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ORIGINAL PAPER

Echinacoside Alleviates Hypoxic-Ischemic Brain Injury in Neonatal Rat by Enhancing Antioxidant Capacity and Inhibiting Apoptosis

Wei Wei[1] · Xiao-bing Lan[1] · Ning Liu[1] · Jia-mei Yang[1] · Juan Du[1,2] · Lin Ma[2] · Wen-jin Zhang[1] · Jian-guo Niu[2] · Tao Sun[2] · Jian-qiang Yu[1,3]

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Abstract

Hypoxic-ischemic brain damage (HIBD) is a leading cause of death and disability in neonatal or perinatal all over the world, seriously affecting children, families and society. Unfortunately, only few satisfactory therapeutic strategies have been developed. It has been demonstrated that Echinacoside (ECH), the major active component of *Cistanches Herba*, exerts many beneficial effects, including antioxidative, anti-apoptosis, and neuroprotective in the traditional medical practice in China. Previous research has demonstrated that ECH plays a protective effect on ischemic brain injury. This study aimed to investigate whether ECH provides neuroprotection against HIBD in neonatal rats. We subjected 120 seven-day-old Sprague–Dawley rats to cerebral hypoxia–ischemia (HI) and randomly divided into the following groups: sham group, HI group and ECH (40, 80 and 160 mg/kg, intraperitoneal) post-administration group. After 48 h of HI, 2,3,5-Triphenyltetrazolium chloride, Hematoxylin-Eosin and Nissl staining were conducted to evaluate the extent of brain damage. Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) activities, total antioxidant capacity (T-AOC), and malondialdehyde (MDA) production were assessed to determine the antioxidant capacity of ECH. TUNEL staining and Western blot analysis was performed to respectively estimate the extent of brain cell apoptosis and the expression level of the apoptosis-related proteins caspase-3, Bax, and Bcl-2. Results showed that ECH remarkably reduced the brain infarct volume and ameliorated the histopathological damage to neurons. ECH post-administration helped recovering the antioxidant enzyme activities and decreasing the MDA production. Furthermore, ECH treatment suppressed neuronal apoptosis in the rats with HIBD was by reduced TUNEL-positive neurons, the caspase-3 levels and increased the Bcl-2/Bax ratio. These results suggested that ECH treatment was beneficial to reducing neuronal damage by attenuating oxidative stress and apoptosis in the brain under HIBD.

Keywords Echinacoside · Oxidative stress · Apoptosis · Hypoxic-ischemic · Neuroprotection

Introduction

Hypoxic-ischemic brain damage (HIBD) is an alarming personal health and socioeconomic problems in perinatal fetus or neonates, which is mainly caused by ischemia, hypoxia, asphyxia results from other damage [1]. HIBD has adverse effects on immature brain, these effects are crucial causes of mortality and chronic neurological disorders [2], such as cerebral palsy, epilepsy, learning disorder, and other neurodevelopmental handicaps [3]. The incidence of HIBD in developed countries ranges from 1 to 8‰ [4], whereas its incidence in developing countries is as high as 26‰ [5]. Currently, hypothermia treatment is the only effective treatment admittedly, but 40–50% of child patients still suffered neurological sequelae and even death after hypothermia treatment [6, 7]. Unfortunately, a variety of conditions, such

Wei Wei and Xiao-bing Lan contributed equally to this work.

✉ Jian-qiang Yu
yujq910315@163.com

[1] Department of Pharmacology, Ningxia Medical University, Yinchuan, Ningxia Hui Autonomous Region 750004, People's Republic of China

[2] Ningxia Key Laboratory of Craniocerebral Diseases of Ningxia Hui Autonomous Region, Ningxia Medical University, Yinchuan, Ningxia Hui Autonomous Region 750004, People's Republic of China

[3] Ningxia Hui Medicine Modern Engineering Research Center and Collaborative Innovation Center, Ningxia Medical University, Yinchuan, Ningxia Hui Autonomous Region 750004, People's Republic of China

as high cost, short therapeutic window limit its application-especially in developing countries. Other treatment methods, such as erythropoietin, melatonin and stem cell transplantation are still in the clinical research process. Although medical workers have various interventions and methods for HIBD, its morbidity and mortality remain high, and the disease severely affects neonates.

With the development of modern medical technology, researchers have an increased understanding of neonatal HIBD pathologies. Although the exact molecular mechanisms of neonatal HIBD are yet to be fully elucidated, considerable evidence indicates that oxidative stress and apoptosis are substantially contributors to the pathogenesis of HIBD [8]. Under physiological conditions, reactive oxygen species (ROS) production and elimination by endogenous antioxidant systems, such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT), are in equilibrium [9, 10], which is responsible for maintaining normal cellular defense systems. The brain is more susceptible than other organs to ROS because of its low antioxidant capacity [11], high oxygen consumption [12, 13] and high lipids and polyunsaturated fatty acid levels [14]. Hypoxia and ischemia (HI) induce the breakdown of cellular defense systems and contribute to the overproduction of ROS in the brain. ROS can oxidize lipids, proteins and DNA, and inactivate key cellular enzymes, resulting in structural and functional cellular changes and leading to neuronal oxidative injury and death.

Apoptosis is a reversible process and can be triggered by oxidative stress. Under several pathologic conditions, such as cerebral HI, neuronal apoptosis is activated, thereby damaging cerebral function [15, 16]. Several caspase family members, including caspase-3, actively induce apoptosis and participate in the final execution phase of apoptosis [17]. In addition, the balance of anti- and pro-apoptotic proteins (Bcl-2/Bax) also regulates cell death [8, 18]. Therefore, intervention methods and therapies with enhanced safety and effectiveness should be developed. For instance, inhibiting caspase-3 expression and regulating Bcl-2/Bax balance have been proposed to help attenuate neonatal HIBD.

