DOI: 10.5137/1019-5149.JTN.23127-18.2
Received: 09.03.2018 / Accepted: 05.09.2018
Published Online: 07.11.2018

Turk Neurosurg, 2018

Original Investigation

miR-24 Reduces Serum Lipid Levels and Inhibits Brain Tissue Cells Apoptosis of Rats with Cerebral Infarction

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ABSTRACT

AIM: To study miR-24 effects on cerebral infarction in rats.

MATERIAL and METHODS: A rat middle cerebral artery occlusion model (MCAO) was constructed. Intracerebroventricular stereotactic injection of miR-24 agomir/antagomir was performed in the rat MCAO model. According to different experiences, rats were divided into normal, sham, MCAO, miR-24 agomir and miR-24 antagomir groups. Serum TCH, HDL and TG levels were detected.

RESULTS: Comparingthe normal and sham groups, we observed decreased relative miR-24 expression (P< 0.05) and increased cerebral infarction area percentage, apoptotic cells and relative caspase-3 protein expression (P< 0.05) in theMCAO, miR-24 agomirand miR-24 antagomir groups. TC, TG and HDL-C levels of the MCAO and miR-24 antagomir groups were higher than those of normal and sham groups (P< 0.05).Compared with the MCAO group, increased relative miR-24 expression (P< 0.05) and decreased TC, TG and HDL-C levels,cerebral infarction area percentage, number of apoptotic cells and caspase-3 expression (P< 0.05) were found in themiR-24 agomir group, contrasting with theobservations from the miR-24 antagomir group.

CONCLUSION: miR-24 reduced serum TCH, HDL and TG levels and inhibited brain tissue cell apoptosis in rats with cerebral infarction.

KEYWORDS: Cerebral infarction, miR-24, Blood lipid, Area of cerebral infarction, Apoptosis, Rat

ABBREVIATION: MCAO: Middle cerebral artery occlusion model.

INTRODUCTION

Cerebral infarction is a type of cerebrovascular disease caused by a cerebral blood supply disorder. Severe disturbance of consciousness, sensory and motor dysfunction, neurodegenerative diseases, and even sudden death often occurat the onset of cerebral infarction (17,18,33). It has been reported that cerebral infarction has become a leading cause of disability and death worldwide because of its high morbidity and mortality (9,12). Cerebral infarction not only severely affects patients' quality of life but also puts tremendous burden and pressure on society and families. Genetic factors and poor habits are two main causes of cerebral infarction (26). According to an epidemiological study, hyperlipidemia and hypertension are two important factors that greatly increase the risk of cerebral infarction (2).

Prevention, timely diagnosis and treatment of cerebral infarction are crucial for reducing the risk of disability and death in patients. Thrombolytic drugs are commonly used in clinical prevention and treatment of cerebral infarction (22). However, in addition to annoying side effects, the fundamental prevention and treatment of cerebral infarction cannot be achieved by these drugs (32). With the development of

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molecular medicine, genetic diagnosis of various diseases is possible for a wide variety of diseases, including tumors (21,31). Prevention and treatment of cerebral infarction at the genetic level are also particularly important. Therefore, there is an urgent need to find effective biomarkers that can be used to diagnose and prognose cerebral infarction. As a type of small molecule RNA, miR-24 was found to be involved in the development and progression of various diseases, such as tumors, coronary heart disease, type 2 diabetes mellitus, and cardiovascular disease (3,4,6,27). In this article, we studied the expression of miR-24 in rat models of cerebral infarction and investigated its effects on cerebral infarction development and brain cell apoptosis. This research provides an important potential therapeutic target for the prevention and treatment of cerebral infarction.

MATERIAL and METHODS

Animals and Groups

Fifty male Sprague-Dawley rats (240-280 g) were housed in a sterile cage at 25°C and a relative humidity of 40%. The food and drinking water were all sterilized. These rats were randomly divided into 5 groups (normal, sham, MCAO, miR-24 agomirand miR-24 antagomir) with 10 rats in each group. No statistical significance in body weight was found among groups.

All animal experiments were approved by our hospital's Medical Ethics Committee.

