A study on the neuroprotective effect of miR-206-3p on Alzheimer's disease mice by regulating brain-derived neurotrophic factor

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Background: Brain-derived neurotrophic factor (BDNF) is involved in the regulation of Alzheimer's disease (AD), but the mechanism is not clear. This study explores the possible mechanism of microRNA-206-3p (miR-206-3p) participating in the neuroprotective effect of AD mice by regulating BDNF.

Methods: 36 SPF grade C57 mice were randomly divided into normal group, model group and miR-206-3p mimics group (intraperitoneal injection of 3 mm miR-206-3p mimics) (n=12). miR-206-3p mimics group was intervened by miR-206-3p mimics on the basis of AD model. The expression of miR-206-3p was detected by Real time quantitative polymerase chain reaction (qPCR). Zea-Longa score and water maze were used for behavioral detection, he was used to observe the morphology of neurons, and immunohistochemical Western blot was used to detect the expression of BDNF protein, and the targeting relationship between miR-206-3p and BDNF was analyzed.

Results: Compared with the model group, the expression level of miR-206-3p in miR-206-3p mimics group was significantly higher (P<0.05). compared with the model group, the Zea-Longa score in miR-206-3p mimics group was significantly lower (P<0.05). The escape latency of miR-206-3p mimics group was significantly shorter than that of model group, and the number of crossing the original platform was significantly more than that of model group (P<0.05). The morphology of neurons in miR-206-3p mimics group was significantly better than that in model group; Immunohistochemistry and Western blot showed that the relative expression of miR-206-3p mimics BDNF protein was significantly increased compared with the model group (P<0.05). Compared with miR-206-3p control group, the luciferase activity at 3' end untranslated area (3' UTR) end of wild-type BDNF in miR-206-3p inhibition group decreased significantly (P<0.01).

Conclusions: miR-206-3p exerts neuroprotective effects on AD mouse neurons by up-regulating BDNF.

Keywords: MicroRNA-206-3p (miR-206-3p); Alzheimer's disease (AD); brain-derived neurotrophic factor (BDNF); neurons

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Introduction

Alzheimer's disease (AD), a fairly common neurodegenerative disorder of the central nervous system, frequently occurs in the elderly and mainly impairs memory, cognitive ability and behavior performance, gradually leading to signs of dementia. It is also known as senile dementia (1,2). Studies (3,4) have demonstrated that the morbidity rate of AD is rising each year. There is also an aging population around the globe. The induced degeneration of memory and cognitive ability seriously affects patients' quality of life and places heavy economic burdens on families and societies.

Brain-derived neurotrophic factor (BDNF) is one of the important members of neurotrophic factor family in the body. It is mainly produced by cerebral cortex and hippocampus, participates in the growth, differentiation and development of neurons, and has a good role in promoting and protecting the nervous system., It is found that the level of BDNF in many patients with nervous system diseases is significantly reduced, and its level is closely related to the learning and memory function of patients; In addition, BDNF is conducive to the survival of neurons in the brain of patients with AD, which is considered to be one of the important targets for the treatment of AD (5-7). As a kind of non-coding RNA, microRNA plays an important role in the body, especially in regulating a variety of physiological and pathological reactions of cells. As an important non-coding RNA, microRNA-206-3p (miR-206-3p) plays an important role in regulating a variety of downstream substances, and then plays a role in regulating a variety of physiological and pathological reactions (8,9). Previous studies have shown that miR-206-3p has a certain anti dementia effect, but its mechanism is not clear, and whether it can play a certain neuroprotective role in patients with AD by regulating BDNF is not clear.

This study sought to investigate the neuroprotective effect of miR-206-3p on AD mice by regulating BDNF. We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-21-6601/rc).

Methods

Laboratory animals

Thirty-six SPF C57 mice {license number: SCXK [Shanghai] 2014-0003} for experiments were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. All the mice were fed a normal diet and sterile filtered water every day at the Laboratory Animal Center under a 12/12 h light/dark cycle and conventional room temperature and humidity. The experiment was carried out according to the project license (No. 2018006) granted by the ethics committee of Gansu Provincial Hospital of Traditional Chinese Medicine, in compliance with Gansu Provincial Hospital of Traditional Chinese Medicine guidelines for the care and use of animals.

