Research Article

Butylphthalide Inhibits TLR4/NF-κB Pathway by Upregulation of miR-21 to Have the Neuroprotective Effect

Lan Zhan,1 Yu Pang,1 Hao Jiang,1 Shicun Zhang,1 Hongwei Jin,1 Jing Chen,2 Cuiying Jia,2 Hongyan Guo,3 and Zhuang Mu4

1Department of Neurology, The Second Affiliated Hospital of Qiqihar Medical University, Qiqihar 161000, China
2Department of Clinical Laboratory, The Second Affiliated Hospital of Qiqihar Medical University, Qiqihar 161000, China
3Department of Biochemistry Teaching and Research, Qiqihar Medical University, Qiqihar 161000, China
4Department of Neurosurgery, The First Hospital of Qiqihar City, Qiqihar 161000, China

Correspondence should be addressed to Hongyan Guo; qyguohongyan5564@qmu.edu.cn and Zhuang Mu; muzhuang006@sina.com

Received 17 November 2021; Revised 6 December 2021; Accepted 10 December 2021; Published 7 January 2022

Academic Editor: Bhagyaveni M.A

Copyright © 2022 Lan Zhan et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Stroke is a disease with the highest incidence rate and the highest mortality rate in the world [1]. Among all the cases, ischemic stroke accounts for about 87%. Most of the patients have the serious functional neurologic disorder, which brings serious economic burden to their families and society [2]. DL-3-n-Butylphthalide (NBP) (chemical formula: C12H14O2, molecular weight: 190.24) is a synthetic compound based on l-3-n-Butylphthalide which is isolated from celery seed. Many clinical studies have shown that NBP is a safe and effective treatment for ischemic stroke. It not only relieves the symptoms of ischemic stroke but also contributes to long-term rehabilitation [3–5].

1. Introduction

Stroke is a disease with the highest incidence rate and the highest mortality rate in the world [1]. Among all the cases, ischemic stroke accounts for about 87%. Most of the patients have the serious functional neurologic disorder, which brings serious economic burden to their families and society [2]. DL-3-n-Butylphthalide (NBP) (chemical formula: C12H14O2, molecular weight: 190.24) is a synthetic compound based on l-3-n-Butylphthalide which is isolated from celery seed. Many clinical studies have shown that NBP is a
platelet phosphodiesterase and increase the level of 3,5-cyclic adenosine monophosphate in platelets, and increase the content of nitric oxide (NO) and PGI2 in cerebral vascular endothelial cells, improving arterial microcirculation [7]. NBP can increase the activities of Na + - K + ATPase and Ca2+ - ATPase in mitochondrial membrane, improve the decrease of neuron mitochondria complex IV activity induced by hypoglycemia and hypoxia, and increase the activities of SOD and glutathione peroxidase (GSH-Px) in mitochondria. It can also decrease the release of cytochrome c and increase the activity of cytochrome c oxidase in mouse cells and inhibit caspase-dependent apoptosis. According to some other studies, NBP can inhibit ROS production by inhibiting nicotinamide adenine dinucleotide (NADH). Therefore, it can improve energy metabolism [16], reduce oxidative damage [17], prevent inflammatory reaction [18], and reduce neuronal apoptosis [19, 20].

Our previous study found that the level of miR-21 in the peripheral blood of ischemic stroke patients treated with Butylphthalide was increased. According to Jiansuo Zhou et al., the level of miR-21 in the plasma of patients with acute cerebral infarction was lower than that of the control group. Studies have confirmed the neuroprotective effect of miR-21 in cerebral ischemia-reperfusion injury and pointed out that MAP2K3 pathway can be used as the therapeutic target [21]. In addition, overexpression of miR-21 can prevent renal ischemic injury [22], which indicates that the level of miR-21 has a protective effect against ischemic diseases. Studies have shown that miR-21 is related to nerve repair and vascular protection after stroke. According to Buller et al.’s in vivo and in vitro experimental studies, the level of miR-21 was significantly increased after ischemic stroke, inhibiting neuronal cell death by reducing Fas ligand (FasL) G (an important cell death-inducing ligand) [23]. miR-21 can participate in vascular remodeling by regulating VSMCs apoptosis and phenotype transformation and play an important role in neointimal formation in the induction model of intimal injury [21, 24]. TLR4/NF-κB pathway plays an important role in ischemia-reperfusion injury. The overexpression of miR-21 can effectively inhibit TLR4/NF-κB pathway, reducing the level of cardiomyocyte apoptosis and the release of inflammatory factors of mice [25]. Other studies have shown that miR-21 can protect against ischemic injury through PDCD4/NF-κB pathway [26]. These results suggest that miR-21 can affect the neurovascular unit remodeling by regulating neuronal apoptosis and angiogenesis. The protective mechanism of Butylphthalide on cerebral ischemia may be achieved by miR-21 regulation. This study aims to clarify the neuroprotective mechanism of Butylphthalide through miR-21.

