The protective effect and its mechanism of 3-n-butylphthalide pretreatment on cerebral ischemia reperfusion injury in rats

R.-Y. YAN¹, S.-J. WANG², G.-T. YAO¹, Z.-G. LIU¹, N. XIAO¹

¹Department of Neurology, Changle People's Hospital, Changle County, Shandong Province, China
²Department of Neurosurgery, Weifang Yidu Central Hospital, Qingzhou City, Shandong Province, China

Ruiyun Yan and Shanjun Wang contributed equally to the work.

Abstract. – OBJECTIVE: To investigate the potential effect of 3-n-butylphthalide (NBP) pretreatment on the cerebral ischemia/reperfusion injury in rats and the relevant mechanism.

MATERIALS AND METHODS: A total of 90 rats was divided into three groups: Sham operation group (Sham group), ischemia-reperfusion group (I-R group), and NBP pretreatment group (NBP group 75 mg·kg⁻¹·d⁻¹ gavage). Pre-treatment was given once a day within 1 week before establishing the rat model of cerebral ischemia-reperfusion injury. The middle cerebral artery occlusion (MCAO) rat models were established with the improved Longa-Zea method on the 7th day after ischemia for 2 h and reperfusion for 24 h in all the rats. We detected the cerebral infarction, the pathologic change of brain, the apoptosis of nerve cell, the production levels of reactive oxygen species (ROS), the content of malonaldehyde (MDA) and the activity of superoxide dismutase (SOD), the water content and the permeability of blood-brain barriers (BBB). In addition, we also observed the expressions of mitogen-activated protein kinase (MAPK, p-38, JNK, ERK1/2) and cleaved caspase-3 in the hippocampus tissues.

RESULTS: Compared with Sham group, we discovered that NBP significantly reduced infarction area, cell apoptosis, BBB damage and water content. Further, we found that NBP could also decrease ROS and MDA, and increase SOD activity in brain tissues of rats with a cerebral ischemia-reperfusion injury. Moreover, results showed that NBP also inhibited the levels p38 and JNK.

CONCLUSIONS: NBP protected the cerebral from I/R injury, providing ideas for the expansion of clinical adaptability of NBP and possible approaches for its application.

Key Words: 3-n-butylphthalide (NBP), Mitogen-activated protein kinase (MAPK), Cerebral ischemia reperfusion injury, Apoptosis.

Introduction

Ischemic cerebrovascular disease (ICVD) commonly and frequently occurs in the elderly people, accounting for 80% of all strokes ¹. With the arrival of an aging society, its incidence rate is increased year by year, and tends to occur in younger people. The disease seriously harms human health and brings a heavy burden to patients’ families and the society. The research regarding the mechanism and prevention of ICVD has always been a hot topic for neurologists. To explore an effective treatment to strengthen the prevention and treatment of such disease, and reduce its occurrence rate, disability and mortality rate, thus improving the quality of life of patients, has become a hot spot in medical research.

Current clinical treatments for ischemic stroke mainly focused to re-perfuse the ischemia area via drugs or early thrombolysis, to restore oxygen and glucose supply in this area ²,³. However, kinds of complex factors, such as excitatory aminoacid toxicity, inflammatory responses, calcium overload and oxidative stress, will lead to a series of pathological cascade reactions ⁴-⁹. These cascade reactions might result in neuronal necrosis or apoptosis and BBB damage. Then, damaged BBB will induce the outflow of plasma proteins and water in the blood capillary, thus leading to hernia formation ¹⁰. At last, these series of injuries consequently developed into cerebral ischemia-reperfusion injury (CIRI). How to reduce the injury is not only a key part of the treatment of ischemic stroke, but also the priority in neuroscience research.

The study on the treatment of CIRI should be started from its complex pathogenesis, so looking for multi-target and multi-link drugs is imperati-
The trade name of 3-n-butylphthalide (NBP) is Butylphthalide Soft Capsule, which is the first Category of State New Drug under the independent intellectual property right of China, and is the first innovative drug applied in ischemic stroke around the world. The drug can improve the local circulation of lesions and alleviate the injury of brain tissues through a number of links, which is the first choice for the treatment of cerebral infarction. Once ischemic cerebrovascular disease occurs, it is difficult to be timely treated, thus leading to poor prognosis. From the perspective of prevention, early intervention in high-risk groups will be an alternative option for such patients.

