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Original Article

The protective effect of butylphthalide on hippocampal mitochondria in epileptic rats

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Abstract: Objective: This study investigates the protective effect of butylphthalide on hippocampal mitochondria in epileptic rats. Methods: (1) A rat model of pentylenetetrazol (PTZ)-kindling was used. (2) Enzymatic activity of respiratory chain complexes II and IV was quantified in the hippocampus. (3) Mitochondrial morphology of the hippocampal CA3 region was observed by transmission electron microscopy; (4) Electrical activity of the rat hippocampal CA3 region was recorded with an electrode in vivo. Results: Compared with the epilepsy (EP) group, the butylphthalide (NBP) group showed significantly improved mitochondrial respiratory chain complex IV activity in epileptic hippocampi [Control group (78.2967 ± 7.6304) mmol Cytc/min/mg; EP group (45.3269 ± 11.6556) mmol Cytc/min/mg; and NBP group (72.1153 ± 15.1009) mmol Cytc/min/mg ($P < 0.01$). In addition, the mitochondrial ultrastructural damage was reduced and the frequency of electrical bursts in the hippocampal CA3 region decreased. Conclusion: Butylphthalide has a protective effect on mitochondrial function in the hippocampus of epileptic rats, thereby reducing the abnormal electrical activity of neurons. It may therefore have potential as an adjuvant in treatment during the active epilepsy phase.

Keywords: Epilepsy, pentylenetetrazol, butylphthalide, hippocampus, mitochondria

Introduction

Some research have found that epilepsy can lead to impaired mitochondrial function, which in turn can lead to a cascade of events that eventually result in structural neuronal damage or neuronal death [1], notably, mitochondrial dysfunction can also lead to epilepsy attacks [2], thus potential for a feed-forward loop exists.

Epileptic seizures are accompanied by abnormal neuronal discharge. As a consequence, the balance of Ca^{2+} and Ca^{2+} binding proteins is lost and significant Ca^{2+} influx compromises intracellular calcium homeostasis. This results in the destruction of macromolecules that maintain the structure and function of cells, and eventually disrupts the mitochondria structure. In addition, the abnormal opening of the mitochondrial permeability transition pore (MPTP) leads to abnormal charge distribution between the inner and outer mitochondrial membranes and lowers the trans-membrane potential. Mitochondrial swelling and outer membrane rupture are also observed, resulting in structural damage and functional decline. When epileptic attacks happen, tonic-clonic seizures result in dramatically increased oxygen consumption and hypoxia which directly impair cellular respiration and mitochondrial function. Mitochondrial damage induces the release of cytochrome-C (Cyt-C) from the inner mitochondrial membrane into the cytoplasm. This can lead to mitochondrial electron transport chain disruptions, inhibition of oxidative phosphorylation, and a decline in ATP production as well as cell death in severe cases. On the other hand, Cyt-C and Apoptosis Protease Activating Factor-1 (Apaf-1) can form a complex that can activate caspase-9 zymogen to initiate the caspase cascade and cause apoptosis [3]. Neuronal death further exacerbates recurrent epileptic seizures, and mitochondrial oxidative stress can also bring about an increase in seizure susceptibility. Therefore, it is necessary to protect mitochondrial function to prevent recurrent seizures.

NBP is a racemic 3-n-butylphthalide compound, it is the active ingredient of and isolated from celery seed. NBP can improve the level of endothelial nitric oxide (NO) and prostacyclin (PGI2) of cerebral blood vessels, reduce intracellular calcium overload, inhibit the release of glutamate (glutamic acid, Glu) and arachidonic acid, reduce free radicals, and improve the activity of antioxidant enzymes; moreover, it can increase the number of capillaries in the ischemic area to protect mitochondrial function [4, 5]. But ylphthalide (NBP) is commonly used for the treatment of ischemic cerebrovascular disease; however, few studies have focused on the effect of NBP in treatment of epilepsy and specifically on its effects on mitochondrial function and electrical activity of neurons after epilepsy. Herein, we explore the impact of NBP on the function and structure of hippocampal mitochondria in a rat model of epilepsy to determine its potential as an europrotective treatment in patients with epilepsy.

Materials and methods

Drugs and reagents

Pentylenetetrazol (PTZ, Sigma, USA); Butylphthalide stock solution (NBP, CSPC NBP Pharmaceutical Co., Ltd., China); Bradford Protein Quantification Kit, colorimetric detection kit of mitochondrial respiratory chain complexes II and IV activity (Genmed Co., Ltd., China); JEM-1200EX transmission electron microscope (JEOL, Japan); stereotaxic apparatus: and BL-420E physiological function detecting system (Chengdu Taimeng Software Co., Ltd, China).

