Original paper

Folia Neuropathologica

The neuroprotective effect of Dl-3-n-butylphthalide in epileptic rats *via* inhibiting endoplasmic reticulum stress

Shuang Tian[1,2], Zhenzhen Qu[1], Huifang Cao[3], Xiaoli Niu[4], Qi Qiao[1], Bing Zhang[2], Lijing Jia[1], Weiping Wang[1]

[1]Department of Neurology, The Second Hospital of Hebei Medical University, Shijiazhuang, Hebei, P. R. China, [2]Department of Neurology, Shijiazhuang People's Hospital, Shijiazhuang, Hebei, P.R. China, [3]Department of Rehabilitation, The Second Hospital of Hebei Medical University, Shijiazhuang, Hebei, P.R. China, [4]Department of Neurology, Hebei General Hospital, Shijiazhuang, Hebei, P.R. China

Folia Neuropathol 2023; 61 (2): 185-195 *DOI: https://doi.org/10.5114/fn.2022.123516*

Abstract

Introduction: *The purpose of this study is to investigate whether Dl-3-n-Butylphthalide (NBP) has a neuroprotective effect on pilocarpine-induced epileptic (EP) rats through endoplasmic reticulum stress (ERS)-mediated apoptosis.*

Material and methods: *The Sprague-Dawley rats were divided into four groups: control (CON), EP, EP + NBP_{60} (NBP 60 mg/kg) and EP + NBP_{120} (NBP 120 mg/kg) groups. After the successful establishment of the temporal lobe EP model using the lithium-pilocarpine, the rats were given NBP for 28 consecutive days in EP + NBP_{60} and EP + NBP_{120} groups. Then, the spontaneous recurrent seizure (SRS) latency, SRS frequency and seizure duration were observed in each group. In order to observe the abnormal discharge of rats, the intracranial electrodes were implanted to monitor the electroencephalogram. Nissl staining was used to observe the damage to the hippocampal CA1 neurons, TUNEL staining was employed to observe hippocampal neuronal apoptosis. Western blot was used to detect the expression of ERS and ERS-mediated apoptotic proteins.*

Results: *NBP_{60} and NBP_{120} decreased SRS frequency (all $p < 0.05$), shortened seizure duration (all $p < 0.05$), and reduced the abnormal discharge of the brain. Nissl staining and TUNEL staining results show that NBP protected the hippocampal neurons from damage (all $p < 0.05$) and inhibited hippocampal neuronal apoptosis in EP rats (all $p < 0.05$). NBP_{60} and NBP_{120} could reduce ERS and ERS-mediated apoptotic protein expression in EP rats (all $p < 0.05$). In addition, the therapeutic effect of NBP on epilepsy in rats is dose-dependent. The SRS frequency of the EP + NBP_{120} group was lower, and the seizure duration was shorter than in the EP + NBP_{60} group (all $p < 0.05$), and there were more neurons in the EP + NBP_{120} group than in the EP + NBP_{60} group ($p < 0.05$).*

Conclusions: *NBP had a significant neuroprotective effect in EP rats. Large doses of NBP are more effective than low doses. The mechanism may be associated with the inhibition of ERS and ERS-mediated apoptosis.*

Key words: *Dl-3-n-Butylphthalide, epilepsy, endoplasmic reticulum stress, apoptosis, neuroprotection.*

Introduction

Epilepsy (EP) is one of the most common neurological disorders. According to the World Health Organization, there are approximately 50 million patients with EP worldwide and approximately six million patients with active EP in China [30]. After standardised and reasonable drug treatment, among newly diagnosed EP patients, 70-80% can be controlled, and approximately 60-70% of patients no longer need treatment with drugs after 2-5 years [31]. However, in approximately 40% of patients with EP, it cannot be completely controlled and progresses to refractory EP [31]. It is of great significance to research and develop effective new drugs for the treatment of EP. Studies have found that oxidative stress, immune-inflammatory responses,

Communicating author:
Weiping Wang, Department of Neurology, The Second Hospital of Hebei Medical University, 215 Heping West Road, Shijiazhuang, Hebei 050000, People's Republic of China, e-mail: wei22ping@163.com

energy metabolism disorders, excitatory glutamate toxicity and calcium homeostasis imbalance are closely related to EP [15]. However, the pathogenesis of EP needs to be fully clarified.

The endoplasmic reticulum (ER) is an important location for protein synthesis, lipid synthesis and intracellular Ca^{2+} storage [29]. When cells are subjected to stimuli, such as hypoxia, inflammation, oxidative stress damage, etc., the normal functions of the ER are destroyed, and a large number of unfolded and misfolded proteins accumulate in the ER lumen, which cause ER stress (ERS) and activate the unfolded protein response (UPR) [28]. Under normal physiological conditions, the transmembrane proteins PKR-like ER kinase (PERK), inositol-requiring enzyme 1α (Ire1α) and transcription factor 6 (ATF6) are combined with the ER chaperone protein binding immunoglobulin (Ig) protein (Bip)/glucose regulated protein (GRP) 78 to maintain stability [9,23]. When ERS occurs, large numbers of unfolded or misfolded proteins in the ER lumen accumulate to compete to bind with Bip, leading to termination of translation and protein synthesis, accelerated degradation of misfolded proteins and the enhanced folding protein ability of ER, thereby maintaining homeostasis [11,34]. However, when the internal and external stimuli persist or the intensity is too high, the unfolded protein response will increase the expression of CHOP, caspase 12 and P-JNK, finally initiating cell apoptosis [32]. ERS is involved in the pathogenesis of a number of diseases, but few studies are available concerning the involvement of ERS in EP.

Dl-3-n-Butylphthalide (NBP) is an extract of Chinese celery seeds which has a neuroprotective effect. Previous studies have confirmed that NBP can improve the blood and oxygen supply to ischemic brain tissue, promote the establishment of vascular collateral circulation in narrow or occluded blood supply areas, protect mitochondrial function in brain cells, improve the permeability of the blood-brain barrier and exert various effects, such as anti-inflammatory and antioxidant effects [2-42], playing a protective role in ischemic cerebrovascular disease, cognitive dysfunction and Parkinson's disease [19-25]. Zheng *et al*. found that in rats with spinal cord damage, NBP reduced the damage to the blood-spinal cord barrier by inhibiting ERS-mediated apoptosis [40]. Niu *et al*. reported that NBP could ameliorate cognitive impairment in VD rats by inhibiting ERS [27]. It can be seen that NBP plays a therapeutic role in a number of diseases by inhibiting excessive ERS. However, there are few reports on the use of NBP in treating EP.

In this study, the use of the EP rat model, through the administration of NBP drug intervention, from the physiological and molecular levels to explore the efficacy of NBP on epilepsy and its possible mechanism. This provides data support for the development of effective drugs for the treatment of epilepsy.

Material and methods

Animals

A total of 57 Sprague-Dawley (SD) male rats (weighing 180-200 g, aged 7-8 weeks) were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. Each rat was housed separately during postoperative status epileptics, and then each group was fed in a separate cage (three rats per cage) after status epilepticus. They were kept in separate cages in 12 h light/dark conditions at room temperature (22-25°C) and had free access to water and food. For adaptation to a novel environment, the rats were exposed freely 3 d before the initiation of modelling. Each rat was reweighed before modelling.

All experiments conformed to the National Institute of Health guidelines for the care and use of laboratory animals and the European Communities Council Directive of 24 November 1986 (86/609/EEC). The study was approved by the Research Ethics Committee of the Second Hospital of the Hebei Medical University (2019-AE049). All efforts were made to minimise animal suffering.

