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Effect of miR-124 on neuronal apoptosis in rats with cerebral infarction through Wnt/β-catenin signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to observe the influence of micro ribonucleic acid (miR)-124 on neuronal apoptosis in rats with cerebral infarction (CI), and to further investigate the underlying mechanism of miR-124 in CI occurrence and development.

MATERIALS AND METHODS: A total of 60 adult male Wistar rats were randomly divided into the Sham group, the CI group and the CI + miR-124 mimics group using a random number table. The focal CI model was established using the suture-occluded method. After successful modeling, miR-124 mimics were stereotactically injected into the lateral ventricle of rats. 24 h after operation, the neurological function of rats in each group was scored using modified neurological severity score (mNSS). Meanwhile, the infarction area of brain tissues was evaluated by the triphenyl tetrazolium chloride (TTC) method. The protein expression levels of apoptosis-related genes, including B-cell lymphoma-2 (Bcl-2), Bcl-2 associated X protein (Bax), C-Caspase and T-Caspase, were detected *via* Western blotting. The expression and location of caspase-3 in brain tissues were detected *via* immunofluorescence staining. Moreover, the level of apoptosis in each group was detected *via* terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. In addition, the expression levels of the Wnt/β-catenin signaling pathway-related proteins were detected *via* Western blotting.

RESULTS: Polymerase Chain Reaction (PCR) results revealed that the expression level of miR-124 in the CI group was significantly decreased when compared with that of the Sham group ($p<0.05$). The mNSS and TTC staining results manifested that injection of miR-124 mimics could significantly reduce the CI-induced neurological deficits and CI area ($p<0.05$). At the same time, the levels of Bax and C-caspase/T-Caspase were significantly decreased, whereas the expression of Bcl-2 was remarkably increased after the injection of miR-124 mimics ($p<0.05$). Besides, the number of apoptotic cells in the CI + miR-124 mimic group was remarkably decreased ($p<0.05$). In addition, miR-124 mimics significantly activated the expression levels of Wnt and β-catenin ($p<0.05$).

CONCLUSIONS: The inhibitory effect of miR-124 on neuronal apoptosis in CI rats is probably related to the activation of the Wnt/β-catenin signaling pathway. Furthermore, miR-124 is expected to be a target drug for the clinical treatment of CI.

Key Words:
MiR-124, Apoptosis, Cerebral infarction (CI), Wnt/β-catenin.

Introduction

Cerebral infarction (CI), namely ischemic stroke, refers to ischemia, hypoxia, necrosis and neurological disorders. It is even characterized by deficits in brain tissues due to abnormal cerebral blood supply[1]. Despite the rapid development of medical technology, CI is still a leading cause of death and disability in the world[2]. Therefore, early diagnosis and treatment of CI are of great significance.

The Wnt/β-catenin signaling pathway plays an important role in mammalian growth and development, cell proliferation and differentiation, as well as energy metabolism[3]. In the CI model, up-regulation of β-catenin expression can significantly increase the regeneration level of neural stem cells[4]. In addition, the activation of Wnt/β-catenin can improve the nerve injury induced by middle cerebral artery ischemia-reperfusion by inhibiting the neuronal autophagy in the CI region of mice[5]. Previous studies have indicated that the activation of the Wnt/β-catenin pathway may be a potential target for CI therapy.

Micro-ribonucleic acid (miRNA) is a group of single-stranded non-coding RNA with 20-24 nt in length, which exists in eukaryotes with regulatory function[6]. MiRNA can regulate the expression of a variety of genes by targeted binding to specif-

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ic genes. Meanwhile, miRNA plays an important role in multiple physiological activities, such as cell proliferation, differentiation and apoptosis[7]. For example, inhibiting miR-155 can significantly improve neurological impairment, reduce CI volume and effectively promote angiogenesis in the ischemic region in CI rats. The possible underlying mechanism may be mediated by the angiotensin type 1 receptor/vascular endothelial growth factor receptor 2 (AT1R/VEGFR2) pathway[8]. Zhao et al[9] have demonstrated that miR-195 can inhibit VEGF-A in a targeted manner. Therefore, inhibiting the expression of miR-195 can remarkable accelerate angiogenesis in the ischemic region. However, no researches have reported the influence of miR-124 on neuronal apoptosis after CI. Therefore, in the present study, Wistar rats were used as objects and the CI model was established. Furthermore, we also investigated the effect of miR-124 on neuronal apoptosis after CI and the possible underlying mechanism.