Experimental reagents and instruments

The experimental reagents and instruments included miR-206-3p mimics (MCE, USA), amyloid beta-peptide 25–35 (Aβ25–35) (Sigma, USA), anti-BDNF primary antibody and secondary antibody (Abcam, USA), an immunohistochemistry kit, a hematoxylin-eosin (HE) staining kit and a quantitative polymerase chain reaction (qPCR) kit (Vazyme, Nanjing, China), a light microscope (Leica, Germany), and fluorescence qPCR instrument (ABI, USA).

Animal grouping and processing

The 36 C57 mice were divided into the normal group (n=12), model group (n=12), and miR-206-3p mimics group (n=12) using a random number table. The experiments were performed after the mice were adaptively fed at the Laboratory Animal Center for 7 d.

The mice in the normal group were raised normally, those in the model group were used to establish the AD model and then intraperitoneally injected with normal saline, and those in the miR-206-3p mimics group were used to establish the AD model and then intraperitoneally injected with miR-206-3p mimics (3 mM). Subsequently, specimens were obtained from each group after 3 consecutive weeks of the intervention.

AD modeling

The AD mice were intraperitoneally injected with 7% chloral hydrate (5 mL/kg). After successful anesthesia, the mice were fixed on a brain stereotaxic instrument, the drill of the instrument was placed at the hippocampus following local disinfection, and Aβ25–35 was injected into a hole drilled on the skull using a micro-syringe to successfully establish the AD model.

Table 1 Zea-Longa scores

Score	Symptom
0 points	No neurological deficits
1 point	Mild: unable to completely extend the right forepaw when the tail is lifted
2 points	Moderate: circles to right/left when walking
3 points	Severe: tumbles to right/left when walking
4 points	Unable to walk spontaneously with loss of consciousness

Specimen acquisition

After the mice were anesthetized successfully, blood was collected from the abdominal aorta. Specimens were obtained from 6 mice in each group. Specifically, the brain tissues were directly removed, flushed with normal saline, placed in EP tubes. and stored at –80 ℃ for subsequent Western blotting (WB) and qPCR assays. As for the remaining 6 mice in each group, specimens were collected by means of perfusion-fixation. After fixation with paraformaldehyde, the brain tissues were taken out and fixed with 4% paraformaldehyde solution for immunohistochemistry and HE staining.

Behavioral examinations

After the intervention, the neurological deficits of the mice were evaluated via the Zea-Longa score according to the symptoms and manifestations of the mice. The Zea-Longa scores are set out in *Table 1*. The mice were also placed in a water maze after the last intervention and allowed to move freely. The escape latency time and crossing platform time of the mice in the water maze were recorded. Later, the platform in the water maze was removed, and the mice were placed at any site in the water maze and allowed free movement. Original platform crossing times and residence times in the original quadrant within 70 s were recorded.

HE staining

The tissues that had been embedded in paraffin in advance were sliced to 5 µm-thick sections, spread in 42 ℃ warm water, collected, and then baked to prepare the paraffin-embedded tissue sections. The sections were then sequentially immersed in xylene solution and graded alcohol for routine deparaffinization and rehydration. Later, the sections were stained with hematoxylin dye for

5 min using the HE staining kit and placed in pure water for 10 min, followed by color separation with 95% ethanol for 5 s, transparentization in xylene for 10 s, and mounting in neutral balsam.

Immunohistochemistry

The tissues that had been embedded in paraffin in advance were sliced to 5 µm-thick sections, spread in 42 ℃ warm water, collected, and then baked to prepare the paraffin-embedded tissue sections. The above sections were then placed in xylene solution and gradient alcohol for routine deparaffinization and rehydration. Next, the sections were soaked in citric acid buffer solution and heated repeatedly in a microwave oven 3 times (for 3 min) and braised for 5 min each time to achieve adequate antigen retrieval. After rinsing, endogenous peroxidase blocker drops were added to the specimens, which were left to react for 10 min and sealed in goat serum that was added dropwise for 20 after rinsing. Subsequently, the goat serum was shaken off, and the specimens were incubated with anti-BDNF primary antibody (1:200) in a refrigerator at 4 ℃ overnight. The next day, the specimens were rinsed, reacted with a secondary antibody solution that was added in drops for 10 min, rinsed sufficiently again, and then reacted with streptavidin-peroxidase solution for 10 min. Color development with DAB then occurred. The nucleus was counterstained using hematoxylin, and then mounted and observed.