Animals

Eighty 8 to 10-week-old healthy adult female Wistar rats (starting weight 228 ± 24 g) were obtained from the Animal Center of the School of Basic Medical Sciences, Jilin University (production license number: SCXK 2003-0001). The rats were housed in separate cages (6 rats per cage). Animal rooms were maintained at a temperature of 21 ± 1°C and a relative humidity of 40-70%, with natural day-night cycle. All rats were randomly assigned to 2 groups, model group (n = 60) and sham group (n = 20). Pentylenetetrazol kindling was used as a rat model of chronic epilepsy in the model group. The model group was subsequently further divided into EP group (receiving PTZ + vehicle) and the NBP treatment group (receiving PTZ + NBP). After receiving treatment for 2 weeks, each group of animals was divided into 3 subgroups to measure respiration chain function in hippocampal mitochondria, to observe the ultrastructure of rat hippocampal neurons, and to detect the electrical activity in the hippocampal CA3 region.

Rat model

In the model group, all rats were intraperitoneally injected each day (9:00-10:00 a.m.) with freshly prepared 1% PTZ saline solution at a concentration of 35 $mg \cdot kg^{-1} \cdot d^{-1}$, and equal volumes of saline were injected intraperitoneally in the control group [6]. Then, behavior changes were observed and recorded for 4 hours after injection. Seizure severity was scored via Racine stages [7]. Rats experiencing grade IV-V seizures for 3 consecutive days were considered fully kindled and were chosen as successful models. Thereafter, these chosen rats were injected with the same dose of PTZ saline solution every 3 days in order to maintain the kindling effect. Rats that were successfully kindled with PTZ were then randomly assigned to the EP group or the NBP group. The NBP group received NBP (diluted with peanut oil) via gavage at a concentration of 80 $mg \cdot kg^{-1} \cdot d^{-1}$, while the EP group was given the same volume of 0.5 $ml \cdot kg^{-1} \cdot d^{-1}$ peanut oil for 2 consecutive weeks.

Determination of respiratory chain complex II and IV activity in hippocampal mitochondria

Rats were fasted for 12 hours, rapidly decapitated, and their brains were removed and placed on ice. The hippocampus was separated for isolating mitochondria into a suspension [8]. The protein concentration of the hippocampal mitochondria suspension was then determined using the Bradford Protein Quantification Kit. Respiratory chain complex II and IV activity was measured with the GENMED colorimetric detection kit, according to the user manual's instructions. The absorbance difference was detected using a 722s visible spectrophotometer, and the value was substituted into the pertinent formula to calculate the activity.

Table 1. Activity of mitochondrial respiratory chain complexes II and IV in the hippocampus (n = 8, $\bar{x} \pm s$)

Group	Activity of Complex II (μmol DCPIP/min/mg)	Activity of Complex IV (μmol Cytc/min/mg)
Control group	1.8661 ± 0.3491	78.2967 ± 7.6304
EP group	1.4697 ± 0.5580	45.3269 ± 11.6556*
NBP group	1.7670 ± 0.3073	72.1153 ± 15.1009Δ

*: compared with the control group $P < 0.001$; Δ: compared with the EP group, $P < 0.05$.

Observation of mitochondrial morphology in hippocampal CA3 region

The rats were anesthetized with 10% chloral hydrate (3.5 $mL \cdot kg^{-1}$). After thoracotomy, rats were perfused transcardially with 100 ml of buffered saline followed by 150 ml of 4% paraformaldehyde (pH 7.04). Brains were removed immediately after perfusion and the hippocampus CA3 region was separated. From the hippocampus, 1 mm × 1 mm × 1 mm of CA3 tissue was fixed in glutaraldehyde and then fixed in 1% osmic acid. The fixed tissue was dehydrated in ethanol and embedded in propylene resin. Tissue was cut into semi-thin sections. Ultrathin sections with a thickness of 50-80 nm were made after precise positioning. The sections were double-stained for electron microscopy with uranyl acetate and lead citrate. Mitochondrial morphology was observed using a JEM-1200 transmission electron microscope.