Materials and Methods

Animal Grouping and Modeling

A total of 60 male Wistar rats aged 12-14 weeks old weighing (275.13±10.31) g were divided into the Sham group (n=20), the CI group (n=20) and the CI + miR-124 mimic group (n=20) using a random number table. There were no statistically significant differences in baseline data (such as week age and body weight) among the three groups. Therefore, the data were comparable among the three groups (p>0.05). The surgical procedure was as follows: 1) the rats were anesthetized and fixed, 2) the left common carotid artery and vagus nerve were isolated, 3) the proximal ends of left common carotid artery and external carotid artery were ligated, 4) a slip knot was made at the distal end of left common carotid artery, 5) the internal carotid artery was isolated and an incision was made at the proximal end of internal carotid artery, 6) the suture was inserted from the incision and pushed forward for about 18 mm until resistance, indicating that the head of the suture had reached the middle cerebral artery, and 7) the incision was sutured and disinfected. After successful establishment of the CI model, miR-124 was injected into the cerebral ventricle using a stereotaxic apparatus. After 24 h, the sample was collected and scored. All operations in rats were approved by the Animal Ethics Committee.

Triphenyltetrazolium Chloride (TTC) Staining

Specific procedures were as follows: 1) fresh brain tissues were placed in the grinding apparatus, followed by frozen in a refrigerator at -20°C for 0.5 h. 2) The brain tissues were sliced into about 2 mm-thick sections, and the number of sections was no more than 6 for each tissue. 3) The sections were placed in and fully contacted with fresh TTC solution (2%) for incubation for no less than 0.5 h. 4) After 0.5 h, the sections were taken, fixed with 4% paraformaldehyde and photographed.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Detectio

Total RNA was extracted from brain tissues in the infarction region according to the instructions of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The concentration and purity of extracted RNA were detected using an ultraviolet spectrophotometer. RNA with absorbance $(A)_{260}/A_{280}$ of 1.8-2.0 could be used. Extracted messenger RNA (mRNA) was synthesized into complementary deoxyribonucleic acid (cDNA) through RT and stored in a refrigerator at -80°C. Specific RT-PCR system was: 2.5 μL 10 × Buffer, 2 μL cDNA, 0.25 μL forward primer (20 μmol/L), 0.25 μL reverse primer (20 μmol/L), 0.5 μL dNTPs (10 mmol/L), 0.5 μL Taq enzyme (2×10^6 U/L) and 19 μL ddH_2O. The amplification system for RT-PCR was the same as above. Primers used in this study were: miR-124, F: 5'-CA-GAGTC-CAAAACGTGTTCTCGCTC-3', R: 5'-CTAGA-CAGATTACACTGTTGAAGGA-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'.

Western Blotting

CI tissues in rats of each group were fully ground in lysis buffer, followed by ultrasonic lysis. The lysis buffer was centrifuged, the supernatant was collected and sub-packaged into an Eppendorf tube (EP). The concentration of the extracted protein was detected using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA) and ultraviolet spectrophotometry. All protein samples were quantified to the same concentration, sub-packaged and stored in a refrigerator at -80°C for subsequent use. Then the extracted proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). The membranes were incubated with primary

antibody at 4°C overnight. Next, the membranes were incubated with the goat anti-rabbit secondary antibody at room temperature for 1 h in the dark. The protein band was scanned and quantified by Odyssey scanner. The protein expression level was finally calculated normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Immunofluorescence

Brain tissue sections were baked in an oven at 60°C for 30 min, followed by dewaxing with xylene (5 min × 3 times), and dehydration with 100% ethanol, 95% ethanol and 70% ethanol for 3 times. The activity of endogenous peroxidase was inhibited using 3% hydrogen peroxide-methanol. Then the sections were sealed with goat serum for 1 h, and incubated with the anti-caspase-3 antibody (diluted at 1:200) at 4°C overnight. After washing with phosphate-buffered saline (PBS) (Beyotime, Shanghai, China) on a shaking table 4 times, the sections were incubated again with fluorescein isothiocyanate-secondary antibody at 37°C for 1 h. The nucleus was stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA). After color development, 6 samples were randomly selected from each group, and 5 visual fields were randomly selected in each sample. Finally, photography was performed under a fluorescence microscope (200×).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Staining

