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ORIGINAL RESEARCH

Pretreatment of Grape Seed Proanthocyanidin Extract Exerts Neuroprotective Effect in Murine Model of Neonatal Hypoxic-ischemic Brain Injury by Its Antiapoptotic Property

Xing Tu[1] · Mengxia Wang[2] · Yilin Liu[3] · Wenyan Zhao[3] · Xuxin Ren[3] · Yuanjun Li[4] · Hongqing Liu[1] · Ziting Gu[1] · Hui Jia[1] · Jing Liu[1] · Guoying Li[1,5] · Li Luo[1]

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Abstract

Grape seed proanthocyanidin extract (GSPE), an active component extracted from the grape, has been reported to demonstrate antioxidant, anti-inflammatory, anticancer, and antiapoptosis effects. However, little is known about the role of GSPE on neonatal hypoxic-ischemic (HI) brain injury. The aim of this study was to evaluate the neuroprotective effect of GSPE pretreatment on neonatal HI brain injury in mice. A modified Rice–Vannucci method was performed to induce neonatal HI brain injury in the 7-day-old mouse pups pretreated with GSPE or vehicle. The infarct volumes were determined by TTC staining. TUNEL staining was used to detect cells apoptosis, and the expressions of apoptosis-related proteins: bax, bcl2, and cleaved caspase-3 were assayed by Western blot. Behavioral tests were also conducted to assess the functional recovery after injury. We showed that the brain damage and neurobehavioral outcomes improvement was observed in GSPE pretreated group. GSPE was proved to suppress apoptosis through inhibition of bax and cleaved caspase-3 expression. It demonstrates that GSPE could alleviate brain damage maybe through its antiapoptotic activity in a neonatal HI brain injury model, and GSPE has the potential to be a new drug for effective prevention of this disorder.

Keywords GSPE · Neonatal hypoxic-ischemic brain injury · Apoptosis · Neuroprotection

Introduction

Neonatal hypoxic-ischemic encephalopathy (HIE) is a common brain injury, which is caused by decreased cerebral perfusion at birth (Ellery et al. 2018). The primary reasons for decreased cerebral perfusion include umbilical cord entanglement, placental detachment, or severe uterine contractions (Confortim et al. 2018). In developed countries, 1–6 of every 1000 newborns are diagnosed with HIE during the perinatal period, and HIE often leads to cerebral palsy, epilepsy, visual impairment, and cognitive impairment (Herz et al. 2018). These catastrophic injuries not only seriously affect the quality of life, but also cause a heavy financial burden to families, society, and the country.

Currently, hypothermic therapy is often used in the treatment of neonatal hypoxic-ischemic brain damage. The efficacy of hypothermic therapy has been demonstrated in newborns with low and moderate brain damage, but efficacy has not been shown to significantly improve severe HIE injuries (Nair and Vhs 2018). Due to limitations in equipment, manpower, and financial resources, the popularity of

✉ Guoying Li
gzlgying820@sina.com

✉ Li Luo
josephluoli@hotmail.com

1 School of Biosciences & Biopharmaceutics, Guangdong Pharmaceutical University, Guangzhou 510006, Guangdong, People's Republic of China

2 Intensive Care Unit, Guangdong No. 2 Provincial People's Hospital, Guangzhou 510317, People's Republic of China

3 School of Clinical Medicine, Guangdong Pharmaceutical University, Guangzhou 510006, Guangdong, People's Republic of China

4 School of Clinical Pharmacy, Guangdong Pharmaceutical University, Guangzhou 510006, Guangdong, People's Republic of China

5 Guangdong Medical Association, Guangzhou 510006, Guangdong, People's Republic of China

hypothermic therapy in developing countries has not been comprehensive. Despite our basic research, the changes in pathophysiology and physical and chemical properties in the brain of HIE model remain unclear. Therefore, it is important to identify a new drug for neonatal HIE therapy, and find a targeted therapy for hypoxic-ischemic brain injury is an urgent task.

Grape seed proanthocyanidin extract (GSPE) is a biological polyphenolic compound with various pharmacologic effects (Hao et al. 2018). GSPE is derived from a wide range of sources and can be extracted from a variety of plants, such as pine bark, cinnamon bark, apple, and lotus. GSPE is recognized as the most potent natural antioxidant in nature (Hao et al. 2018; Stoupi et al. 2010). Compared with traditional Western medicine, naturally active medicines have low production costs, fewer side effects, and a wide range of sources. Therefore, naturally active medicines are very popular in drug research. GSPE has antioxidant, free radical scavenging, anti-inflammatory, and other biological activities (Zhang et al. 2018; Lu et al. 2018). It has been proven that the antioxidant capacity of GSPE is 50 times that of vitamin E and 20 times that of vitamin C (Fracassetti et al. 2013).

