THE EFFECT OF MIR-206 ON MOTOR AND COGNITIVE FUNCTION IN RATS WITH CEREBRAL INFARCTION BY DOWN REGULATING BDNF

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ABSTRACT

Objective: *To explore the relationship between miR-26 and brain-derived neurotrophic factor (BDNF), and the role of miR-26 in the neural function of rats with cerebral infarction (CI).*

Methods: *The CI rat model was constructed by using cerebral artery ischemia/reperfusion. The triphenyltetrazolium chloride (TTC) method was used to detect the infarct area of rats in each group to determine whether the model was successfully constructed. At the same time, RT-qPCR was used to detect the transcription level of miR-206 and BDNF in the brain tissue of rats in each group. We then tested the luciferase activity of co-transfected miR-206 and BDNF 3`UTR plasmid ha cells by using double luciferase assay. To further verify the relationship between miR-206 and BDNF and the regulatory mechanism, we constructed the low expression HA cells of miR-206 by using an miR-206 inhibitor and constructed the in vitro model by anoxia/reoxygenation. Meanwhile, RT-qPCR was used to detect the transcription level of miR-206 and BDNF in each group of cells, and Western Blotting and TURNEL staining was used to detect the apoptosis level of each group of cells, as well as the expression of related apoptosis proteins and TrkB-Akt pathway related proteins. To further explore the role of miR-206 and BDNF in the motor and cognitive function recovery of rats with CI, we injected BDNF into the brain to intervene in the animal model, and detected the CI area of the model group and BDNF group by the TTC method, following which we observed the motor and cognitive ability recovery level of rats by neurological deficit score.*

Results: *First, we confirmed the successful implementation of the brain infarction rat model through a TTC experiment. At the same time, RT-qPCR showed that the BDNF level was significantly down regulated and the miR-206 level was down regulated, which was negatively correlated. In addition, luciferase report showed that miR-206 could directly exert negative regulation on BDNF. RT-qPCR results revealed that the BDNF expression of hypoxia/reoxygenation HA cells was significantly up-regulated by the miR-206 inhibitor, whereas turn results showed that the apoptosis level of miR-206 inhibitor group was significantly reduced, and Western Blotting results showed that the ratio of Bax/Bcl-2 of apoptosis protein was significantly reduced, with the expression of TrkB and pAkt being significantly increased. Furthermore, the animal level indicated that the injection of BDNF into the brain could significantly improve the area of CI and promote the recovery of motor and cognitive function in rats with CI.*

Conclusion: *miR-206 can inhibit the recovery of motor and cognitive function in rats with CI by down regulating the BDNF and TrkB-Akt pathway.*

Keywords: *Cerebral infarction, miR-206, BDNF, Motor and cognitive function.*

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Introduction

Cerebral infarction (CI) is one of the most common clinical cardiovascular and cerebrovascular diseases, in which the common form is stroke, which has a high incidence and mortality, leading to approximately 75% patients being disabled patients to different degrees and imposing a heavy economic burden on the society[1, 2]. The pathological basis of CI is related to the interruption of cerebral blood supply, cerebral malacia and cerebral ischemic necrosis caused by local ischemia and hypoxia[3]. Because CI can lead to brain neuron necrosis, resulting in neurological dysfunction, threatening human health and affecting the quality of life, it is very important to provide a powerful treatment method to promote angiogenesis in the early stage of CI(4). Brain-derived neurotrophic factor (BDNF) belongs to the family of neurotrophic growth factors which are secreted by brain cells and abundant in the brain, and plays an

important role in regulating the survival, growth and differentiation of neurons[5]. BDNF can promote the maturation of neurons and glia, participate in the differentiation of axons and dendrites, and improve the survival rate of neurons[6, 7]. Up regulation of BDNF is believed to have beneficial effects on many nervous system diseases[8, 9]. In addition, BDNF plays an important role in learning, memory and sensory motor recovery by promoting the plasticity of synapses and axons[10]. BDNF appears in the early stage of nerve development and contributes to nerve repair by promoting neurogenesis and angiogenesis[11].

