



Ferulic acid ameliorates pentylenetetrazol-induced seizures by reducing neuron cell death

Shu-Hong Zhang^{a,1}, Donghai Liu^{a,1}, Qingyun Hu^b, Jinling Zhu^{a,*}, Shuqiu Wang^c, Shaobo Zhou^{c,d,**}

^a Department of Biology, Jiamusi University, Jiamusi, Heilongjiang, 154007, PR China

^b Department of Anatomy, Jiamusi University, Jiamusi, Heilongjiang, 154007, PR China

^c Department of Pathophysiology, School of Basic Medicine, Jiamusi University, Jiamusi, Heilongjiang, 154007, PR China

^d School of Life Sciences, Institute of Biomedical and Environmental Science and Technology, University of Bedfordshire, Luton LU1 3JU, UK

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ABSTRACT

To investigate the neuroprotective effect of ferulic acid (FA) in a pentylenetetrazol (PTZ)-induced seizures model in rat, the motor response, spatial learning ability and memory capability of the rats were assessed. Both the antioxidation and anti-apoptosis pathways were also investigated. In this study, male *Wistar* rats were randomly divided into 3 groups (n = 12 in each group). For 28 days, the rats were administered saline alone (i.p. normal saline, NS group), PTZ (40 mg/kg, i.p., PTZ group) once daily to induce seizures, or FA (i.p. 60 mg/kg) 20 min before being given PTZ (40 mg/kg, i.p., FA + PTZ group) to assess the neuroprotective effect of FA. The motor response of the rats was analysed with the Racine scale. The spatial learning and memory capacity of the rats were assessed by the Morris water maze test. The superoxide dismutase (SOD) activity and malondialdehyde (MDA) content were measured, and both in situ staining with the DNA-binding bisbenzimidazole Hoechst 33258 and TUNEL assays were used to assess apoptosis. Western blotting was used to further analyse the expression of Apaf-1, caspase-9, caspase-3, Bcl-2, Bid, Bax, cleaved caspase-3 and cytochrome c. The results showed that compared to the those of the PTZ group, FA pre-treatment significantly ($p < 0.01$) reduced the Racine scores starting at day 4, prolonged the latency of the onset of seizure at day 28, reduced the escape latency period starting at day 2, increased the frequency of crossing the platform location, increased the SOD activity, reduced the MDA content and apoptosis percentage, and upregulated the Bcl-2 levels whilst downregulating the Bax, cytochrome c, Apaf-1, caspase-9, caspase-3, cleaved caspase-3 and Bid expression levels. This study demonstrated that pre-treatment with FA exerts strong neuroprotective effects by reducing the motor response and by improving spatial learning ability and memory capacity. The neuroprotective effect may be a result of a reduction in neuron cell death that occurs via the antioxidative and anti-apoptotic pathways.

1. Introduction

Epilepsy is a chronic recurrent brain dysfunctional syndrome with multiple causes. Epilepsy is characterized by the abnormal discharge of brain neurons and has been listed by the WHO as one of the five major neuropsychiatric diseases that need to be prevented and controlled. Epilepsy seriously affects both health and the quality of life. At present, the pathogenesis of epilepsy has not yet been fully elucidated. Epilepsy has been linked to neuron cell death, dysfunction, neuronal degeneration, and neuroinflammation, among other effects (Mc Namara et al.,

2006). One of the causes of epilepsy is oxidative damage to neurons. Currently, among commonly used drugs for the control of epileptic seizures include carbamazepine phenobarbital, phenytoin sodium, and sodium valproate, but approximately one-third of epileptic patients still cannot control their disease using these treatments (Kwan and Brodie, 2000). Despite the introduction of a new generation of antiepileptic drugs, drug resistance is still one of the biggest challenges in the treatment of epilepsy (Tang et al., 2017). Therefore, there is an urgent need to find new therapeutic targets and to develop new antiepileptic drugs.

* Corresponding author at: Department of Biology, Jiamusi University, Jiamusi, Heilongjiang, 154007, PR China.

** Corresponding author at: School of Life Sciences, Institute of Biomedical and Environmental Science and Technology, University of Bedfordshire, Luton LU1 3JU, UK.