Kong et al. (2017) reported that GSPE can be used in ischemia–reperfusion models. GSPE treatment clearly improved cognitive function and reduced pathologic damage in the brain of adult rats (Kong et al., 2017). However, few studies have addressed whether GSPE can be used for hypoxic-ischemic (HI) brain injury in neonatal mice. The current study was designed to investigate the neuroprotective effects of GSPE pretreatment on HIE models and its potential mechanism. We explored the expression levels of apoptosis-related proteins (bax/bcl2/cleaved-caspase-3) through immunofluorescence and western blot. TUNEL staining was used to reveal the numbers of apoptotic cells, In addition, the neurobehavioral tests of HIE newborn mice were also studied. The results in our research show that GSPE ameliorates brain injury with evident improvement in neurobehavioral outcomes when administered before HI injury through its antiapoptotic role.

Results

GSPE Pretreatment Reduced Brain Infarct Volume

To determine whether or not GSPE pretreatment protects newborn mice after HIE injury, pups were pretreated with GSPE before right common carotid artery (CCA) occlusion. The brain infarct volume was measured, and the degree of brain damage in mice was assessed by means of TTC staining (Fig. 1a). There was no infarction in the sham group; however, the infarct volume was remarkably reduced from $44.67 \pm 2.03\%$ in the HI group to $21.67 \pm 0.33\%$ in the GSPE + HI group (Fig. 1b). GSPE pretreatment clearly reduced brain infarct volume in the neonatal HI brain injury model.

GSPE Pretreatment Attenuates HI-Induced Neuronal Cell Apoptosis and Inhibits Apoptosis-Related Protein Expression

To verify whether or not GSPE reduced apoptosis, the TUNEL assay and Western blot were performed. TUNEL-positive cells were labeled with apoptotic cells. Compared to the sham group, the number of TUNEL-positive cells in the brain increased in the HI group; however, the pups were pretreated with GSPE, and the number of TUNEL-positive cells decreased significantly (Fig. 2).

In addition, apoptosis-related protein (bax, bcl-2, and cleaved-caspase-3) expression was assessed by the Western blot. Compared to the HI group, the expressions of proapoptotic proteins, bax and cleaved-caspase-3, were attenuated and expression of the antiapoptosis protein, bcl-2, was enhanced in the GSPE + HI group (Fig. 3). Furthermore, in the GSPE + HI group, the ratio of bcl2-to-bax increased in the brain compared with the HI group.

These data indicated that GSPE pretreatment attenuated the HI-induced neuronal cell apoptosis and inhibited proapoptosis-related protein expression; antiapoptosis-related proteins were activated.

GSPE Pretreatment Improved the Neurobehavioral Outcomes of Mice After HI Brain Injury

Next, we explored the behavioral effects of GSPE in HI brain injury mice by measuring the body weight and neurobehavioral (righting reflex, geotaxis reflex, and grip test) on days 1, 3, and 7 after HIE injury.

Compared with the HI group, the GSPE + HI group gained more body weight on days 3 and 7 after the HIE injury (Fig. 4a). The righting reflex and geotaxis reflex test scores were significantly decreased in the GSPE + HI group compared with the HI group on days 3 and 7 after the HI brain injury (Fig. 4b, c). In contrast, compared with the HI group, the GSPE + HI group had higher scores on days 3 and 7 after the HI injury (Fig. 4d). These data suggest that GSPE had a positive effect on the overall condition and general neurobehavior in HI brain injury mice.

Discussion

The pathogenesis of HIE is very complicated. At present, low-temperature therapy used in clinical practice can only achieve a good therapeutic effect on neonates with