Material

Dl-3-n-Butylphthalide was provided by the CSPC NBP Pharmaceutical Co., Ltd. in the form of a yellow, oily liquid with a purity > 99%. It was mixed with corn oil for use in the EP + NBP groups.

Experimental grouping and drug intervention

The experiment was randomly divided into four groups: the control group (CON, $n = 12$), EP group ($n = 15$), EP + NBP 60 mg/kg group (EP + NBP_{60}, $n = 15$) and EP + NBP 120 mg/kg group (EP + NBP_{120}, $n = 15$).

Starting from the day after the successful establishment of the rat model, the rats were weighed daily, and the EP + NBP_{60} and EP + NBP_{120} groups were given the corresponding dose of NBP (60 mg/kg and 120 mg/kg, respectively) by gavage from the 7^{th} day post-surgery for 28 days, once a day. The CON and EP groups were given an equal volume of corn oil daily for 28 days, once a day.

The preparation of the temporal lobe EP rat model

For the EP, EP + NBP_{60} and EP + NBP_{120} groups, a total of 45 SD rats were weighed and lithium chloride (127 mg/kg; Sigma, St. Louis, USA) was injected by intra-

peritoneal injection (IP) 20 h prior to the administration of pilocarpine (PILO; 50 mg/kg IP; Sigma, St. Louis, USA). To reduce the peripheral reaction of PILO, atropine sulfate (1 mg/kg IP; Tianjin Jinyao Pharmaceutical Co., Ltd, Tianjin, China) was administered 30 min before the PILO injection. Seizures were assessed according to the Racine classification standard (level 0 – no convulsion, level I – chewing movement, level II – rhythmic nodding, level III – unilateral forelimb clonus, level IV – rearing with bilateral forelimb clonus and level V – generalised tonic-clonic seizure, loss of balance and even falls). When the rats showed a lasting level IV or above, they were identified as having started status epilepticus (SE). After 60 min of continuous seizure activity, seizures were terminated using 2% sodium pentobarbital. Rats without successful SE (5 rats) and dead rats (4 rats) during modelling process were excluded from the experiment [17]. The rest of SE rats were randomly divided into 3 groups, the EP group (n = 12), EP + NBP_{60} group (n = 12) and EP + NBP_{120} group (n = 12). In the control group, a 0.9% sodium chloride solution was used to replace the PILO injection, and the procedure was performed as described for the model group.

Monitoring of spontaneous recurrent seizure latency, SRS frequency and seizure duration, intracranial electrode implantation and electroencephalogram monitoring

At 7 d of NBP gavage, the rats were placed in separate transparent cages, and video monitoring equipment was used to monitor the spontaneous recurrent seizure (SRS) latency, SRS frequency and seizure duration in the rats.

At 21 d of NBP gavage, six rats were randomly selected from each group, and after anaesthesia, each rat was fixed to a stereotaxic apparatus (type: 68027; RWD, Shenzhen, China). After routine skin preparation and disinfection, the top of the skull was cut and exposed, and the surgical site was marked at the anterior and posterior fontanelles. A hole was drilled into the skull 2.0 mm anterior and 2.0 mm lateral to the bregma, an anchoring screw was placed and the recording electrode was connected. Two holes were drilled separately in the left and right occipital bones of the rat, an anchoring screw was placed in each of these locations and the reference and ground electrodes were connected. Dental cement was used to fix the electrodes. After the operation, the drugs and corn oil were given regularly *via* gavage, and the follow-up experiment was conducted 4 d postoperatively. The implanted electrode was connected to the electroencephalograph (PL3516, AD Instruments, Australia; DP304, Warner Instruments, USA) for 3 consecutive days to monitor and record the electroencephalogram (EEG) changes during the seizures. An epileptiform seizure was defined as a seizure when the EEG revealed sharp waves, spike waves, sharp (spine) slow comprehensive waves and multiple spike slow waves and when video monitoring showed the symptoms of the attacks.

Nissl staining

After EEG monitoring, six rats were randomly selected from each group, anesthetized with 2% sodium pentobarbital and perfused with 4% paraformaldehyde. The rats were decapitated and the brains were fixed in 4% paraformaldehyde for 24 h at a low temperature. After the wax blocks of brain were prepared, the hippocampus was continuously cut into coronary slices of 5 µm, and a total of five slices were selected from each group for Nissl staining to observe the degree of hippocampal neuron damage. Slices were dewaxed in xylene and rehydrated in gradient alcohol. Then, slices were stained with 1% thionine solution for 3-5 min, rinsed in distilled water, dehydrated in gradient alcohol, transparented in xylene and sealed with neutral balsam. Microscopic examination was performed under Olympus BX51 optical microscope (Olympus, Tokyo, Japan).

Terminal deoxynucleotidyl transferase dUTP nick-end labelling staining

The staining was carried out using an *in situ* cell death detection kit (Roche, Basel, Switzerland) in accordance with the manufacturer's instructions. In short, the slices underwent dewaxing, rehydration, proteinase K incubation at 37°C for 30 min and rinsing with phosphate buffered saline (PBS). A 50 µl transferase dUTP nick-end labelling (TUNEL) reaction mixture was added, and the samples were incubated for 1 h at 37°C in a dark, humidified chamber, then incubated for 30 min in 50 µl converter-POD at 37°C in a dark, humidified chamber. After washing with PBS, DAB was displayed for colour, and haematoxylin was redyed for dehydration, transparency and sealing. The apoptotic cells in the hippocampal CA1 were observed and photographed using the Olympus BX51 optical microscope (Olympus, Tokyo, Japan). The Image J software was used to count TUNEL-positive cells in the hippocampal CA1 and calculate the percentage of TUNEL-positive cells, i.e., the proportion of TUNEL-positive cells to the total number of cells.

Western blot

The total protein was extracted using a protein extraction kit, and the protein concentration was mea-

sured using the BCA method. A total of 70 g of the protein was taken for denaturation, followed by vertical gel electrophoresis using 10% SDS/PAGE and transferred to the PVDF membranes. They were blocked in 5% skimmed milk powder at room temperature for 1 h and incubated with the primary antibodies anti-GRP78 (1 : 1000; Cell Signaling Technology, Boston, USA), anti-PERK (1 : 1000; Cell Signaling Technology, Boston, USA), anti-P-PERK (1 : 1000; Cell Signaling Technology, Boston, USA), anti-eIF2α (1 : 1000; Cell Signaling Technology, Boston, USA), anti-P-eIF2α (1 : 1000; Cell Signaling Technology, Boston, USA), anti-Ire1α (1 : 1000; Cell Signaling Technology, Boston, USA), anti-P-Ire1α (1 : 1000; Abcam, Cambridge, UK), anti-ATF6 (1 : 500; Abcam, Cambridge, UK), anti-CHOP (1 : 1000; Cell Signaling Technology, Boston, USA), anti-JNK (1 : 1000; Cell Signaling Technology, Boston, USA), anti-P-JNK (1 : 1000; Cell Signaling Technology, Boston, USA), anti-Bcl-2 (1 : 500; Abcam, Cambridge, UK), anti-Bax (1 : 1000; Cell Signaling Technology, Boston, USA), anti-caspase 12 (1 : 1000; Cell Signaling Technology, Boston, USA) and anti-c-caspase 3 (1 : 1000; Cell Signaling Technology, Boston, USA) overnight at 4°C and then rinsed with PBS. They were then incubated in the goat anti-rabbit IgG fluorescent antibody (1 : 10,000, Rockland, Limerick, USA) at room temperature in the dark for 1 h, then rinsed with PBS. The Odyssey far-infrared fluorescence scanning imaging system (LI-COR, Lincoln, USA) was used to scan and measure the optical density of the target protein per unit length, which was then compared with the optical density value of GAPDH (1 : 1000, Cell Signaling Technology, Boston, USA) for statistical analysis.