Establishment of Rat Middle Cerebral Artery Occlusion Model (MCAO)

Rats were anesthetized with ether before fixation on the operating table in the supine position. Respiratory secretions were promptly cleared to prevent airway obstruction. Continuous inhalation of ether was implemented throughout the surgical procedure. An incision of approximately 2 cm in length was made in the middle of the neck to expose the carotid sheath after disinfection. The common carotid artery, external carotid artery and internal carotid artery were carefully separated. The common carotid artery was ligated proximally and a loose knot was made at the distal end of the common carotid artery. After the external carotid artery was ligated at the bifurcation of the internal carotid artery and external carotid artery, another loose knot was made at the proximal end of the internal carotid artery, and a microarterial clamp was used to grasp the distal end of the internal carotid artery. A small incision was made on the common carotid artery 3-5 mm away from the bifurcation of the artery. A thread plug was inserted into the internal carotid artery from this small incision. Then, the loose knot at the distal end of the common carotid arterywas slightly tightened. The microartery clamp at the internal carotid artery was released, and the thread plug was slowly advanced approximately 18 mm from the carotid bifurcation until mild resistance was felt. Loose knots on the common carotid artery and internal carotid artery were tightened, followed by cutting off extra part of the thread plug. Finally, the wound was sutured layer by layer. It should be noted that rats in the sham group were subjected to the same surgical procedure without the insertion of a thread plug, while rats in the normal group did not undergo any surgical treatment. All rats were placed in a warm box with free access to food and water.

Screening of MCAO Model Rats by Zea Longa

After the rats woke, the Zea Longa scoring method was used for neurological evaluation to screen for rats with MCAO. The criteria for scoring were as follows: 0 points, rats were without neurological deficit; 1 point, rats did not fully extend the left front paw; 2 points, rats circled to the left when walking; 3 points, rats fell to the left when walking; 4 points, rats did not self-motivated and lost consciousness; 5 points, rats died. Rats with a Zea Longa score of 2-3 were included in the study. Finally, 9 rats ineach of the MCAO, miR-24 agomirand miR-24 antagomir groups were screened. In addition, rats in the normal and sham groups were not evaluated by Zea Longa, and 9 rats in each group were randomly enrolled in this study.

Intracerebroventricular Stereotactic Injection ofmiR-24 Agomir/Antagomir in the Rat MCAO Model

For rats in miR-24 agomirand miR-24 antagomir groups, intracerebro ventricular stereotactic injection of miR-24 agomiror miR-24 antagomir was performed 2 h after the MCAO model was established. Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.4 mL/100 g) before their heads were fixed on the brain stereotaxic instrument. Then, iodophor and 75% alcohol were used to disinfect the skin after the hair on the head was removed. An opening of approximately 1.5 cm was cut along the midline of the head to expose the skull around the culvert. A dental drill was used to drill through the skull 0.8 mm behind the front chimney and 1.6 mm to the right with the culvert. After positioning on the stereotaxic instrument again, a microsyringe was used to puncture at a depth of 3.5 mm with an injection speed of 1 μL/min. The total volume of the injection was 10 μL. Ten minutes after injection was completed, the microsyringe was slowly pulled out. The opening of approximately 1.5 cm was sutured after the injection hole was closed with bone wax. Rats were then placed in a warm box with free access to food and water.

Detection of Serum TCH, HDL and TG

After feeding for 7 days, 3 rats in each group were randomly selected for fasting blood experiments. The levels of serum TCH, HDL and TG were measured by an automatic biochemical analyzer.

Detection of Cerebral Infarction Area by the TTC Method

Three rats in each group were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.4 mL/100g) 24 h after the MCAO procedures were completed. They underwent decapitation rapidly, and the skullswere removed by a rongeur. Whole brain tissues of rats were obtained using a spoon and were sectioned after being placed in a -20°C freezer for 10-20 min. These brain tissues were subjected to continuous coronal slices from the frontal to occipital lobes with a thickness of approximately 2 mm. TTC solution (2%, 1 mL) was added to the brain tissue sections for a 10 min incubation at 37°C

in darkness. Infarcted brain tissues were white while normal brain tissues were red after TTC staining.

