**DL-3-n-Butylphthalide (NBP) Provides Neuroprotection in the Mice Models After Traumatic Brain Injury via Nrf2-ARE Signaling Pathway**

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**Abstract** The present study was aimed to evaluate the neuroprotective effects of NBP in the mice models of TBI, as well as the possible role of Nrf2-ARE pathways in the assumptive neuroprotection. In mice, a modified Marimrou’s weight-drop model was employed to induce TBI. ICR mice were randomly assigned to four experimental groups: sham, TBI, TBI+vehicle (V) and TBI+NBP. NBP (100 mg/kg) was administrated via an intraperitoneal (i.p.) injection at 1 h following TBI. The administration of NBP significantly ameliorated the effects of the brain injury, including neurological deficits, brain water content, and cortical neuronal apoptosis. Furthermore, the level of malondialdehyde and the activity of superoxide dismutase (SOD) paired with glutathione peroxidase (GPx) were restored in the NBP treatment group. NBP promoted the translocation of Nrf2 protein from the cytoplasm to the nucleus markedly, increased the expressions of Nrf2-ARE pathway-related downstream factors, including hemeoxygenase-1 (HO-1) and NAD(P)H:quinone oxidoreductase 1 (NQO1), and prevented the decline of antioxidant enzyme activities, including SOD and GPx. NBP enhanced the translocation of Nrf2 to the nucleus from the cytoplasm, verified by a western blot, immunofluorescence. Additionally, it upregulated the expression of the Nrf2 downstream factors such as HO-1 and NQO1 were also confirmed via a western blot and real-time quantitative polymerase chain reaction. In conclusion, NBP administration may increase the activities of antioxidant enzymes and attenuate brain injury in a TBI model, potentially via the mediation of the Nrf2-ARE pathway.

**Keywords** dl-3-n-Butylphthalide · Neuroprotection · Traumatic brain injury · Nrf2-ARE signaling pathway · Mice model

**Abbreviations**

NBP          dl-3-n-Butylphthalide
Nrf2         Nuclear factor erythroid 2-related factor 2
ARE          Antioxidant response element
TBI          Traumatic brain injury
TBI+V        Traumatic brain injury+vehicle
TBI+NBP      Traumatic brain injury+dl-3-n-Butylphthalide
MDA          Malondialdehyde
SOD          Superoxide dismutase
GPx          Glutathione peroxidase
HO-1         Hemeoxygenase-1
NQO1         NAD(P)H:quinone oxidoreductase 1
i.p          Intraperitoneal injection
RT-qPCR      Real-time quantitative polymerase chain reaction

**Introduction**

dl-3-n-Butylphthalide (NBP) is a synthesized compound based on l-3-n-Butylphthalide that was extracted from seeds of Apium graveolens. Previous research has indicated that NBP is effective in the treatment of cerebral...
ischemia, possibly due to its effect on improving regional cerebral blood flow [1] and brain energy metabolism [2]. Additionally, in experimentally induced ischemic stroke, NBP decreased the severity of brain edema [3, 4], and reduced neuronal cell death [5, 6]. Neuroprotective effects of NBP have been documented in other experimentally induced brain injury. For example, NBP relieved hypoxia-induced damage in vitro [7], and enhanced the ability of learning and memory in animal models of Alzheimer’s disease [8], and prolonged animal survival in mouse model of amyotrophic lateral sclerosis [9]. The State Food and Drug Administration of China has approved NBP for clinical treatment with ischemic stroke patients in 2002. Thus NBP plays an important role in protecting ischemic brain from further secondary damage. The cerebral edema and ischemia is a secondary development to neurotrauma that contributes greatly to the pathological consequences of the traumatic insult. Possibly, NBP may play a protective effect on traumatic brain injury.