Observation of the electrical activity of hippocampal CA3 cell populations

The rats were anesthetized with 10% chloral hydrate. The hair on the head and neck was shaved. Ethyl alcohol at 75% concentration was then used to disinfect the skin. The scalp was incised along the parietal midline, exposing the skull. The rats were fixed in a stereotaxic apparatus after hydrogen peroxide treatment of the skull surface. According to the "three-dimensional atlas of the rat brain [9]", a hole was drilled through the corresponding parts of the skull. Electrodes were placed on the basal dendrites of the right anterior dorsal hippocampus CA3 region (P: -3.0 mm, R: -2.5 mm, H: -3.4 mm) to record the local electrical activity. The deep brain electrical potential was fed into a BL-420 biological function system to collect and analyze the electrical activity in the rat CA3 region. In order to observe the high-frequency components of the deep brain electrical activity, the time constant (T), the high-frequency filter (F) were set at 0.01 s and 300 Hz, respectively. Volatility ≥ 3 times the background volatility, phase ≥ 400 ms, and wave group spacing ≥ 800 ms were the criteria for identifying bursts of electrical activity [10]. Measurements were recorded for 18 minutes and used to calculate the frequency of attacks.

Statistical analysis

Using SPSS17.0 statistical software, the comparison of activity of mitochondrial respiratory chain complexes II and IV in the hippocampus between groups was expressed as means ± SEM ($x \pm s$). All data were compared by post-hoc test. The statistical significance level was set at $P < 0.05$ for all tests.

Results

Epilepsy model

After 3 weeks of PTZ kindling, the success rate was 78.3% in the model group, the mortality rate was 10%, and 11.7% of the rats never reached the target score for successful kindling. The death of rats was mainly caused by acute pulmonary edema. Spontaneous seizures can be observed in successfully kindled models. Seizure frequency was 1-3 times a week. Most of the rats had I-II grade seizures, with occasional grade IV seizures which were less than 1 minute in duration. Irritability was found in individual rats. During the process of administering NBP or vehicle, there was no significant difference in the number of seizures between rats from the EP and NBP groups. Rats in the sham group had no seizures.

*Activity of mitochondrial respiratory chain complexes II and IV (**Table 1**)*

Compared with hippocampal complex IV activity in the sham group (78.2967 ± 7.6304 mmol $Cytc \cdot min^{-1} \cdot mg^{-1}$), activity declined significantly in the EP group (45.3269 ± 11.6556 mmol $Cytc \cdot min^{-1} \cdot mg^{-1}$; $P = 0.00019$). Treatment with NBP resulted in significant restoration of complex IV activity (72.1153 ± 15.1009 mmol $Cytc \cdot min^{-1} \cdot mg^{-1}$), compared with the EP group ($P = 0.001$). Complex II activity declined numerically in the EP and NBP groups when compared