MicroRNA (miRNA) is a new type of endogenous small non coding RNA, which consists of approximately 18-24 nucleotides. miRNA controls gene expression by targeting mRNA degradation or translation inhibition. Since the discovery of the first miRNA lin-4, research on miRNA has increased exponentially[12]. So far, the role of miRNA has been proved in many pathophysiological processes including stroke. Takuma et al. first reported the miRNA expression profile of cerebral ischemia in brain and blood[13]. Since then, several studies have been carried out on miRNA expression in stroke using human samples and animal models[14, 15]. These reports suggest that miRNA is a biomarker in the diagnosis and prognosis of cerebral ischemia and the potential function of therapeutic targets. However, the expression of miRNA in the process of stroke recovery is still poorly understood[16, 17].

In this study, we found that there was a negative correlation between the level of miR-206 and BDNF in the brain tissue of CI model rats. The luciferase reporter gene experiment showed that miR-206 can regulate the expression of BDNF by binding to its 3`UTR. At the same time, inhibiting the expression of miR-206 can inhibit the apoptosis of hypoxia/reoxygenation HA cells by promoting the expression of BDNF and activating the TrkB-Akt pathway. Similarly, the brain injection of BDNF can improve the recovery of motor and cognitive function and reduce the area of CI. In conclusion, our results show that miR-206 can inhibit the recovery of motor and cognitive function in rats with CI by down regulating BDNF and inhibiting the TrkB-Akt pathway.

Methods

Experimental animals and cells

SD rats used in the experiment were provided by the experimental animal centre of Yangzhou University. The rats were randomly divided into three groups and fed with water freely under appropriate temperature, humidity and light conditions, and fasted 12 hours before operation. Control group (n = 40): the middle cerebral artery and bilateral carotid artery of rats was exposed without other treatment. Model group (n = 40): the occlusion of the left middle cerebral artery and left internal carotid artery was temporarily 1.5 hours.

The human astrocytes (HA) used in the experiment were from the cell research institute of the Shanghai Academy of Sciences and were cultured in DMEM/F12 medium containing 10% foetal bovine serum and 100 μl/ml penicillin streptomycin.

Control random sequence plasmids and miR-206 inhibitors were transfected according to the manufacturer's protocol.

Establishment of CI rat model

Rats were intraperitoneally injected with 10% chloral hydrate (400 mg / kg, Qingdao yulonghai Manufacturing Co., Ltd., China). After anaesthesia, the right side of the rats was placed down on the operating table, the reserved skin was selected in front of 1/3 of the left ear and the corner of the eye, and an incision of approximately 1.5cm was cut perpendicular to the joint. A 2mm^2 bone hole was then drilled at the front junction of the scaly part of the temporal bone and the arch with a low-speed drill. In the drilling operation, the isotonic saline was infused into the local bone surface to prevent thermal damage to the brain tissue being produced in the drilling process.

Finally, the thin layer of the inner temporal bone plate was reserved to prevent the thermal and mechanical operation on the cerebral cortex. The thin inner plate was torn off, the dura mater exposed and gently torn off, and then the left side of the middle cerebral artery exposed. Before branching, the distal cerebral vein was ligated and the end of the cerebral artery with surgical line. After successful ligation, gelatin sponge was used to cover the cortical surface and bony foramen, and temporal muscle was reset. The whole operation was gentle to avoid damage to brain tissue and blood vessels. After ligation of the middle cerebral artery, the rats were supine, and the incision was made in the right middle of the neck to expose the left carotid artery, which was sutured and ligated. The right carotid artery was exposed, and the contralateral carotid artery was clamped for 1.5 h. The operation site was washed and sutured with 30% hydrogen peroxide, and the skin incision was detoxified. After the rats were conscious, they were fed with water and kept continuously.

Neurological deficit score

On the 15th day after injection, neurological function was evaluated in the BDNF model group and CI model group.