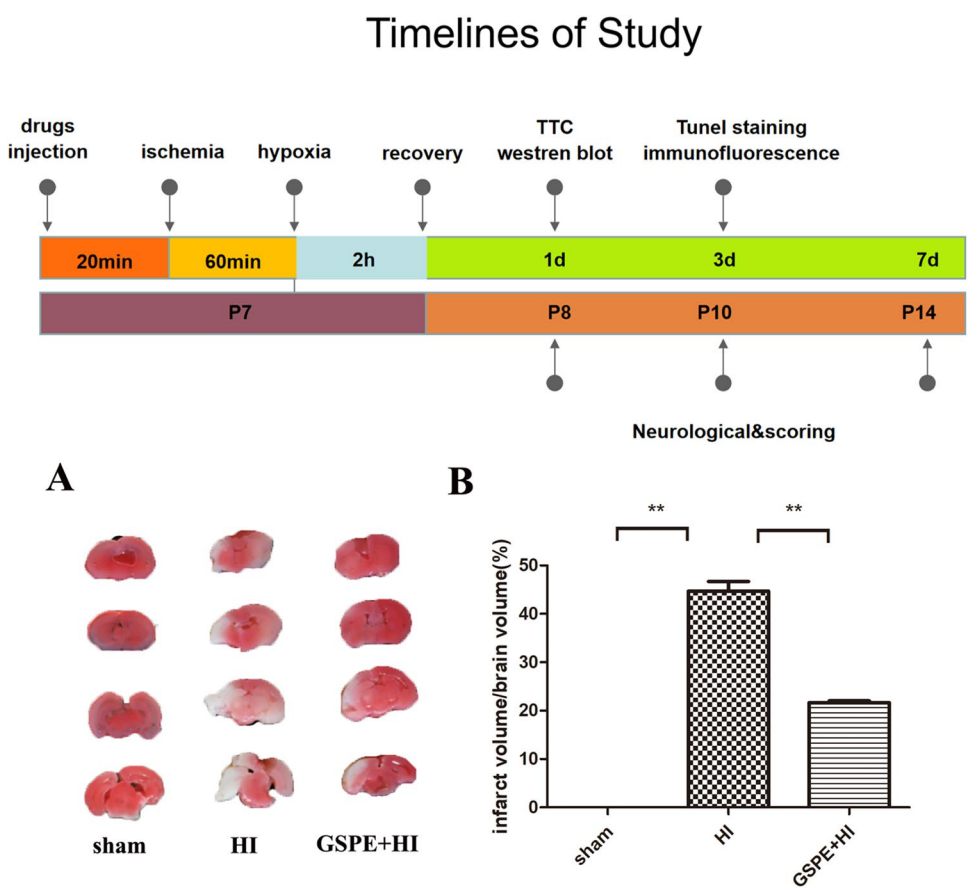

Fig. 1 GSPE reducing the volume of cerebral infarction in neonatal hypoxic-ischemic brains. Timelines of Study; ischemia = the common carotid artery was coagulated using an electric knife; hypoxia = 8% O_2 +92% N_2 (120 min); recovery = pups were returned to their dams. **a** Representative TTC stained the volume of cerebral infarction from the GSPE pretreatment at 24-h post-HIE injury (red area = normal brain tissue; white area = infarct tissue). **b** Quantitative analysis of infarct volume. The results are represented by infarct volume/brain volume (%) compared to the HI group. Mean percentage of infarct volume was significantly reduced in the GSPE + HI group (**$P < 0.01$; one-way ANOVA followed by Bonferroni test). $N = 3$ in each group

low-to-moderate HIE. Nevertheless, there is still no effective clinical treatment for severe brain injuries (Shankaran et al. 2016). Thus, to search for new therapeutic targets in clinical practice, an in-depth understanding of the pathogenesis and pathologic changes of HIE are urgently needed. In this study, the reasons we chose the 7-day-old pups to establish the HIE model is that the brains of young mice are similar with respect to the histologic structure of 32–34-week fetuses or newborns (Bonnin et al. 2011). Studies have shown that the brain injury caused by surgery and hypoxia is mainly limited to the cerebral cortex and hippocampus on the side of carotid artery ligation (Towfighi et al. 1997), and rarely occurs on the opposite side. This statement has been preliminarily verified by TTC staining experiments in the early stage.

GSPE is a polyphenolic compound deriving from flavanols, which is widely distributed in red grape seeds, including catechins, epicatechins, and gallic acid (Muzzi et al. 2018). Common effects of GSPE include antioxidation, anti-inflammatory, and anticancer, and is also helpful to reduce mitochondrial damage and apoptosis (Tousson et al. 2018). In this study, we pretreated the model mice with a GSPE intraperitoneal injection. Based on the results of TTC staining, a white infarct was observed in the HI group, and the area of the infarct was reduced in the GSPE + HI group. This finding not only showed that our modeling was successful, but also showed that GSPE therapy reduced the volume of cerebral infarction. Our study also demonstrated that GSPE can inhibit apoptosis by suppressing activation of bax and cleaved caspase-3. Moreover, GSPE had a positive protective effect on the general condition and neurobehavioral recovery in HI brain injury mice. For these reasons, GSPE might be an effective neuroprotective agent that mitigates the HI brain injury and inhibits apoptosis.