Echinacoside (ECH; Fig. 1), a natural phenylethanoid glycoside, is the major active component of *Cistanches Herba*, which has been reported to possess different pharmacological activities, including strong antioxidant property [19] anti-inflammatory activity [20], anti-apoptosis [21] and neuroprotective effects [19]. Recently, ECH shows neuroprotective activities in various in vitro and in vivo brain injury models. ECH decreases MDA contents and increases SOD and GSH-Px activities in MCAO-induced ischemia brain injury [22]. Du et al. also verified that ECH protects the brain against ischemic injury by reducing apoptosis in nerve cells [23]. However, in vivo research on ECH in HIBD is scarce. Therefore, it was speculated that ECH might exert

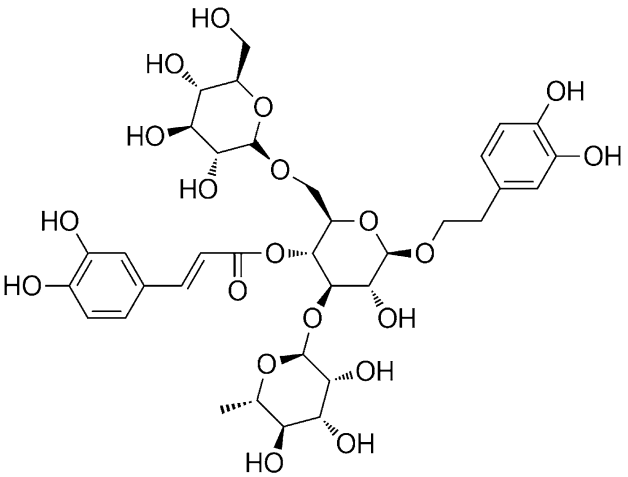

Fig. 1 Structure of Echinacoside (ECH). The molecular formula for ECH is $C_{35}H_{46}O_{20}$ and the molecular weight is 786.73

protective properties on neonatal HIBD. To test this hypothesis, the present experiment was conducted to investigate the potential neuroprotective effects of post-administration of ECH on neonatal HIBD using a neonatal rat model of hypoxic-ischemic as well as propose this neuroprotective effects may by enhancing antioxidant capacity and inhibiting apoptosis.

Materials and Methods

Animals

A total of 120 seven-day old male and female Sprague–Dawley rats weighing 12–17 g were acquired from the Experimental Animal Center of Ningxia Medical University (Certificate number: SCXK Ningxia 2015-0001). The animals were housed in a temperature (22–24 °C) and humidity (40–70%) controlled environment under a 12 h/12 h light/dark cycle with food and water available adlibitum. All surgeries and sample collection were carried out under diethyl ether anesthesia to minimize suffering and the number of animals used. The experiments were carried out in accordance with the current guidelines for the care of laboratory animals in Ningxia Medical University. All the researchers who performed animal testing were blinded.

HIBD Surgical Procedures

Induction of cerebral ischemia-hypoxia was performed by a procedure demonstrated previously [24]. In brief, 7-day-old Sprague–Dawley rats (male/female ratio, 1:1) were narcotized by ether inhalation. Under sterile neck operation, the left common carotid artery was separated, ligated and

sutured with 6-0 silk permanently. Every surgery was completed within 5 min. The pups were allowed to recover and were returned to cage with their mothers and for 1.5 h. Then, the pups were placed in a low-oxygen chamber with 8% O_2, 92% N_2 for 2.5 h at a constant temperature of 37 °C. The left carotid arteries were only exposed without undergoing hypoxic-ischemic in sham operated rats. Previous experiments showed that brain tissue suffers moderate damage at 48 h time point [25]. Therefore, the pups were sacrificed at 48 h after HIBD for the following experiments.

Drug Administration and Experimental Groups

ECH (purity = 99.43%, Batch NO: S-003-170119), white powder, was purchased from Beijing Zhongke quality inspection Biotechnology Co., Ltd., China and dissolved in normal saline (NS; 0.9% NaCl) before use. Drugs were administered intraperitoneally in an application volume of 0.1 ml/10 g body weight. The selection of ECH dose was based on our preliminary experiment and the references [26, 27]. Animals were randomly divided into 5 groups of 24 rats each (6 for infarct volume, 6 for histopathology analysis, 6 for oxidative stress biomarkers and MDA content and 6 for Western blot) as follows: control (sham surgery) group; cerebral hypoxia-ischemia (HI) group; hypoxia-ischemia (HI) with ECH (40, 80 or 160 mg/kg) group. ECH was intraperitoneally injected every 12 h after HI for two consecutive days while the sham and hypoxia-ischemia groups received NS under similar conditions.

Measurement of Infarct Volume

Pups were deeply anesthetized and the brains were removed subsequently on ice. After freezing at − 20 °C for 20 min, the brain tissues were sectioned into 2-mm sections and incubated into 2% solution of 2,3,5-triphenyl tetrazolium chloride (TTC) (Sigma, USA) for 30 min at 37 °C followed by immersion in 4% formaldehyde solution overnight and photographed to elucidate the infarct damage. The infarct volumes were calculated through image analysis software (Image-Pro Plus, USA). The infarcted tissue size was distorted by cerebral edema, which the accuracy and objectivity was affected [28]. To this end, the following formula was used to determine the area of cerebral infarction accurately: Brain infarction rate (%) = (*normal hemisphere volume—non-infarct volume of the infarct side*)*/normal hemisphere volume* × 100 [29].

Histopathological Analysis

For pathologic studies, pups were anesthetized and perfused with ice NS followed by 10% cold formalin. Samples of brain tissue were submerged immediately for 24 h (10% cold formalin). Every tissue samples was dehydrated in alcohol gradient, cleaned in xylene and embedded in paraffin. Successive brain coronal slices (5 μm thick) were made by microtome (Leica, Germany), subsequently, the sections were taken for deparaffinization and rehydration for histopathologic staining.