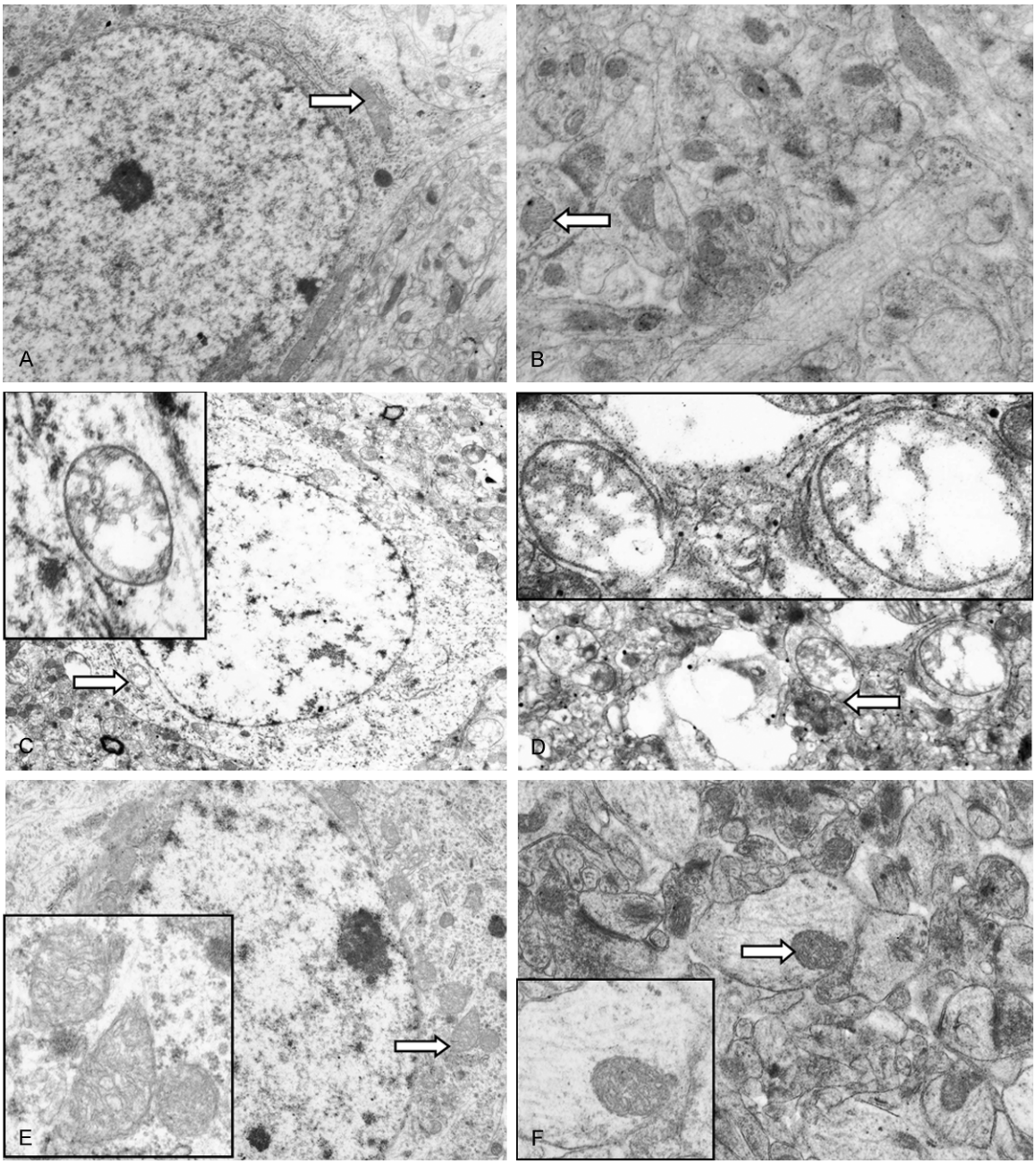

Figure 1. Ultrastructure of rat hippocampal CA3 region in different groups. (A, B) Control group; (C, D) EP group; (E, F) NBP group. (A, E) Original drawing bar = 1 μm, local zoom bar = 200 nm; (C) Original drawing bar = 2 μm, local zoom bar = 200 nm; (B, D, F) Original drawing bar = 500 nm, local zoom bar = 200 nm. Mitochondria are indicated by a white arrow. In the sham group (A, B), in the region of hippocampal CA3, neurons had abundant nuclear matrices and a high number of organelles, neuropil was arranged closely with an abundant number of synapses. Mitochondrial membranes were complete and their ridge structure was clear. In the EP group (C, D), some neurons were swollen, nuclear chromatin was reduced, cavitation of cytoplasmic matrix was observed, and organelles were reduced. Some neurons exhibited dense, dark cell-like organelles which were poorly distinguished. In addition, cavitation of neuropil occurred and the number of synapses significantly decreased. Some pyknotic and cavitated mitochondria were found. In the NBP group (E, F), nuclear chromatin in the neurons was still abundant. Organelles were abundant and dark cells were rarely seen. Neuropil and synapses were relatively abundant. Mitochondrial membrane was complete and the ridge structure was clear. Some dense mitochondria were also observed.