Traumatic brain injury (TBI) not only causes primary mechanical injury of brain cells but also initiates secondary damage that occurs immediately following the primary injury. There are several pathological processes that are responsible for the neuronal death in the secondary damage of TBI, including glutamate excitotoxicity, loss of ionic homeostasis, inflammatory reaction, and oxidative stress [10–12]. Among these processes, oxidative stress plays a crucial role in secondary damage. The production of reactive oxygen species produced from oxidative stress and the exhaustion of the endogenous antioxidant system induce the oxidation of nucleic acids, and DNA breakdown, thereby inducing further cell damage [13]. In other words, oxidative stress causes cerebral cell death.

Nuclear factor erythroid 2-related factor 2 (Nrf2), which is a knowable transcription factor, locates in cytoplasm and is bound to its inhibitor, Kelch-like ECH associated protein 1 (Keap1), which associates with the actin cytoskeleton and prevents Nrf2 from entering the nucleus. When a damaged neuron cell is stimulated by some protective factors, nrf2 activates the antioxidant response and initiates transcription of a large scale of genes, which are able to counteract the harmful effects of oxidative stress, thus rebuilding intracellular homeostasis [14]. Heme oxygenase 1 (HO-1) and NADPH: quinine oxidoreductase 1 (NQO-1), being the most well known downstream genes of Nrf2 are able to restore intracellular redox-balancing [15].

Previous studies in our laboratory [16–18] have demonstrated that some drugs protect brain from damage of oxidative stress in experimental traumatic brain injury mainly through the Nrf2-ARE signaling pathway. The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) is a key protein that controls the redox state of the cell in oxidative stress [19].

In the present study, we investigated the potential function of NBP in the reduction of oxidative stress by enhancing the activities of antioxidant enzymes in an experimental model of TBI in mice, as well as the role of the Nrf2-ARE signaling pathway within this process.

### Experimental Procedure

#### Experimental Animals

Adult male ICR mice (6–8 weeks) (Experiment Animal Centre of Nanjing Medical University, Jiangsu, China) 28–32 g were used in this study. Mice were housed at 23 ± 1 in humidity-controlled animal quarters with a 12-h light/dark cycle with free access to food and water and were acclimatized for at least 2 days before any experiment. Experiment protocols were approved by the Animal Care and Use Committee of South Medical University and conformed to the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health.

#### Drugs Employed

Synthesized dl-3-n-butyphthalide (NBP) was purchased from Sigma, St. Louis, MO. It was dissolved in DMSO (Sigma, St. Louis, MO) before dilution in the culture medium. The final concentration of DMSO was 0.2% [20]. Varying doses of NBP or equal volume of 0.2% DMSO were injected into peritoneal cavity. See below for information about antibodies and other reagents.

#### Models of TBI

The animal model of TBI used in this study was based on Marmarou’s weight-drop model previously described by Flierl et al [21]. At first, mice were anesthetized with an intraperitoneal injection of chloralhydrate (1%, 5 mL/kg) and then placed onto the platform directly under the hit of the weight-drop device. Then, a 1.0 cm midline longitudinal scalp incision was made to expose the partial skull. After locating the left anterior frontal area as the impact crater (1.5 mm lateral to the midline on the midcoronal plane), a 200 g weight with a height of 2.5 cm was vertically released along a stainless steel string onto the skull. The scalp wound was closed with standard suture material, and the mice were returned to cages, where they had free access to water and food. Sham-injured animals underwent the same procedures, although they did not undergo the weight drop.
Experimental Protocol

Mice were randomly assigned to four experimental groups: sham, TBI, TBI+vehicle and TBI+NBP. Mice of TBI+NBP group received injections of NBP (100 mg/kg diluted in 10mL/kg 0.2% DMSO, i.p.) 1 h after the onset of TBI. This dose of NBP administration was based on study on neuroprotection of NBP in a focal cerebral ischemia mice model [20], where the mice of TBI+vehicle group received equal volumes of 0.2% DMSO (i.p) 1 h after TBI (10 mL/kg). Then, all the mice were returned to separate cages where water and food were supplied freely.