2. Methods

2.1. Animal Model. Male C57BL/6 mice aged 8–10 weeks were purchased from Shanghai Lab Animal Research Center and fed in SPF animal room. The mice were divided into three groups: sham operation group, MCAO group, and MCAO + Butylphthalide-treated group, with 5 mice in each group. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). After midline skin incision, the left common carotid artery was exposed and electrocautery was performed on its branches. Two centimeters were slowly pushed from the external carotid artery bifurcation to the internal carotid artery, and a 6–0 round nylon suture was used. After 60 minutes of proximal MCA obstruction, blood flow was restored after the removal of the suture. In the sham group, the carotid artery was exposed without middle cerebral artery ligation. The mice in MCAO + Butylphthalide-treated group were administered with 70 mg/kg Butylphthalide injection intraperitoneally after cerebral ischemia-reperfusion once a day. The normal saline with the same volume was administered intraperitoneally for the mice in MCAO group and sham group. After a 60-minute MCAO, blood perfusion was restored for 72 hours. During the operation, the rectal temperature was controlled at 37.0 ± 0.5°C with heating pad. With ischemic injury, mice were placed in a ventilated incubator at 24.0 ± 0.5°C. After 72 hours of reperfusion, all mice had the functional neurologic impairment scoring, and then the animals were killed and their brains were taken out quickly to confirm the infarct. According to Longa’s 5-grade scoring standard, the functional neurologic impairment was assessed as “no symptoms of functional neurologic impairment” [0], “the contralateral forepaw of cerebral ischemia could not be fully extended” [1], “turning to the opposite side” [2], “dumping to the opposite side” [3], “involuntary walking and unconsciousness” [4], and “death” [5]. All procedures for the use of laboratory animals were approved by the animal ethics committee of the Second Affiliated Hospital of Qiqihar Medical University. The protocol fully complies with the Guide for Care and Use of Laboratory Animals of National Institutes of Health (NIH publication 85–23, revised in 1996).

2.2. Calculation of Infarct Volume and Brain Loss Volume. The infarct volume was measured with 2,3,5-triphenyltetrazolium chloride (TTC) [27]. The brain was cut into four coronal sections with the thickness of 0.2–0.3 cm. The slices were immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) for 30 minutes at 37°C, and the images were scanned into a computer to be measured with imaging analysis software (Image J, 1.52a). The infarct volume (cm³) of each slice was equal to the infarct area (cm²) multiplied by the slice thickness (0.2 cm or 0.3 cm). The total infarct volume of each brain was then calculated by summing the infarct volumes of all slices. In order to minimize the effect of edema on the accuracy of infarct volume measurement, the final infarct volume was corrected with a coefficient equal to the ratio of nonischemic hemisphere volume to the ischemic one. Hematoxylin and Eosin (H&E) staining was performed to detect brain loss. Brain loss was measured by subtracting the nonlesion area of the ipsilateral hemisphere from the contralateral hemisphere. The volume of tissue loss was calculated with the lesion area of six parts and analyzed with Image J (1.52a).

2.3. Cell Culture and Oxygen-Glucose Deprivation/Reoxyge-
nation (OGD/R) Cell Model. Neuro2A was purchased from American Type Culture Collection (ATCC, Shanghai,
Cells were cultured under normal conditions (95% air, 5% CO2) with conventional high-glucose DMEM containing 10% FBS (Gibco) in an incubator at 37°C. As the density of the cells grew to 50%, the old medium was replaced with Hank’s Balanced Salt (HBSS; glucose concentration = 0 mg/dL) and the medium was transferred to the anoxic chamber (95% nitrogen and 5% CO2) for 3 hours. At the end of OGD phase, the medium was replaced with the original complete medium and cultured under normal conditions for 24-hour reoxygenation. Cells without OGD/R treatment were used as a control. After OGD/R, the medium in Butylphthalide-treated group was replaced with a complete medium containing 10 μM Butylphthalide. All chemicals/drugs have been sterilized.