The half-life of a drug has an effect on the treatment of related diseases. Several studies have demonstrated that the most abundant in GSPE include gallic acid (GA), catechin (C), epicatechin (EC) (Margalef et al. 2014; Yamakoshi et al. 2002; Arola-Arnal et al. 2013). Ferruzzi and his colleagues studied the bioavailability of GSPE and found that GA, EC, and C were absorbed within 1–2 h after ingestion; and clearance from plasma was also rapid and its concentration decreased back to baseline 6–8 h after ingestion with a monophasic response; and GSPE has a half-life of about 3–4 h (Ferruzzi et al. 2009). The results of Wu (Wu et al.; 2012) also support this view. In the present study, the GSPE + HI mice were received GSPE 20 min before the ischemic surgery, the whole process of hypoxic-ischemic was within the half-life of GSPE. In other words, GSPE could continue to play a protective role in the process of hypoxic-ischemic.

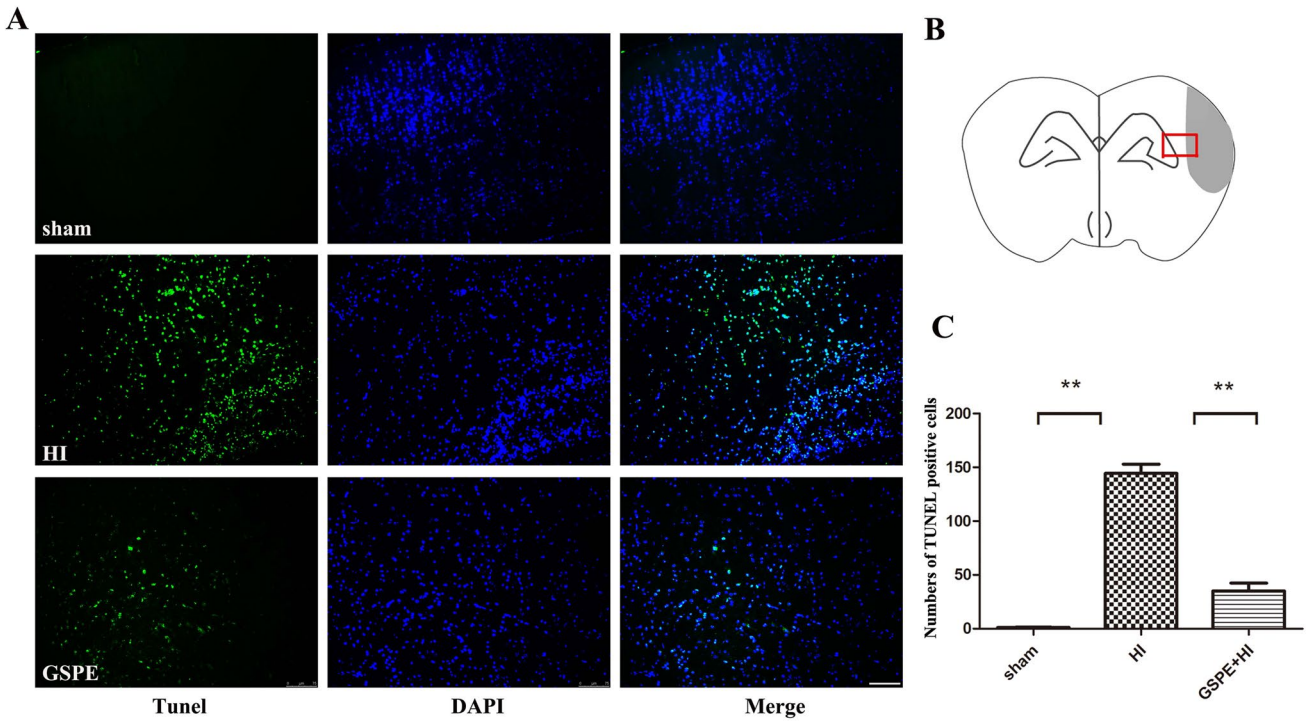

Fig. 2 GSPE pretreatment reducing the expression of TUNEL-positive cells after HIE injury. **a** Representative micrographs showing TUNEL staining of sham group, HI group, and GSPE + HI group of TUNEL-positive cells (green) in the ipsilateral cerebral hemisphere 3 days after HI injury. Scale bar = 75 μm. **b** Red box: TUNEL staining area. **c** Quantitative analysis of TUNEL-positive cells. GSPE pretreatment significantly reduced the number of TUNEL-positive cells compared to the HI group. $^{**}P < 0.01$. $N = 6$ in each group