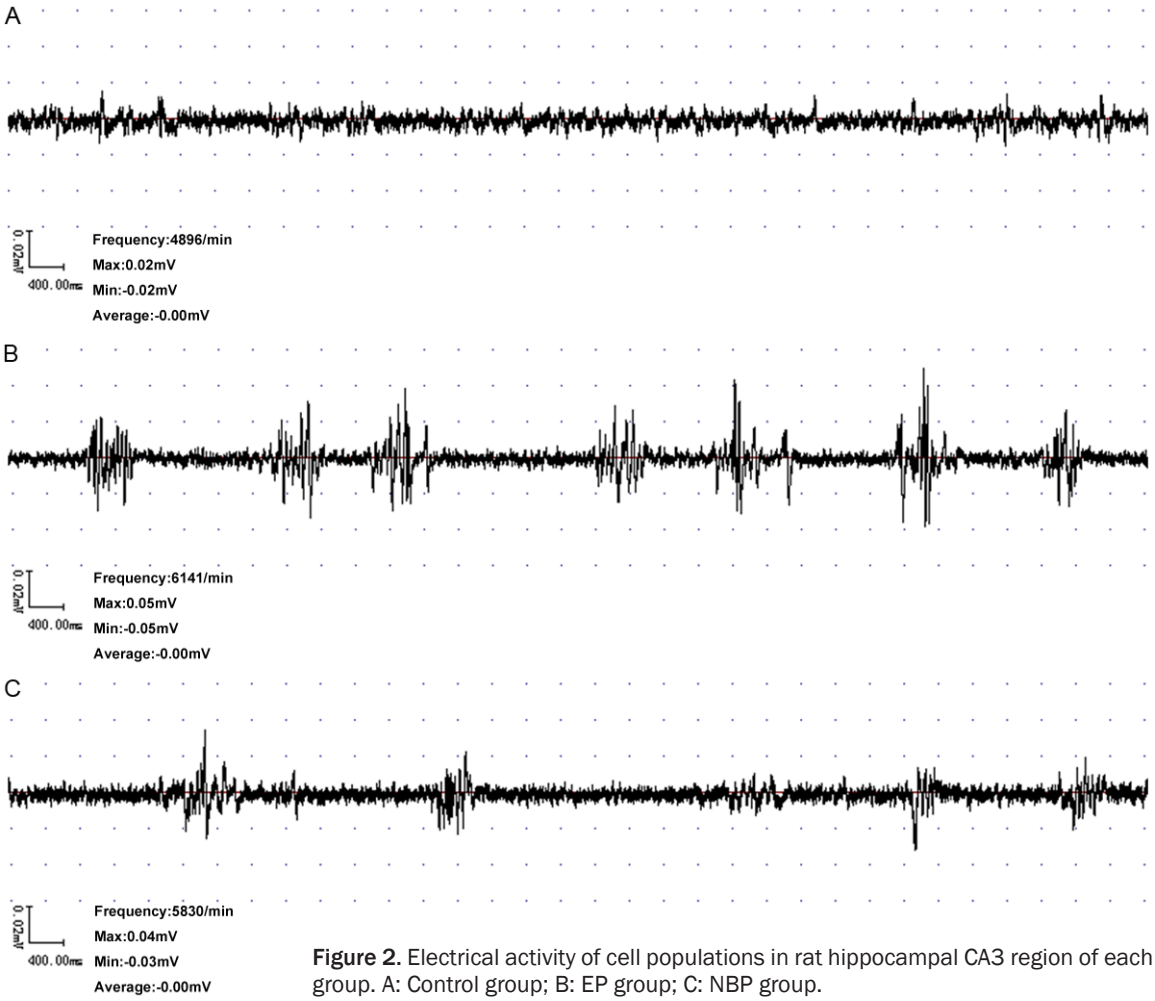

Figure 2. Electrical activity of cell populations in rat hippocampal CA3 region of each group. A: Control group; B: EP group; C: NBP group.

with the sham group. However, none of the differences were statistically significant.

*Ultrastructure of hippocampal CA3 region (**Figure 1**)*

In the sham group (**Figure 1A**, **1B**), in the region of hippocampal CA3, neurons had abundant nuclear matrices and a high number of organelles, neuropil was arranged closely with an abundant number of synapses. Mitochondrial membranes were complete and their ridge structure was clear. In the EP group (**Figure 1C**, **1D**), some neurons were swollen, nuclear chromatin was reduced, cavitation of cytoplasmic matrix was observed, and organelles were reduced. Some neurons exhibited dense, dark cell-like organelles which were poorly distinguished. In addition, cavitation of neuropil occurred and the number of synapses significantly decreased. Some pyknotic and cavitated mitochondria were found. In the NBP group (**Figure 1E**, **1F**), nuclear chromatin in the neurons was still abundant. Organelles were abundant and dark cells were rarely seen. Neuropil and synapses were relatively abundant. Mitochondrial membrane was complete and the ridge structure was clear. Some dense mitochondria were also observed.

*Electrical activity in the hippocampal CA3 region (**Figure 2**)*

The electrical activity in the hippocampal CA3 region stabilized after 4 minutes of continuous recording. Therefore, we quantified the sum of burst electrical activity starting from 5 minutes and 30 seconds of continuous recording. In the control group (A), occasional burst electrical activity was recorded (0.89 times/min) with low volatility; however, in the EP group (B), large numbers of clustered spike waves were detected (13.3 times/min). In the NBP group (C), only a small amount of visible bursting electrical

activity (3.00 times/min) was detected and the volatility was low.