Paraformaldehyde Perfusion

Three rats in each group were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.4 mL/100 g) 24 h after MCAO procedures were completed. The upper abdomen and chest were opened with scissors to fully expose the heart. A perforating puncture needle was inserted into the left ventricle until the ascending aorta at the apical position. It should be noted that the heart and aorta could not be punctured. The perforating puncture needle was fixed using forcepsin the apical position. Saline with a total volume of 200-300 mL was continuously perfused after the right atrial appendage was opened. When the perfusion fluid flowing out from the right atrial appendage became clear and the color of liver and limbs turned white, 4% paraformaldehyde solution was used instead of saline for continuous perfusion. The perfusion speed was slowed after the liver was stiff. The total 4% paraformaldehyde perfusion volume was approximately 300-400 mL. After perfusion, the skull was opened, and the intact rat brain was removed and fixed with 4% paraformaldehyde.

qRT-PCR Detection of miR-24 Expression in Brain Tissue

Brain tissues of rats in each group were ground into small particles in liquid nitrogen. Total RNA from these brain tissues was extracted by themiRNeasy Mini Kit (obtained from Qiagen, Germany). Then, qRT-PCR was conducted with a 20 μL reaction system with 1 μL cDNA, 1 μL forward primer and 1 μL reverse primer. The reaction procedure was as follows: denaturation at 95°C for 10 s, reannealing at 60°C for 20 s, extension at 72°C for 34 s. The reaction procedure was cycled 38 times. Data analysis was processed by the 2-ΔΔCt method. The primer sequences of miR-24 and its internal reference (U6) are shown in Table I.

Detection of Apoptosis by Tunel Staining

Tissue sections of rats in each group were deparaffinized with xylene and rehydrated with gradient ethanol, followed by Tunel staining for apoptosis detection. The detection process was carried out in strict accordance with the instructions of the Tunel kit (purchased from Beijing Boao Sen Biological Engineering Co., Ltd.). Cells that presented brown particles in the nucleus or cytoplasm were considered Tunel-positive apoptotic cells. Five sections of each rat brain tissue were randomly selected, and 2 fields were randomly selected under a microscope for Tunel-positive cell counting. The average number of Tunel-positive cells was calculated. The greater the average number of Tunel-positive cells, the more severe the apoptosis.

Table I: Primer Sequences of miR-24 and Its Internal Reference

Name of primer	Sequences
miR-24-F	CAGAGATCTAAGTCGTGTGAAATCATGTG
miR-24-R	TAAGTCGACAACAGGGTTTTCCAAGTCTA
U6-F	GTGCAGGGTCCGAGGT
U6-R	CTCGCTTCGGCAGCACA

Detection of Caspase-3 by Immunohistochemistry

Five consecutive sections of each brain tissue were randomly selected. After dewaxing and rehydration, these sections were placed in boiling 0.01 M citrate buffer for antigen retrieval. H_2O_2 (3%) was used to eliminate endogenous peroxidase activity for 15 min. After washing 3 times with PBS, normal goat serum blocking solution was used for incubation at room temperature for 15 min. Then, rabbit anti-human caspase-3 polyclonal antibody (1: 100, Santa Cruz, US) was added for a 12 h incubation at 4°C. Secondary antibody was then added for a15 min incubation at 37°C, followed by 3 washes with PBS. Then, horse radish enzyme-labeled streptavidin working solution was added for an additional 15 min incubation at 37°C. Hematoxylin counterstain was carried out after the DAB color reaction. After dehydration, these sections were sealed with a neutral gland. Under the microscope, 5 fields of each section were randomly selected for caspase-3-positive cell counting. Caspase-3 was mainly expressed in the cytoplasm, and brownish yellow particles were found in caspase-3-positive cells.

Detection of Caspase-3 Protein by Western Blot

Brain tissues were washed 3 times with precooled PBS buffer and then RIPA lysis buffer (Santa Cruz, US) was used to lyse cells to obtain total protein concentration. Total protein was separated by SDS-PAGE for 2 h at 4°C and transferred onto a PVDF membrane. After blockingwith 5% skim milk for 1-2 h at room temperature, the membrane was placed in an incubator for a 12 h incubation at 4°C with mouse anti-human caspase-3 monoclonal antibody (1:1000, Santa Cruz, USA). Subsequently, horseradish peroxidase-labeled secondary antibody (1:5000, Beijing Zhong Shan Biotechnology Co., Ltd., China) was used during a 1 h incubation at room temperature. Chemiluminescence and data analysis was performed after the membrane was washed 3 times with TBST. In this study, GAPDH was used as an internal reference.