E-mail addresses: 13845414150@139.com (J. Zhu), shaobo.zhou@beds.ac.uk (S. Zhou).

¹ Equal Contribution.

Ferulic acid (4-hydroxy-3-methoxycinnamic acid, FA) is one of the derivatives of cinnamic acid. FA belongs to the hydroxycinnamic acid family and is extracted from *Ferula asafoetida*, where it is highly abundant in the leaves and seeds and its dry weight can reach approximately 2 g/kg in its richest granular form (Sosulski et al., 1982; Lempereur et al., 1997). FA also exists in grains such as brown rice, whole wheat and oats (Lempereur et al., 1997). FA is widely used as a medicinal material, and as a natural antioxidant, FA can prevent substrates such as lipids, proteins and DNA from being oxidized (Rice-Evans et al., 1996; Kroon and Williamson, 1999). In recent years, FA has been used in the prevention of cardiovascular, cerebrovascular and neurological diseases (Salazar-López et al., 2017; Hong et al., 2016; Barone et al., 2009; Sgarbossa et al., 2015). FA also ameliorated comorbid depression caused by neuroinflammation by restoring circulating corticosterone levels, decreased proinflammatory cytokines (IL-1 β , TNF- α) and indoleamine 2, 3-dioxygenase activity in mice brain (Singh et al., 2017). FA shows neuroprotective effects against ischaemia/reperfusion-induced brain injury and attenuates memory impairment in rats (Ren et al., 2017), as well as protecting against rotenone-induced degenerative changes in a rat model of Parkinson's disease (Ojha et al., 2015). FA has shown to have a strong cytoprotective ability by scavenging free radicals (Ogiwara et al., 2002), activating cellular anti-oxidative stress responses and acting as an anti-inflammatory in hypertensive rats (Alam et al., 2013; Mancuso and Santangelo, 2014; Hassanzadeh et al., 2017). FA improves cognitive function (Hassanzadeh et al., 2017; Deepa et al., 2012) in response to pentylentetrazol (PTZ), a γ -aminobutyric acid type A (GABA(A)) receptor blocker that induces seizure. Its ester, isopentyl ferulate, showed the anti-seizure effect in mice induced by both pilocarpine and pentylentetrazole, but this effect can be blocked by flumazenil, which suggested the effect may be through the benzodiazepine-binding site of the GABA a receptor (Machado et al., 2015). However, there is no significant changes on locomotor activity and motor coordination. FA improves odour-taste reward associative memory scores in larval *Drosophila* and prevents the age-related decline of this appetitive memory in adult flies. FA increases excitability in mouse hippocampal CA1 neurons, which leads to more stable context-shock aversive associative memory in 3-month-old mice and increases memory scores in > 2-year-old mice (Michels et al., 2018). Seizures increased the risk of memory loss, e.g., the IQ score in children with seizures is lower than that in the normal population (Rayner et al., 2016), or of having learning, cognitive and behavioural problems (Bell et al., 2011). However, there are no reports about the effect of FA on these abilities thus far. The Morris water maze test is often used to assess spatially related forms of learning and memory in rodents (Vorhees and Williams, 2015). Therefore, the Morris water maze test was used to assess FA pre-treatment on these abilities in a PTZ-induced seizure model in rat in this study.

Oxidative stress in neurons is generated by the persistent imbalance between the generation of toxic reactive oxygen species (ROS) and the antioxidative defences of aerobic metabolism (Shin et al., 2011). The massive production of ROS can lead to the significant destruction of cell structure and function, which leads to the progression of epileptic seizures (Azam et al., 2010; Sun et al., 2017). Therefore, ROS reduction can improve mitochondrial function in the hippocampus (Simeone et al., 2014). Pre- and post-treatment with FA counteract seizures in rats induced by PTZ, attenuate oxidative stress, e.g. reduced the MDA levels and increased GSH levels, and upregulation of heat shock protein 70, connexin 43, and monoamines in the hippocampus (Hussein et al., 2017). Mitochondria are involved in both ROS production and neuron apoptosis (Balaban et al., 2005). Approximately 90% of ROS are generated by the mitochondria (Yang et al., 1997). Oxidative stress can trigger apoptosis through the mitochondrial pathway. Increasing the ratio of Bax/Bcl-2 expression in the mitochondrion leads to a decrease in mitochondrial membrane potential, the release of cytochrome c, and the activation of caspase-induced apoptosis (Galluzzi et al., 2012a).