Drug release in the central nervous system (CNS) is primarily restricted by the blood–brain barrier (BBB), a goalkeeper of tight junctions and cerebral vascular endothelial cells regulating communication between the blood flow and brain tissue (Pruitt et al. 2017; Zhao et al. 2018; Yang et al. 2012). Recent studies have suggested that GSPE might exert neuroprotective properties (Strathearn et al. 2014; Kim et al. 2006; Devi et al. 2006), so it was necessary to confirm whether GSPE components are accessible to the brain to exert these effects. Prasain have reported the identification of proanthocyanidins (catechin and epicatechin) in rat plasma after oral administration of GSPE using liquid chromatography tandem mass spectrometry (LC–MS/MS). The results demonstrated that monomeric catechin (C) and epicatechin (EC) were shown to have crossed the BBB and gained entry to the brain in GSPE-treated rats and may be responsible for the neuroprotective effects of GSPE (Prasain et al. 2009). Moreover, it also has been reported that when HIE injury occurs, the normal function of the BBB of newborn rats is destroyed, and the permeability of the blood–brain barrier becomes larger (Liu et al. 2015). This condition also provides an opportunity for GSPE to successfully pass the BBB into brain. Furthermore, we also proved that GSPE can reduce the volume of cerebral infarction in neonatal hypoxic-ischemic brains. It suggests that the potential mechanism of GSPE may be related to its ability to cross the BBB.

When HI brain injury occurs, three different morphologic features of cell death in neuronal cells were mainly observed: necrosis; apoptosis; and autophagy (Northington et al. 2011). Necrosis was considered to be the structural and mechanical basis of cell degeneration during HI brain injury in early studies (Northington et al. 2011). With further research, apoptosis- or autophagy-mediated programmed cell death as a key role in HI brain injury-induced cell degeneration in recent years (Hu et al. 2017; Gamdzyk et al. 2018). To explore whether the GSPE protection against HI-induced brain injury is related with the antiapoptosis or not, we detected the cell apoptosis and expressions of bax and cleavage caspase-3. The results of TUNEL staining and western blot analysis showed that the numbers of apoptotic cells, bax, and cleaved caspase-3 were decreased in HI brain injury mice, which were pretreated with GSPE. Together, these data demonstrate that GSPE can inhibit apoptosis by preventing the activation of bax and caspase-3. It is also reported that bax and bcl-2 proteins are involved in the regulation of neurons after HIE, and the downturn of bax and the upregulation of