Statistical analysis

Data are expressed as mean ± standard deviation, SPSS 23.0 was used for statistical analysis, and GraphPad Prism 6 was used to generate the figures. All data were tested for normality and homogeneity of variance using Levene's test. Measurement data were analysed using one-way analysis of variance for intergroup comparison. For data of SRS latency, SRS frequency, seizure duration and protein levels (with homogenous variance), the intergroup comparison was performed using the LSD test, and for data of Nissl staining and TUNEL staining (with heterogeneous variance), the Tamhane's T2 test was used. $P < 0.05$ indicated a statistical difference, and $p < 0.01$ indicated a significant statistical difference.

Results

NBP reduced the SRS frequency, shortened the seizure duration and reduced the EEG interictal discharge in EP rats

As shown in Table I, the rats in each group were subjected to behavioural observation 7 days after SE. There was no significant difference in the SRS latency between the EP, EP + NBP_{60} and EP + NBP_{120} groups ($p > 0.05$). When compared with the EP group, the SRS frequency was lower in the EP + NBP_{60} and EP + NBP_{120} groups ($p < 0.05$), and the seizure duration was shorter ($p < 0.05$). When compared with the EP + NBP_{60} group, the SRS frequency was lower, and the seizure duration was shorter in the EP + NBP_{120} group ($p < 0.05$).

As shown in Figure 1, the EEG of the CON group was mainly α or β rhythms with a frequency of 5-10 Hz and a wave amplitude < 100 uV. Interictal discharge in EP rats refers to the appearance of regular spikes with EP wave frequency < 2 Hz and amplitude ≥ 3 times the EEG baseline. The interictal epileptiform discharge was observed in the EEG and recorded. When compared with the CON group, the interictal discharge was significantly increased in the EP, EP + NBP_{60} and EP + NBP_{120} groups, but the interictal discharge was less frequent in the EP + NBP_{60} and EP + NBP_{120} groups than in the EP group and less frequent in the EP + NBP_{120} group than in the EP + NBP_{60} group.

NBP reduced hippocampal neuron damage in EP rats

The result of Nissl staining is shown in Figure 2. The hippocampi in the CON group were abundant and full, and the pyramidal cells in the CA1 region had normal morphology, i.e., they were well arranged and uniformly stained with large round nuclei, obvious nucleoli and abundant cytoplasmic Nissl bodies. The hippocampi

Table I. The effect of Dl-3-n-Butylphthalide on seizure of PILO-induced epileptic rats. Values are expressed as the means ± SEM ($n = 12$ per group)

Groups	Number of rats	SRS latency (day)	SRS frequency (per day)	Seizure duration (s)
CON	12	–	–	–
EP	12	12.00 ±3.74	2.53 ±0.36	33.41 ±5.78
EP + NBP_{60}	12	14.00 ±3.03	1.91 ±0.57*	27.71 ±3.45*
EP + NBP_{120}	12	15.33 ±3.78	1.26 ±0.38*#	22.67 ±3.32*#

$p < 0.05$ vs. the EP group, #$p < 0.05$ vs. the EP + NBP_{60} group

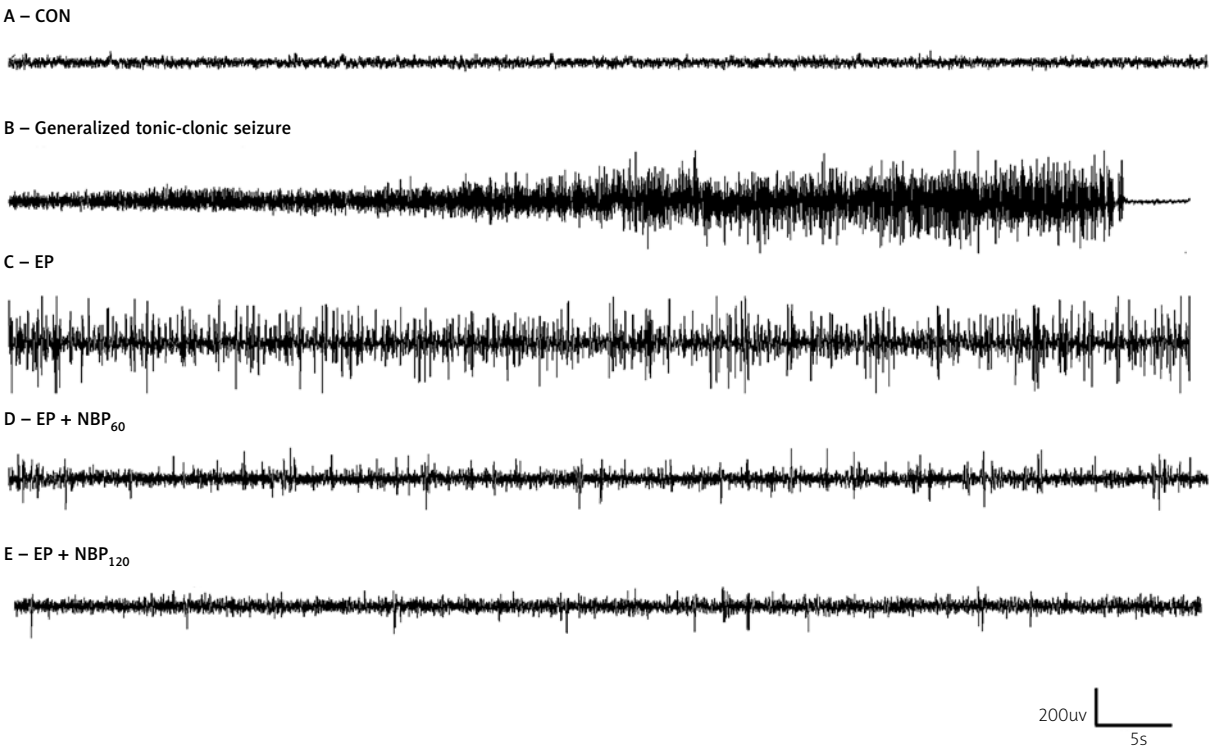

Fig. 1. The effect of Dl-3-n-Butylphthalide treatment on brain electrical discharge in EP rats (n = 6 per group). CON – control group, EP – epileptic group, EP + NBP_{60} – epileptic with 60 mg/kg NBP group, EP + NBP_{120} – epileptic with 120 mg/kg NBP group.

in the EP group were significantly atrophic, the neurons in the CA1 were sparsely arranged and significantly reduced in number when compared with the CON group, and the nuclei were pyknotic with unclear nucleoli, smaller cell volume and significantly reduced Nissl bodies. The hippocampi in the EP + NBP_{60} group and EP + NBP_{120} group were relatively more abundant than those in the EP group, and the pyramidal cells in the CA1 had more regular morphology, were greater in number and had more Nissl bodies (Fig. 2A).

When compared with the CON group, the number of normal neurons in the EP, EP + NBP_{60} and EP + NBP_{120} groups was significantly lower ($p < 0.01$). The EP + NBP_{60} and EP + NBP_{120} groups had more nerve cells than the EP group ($p < 0.05$), and there were more neurons in the EP + NBP_{120} group than in the EP + NBP_{60} group ($p < 0.05$) (Fig. 2B).