Neurological Evaluation

The neurologic status of the mice was evaluated at 1 h (onset of drug administration), 1, and 3 days following TBI using the neurological severity score (NSS) method with reference to the fill Flierl et al [21]. Every mouse was required to perform 10 different tasks that represented for motor function, balance, and alertness. One point was given for failing to perform each of the tasks; thus 0 = minimum deficit and 10 = maximum deficit. A lower score demonstrated less neurological deficits. The severity of injury is defined by the initial NSS, evaluated 1 h after TBI. Testing was performed by an investigator who was blind to the experimental groups. The sequence of the behavioral tasks was randomized.

Brain Tissue Preparation

The mice were anesthetized with a solution of chloral hydrate at 24 h after TBI and perfused intracardially with 30–40 mL of cold (4°C) heparinized 0.9% saline to isolate the protein. The left (ipsilateral) cerebral cortex peri-contusion was collected, frozen immediately in liquid nitrogen, and then transferred to a −80 °C freezer until use. For fluorescence immunoassay, Nissl staining, and the terminal deoxynucleotidyl transferase-mediated biotinylated deoxyuridine triphosphate nick-end labeling (TUNEL) assay, animals were also euthanized 24 h after TBI. After being deeply anesthetized with chloralhydrate, animals were perfused intracardially with 30–40 mL of cold (4°C) heparinized 0.9% saline followed by 20–30 mL of cold 4% paraformaldehyde. The entire brain was removed and immersed in 4% paraformaldehyde overnight and then was paraffin embedded. Coronal sections of brain tissue were cut at a 4 μm thickness consecutively and were separately sent for fluorescence immunoassay, Nissl staining, and TUNEL assay.

Brain Water Content

Twenty-four hours after TBI, mice brains were removed and placed on a cooled brain matrix. The brain stems and cerebella were removed, and the left cerebral hemispheres were separated. In order to obtain the wet weight (WW) the brain cortical samples were harvested, and immediately weighed. The samples were then dried in an oven for 72 h at 80°C and weighed again to obtain the dry weight (DW). Water content was calculated as [(WW − DW) × 100]÷WW.

Nissl Staining

Coronal sections of brain tissue (4 μm thick) were stained with cresyl violet as previously described [22]. Normal neurons had large cell bodies and cytoplasmic volume, with one or two large, round nuclei. In contrast, damaged cells were identified as those with shrunken cell bodies, condensed nuclei, and dark cytoplasm containing many empty vesicles. Histological examination was performed by two observers who were blind to the group assignment. 10 sections from each animal were used for quantification. Six random fields (×400) in every coronary section were chosen bilaterally, and the mean number of damaged neurons in the six views was regarded as the data of apoptotic neurons of every section.

TUNEL Staining and Cell Counting

The formalin-fixed tissues were embedded in paraffin and sectioned at 4 μm thickness with a microtome. The sections were identified for apoptotic cells by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method. In situ cell death detection Kit POD (ISCDD, Boehringer Mannheim, Germany) was used. The procedures ran according to protocol of the kit and the other references. Sections were deparaffinized, rehydrated, and washed with distilled water briefly. The tissues were digested with 20 g/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) at room temperature for 15 min. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide/methanol in PBS at 37°C for 30 min. The sections then were incubated with terminal deoxynucleotidyl transferase at 37°C for 60 min to add the dioxigenin-conjugatd dUTP to the 3’-OH ends of fragmented DNA. Anti-digoxigenin antibody peroxidase was applied to the sections to detect the labeled nucleotides. The sections were stained with DAB and counterstained slightly with hematoxylin. The positive cells were identified, counted, and analyzed under the light microscope by an investigator blind to the grouping. Apoptotic neuron counting was also restricted to the cerebral cortex.
contusion distance around 3.0 mm. A total of 10 sections from each animal were used for quantification. Six random high power fields (×400) in each coronary section were chosen bilaterally, and the mean number of apoptotic neurons in the six views was regarded as the data of each section. The final average proportion of apoptotic neurons of the sections was regarded as the data for each sample and the severity of brain damage was evaluated by apoptotic index, defined as the average percentage of TUNEL-positive neurons.

