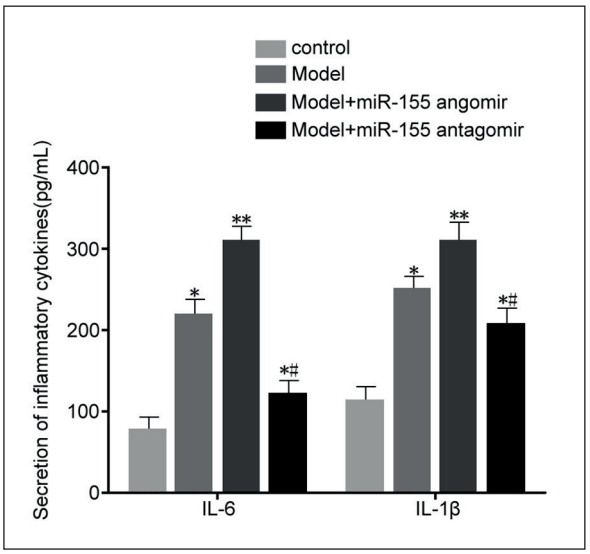

Figure 7. Effect of regulating miRNA-155 on the secretion of inflammatory factors in the brain tissue of rats with cerebral ischemia. Compared with the sham operation group, $*p<0.05$, $**p<0.05$; compared with the model group, $^{\#}p<0.05$.

people. Ischemia and hypoxia lead to the loss of nutritional supply to the brain, resulting in central nervous disease with focal brain tissue damage[20]. MiRNA-155 plays a role in inflammation, autoimmune diseases, and cardiovascular diseases. Increasing expression of miRNA-155 can promote inflammatory response, while inhibiting miRNA-155 can suppress inflammatory response. It can increase endothelial NOS and reduce vascular inflammatory response by inhibiting the inflammatory response[17,18]. This study confirmed that miRNA-155 expression increases during cerebral ischemia, Caspase 3 activity increases, and inflammatory factor secretion is promoted; miRNA-155 agonist further exacerbates the above changes, and miRNA-155 inhibitor can inhibit miRNA-155 expression, Caspase 3 activity and inflammatory factor secretion, suggesting that it is involved in the progression of cerebral ischemic inflammation.

The central neurotransmitters in the brain include amino acid and monoamine neurotransmitters. The content and proportion of neurotransmitters are involved in the maintenance of central nervous system function, especially the release of amino acid transmitters has a more important role[21,22]. Amino acid transmitters include excitatory amino acids and inhibitory amino acids. Glutamic acid (Glu) is an excitatory amino acid and is generally used as an excitatory transmitter to maintain normal physiological activities of cells. However, if the concentration is significantly increased, it can lead to neurotoxicity[23]. Inhibitory amino acids are mainly GABA, which inhibits the role of neurotransmitters and reduces excitatory amino acids, thereby protecting neurons from ischemic animal models from toxic effects[19]. In the process of neurons transmitting information, special channels are required to allow potassium ions to pass, otherwise it will cause epilepsy, depression and other diseases. Among them, the newly discovered important potassium channel (HCN channel) is located in the cell membrane where a filtering pores region can be formed, which selectively allow potassium ions to pass through. This process is regulated by the signal molecule cAMP. The signal-driven potassium channels are

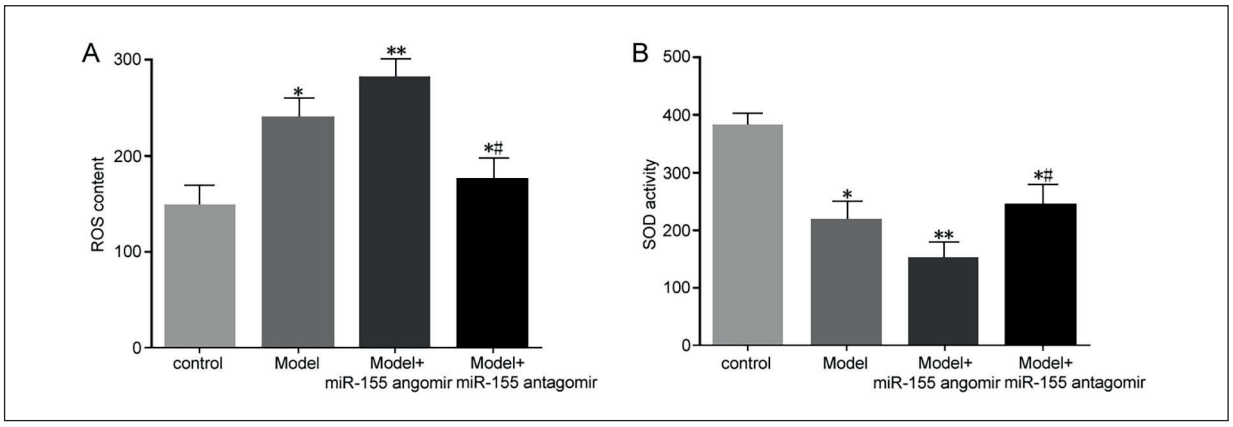

Figure 8. Effect of regulating miRNA-155 on oxidative stress in brain tissue of rats with cerebral ischemia. **A,** ROS content changes. **B,** SOD activity analysis, compared with sham operation group, $*p<0.05$, $**p<0.05$; compared with model group, $^{\#}p<0.05$.

also known as "pacer channels." They not only participate in the formation of heart rhythms, but also involve in the rhythmic excitement of nerve cells[24]. Increased ROS content and decreased SOD activity during oxidative stress causes lipid peroxidation, abnormal protein expression and oxidization of DNA, resulting in increased nerve damage[25]. This study confirms that miRNA-155 expression in cerebral ischemic brain tissue increases, inhibits HCN channel expression, increases ROS content, decreases SOD activity, increases Glu content, and decreases GABA content; and transfection miRNA-155 agonists into rats with cerebral ischemia further aggravated the above changes, suggesting that miRNA-155 targets nerve cell pacing ion channels to participate in regulating the release of amino acid transmitters, promoting cell apoptosis and increasing neurological scores, thereby aggravating cerebral ischemic injury. The addition of miRNA-155 inhibitors in cerebral ischemic rats can upregulate HCN channels, regulate oxidative stress, and inhibit inflammation, and thus reducing Glu content, while increasing GABA content, inhibiting apoptotic activity, thereby improving the neural function score and alleviating the progress of cerebral hemorrhage. Our study is consistent with a previous study[26] showing a role of microRNA-155 in modifying neuroinflammation and γ-aminobutyric acid transporters in specific central regions after post-ischaemic seizures. However, the main novelty of our study is the findings of elevated Caspase 3 activity and reduced SOD activity in the model group, which are further promoted by miRNA-155 agonist and inhibited by miRNA-155 inhibitor. All these were not studied in the previous study[26].

Conclusions

The expression of miRNA-155 is increased in cerebral ischemia. Regulation of miRNA-155 expression can target neural cell pacing ion channels HCN channels, regulate the release of amino acid transmitters, inhibit inflammation and oxidative stress responses, thereby alleviating the progression of cerebral ischemia. Our study for the first time demonstrates the critical role of miRNA-155 in the pathogenesis of cerebral ischemia via targeting HCN channels, indicating that it might be a novel therapeutic target for the treatment of cerebral ischemia, which is the novelty of our study.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding

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