Walking evaluation of balance beam

A square wood with a length of 150 cm and a width of 2 cm is used as the balance beam, and is placed horizontally at a distance of 10 cm from the ground, on which the rats can crawl.

The scores are as follows:

• 0 points: the rats scan, jump and move the balance beam without dropping;

• 1 point: the rats can jump and move on the balance beam with a drop rate of less than 50%;

• 2 points: the rats can jump and move the balance beam with a drop rate of more than 50%;

• 3 points: the rats can jump on the balance beam with the help of healthy limbs but cannot move forward;

• 4 points: the rats cannot move and will fall down;

• 5 points: the mouse falls off the balance beam immediately.

Rotating rod travel evaluation

The wooden rod was fixed on the rotator and rotated clockwise and anticlockwise alternately.

The scoring is as follows:

• 0 points: the rats can move in the process of rod rotation;

• 1 points: the rats will not fall off the rod, and the duration is ≥60s;

• 2 points: the rats will not fall off the rod until the beginning of rotation;

• 3 points: once the rotation starts, the rats will fall off the rod.

Screen test evaluation

The screen is a 50×40 cm mesh belt with a mesh of 1×1 cm and a height of 80 cm from the ground; 15 cm thick sponge is laid under the screen.

First, place the sieve horizontally and place the rat on the sieve, then raise one side of the sieve, then slowly raise the sieve to the vertical position within 2 s and hold for 5 s.

The score is as follows:

• 0 points: the rat's forepaw can grasp the screen for about 5S, during which time the rat will not fall;

• score 1 points: the rat can temporarily grasp the screen and slide a distance, but it will not fall off the screen;

• score 2 points: the rat will fall off the screen within 5 seconds;

• 3 points: when the screen rotates, the rat immediately falls off the screen.

Western blotting

Protein lysates were prepared from tissues or cells and dissolved by 10% SDS-PAGE. The protein samples were transferred to a PVDF membrane and sealed with 5% skimmed milk for 2 hours. The membrane was washed three times with tris buffer brine containing Tween 20 (TBST) and incubated overnight with anti-human BDNF primary antibody (AV41970; Sigma-Aldrich) at 4°C. The membrane was washed again with TBST and incubated at room temperature. HRP labelled antibodies were incubated for 1 h, and DAB (3,3-diaminobiphenylamine; Sigma-Aldrich) was used to observe the signal. Actin was used as a control.

RT-qPCR

From each group, 100 mg of brain tissue was taken and put into a glass homogenizer. Trizol reagent (3ml) was added and tissue RNA extracted according to the instructions of the RNA Extraction Kit (Shanghai Biotechnology Co., Ltd.). The reverse reaction conditions were 70oC for 5 min, 42oC for 60 min and 70oC for 10 min. The primer sequences used for amplification were as follows: GAPDH forward 5'-CCCACTCCTCCACCTTTGAC-3'; reverse 5'-CCACCACCCTGTTGCTGTAG-3'; BDNF forward 5'-GTCCACGGACAAGGCAA-3'; reverse 5'-AGG GACGTCGTCGTCAGAC-3'; miR-206 forward 5'-CGGGCTTGTGGAATGGTAAGC-3', and reverse 5'-GCTTCGGCAGCACATATACTAAAAT-3'. The reaction conditions were: 94oC denaturation for 30 s, 56oC annealing for 45 s, 72oC extension for 45 s and 30 cycles in total. PCR products were detected by agarose gel electrophoresis.

TTC staining

Twenty-four hours after operation, TTC staining was performed on 10 rats in each group, and the brain stem was removed. The brain was placed in a refrigerator at -15°C for about 30 minutes, then removed and left at room temperature for 30 seconds. After the slide was separated from the brain tissue, the cerebellum was cut and discarded, and the brain tissue fixed on the tray with glue.