Hematoxylin and Eosin (HE) Staining

Tissue specimens were stained with hematoxylin and eosin (HE) and histopathological changes of brain tissue were observed using light microscopy (Olympus BX-51, Japan) at a magnification of 200 × and 400 × and images were acquired.

Nissl Staining

The sections were immersed in cresyl violet stain for 1 h at 56 °C oven, washed quickly in distilled water, and then placed in a Nissl differentiation solution until the best results were observed under microscope. Finally, the sections were cleared in pure xylene, and then mounted with neutral gum for light microscopy (Olympus BX-51, Japan) at magnifications of 200 × and 400 ×. The degree of brain tissue specimens damage of the cerebral hippocampus CA3, CA1 and the cortex of HE and Nissl staining using a five-point score: 0, normal; 1, damaged neurons were < 25%; 2, damaged neurons were 25–50%; 3, damaged neurons were 50–75%; and 4, damaged neurons were > 75%.

TUNEL Staining

The operation of the TUNEL staining was implemented by using the commercial kit as previously reported [30]. In short, the sections were permeated with proteinase K for 15 min. Then, the DNA fragmentation of apoptotic and necrotic cells bound to the terminal deoxynucleotidyl transferase (TdT) enzyme in a reaction mixture. After rinsing with PBS for 15 min, cerebral slides were mounted with 4′,6-diamidino-2-phenylindole (DAPI) for 10 min at room temperature. Ultimately, the apoptotic neurons of each section in 6 cortical fields were photographed randomly with an Olympus fluorescence microscope (Olympus FV1000, Japan) at a constant magnification of 400 ×. The following formula was used to calculate the rate of apoptosis: *apoptotic neurons amount/total neurons amount* × *100%*.

Assays the Level of Oxidative Stress in Brain Tissue

The activities of SOD, GSH-Px, CAT, T-AOC and the levels of malondialdehyde (MDA) were measured 48 h after HIBD. The brains were removed quickly, washed in 4 °C NS and preserved in the refrigerator at − 80 °C. The following

biochemical tests were completed within 48 h. The protocol of the level of oxidative stress was implemented by using the commercial kit as previously reported [27].

Activities of Antioxidant Enzymes

The ischemic hemispheres were placed into glass homogenizer with 0.9% NS for 15 min to prepare a 10% (w/v) brain homogenate. The homogenates were centrifuged at 2500 rpm for 10 min (4 °C). Then, the supernatants were analyzed brain tissue protein concentration and the activities of SOD, GSH-PX, CAT and T-AOC according to the instructions of the assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and using a microplate reader (1510, Thermo Fisher Scientific, Waltham, MA, USA). One unit of SOD activity was defined as the quantity required to inhibit the rate of reduction of cytochrome c by 50%. The absorbance change at 550 nm was monitored. SOD activity is expressed as units/mg protein (U/mg prot). One unit of GSH-PX activity was defined as the GSH-PX in 1 mg protein that led to the decrease of 1 mmol/l glutathione (GSH) in the reactive system. GSH-PX activity was expressed as U/mg prot. The CAT decomposition reaction of hydrogen peroxide can be terminated by adding ammonium molybdate, which generated a light yellow complex compound. The absorbance change at 405 nm to measure its generation can calculate the CAT activity. CAT activity was expressed as U/mg prot. T-AOC reflects the overall cellular endogenous antioxidative capability including both enzymatic and nonenzymatic antioxidants. All these antioxidants were assessed using the chromogenic reagent 2, 20-azinobis (3-ethylbenzthiazoline-6- sulfonic acid) (ABTS) with maximal absorbance at 532 nm. The T-AOC is expressed as U/mg prot.

Lipid Peroxidation (MDA) Measurement

As the produce of lipid peroxidation, the malondialdehyde (MDA, Jiancheng Bioengineering Institute, Jiangsu, China) content in the brain tissue was determined using the thiobarbituric acid method, because thiobarbituric acid reacts with reactive substances to generate a red-colored complex whose absorbance is measured at 532 nm. Assay results were normalized to the protein concentration in each sample and expressed as nmol/mg protein.

Western Blot Analysis

Western blot was carried out as described previously [31]. Briefly, the ischemic brain of rat pups were isolated in ice rapidly, weighed and homogenized in a 1:10 (w/v) ice-cold lysis buffer in glass homogenizers(Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Then supernatant obtained by centrifugation of tissue homogenates at 12,000×*g* for 10 min at 4 °C, which was extracted the total protein. According to manufacturer's instruction (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), BCA method was utilized for the protein concentration of the samples. Equal amount of protein lysate (50 μg) in each group was separated by 12% sodium dodecyl sulfateepolyacrylamide gel electrophoresis (SDS-PAGE). The proteins on the gel were subsequently transferred onto polyvinylidene fluoride (PVDF) membranes (200 mA, 2 h). Then, membranes was blocking with 5% skim milk powder under shaking for 1 h at room temperature and incubated overnight at 4 °C with the primary antibodies: caspase-3, Bcl-2 and Bax (caspase-3, 1:1000, 19677-1-AP; Bax, 1:500, 50599-2-Ig; β-actin, 1:2000, 20536-1-AP; Proteintech Group, USA. Bcl-2, 1:500, ab182858; Abcam, U.K). After washing with PBST (containing 20% Tween-20), the PVDF membranes were incubated with horseradish peroxidase-conjugated Affinipure goat anti-rabbit IgG (1:2000, SA00001-2; Proteintech Group, USA) for 2 h at room temperature and followed by three PBST washes. The protein bands were visualized by electrochemiluminescence and observed by a Western blot detection system (Bio-Rad Laboratories, USA). Finally, the protein band was analyzed by Quantity One software.

Data Analysis

Data were expressed as mean ± SEM, and the statistical analysis of the results was evaluated by one-way ANOVA followed by Dunett's test. $p < 0.05$ was considered statistically significant.