WB assay

The cardiac tissues that had been preserved at an ultra-low temperature were placed in an ice bath to which lysis buffer had been added for 1 h, and centrifuged at 14,000 g for 10 min. Later, the protein was quantified using the BCA method, and the protein concentration

Table 2 Primer sequences

Name	Primer sequence
miR-206-3p	Forward primer: 5'-TTGGGGGTTAGGTTTGGGTTTTTAC-3'
	Reverse primer: 5'-CGTAAACGAAATTCGACCCCACTA-3'
GADPH	Forward primer: 5'-ACGGCAAGTTCAACGGCACAG-3'
	Reverse primer: 5'-GAAGACGCCAGTAGACTCCACGAC-3'

miR-206-3p, microRNA-206-3p; GADPH, glyceraldehyde 3-phosphate dehydrogenase.

of the tissues was calculated based on the absorbance and standard curve obtained from a microplate reader. Subsequently, the proteins in the tissue specimens were denatured and then separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis, which was terminated when the marker proteins reached the bottom of the glass plate in a straight line. Next, the proteins were transferred onto a polyvinylidene fluoride membrane and sealed in blocking buffer for 1.5 h, after which anti-BDNF primary antibody (1:1,000) and secondary antibody (1:1,000) were sequentially added. Finally, the images were developed using chemiluminescent reagent in the dark for 1 min after rinsing.

qPCR assay

RNA extraction reagent was added to the cardiac tissues, which had been stored for use, to extract the total RNA in the specimens. Next, the total RNA extracted was reverse transcribed into complementary DNA (cDNA) using a reverse transcription kit and a 20 μL reaction system. Reaction conditions: reaction at 53 ℃ for 5 min, pre-denaturation at 95 ℃ for 10 min, denaturation at 95 ℃ for 10 s, and annealing at 62 ℃ for 30 s; 35 cycles in total. The ΔCt value was first calculated, and the difference in the expression of the target genes was then calculated. The detailed primer sequences are shown in *Table 2*.

Luciferase reporter assay

BV2 microglia were cultured to construct the 3' end untranslated area (3' UTR) region wild-type reporter gene plasmid of BDNF gene and the mutant gene reporter plasmid of miR-206-3p binding site with BDNF region. At the same time, miR-206-3p control and miR-206-3p inhibition were transfected and cultured for 24 hours to detect its luciferase activity. Each group is provided with 6 multiple holes.

Statistical methods

In this study, SPSS 20.0 software was used for the statistical analysis. The enumeration data are presented as mean ± standard deviation. The data that had a normal distribution and homogeneity of variance were tested using a *t*-test. The data that had a normal distribution and heterogeneity of variance were tested using a corrected *t*-test. The data that did not have a normal distribution and homogeneity of variance were tested using a non-parametric test. The rank-sum test was used for the ranked data. The chi-square test was used for the enumeration data.

Results

miR-206-3p expression

Compared with the normal group, the expression of miR-206-3p in the model group decreased significantly (P<0.05); compared with the model group, the expression level of miR-206-3p in the miR-206-3p mimics group increased significantly (P<0.05) (see *Table 3*).

Zea-Longa scores

The Zea-Longa score was the lowest in the normal group and the highest in the model group (see *Figure 1*). Additionally, the Zea-Longa score in the model group and miR-206-3p mimics group was significantly increased compared to that of the normal group (P<0.05). Conversely, it was significantly decreased in the miR-206-3p mimics group compared to that of the model group (P<0.05).

