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Protective effects of MiR-146b in cerebral infarction via targeting SIRT1/FOXO1 signaling pathway

Ren Yang[1], Chen Zeng[2*]

[1] Department of Neurosurgery, The Affiliated Hospital of Guizhou Medical University, Guizhou, China
[2] Center for Drug Clinical Trials, The Affiliated Hospital of Guizhou Medical University, Guizhou, China

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ABSTRACT

To observe the therapeutic effect of micro ribonucleic acid (miR)-146b on brain tissue injury in rats with cerebral infarction (CI) by regulating the Sirtuin 1 (SIRT1)/forkhead box protein O1 (FOXO1) signaling pathway, a rat model of CI was established. Lentiviral cells were used to transfect and silence or overexpress miR-146b. The rats were divided into the miR-146b inhibitor group (Inhibitors), miR-146b mimic group (Mimics) and normal group (Control). Then quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the transfection rate of miR-146b in rat brain tissues in each group. The improved method was adopted to score the nerves of rats, and the infarction volume of rats in each group was determined. Subsequently, the levels of superoxide dismutase (SOD) and reactive oxygen species (ROS) in the brain tissues in each group were measured *via* enzyme-linked immunosorbent assay (ELISA), the apoptosis of nerve cells in the brain tissues was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), and glial fibrillary acidic protein (GFAP), S100β gene and SIRT1/FOXO1 pathway-related genes and proteins in the brain tissues were determined through qRT-PCR and Western blotting. MiR-146b exhibited a high expression in Mimics and an extremely low expression in Inhibitors. Rats in Mimics were normal in movement, and their neurological scores were close to those in Control. Rats in Inhibitors could walk normally, and their neurological scores were notably higher than those in other groups ($P<0.05$). In addition, Inhibitors had a remarkably larger CI volume ($P<0.05$), a remarkably increased ROS level and a significantly reduced SOD level compared with those in other groups. Moreover, TUNEL staining results manifested that apoptotic cells, especially glial cells, were notably increased in Inhibitors compared with those in Mimics. Besides, the messenger RNA (mRNA) expression levels of S100β and GFAP in Inhibitors were higher than those in other groups ($P<0.05$). SIRT1 and FOXO1 genes were increased in Mimics, which were close to those in Control. According to Western blotting results, the protein expression levels of SIRT1 and FOXO1 in Mimics were notably higher than those in Inhibitors. MiR-146b can play a protective role in CI rats by activating the SIRT1/FOXO1 signaling pathway, lowering the oxidative stress level and reducing brain tissue apoptosis.

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Introduction

Acute cerebral infarction (ACI) is a common clinical cardiovascular and cerebrovascular disease in the nervous system (1). An epidemiological investigation reveals that the incidence rate of CI in China shows an uptrend. CI has attracted extensive attention due to its high disability and mortality rates and is becoming one of the major challenges to public health (2). CI is an ischemic disease caused by transient or permanent reduction in the cerebral blood flow in the main cerebral artery range (3). It can lead to irreversible degeneration and necrosis of brain neurons, thereby resulting in a train of neurological dysfunctions. Therefore, it is of great significance to find effective means to promote local angiogenesis in the CI area at an early stage and to resume tissue perfusion for the prognosis of patients with CI (4). Plasminogen activators in the thrombolytic recombinant tissues are currently regarded as the most effective drugs for clinical treatment of ACI, but they have more side effects, so there is still a need to find an effective treatment method (5). The pathological process and mechanism of ACI are fairly complex and remain unclear. A recent study has revealed that the blood-brain barrier and neurovascular rupture induced by oxidative stress are the primary mechanisms leading to hemorrhagic CI (6). Injury and inflammation caused by free radicals, the apoptosis of nerve cells and the metabolic abnormalities in brain tissues are all involved in the occurrence and development of ACI, but there is still no effective treatment scheme (7,8). Angiogenesis can attenuate the perfusion in the CI area so as to stimulate the regeneration of the nervous centralis, thus laying a foundation for the recovery of cranial nerve cells (9). However, the body's response still cannot improve the blood supply reconstruction and cerebral tissue perfusion in the CI area when CI occurs. Oxidative stress is a vital mechanism of ACI injury, and reactive oxygen species (ROS) is a pivotal component of tissue damage after ischemic injury (10). Nevertheless, although a lot of time and energy has been spent, successful intervention measures in the treatment of CI-related neurological diseases are still not sufficient. Hence, there is an urgent need for novel treatment methods

* Corresponding author. Email: 18786703085@163.com

to provide a basis for the treatment and prevention of CI and its complications.