**Determination of Malondialdehyde (MDA), Superoxide Dismutase (SOD), and Glutathione Peroxidase (GPx)**

The left cerebral cortex tissue was homogenized in 2 ml of 10 mM phosphate buffer (PBS pH7.4). After centrifugation at 12,000×g for 20 min, the MDA, SOD, and GPx content in the supernatant was measured spectrophotometrically with the appropriate kits (Nanjing Key genentc Biochemistry Co. Nanjing, China). Total protein concentrations were determined by the Bradford method. The contents of MDA, SOD, and GPx were expressed as the nmol/mg protein, the U/mg protein, and the U/mg protein, respectively.

**Western Blot Analysis**

Quinone oxidoreductase 1 (NQO-1), nuclear and cytoplasmic proteins were extracted according to the instructions of the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotech Inc., Nantong, China) to measure the nuclear factor erythroid 2 (Nrf2), hemeoxygenase-1 (HO-1) and NAD(P)H. After the protein concentrations were calculated using the Bradford method, equal protein amounts (50 µg) per lane were separated by 10% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein bands were blocked for 2 h in blocking buffer (Tris buffered saline/0.05% Tween 20) containing 5% skim milk). The nuclear protein was incubated overnight at 4 °C with rabbit monoclonal anti-Nrf2 (1:100, Abcam, Cambridge, MA, USA) and rabbit anti-Histone 3 (1:1000, Cell Signaling Technology, Beverly, MA, USA) in blocking buffer. The cytoplasmic protein was incubated overnight at 4 °C with rabbit anti-Nrf2 (1:1000, Abcam, Cambridge, MA, USA) and rabbit anti-Histone 3 (1:1000, Cell Signaling Technology, Beverly, MA, USA) in blocking buffer. The cytoplasmic protein was incubated overnight at 4 °C with rabbit anti-Nrf2 (1:1000, Abcam, Cambridge, MA, USA) and rabbit anti-Histone 3 (1:1000, Cell Signaling Technology, Beverly, MA, USA) in blocking buffer. After being washed with TBST (3×10 min), the membranes were incubated with goat anti-rabbit horseradish peroxidase (HRP)-conjugated IgG (1:5000, Bioworld Technology, St. Louis Park, MN, USA) for 2 h at room temperature. The protein bands were visualized by enhanced chemiluminescence (ECL) Western blot detection reagents (Millipore, Billerica, MA, USA) and exposure to X-ray film. Developed films were digitized on an Epson Perfection 2480 scanner (Seiko Corp., Nagano, Japan). Band density was quantified using Un-Scan-It 6.1 software (Silk Scientific Inc., Orem, UT, USA); data was normalized to β-actin or Histone 3.

**Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)**

Total RNA was extracted from ipsilateral cortex samples with RNAiso Plus (TaKaRa Bio, Dalian, China). The concentration and purity of total RNA was determined with a spectrophotometer (OD260/280 1.8–2.0) and 1% agarose gel electrophoresis. To avoid RNA degradation, a portion of of the RNA was immediately reverse transcribed to cDNA with the PrimeScript RT reagent kit (TaKaRa Bio, Dalian, China), and the surplus RNA was kept at −80°C. Te primers were designed according to PubMed GenBank and synthesized by Invitrogen Life Technologies (Shanghai, China), designed according to PubMed GenBank and synthesized by Invitrogen Life Technologies (Shanghai, China). The primer sequences were as follows:

- NQO1: F: 5′-CAT TCT GAA AGG CTG GTT TGA-3′; R: 5′-CTAGC TTTGATCGTGTTCG-3′;
- HO-1: F: 5′-ATCGTGTCGATGACACT-3′; R: 5′-CCAACACTGTTTACATGGC-3′;
- β-actin: F: 5′-AGTGTGACGGTGAATCGGTA-3′; R: 5′-GCCAGAGCTATGCTCTTCT-3′.