Brain tissue sections were baked in an oven at 60°C for 30 min, followed by dewaxing with xylene (5 min × 3 times), and dehydration with 100% ethanol, 95% ethanol and 70% ethanol 3 times. Then the sections were incubated with protein kinase K for 0.5 h and washed with PBS. After that, the sections were reacted with terminal deoxynucleotidyl transferase and Luciferase-labeled dUTP at 37°C for 1 h. The horseradish peroxidase-labeled specific antibody was added for incubation again in a 37°C incubator for 1 h. After the nucleus was stained with DAPI, the sections were photographed and counted under a fluorescence microscope.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for all statistical analysis. Measurement data were expressed as mean ± standard deviation. *t*-test was used to compare the differences between the two groups. $p<0.05$ was considered statistically significant.

Results

Expression of MiR-124 in Brain Tissues of each Group

RT-PCR results revealed that the expression of miR-124 in brain tissues of the CI group was significantly decreased than that of the Sham group ($p<0.05$). 24 h after injection of miR-124 mimics into the lateral ventricle, the expression of miR-124 in the CI region was significantly increased ($p<0.05$) (Figure 1). This indicated the successful establishment of the miR-124 overexpression model.

Postoperative Modified Neurological Severity Score (mNSS) of Rats in Each Group

At 24 h after operation, neurological deficits were found in all groups except for the Sham group, including reduced activity, depression, inability to walk straight as well as leaning or rotating to one side. Therefore, the neurological function of rats in each group was scored using the mNSS (modified neurological severity score) scale. The results showed that the score in the CI

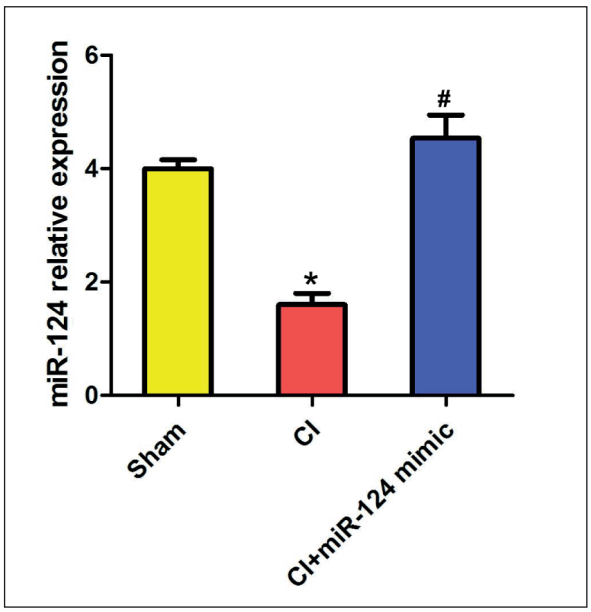

Figure 1. Expression level of miR-124 in the CI region in different groups. Sham: Sham operation, CI: CI group, CI + miR-124 mimics: miR-124 intervention group. $p<0.05$ *vs.* *Sham group and vs. #CI group.

Table I. mNSS of rats in each group after CI (n=20).

Group	Score ($\bar{\chi}$±s)	F	*p*
Sham	0	221.3	0.00
CI	15.12 ±1.21		
CI + miR-124 mimic	5.21±0.44		

group was significantly higher than that of the Sham group ($p<0.05$). However, it was significantly reduced after injection of miR-124 mimics ($p<0.05$) (Table I and Figure 2). These results suggested that miR-124 could improve CI-induced neurological dysfunction.

TTC Staining of Rats in Each Group After CI

After TTC staining, brain tissues in CI region displayed grey white color, while those in non-CI region showed red color. TTC staining results manifested that there was no infarction region in brain tissues of the Sham group, whereas significant CI was found in the CI group. Meanwhile, there was also a certain infarction region in brain tissues of the CI + miR-124 mimics group. However, the scope of infarction region in the CI + miR-124 mimics group was significantly smaller than that of the CI group (Figure 3), indicating that miR-124 could alleviate CI caused by middle cerebral artery ischemia to a certain degree.