TUNEL staining

TUNEL staining was carried out according to the instructions provided by the manufacturer (Roche, Basel, Switzerland). The apoptosis index was defined as the average percentage of TUNEL positive cells relative to the total number of cells.

Statistical methods

The data were analysed with Graphpad 8.3.0 statistical software and expressed as mean ± standard deviation. The difference was statistically significant (P<0.05).

Results

The expression of BDNF and miR-206 was negatively correlated in rats with CI

To explore the related pathological mechanism of CI in rats, we constructed the CI model by cerebral artery ischemia/reperfusion and measured the CI area of blank control group and CI model group by TCC method to determine whether the model was successfully constructed.

The results showed that the CI area of model group was significantly higher than that of control group (Figure A). Meanwhile, we detected the vacancy by RT-qPCR experiment. The results showed that the expression of BDNF and miR-206 in the brain tissue of the model group was significantly lower than that of the blank group, whereas the level of miR-206 was significantly higher than that of the blank group, which was negatively correlated (Figure B and C).

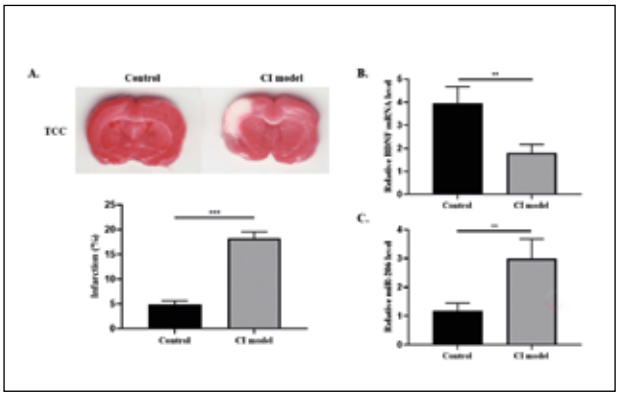

Figure 1: The expression of BDNF and miR-206 in CI rats is negatively correlated.
*A. The CI levels of the blank control group and CI model group rats were measured by TTC method, and the infarct area was calculated. B. RT-qPCR was used to detect the transcription level of BDNF in the brain tissues of rats in the blank control group and CI model group. C. RT-qPCR was used to detect miR-206 transcription levels in the brain tissues of rats in the blank control group and CI model group. **, p<0.01; ***, p<0.001.*

BDNF is the direct target of miR-206

To explore the relationship between miR-206 and BDNF, we designed the following possible binding sequences of BDNF and miR-206 (Figure A). At the same time, we carried out luciferase experiments to determine whether there is a relationship between miR-206 and BDNF. By amplifying the 3`UTR of human BDNF and cloning the amplified fragment into pmirGLO vector, the target region predicted by miR-206 (UGUAAGG) in the 3`UTR of BDNF was mutated. The results demonstrated that the luciferase activity of miR-206 group was significantly lower than that of the control group, which indicated that the expression of BDNF was negatively regulated by miR-206, and that the luciferase activity could be restored by co-transfection of mut-BDNF 3`UTR plasmid and miR-206 mimic into HA cells, suggesting that BDNF was the direct target of miR-206 Mark (Figure B).

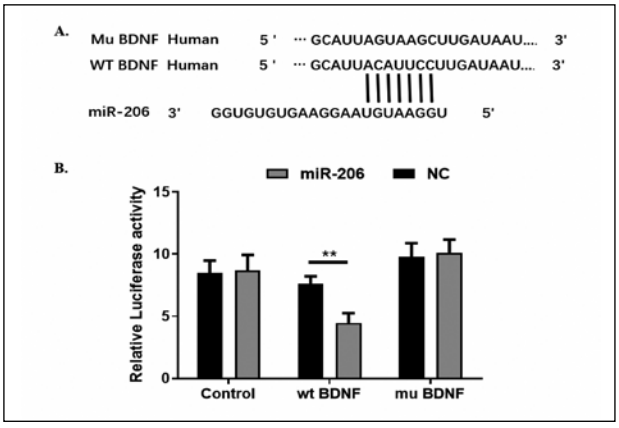

Figure 2: BDNF is a direct target of miR-206.
*A. The possible binding sequence of miR-206 to human BDNF 3`UTR region. B. The luciferase reporter assay was used to detect the luciferase activity of HA cells by co-transfection of miR-206 and BDNF 3`UTR plasmid. **, p<0.01.*

miR-206 inhibitors inhibit the hypoxia/reoxygenation induced apoptosis of HA cells by up regulating BDNF expression