The quantitative real-time PCR analysis was performed using the Mx3000P System (Strata gene, San Diego, CA, USA), with applied real-time SYBR Green PCR technology. The PCR amplification program consisted of an initial denaturation step of 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, and a 30 s annealing and elongation step at 60°C. All samples were analyzed in triplicate. β-actin was used as an endogenous reference ‘housekeeping’ gene.

**Fluorescence Immunoassay**

Nrf2 expression translocated from cytoplasm to nuclear was evaluated by fluorescence immunoassay. Brain tissue samples were fixed in formalin for 24 h and embedded in paraffin. Sections were cut at a 4 µm thickness consecutively and deparaffinized in xylene, dehydrated in a graded series of ethanol, subjected to antigen retrieval in citrate buffer (pH 6.0) for 30 min in a 37°C chamber, and the finally washed in PBS. The sections were quenched in 3% hydrogen peroxide and blocked with PBS containing 10% goat serum (Sigma-Aldrich) for 1 h at 37°C, then incubated with rabbit monoclonal anti-Nrf2 (1:100, Abcam, Cambridge, MA, USA), overnight at 4°C, followed by three
15-min washes in PBS and incubation with Alexa Fluor 594 (1:200; Invitrogen, Grand Island, NY, USA) for 1 h at room temperature, respectively. After three washes in PBS, the slides were counterstained with DAPI for 2 min. Cover slips were applied with mounting media. Fluorescence was captured on an Olympus IX71 inverted microscope system and analyzed by Image-Pro Plus 6.0 software (Media Cybernetics, USA). The specificity of the immunofluorescence reaction was evaluated by replacement of the primary antibody with PBS.

Statistical Analyses

SPSS21.0 (SPSS Inc., Chicago) was used for the statistical analyses. Each experiment was repeated at least three times, and the data are expressed as the mean ± SEM. The measurements were subjected to student’s t-test and between multiple groups using ANOVA analysis. Differences between experimental groups were determined by Fisher’s LSD posttest. Significance was assigned at $P < 0.05$. Every experiment was repeated at least three times.

Results

Di-3-n-Butylphthalide (NBP) Decreased Neuronal Deficits and Alleviated Cerebral Edema in the Brain After TBI

To investigate the neuroprotective role of NBP, we employed the NSS scores to evaluate the neurobehavioral function after TBI. Initially, all mice were trained 1 day before TBI. The control group showed no difference among time points in the study (data not shown), and also no difference was found between the TBI group and the TBI+V group (Fig. 1a, ns). However, as shown in Fig. 1a, the NSS score of NBP (100 mg/kg) treated mice post TBI was significantly better than that of the TBI or TBI+V group mice at 24 h ($P < 0.01$). Additionally, at 72 h, the significant difference was still apparent (Fig. 1a, $P < 0.01$), which also indicated neuroprotective effects of NBP on mice following TBI.

The brain water content of mice was calculated to confirm the protective effects of NBP at the macroscopic level. As shown in Fig. 1b, mice in the TBI groups or TBI+V groups had significantly more brain water content ($P < 0.05$) when compared with the control group. The brain water content did not differ significantly between the TBI and the TBI+vehicle groups (ns). Nevertheless, groups treated with NBP had significantly less brain water content than the TBI+vehicle group (Fig. 1b, $P < 0.05$).

NBP Inhibited Neuronal Apoptosis in Mice Following TBI

To determine whether the neuroprotective effects of NBP can be detected on histopathology level, we used Nissl staining and TUNEL to examine the apoptotic cells in the brain tissues. Neuron morphology was examined by Nissl staining. While neurons in the sham group were intact and clear (Fig. 2a), in the TBI and TBI+V group most neurons were damaged, exhibiting diverse changes including triangular or oval nuclei, shrunken cytoplasm, and swollen cell bodies (Fig. 2a, arrows). An improvement in neuronal morphology and neuronal apoptosis was observed in mice within the TBI+NBP group as compared to TBI+V group ($P < 0.05$, Fig. 2a, c). Additionally there was no statistical