Apoptosis can be divided into either the intrinsic apoptotic pathway or the receptor-mediated extrinsic pathway. The intrinsic apoptotic pathway is caused by internal stimuli such as DNA injury, oxidative stress, hypoxia, cytoplasmic Ca²⁺ overload and endoplasmic reticulum stress. These signals trigger a critical event called mitochondrial outer membrane permeabilization (MOMP), which promotes the release of apoptotic cytochrome c into the cytoplasm and eventually leads to cell death due to caspase activation (Galluzzi et al., 2012b). The interaction between Bcl-2 family proteins, indicated by the Bcl-2 homology 3 (BH3) region, regulates MOMP and thereby converts cellular stress into apoptosis (Kale and Osterlund, 2018; Kalkavan and Green, 2018). Even though a study showed the antioxidant and antiepileptic effect of FA pre-treatment in rats with PTZ-induced seizure (Hassanzadeh et al., 2017), there have been no studies on its effect on the apoptosis pathway. Thus, the oxidation and the changes in the apoptotic indexes, together with the expression of Apaf-1, caspase-9, caspase-3, Bid, and cleaved caspase-3 were thoroughly investigated here. This study focuses on the hippocampus because 1 day after PTZ injection, the hippocampal neurons became more excitable (Postnikova et al., 2019), and 4 days after PTZ injection, rats' electroencephalography was affected, γ -Aminobutyric acid B receptor (GABABR) expression was decreased and neuronal apoptosis was induced in the hippocampal part of the brain (Naseer et al., 2013). Furthermore, prolonged and repeated seizures affected the viability and morphological changes of neurons in the hippocampus up to 1 week after PTZ treatment (Vasilev et al., 2018). The number of cells with structural and functional abnormalities in the hippocampus after PTZ-induced seizures decreased in the following order: CA1 > CA3b, c > hilus > dentate gyrus (DG) (Vasilev et al., 2018).

In this study, rats were given an FA pre-treatment 20 min before PTZ injection to investigate the neuroprotective effects of FA by assessing their seizures, spatial learning ability and memory capacity; the potential mechanisms underlying above changes were further explored by analysis of FA's anti-oxidation and anti-apoptosis pathways.

2. Methods

2.1. Chemicals and reagents

The reagents and suppliers included the following: PTZ and iso-flurane (Sigma, USA); FA (Shanghai Yuanye Biotechnology Co., Ltd. China); total SOD kit, Hoechst 33258 staining kit and BCA protein assay kit (Shanghai Beyotime Biotechnology Co. Ltd.China); Bid antibody, Bax antibody, Bcl-2 antibody, Apaf-1 antibody, caspase-3 antibody and caspase-9 antibody (Affinity, USA); MDA kit (Nanjing Institute of Biotechnology China); β -actin, goat anti-mouse IgG, goat anti-rabbit IgG (Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd., China); and TUNEL cell apoptosis detection kit (Shenyang Wanlei Institute of Biotechnology, China)

2.2. Animal groups and treatments

Male Wistar rats (body weight, 240 \pm 20 g, n = 36) were obtained from Harbin Medical Laboratory and were housed in the Animal Experimental Centre of Jiamusi University, China. The study was approved by the Ethics Committee of Jiamusi University. Rats were able to access food and water freely in a controlled environment with a 12 h dark/light cycle for 7 days before starting the experiment. All procedures complied with international regulations, minimizing the number of animals and avoiding suffering.

Rats were randomly divided into 3 groups (12 in each group): (1) normal saline (NS group), treated intraperitoneally (*i.p.*) with saline alone; (2) PTZ (seizures) group, treated with PTZ (40 mg/kg, *i.p.*, dissolved at a concentration of 0.36 mol/L) to induce seizures; (3) FA treatment (PTZ + FA) group, before the injection of PTZ, mice were treated with FA (*i.p.*, 60 mg/kg, dissolved at a concentration of

Table 1
Racine scale.