2.4. Cell Transfection. MiR-21 mimics, miR-21 inhibitor, and negative control (miR-NC or anti-miR-NC) were purchased from GenePharma. When the density of Neuro2A cells grew to 70%, Lipofectamine 2000 Reagent (Invitrogen, USA) was used to transfect the cells with miR-211 mimics or inhibitor or negative control according to the operating procedure of the manufacturer, and the final concentration was 50 nM. The cells were harvested 24 hours after transfection for further study, and then the above cells were placed in OGD/R.

2.5. Real-Time Fluorescence Quantitative PCR. Total RNA and miRNA were extracted from cerebral infarction tissues and Neuro2A cells with miRNeasy Mini Kit (Qiagen, Germany). TaqMan microRNA Reverse Transcription Kit (California, Applied Biosystems) was used to obtain cDNA, and TaqMan miRNA Kit (Applied Biosystems) was used for qRT-PCR. The reaction procedure was as follows: initial denaturation: 95°C, 30 sec; circular reaction: 60°C, 20 sec, 40 cycles. U6 small nuclear RNA (U6 snRNA) was used as a standardized endogenous control. SYBR Green mix (Taq-Man) was used to perform qRT-PCR on 7900HT rapid real-time PCR system (Applied Biosystems). U6 was used as the internal control and the changes of miRNA level were quantified by 2−ΔΔCT. The primer sequence is shown in Table 1.

2.6. MTT Assay. Neuro2a cells were seeded into a 96-well plate at a density of 105. After overnight culture, transfection or Butylphthalide treatment was given according to the requirements of each group with MTT cell proliferation and cytotoxicity detection kit (Beyotime). Then 10 μL MTT solution was added to each well and they were continued to be incubated for 4 hours in the cell incubator. 100 μL formazan solution was added into each well. After mixing, they were incubated with horse radish peroxidase labeled goat anti-rabbit IgG (Beyotime, A0216, 1: 5000) for 1 hour at room temperature. Enhanced chemiluminescence detection system (Bio-Rad, Hercules, CA, USA) was used to detect protein bands, and Image Lab software (Bio-Rad) was used to quantify protein bands.

2.7. WB Experiment. The cells were sonicated for 1 minute in the precooled RIPA lysate (Beyotime) and centrifuged at 12,000 g for 10 minutes at 4°C and the supernatant was stored at −80°C. The protein concentration in the sample was determined with BCA Protein Assay Kit (Beyotime). Electrophoretic separation was performed for the extracted protein with 10% SDS-polyacrylamide gel and the protein was transferred to PVDF (Millipore, Bedford, MA, USA). The membrane was sealed in TBST buffer containing 5% skim milk for 1 hour and then incubated overnight at 4°C with the following primary antibodies: anti-mouse-p-trl4 (Santa Cruz, 1:1000), anti-mouse TLR4 (CST, 1:1000), anti-mouse-p-nf-κ B (Santa Cruz, 1:1000), anti-mouse-NF-κ B (Santa Cruz, 1:1000), anti-mouse-caspse3 (abcam,1:1000), anti-mouse-Bax (abcam,1:1000); anti-mouse-Bcl-2 (abcam,1:1000), and anti-mouse-GAPDH (Santa Cruz, 1: 1000). Then the membrane was washed with TBST and incubated with horse radish peroxidase labeled goat anti-rabbit IgG (Beyotime, A0216, 1: 5000) for 1 hour at room temperature. Enhanced chemiluminescence detection system (Bio-Rad, Hercules, CA, USA) was used to detect protein bands, and Image Lab software (Bio-Rad) was used to quantify protein bands.

2.8. Determination of Intracellular ROS. The cultured cells were digested with 0.04% Trypsin pancreatin and centrifuged at 1000 rpm for 5 minutes. DCFH-DA was diluted with serum-free medium at the ratio of 1:1000 through reactive oxygen species detection kit (Beyotime) to make the final concentration of 10 μmol/L. The collected cells were suspended in diluted DCFH-DA and incubated for 20 minutes in the cell incubator at 37°C. The solution was mixed every 3–5 minutes to make the probe fully in contact with the cell. The cells were washed three times with serum-free cell culture medium to remove DCFH-DA from the cells. The production of ROS was detected with flow cytometry and quantified with CellQuest.