Fig. 1 Administration of NBP protected mice against secondary brain injury after TBI (a, b). Mice were subjected to TBI and then received 100 mg/kg of NBP or vehicle 1 h after TBI. NSS score was evaluated at 1, 1 and 3 days after TBI, while the brain water content was examined at 24 h after TBI. a TBI+NBP group had an improved neurobehavioral function at 1 or 3 days post TBI. b Mice subjected to TBI or treated with vehicle had increased brain water content as compared with the sham group. Brain water content was significantly lower in the groups with administration of NBP than the vehicle-treated group. Data are presented as mean ± SEM. n=6 per group. *$p < 0.01$ versus sham group; ns, no statistical significance versus TBI group; #$p < 0.05$, ##$p < 0.01$ versus TBI group

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significance detected between TBI group versus TBI+V group (ns, Fig. 2a, c).

TUNEL staining was used to examine neural cell in injured brain tissue. TUNEL-positive cells were observed at a low frequency in the brains of mice in the sham group (Fig. 2b, arrow). The apoptotic index was increased in the TBI group or TBI+vehicle as compared to control group ($P < 0.01$) (Fig. 2b, d) but was reduced in the TBI+NBP group ($P < 0.05$ vs. TBI+vehicle, Fig. 2b, d). These results indicate that NBP blocks neural apoptosis in the brain surrounding the cortical contusion induced by TBI.

**NBP Alleviated Oxidative Stress Caused by TBI**

To determine whether the neuroprotective effect of NBP was derived from its ability to alleviate the oxidative stress caused by TBI, malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPx) were analyzed in brain tissue as an indicator of lipid peroxidation and the activities of antioxidant enzymes, respectively. The TBI and TBI+V groups exhibited an elevation of MDA, which was significantly different from the control groups (Fig. 3a. $P < 0.05$). However, the level of cortical MDA in mice treated with NBP was significantly reduced when compared with the TBI+V group (Fig. 3a. $P < 0.05$), and no differences were detected between the TBI and TBI+vehicle groups.

SOD and GPx are antioxidant enzymes responsible for scavenging metabolites generated by free radicals. As shown in Fig. 3b, c, SOD and GPx were significantly lower in the TBI and TBI+V groups compared with the sham group ($P < 0.01$ and $P < 0.01$, respectively), and there were no differences between the TBI and TBI+V groups. After the administrated NBP, the GPx and SOD level of mice increased more than the TBI and TBI+V groups ($P < 0.05$).

**NBP Accelerated Translocation of Nrf2 from Cytoplasm to Nucleus**

It had been proven from the above results that NBP significantly increased the activities of the antioxidant enzymes following TBI. However, the underlying molecular mechanisms for it remain unclear. Since Nrf2 plays a key role in the activation of antioxidant enzymes, it was reasonable to hypothesize that NBP might activate Nrf2, thereby enhancing the activities of the antioxidant enzymes.
The well-established, classic activation pattern of Nrf2 is the translocation from the cytoplasm to the nucleus. The expression of cytoplasmic Nrf2 and nuclear Nrf2 were investigated through western blotting, respectively. The results demonstrated that, both TBI and NBP administration were inducers of Nrf2 nuclear translocation (Fig. 4a, b) when compared with the control group. In addition, compared with the vehicle treated group, the NBP treated group had a significantly increased expression of nuclear Nrf2 and a reduced expression of cytoplasmic Nrf2 ($P<0.01$ and $P<0.01$, respectively; Fig. 4a, b). There were no differences between the TBI or the TBI+V groups, indicating that NBP promoted Nrf2 nuclear translocation.

This effect was also confirmed by the observations from immunofluorescence of nrf2 and DAPI. The Nrf2 and DAPI overlap expression of TBI or the TBI+V groups were weaker when compared with the control group. In addition, compared with the vehicle treated group, the NBP treated group had a significantly increased expression of nuclear Nrf2 and a reduced expression of cytoplasmic Nrf2 ($P<0.01$ and $P<0.01$, respectively; Fig. 4a, b). There were no differences between the TBI or the TBI+NBP groups, indicating that NBP promoted Nrf2 nuclear translocation.