Statistical Analysis

All data were expressed as the means ± SD and processed bySPSS 18.0 statistical software. The comparison between the two groups was performed using the t-test, and $P< 0.05$ was considered as statistically significant.

RESULTS

Downregulation of miR-24 in MCAO Model Rats

We examined the relative expression of miR-24 in brain tissues of rats in each group by qRT-PCR. The results showed that compared with normal and sham groups, the relative expression of miR-24 in the MCAO, miR-24 agomirand miR-24 antagomir groups were markedly decreased ($P<0.05$), illustrating that miR-24 was downregulated in rats with cerebral infarction. In addition, compared with the relative expression of miR-24 in theMCAO group, the miR-24 agomir group was much higher ($P< 0.05$) and the miR-24 antagomir group was

obviously lower (P< 0.05) (Figure 1), suggesting that miR-24 agomir or antagomir could effectively regulate the expression of miR-24.

miR-24 Decreased Serum TC, TG and HDL-C Levels in MCAO Model Rats

To investigate the effect of miR-24 on blood lipids of rats with cerebral infarction, the fasting serum TC, TG and HDL-C levels of rats in each group were detected. TC, TG and HDL-Clevels of rats in the MCAO and miR-24 antagomir groups were markedly higher than those in the normal and sham groups (P< 0.05). However, no statistically significant difference was found in TC, TG and HDL-C levels among the miR-24 agomir, normal and sham groups. Furthermore, we also found that compared with the MCAO group, TC, TG and HDL-C levels of rats in the miR-24 agomir group were decreased (P< 0.05), while those in the miR-24 antagomir group were obviously increased (P< 0.05) (Figure 2A-C). These results revealed that for rats with cerebral infarction, serum TC, TG and HDL-C levels increased, and miR-24 reduced the serum lipid levels.

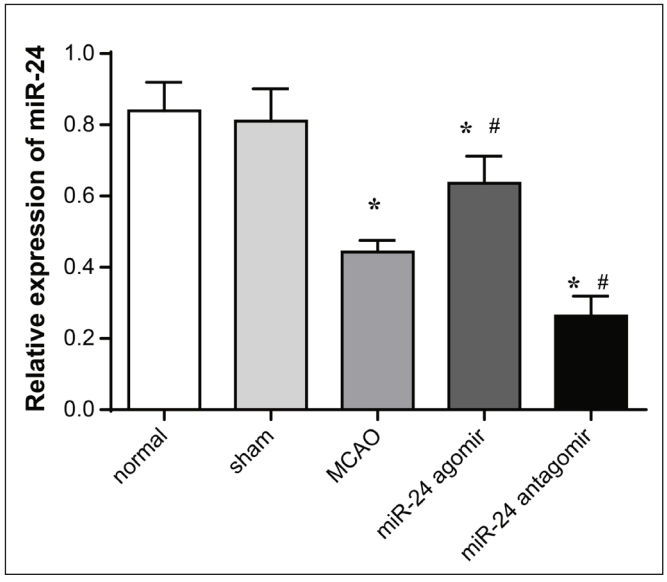

Figure 1: Relative expression of miR-24 in brain tissues of rats in each group.
*P< 0.05 when compared with the normal or sham groups; #P< 0.05 when compared with the MCAO group.

miR-24 Inhibited the Development of Cerebral Infarction

TTC staining was used to detect the cerebral infarction area of rats in each group to assess miR-24 impact on cerebral infarction development. After TTC staining, normal brain tissues were red and infarcted brain tissues were white. Cerebral infarction was not found in rats in the normal and sham groups, while different degrees of cerebral infarction occurred in rats of the other three groups. Compared with the MCAO group, the cerebral infarction area percentage of rats in the miR-24 agomir group was much smaller (P< 0.05), while the percentage in the miR-24 antagomir group was significantly larger (P< 0.05) (Figure 3). miR-24 effectively relievedrat cerebral infarction development.