According to a study, the functions of microribonucleic acids (miRNAs) in the pathogenesis of different diseases have been fully investigated, and miRNAs are proven to exert effects on physiological diseases and various other diseases (11). MiRNAs are non-coding RNAs that participate in the specific regulation of protein-coding and non-coding genes. Increasingly more studies have proved that miRNAs may regulate 1/3 of human genes (12). In addition, miRNAs participate in adjusting many processes such as cell cycle, metabolism and various immune responses (13). They have become crucial gene expression regulators in numerous diseases, and their regulatory networks have attracted much attention in recent years (14). A study has manifested that miR-146 is down-regulated at the early stage of liver ischemia/reperfusion (I/R). The increases in IRAK1 and TRAF6 lead to the decreased level of miR-146 at 24 h after reperfusion, and the expressions of IRAK1 and TRAF6 reach the peak at 6 h (15). MiR-146 has two copies, namely, miR-146a and miR-146b. They have very similar sequences, and their targets are the same transcript (16). However, the underlying effect of miR-146b on CI in rats remains obscure and needs to be further studied.

Sirtuin 1 (SIRT1) and forkhead box protein O1 (FOXO1) interact with diversified target genes and become a hot topic in research on many brain tissue-related diseases. The protection mechanism of SIRT1 has close correlations with various substrates. FOXO1 is a target of SIRT1 and plays a vital role in intracellular homeostasis. It displays a high expression in the hippocampus and exerts a crucial effect on the regulation of hippocampal neuronal homeostasis (17). Besides, the SIRT1/FOXO1 signaling pathway exerts indispensable antioxidant and anti-apoptotic effects on I/R diseases (18). A recent study has indicated that SIRT1 is important in DNA damage repair, apoptosis inhibition and oxidative stress. FOXO1 may enhance cell resistance to oxidative stress via SIRT1 deacetylation under ischemia or hypoxia conditions, which is mainly completed by Mn-superoxide dismutase (SOD) capable of scavenging ROS (19). Brunet found that SIRT1 deacetylation strengthens FOXO1's ability to induce cell cycle arrest and antioxidant stress, but suppresses its ability to induce cell death (20). However, the specific action mechanisms of the SIRT1/FOXO1 signaling pathway on CI, the apoptosis of nerve cells and oxidative stress remain unclear. In this study, therefore, the effects of the SIRT1/FOXO1 signaling pathway on CI, the apoptosis of nerve cells and oxidative stress were explored using various molecular means, so as to provide an experimental and theoretical basis for the treatment and prevention of CI through the SIRT1/FOXO1 signaling pathway.

It was proposed in this study that miR-146b can exert therapeutic effects on CI in rats through the SIRT1/FOXO1 signaling pathway, and the changes in SIRT1/FOXO1 pathway-related genes and proteins in the tissues were planned to be detected through a classical rat model of CI and the neurological function test via quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western blotting, thus revealing the therapeutic effect of miR-146b on CI in rats and providing an experimental basis for further research and development of new drugs.

Materials and Methods

Animal grouping and model establishment

Specific Pathogen Free (SPF) male Sprague Dawley (SD) rats were purchased from the Shanghai Institutes for Biological Sciences (Shanghai, China). All experiments were approved by the Animal Ethics Committee of our hospital. All the rats were placed in conventional cages, had free access to food and water, and were reared adaptively for one week. A total of 20 rats were used to establish the CI model, and then they were anesthetized using loral hydrate (400 mg/kg) and placed in the dorsal position. Through the midline incision of the neck, the silicone rubber was gently pushed from the external carotid artery (ECA) to the cerebral medium-sized artery and finally to the internal carotid artery (about 17-20 mm) until slight resistance and a sharp drop in blood flow occurred, which should be tracked and observed at any time. At 2 h after the infarction of the cerebral medium-sized artery, nerve fibers were gradually drawn out. Ultimately, the wound was sutured, and the rats were placed back into a warm cage. The rats in the normal group (Control) received suture treatment after the operation. During transfection and overexpression, gene-specific primers were amplified, and the purified and recycled reaction products and vector fragments were connected under the action of T4 DNA. Then the connected products were transformed into competent cells, miR-146b was expressed, transferred to adenovirus vectors and transfected into rats. Subsequently, the rats in each group were anesthetized and killed, and the brain tissues were taken out.