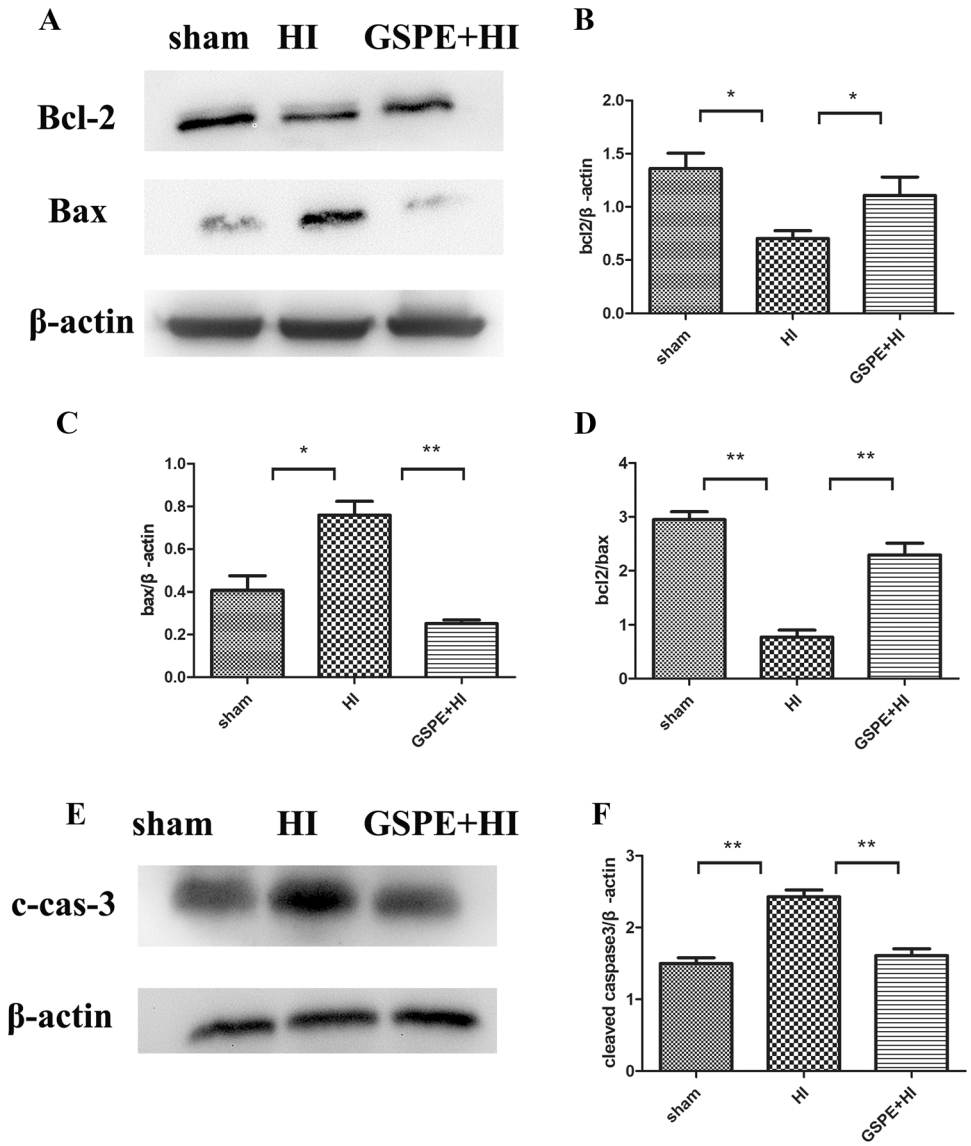

Fig. 3 GSPE pretreatment attenuating the apoptosis induced in neonatal hypoxic-ischemic brains. Representative Western blots and quantitation data of apoptosis-related proteins (bcl-2, bax, cleaved-caspase-3) in the ipsilateral cerebral hemisphere 24 h after HI injury. **a, e** The levels of the apoptosis-related proteins are reported as the value normalized to β-actin for each sample. **b** Compared to the HI group, the mean percentage of bcl-2 was significantly induced in the GSPE + HI group, **c, f** and mean percentage of bax and cleaved-caspase-3 were significantly reduced in the GSPE + HI group. **d** The mean of bax/bcl-2 was declined in the HI group than that in the GSPE + HI group (*$P < 0.05$; **$P < 0.01$; one-way ANOVA followed by Bonferroni test). $N = 3$ in each group

bcl-2 inhibit neonatal neuronal apoptosis (Liang et al. 2007; Fang et al. 2018). The results of our research are consistent with the previous view.

However, our current research demonstrated the neuroprotective effects of GSPE on neonatal HI brain injury from the perspective of apoptosis, but did not explore the effects of autophagy. The pros and cons of autophagy on cells depends on the level of activation, stimulation, and cell types (Wang et al. 2008). In fact, apoptosis and autophagy are often inseparable when HI brain injury occurs (Zheng et al. 2018; Fang et al. 2017). According to previous results, more autophagic bodies can be observed in HI-injured brains (Li et al. 2010), but others had opposite view (Zheng et al. 2018). This may be brought about by the controversy between the dual roles of autophagy and the ambiguity between the term "autophagic cell death" (Clarke and Puyal 2012; Yuan and Kroemer 2010). A further study is needed to demonstrate whether or not GSPE can exert neuroprotective effects on HIE models by affecting autophagy of neurons.

In summary, GSPE has been well applied to antioxidation, anti-inflammatory, and anticancer. Our results showed that GSPE can also be applied to a model of HI brain injury in neonatal mice and significantly decreased cerebral infarct volumes. In a model of HIE, the apoptotic signaling pathway was activated, and GSPE pretreatment inhibited the apoptotic signaling pathway and reduced the death of cells, which suggested that GSPE might be a potential therapeutic agent for treatment or preventing neonatal HI brain injury.