2.9. Statistical Analysis. SPSS 22.0 was used for statistical analysis. All experimental results were expressed as mean± standard deviation. The difference of quantitative data between the two groups was compared with unpaired t-test. The quantitative data among multiple groups was analyzed with ANOVA. Bonferroni method was used for pairwise comparison between the two groups. P < 0.05 was considered as significant difference.

3. Results

3.1. Butylphthalide Improves Neurological Function Score and Reduces Cerebral Infarction Area in the Mouse Model of MCAO. In order to clarify the neuroprotective effect of Butylphthalide on ischemic stroke, the mouse model of MCAO was established. On the day of cerebral ischemia-reperfusion, 70 mg/kg Butylphthalide injection was administered intraperitoneally. The neurological function score and infarct area of the mice in MCAO group and MCAO + Butylphthalide-treated group were compared after
3-day administration in a row. It was found that the functional neurologic impairment score of the mice in the MCAO + Butylphthalide-treated group was significantly lower than that in MCAO group (Figure 1(a), \( P < 0.01 \)). According to the calculated infarct size and brain loss volume, it was found that the infarct volume and brain loss volume of the mice in the MCAO + Butylphthalide-treated group were significantly lower than those in MCAO group (Figures 1(b) and 1(c); \( P < 0.05; P < 0.01 \)). The level of miR-21 in infarcted brain tissue was further explored and it was found that the level of miR-21 of the mice in MCAO group was significantly lower than that in sham group (\( P < 0.001 \)), while the level of miR-21 of the mice in Butylphthalide-treated group was significantly higher than that in MCAO group (\( P < 0.01 \)), and there was no statistical difference between MCAO group and sham group (Figure 1(d)). According to the correlation analysis of neurological function score and miR-21 level, it was found that there was a negative correlation between them (Figure 1(e), \( r = -0.8933, P < 0.001 \)).

### Table 1: The primer sequence of miR-21 and U6.

| Gene      | Forward (5'→3')          | Reverse (5'→3')        |
|-----------|--------------------------|------------------------|
| miR-21    | AGGGCCCCTCTGAACCCTAAG3    | CCAGGCGGATACAGGGAAC3    |
| U6        | GCTTCGCCCAGCACATACTAAAT  | CGCTTCAGGAATTGGGTACAT3  |

#### 3.3. Butylphthalide and miR-21 Mimics Inhibit the OGD/R Apoptosis of Neuro2A Cells. 

**Injury of Neuro2A Cells.** WB method was used to detect apoptosis-related proteins. It was found that the proportion of Bcl-2/Bax in OGD/R + Butylphthalide group and OGD/R + miR-21 mimics group was significantly higher than that in OGD/R + mir-nc group, and lower than that in control group (Figures 3(a) and 3(b); \( P < 0.05 \)). The proportion of caspase-3/GAPDH in these two groups was significantly lower than that in OGD/R + mir-NC group and higher than that in control group (Figures 3(a) and 3(c); \( P < 0.001 \)), indicating that Butylphthalide treatment and overexpression of miR-21 could inhibit OGD/R-induced apoptosis.

#### 3.4. Butylphthalide Exerts the Neuroprotective Effect through TLR4/NF-κB Pathway. 

In order to clarify the specific mechanism of neuroprotective effect of Butylphthalide through miR-21, WB method was used to detect the expression of miR-21-related signaling pathway, TLR4/NF-κB. The results showed that the p-TLR4/TLR4 level in OGD/R + Butylphthalide group and OGD/R + miR-21 mimics group was significantly lower than that in OGD/R + mir-NC group, and higher than that in control group. And the p-TLR4/TLR4 level in OGD/R + mir-NC group was significantly lower than that in control group (Figures 4(a) and 4(b); \( P < 0.05 \)). The level of p-NF-κB/NF-κB in OGD/R + Butylphthalide group and OGD/R + miR-21 mimics group was significantly decreased compared with that in OGD/R + mir-NC group (Figures 4(a) and 4(c); \( P < 0.001 \)); the level of p-NF-κB/NF-κB in OGD/R + Butylphthalide group was similar to that in OGD/R + mir-NC group and that in OGD/R + mir-NC group was higher than that in control group (Figures 4(a) and 4(c); \( P < 0.001 \)).