miR-24 Inhibited Apoptosis of Rat Cerebral Infarction Tissue Cells

Apoptosis of rat cerebral infarction tissue cells was detected by the Tunel method. Significantly more apoptotic cells were found in brain tissues of rats in the MCAO, miR-24 agomirand miR-24 antagomir groups when compared with apoptotic cells in the normal and sham groups (P< 0.05). Meanwhile, compared with the MCAO group, the number of apoptotic cells decreased in the miR-24 agomir group (P< 0.05) but markedly increased inthe miR-24 antagomir group (P< 0.05) (Figure 4), illustrating that miR-24 effectively alleviated the apoptosis of cerebral infarction in rat brain cells.

miR-24 Inhibited the Expression of Caspase-3 Protein

Our further research showed that the relative caspase-3 protein expression inthe brain tissues of rats in the MCAO, miR-24 agomirand miR-24 antagomir groups was significantly higher than expression in the normal and sham groups (P< 0.05). However, compared with the MCAO group, relative caspase-3 protein expression decreased in the miR-24

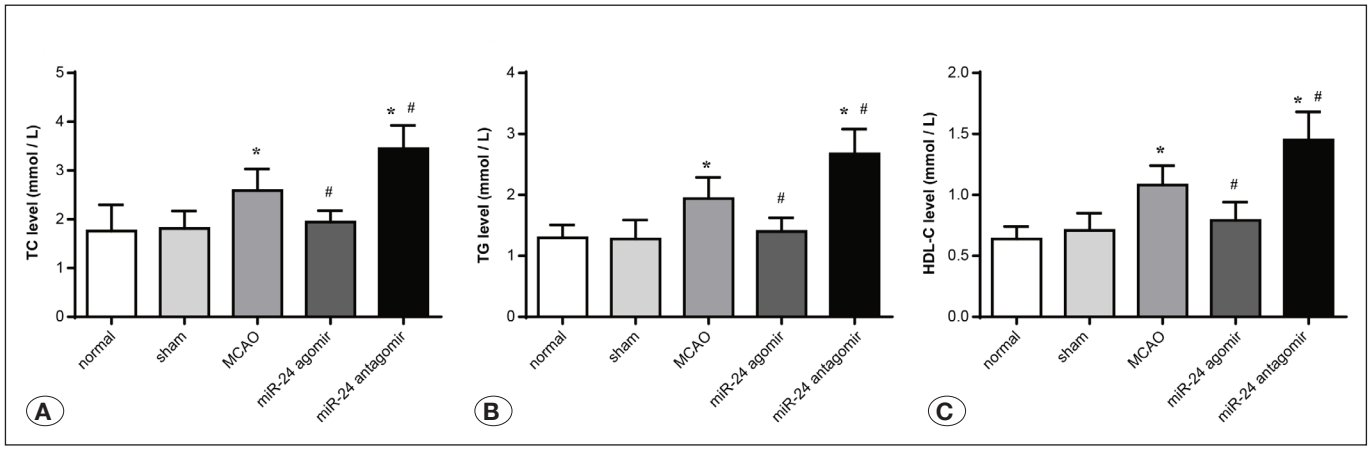

Figure 2: SerumTC, TG and HDL-C levels of rats in each group. *P< 0.05 when compared with the normal or sham groups; # P< 0.05 when compared with the MCAO group.

agomir group (P< 0.05) and greatly increased in the miR-24 antagomir group (P< 0.05) (Figure 5A). According to the immunohistochemistry, caspase-3, mainly in the cytoplasm, was more highly expressed in MCAO rat brain tissue cells. We observed that in the brain tissues of the MCAO, miR-24 agomir and miR-24 antagomir groups, the number of caspase-3-positive cells was significantly greater than the number of positive cells in the normal and sham groups (P< 0.05). Compared with the MCAO group, the number of caspase-3-positive cells in the miR-24 agomir group decreased (P< 0.05), while the number of positive cells was significantly increased in the miR-24 antagomir group (P< 0.05) (Figure 5B). All of these results suggested that caspase-3 was upregulated in brain tissue of rats with cerebral infarction, while miR-24 had an inhibitory effect on the expression of caspase-3.

DISCUSSION

In this study, we constructed a rat MCAO model and regulated miR-24 expression through intracerebroventricular stereotactic injection of miR-24 agomir/antagomir in our MCAO model. The results revealed that miR-24 was downregulated in the MCAO rats, and upregulation of miR-24 effectively decreased serum TC, TG and HDL-C levels and inhibited cerebral infarction development, apoptosis in rat cerebral infarction tissue cells and caspase-3 expression.