Results

Determination of ECH Dosage

To study the neuroprotective effect of ECH on HIBD neonatal rat and further to explore the effective dosage, an additional seven-day old male and female 42 Sprague–Dawley rats were employed to TTC staining in pre-experiment. Rats were divided into 7 groups of 6 rats each as sham group, HI group, HI + ECH group (20, 40, 80, 160, 240 mg/kg). The results of TTC staining at 48 h after hypoxic-ischemic brain damage performed as Fig. 2A, B. The results showed that the infarct volume of the injured hemisphere of the brain increased significantly after the ischemic hypoxic brain damage ($p < 0.01$). Compared with HI group, intraperitoneal administration with ECH 20 or 40 mg/kg were slight almost no effect ($p > 0.05$), the three doses of 80, 160 and 240 mg/Kg could significantly reduce the volume of cerebral infarction ($p < 0.01$). However, compared with ECH (80 mg/Kg), 160 mg/Kg of ECH reduced infarct volume ($p < 0.05$), and

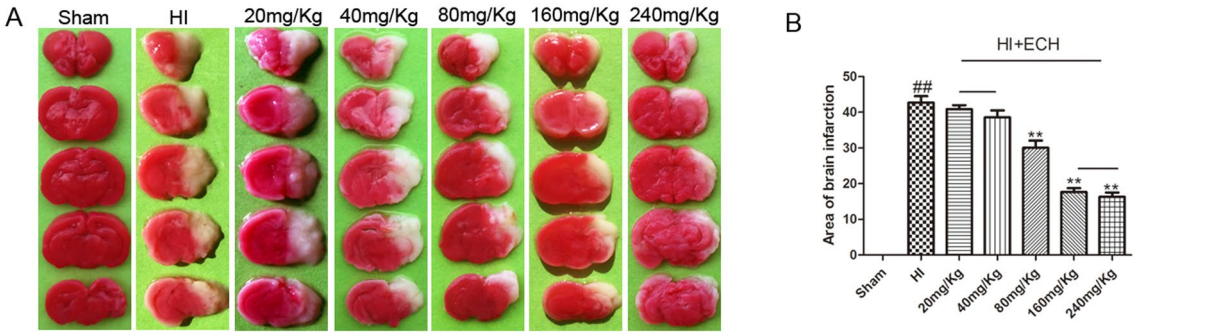

Fig. 2 Protective effect of ECH against hypoxic-ischemic brain damage in neonatal rats in preliminary experiments. **A** TTC staining of representative coronal sections at 48 h after hypoxic-ischemic brain damage. **B** Quantitative analysis of infarct volumes at 48 h after hypoxic-ischemic brain damage. Data are expressed as mean ± SEM (n = 6). $^{\#\#}p < 0.01$ versus the Sham group and $^{**}p < 0.01$ versus the HI group

there was no significant difference between 160 mg/Kg and 240 mg/Kg of ECH. Therefore, ECH (40, 80 and 160 mg/kg) was chosen as the lowest, medium and highest dosage in formal study.

ECH Post-treatment Against Hypoxia-Ischemia Induced Brain Infarct Volume

Results of the TTC-stained brain tissue were illustrated in Fig. 3A. Before hypoxia–ischemia, No cerebral infarction was observed. In the HI group, an obvious infract area (41.35 ± 4.03%) was observed and total infarct volume was significantly reduced in the ECH groups (80 and 160 mg/kg, 28.93 ± 2.92% and 12.9 ± 2.38%) compared with the HI group (Fig. 3B, $p < 0.01$).

ECH Post-treatment Against Hypoxia-Ischemia Induced Pathological Changes

Neurons in Sham group remained intact, rich, well-arranged and the staining was uniform in the hippocampal and cortex region (Fig. 4A, B). However, in the HI group, neurons showed karyopyknosis, interstitial swollen, even neuronal structure and Nissl's body disappeared (Fig. 4B). Post-treatment with ECH (160 mg/kg) was significantly alleviated neuronal injury after hypoxia–ischemia, whereas the density of neurons significantly increased relative to those in HI group (Fig. 4C, $p < 0.01$).

Effects of ECH Post-treatment on Neuronal Apoptosis After HIBD

As shown in Fig. 5A, a large number of TUNEL positive cells were densely observed in the HI-treated rats, while, it was observed rarely in sham group. In comparison with the HI group, post-treatment with ECH (160 mg/kg) substantially reduced the TUNEL positive cells (Fig. 5B, $p < 0.01$).

ECH Post-treatment Altered Oxidative Stress Increase Induced by Hypoxia-Ischemia

To demonstrate the effect of ECH on the oxidative stress induced by HIBD, several antioxidant enzymes activities and MDA contents were measured 48 h after HIBD and shown in Fig. 6. Results indicated that SOD, GSH-Px, CAT and T-AOC activities significantly decreased and the contents

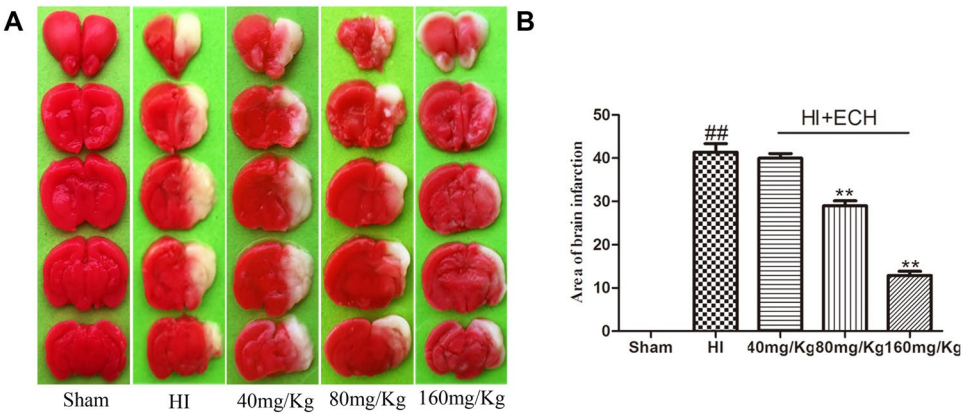

Fig. 3 Protective effect of ECH against hypoxic-ischemic brain damage in neonatal rats. **A** TTC staining of representative coronal sections at 48 h after hypoxic-ischemic brain damage. **B** Quantitative analysis of infarct volumes at 48 h after hypoxic-ischemic brain damage. Data are expressed as mean ± SEM (n = 6). $^{\#\#}p < 0.01$ versus the sham group and $^{**}p < 0.01$ versus the HI group