NBP inhibited hippocampal neuronal apoptosis in EP rats

TUNEL staining was used to observe the apoptosis of the hippocampal CA1 neurons in each group (Fig. 3A). TUNEL staining results showed that the percentage of TUNEL-positive cells in the hippocampal CA1 region in the EP group was significantly higher than in the CON group ($p < 0.01$). After NBP intervention, the percentage of TUNEL-positive cells in the CA1 in the EP + NBP_{60} and EP + NBP_{120} groups was lower than in the EP group ($p < 0.05$), and the percentage of TUNEL-positive cells in the EP + NBP_{120} group was lower than in the EP + NBP_{60} group ($p < 0.05$) (Fig. 3B).

NBP reduced ERS and ERS-related apoptotic protein expression in EP rats

The Western blot method was used to detect ERS and the expression of ERS-related apoptotic proteins, such as GRP78, PERK, P-PERK, eIF2α, P-eIF2α, Ire1α, P-Ire1α, ATF6, CHOP, JNK, P-JNK, caspase 12, c-caspase 3, Bcl-2 and Bax in the hippocampi of the rats from each group. When compared with the CON group, the expression of GRP78, P-PERK, P-eIF2α, P-Ire1α, ATF6, CHOP, P-JNK, caspase 12, c-caspase 3 and Bax was higher in the EP group ($p < 0.01$), and the expression of anti-apoptotic protein Bcl-2 was lower ($p < 0.01$). When compared with the EP group, the expression of GRP78, P-PERK, P-eIF2α, P-Ire1α, ATF6, CHOP, P-JNK, caspase 12, c-caspase 3 and Bax was lower in the EP + NBP_{60} group ($p < 0.05$), and the expression of Bcl-2 was higher ($p < 0.05$). The expression of P-PERK, P-eIF2α, P-Ire1α, ATF6, CHOP, P-JNK, caspase 12 and c-caspase 3 was

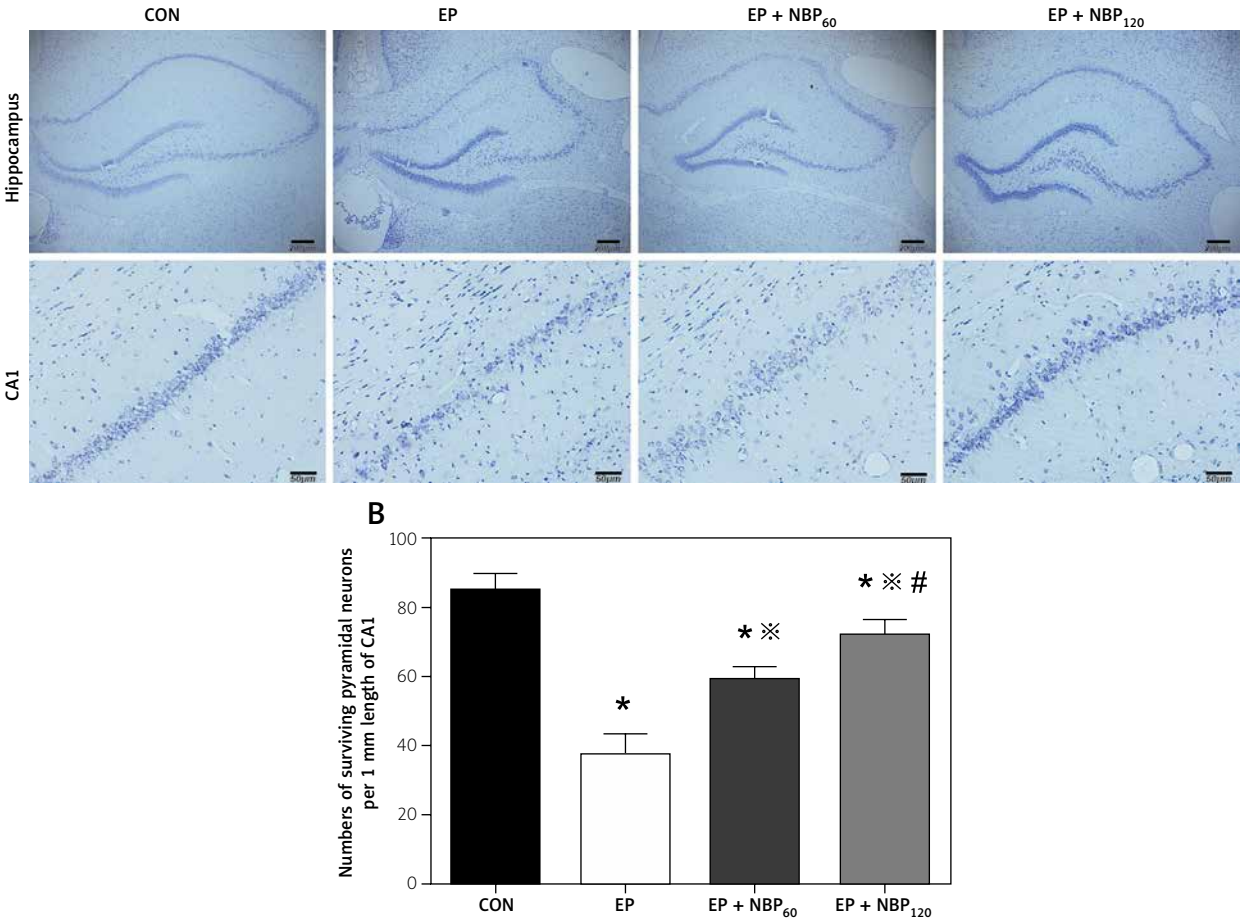

Fig. 2. A) Nissl staining results of hippocampal lesions of rats in each group to observe the number of neurons in the hippocampal CA1. Magnification: Hippocampus, 40×; CA1, 200×; **B**) The number of neurons in the CON group is 85.16 ±4.62, in the EP group 37.40 ±5.89, in the EP + NBP_{60} group 59.00 ±3.82 and in the EP + NBP_{120} group 72.00 ±4.47. CON – control group, EP – epileptic group, EP + NBP_{60} – epileptic with 60 mg/kg NBP group, EP + NBP_{120} – epileptic with 120 mg/kg NBP group. Values are expressed as the means ± SEM (n = 6 per group; $^{*}p < 0.01$ vs. the CON group, $^{※}p < 0.05$ vs. the EP group and $^{\#}p < 0.05$ vs. the EP + NBP_{60} group).

more significantly lower in the EP + NBP_{120} group than in the EP + NBP_{60} group ($p < 0.05$), and the expression of Bcl-2 was higher ($p < 0.05$). No significant difference was found in the protein expressions of PERK, eIF2α, Ire1α and JNK between the groups (Fig. 4).