Water maze test

As *Figure 2* shows, the model group and miR-206-3p mimics group had a significantly longer escape latency time and took significantly fewer times to cross the original platform than the normal group (P<0.05). Additionally,

Table 3 expression of miR-206-3p in each group ($\bar{x}\pm s$)

Grouping	N	miR-206-3p
Normal group	12	3.39±0.86
Model group	12	1.62±0.58*
miR-206-3p mimics group	12	2.29±0.63#
F	–	19.520
P	–	<0.001

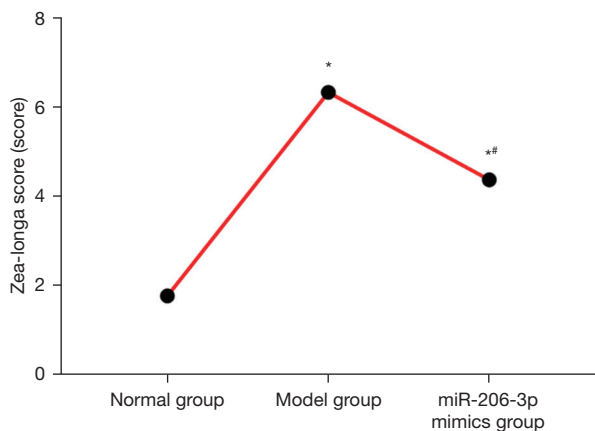*$P<0.05$ vs. normal group; #$P<0.05$ vs. model group. miR-206-3p, microRNA-206-3p.

Figure 1 Zea-Longa scores for each group. *$P<0.05$ vs. normal group; #$P<0.05$ vs. model group. miR-206-3p, microRNA-206-3p.

the miR-206-3p mimics group had a significantly shorter escape latency time and took significantly more times crossing the original platform than the model group ($P<0.05$).

HE staining

In the normal group, the neuronal morphology was normal and there was a large number of Nissl bodies. In the model group, the neurons were damaged and the Nissl bodies were decomposed. However, the neuronal morphology was improved in the miR-206-3p mimics group (see *Figure 3*).

Immunohistochemistry

As *Figure 4A* shows, the positive expression of BDNF was low in the normal group but high in the other groups. The statistical results (see *Figure 4B*) revealed that the average optical density of the positive expression of BDNF was
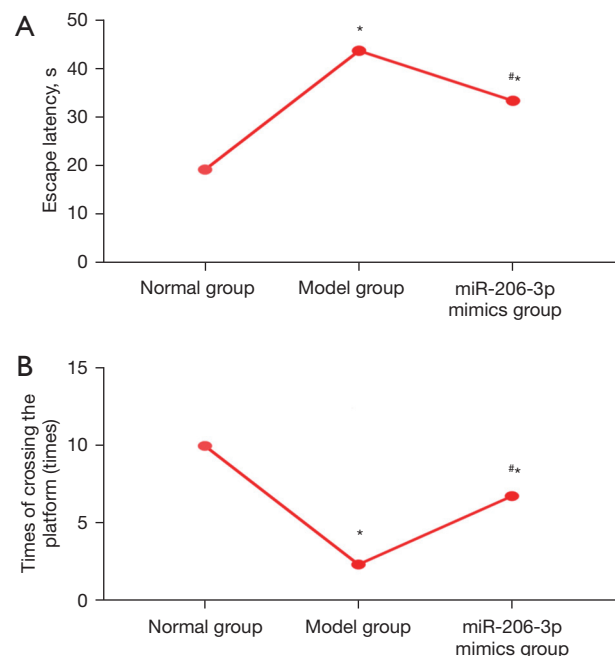
Figure 2 Water maze test results for each group of mice. (A) Escape latency times for each group; (B) platform crossing times for each group. *$P<0.05$ vs. normal group; #$P<0.05$ vs. model group. miR-206-3p, microRNA-206-3p.

significantly higher in the model group and miR-206-3p mimics group than the normal group ($P<0.05$), and significantly higher in the miR-206-3p mimics group than the model group ($P<0.05$).