**NBP Upregulated the Expression of HO-1 and NQO-1 (Nrf2 Downstream Factors)**

The observations demonstrated that NBP was able to activate Nrf2 and provide neuroprotection against TBI. Thus, we hypothesized that NBP might also regulate the Nrf2 downstream pathway. Therefore, the expression of downstream factors in this pathway were further investigated. Both HO-1 and NQO-1 proteins were measured by Western blot testing. The results indicated both HO-1 and NQO-1 proteins were upregulated after TBI ($P<0.05$, $P<0.001$ versus control group separately. Fig. 5a–d). In addition, the administration of NBP following TBI further enhanced protein expression compared with the vehicle treated group ($P<0.001$, $P<0.01$ separately. Fig. 5a–d). Additionally, there were no differences found between the TBI or the TBI+NBP groups.

Moreover, the NBP enhanced the expression of HO-1 and NQO-1 compared with the TBI and TBI+V groups ($P<0.001$, $P<0.01$ separately. Fig. 5e, f), which is consistent with the protein changes at the mRNA level. There was no difference between the TBI and TBI+V groups, which had more protein expressions than the control group ($P<0.01$, $P<0.01$ separately. Fig. 5e, f). These results indicated that NBP induced more expression of Nrf2 downstream factors with regards to both proteins and mRNA levels through the activation of the Nrf2-ARE signal pathway.

**Discussion**

NBP possesses a multiplicity of important biological properties, including anti-oxidative [23, 24] in model of Parkinson’s disease, anti-apoptotic [25] in diabetic rats, and anti-inflammatory in ischemic rats [26]. Additionally, NBP has been engineered to relieve brain edema in mice of close head injury, although the research had not explained the inner mechanism [27]. These beneficial effects led us to hypothesize the inner mechanism by which NBP protects the brain from oxidative insults. In our study, animal model was intraperitoneally injected with NBP, 1 h after TBI.
Fig. 4 The results of western blot and immunofluorescent assay definitely demonstrate that NBP promotes Nrf2 translocation from cytoplasm to nucleus (c, arrows). NBP significantly increased the level of Nrf2 in the nucleus (b), and consequently reduced the level of Nrf2 in the cytoplasm (a). β-actin and H3 were used as protein loading control to Nrf2 expression in the cytoplasm and nuclear separately. The Nrf2 staining indicating the aim proteins (red) and DAPI staining indicating the nucleus (blue). The merged immunofluorescent images with Nrf2 and DAPI represented for Nrf2 translocation from cytoplasm to nucleus (c, arrows). The white arrows indicate expressions and overlaps of Nrf2 and DAPI. Data are presented as mean ± SEM, n = 6 per group; *P < 0.05, **P < 0.01 versus sham group; ns, no statistical significance versus TBI group; ##P < 0.01 versus TBI+V group. Scale bar 100 μm.
Marmarou’s weight-drop model was used in the animal model [21], which is a well-documented model of closed head injury in mice. The result showed that treatment with NBP after TBI relieved brain contusion-induced oxidative stress, alleviated brain edema, and improved neurological function scores in TBI. In addition, NBP treatment activated antioxidant enzymes, including SOD, GPx, HO-1 and NQO1 by means of the Nrf2-ARE pathway. To our knowledge, it was necessary that study of evaluating the effects of NBP in the adjustment of the activity of the Nrf2-ARE signaling pathway in a TBI model was initiated.

In the present study, ICR type mice were utilized to investigate the neuroprotective effects of NBP after TBI. The main results are summarized as follows. (1) Experimental mice were protected by NBP administration through improving neurobehavioral performance, relieving cerebral edema, and suppressing neuronal apoptosis. (2) The oxidative stress state induced by TBI was alleviated after treatment with NBP in mice, and represented by the level of MDA, the activity of SOD and GPx. (3) NBP promoted a translocation of Nrf2 from cytoplasm to nucleus, and then successively increased the expression of downstream enzymes.
factors, HO-1 and NQO1, on mRNA and protein levels caused by the elevated binding ability of Nrf2 to AREs enhancers.