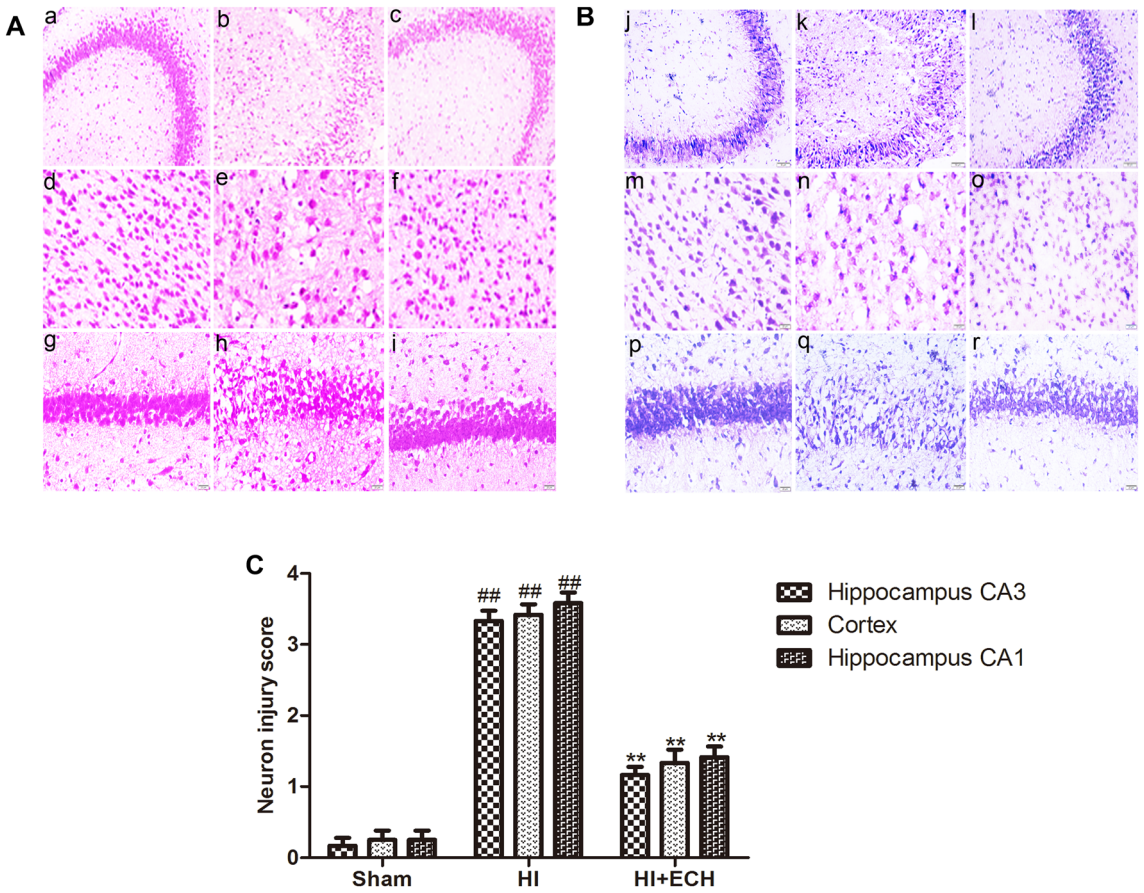

Fig. 4 Effect of ECH post-treatment on histomorphological alterations of ischemic cerebral hippocampus CA3 (×200), hippocampus CA1 (×400) and cerebral cortex (×400) at 48 h after hypoxic-ischemic brain damage. **A** HE staining (a, d, g) sham group; b, e, h HI group; c, f, i HI + ECH (160 mg/Kg) group; a–c Hippocampus CA3; d–f cerebral cortex; g–i Hippocampus CA1. **B** Nissl staining, j, m, p sham group; k, n, q HI group; l, o, r HI + ECH (160 mg/Kg) group; j–l Hippocampus CA3; m–o: cerebral cortex, p–r Hippocampus CA1. **C** Quantification of neurons damage is shown as injury score. Data are expressed as mean ± SEM (n = 6). $^{\#\#}p < 0.01$ versus the sham group and $^{**}p < 0.01$ versus the HI group