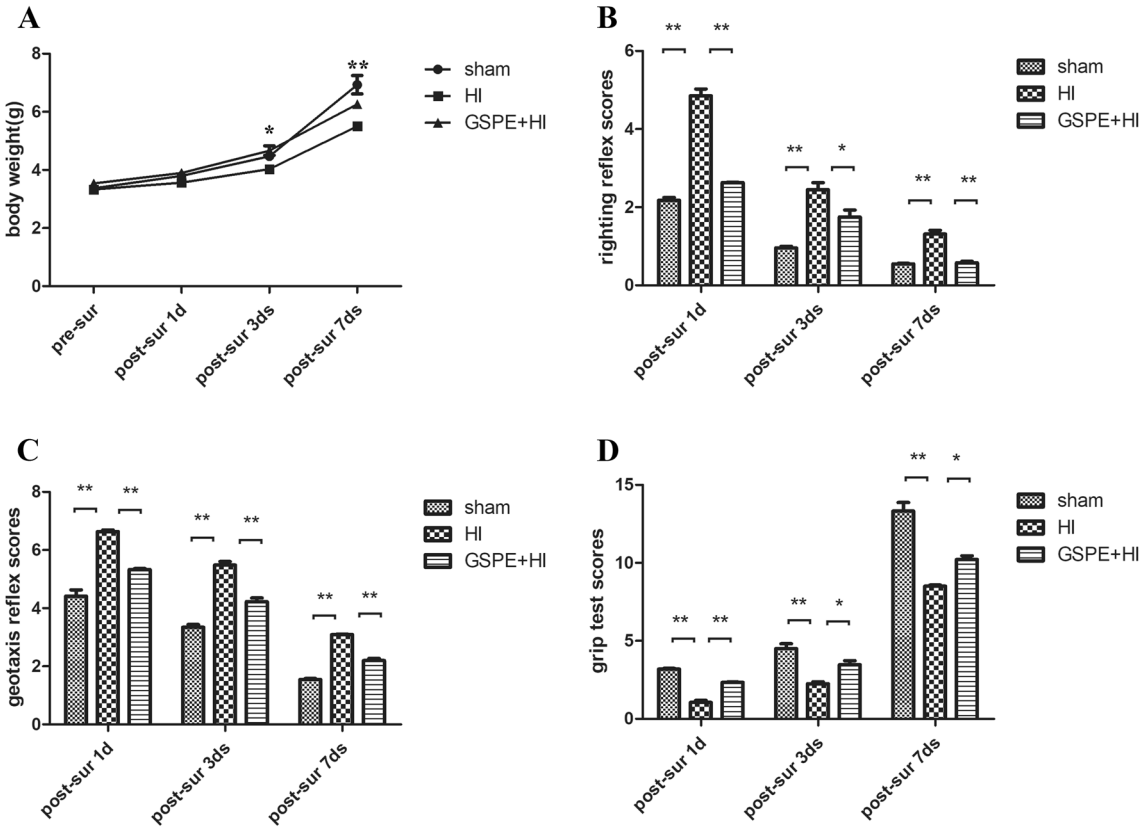

Fig. 4 GSPE pretreatment improvement in the overall condition and neurobehavior of mice after hypoxic-ischemic brain damage. **a** Compared to the HI group at post-sur 3d and post-sur 7d, the GSPE + HI group gained more body weight (*$P < 0.05$; **$P < 0.01$). **b–d** At post-sur 1, 3, and 7d, GSPE pretreatment improved the **b** righting reflex, **c** geotaxis reflex, **d** grip test of mice after HIE (*$P < 0.05$; **$P < 0.01$; one-way ANOVA followed by Bonferroni test). $N = 8$ in each group

Materials and Methods

Neonatal Hypoxia–Ischemia Injury Model and GSPE Administration

All animals were purchased from the Experimental Animal Center of Sun Yat-Sen University (Guangzhou, Guandong), and approved by the Experimental Laboratory Animal Committee of Guangdong Pharmaceutical University (Permit No: gdpulac2017175), and conformed to the Chinese Council on Animal Care guidelines.

The day of birth of C57BL/6 mice pups was designated day 0 (P0); postnatal 7-day-old (P7) pups were used in the subsequent experiments. A total of 60 pups were used in this study. The mouse pups were divided randomly into three groups with 20 animals in each group for the following experiments (TTC staining = 3 animals, western blot = 3 animals, TUNEL staining = 6 animals, behavior tests = 8 animals). A HI brain injury model was established in P7 pups using the Rice–Vannucci method (13), with some modifications. In brief, the P7 pups were anesthetized with isoflurane gas. After the pups were comatose, the right common carotid artery (CCA) was identified under a microscope and electrocoagulated with an electric knife. After the surgery, the pups were returned to the mother for breastfeeding and warmed back for 1 h in their dam. The pups were then placed in an oxygen-deficient box (gas mixture of 8% O_2 and 92% N_2 at a flow rate of 2 l/min). Pups were kept in a hypoxia chamber for 2 h by placing them in hypoxia chamber partially submerged in a water bath of 37 °C for maintaining a constant thermal environment. Sham group pups underwent anesthesia, and the right CCA was exposed as in the HI group, but there was no ligation or exposure to hypoxia.

GSPE (Meilun Biological, Dalian, China. Cat: MB2056) was dissolved in PBS solution at a concentration of 1.25 mg/ml at 4 °C for future use. Twenty minutes before the surgery, the GSPE + HI mice were injected intraperitoneally at a dose of 30 mg/kg (HI group received the same dose of PBS intraperitoneally).

2,3,5-Triphenyltetrazolium chloride (TTC) Staining

Twenty-four hours after the HI injury, TTC (Solarbio, Beijing, China. Cat: G3005) staining was performed on pup

brain slices. The pups were anesthetized with 10% chloral hydrate (Cat. No. 302-17-0; Sigma-Aldrich; Merck KGaA), and the brain was removed quickly. We sectioned the pup brains at 2-mm intervals into four coronal slices. The brain slices were soaked in 1% TTC solution for approximately 20 min at room temperature. TTC stains normal tissues red and the infarct tissue is white. The infarct tissue was measured by Image J software (version 1.8.0; National Institutes of Health, Bethesda, MD, USA), and the percentage of infarction (infarct ratio) was calculated with the following formula: {corrected infarct volume = [contralateral hemisphere volume − (ipsilateral hemisphere volume − infarct volume)]/contralateral hemisphere volume × 100%; Kong et al. 2017}.