Racine score	Behavioural characteristics
0	No behavioural changes
1	Facial movements, ear and whisker twitching
2	Unilateral forelimb convulsions
3	Bilateral forelimb complete convulsions
4	Clonic convulsion
5	Generalized clonic-tonic seizures

0.31 mol/L), and 20 min later the rats were treated with PTZ (40 mg/kg, *i.p.*). Drug treatments were administered once daily for 28 days. The dose of FA (60 mg/kg) was based on the following studies (Mancuso and Santangelo, 2014; Hassanzadeh et al., 2017; Zhang et al., 2018) with a modification. There was no effect of pre-treatment with ferulic acid (50 mg/kg), but there were significant effects at doses of 75 mg/kg and 100 mg/kg, and there were no differences between these two doses (Mancuso and Santangelo, 2014; Hassanzadeh et al., 2017). Fifty mg/kg FA caused the attenuation of inflammation in lipopolysaccharide-induced rat acute respiratory distress syndrome (Zhang et al., 2018). The time of FA pre-treatment and the treatment duration were based on a previous study (Hassanzadeh et al., 2017), but our treatment period was 10 days longer than their 18-day treatment, as they failed to show an improvement of locomotive activities. The dose of PTZ (40 mg/kg, *i.p.*) has been routinely used in many studies (Ogiwara et al., 2002; Alam et al., 2013; Zhang et al., 2018).

The seizure intensity was scored based on the Racine scale (Racine, 1972) (Table 1). The behaviour was recorded every 4 days, and the seizure latency analysis was based on data recorded at day 28. On day 29, 24 h after the final drug administration, all rats underwent Morris water maze tests to assess learning and memory capacity for 5 days. On day 35, rats were anaesthetized with inhaled isoflurane (5% for induction and 2% for maintenance). Six rats from each group were subjected to transcardiac perfusion, and their cerebral hemispheres were removed and fixed in 4% paraformaldehyde solution. The remaining 6 rats were decapitated, and their hippocampus was removed and stored in liquid nitrogen for further analysis.

2.3. Morris water maze test

All rats were subjected to the Morris water maze test to evaluate spatial learning and memory capacity (Vorhees and Williams, 2006). The water maze is a circular pool with a diameter of 130 cm, a height of 50 cm and a depth of 30 cm. The water maze is divided into four quadrants, denoted as SW, NW, SE and NE. A circular transparent platform with a diameter of 9 cm and a height of 29 cm is placed in the middle of the SE quadrant. The top of the platform was 1 cm below the water surface (Fig. 2). A video camera with a display system was placed above the maze to record the motion trajectory of the rat. The position of the circular transparent platform remained unchanged during training.

The Morris test was conducted in two phases. Phase 1 was the place navigation, a test to measure the learning and memory of rats. The experiment lasted for 5 days. On the first day, rats were placed on the platform for 15 s, so that they were familiar with the environment. The rats were allowed to swim for 2 min. Starting on day 2, the rats were divided into a morning and an afternoon session daily for 3 days. In each session, the rat was trained twice, for 2 min each time. During training, a rat was faced towards the pool wall at one of the 4 quadrant points and was then placed into the water. The rat was then allowed to swim towards the underwater platform from each of the four different quadrants. If the rats could reach the platform and stay for 2 s, it was regarded as a success in finding the hidden platform. The route map of the rats climbing onto the platform was observed, and the escape latency (the time it takes to find the platform) was recorded. Each rat was

trained 4 times from each of the four quadrants. If a rat did not find the platform at the end of 120 s, it would be guided to the platform and placed on the platform for 15 s. Escape latency was recorded as 120 s, and the training interval was 60 s. Phase 2, the spatial probe test, was used to measure the ability of rats to learn the spatial position of the platform after memorizing the position of the platform. After the rat completed the 16th training session, the platform was removed on the 5th day. For the 17th test, the rat was placed in the water at any one of the quadrant points. The number of times that a rat crossed the original platform location (target area) within 120 s was recorded. Milk powder (8%) was added into water to make the water opaque, and the water temperature was controlled at 20–21 °C.