Transfection of miR-146b into each group of cells

In order to further analyze the role of miR-146b in CI, miR-146b was transfected into the rats by adenoviruses, after which the transfection rate of miR-146b in CI was examined via qRT-PCR, in order to prepare for the subsequent study on the molecular action mechanism of miR-146b on CI.

Neurological scores of the rats in each group

Before execution of the rats in each group, their improved neurological functions were scored by 3-5 people and evaluated by multiple people, followed by averaging. The specific scoring details are shown in Table 1. Rats with other symptoms in clinical manifestations during the experiment were discarded, and new rats were supplemented randomly.

Determination of the CI volume

After the rats in each group selected were under deep anesthesia with pentobarbital sodium, the skin was cut along the middle part of the rat neck with a piece of surgical scissors to quickly and completely separate the brain tissues. Then the brain tissues were weighed and sliced. Each brain tissue with a thickness of about 2 mm was stained by 2, 3, 5-Triphenyte-trazoliumchloride (TCC) in a thermostatic water bath (at 37°C) away from light for 30 min. After that, the stained tissue slices were fixed in 4% paraformaldehyde. Finally, CI was observed, and the infarction volume was calculated according to the thickness and the infarction area of each slice.

Detection of apoptosis via terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

The paraffin-embedded slices were subjected to apoptosis detection using an apoptosis detection kit purchased from Roche (Basel, Switzerland). The specific steps are as follows: The paraffin-embedded slices were deparaffinized, washed with phosphate buffer saline (PBS), added with protease K working solution and immersed in the sealing solution, followed by fixation and rinsing. Thereafter, 0.1% Triton X-100 was employed for permeation, and TUNEL detection kits were used for FITC terminal labeling on the DNA of the apoptotic segments. Ultimately, the images of FITC-labeled TUNEL-positive cells were observed under a fluorescence microscope, and 10 fields of view were taken to count TUNEL-positive cells.

Measurement of the levels of the antioxidant indexes SOD and ROS in the brain tissue by enzyme-linked immunosorbent assay (ELISA)

After the rats were anesthetized and killed, their brain tissues were harvested and washed with normal saline. Then 0.5 g brain tissues were taken, smashed with a homogenizer containing prepared tissue lysate and centrifuged at 1200×g. After that, the supernatant was taken, and changes in SOD and ROS levels were detected. At last, the absorbance of each index in the rats was determined using a microplate reader, and the standard curve was plotted to analyze the changes in the index levels according to the instructions.

Detection of the expression levels of related genes via RT-PCR

(1) Brain tissue homogenate of the rats in each group was taken, and the total RNA was extracted using the TRIzol kit (Invitrogen, Carlsbad, CA, USA). The concentration, purity and integrity of RNAs were examined via UV spectrophotometry and agarose gel electrophoresis. After the RNA quality was ensured, mRNAs were reversely transcribed into complementary deoxyribonucleic acids (cDNAs) and stored in a refrigerator at -80°C. (2) Primer amplification was carried out for 40 cycles in total according to a 20 μL amplification system consisting of cDNAs (2 μL), qPCR mixes (10 μL), primers (2 μL) and ddH_2O (6 μL). The primer sequences of the target gene and glyceraldheyde 3-phosphate dehydrogenase (GAPDH) internal reference were designed according to the sequences on GenBank, and the expression of the target gene was detected by qRT-PCR. The specific primer sequences are shown in Table 2. The relative expression level of related genes in the rat brain tissues in each group was calculated via the $2^{-\Delta\Delta Ct}$ method. The Ct value of each model had a linear relationship with the logarithm of the initial copy number of the model, namely, the more copies, the smaller the Ct value.