Neuronal Apoptosis of Rats in Each Group After CI

To further evaluate the influence of miR-124 intervention on neuronal apoptosis of rats after CI, the expression of apoptosis-related proteins in each

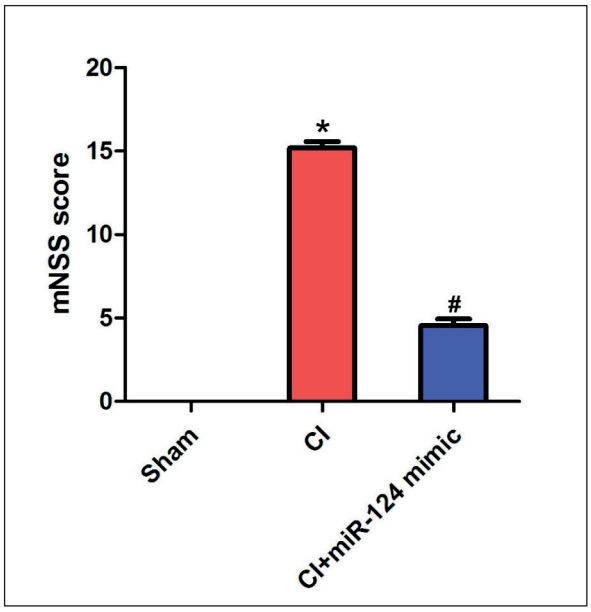

Figure 2. The mNSS of rats in different groups. Sham: Sham operation, CI: CI group, CI + miR-124 mimics: miR-124 intervention group. $p<0.05$ *vs.* *the Sham group and *vs.* #CI group.

group was detected *via* Western blotting (Figure 4A-4D). Meanwhile, the expression and location of caspase-3 in nerve cells were detected *via* immunofluorescence staining (Figure 4E). The results showed that miR-124 could significantly inhibit the expression of pro-apoptosis gene B-cell lymphoma-2 associated X protein (Bax) and caspase-3 cleavage. Moreover, miR-124 remarkably promoted the expression of anti-apoptosis gene Bcl-2. The above results suggested that miR-124 could inhibit CI-induced neuronal apoptosis.

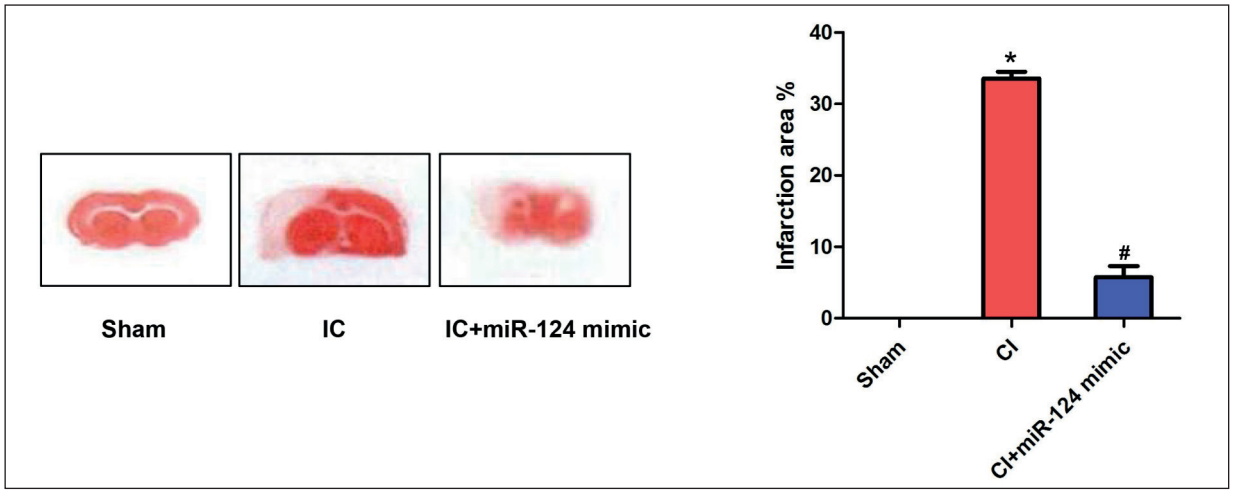

Figure 3. TTC staining of rats in different groups after CI. Sham: Sham operation, CI: CI group, CI + miR-124 mimics: the miR-124 intervention group. $p<0.05$ *vs.* *Sham group and *vs.* #CI group.