2.4. Measurement of SOD activity and MDA content in the hippocampus

Both the activity of SOD (U/mg protein) and MDA content (nmol/g protein) in the hippocampus of rats was measured with commercial kits. The protein concentration was determined by using a BCA protein assay kit (Biagini et al., 1995).

2.5. Hoechst 33258 measurement of the apoptosis of hippocampal neurons

The apoptosis of hippocampal neurons was measured with the method described by Huang et al. (2016). Briefly, the hippocampus was fixed with 4% paraformaldehyde and was dehydrated, embedded in paraffin and was then sectioned (5 µm) and baked (56 °C, 24 h). The sections were deparaffinized with xylene and an alcohol gradient, incubated in citrate buffer (*pH* = 6.0) for 15 min at 97 °C, and then washed twice with PBS, for 3 min each. Then, the slides were incubated in a solution of Hoechst 33258 stain for 5 min and washed twice with PBS. Finally, a drop of mounting solution and a clean coverslip was added onto the specimen slide, while avoiding air bubbles. The fluorescence intensity was detected with an excitation wavelength of 350 nm and an emission wavelength of 460 nm. After Hoechst staining, apoptotic nuclei appear condensed and light blue (blue-white). The Ipp6.0 software (USA, Media Cybernetics) was used to assess the number of positive cells and total cells in the same area, and the number of positive cells/total cells × 100% was used to calculate the rate of apoptosis.

2.6. TUNEL assay to assess the apoptosis of hippocampal neurons

The paraffin sections of hippocampal tissues were deparaffinized with xylene and an alcohol gradient and then hydrated with citrate buffer (*pH* = 6.0) for 8 min at 97 °C, followed by washing twice with PBS for 3 min each. Proteinase K was added and incubated for 20 min at room temperature, followed by washing five times with PBS for 3 min each. A drop of 3% H₂O₂ in methanol was added and incubated for 15 min at room temperature. Then, TUNEL solution was added and incubated at 37 °C for 70 min in the dark, followed by washing three times with PBS for 3 min each. The nuclei were counterstained with DAPI solution and incubated for 5 min at room temperature, followed by washing three times with PBS for 3 min each. Then, a drop of anti-fluorescent quencher was applied and covered with a clean cover slip. Slides were scanned using an OLYMPUS laser confocal microscope (400x magnification; DAPI excitation at 358 nm; blue light for TUNEL at 488 nm). The Ipp6.0 software was used to assess the number of positive cells and total cells in the same area, and the number of positive cells/total cells × 100% was used to calculate the rate of apoptosis.

2.7. Western blot assay for the protein expression of Apaf-1, caspase-9, caspase-3, Bax, Bcl-2, Bid and cytochrome c

The hippocampal tissue was removed from liquid nitrogen and placed in a 2 ml Eppendorf tube. Magnetic beads and Western blot cell lysis buffer were added. The tissue was crushed and homogenized with a tissue grinder, allowed to stand at 4 °C for 30 min and then

centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was collected. The protein concentration was measured by a BCA assay, 5 × SDS-PAGE protein loading buffer was added and the samples were placed in a 95 °C water bath for 10 min and then cooled at room temperature. Ten milligrams of protein sample was loaded onto the SDS gel. Proteins were separated by SDS gel electrophoresis. After the separation was completed, the proteins were transferred onto PVDF membranes. Then, the PVDF membrane was blocked at 37 °C in a 5% skim milk powder TBS solution for 1.5 h. Incubation was performed with the primary antibody (β -actin 1:1000, Bax 1:500, Bcl-2 1:500, Bid 1:500, Apaf-1 1:500, caspase-3 1:500, caspase-9 1:200, and cytochrome c 1:500) overnight at 4 °C. On the second day, the membrane was washed 3 times with TBST (5 min/wash). An additional incubation was performed with a secondary antibody (HRP-labelled goat anti-mouse IgG 1:1000 and HRP-labelled goat anti-rabbit IgG 1:1000) for 1 h at room temperature. The membranes were then washed 3 times with TBST (5 min/wash). A drop of ECL were placed on the PVDF membrane, and the protein bands were visualized by using an electrochemiluminescent system.