Western blotting

The left hemisphere brain tissues were taken and quickly frozen at -80°C. Then the frozen samples were weighed and broken on ice. After that, the tissues were added with protease inhibitors and improved radio-immunoprecipitation assay (RIPA) buffer and incubated in the refrigerator for full lysis to release tissue proteins (Beyotime, Shanghai, China). After centrifugation, the supernatant was collected. Eventually, the protein concentration was calculated according to bicinchoninic acid (BCA) kit instructions (Beyotime, Shanghai, China). Subsequently, the samples were loaded, and the proteins were separated on 12% gel and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) that were then blocked in 5% skim milk for 1.5 h at room temperature. Afterwards, the primary antibody and the secondary antibody were applied at the ratio of 1: 1000 for Western blotting. Lastly, the gel imaging system was utilized for color development and imaging, GAPDH was used to correct the protein level to be detected, and the gray value of protein bands was analyzed. The experiment was repea-

Table 1. Specific scoring details.

Score	Behavior
0	Rats are in a normal state and walk independently
1	Rats rotate to the opposite side of the lesion under the free-walking state
2	Rats grasp their tails and rotate to the left
3	Rats topple to the left in the rest state
4	Rats cannot walk on their own and are then in the rolling state.

Table 2. Primer sequences of each index in RT-PCR.

Target gene	Primer sequence (5'-3')
GAPDH	F: 5'-TGACTTCAACAGCGACACCCA-3' R: 5'-CACCCTGTTGCTGTAGCCAAA-3'
S100β	F: 5'-GGTGGTCATATGACAAAACTTGAAGAG-3' R: 5'-GGTGGTACTAGTGCATCTCCCGTGATTT-3’
Glial fibrillary acidic protein (GFAP)	F: 5'-AGAATTCGACGAGGACGACAAGGAGAGGA-3' R: 5'-ACTCGAGTCACATCACATCCTTGTGCTC-3'
SIRT1	F: 5'-GCAACAGCATCTTGCCTGAT-3' R: 5'-GTGCTACTGGTCTCACTT-3'
FOXO1	F: 5'-CCCAGGCCGGAGTTTAACC-3' R: 5'-GTTGCTCATAAAGTCGGTGCT -3'
MiR-146b	F: 5'-CGTATCCAGTGCAGGGTCCGA-3' R: 5'-TTCGCACTGGATACGACCCCC-3'

ted three times.

Statistical analysis

The data originally recorded in the experiment were processed using Statistical Product and Service Solutions (SPSS) 20.0 (SPSS, Chicago, IL, USA) analysis software, and multiple comparisons were made for the data. The experimental results were expressed by mean ± standard deviation ($\bar{x}$±SD), and $P<0.05$ represented that the difference was statistically significant. Each experiment was repeated at least three times, and the histogram was plotted using GraphPad Prism 7.0 (La Jolla, CA, USA).

Results

Transfection results of miR-146b in each group of rats

In order to observe the transfection rate of miR-146b in each group of rats, its gene expression level was detected. The results (Figure 1) revealed that the miR-146b expression level in Mimics was significantly increased, while that in Inhibitors was notably decreased ($P<0.05$), indicating that miR-146b with a suitable transfection rate can be applied for subsequent relevant verification tests.

Neurological scores of the rats in each group

In Inhibitors, the rats suffered from partial inability to walk and general contralateral flexion, with a relatively high neurological score. No abnormality was found in Control. The conditions in Mimics were markedly better than those in Inhibitors since the rats in the former could walk normally without other neurological symptoms, and their neurological score was almost close to that in Control ($P<0.05$). The specific score is shown in Figure 2.

CI volume

As shown in Figure 3, the CI volume in Mimics was relatively low and basically close to that in Control, while the volume in Inhibitors was remarkably increased ($P<0.05$), suggesting that infarction occurs in a large number of rat brain tissues after CI.