### 4. Discussion

In this study, the possible mechanism of the neuroprotective effect of Butylphthalide was discussed. The previous study found that the level of miR-21 in the peripheral blood of ischemic stroke patients treated with butylphthalide was increased. In order to further clarify this phenomenon, the mouse model of a focal middle cerebral artery occlusion (MCAO) was established and it was found that the level of miR-21 in brain tissue of the mouse model of MCAO treated with Butylphthalide was significantly increased. What is more, Butylphthalide reduced the functional neurologic
impairment score and infarct volume, showing neuroprotective effect. Studies have shown that its main mechanism is attributable to its anti-inflammatory and antiapoptotic properties [28, 29]. In addition, studies found that NBP can increase the local blood flow after cerebral ischemia, which may be beneficial for penumbral rescue [6]. It has been reported that NBP can reduce the activation of caspase-3 and caspase-9 and inhibit apoptosis after ischemic injury [19]. According to an in vitro study, NBP can protect cerebral microvascular endothelial cells from the injury induced by oxygen-glucose deprivation by upregulating the expression of Bcl-2 [30]. Another study found that NBP can reduce brain edema and downregulate RhoA expression, which is a downstream effector of RAS and can regulate the contraction of smooth muscle cells. The protective effect of NBP on endothelial cells and blood-brain barrier showed that NBP could recover the ischemic injury induced by angiotensin [31]. Fangfang Li et al. found that NBP treatment can reduce the volume of cerebral infarction 1 day after MCAO and significantly reduced the volume of cerebral infarction 3 days after MCAO [32]. In addition, Butylphthalide can also improve the functional neurologic impairment of mice. Butylphthalide can downregulate the expression of MMP-9, upregulate the expression of claudin-5, increase the expression of VEGF and GFAP, improve the ultrastructure of capillary endothelial cells of BBBs, and

![Figure 1](image-url)
Figure 2: Effects of Butylphthalide treatment and miR-21 overexpression on the proliferation of Neuro2A cells and ROS production. (a) qPCR method was used to detect the level of miR-21 in Neuro2A cells. The level of miR-21 in OGD/R + Butylphthalide group was significantly higher than that in OGD/R + Butylphthalide + miR-21 inhibitor group and control group. (b) MTT assay was used to detect the proliferation rate of Neuro2A. The proliferation rate of Neuro2A in OGD/R + Butylphthalide group was higher than that in OGD/R + Butylphthalide + miR-21 inhibitor group and control group, but there was no significant difference between OGD/R + Butylphthalide + miR-21 inhibitor group and control group. (c) Compared with OGD/R + Butylphthalide + miR-21 inhibitor group and OGD/R + miR-NC group, the ROS activity in OGD/R + Butylphthalide + miR-21 inhibitor group was significantly decreased, but there was no significant difference between OGD/R + Butylphthalide + miR-21 inhibitor group and OGD/R + miR-NC group. **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 3: Continued.
Figure 3: Effects of Butylphthalide treatment and miR-21 overexpression on Neuro2A apoptosis. (a) The protein expression levels of Bcl-2, Bax, caspase-3, and GAPDH in OGD/R + Butylphthalide group, OGD/R + miR-21 mimics group, OGD/R + miR-NC group, and control group. (b) Statistical results showed that the proportion of Bcl-2/Bax in OGD/R + Butylphthalide group and OGD/R + miR-21 mimics group was significantly higher than that in OGD/R + miR-NC group, and the proportion of Bcl-2/Bax in OGD/R + miR-NC group was lower than that in control group. (c) Statistical results showed that the proportion of caspase-3/GAPDH in OGD/R + Butylphthalide group and OGD/R + miR-21 mimics group was significantly lower than that in OGD/R + miR-NC group, and the proportion of caspase-3/GAPDH in OGD/R + miR-NC group was higher than that in control group. * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \), **** \( P < 0.0001 \).

Figure 4: Continued.
increase the expression of Nrf-2 and HO-1, having a protective effect on the brain of the mice [33]. In spontaneously hypertensive rats (SHR), NBP significantly reduced the functional neurologic impairment score and infarct volume and minimized the pathological changes of penumbra compared with placebo-treated control group [34]. All the above studies indicate that Butylphthalide plays a positive role in the neuroprotective effect of ischemic stroke, which also supports the results of this study. As the mechanism of action of Butylphthalide is diverse, further study is required.