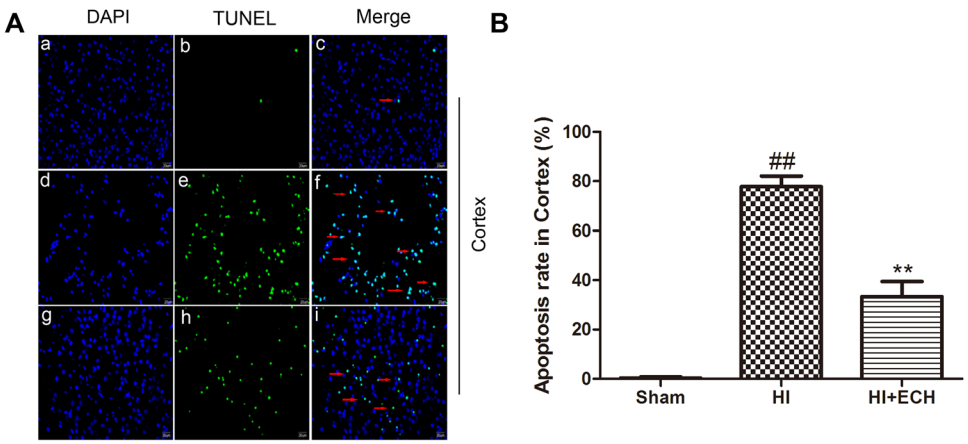

Fig. 5 Effect of ECH post-treatment for dead neuronal in the ischemic cerebral cortex (×400) at 48 h after hypoxic-ischemic brain damage. **A** Representative fluorescence photos of TUNEL staining in the ischemic cerebral cortex, a–c sham group; d–f HI group; g–i HI + ECH (160 mg/Kg) group. **B** Quantitative analysis of the TUNEL-positive cells. Data are expressed as mean ± SEM (n = 6). $^{\#\#}p < 0.01$ versus the sham group and $^{**}p < 0.01$ versus the HI group

of MDA significantly increased in the HI group compared with the sham group ($p < 0.01$ for all). However, the ECH (160 mg/kg) treatment group remarkably restored SOD (Fig. 6A), GSH-px (Fig. 6B), CAT (Fig. 6C) and T-AOC (Fig. 6D) enzyme activities ($p < 0.01$ for all). The level of

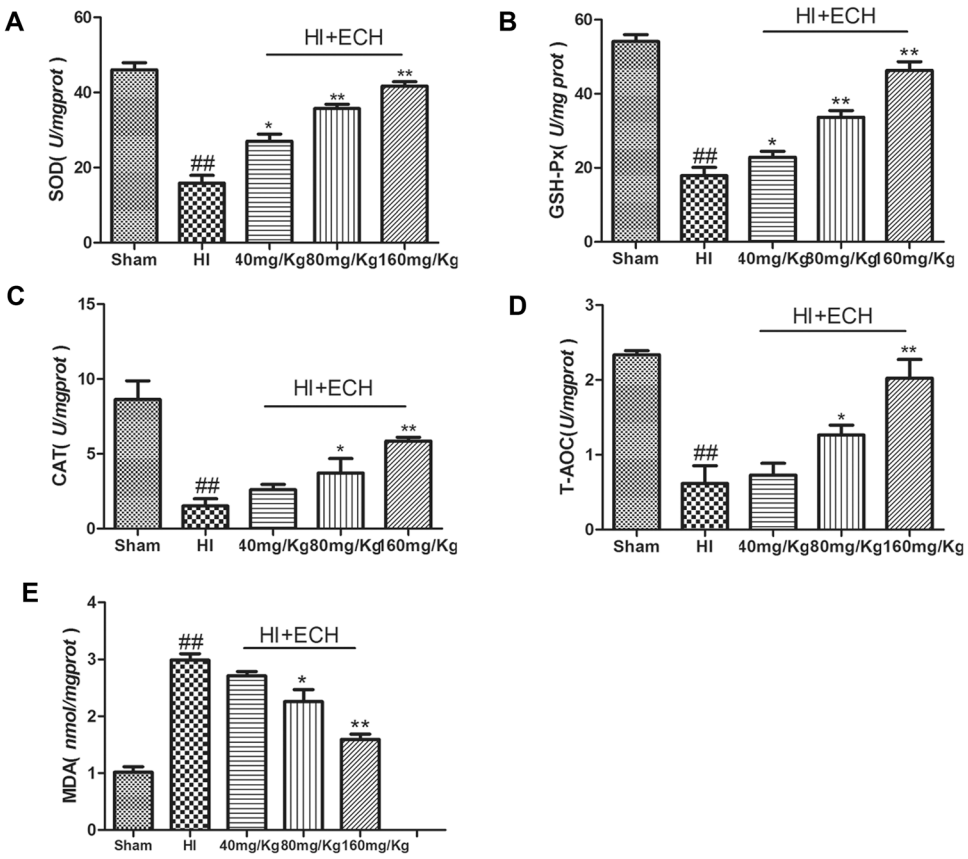

Fig. 6 ECH attenuates oxidative stress after hypoxic-ischemic brain damage in neonatal rats. **A**–**D** Effect of ECH on the activities of SOD, GSH-Px, CAT, T-AOC at 48 h after hypoxic-ischemic brain damage. **E** Effect of ECH on the content of MDA at 48 h after hypoxic-ischemic brain damage. Data are expressed as mean ± SEM (n = 6). $^{\#\#}p < 0.01$ versus the sham group and $^{*}p < 0.05$, $^{**}p < 0.01$ versus the HI group

MDA significantly attenuated after the post-administration of ECH (Fig. 6E, $p < 0.01$).

Effect of ECH Post-treatment on Apoptosis-Related Proteins

To determine the effect of ECH on apoptosis in the ischemia hemisphere after HIBD, as shown in Figs. 7, 8 and 9, we test the protein levels of caspase-3, Bcl-2 and Bax at 48 h after HIBD. HI significantly decreased the protein levels of Bcl-2 and ratio of Bcl-2/Bax, which were obviously restored by the 160 mg/kg ECH post-treatment ($p < 0.05$, Figs. 8B, 9C). Contrastly, caspase-3 and Bax were at a low level in sham group, and significantly increased in HI group ($p < 0.01$, Figs. 7B, 9B), while in the ECH post-treatment groups, the increased expressions of these proteins were significantly lower than those of the HI group ($p < 0.01$, Figs. 7B, 9B).