Expression of relevant proteins determined via WB assay

As *Figure 5A* shows, there were few protein expressions of BDNF in the normal group and there were more protein expressions of BDNF in the other groups. The statistical results (see *Figure 5B*) showed that the relative protein
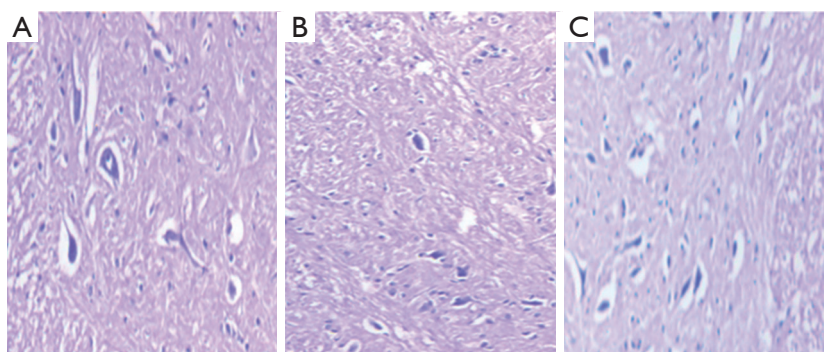
Figure 3 Neuronal morphology observed via HE staining (×200). (A) Normal group; (B) model group; (C) miR-206-3p mimics group. HE, hematoxylin-eosin; miR-206-3p, microRNA-206-3p.
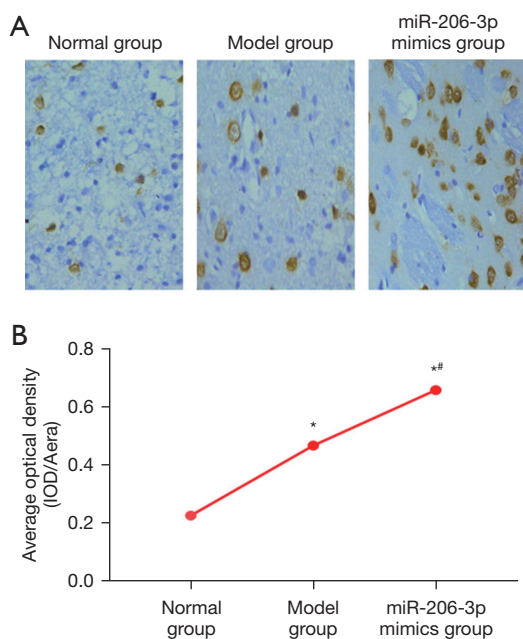

Figure 4 BDNF expression detected via immunohistochemistry. (A; ×200) Positive expression of BDNF detected via immunohistochemistry; (B) average optical density of the positive expression of BDNF for each group. *P<0.05 *vs.* normal group; #P<0.05 *vs.* model group. BDNF, brain-derived neurotrophic factor; miR-206-3p, microRNA-206-3p; IOD, integrated optical density.

expression of BDNF was significantly more increased in the model group and miR-206-3p mimics group than the normal group (P<0.05). Further, the relative protein expression of BDNF was also significantly more increased in the miR-206-3p mimics group than the model group (P<0.05).
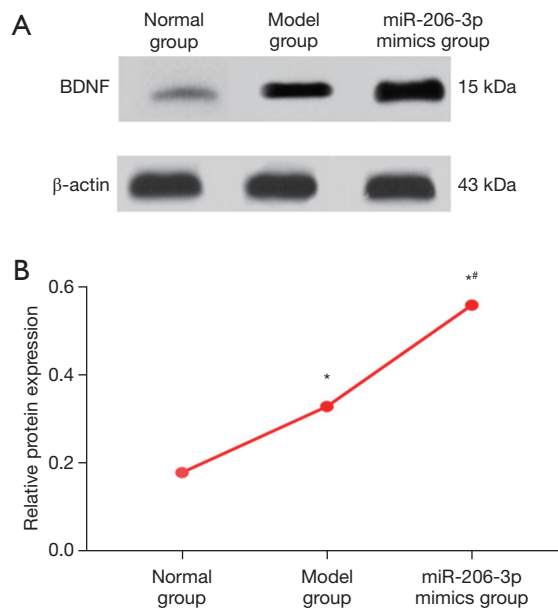
Figure 5 Protein expression of BDNF determined via WB assay. (A) Protein expression of BDNF detected via WB assay; (B) relative protein expression of BDNF for each group. *P<0.05 *vs.* normal group; #P<0.05 *vs.* model group. BDNF, brain-derived neurotrophic factor; WB, Western blotting; miR-206-3p, microRNA-206-3p.

mRNA expression examined by qPCR assay

As *Figure 6* shows, the model group and miR-206-3p mimics group had a significantly higher expression level of miR-206-3p than the normal group (P<0.05). Finally, the miR-206-3p mimics group had a significantly higher relative expression level of miR-206-3p than the model (P<0.05).