2.8. Statistical analysis

SPSS 24.0 software was used for the data analysis. The experimental data are expressed as the mean \pm SD. For the analysis of variance test, when the variance was homogenous, One-way analysis of variance test was used; when the variance was not uniform, Welch's method was used for comparison, and Fisher's least significant difference was used for multiple comparison post hoc tests. Differences were considered statistically significant when *, $p < 0.01$ and **, $p < 0.01$.

3. Results

3.1. Racine score and seizure latency

In our study, rats treated with PTZ (40 mg/kg) daily had increased seizure scores compared to those of the controls (Fig. 1A), which subsequently led to systemic clonic seizures. However, when rats were pre-treated with FA (60 mg/kg) 20 min before PTZ injection, the Racine score was significantly reduced, and the latency to seizure was significantly increased, compared to those of the PTZ group ($p < 0.01$).

3.2. Morris water maze test

The results of the behavioural follow-up testing revealed that the escape latency of all groups of rats decreased gradually (Fig. 2A) during the training period. Compared with that in the PTZ group, the escape latency, which is the time to find the platform, in the PTZ + FA group was significantly reduced at days 2, 3 and 4 ($p < 0.01$, Fig. 2A). In the spatial exploration experiment, the frequency of crossing the platform location in the PTZ + FA group was significantly higher than that in the PTZ group ($p < 0.01$, Fig. 2B). These results indicate that FA can

reduce the damage to both spatial cognition and memory in rats subjected to PTZ-induced seizures.

3.3. Superoxide dismutase (SOD) activity and malondialdehyde (MDA) content

Compared with that in the PTZ group, the SOD activity in the PTZ + FA group was significantly increased ($p < 0.01$, Fig. 3A). Moreover, the SOD activity in the PTZ and NS groups were also significantly different ($p < 0.01$, Fig. 3A). Compared with that in the PTZ group, the MDA content in the PTZ + FA group decreased significantly ($p < 0.01$, Fig. 3B). There was also a statistically significant difference between the MDA content in the PTZ group and the NS group ($p < 0.01$, Fig. 3B). This indicated that FA has the ability to reduce oxidative stress in the hippocampal tissue of rats with seizures.

3.4. Apoptosis percentage in hippocampal neurons

As shown in Fig. 4A, all of the cells stained blue with DAPI, and only apoptotic cells stained green in the TUNEL assay. Compared to that in the PTZ group, the apoptosis percentage (calculated by green cells/blue cells \times 100%) in both the dentate gyrus (DG) (Fig. 4B) and CA1 (Fig. 4C) in both the PTZ + FA group and NS group was significantly reduced ($p < 0.05$). The Hoechst 33258 staining results are shown in Fig. 4D. The cells that stained blue are normal cells, while the cells that stained blue-white are apoptotic cells. Compared to that in the PTZ group, the apoptosis percentage in both the DG and CA1 in both the PTZ + FA and NS groups was significantly reduced ($p < 0.05$, Fig. 4E, F). Both of these results demonstrated that FA could prevent the apoptosis of neurons in the hippocampus.

3.5. The expression of Apaf-1, caspase-9, caspase-3, Bcl-2, Bid, Bax, cleaved caspase-3 and cytochrome c

Western blot results showing the expression of Apaf-1, caspase-9, caspase-3, Bcl-2, Bid, Bax, cleaved caspase-3, cytochrome c and Bax/Bcl-2 are shown in Fig. 5A. The ratio of the density of each individual protein band to the density of β -actin was analysed and is presented in Fig. 5B-J. Compared to that in the PTZ group, the protein expression of Apaf-1, caspase-9, caspase-3, Bid, Bax, cleaved caspase-3, cytochrome c and Bax/Bcl-2 in the PTZ + FA group was significantly reduced ($p < 0.05$). Moreover, the expression in the PTZ group and the NS group was a significantly different ($p < 0.05$). Compared to that in the PTZ group, Bcl-2 protein expression in the PTZ+FA group increased significantly ($p < 0.05$), whilst there was also a statistically significant difference between the PTZ and NS groups ($p < 0.05$).