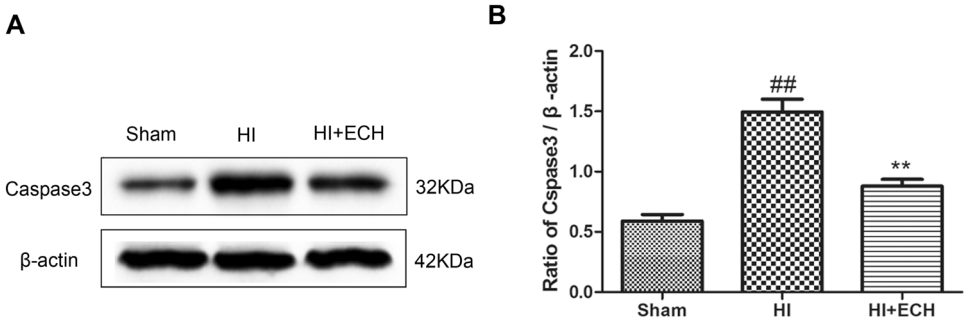

Fig. 7 Effects of ECH on caspase-3 protein expression. **A** Representative Western blot band of caspase-3 protein expression in the ischemic brain at 48 h after hypoxic-ischemic brain damage. **B** Effect of ECH on the caspase-3 protein expression at 48 h after hypoxic-ischemic brain damage. Data are expressed as mean ± SEM (n = 6). $^{\#\#}p < 0.01$ versus the sham group and $^{**}p < 0.01$ versus the HI group

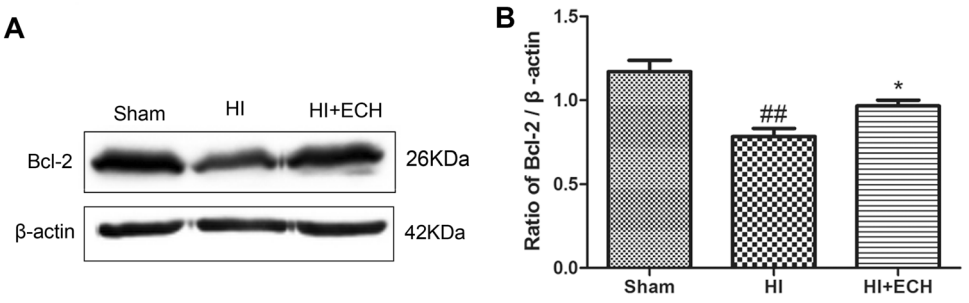

Fig. 8 Effects of ECH on Bcl-2 protein expression. **A** Representative Western blot band of Bcl-2 protein expression in the ischemic brain at 48 h after hypoxic-ischemic brain damage. **B** Effect of ECH on the Bcl-2 protein expression at 48 h after hypoxic-ischemic brain damage. Data are expressed as mean ± SEM (n = 6). $^{\#\#}p < 0.01$ versus the sham group and $^{*}p < 0.05$ versus the HI group

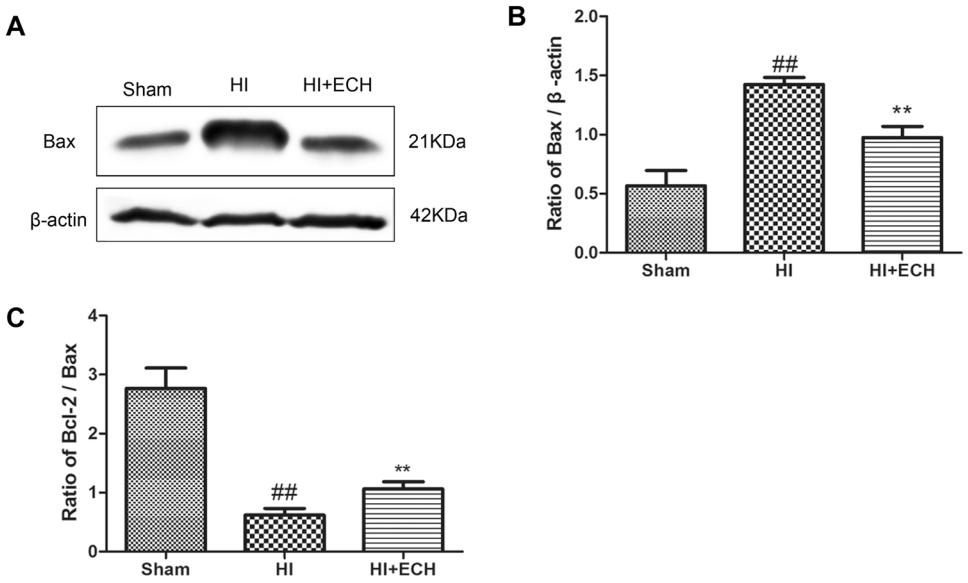

Fig. 9 Effects of ECH on Bax protein expression. **A** Representative Western blot band of Bax protein expression in the ischemic brain at 48 h after hypoxic-ischemic brain damage. **B** Effect of ECH on the Bax protein expression at 48 h after hypoxic-ischemic brain damage. C Effect of ECH on the ratio of Bcl-2/Bax at 48 h after hypoxic-ischemic brain damage. Data are expressed as mean ± SEM (n = 6). $^{\#\#}p < 0.01$ versus the sham group and $^{**}p < 0.01$ versus the HI group

Discussion

The present data showed that ECH post-treatment significantly decreased not only the brain infarct volume but also the HIBD-induced neuronal neurodegeneration and death. Administering ECH could restore the activities of the endogenous antioxidant enzyme system and attenuate the level of lipid peroxidation. The number of apoptotic neurons and the expression of apoptosis-related proteins were reversed by ECH post-administration following cerebral HI. Our present study indicated that ECH elicited a neuroprotective effect by suppressing oxidative stress and apoptosis in a neonatal HIBD rat model.