Discussion

Dl-3-n-Butylphthalide is widely used in the treatment of acute ischemic cerebrovascular disease in clinical practice in China and intervenes in the pathophysiological processes of cerebral ischemia. NBP can improve the symptoms of ischemic stroke and help in long-term recovery [37]. Zhou *et al*. found in a randomized controlled clinical study that the continuous use of NBP for six months could significantly improve the symptoms of patients with PD and slow the progression of the disease [41]. Subsequent studies found that NBP could improve cognitive impairment in vascular dementia (VD) rats [33]. Han *et al*. found that NBP could reduce the level of the excitatory neurotransmitter glutamate in the brain of EP mice [12]. Yang *et al*. reported that NBP could reduce the amplitude of the miniature excitatory postsynaptic current and the EP-like electrical activity in EP rats [39]. In this experiment, NBP was used to intervene in PILO-induced EP rats. The results indicated that NBP can reduce EP seizures, reduce abnormal discharge of the brain, protect hippocampal neurons from injury, and inhibit apoptosis of hippocampal neurons. The above

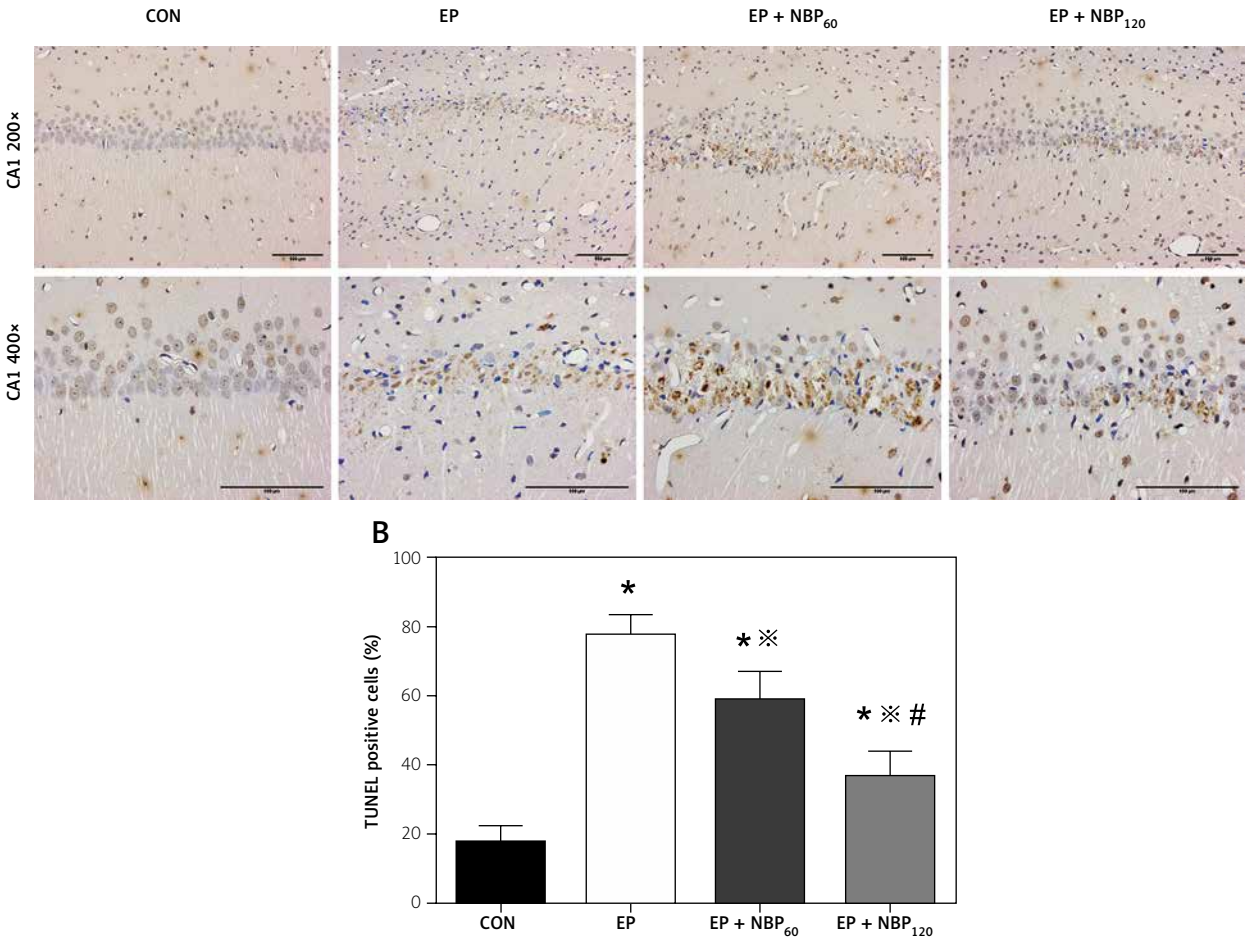

Fig. 3. A) The effect of NBP on hippocampal CA1 neuronal apoptosis of PILO-induced EP rats detected using TUNEL staining. **B**) The percentage of TUNEL-positive cells in the CON group is 17.50 ±4.76, in the EP group 77.84 ±5.25, in the EP + NBP_{60} group 58.72 ±7.99 and in the EP + NBP_{120} group 36.59 ±7.14. CON – control group, EP – epileptic group, EP + NBP_{60} – epileptic with 60 mg/kg NBP group; EP + NBP_{120} – epileptic with 120 mg/kg NBP group. Values are expressed as the means ± SEM (n = 6 per group; $^{*}p < 0.01$ vs. the CON group, $^{※}p < 0.05$ vs. the EP group and $^{\#}p < 0.05$ vs. the EP + NBP_{60} group.

effects were more obvious in the EP + NBP_{120} group than in the EP + NBP_{60} group. These results show that NBP has a definite neuroprotective effect on EP rats, and there is a dose-effect relationship.

The ER is an important organelle that maintains normal cell function. When cells are stimulated by some kinds of stimulation, GRP78 was isolated from three transmembrane proteins, PERK/eIF2α, Ire1α/XBP-1 and ATF6 pathway are activated [1,4]. PERK and Ire1α homodimerise and activate *via* autophosphorylation, and activated P-PERK phosphorylates the downstream translation initiation factor eIF2α, leading to a translation halt and reduced protein synthesis [6,24]. Ire1α is activated *via* autophosphorylation, which leads to a frameshift of protein translation and the production of active transcription factor XBP-1s, which enhances the transcription of ER chaperone genes and recovery of ER stability [10-16]. ATF6 is transported to the Golgi apparatus and is cleaved by its endoprotease to produce an active transcription factor ATF6 (P50) that enters the nucleus, binds to the ER stress response element (ERSE) and expresses ER-related genes to restore ER stability [7,36]. When the internal and external stimuli persist or the intensity is too high, UPR will eventually initiate apoptosis [13]. P-eIF2α activates ATF4 transcription while reducing protein synthesis, thereby inducing CHOP expression [14]. *In vivo* and *in vitro* studies have shown that CHOP is the main regulator of ERS-induced apop-