Apoptosis detected via TUNEL assay

As shown in Figure 4, no obvious positive cells were found in Control. The number of TUNEL-positive cells in the Inhibitors group was markedly larger than that in Control, mainly distributed around the hemorrhage foci and composed of glial cells ($P<0.05$). It can be seen that CI promotes the abnormal apoptosis of nerve cells.

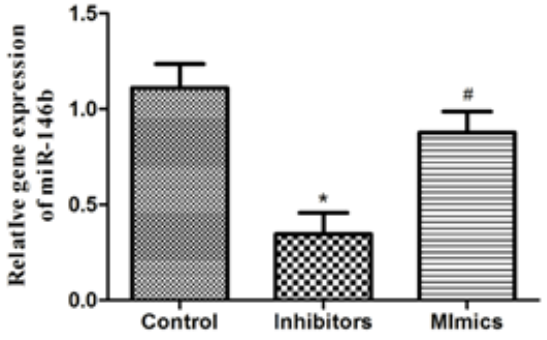

Figure 1. MiR-146b transfection rate. The expression of miR-146b is increased in Mimics but decreased in Inhibitors ($P<0.05$). *$P<0.05$ vs. Control, and #$P<0.05$ vs. Inhibitors.

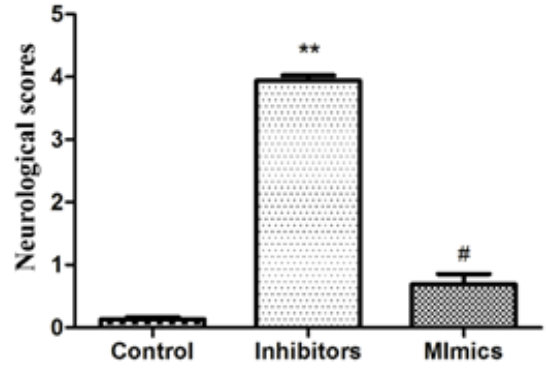

Figure 2. Neurological score. In Inhibitors, the rats suffer from a partial inability to walk and general contralateral flexion. No abnormality is found in Control. The conditions in Mimics are markedly better than those in Inhibitors since the rats in the former can walk normally without other neurological symptoms ($P<0.05$). *$P<0.05$ vs. Control, and #$P<0.05$ vs. Inhibitors.

Figure 3. Determination results of the CI volume. The CI volume in Mimics is relatively low and basically close to that in Control, while the volume in Inhibitors iss remarkably increased ($P<0.05$). *$P<0.05$ vs. Control, and #$P<0.05$ vs. Inhibitors.

Levels of the antioxidant indexes SOD and ROS in the brain tissues measured through ELISA

The results of SOD and ROS (Figure 5) manifested that the level of ROS was elevated in Inhibitors ($P<0.05$), but reduced in Mimics ($P<0.05$). However, SOD expression showed an opposite trend ($P<0.05$).

Expression levels of related genes examined via RT-PCR

In comparison with those in Control, the levels of GFAP and S100β in Inhibitors were prominently raised ($P<0.05$), but SIRT1 and FOXO1 expressions declined significantly ($P<0.05$). The opposite results were obtained in Mimics, which were close to those in Control ($P>0.05$) (Figure 6).

Western blotting results

Compared with Control, Inhibitors had significantly decreased protein expression levels of SIRT1 and FOXO1 ($P<0.05$). In Mimics, these levels exhibited an opposite trend and were close to those in Control ($P>0.05$) (Figure 7).

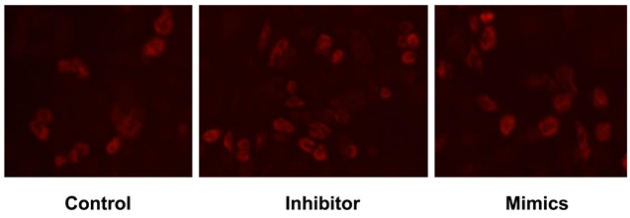

Figure 4. TUNEL staining. No obvious positive cells are found in Mimics, and positive cells in Inhibitors were increased significantly (P<0.05).