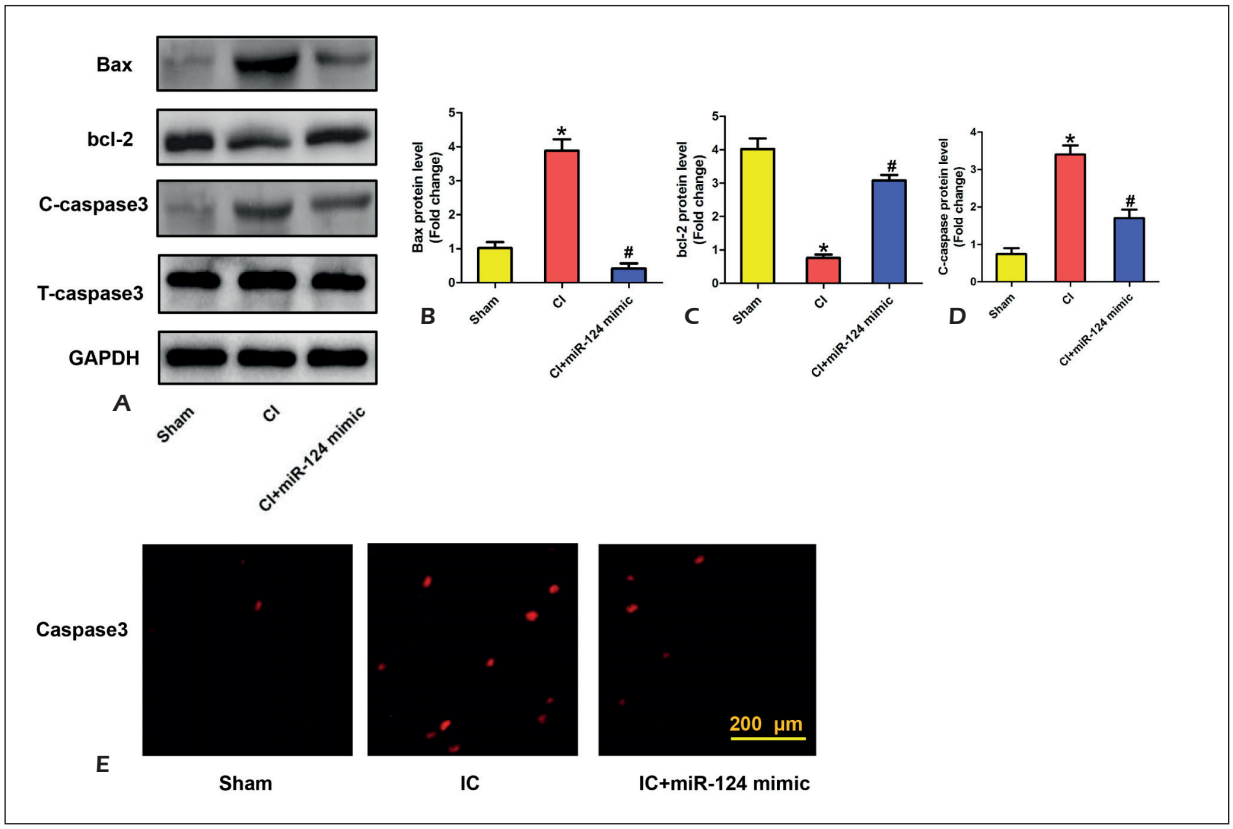

Figure 4. Neuronal apoptosis of rats in different groups after CI. MiR-124 could markedly decrease the expression of the pro-apoptosis gene Bax and caspase-3 cleavage, whereas significantly increase the expression of the anti-apoptosis gene Bcl-2 via Western blot ***(A-D)*** and immunohistochemistry ***(E)***. Sham: Sham operation, CI: CI group, CI + miR-124 mimics: miR-124 intervention group. $p<0.05$ *vs.* *Sham group and *vs.* #CI group. Red fluorescence: caspase-3.

Comparison of TUNEL Results Among Groups

To further explore the effect of miR-124 on neuronal apoptosis, TUNEL staining was performed in the brain tissues of rats in each group. The number of apoptotic neurons was quantified. Results manifested that the number of apoptotic neurons in the CI group was about 13 times that of the Sham group. Meanwhile, miR-124 intervention could significantly reduce the neuronal apoptosis to 6 times as that of the Sham group ($p<0.05$) (Figure 5), proving once again that miR-124 had an anti-apoptotic effect on nerve cells.