4. Discussion

This study investigated the preventive effect of FA in seizures, especially focusing on apoptosis in the rat hippocampus when the rats

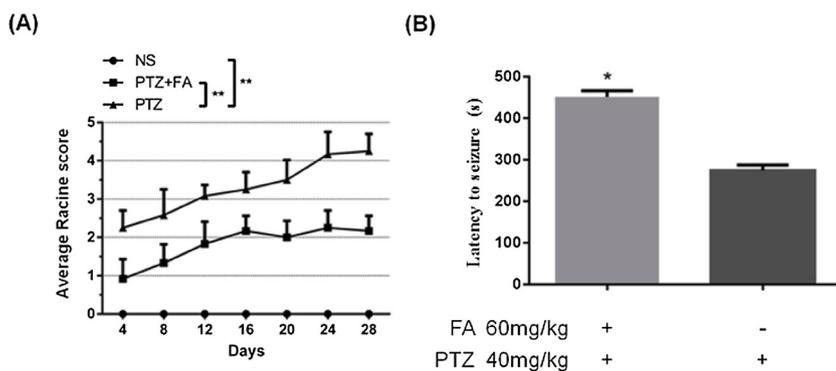


Fig. 1. Racine scores (A) during the experimental period, data are represented as the mean \pm SD, $n = 12$ /group; and latency to seizures (B) at day 28, each column represents the mean \pm SD, $n = 12$ /group, **, $p < 0.01$ compared all data points of among three groups (A) and *, $p < 0.05$ between PTZ + FA group and the PTZ group (B).

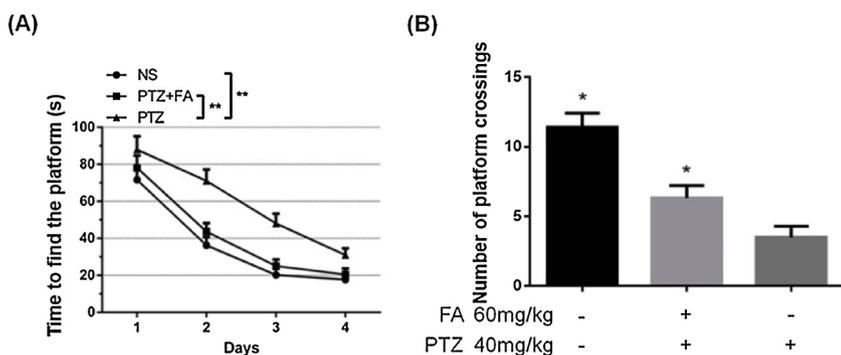


Fig. 2. The escape latency (the time to find the platform, in seconds) (A) data of the rats are represented as the mean \pm SD, $n = 12$ /group; the number of platform crossings were measured (B), and each column represents the mean \pm SD, $n = 12$ /group, *, $p < 0.05$ and **, $p < 0.01$ compared all data points of PZ + FA group to the PTZ group.

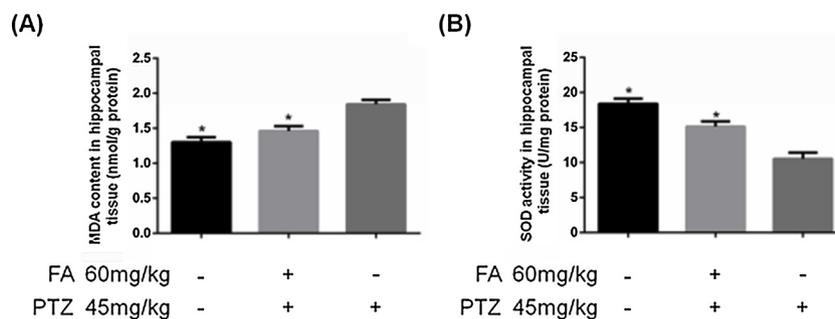


Fig. 3. The SOD activities (A) and MDA content (B) in the hippocampus of the three groups are indicated. Each column represents the mean \pm SD, $n = 6$ /group, *, $p < 0.05$ compared to the PTZ group.

were treated with FA 20 min before being treated with PTZ. The results showed that FA pretreatments (1) significantly reduced the seizure from day 4; (2) significantly increased the seizure latency at day 28; and (3) significantly reduced the escape latency period from day 2 and increased the frequency of crossing the platform location, which suggests a significant improvement of spatial cognition and memory. All of these effects may have occurred via the following mechanisms: (a) FA exhibited strong antioxidant ability via a significant increase in the SOD activity and a decrease in the MDA content in the hippocampus; and (b) FA significantly reduced apoptosis of the hippocampal neurons. Thus, the study demonstrated that FA targeted the intrinsic apoptosis pathway of neurons to prevent neuronal apoptosis in the hippocampus and improved spatial learning and memory in rats with seizures.