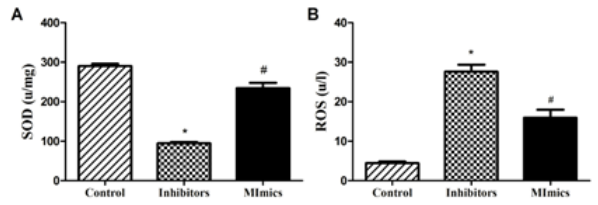

Figure 5. Antioxidant indexes. The level of ROS is raised in Inhibitors (P<0.05) but lowered in Mimics (P<0.05), opposite to the results of SOD expression (P<0.05). *P<0.05 vs. Control, and #P<0.05 vs. Inhibitors.

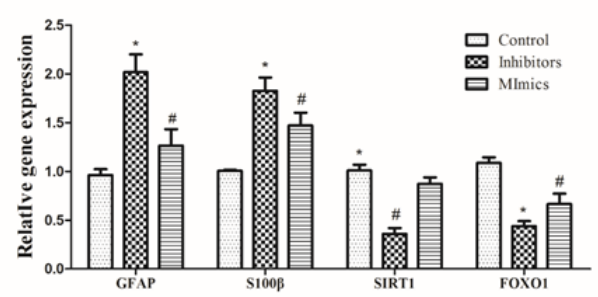

Figure 6. Detection results of related genes via qRT-PCR. The levels of GFAP and S100β in Inhibitors are increased significantly (P<0.05), whereas SIRT1 and FOXO1 expressions are decreased remarkably (P<0.05). *P<0.05 vs. Control, and #P<0.05 vs. Inhibitors.

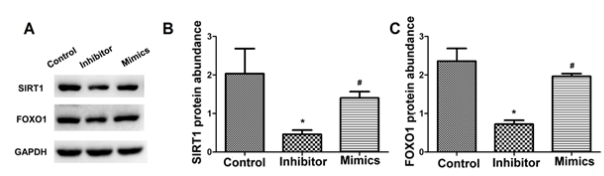

Figure 7. Detection results of the signaling pathway-related proteins. The SIRT1 and FOXO1 protein expressions in Inhibitors are notably reduced (P<0.05), which is contrary to those in Mimics. *P<0.05 vs. Control, and #P<0.05 vs. Inhibitors.

Discussion

ACI is a common clinical cardiovascular and cerebrovascular disease in the nervous system. CI can lead to irreversible degeneration and necrosis of brain neurons, thereby resulting in a train of neurological dysfunctions. Although some therapeutic drugs can interfere with the development of CI currently, they are seldom used clinically due to their side effects and difficult metabolism, so there is still a need to find an effective treatment method (21). The pathological process and mechanism of ACI are fairly complex and remain unclear. However, the body's response still cannot improve the blood supply reconstruction and brain tissue perfusion in the CI area when CI occurs. It is urgent to better and more effectively treat such CI diseases. CI recovery is a complex and dynamic process regulated by various cellular components and cytokines, and this process is affected by many genes and other regulatory factors (22). Therefore, it is crucial to deeply understand the specific molecular regulatory network of CI for its treatment. Despite the fact that a great deal of time and energy has been spent, there is still a lack of successful intervention measures for the treatment of CI-related neurological diseases. Hence, there is an urgent need for novel treatment methods to provide a basis for the treatment and prevention of CI and its complications. Recent studies have demonstrated that miRNAs exert vital effects on physiological homeostasis and health and various diseases. They have become pivotal gene expression regulators in numerous diseases, and their regulatory networks have attracted much attention in recent years (23). According to a study, miR-146b plays a regulatory role in CI, but the underlying effect of miR-146b on CI in rats remains obscure, which needs to be further studied. In this study, a typical rat model of CI was established, and the effect of miR-146b on CI was analyzed by silencing or overexpressing miR-146 using RNA interference technology. In order to observe the transfection rate of miR-146b in each group of rats, its gene expression level was detected. The results revealed that the miR-146b expression level was significantly increased in Mimics but notably decreased in Inhibitors, indicating that miR-146b with an appropriate transfection rate can be utilized for subsequent relevant verification tests. During CI, chronic neurological dysfunction, including cerebral palsy, and cognitive and other defects, will occur. The neurological function of the rats in each group was scored. It was found that in Inhibitors, the rats suffered from partial inability to walk and general contralateral flexion, with a relatively high neurological score. No abnormality was discovered in Control. The conditions in Mimics were markedly better than those in Inhibitors since the rats in the former could walk normally without other neurological symptoms, and their neurological score was almost close to that in Control. Moreover, the severity of the disease was evaluated by measuring the CI volume, the results of which verified that the CI volume in Mimics was relatively low and basically close to that in Control, while this volume in Inhibitors was remarkably increased, suggesting that infarction occurs in a large number of rat brain tissues after CI. The above findings indicate that CI is able to trigger brain tissue injury, whose development can be resisted by miR-146b, but the specific mechanism of miR-146b needs to be further investigated. Therefore, gene and protein experiments were further carried out to verify the results.