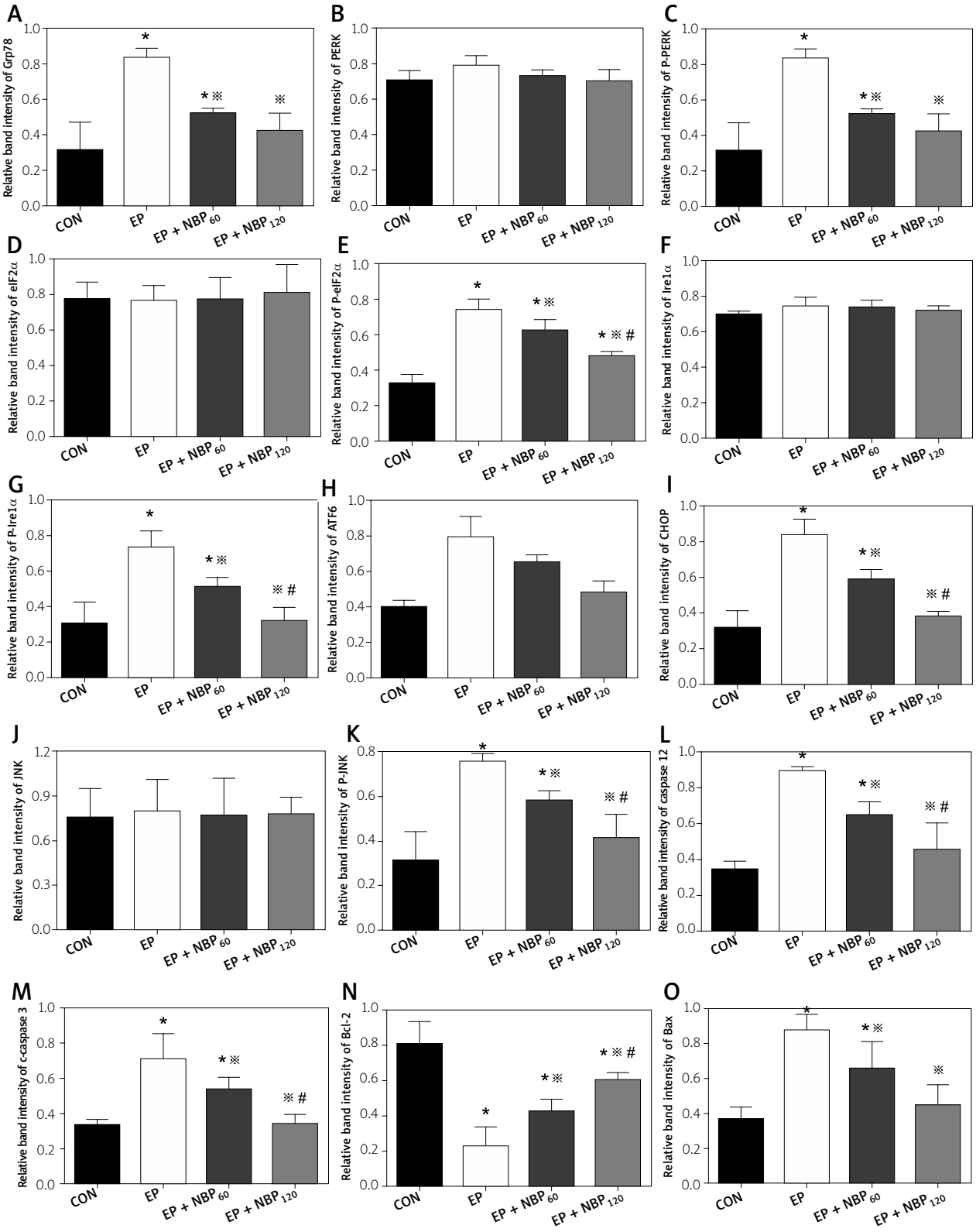

Fig. 4. The effects of NBP treatment on the expression of GRP78, PERK, P-PERK, eIF2α, P-eIF2α, Ire1α, P-Ire1α, ATF6, CHOP, JNK, P-JNK, caspase 12, c-caspase 3, Bcl-2 and Bax in the hippocampus. **A-O**) The quantitative analyses of GRP78, PERK, P-PERK, eIF2α, P-eIF2α, Ire1α, P-Ire1α, ATF6, CHOP, JNK, P-JNK, caspase 12, c-caspase 3, Bcl-2 and Bax. CON–control group, EP – epileptic group, EP + NBP_{60} – epileptic with 60 mg/kg NBP group, EP + NBP_{120} – epileptic with 120 mg/kg NBP group. Data are expressed as the means ± SEM (n = 6 per group; $^{*}p < 0.01$ vs. the CON group, $^{※}p < 0.05$ vs. the EP group and $^{\#}p < 0.05$ vs. the EP + NBP_{60} group).

tosis, and CHOP-induced apoptosis plays a key role in ERS. Highly expressed CHOP can reduce the expression of the anti-apoptotic protein Bcl-2 through transcriptional inhibition and promote the expression of Bax, BIM and BID, thus regulating cell apoptosis [21]. In the case of severe or sustained ERS, P-Ire1α interacts with tumour necrosis factor receptor-associated factor 2 (TRAF2) to form a complex, which activates the downstream JNK signalling pathway, promotes the release of cytochrome C from mitochondria and the activation of caspase 9, leading to cell apoptosis [5]. The P-Ire1a/TRAF2 complex induces and activates caspase 12 on the surface of the ER cell membrane. The activated caspase 12 successively activates its downstream caspase 9 and caspase 3, eventually leading to cell apoptosis [20]. Activated ATF6 enters the nucleus and combines with specific ERSE to initiate the expression of apoptosis-related CHOP and other genes [18].

In this study, we used a chronic EP model induced by PILO, which is currently recognized as an ideal model for human temporal lobe EP [3-26]. After the successful induction of SE, the rats were given an intragastric administration of NBP for 28 consecutive days. While the rats were in the stage of chronic EP, the SRSs were observed, and the expression of ERS-related proteins was detected. When compared with the CON group, we found that the expression of ERS and ERS-mediated apoptotic proteins, including GRP78, P-PERK, P-eIF2α, P-Ire1α, ATF6, CHOP, P-JNK, caspase 12, c-caspase 3 and Bax was significantly higher in the hippocampi of rats with EP, while the expression of anti-apoptotic protein Bcl-2 was lower. The results indicated that ERS and ERS-mediated apoptosis were involved in the pathogenesis of EP. Akitaka Yamamoto *et al*. found that ERS and ERS-related apoptotic proteins GRP78 and caspase 9 were higher in the removed tissues of patients with temporal lobe EP than in healthy patients [38]. Zhu *et al*. reported that ERS developed in the hippocampi of C57 mice with chronic EP induced by PTZ, and the expression of GRP78 and CHOP was higher than in mice without EP [43]. Fu *et al*. found that the expressions of GRP78, p-eIF2a and CHOP were higher in C57 mice with acute EP induced by pentylenetetrazol after general tonic-clonic seizures, and sodium valproate could inhibit the high expression of ERS-related apoptotic protein in the hippocampi of EP mice to prolong the latency of seizures, thereby exerting a neuroprotective effect [8]. In contrast to the aforementioned studies, our study is the first to investigate the ERS and the mechanism of ERS-mediated apoptosis in rat models of chronic temporal lobe EP induced by PILO. Western blot revealed that after NBP intervention in EP rats, highly expressed ERS and ERS-mediated apoptotic proteins in the hippocampi of the rats, including GRP78, P-PERK,