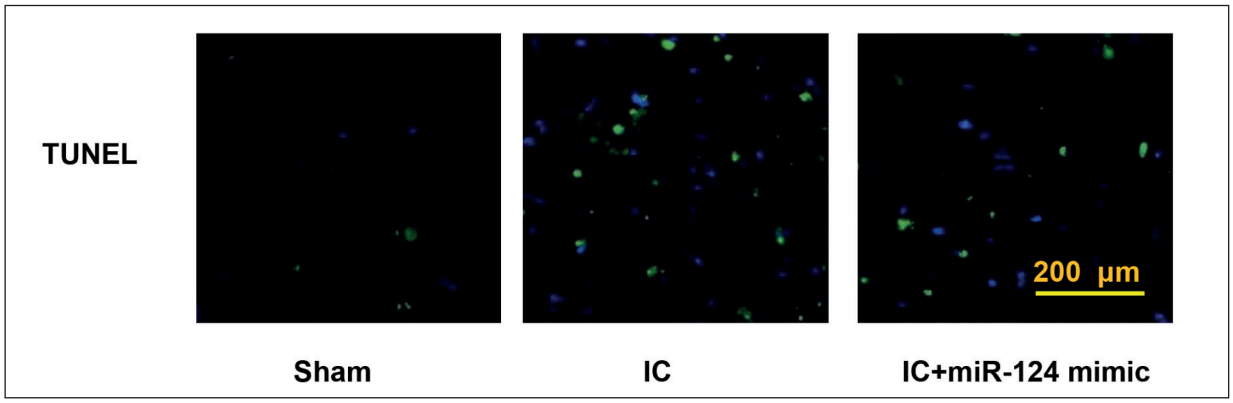

Figure 5. TUNEL staining of neurons in rats of different groups after CI. Sham: the Sham operation, CI: CI group, CI + miR-124 mimics: miR-124 intervention group. Green fluorescence: TUNEL (+), blue fluorescence: cell nucleus.

Regulatory Effect of MiR-124 on Wnt/β-Catenin Signaling Pathway

Considering the important role of the Wnt/β-catenin signaling pathway in cerebrovascular diseases, whether the inhibition of the Wnt/β-catenin signaling pathway in CI can be regulated by miR-124 is another question. Therefore, we detected the protein expression levels of Wnt and β-catenin in each group *via* Western blotting. Results indicated that the expression levels of Wnt and β-catenin in the CI group were significantly lower than that of the Sham group ($p<0.05$). After miR-124 intervention, the inhibitory effects on Wnt and β-catenin could be reversed ($p<0.05$) (Figure 6). These results further clarified that the regulatory effect of miR-124 on neuronal apoptosis in CI rats was mediated by the Wnt/β-catenin signaling pathway.

Discussion

CI is one of the most common cerebrovascular diseases, whose mortality rate has increased year by year. The occurrence and development of CI are closely related to genetic changes[9]. With the development of modern molecular biology and bioinformatics, it has been proved[10,11] that increasingly more genes, RNAs and proteins are involved in the occurrence and development of CI. Current studies[12,13] have found that neuronal apoptosis plays an extremely important role in the cerebral ischemia-reperfusion injury. Meanwhile, the level of apoptosis directly determines the severity and prognosis of the disease. In this study, Wistar rats were used as experimental subjects. The middle cerebral artery was embolized by the suture-occluded method to simulate the attack of CI in clinic. At the same time, miR-124 was injected into the lateral ventricle. Our findings clarified that miR-124 could activate the Wnt/β-catenin signaling pathway, ultimately inhibiting neuronal apoptosis during CI.

Apoptosis, also known as programmed death, refers to gene-controlled autonomic ordered cell death to maintain homeostasis under physiological or pathological conditions[14]. Sung et al[15] have demonstrated that various apoptosis-inducing signals are activated during CI. This may induce neuronal edema, apoptosis or necrosis in the infarction region. Particularly, neurons die in the form of necrosis in the first 6 h after CI, eventually dying in the form of apoptosis. Therefore, in this study, the samples were collected at 24 h after CI to further study the effect of miR-124 on neuronal apoptosis. During apoptosis, cell fate is directly determined by the levels of Bax and Bcl-2. When the pro-apoptosis gene Bax is dominated, programmed cell death occurs. On the contrary, when the expression of the anti-apoptosis gene Bcl-2 is increased, apoptosis will be inhibited[16]. In this study, miR-124 remarkably up-regulated the expression of the anti-apoptosis gene Bcl-2, whereas inhibited the level of the pro-apoptosis gene Bax, ultimately inhibiting CI caused by ischemia and hypoxia.