Discussion

PTZ is an antagonist of the γ-amino butyric acid receptor which can bind to the channel of Chloridion, lead to depolarization of the cell membrane, therefore result in abnormally high levels of brain excitability by prolonged or repeated sub-convulsive stimulation [11]. The PTZ-kindled epileptic rat model replicates certain features of human epilepsy, including classical changes of praxiology and neurological pathology [12]. It is a useful model for the study of human epilepsy and neuronal damage [13]. Our study had clarified that the model can manifestate the characters of epilepsy such as spontaneous seizures, recurrent and long-term attacks. Besides, we had found the classical pathological changes of epilepsies: hippocampal injury [14], mitochondrial dysfunction and ultrastructure injuries [15].

Some research [1, 16, 17] had found that epilepsy can lead to damage of mitochondrial ultrastructure and function, as well as decreased activity of respiratory chain complex. The core structure of complex IV is highly conserved throughout evolution. All three subunits are encoded by mtDNA. Because the mtDNA is exposed in the mitochondrial matrix and lacks protein protection and damage repair mechanisms, it is vulnerable to destruction via hypoxia, superoxide radicals, oxidative stress, and other factors. Such damage may affect the structure and function of mitochondrial respiratory chain complexes and lead to an increase in electron leakage and to inhibition of energy metabolism. Complex II which is composed of four subunits, is coded by nDNA. In this study, the activity of complex IV in the EP group was significantly lower than in the sham group, and there was obvious mitochondrial ultrastructural damage, however, there was no significantly change of the activity of complex II. These results suggested that Complex IV which is coded by mtDNA is more sensitive to energy metabolism disorder resulted by epilepsy than Complex II coded by nDNA. The decreased activity of Complex IV could be a sign of mitochondrial dysfunction. Abnormal electrical activity of the hippocampal CA3 cell had been detected in the EP group, which is accordance with the manifestations of epilepsy in human. This results had also clarified that the model is a good choice for the research of epilepsy.

Abnormal electrical activity of the hippocampal CA3 cell populations in the NBP group was lighter than that in the EP group, which indicates that mitochondrial function and epilepsy are related and that NBP could possibly reduce epileptic seizures by reducing the damage to mitochondrial structure and function.

NBP is a racemic 3-n-butyphthalide compound, it is the active ingredient of and isolated from celery seed, it is a resistance to cerebral ischemia [18, 19], obviously increasing the quantities of ATP and creatine phosphate, and significantly decrease the injury of mitochondria structure and function induced by ATP depletion. In addition, it has been confirmed that NBP has anti-convulsion effect in a variety of epilepsy models [20]. In our study, the activity of Complex IV decreased significantly lower in the NBP group than that in the EP group, which is accompanied with the abnormal electrical activity detected in the hippocampal CA3 region cell populations, moreover, NBP can reduce the destruction of mitochondrial ultrastructure in the hippocampus of epilepsy rats, and maintain the integrity of the mitochondrial membranes. The possible mechanism is as follows: NBP reducing abnormal opening of the MPTP, inhibiting the release of cytochrome-C (Cyt-C) from the inner mitochondrial membrane into the cytoplasm, stabilizing the mitochondrial membrane. Because the release of Cyt-C can lead to mitochondrial electron transport chain disruptions, inhibition of oxidative phosphorylation, and a decline in ATP production as well as cell death in severe cases. On the other hand, Cyt-C and Apoptosis Protease Activating Factor-1 (Apaf-1) can form a complex that can activate caspase-9 zymogen to initiate the caspase cascade and cause apoptosis. Neuronal death further exacerbates recurrent epileptic seizures, and mitochondrial oxidative stress can also bring about an increase in seizure susceptibility. NBP may also have free-radical scavenging activity and the ability to enhance the activity of antioxidant enzymes to restore mitochondrial energy metabolism and protect mitochondrial function. When epileptic attacks happen, seizures result in dramatic increases in oxygen consumption and cause hypoxia in neurons which directly cause secondary brain injury. NBP can increase the activity of antioxidant

enzymes in brain tissue, improve mitochondrial energy metabolism, increase the expression of hypoxia-inducible factors (HIF-1α), and promote the expression of vascular endothelial growth factor (VEGF), thereby preventing anti-hypoxic injury.

In summary, recurrent seizures may be associated with mitochondrial damage after seizures; therefore, in treatment of epilepsy, it is important to not only control epileptic seizures but also to pay attention to the protection of neurons. NBP can protect mitochondrial structure and complex IV activity. These results suggest that NBP can become a new approach in adjuvant treatment of epilepsy.

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Disclosure of conflict of interest

None.

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