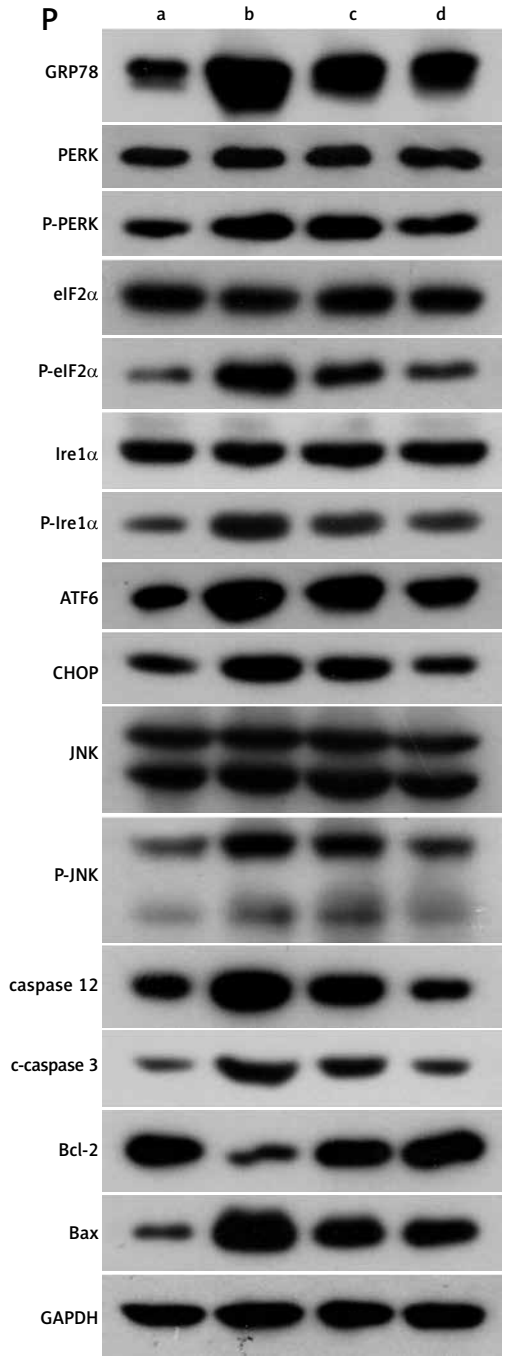

Fig. 4. Cont. The effects of NBP treatment on the expression of GRP78, PERK, P-PERK, eIF2α, P-eIF2α, Ire1α, P-Ire1α, ATF6, CHOP, JNK, P-JNK, caspase 12, c-caspase 3, Bcl-2 and Bax in the hippocampus. **P**) The Western blot of GRP78, PERK, P-PERK, eIF2α, P-eIF2α, Ire1α, P-Ire1α, ATF6, CHOP, JNK, P-JNK, caspase 12, c-caspase 3, Bcl-2 and Bax in the CON, EP, EP + NBP_{60} and EP + NBP_{120} groups. CON – control group, EP – epileptic group, EP + NBP_{60} – epileptic with 60 mg/kg NBP group, EP + NBP_{120} – epileptic with 120 mg/kg NBP group.

P-eIF2α, P-Ire1α, ATF6, CHOP, P-JNK, caspase 12, c-caspase 3 and Bax, decreased, whereas the expression of the anti-apoptotic protein Bcl-2 increased. The change was more significant in the EP + NBP_{120} group than in the EP + NBP_{60} group. Therefore, NBP can protect neurons by inhibiting ERS and reducing ERS-mediated apoptosis in EP rats. Liao *et al*. found that NBP could improve the anxiety and depression behaviours caused by Adriamycin by inhibiting ERS and ERS-mediated apoptosis [22].

There are also limitations and deficiencies in this study. We only studied and observed the SRS and mechanism of chronic epilepsy after NBP intervention, but did not do any work about the cognitive impairment, affective disorder and other accompanying symptoms of the EP rats. It can be seen that our research on the neuroprotective effect of NBP on epilepsy is not comprehensive enough, which is the limitation of this experimental study. Besides, 4 rats died after intracranial electrode implantation operation, reminding us of the need to improve the proficiency in further studies.

Conclusions

Dl-3-n-Butylphthalide treatment after SE can reduce SRS frequency in PILO-induced chronic EP, shorten the duration of seizures, and reduce interictal discharges. NBP treatment protects the hippocampal neurons from damage after SRS, inhibits hippocampal neuronal apoptosis in EP rats, and can reduce the overexpression of ERS and ERS-mediated apoptosis protein in the hippocampus of EP rats. There is a dose-effect relationship, which means that large doses of NBP are more effective than low does. The above finding showed that NBP has a clear neuroprotective effect on EP rats. B the above, NBP may become a new treatment method for epilepsy patients.

Acknowledgements

The authors wish to thank Li Chunyan, academician of the Key Laboratory of Neurology, the Second Hospital of Hebei Medical University, for his valuable help in providing perfect experimental equipment and conditions.

Funding

This work was supported by the National Natural Science Foundation of China (81671292) and the Natural Science Foundation of Hebei Province (H2018206435).

Statement of Ethics

This study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the Second Hospital of Hebei Medical University.

Disclosure

The authors report no conflict of interest.

References

1. Apostolou E, Moustardas P, Iwawaki T, Tzioufas AG, Spyrou G. Ablation of the chaperone protein ERdj5 results in a Sjögren's syndrome-like phenotype in mice, consistent with an upregulated unfolded protein response in human patients. Front Immunol 2019; 10: 506.
2. Bi MJ, Zhang MW, Guo DD, Bi WK, Liu B, Zou Y, Li Q. N-Butylphthalide alleviates blood-brain barrier impairment in rats exposed to carbon monoxide. Front Pharmacol 2016; 7: 394.
3. Cao SS, Kaufman RJ. Endoplasmic reticulum stress and oxidative stress in cell fate decision and human disease. Antioxid Redox Signal 2014; 21: 396-413.
4. Carrara M, Prischi F, Nowak P R, Kopp C, Ali MM. Noncanonical binding of BiP ATPase domain to Ire1 and Perk is dissociated by unfolded protein CH1 to initiate ER stress signaling. Elife 2015; 4: e03522.
5. Chen C, Lv Q, Li Y, Jin YH. The anti-tumor effect and underlying apoptotic mechanism of ginsenoside Rk1 and Rg5 in human liver cancer cells. Molecules 2021; 26: 3926.
6. Chen KY, Chen YJ, Cheng CJ, Jhan KY, Wang LC. Excretory/secretory products of Angiostrongylus cantonensis fifth-stage larvae induce endoplasmic reticulum stress via the Sonic hedgehog pathway in mouse astrocytes. Parasit Vectors 2020; 13: 317.
7. Chen QG, Men YJ, Wang D, Xu DQ, Liu SY, Xiao SB, Fang LR. Porcine reproductive and respiratory syndrome virus infection induces endoplasmic reticulum stress, facilitates virus replication, and contributes to autophagy and apoptosis. Sci Rep 2020; 10: 13131.
8. Fu J, Peng L, Wang W, He H, Zeng S, Chen TC, Chen Y. Sodium valproate reduces neuronal apoptosis in acute pentylenetetrzole-induced seizures via inhibiting ER stress. Neurochem Res 2019; 44: 2517-2526.
9. Ghemrawi R, Khair M. Endoplasmic reticulum stress and unfolded protein response in neurodegenerative diseases. Int J Mol Sci 2020; 21: 6127.
10. Govindarajan S, Gaublomme D, Van Der Cruyssen R, Verheugen E, Gassen SV, Saeys Y. Stabilization of cytokine mRNAs in iNKT cells requires the serine-threonine kinase IRE1alpha. Nat Commun 2018; 9: 5340.
11. Guan LY, Zhan ZG, Yang YZ, Miao Y, Huang X, Ding M. Alleviating chronic ER stress by p38-Ire1-Xbp1 pathway and insulin-associated autophagy in C. elegans neurons. PLoS Genet 2020; 16: e1008704.
12. Han L, Wang QY, Liu XM. The effect of butylphthalide on amino acid in the brain of epileptic mice. Minerva Pediatr 2016; Online ahead of print.
13. Hetz C. The unfolded protein response: controlling cell fate decisions under ER stress and beyond. Nat Rev Mol Cell Biol 2012; 13: 89-102.
14. Hu H, Tian MX, Ding C, Yu SQ. The C/EBP homologous protein (CHOP) transcription factor functions in endoplasmic reticulum stress-induced apoptosis and microbial infection. Front Immunol 2018; 9: 3083.
15. Hui YY, Ahmad N, Makmor-Bakry M. Pathogenesis of epilepsy: challenges in animal models. Iran J Basic Med Sci 2013; 16: 1119-1132.
16. Korennykh A, Walter P. Structural basis of the unfolded protein response. Annu Rev Cell Dev Biol 2012; 28: 251-277.