HIBD is a brain lesion caused by decreased cerebral blood flow and hypoxia in the perinatal period in neonates, the pathogenesis of this condition is still not elucidated and no acknowledged treatment is currently available for HIBD apart from hypothermia [6]. Experimental models of HIBD have been of a great value for effectiveness of therapeutic interventions and determining mechanisms underlying disease [32]. A modified Rice [33] model is most often used for HIBD and has been well proved by far. Seven-day-old rats are typically selected because the maturity of their CNS is similar to that of a near-term human fetus or infant [34]. Therefore, we used this model to investigate the neuroprotective effect of ECH. Drugs penetrate the blood-brain barrier and the corresponding pharmacological effects will not be exerted until the central nervous system has a certain concentration. ECH has been proven that can easily cross the blood-brain barrier and enter the central nervous system after systemic administration [35]. In our pre-experiment, we evaluated the effects of different doses of ECH on cerebral infarction via TTC staining in neonatal rat. The results showed that ECH dose-dependently attenuated the cerebral infarct volume induced by hypoxia-ischemia and the optimal dosage range is between 40 mg/Kg and 160 mg/Kg. In the formal study, TTC staining revealed pale infarct areas in the HI group, indicating that the HIBD model was successfully

established in neonatal rats. ECH (160 mg/Kg) significantly decreased the brain infarct volume in the neonatal rats induced by HIBD, suggesting that ECH might play a neuroprotective role in reducing brain injury. Morphological tests, such as hematoxylin-eosin (HE) and Nissl staining, further provided convincing evidence.

HE and Nissl staining usually provide an estimate of the extent of damage and the number of necrotic neurons. As we know, the hippocampus and cortex are susceptible to ischemia and hypoxia injury [36], therefore, the above regions were selected to observe the morphological changes of neurons in this study. The neurons in the hippocampus CA3, cortex and the hippocampus CA1 were observed with the following prominent morphological injuries in the HI group 48 h after HIBD: loss of neurons and Nissl bodies, karyopyknosis, and vacuolation. However, ECH (160 mg/Kg) restored the damaged neurons, further confirming that ECH had a neuroprotective effect.

Oxidative stress plays a vital role in HI-induced neuronal damage and mainly occurs through the generation of oxygen free radicals [37], such as: superoxide anions, hydroxyl radicals and hydrogen peroxide. Under physiological conditions, oxidative stress occurs when the balance between oxygen free radicals and endogenous antioxidant enzymes is disrupted. The generation of oxygen free radicals leads to lipid peroxidation, protein breakdown, and DNA damage, which also result in brain damage after HI [38, 39]. On the other hand, the antioxidant system, which consists of SOD, GSH-Px, CAT, and T-AOC, consequently becomes inhibited and inefficient to scavenge excessive oxygen free radicals, thereby exacerbating the severity of brain injury [40]. In the present study, post-treatment with ECH (160 mg/Kg) antagonized the HI-induced decrease in SOD, GSH-px, and CAT activities and T-AOC. Concomitantly, the increased MDA level was reduced by ECH, demonstrating that ECH could alleviate HIBD in neonatal rats at least partly perhaps due to increasing antioxidant enzyme activities and decreasing lipid peroxide.

Oxidative stress leads to nerve cellular dysfunction and death, and the main death outcomes following HIBD involve neuronal apoptosis [41, 42]. In this study, we also detected the changes in TUNEL-positive cells. The number of the TUNEL-positive cells significantly increased after HIBD in the brain cortex. The cortex of the rats administered with 160 mg/kg ECH showed a substantially decreased number of TUNEL-positive cells. This finding also supported the histological results and indicated that ECH exerted anti-apoptotic effects. In addition, the apoptosis-related proteins, such as caspase-3, Bcl-2, and Bax, were detected in the ischemic hemisphere to clarify the relationship between the protective effects of ECH and apoptosis. Bcl-2 family members are key regulators of apoptosis, which involves pro- and anti-apoptotic activities [43]. Bcl-2/Bax can indicate the survival or death of neurons by governing mitochondrial membrane permeabilization [44]. Consistent with previous findings [45], our results indicated that 160 mg/kg ECH could rescue the increased Bcl-2/Bax ratio and decreased caspase-3 expression induced by HIBD. These results suggested that ECH could inhibit neuronal apoptosis and exert a neuroprotective effect.

HIBD is the result of a combination of various cellular and molecular mechanisms, there are different stages of pathological change after the onset of HIBD, and there are different stages "Treatment time window". Future study will explore different time courses and different stages post-treatment for ECH, and evaluate its optimal therapeutic window for neonatal HIBD. In addition, it also needs to be solved further whether the time for the first application of ECH after HIBD can be delayed. The present experiments are only basic research aimed at finding potential, safe and effective drugs against HIBD, these explorations are part of further research. Actually, there is still a significant amount of work to be done to apply ECH to clinical practice. Hypothermia treatment is the standard of care for neonatal HIBD admittedly. However, the short therapeutic window and other reasons limit its clinical application. In addition, the pathogenesis and clinical features of HIBD is complex. Current studies have shown that combination of drugs and hypothermia has more effects for HIBD [46, 47]. To making the neuroprotective effects of ECH more clinical applicability, combinational therapies with hypothermia should be further considered and explored to reach more targets of HIBD [2] and enhance the neuroprotection of ECH. Clinically, there is a lack of drugs for the treatment of HIBD, so it is still urgent to find safe and effective drugs from natural products.

In conclusion, this study is the first to report that ECH, a compound with neuroprotective properties, which may exerts neuroprotective effects on a rat HIBD model by increasing antioxidant activity and inhibiting apoptosis. ECH has a potential therapeutic efficacy in HIBD treatment. Molecular docking and study analysis of Li MQ et al. [48] showed that ECH could bind to Keap1, led to the Nrf2 nuclear translocation, protect PC12 cell against cytotoxicity. Therefore, it is reasonable to assume that ECH may induce Nrf2 activation, enhance antioxidant capacity, decrease the production of ROS, which alleviated oxidative stress and promoted neuron survival. However, this study is a preliminary investigation of the neuroprotective effect of ECH, more neuroprotection effects and relevant various cell survival mechanism of ECH on HIBD needs to be explored in the following work.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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