It should be noted that the level of miR-21 in the infarcted brain tissue was significantly decreased, and the level of miR-21 in the infarcted brain tissue of the mice in Butylphthalide-treated group was increased. Moreover, it was found that there was a negative correlation between the level of miR-21 and the neurological function score, indicating that miR-21 played an important role. Jiansuo Zhou et al. believed that the level of miR-21 in the plasma of patients with acute cerebral infarction was lower than that in control group [34]. All the above studies indicate that Butylphthalide plays a positive role in the neuroprotective effect of ischemic stroke, which also supports the results of this study. As the mechanism of action of Butylphthalide is diverse, further study is required.

Figure 4: Effects of Butylphthalide treatment and miR-21 overexpression on TLR4/NF-κB of Neuro2A cells. (a) The protein expression levels of p-TLR4, p-NF-κB, and NF-κB in OGD/R + Butylphthalide group, OGD/R + miR-21 mimics group, OGD/R + miR-NC group, and control group. (b) Statistical results showed that the proportion of p-TLR4/TLR4 in OGD/R + Butylphthalide group and OGD/R + miR-21 mimics group was significantly lower than that in OGD/R + miR-NC group, and the proportion of p-TLR4/TLR4 in OGD/R + miR-NC group was higher than that in control group. (c) Statistical results showed that the proportion of p-NF-κB/NF-κB in OGD/R + Butylphthalide group and OGD/R + miR-21 mimics group was significantly lower than that in OGD/R + miR-NC group, and the proportion of p-NF-κB/NF-κB in OGD/R + miR-NC group was higher than that in control group.

5. Conclusion

Further exploration was made on the specific mechanism of neuroprotective effect of Butylphthalide on MCAO by regulating miR-21. The experimental results showed that Butylphthalide could upregulate the survival rate of OGD/R cells, and the protective effect of upregulating the survival rate of Neuro2A cells was counteracted by inhibition of miR-21 expression with Butylphthalide treatment. Similarly, Butylphthalide treatment inhibited the production of reactive oxygen species and the cytotoxicity after OGD/R. However, inhibition of miR-21 expression significantly increased the cytotoxic reaction. In addition, it has been reported that ROS is involved in apoptosis [37]. Our results showed that Butylphthalide and miR-21 can inhibit caspase-3 and increase the proportion of Bcl-2/Bax, indicating that Butylphthalide can inhibit OGD/R-induced apoptosis through miR-21.

The expression of miR-21 can effectively inhibit TLR4/NF-κB pathway, reducing the level of apoptosis and the
release of inflammatory factors (25). We also found that Butylphthalide and miR-21 could inhibit p-TLR4/TLR4 and p-NF-κB/NF-κB in OGD/R model, which verified the effect of miR-21 on TLR4/NF-κB in ischemic stroke. Butylphthalide may act on this pathway through miR-21, further affecting the apoptosis and activity of nerve cells to show its neuroprotective effect.

In conclusion, Butylphthalide has neuroprotective effect on the mouse model of MCAO. It may upregulate the level of miR-21 to inhibit neuronal apoptosis and ROS production and improve the proliferation activity. The specific mechanism may lie in inhibiting TLR4/NF-κB pathway.

Data Availability
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Ethical Approval
The study was approved by the Ethics Committee of the Second Affiliated Hospital of Qiqihar Medical University.

Consent
Not applicable.

Conflicts of Interest
The authors declare that they have no conflicts of interest.

Authors’ Contributions
L. Zhan, Y. Pang, and H. Guo led the conception and design of this study. L. Zhan, H. Jiang, S. Zhang, H. Jin, J. Chen, C. Jia, and Z. Mu were responsible for the data collection and analysis. Y. Pang, H. Jiang, J. Chen, C. Jia, and H. Guo were in charge of interpreting the data and drafting the manuscript. L. Zhan, Y. Pang, and Z. Mu made revision from critical perspective for important intellectual content. The final version was read and adopted by all the authors.

Acknowledgments
This study was funded by Clinical Research Fund Project of Qiqihar Academy of Medical Sciences (QMSI2020L-04).