TUNEL Staining

On the third day after HI injury, the mice were deeply anesthetized with 10% chloral hydrate. Then, the mice were perfused with 0.9% saline transcardially and fixed with 4% paraformaldehyde. Next, the brains were carefully removed and placed in a 4% paraformaldehyde solution overnight at 4 °C. After rinsing the brain tissue under the running water for 24 h the next day, the brain tissue was placed in a tissue dehydrator for gradient dehydration, paraffin-embedded, and sectioned coronally into 5-μm slices for subsequent staining.

A TUNEL Apoptosis Detection Kit (fluorescence) was used to detect apoptotic cells (Wanleibio, Shanghai, China. Cat: WLA030a). According to the standard protocol, the appropriate slices were selected for TUNEL assay and gradient dewaxing of those with xylene and ethanol solution. Fifty microliters of 3% H_2O_2 [30% H_2O_2: methanol (1:5)] were added to each brain slice and incubate for 10 min. Then, 50 μl of TUNEL reaction buffer (configuration solution according to the manufacturer's instructions) was added to each tissue and also incubated at 37 °C for 90 min in the dark. Next, the brain slices were again stained with DAPI dye for 5 min, and antifluorescence quencher was added to each brain slice. The brain slices were filmed with a fluorescence microscope (BX51; Olympus Corporation, Tokyo, Japan) within 24 h. ImageJ software was used for apoptotic cell counting and the results were analyzed using SPSS.19.0 software.

Western Blot

Mice were deeply anesthetized and sacrificed by decapitation 24 h after HI injury. Brain tissue was removed and placed in a − 80 °C refrigerator. A high-speed tissue grinder was adopted to grind the brain tissue into a tissue homogenate, and the lysate and PMSF were added to the tissue homogenate. The cells were disrupted by a sonic cell disruptor and centrifuged for 20 min (12,000 rpm/min at 4 °C). The supernatant was reserved for subsequent experiments, then a 20-μl sample was used for protein quantitation.

An equal amount of protein (20 μl/well) was loaded, concentrated on a 12% SDS-PAGE gel, and then transferred to a PVDF membrane. After blocking with 5% skim milk powder for 1 h, the membranes were incubated with primary antibodies targeting the following proteins: bax (1:500; Affinity Biosciences, Cincinnati, OH, USA. Cat: AF0120); bcl-2 (1:500; Affinity Biosciences. Cat: AF6139); and cleaved-caspase-3 (1:500; Affinity Biosciences. Cat: AF7022). After the primary antibody was added, the membranes were placed in a refrigerator at 4 °C overnight. The next day, the membranes were removed from the refrigerator, and secondary antibody (1:10,000; EarthOx, San Francisco, CA, USA. Cat: E030120-01) was added. β-Actin (1:500; Affinity Biosciences. Cat: T0022) was used as an internal reference protein. The signals were visualized by a chemiluminescence imaging and analysis system (Tanon, Shanghai, China). All of the experiments were repeated at least 3 times. Image J software was adopted to perform optical density analysis on strips and SPSS 19.0 was used to analysis data.

Neurobehavioral Assessments

A behavioral assessment was performed on the mice in each group on days 1, 3, and 7 after HI. There are three major types of behavior we observed, righting reflex (assess the recovery of the mice brain; Shen et al. 1991); geotaxis reflex (assess the vestibular and/or proprioceptive functions; Chen et al. 2015); and grip test (assess the fore limb grip strength and executive force; Xiao et al. 2014). In addition, the body weight of mice was also measured on the day of surgery as well as days 1, 3, and 7 after the surgery.

Statistical Analysis

All numerical data are expressed as the mean ± SEM. Data were analyzed using SPSS 19.0 software and Graphpad Prism (version 4.0; Graphpad software, San Diego, CA, USA). Statistical significance was assessed using one-way analysis of variance (ANOVA), followed by a Bonferroni test if more than two groups were involved. P value < 0.05 was considered statistically significant.

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Author Contributions Xing Tu contributed to the general administration, statistical analysis, manuscript writing; Yilin Liu, Wenyan Zhao, Hongqing Liu, and Yuanjun Li assisted in the completion of Western blotting, behavioral tests, etc.; Mengxia Wang, Ziting Gu, and Hui Jia assisted in the completion of Western blotting, immunoluorescence, etc.; Li Luo established the animal models and providing technical guidance; and Jing Liu and Guoying Li provided technical guidance.

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Compliance with Ethical Standards

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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