Since the discovery of the first miRNA in 1993, it has been extensively studied in the medical field, and the role of miRNAs in various diseases has also been well characterized (1). miRNAs are a class of single-stranded small RNAs with 19-25 nucleotides, which are involved in a series of processes such as cell development, proliferation, differentiation, apoptosis, inflammation and tumorigenesis (5,25). miRNAs play a very important role in physiological and pathological processes. Sempere et al. (19) first detected seven specific brain tissue miRNAs in human and mouse neurons, namely, miR-9, miR-124a, miR-124b, miR-129, miR-135, miR-153, and miR-183. They showed for the first time that miRNAs were closely related to cerebral infarction. miRNAs were also closely associated with many risk factors for cerebral infarction. Hypertension is an important independent risk factor for cerebral infarction, which can directly cause a wide range of cerebral atherosclerosis (7,11). Thrombosis, which occurs on the basis of atherosclerosis, is one of the most common causes of cerebral infarction (24). It was reported that miR-155 was negatively correlated with elevated blood pressure by acting on the RAAS system. In addition, miR-124 and miR-

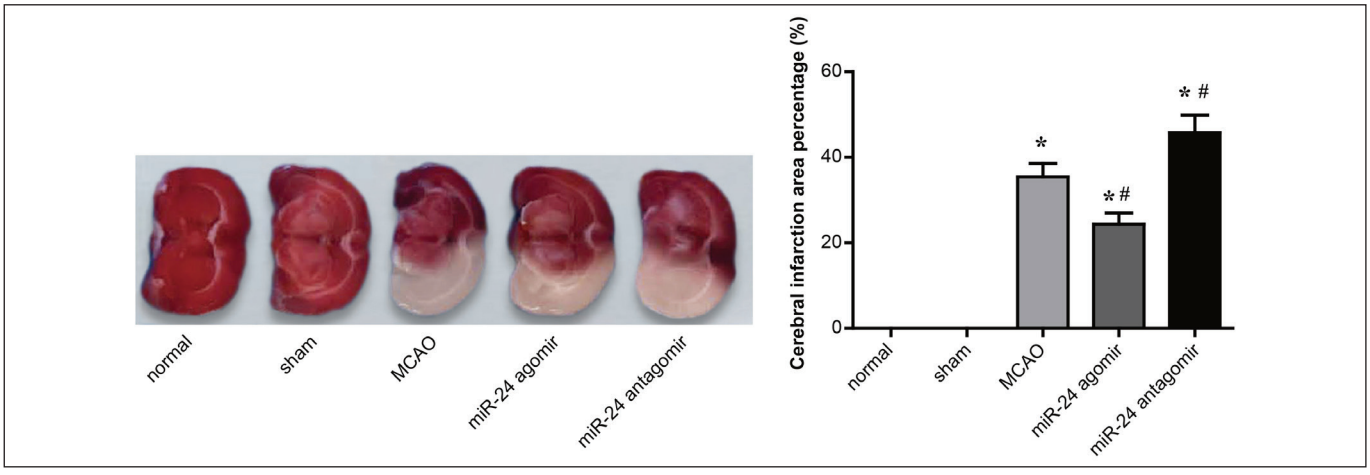

Figure 3: Percentage of cerebral infarction area of rats in each group (%). *P< 0.05 when compared with the normal or sham groups; # P< 0.05 when compared with the MCAO group.