To further explore the relationship between miR-206 and BDNF and its role in anoxic/reoxygenated HA cells, we used miR-206 inhibitors to interfere. RT-qPCR results showed that miR-206 inhibitors could significantly inhibit the expression of miR-206 in anoxic/reoxygenated ha cells and significantly improve the expression of BDNF in anoxic/reoxygenated ha cells (Figure A and B). At the same time, the results of TURNEL staining and Western Blotting showed that the miR-206 inhibitor significantly reduced the apoptosis level of hypoxia/reoxygenation HA cells, significantly reduced the proportion of Bax/Bcl2, and increased the protein expression level of BDNF (Figure C and figure D).

In addition, the results showed that the miR-206 inhibitor could significantly increase the expression of TrkB and p-Akt in hypoxia/reoxygenation ha cells (Figure E).

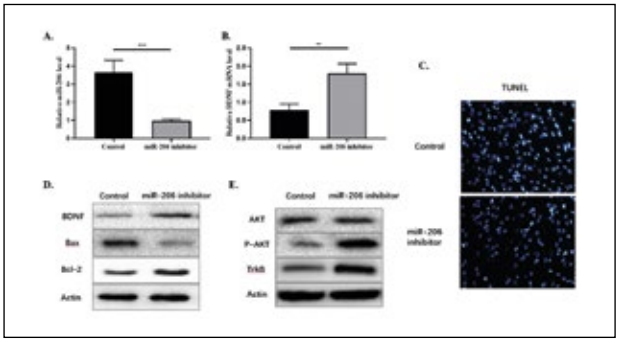

Figure 3: miR-206 inhibitors inhibit hypoxia / reoxygenation apoptosis by up-regulating BDNF expression.
*A & B. The transcription levels of miR-206 and BDNF in HA cells treated with control scrambled plasmids and miR-206 inhibitors were detected by RT-qPCR. C. The apoptosis level of HA cells treated with control scrambled plasmid and miR-206 inhibitor was detected by TUNEL staining. D & E. Western blotting was used to detect the expression levels of apoptotic proteins Bax/Bcl-2 and AKT, pAKT, TrkB in HA cells treated with control scrambled plasmid and miR-206 inhibitor. **, p<0.01; ***, p<0.001.*

BDNF can promote the recovery of motor and cognitive function in rats with CI

To further explore the role of miR-206 and BDNF in the motor and cognitive function recovery of rats with CI, we intervened via the intracerebral injection of BDNF. The results of the TTC showed that the area of CI in the BDNF intervention group decreased significantly (Figure A). At the same time, we evaluated the neurological deficit of rats in each experimental group. The results demonstrated that the BDNF intervention group significantly promoted the motor and cognitive function recovery of rats with the CI model (Figure B, figure C and Figure D).

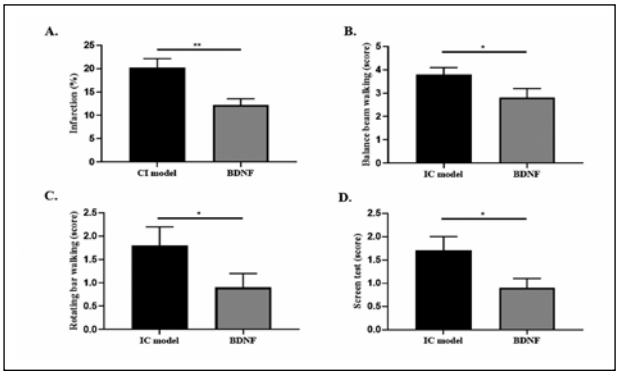

Figure 4: BDNF promotes motor and cognitive function recovery in rats with CI.
A TTC test was used to detect the CI area in rats with the CI model group and rats with the BDNF intervention group, and neurological deficit scores in rats with the CI model group and BDNF intervention group.