The SIRT1/FOXO1 signaling pathway exerts indispensable antioxidant and anti-apoptotic effects on I/R diseases (24). FOXO1 is a target of SIRT1 and plays a vital role in intracellular homeostasis. A recent study has indicated that SIRT1 plays a role in DNA damage repair, apoptosis inhibition and oxidative stress. FOXO1 may enhance cell resistance to oxidative stress *via* SIRT1 deacetylation under ischemia or hypoxia conditions and is capable of scavenging ROS (25). However, the specific action mechanisms of the SIRT1/FOXO1 signaling pathway on CI,

the apoptosis of nerve cells and oxidative stress remain unclear. In this study, therefore, it was proposed that miR-146b can exert therapeutic effects on CI in rats through the SIRT1/FOXO1 signaling pathway. Besides, the effects of the SIRT1/FOXO1 signaling pathway on CI, the apoptosis of nerve cells and oxidative stress were explored by the classical rat model of CI and using various molecular means, so as to provide an experimental and theoretical basis for the treatment and prevention of CI through the SIRT1/FOXO1 signaling pathway. The role of oxidative stress in CI has received extensive attention, and SOD is widely present, the action of which can be resisted by MDA to produce cytotoxicity. ROS is a vital component of tissue damage after ischemic injury (26). It was discovered in this study that the ROS level in Inhibitors was significantly higher than that in other groups, while the opposite result was found in the SOD expression, indicating that CI is alleviated after the overexpression of miR-146b. Apoptosis, as the body's defense force, is a metabolic pathway that can maintain cell stability. However, the mechanism of apoptosis in the physiological metabolism of organisms has not been fully explored and clarified, but the mechanism and approach can be used as important guidelines for various diseases such as tumors, CI injury and other clinically related diseases (27). TUNEL results manifested that the level of apoptosis in Inhibitors was notably higher than that in other groups, while the apoptosis in Mimics was obviously reduced. It can be seen that significant apoptosis occurs in cells during CI. S100β can be used as a specific molecule for brain injury. Glial cells are toxic to the central nervous system when activated by exogenous or endogenous ligands (28). GFAP, as a specific astrocyte marker, can rapidly activate microglia and astrocytes due to its increased specificity (29-31). This study showed that GFAP and S100β mRNAs were evidently raised in Inhibitors ($P<0.05$), but remarkably reduced in Mimics, which are consistent with the above research results. Additionally, the detection of pathway-related genes and proteins illustrated that the expression levels of SIRT1 and FOXO1 in Inhibitors declined markedly ($P<0.05$), while those in Mimics showed an opposite trend and were close to those in Control. However, the expression trend of proteins in Inhibitors and Mimics was identical. The above findings are confirmed by the research of Nia et al. (32) and Tian et al. (33) and consistent with their research results. Furthermore, it can be concluded from the above research that miR-146b can exert therapeutic effects on CI in rats through the SIRT1/FOXO1 signaling pathway.

To sum up, a series of pathological changes such as cell oxidative stress, cell apoptosis and neurological dysfunction will occur during CI, but miR-146b is able to repress its occurrence *via* the SIRT1/FOXO1 signaling pathway. Besides, further effects can be explored through more molecular means.

Conclusions

In a word, this study lays a foundation for the theory of preventing and treating CI and provides new ideas and an experimental basis for further research.

Conflict of Interest

The authors declared no conflict of interest.

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