In this study, a CIRI model was established using the suture method. The changes after CIRI were explored by observing brain tissue infarction size and neuron morphological changes. To provide a basis for the mechanism study, ROS levels, SOD activity, MDA content, the permeability of BBB, the expression of mitogen-activated protein kinases (MAPKs) and neuronal apoptosis were also measured. Based on these finding, we aimed to provide a more reliable theoretical and experimental basis for the clinical application of NBP in the prevention of the ischemic cerebrovascular disease.

Materials and Methods

Materials

The synthesized dl-3-n-butylphthalide (NBP) was provided by Weifang Yidu Central Hospital (Weifang, China). It was dissolved in normal saline. The content of MDA and the activity of SOD were detected with ELISA detection kits provided by Beyotime Institute of Biotechnology (Shanghai, China). The antibodies of MAPK and cleaved Caspase-3 were purchased from the Cell Signaling Technology (Danvers, MA, USA). All other chemicals and materials were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Model Establishment and Screening

The study was approved by the Animal Ethics Committee of Weifang Yidu Central Hospital Animal Center. 90 healthy adult Sprague-Dawley (SD) rats, weighing range from 180-220 g, were fed for 1 week in ventilated cages at 18-22°C and 30-50% humidity with free food and water to adapt to the feeding environment. In our study, 90 rats were divided into three groups: Sham operation group (Sham group), ischemia-reperfusion group (I-R group) and NBP pretreatment group (I-R+NBP group) randomly. The rats in I-R+NBP group were pre-treated with gavage administration of the NBP at 75 mg/kg once a day for 7 days, rats in the other two groups were treated with the same amount of saline. 24 h after the last administration, rats from the I-R group and the I-R+NBP group were inflicted to focal cerebral ischemia/reperfusion injury via occlusion of the middle cerebral artery using the improved Longa-Zea method[11]. In brief, the animals were incised in the midline of the neck and the soft tissues were retracted. The right common carotid artery (CCA) was identified, and it was followed toward the rostral portion, which bifurcated into the external carotid artery (ECA) and the internal carotid artery (ICA). The intraluminal embolus was inserted past the ECA stump into the ICA (17-19 mm) until a slight resistance was felt. At this moment, the embolus was blocked by the origin of the right MCA. After 2 h, the embolus was removed carefully to allow MCA reperfusion for 24 h. Rats in Sham group suffered the same procedure but did not receive embolus insertion.

Measurement of Brain Water Content

The brain water content was determined to access the brain edema, which is according to the wet-dry method[12]. In brief, the brains of rats were immediately acquired after the reperfusion. A neutral filter paper was used to absorb and remove blood stains from the brain. The hemispheres were dissected, an electronic scale was used to detect the wet weight of the tissue (wet weight). Subsequently, tissues were dried overnight in a desiccating oven and adapted to the dry weight. The brain water content was calculated with the formula: brain water content (g) = (wet weight - dry weight).

Measurement of Infarct Volume

After the brains were harvested, they were sliced into 1-mm coronal sections after 24 h reperfusion. The cortical infarct volume was measured by 2,3,5-triphenyltetrazolium chloride (TTC) according to manufacturer’s instructions; briefly, the sections were staining with 2% TTC in phosphate-buffered saline (pH 7.4) at 37°C for 20 min. After they were stored by formalin, the cross-sectional area of the TTC-unstained region was determined.

Measurement of BBB Permeability

The blood-brain barrier (BBB) integrity was detected by using Evans Blue injection[13]. In brief,
after 24 h of reperfusion, the animals were immediately anesthetized and injected with 2% Evans Blue solution (4 mL/kg) by intravenous injection into the jugular vein. After incubation with Evans Blue solution for 30 min, the brains were removed, separated, and homogenized in dimethyl sulfoxide (DMSO). The samples were centrifuged at 12,000 g for 30 min, the supernatants were adapted and measured.

Measurement of ROS level
The Reactive oxygen species (ROS) was measured with oxidation-sensitive 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) dye. After 24 h of perfusion, the brains were removed and cut into coronal sections at the same level (2 µm). Preparation for total lysis buffer with protease inhibitor cocktail, 300 µL of lysis buffer/100 mg of sample, was added. The samples were homogenized and centrifuged at 12,000 g for 15 min at 4°C and the supernatant was collected. A volume of 50 µL of the supernatant was placed in a 96-well plate and incubated with 10 µL of DCFH-DA solution for 25 min in the dark. The samples were measured by using a fluorescent microplate reader at an absorbance of was 450 nm to calculate the ROS level.