References
[1] A. G. Thrift, T. Thayabaranathan, G. Howard et al., “Global stroke statistics,” International Journal of Stroke, vol. 12, no. 1, pp. 13–32, 2017.
[2] E. J. Benjamin, M. J. Blaha, S. E. Chiuve et al., “Heart disease and stroke statistics-2017 update: a report from the American heart association,” Circulation, vol. 135, pp. e146–e603, 2017.
[3] L.-X. Xue, T. Zhang, Y.-W. Zhao, Z. Geng, J.-J. Chen, and H. Chen, “Efficacy and safety comparison of DL-3-n-butylphthalide and Cerebrolysin: effects on neurological and behavioral outcomes in acute ischemic stroke,” Experimental and Therapeutic Medicine, vol. 11, no. 5, pp. 2015–2020, 2016.
[4] L. Y. Cui, Y. C. Zhu, S. Gao et al., “Ninety-day administration of dl-3-n-butylphthalide for acute ischemic stroke: a randomized, double-blind trial,” Chinese Medical Journal, vol. 126, pp. 3405–3410, 2013.
[5] H. Zhao, W. Yun, Q. Zhang et al., “Mobilization of circulating endothelial progenitor cells by dl-3-n-Butylphthalide in acute ischemic stroke patients,” Journal of Stroke and Cerebrovascular Diseases, vol. 25, no. 4, pp. 752–760, 2016.
[6] C. H. Yan, Y. P. Feng, and J. T. Zhang, “Effects of dl-3-n-butylphthalide on regional cerebral blood flow in right middle cerebral artery occlusion rats,” Zhongguo yao li xue bao = Acta pharmacologica Sinica, vol. 19, pp. 117–120, 1998.
[7] C.-L. Liu, S.-J. Liao, J.-S. Zeng et al., “dl-3n-butylphthalide prevents stroke via improvement of cerebral microvessels in RH RSP,” Journal of the Neurological Sciences, vol. 260, no. 1-2, pp. 106–113, 2007.
[8] Z. Z. Chong and Y. P. Feng, “dl-3-n-butylphthalide attenuates reperfusion-induced blood-brain barrier damage after focal cerebral ischemia in rats,” Zhongguo yao li xue bao = Acta pharmacologica Sinica, vol. 20, pp. 696–700, 1999.
[9] Y. Feng, X. Zeng, Y. Feng, and X. Wang, “Antiplatelet and antithrombotic activity of L-3-n-butylphthalide in rats,” Journal of Cardiovascular Pharmacology, vol. 43, no. 6, pp. 876–881, 2004.
[10] X. G. Liu and Y. P. Feng, “[Protective effect of dl-3-n-butylphthalide on ischemic neurological damage and abnormal behavior in rats subjected to focal ischemia],” Yao Xue Xue Bao, vol. 30, pp. 896–903, 1995.
[11] J. F. Lin and Y. P. Feng, “[Effect of dl-3-n-butylphthalide on delayed neuronal damage after focal cerebral ischemia and intrasynaptosomes calcium in rats],” Yao Xue Xue Bao, vol. 31, pp. 166–170, 1996.
[12] L. Y. Zhang and Y. P. Feng, “[Effect of dl-3-n-butylphthalide (NBP) on life span and neurological deficit in SHRsp rats],” Yao Xue Xue Bao, vol. 31, pp. 18–23, 1996.
[13] Z. Chong and Y. Feng, “Protective effects of dl-3-n-butylphthalide on changes of regional cerebral blood flow and blood-brain barrier damage following experimental subarachnoid hemorrhage,” Chinese Medical Journal, vol. 111, pp. 858–860, 1998.
[14] J. Ye, L. Zhai, S. Zhang et al., “dl-3-n-butylphthalide inhibits platelet activation via inhibition of cPLA2-mediated TXA2-synthesis and phosphodiesterase,” Platelets, vol. 26, no. 8, pp. 736–744, 2015.
[15] Z. Z. Chong and Y. P. Feng, “Effects of dl-3-n-butylphthalide on production of TXB2 and 6-keto-PGF1 alpha in rat brain during focal cerebral ischemia and reperfusion,” Zhongguo yao li xue bao = Acta pharmacologica Sinica, vol. 18, pp. 505–508, 1997.
[16] Y. P. Feng, D. Hu, and L. Y. Zhang, “[Effect of DL-buty1phthalide (NBP) on mouse brain energy metabolism in complete brain ischemia induced by decapitation],” Yao Xue Xue Bao, vol. 30, pp. 741–744, 1995.
[17] G. X. Dong and Y. P. Feng, “Antithrombotic effects of NBP on experimental subarachnoid hemorrhage,” Zhongguo yao li xue bao = Acta pharmacologica Sinica, vol. 21, pp. 433–438, 2000.
[18] H. L. Xu and Y. P. Feng, “Inhibitory effects of DL-3n-butylphthalide on swelling following focal ischemic brain injury in rats,” Acta Pharmacologica Sinica, vol. 21, pp. 433–438, 2000.
[19] J. Li, Y. Li, M. Ogle et al., “DL-3-n-butylphthalide prevents neuronal cell death after focal cerebral ischemia in mice via the JNK pathway,” Brain Research, vol. 1359, pp. 216–226, 2010.
[20] Q. Chang and X. L. Wang, "Effects of chiral 3-n-butylphthalide on apoptosis induced by transient focal cerebral ischemia in rats," *Acta Pharmacologica Sinica*, vol. 24, pp. 796–804, 2003.