17. Leite JP, Garcia-Cairasco N, Cavalheiro EA. New insights from the use of pilocarpine and kainate models. Epilepsy Res 2002; 50: 93-103.
18. Li D, Wang WJ, Wang YZ, Wang Y, Li Y. Lobaplatin promotes (125) I-induced apoptosis and inhibition of proliferation in hepatocellular carcinoma by upregulating PERK-eIF2α-ATF4-CHOP pathway. Cell Death Dis 2019; 10: 744.
19. Li HY, Wang HQ, Zhang L, Wang MS, Li YF. Dl-3-n-Butylphthalide alleviates behavioral and cognitive symptoms via modulating mitochondrial dynamics in the A53T-alpha-synuclein mouse model of Parkinson's disease. Front Neurosci 2021; 15: 647266.
20. Li HT, Zhang XR, Qi X, Zhu X, Cheng LM. Icariin inhibits endoplasmic reticulum stress-induced neuronal apoptosis after spinal cord injury through modulating the PI3K/AKT signaling pathway. Int J Biol Sci 2019; 15: 277-286.
21. Li YM, Guo YS, Tang J, Jiang JL, Chen ZN. New insights into the roles of CHOP-induced apoptosis in ER stress. Acta Biochim Biophys Sin (Shanghai) 2014; 46: 629-640.
22. Liao DH, Xiang DX, Dang RL, Xu PF, Wang JM, Han WX, Fu YZ, Yao DW, Cao LZ, Jiang P. Neuroprotective effects of dl-3-n-Butylphthalide against doxorubicin-induced neuroinflammation, oxidative stress, endoplasmic reticulum stress, and behavioral changes. Oxid Med Cell Longev 2018; 2018: 9125601.
23. Liu Y, Wang XM, Zhen Z, Yu YB, Qiu YW, Xiang WS. GRP78 regulates milk biosynthesis and the proliferation of bovinemammaryepithelial cells through the mTOR signaling pathway. Cell Mol Biol Lett 2019; 24: 57.
24. Luhr M, Torgersen M L, Szalai P, Szalai P, Hashim A, Brech A, Staerk J, Engedal N. The kinase PERK and the transcription factor ATF4 play distinct and essential roles in autophagy resulting from tunicamycin-induced ER stress. J Biol Chem 2019; 294: 8197-8217.
25. Lv CN, Ma QY, Han B, Li J, Geng Y, Zhang XM, Wang MW. Long-term DL-3-n-Butylphthalide treatment alleviates cognitive impairment correlate with improving synaptic plasticity in SAMP8 mice. Front Aging Neurosci 2018; 10: 200.
26. Nirwan N, Vyas P, Vohora D. Animal models of status epilepticus and temporal lobe epilepsy: a narrative review. Rev Neurosci 2018; 29: 757-770.
27. Niu XL, Jiang X, Xu GD, Zheng GM, Tang ZP, Yin N. DL-3-n-butylphthalide alleviates vascular cognitive impairment by regulating endoplasmic reticulum stress and the Shh/Ptch1 signaling-pathway in rats. J Cell Physiol 2019; 234: 12604-12614.
28. Oakes SA, Papa FR. The role of endoplasmic reticulum stress in human pathology. Annu Rev Pathol 2015; 10: 173-194.
29. Ruan YX, Zeng JJ, Jin QK, Chu MP, Ji KT, Wang ZY, Li L. Endoplasmic reticulum stress serves an important role in cardiac ischemia/reperfusion injury (Review). Exp Ther Med 2020; 20: 268.
30. Savage N. Epidemiology: The complexities of epilepsy. Nature 2014; 511: S2-3.
31. Sethi AK, Singh V, Chaurasia RN, Joshi D, Pathak A, Rath S, Mishra A, Mishra VN. Study of knowledge, attitude, and practice among epilepsy patients in North India. J Neurosci Rural Pract 2020; 11: 278-285.
32. Tabas I, Ron D. Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. Nat Cell Biol 2011; 13: 184-190.
33. Tian AY, Ma XC, Li H, Zhang RW. Dl-3n-butylphthalide improves spatial learning and memory in rats with vascular dementia by reducing autophagy via regulation of the mTOR signaling pathway. Exp Ther Med 2020; 19: 1940-1946.
34. Wang S, Kaufman RJ. The impact of the unfolded protein response on human disease. J Cell Biol 2012; 197: 857-867.
35. Wang S, Ma F, Huang LJ, Zhang Y, Peng YC, Xing CH, Feng YP, Wang XL, Peng Y. Dl-3-n-Butylphthalide (NBP): a promising therapeutic agent for ischemic stroke. CNS Neurol Disord Drug Targets 2018; 17: 338-347.
36. Wires ES, Trychta KA, Kennedy LM, Harvey BK. The function of KDEL receptors as UPR genes in disease. Int J Mol Sci 2021; 22: 5436.
37. Yang LC, Li J, Xu SF, Cai J, Lei H, Liu DM, Zhang M, Rong XF, Cui DD, Wang L, Peng Y, Wang XL. L-3-n-butylphthalide promotes neurogenesis and neuroplasticity in cerebral ischemic rats. CNS Neurosci Ther 2015; 21: 733-741.
38. Yamamoto A, Murphy N, Schindler CK, So NK, Stohr S, Taki W, Prehn JHM, Henshall DC. Endoplasmic reticulum stress and apoptosis signaling in human temporal lobe epilepsy. J Neuropathol Exp Neurol 2006; 65: 217-225.
39. Yang Q, Hu YD, Wang XF, Zheng FS. Dl-3n-butylphthalide reduces epileptiform activity through GluA2-lacking calcium-permeable AMPARs in epilepsy models. Oncotarget 2017; 8: 98242-98257.
40. Zheng BB, Zhou YL, Zhang HY, Yang GY, Hong GH, Han DD, Wang QQ, He ZL, Liu YL, Wu FZ, Zhang X, Tong SL, Xu HZ, Xiao J. Dl-3-n-butylphthalide prevents the disruption of blood-spinal cord barrier via inhibiting endoplasmic reticulum stress following spinal cord injury. Int J Biol Sci 2017; 13: 1520-1531.
41. Zhou HY, Ye M, Xu WF, Yu ML, Liu XL, Chen YH. DL-3-n-butylphthalide therapy for Parkinson's disease: A randomized controlled trial. Exp Ther Med 2019; 17: 3800-3806.
42. Zhou PT, Wang LP, Qu MJ, Shen H, Zheng HR, Deng LD, Ma YY, Wang YY, Wang YT, Tang YH, Tian HL, Zhang ZJ, Yang GY. Dl-3-N-butylphthalide promotes angiogenesis and upregulates sonic hedgehog expression after cerebral ischemia in rats. CNS Neurosci Ther 2019; 25: 748-758.
43. Zhu XJ, Dong JD, Han B, Huang RR, Zhang AF, Xia ZR, Chang HH, Chao J, Yao HH. Neuronal nitric oxide synthase contributes to PTZ kindling epilepsy-induced hippocampal endoplasmic reticulum stress and oxidative damage. Front Cell Neurosci 2017; 11: 377.