Detection of MDA Content and SOD Activity
After, the brain was removed and homogenized in ice-cold normal saline. The samples were centrifuged at 3000 rpm for 20 min. The supernatants were collected to determine the levels of malondialdehyde (MDA) and superoxide dismutase (SOD) by using the MDA and SOD ELISA kit.

Histopathological Examination
Hematoxylin eosin (HE) staining was used to exhibit the morphological features of injured neurons in the cerebral cortex. After reperfusion, tissue samples were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned. Then, the sections were counterstained with hematoxylin-eosin, and photographed with microscope.

Measurement of Cell Apoptosis
The apoptosis was detected by TUNEL assay according to the manufacturer (Roche, Switzerland). Horseradish peroxidase (HRP)-mediated diaminobenzidine reaction was used to visualize the TUNEL-positive cells. Following the counterstain, fields were photographed and randomly selected. The number of TUNEL-positive cells was expressed as a percentage of the total number of nuclei.

Western Blot Analysis
The brain tissues were collected and homogenized on ice. Then lysis buffer was added to the samples. Protein centration was measured by bicinchoninic acid (BCA) method. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the tissue lysates. Primary antibodies against p38 MAPK, phospho-p38 MAPK, ERK, phospho-ERK, JNK, phospho-JNK, and cleaved caspase-3 antibodies were purchased from Abcam (Cambridge, MA, USA). The samples were incubated with primary antibodies at 4°C overnight and, then, incubated with secondary HRP-conjugated antibodies for 1 h. The membrane was, then, incubated with enhanced chemiluminescence (ECL) (Millipore, Billerica, MA, USA) for luminescence generation. The proteins were visualized and detected. β-actin was served as internal control.

Statistical Analysis
All results are presented as the means ± standard deviation (SD). The statistical analyses were performed using both GraphPad Prism 6.02 (GraphPad Software, La Jolla, CA, USA) and PASW Statistics 18.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to compare differences among groups. An unpaired t-test was used for comparisons between 2 groups. A value of p<0.05 was considered statistically significant.

Results
Effects of NBP on the Brain Water Content After I/R
To evaluate brain edema, brain water content was determined. Results showed that brain water content significantly increased in the I-R groups compared with the Sham group. In contrast, NBP pre-treatment significantly decreased brain water content (Figure 1A).

Effects of NBP on the BBB Permeability After I/R
Evans Blue extravasation was used as an indicator for the BBB breakdown. Compared with the Sham group, EB contents in brain tissues were increased significantly in I-R groups. Meanwhile, pretreated with the NBP, significant decrease of EB contents was also observed (Figure 1B).
Effects of NBP on the Cerebral Infarction After I/R

The detection by TTC showed that the percentage of infarct volume was found to have increased in the I-R groups, while NBP could reduce the infarct volume significantly compared to the I-R group (Figure 1C).

Effects of NBP on the Level of ROS, SOD Activity and MDA Content After I/R

By comparing with the Sham group, the ROS level and MDA content was increased remarkably and the SOD activities decreased in the I-R groups. NBP treatment significantly reduced ROS and MDA, and increased SOD activity. Therefore, NBP could remarkably remit the oxidative stress (Figure 1D-F).

Effect of NBP on Morphology Change After I/R

HE staining results showed that the neurons in the cerebral cortex in the sham group were uniformly distributed and well organized. However, in the I-R groups, the neurons were arranged irregularly, exhibiting deeply colored and condensed nuclei. While treatment with NBP could significantly alleviate these neuronal changes (Figure 2).

Effect of NBP on Cell Apoptosis After I/R

As detection by Western blot, we could see the release level of cleaved caspase-3 was significantly increased in I-R groups in comparison with Sham group. Cleaved caspase-3 is a characteristic sign of apoptosis. Intervention with NBP prevented cleaved-Caspase-3 activation. TUNEL staining was performed for further evaluation. Results showed that NBP treatment markedly reduced neurons apoptosis compared with the Sham group (Figure 3).

Effect of NBP on MAPK Phosphorylation After I/R

The MAPK family comprises the following 3 primary subfamilies: ERK1/2, JNK, and p38. The phosphorylation of p38 and JNK levels were both gained in I-R groups compared with...
Sham. However, ERK was not affected in this series of interventions. NBP administration prevented the increases in p38 and JNK phosphorylation (Figure 4).