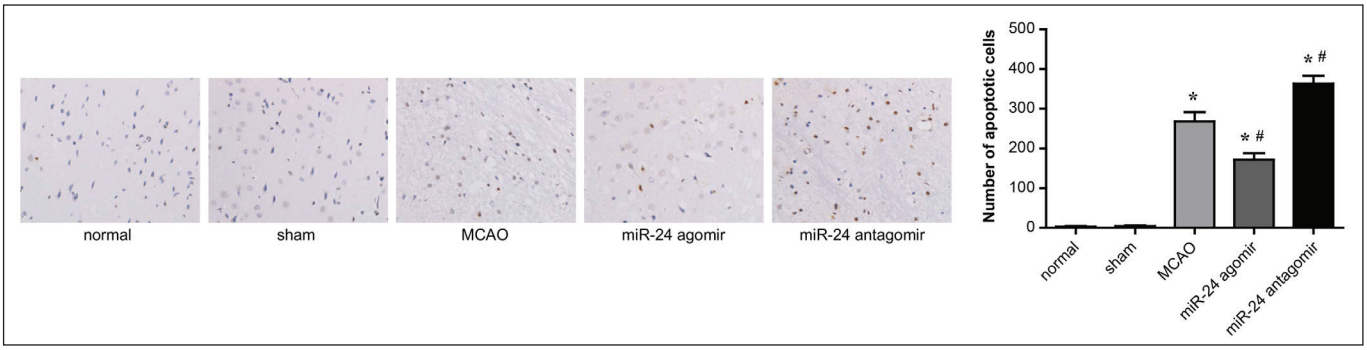

Figure 4: The number of apoptotic cells in rat brain tissues of each group. * P< 0.05 when compared with the normal or sham groups; # P< 0.05 when compared with the MCAO group.