MiR-124, a member of the miRNA family, is highly expressed in the central nervous system of human and various mammals. On the one hand, miR-124 promotes neuronal development,

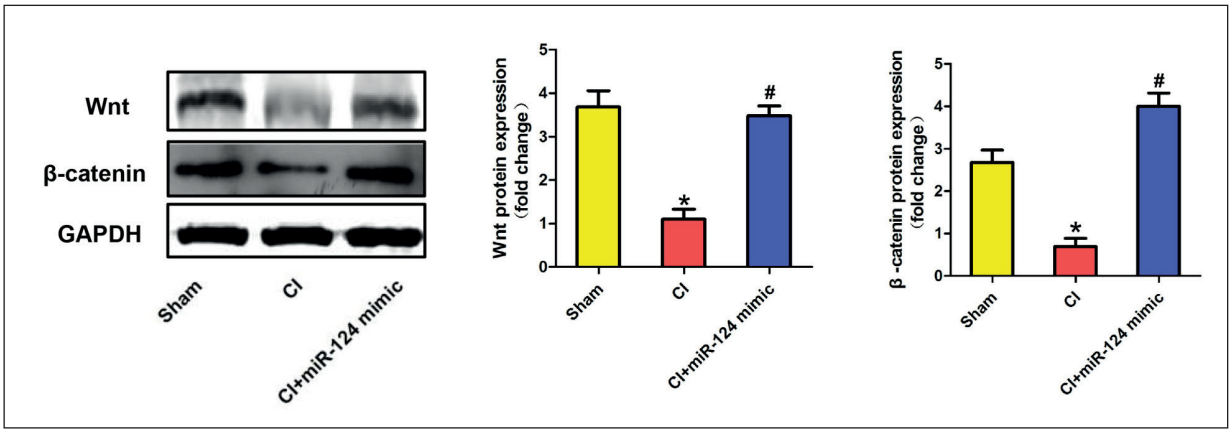

Figure 6. Expression of the Wnt/β-catenin signaling pathway in rats of different groups after CI. Sham: Sham operation, CI: CI group, CI + miR-124 mimics: miR-124 intervention group. $p<0.05$ *vs.* *Sham group and *vs.* #CI group.

including promoting neurite extension, inducing differentiation of stem cells into neurons and facilitating maturation of neurogliacyte. On the other hand, miR-124 can be involved in the repair of damaged neurons[17]. For example, the expression level of miR-124 in the cerebral injury area is significantly declined in the mouse model of cerebral hemorrhage. Meanwhile, the expressions of pro-inflammatory factors are also markedly down-regulated. However, the expression levels of anti-inflammatory factors are remarkably increased in the cerebral injury area after injection of miR-124 mimics into the ventricle. Yu et al[18] have demonstrated that such protective effect of miR-124 may be related to its targeted inhibition on C/EBP-α[18]. Jiao et al[19] have suggested that miR-124 can activate the Wnt/β-catenin signaling pathway through targeted inhibition on DACT1 expression, ultimately promoting the differentiation of neural stem cells into neurons. In this study, we also found that miR-124 activated the Wnt/β-catenin signaling pathway. Interestingly, miR-124 inhibits Capn4 in a targeted manner, thereby inhibiting the Wnt/β-catenin signaling pathway in nasopharyngeal carcinoma cells[20]. It is speculated that such difference is due to that the targeted inhibition of miR-124 on target genes is different in distinct disease models. In addition, different target genes may exert completely opposite effects on the Wnt/β-catenin signaling pathway.

However, there were still some deficiencies in this experiment as follows: (1) the conclusion was not verified *via* cell experiments. (2) The direct target of miR-124 in neurons was not investigated. (3) The initiation pathways of apoptosis are different. Therefore, is the initiation of neuronal apoptosis dependent on external pathways (such as Fas or tumor necrosis factor pathway) or mitochondrial pathway? The above scientific issues remained to be further explored. In spite of these deficiencies, this study still revealed that miR-124 exerted an important regulatory effect on neuronal apoptosis in CI.

Conclusions

We showed that miR-124 can inhibit neuronal apoptosis in the CI region in rats by activating the Wnt/β-catenin signaling pathway, ultimately exerting a protective effect. Furthermore, miR-124 is expected to be a targeted drug for the treatment of CI in the future.

Conflict of Interests

The authors declare that they have no conflict of interest.

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