[21] X. Yao, Y. Wang, and D. Zhang, "microRNA-21 confers neuroprotection against cerebral ischemia-reperfusion injury and alleviates blood-brain barrier disruption in rats via the MAPK signaling pathway," *Journal of Molecular Neuroscience*, vol. 65, no. 1, pp. 43–53, 2018.

[22] N. Song, T. Zhang, X. Xu et al., "miR-21 protects against ischemia/reperfusion-induced acute kidney injury by preventing epithelial cell apoptosis and inhibiting dendritic cell maturation," *Frontiers in Physiology*, vol. 9, p. 790, 2018.

[23] B. Buller, X. Liu, X. Wang et al., "MicroRNA-21 protects neurons from ischemic death," *FEBS Journal*, vol. 277, no. 20, pp. 4299–4307, 2010.

[24] J. Zhu, B. Liu, Z. Wang et al., "Exosomes from nicotine-stimulated macrophages accelerate atherosclerosis through miR-21-3p/PTEN-mediated VSMC migration and proliferation," *Theranostics*, vol. 9, no. 23, pp. 6901–6919, 2019.

[25] Y. Q. Pan, J. Li, Y. C. Li, J. Li, and J. F. Lin, "Effect of miR-21/TLR4/NF-kappaB pathway on myocardial apoptosis in rats with myocardial ischemia-reperfusion," *Journal of Molecular Neuroscience*, vol. 65, no. 1, pp. 43–53, 2018.

[26] H. Yan, J. Rao, J. Yuan et al., "Long non-coding RNA MEG3 functions as a competing endogenous RNA to regulate ischemic neuronal death by targeting miR-21/PDCD4 signaling pathway," *Cell Research*, vol. 32, no. 1, pp. 235–242, 2013.

[27] L. Zhang, W.-h. Amy Yu, J. Y.-X. Wang et al., "DL-3-n-Butylphthalide, an anti-oxidant agent, prevents neurological deficits and cerebral injury following stroke per functional analysis, magnetic resonance imaging and histological assessment," *Current Neurovascular Research*, vol. 9, no. 3, pp. 167–175, 2012.

[28] J. Hu, Q. Wen, Y. Wu, B. Li, and P. Gao, "The effect of butylphthalide on the brain edema, blood-brain barrier of rats after focal cerebral infarction and the expression of Rho A," *Cell Biochemistry and Biophysics*, vol. 69, no. 2, pp. 363–368, 2014.

[29] F. Li, Q. Ma, H. Zhao et al., "L-3-n-Butylphthalide reduces ischemic stroke injury and increases M2 microglial polarization," *Metabolic Brain Disease*, vol. 33, no. 6, pp. 1995–2003, 2018.

[30] J. Zhou and J. Zhang, "Identification of miRNA-21 and miRNA-24 in plasma as potential early stage markers of acute cerebral infarction," *Molecular Medicine Reports*, vol. 10, no. 2, pp. 971–976, 2014.

[31] W. Wang, D.-B. Li, R.-Y. Li et al., "Diagnosis of hyperacute and acute ischaemic stroke: the potential utility of exosomal MicroRNA-21-5p and MicroRNA-30a-5p," *Cerebrovascular Diseases*, vol. 45, no. 5-6, pp. 204–212, 2018.

[32] J. Lu, Y. Xue, Y. Wang et al., "Retracted article: CiRS-126 inhibits proliferation of ovarian granulosa cells through targeting the miR-21-PDCD4-ROS axis in a polycystic ovarian syndrome model," *Cell and Tissue Research*, vol. 381, no. 1, pp. 189–201, 2020.