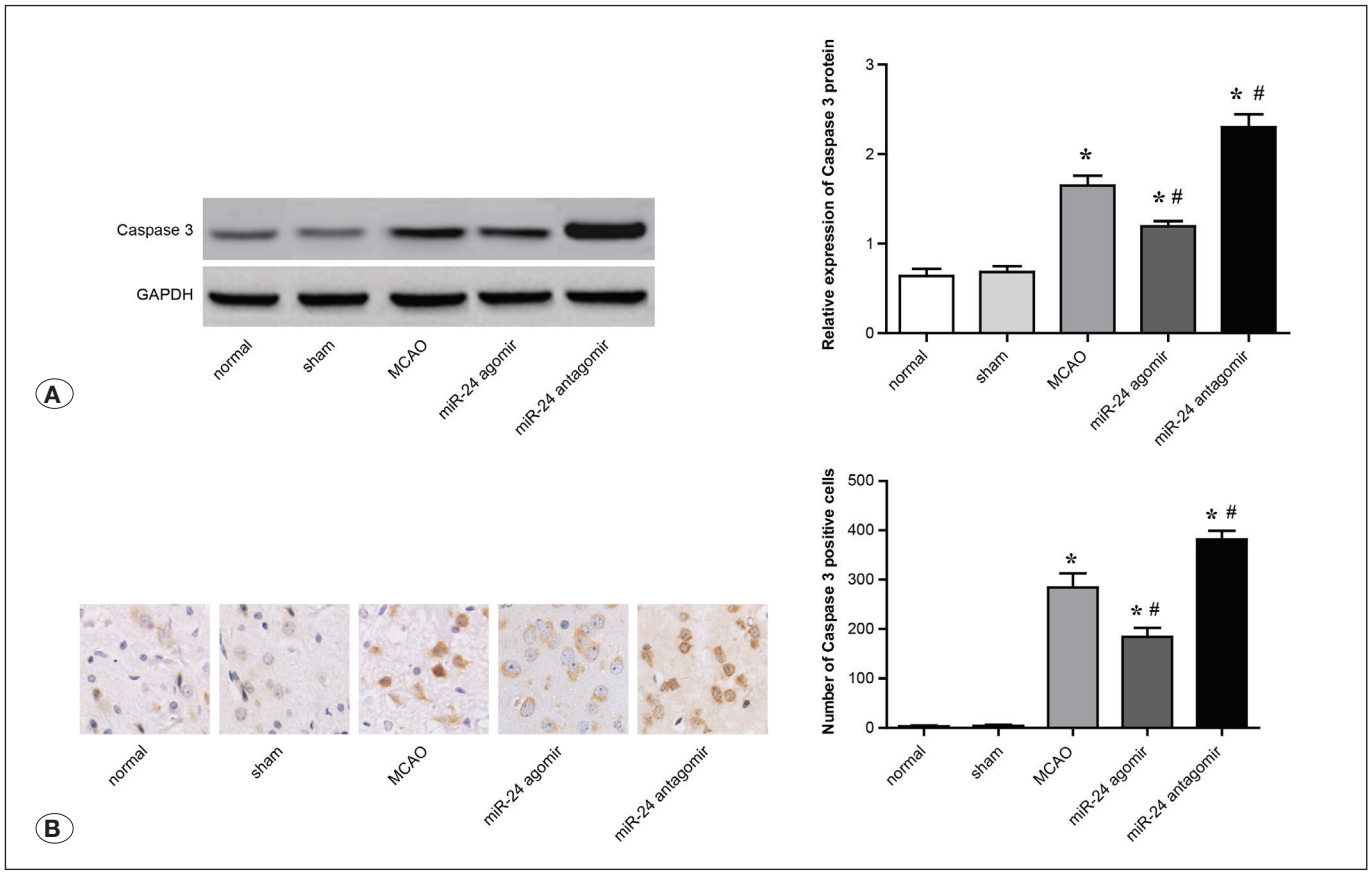

Figure 5: Caspase-3 expression in rat brain tissues of each group. **A)** Detection of caspase-3 expression in rat brain tissues by Western blot; **B)** Detection of caspase-3 expression in rat brain tissues by immunohistochemistry. *P< 0.05 when compared with the normal or sham groups; **#** P< 0.05 when compared with the MCAO group.

135a also participated in the regulation of blood pressure by inhibiting the mineralocorticoid receptor gene (16). Jackson et al. showed that low expression of miR-181a may be involved in the regulation of blood pressure by promoting sympathetic activity and inducing renin synthesis (10). Yang et al. found that miR-505 could be used as a new circulating marker of hypertension, which was a potential biological marker of hypertension (28). Diabetes was another recognized risk factor for cerebral infarction, and strict control of diabetes was an important factor in preventing cerebral infarction (29). Ortega et al. revealed that expression of miR-140-5p and miR-222 in type 2 diabetes mellitus was associated with insulin resistance (13). Párrizas et al. suggested that expression of miR-192 and-193b could predict the occurrence of type 2 diabetes (14). Furthermore, microRNA was also closely related to the metabolism of blood lipids. Lipids deposited on the inner wall of blood vessels when the blood lipids levels increased, eventually leading to an increased risk of cerebral infarction (30). miR-122 was the first microRNA found to regulate lipid metabolism, which significantly decreased serum cholesterol, triglycerides, and very low-density lipoproteins (20). Wang et al. demonstrated that miR-370 regulated the degradation of low-density lipoprotein by acting on the target gene OLR1 (23). Iraniet al. found that miR-30c reduced the plasma cholesterol in mice and relieve atherosclerosis (8).

In this research, we found that miR-24 was another potential biomarker of cerebral infarction. Its expression was significantly downregulated in the brain tissues of cerebral infarction rats. We also observed that miR-24 decreased serum TC, TG and HDL-C levels and inhibit cerebral infarction development and brain tissue cells apoptosis. Pan et al. found that miR-24 was reduced in cardiomyocytes after the onset of acute myocardial infarction, and miR-24 upregulation played a critical role in decreasing Bcl-2 interacting mediator expression. They thought that up regulation of miR-24 was an important way to reduce cardiomyocyte apoptosis and improve cardiac function (15). Liu et al. revealed down regulation of miR-24 in acute myocardial infarction and further found that percutaneous coronary intervention increased the expression of miR-24. In addition, they also found that overexpression of miR-24 improved cardiac function through decreasing type I collagen as well as regulating apoptosis imbalance and alleviating myocardial ischemia reperfusion. Therefore, they postulated the use of miR-24 as a cardiac function indicator for acute myocardial infarction (12). In this article, we also suggested that miR-24 could be used as a potential biomarker for the prevention and treatment of cerebral infarction.

However, this study still has some limitations. First, the application of miR-24 in the diagnosis of cerebral infarction

is still in the exploratory stage, and miR-24 expression levels are not exactly the same using different detection methods. Second, miR-24 can be measured from tissues, plasma and serum. Thus, its expression level may be different if the sample types used are different. Finally, the target genes of miR-24 in cerebral infarction are not clear from this study; this will also be the focus of our follow-up study.

CONCLUSIONS

In short, our research showed that miR-24 was downregulated in rats with cerebral infarction. Upregulation of miR-24 reduced serum TCH, HDL and TG levels and inhibited brain tissue cells apoptosis of rats with cerebral infarction. miR-24 is a potential biomarker for the prevention and treatment of cerebral infarction.

FUNDING

This research did not receive any specific grant from funding agencies in the public, commercial, or nonprofit sectors.

COMPETING INTEREST

On behalf of all authors, the corresponding author states that there are no conflicts of interest.

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