**Discussion**

With the continuous improvement of CIRI research, more and more data show that effects of...
the oxidative stress in ischemic cerebrovascular disease cannot be underestimated. In the early stage of cerebral ischemia, especially after reperfusion, oxygen free radicals, reactive oxygen species (ROS) are significantly increased, which can quickly attack molecules of other compounds to produce new free radicals due to its strong chemical activity. Then new free radicals produce more radicals by this way, which is called the free radical chain reaction.

Lipid peroxidation and MDAs deposition might be the results of this reaction, which may consequently lead to neuronal abnormal metabolism, membrane receptors damage and even cell death. Under physiological conditions, there are a series of free radicals in the body that can clear radical enzymes such as SOD. When the amount of the generated free radicals is small, they will be quickly removed by the free radical scavenging system without causing tissue cell damage; during the cerebral ischemia reperfusion, ROS is significantly increased, and SOD and other scavenger enzymes are excessively consumed, resulting in decreased activity and causing oxidative stress injuries of tissue cells.

Apoptosis, also known as the programmed cell death, is a “waterfall” activation process regulated by endogenous genes, enzymes and signal transduction pathways, and it is considered as the main way of the delayed neuronal

**Figure 4.** Effects of NBP on the phosphorylation of kinase in MAPK signal pathways (including p-38, JNK and ERK). NBP administration prevents the p38 and JNK phosphorylation. Representative Western blots and densitometry data for the levels of phospho-p38/p38, phospho-ERK/ERK, and phospho-JNK/JNK in each group. PFC administration prevented the increases in p38 and JNK phosphorylation. However, cerebral ischemia-reperfusion injury did not induce the phosphorylation of ERK. (**p<0.01, ***p<0.001, vs. sham group; **p<0.05, ***p<0.01, vs. I-R group, respectively).
death after cerebral ischemia-reperfusion. Since apoptosis has been testified to be involved in the process of pathological injuries of ischemic cerebrovascular disease, apoptosis has become the focus of people’s attention, and antagonistic apoptosis has brought new hope for the treatment of ischemic cerebrovascular disease. MKPK is an important transmembrane signaling pathway, mainly including three important MAPK cascade reactions: p38, extracellular signal-regulated kinase 1/2 (ERK1/2) and c-Jun N-terminal kinase (JNK), which are involved and play an important role in cell growth, proliferation and apoptosis processes. p38, ERK1/2 and JNK are sensitive to ROS stimulation\(^{21,22}\). Recent experiments have shown that ROS can activate MAPK and lead to apoptosis\(^{23,24}\). Besides, our experimental results prove the same outcome, confirming that ROS produced by ischemia-reperfusion can activate MAPK pathway and further induce the activation of apoptosis protein caspase-3, thus leading to neuronal apoptosis.

Brain edema is a serious complication of brain tissue ischemia-reperfusion, which can aggravate the symptoms of cranial hypertension, lead to encephalopathy in severe cases, and even threaten life\(^{25}\). CIRI-induced cerebral edemas are mostly angiogenic cerebral edemas, characterized by the accumulation of edematous fluid in the extracellular space caused by the increased permeability of BBB\(^{26}\). The increased BBB permeability will enable water molecules, plasma and other ingredients to enter into brain tissues, leading to brain edema, and some inflammatory factors will directly damage the neurons through BBB, aggravating cerebral ischemic injuries\(^{27}\).

Effects of NBP have been widely demonstrated in the treatment of ischemic cerebrovascular diseases. However, it is hard for patients to receive timely medical treatments. These patients might suffer serious sequelae, which not only affect their quality of life, but also burden their families and the society. Therefore, our research on the prevention of neurological disease has far-reaching significance. NBP was preventively applied for CIRI to investigate its effect on the lesion.

**Conclusions**

The results of this study provide a practical clinical significance for the prevention and treatment of ischemic cerebrovascular disease, which brings immeasurable economic and social benefits, thus providing ideas for the expansion of clinical adaptability of NBP and possible approaches for the application of new drugs.

**Conflict of interest**

The authors declare no conflicts of interest.

**References**

10. Panahpour H, Niekooeian AA, Dehghani GA. Candesartan attenuates ischemic brain edema and protects the blood-brain barrier integrity from ische-