Discussion

Stroke is one of the major causes of global death and adult disability. The possible strategies for the treatment of ischemic stroke include intravenous thrombolysis (rt-PA), neuroprotection and neuronal repair. Although rt-PA is the only approved treatment for ischemic stroke, the risk of bleeding and its narrow treatment window (4-5 hours) limit the number of stroke patients that can receive such treatment (5-10%)[18-20]. So far, many of the neuroprotective agents that have been developed and tested in clinical trials have failed to produce satisfactory results. Although neuronal repair therapy has become a feasible treatment option, its progress has been hindered due to the lack of a complete understanding of the basic mechanism[21, 22].

miR-206 is a muscle specific miRNA and a key regulator of muscle cell proliferation, differentiation, apoptosis, migration and angiogenesis[23-26].

Although the expression of miR-206 in a normal brain is poor (unable to detect the level), the high level expression of miR-206 has been observed in AD patients and AD transgenic mouse models, and the overexpression of miR-206 in the cultured primary mouse hippocampal neurons results in the decrease of dendritic spine density[27]. Accordingly, low density dendritic spines have also been observed in the primary hippocampal neurons of an AD model[28]. Studies have shown that miR-206 is involved in the pathogenesis of AD by inhibiting BDNF expression[29]. This study further proved that the intranasal administration of the miR-206 inhibitor improved the memory function and increased the expression of BDNF in the brain. The injection of the miR-206 inhibitor can enhance the memory function, synaptic density and neurogenesis of AD (Tg2576) mice (30). In another study, overexpression of miR-206 was found to inhibit the activity of neural cells[30]. The abnormal development of neural cells induced by miR-206 is believed to be mediated in part by inhibiting the transcription and translation of Orthobox 2 (Otx2) mRNA, which plays an important role in neurogenesis[31, 32]. In the process of abnormal development of neural cells, miR-206 mainly affects cell viability and apoptosis by regulating the expression of Otx2[33]. In summary, miR-206 plays a crucial role in brain nerve function.

In this study, we used the ischemia/reperfusion cerebral artery to construct the CI model of rats and detected the CI area of rats in each group by TTC staining. The results showed that the CI area of rats

in the model group was significantly higher than that in the control group. At the same time, RT-qPCR results showed that BDNF level was significantly down regulated and miR-206 level was down regulated, which was negatively correlated. Furthermore, luciferase experiments showed that miR-206 could directly affect BDNF to produce negative regulation. To further verify the relationship between miR-206 and BDNF, we constructed hypoxia/reoxygenation ha cells with low expression of miR-206 by using miR-206 inhibitors. RT-qPCR results showed that the expression of BDNF in hypoxia/reoxygenation ha cells was significantly up-regulated by using miR-206 inhibitors. The turn results revealed that the apoptosis level of miR-206 inhibitor group was significantly reduced, whereas the Western Blotting showed that the ratio of Bax/Bcl-2 of apoptosis protein was significantly reduced and the expression of TrkB and pAkt was significantly increased.

The above results demonstrate that miR-206 may inhibit the TrkB-Akt signal transduction by inhibiting the expression of BDNF, thus promoting the apoptosis of hypoxia/reoxygenation HA cells. At the same time, we used the intracerebral injection of BDNF to verify the animal level. The results showed that the intracerebral injection of BDNF could significantly improve the area of CI in rats and promote the recovery of motor and cognitive function in rats with CI. In conclusion, our study shows that miR-206 inhibits the TrkB-PI3K/Akt pathway by down regulating BDNF, thus inhibiting motor and cognitive function recovery in rats with CI.

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