Luciferase activity expression

Compared with miR-206-3p control group, the luciferase activity at 3' UTR end of wild-type BDNF in miR-206-3p inhibition group decreased significantly (P<0.01); there was no significant difference in luciferase activity at 3' UTR end of mutant BDNF between the two groups (P>0.05) (*See Table 4*).

Discussion

As a relatively common neurodegenerative disorder of the central nervous system in clinic, AD is characterized by chronic progressive cognitive and memory impairment, and changes in personality behavior. Studies (10-12) have shown that the incidence rate of AD is increasing each year, and the disease is highly prevalent among elderly people. However, as the pathogenesis of AD is very complex and unclear, it is crucial to seek an efficacious therapeutic method for AD to improve the memory, cognitive ability, and personality behavior of AD patients. Neuronal damage is currently thought to be a key pathological response in the pathogenesis of AD (3,4,13-16). Various factors may lead to neuronal damage in the brain, including neuroinflammation, apoptosis, and Aβ accumulation, which can cause a series of pathological changes, such as necrosis and axonal demyelination by inducing neuronal damage, thus affecting the repair of the nervous system after damage and triggering changes in the memory, cognitive ability, and personality behavior of patients. Hence, restoring neuronal damage in the course of AD and protecting neurons are important to the treatment of AD.

BDNF, a vital member of the neurotrophin family of organisms, plays crucial roles in promoting neuron repair, facilitating neuron regeneration, and ameliorating axonal repair. Research (17-20) has shown that exogenous BDNF can effectively accelerate neuron regeneration and repair at the site of a central nervous system injury, exerting important neuroprotective effects. As a category of non-coding RNAs, miRNAs have vital roles in regulating multiple downstream signaling pathways and substance expressions in the body and participate in a variety of physiological and pathological responses. miR-206-3p, an essential member of the miRNA family, also controls the expressions of many downstream proteins and genes and plays crucial physiological and pathological roles.

In this study, we found that BNDF was highly expressed in the brain tissues of AD mice and involved in the protective effect on neurons. Similarly, miR-206-3p was highly expressed in the brain tissues of AD mice. Thus, miR-206-3p appears to be involved in the regulation of BDNF after the onset of AD, thereby protecting neurons. In addition, exogenous miR-206-3p further enhances the BDNF expression, ameliorates the neuronal morphology, and improves the cognitive ability and memory of AD mice. Thus, miR-206-3p exerts a neuroprotective effect on the neurons of AD mice by upregulating BDNF.
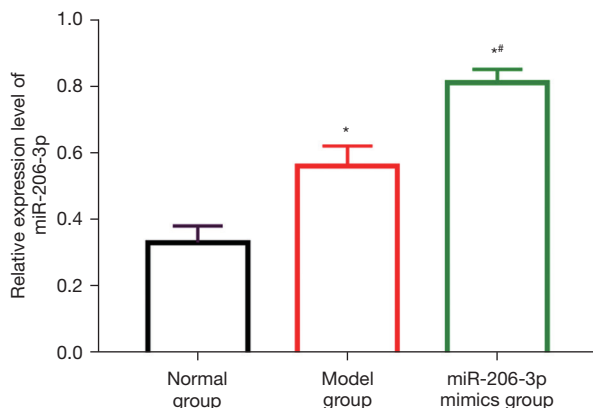

Figure 6 Relative expression level of miR-206-3p for each group. *P<0.05 *vs.* normal group; #P<0.05 *vs.* model group. miR-206-3p, microRNA-206-3p.

Table 4 Luciferase activity of miR-206-3p and 3' UTR end of BDNF detected by double luciferase report experiment ($\bar{x}\pm s$)

Grouping	N	Wild type	Mutant
miR-206-3p control	6	1.00±0.05	1.01±0.15
miR-206-3p inhibition	6	0.47±0.08	1.07±0.06
t	–	13.761	0.910
P	–	<0.001	0.384

miR-206-3p, microRNA-206-3p; 3' UTR, 3' end untranslated area; BDNF, brain-derived neurotrophic factor.

Acknowledgments

Footnote

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