The modified weight-drop model has been thought to be an appropriate imitation in the study of TBI and induced oxidative stress. The content of oxidative products (MDA) were increased while the activity of antioxidant enzymes (SOD and GPx) were reduced around the contusion cortex at the 24 h after the TBI. This phenomenon showed that TBI caused oxidative stress reaction. Furthermore, the fact that NBP alleviated the oxidative insult of TBI also confirmed the existence of oxidative stress in this model.

The protective effects of NBP in neurons via its antioxidative characteristics have been shown in many studies, demonstrating that NBP acts as an inducer of antioxidant enzymes [23, 24, 28]. The consecutive pathological processes after traumatic brain injury include oxidative stress, neurotoxicity, inflammatory reaction, and vascular abnormalities. Several oxidants and their derivatives are generated after TBI, including superoxide anions and hydroxyl radicals [29]. This enhanced production of reactive oxygen species and MDA along with exhausted antioxidant defense enzymes, such as SOD and GPx, caused oxidative stress [30]. However, the underlying molecular mechanisms related to NBP and antioxidant enzymes in TBI are still unknown.

To confirm that the Nrf2-ARE cell signal pathway is involved in the neuroprotective role of NBP, we investigated the changes in the Nrf2-ARE signaling pathway after treatment with NBP. The present data displayed the translocation of Nrf2 from the cytoplasm to the nucleus after TBI, NBP further promoted this translocation. Furthermore, this finding is consistent with the classic pattern of Nrf2 activation in protein expression, including translocation from the cytoplasm to the nucleus [16]. NBP-triggered enhancement of expressions of Nrf2 is involved in the usage of delaying the onset and progression of diabetic cataract in rat diabetic model [31], as well as the treatment of AD mouse model [32].

NBP is a widely used clinically therapeutic drug for ischemic stroke. It is a multiple-target neuroprotective agent that significantly reduces oxidative damages, improves mitochondrial function, neuronal apoptosis, and inhibits inflammation [33, 34]. Additionally, Professor Feng had reported that NBP upregulated the expressions of Nrf2 and its downstream antioxidants enzyme, heme oxygenase-1 (HO-1) in a mouse model of amyotrophic lateral sclerosis [9].

Nrf2/ARE pathway plays an important role in adapt to oxidative stress through up-regulating phase II detoxifying enzymes. Previous studies have demonstrated that polychlorinated biphenyl quinone exposure caused antioxidant function via Nrf2/ARE pathway activation, HO-1 and NQO1 highly expression [35]. NBP had a protection effect through up-regulation of NF-E2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) in the spinal cord of Tg mice [9]. The present study demonstrated, that exposure to NBP after TBI of mice model resulted in a significant increase in Nrf2 translocation from the cytoplasm to the nucleus, as well as the Nrf2 targeting genes, NQO1, and HO-1. Moreover, NBP induced additional expression of Nrf2 downstream factors with regards to both mRNA levels and proteins through the activation of the Nrf2-ARE signal pathway.

Furthermore, the study of immunofluorescence analysis identified the accumulation of Nrf2 in nucleus of mice treated with NBP after TBI. The increased Nrf2 induced more combination with protein Keap1 in nucleus. After the formation of complex with protein Keap1, the phase II detoxifying enzymes were activated and significant up-regulation of the mRNA level of antioxidant enzymes (NQO1 and HO-1 [36–39]), which developed a neuro-protective effect at last.

In summary, the present study demonstrated that NBP attenuated the oxidative reaction by enhancing the expressions and activities of antioxidant enzymes in a TBI model. Furthermore, our date showed that NBP exerted neuroprotection against TBI by combating with oxidative stress at least partially via the translocation of Nrf2 from cytoplasm to nucleus and activation of downstream proteins, NQO1 and HO-1. However, further study is still needed to elucidate the underlying mechanism of Nrf2 translocation after NBP administration, especially when Nrf2 knocked out mice are involved.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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