FA has been shown to have many pharmacological properties, including proliferative, anti-inflammatory, antioxidative and neuroprotective activities in neuronal progenitor cells (Yabe et al., 2010; Kim et al., 2015). FA was also well tolerated in the treatment of cardiovascular and cerebrovascular diseases (Sang et al., 2017; Alam et al., 2013). Recently, studies have demonstrated the anti-inflammatory and antioxidative effects of FA in epilepsy (Deepa et al., 2012), as well as its antiepileptic effect and its ability to improve cognition but not locomotor activity (Hassanzadeh et al., 2017). PTZ is a central nervous system stimulant that has an effect on the entire cerebrospinal axis. PTZ can trigger whole-body tonic-clonic seizures; thus, PTZ has often been used as a model for studying seizures (Dhir, 2012; Atinga et al., 2015). This study further confirmed that the repeated use of PTZ at a convulsive dosage caused seizures based on the behavioural assessment (Racine, 1972), but pretreatments with FA reduced the seizures' severity. Under normal physiological conditions, the endogenous antioxidative system in the rat hippocampus can scavenge the normal production of free radicals (Atinga et al., 2015). In the pathological state of PTZ-induced seizures, the overproduction of free radicals disturbs and destroys the antioxidative system and causes oxidative damage (Postnikova et al., 2019). By comparing the results from the PTZ + FA group and the PTZ group, it was demonstrated that FA reduced the level of seizures and prolonged the latency to first seizure in a PTZ-induced seizure model. These findings are consistent with a

previous study¹⁷. Mitochondria are the major source of their oxidative stress, and ROS accumulation is associated with many diseases, including neurological diseases. The SOD activity and MDA content are commonly used to assess oxidative stress. Our results showed that FA increased the level of SOD and decreased the level of MDA in the hippocampus, further confirming the results of Hassanzadeh et al. (2017) and support the idea of FA protecting neuron from free-radical damage.

The Morris water maze test is considered a standard experiment for studying spatial learning and memory. In both the behavioural tracking test and spatial exploration test, rats with seizures showed severely impaired spatial learning and memory capacity, which is consistent with the findings of Frisch (Frisch et al., 2007). The comparison of the results from the PTZ + FA and PTZ groups confirm the results of Hassanzadeh et al. (2017) that FA can significantly improve the spatial learning and memory capacity of rats. These effects might also have been caused by the antioxidant effect of FA. As oxidative stress injury caused by ROS is a key factor leading to a memory disorder (Chong et al., 2005), the increased SOD activities and decreased MDA with FA pre-treatment further demonstrated that FA has strong antioxidative activity and the ability to scavenge free radicals^{6,15}. This finding showed the improvement the learning and memory ability of rats with seizures, which is consistent with the view of de Chaves (De Chaves and Narayanaswami, 2008). However, these results could not necessarily be interpreted to indicate that FA may promote the regeneration of neurons by eliminating lipid oxidation and oxygen free radicals, to verify this, more study is required. MDA is a marker of lipid peroxidation. When the MDA content is higher, the degree of oxidative stress is more severe; anti-oxidative enzymes, which are responsible for eliminating free radicals such as superoxide and hydrogen peroxide, play a defensive role against oxidation. SOD, an antioxidative enzyme, has a strong antioxidative ability (Camkurt et al., 2016; Su et al., 2014). The results of this study that showed that there was increased MDA content and reduced SOD activity in PTZ-induced rats are also consistent with the results of Fartseva and Xiang (Frantseva et al., 2000; Xiang and Jiang, 2014). However, when comparing MDA content and SOD activity among the groups, the results showed that pre-treatment with FA reduced MDA generation and improved SOD activities compared to those

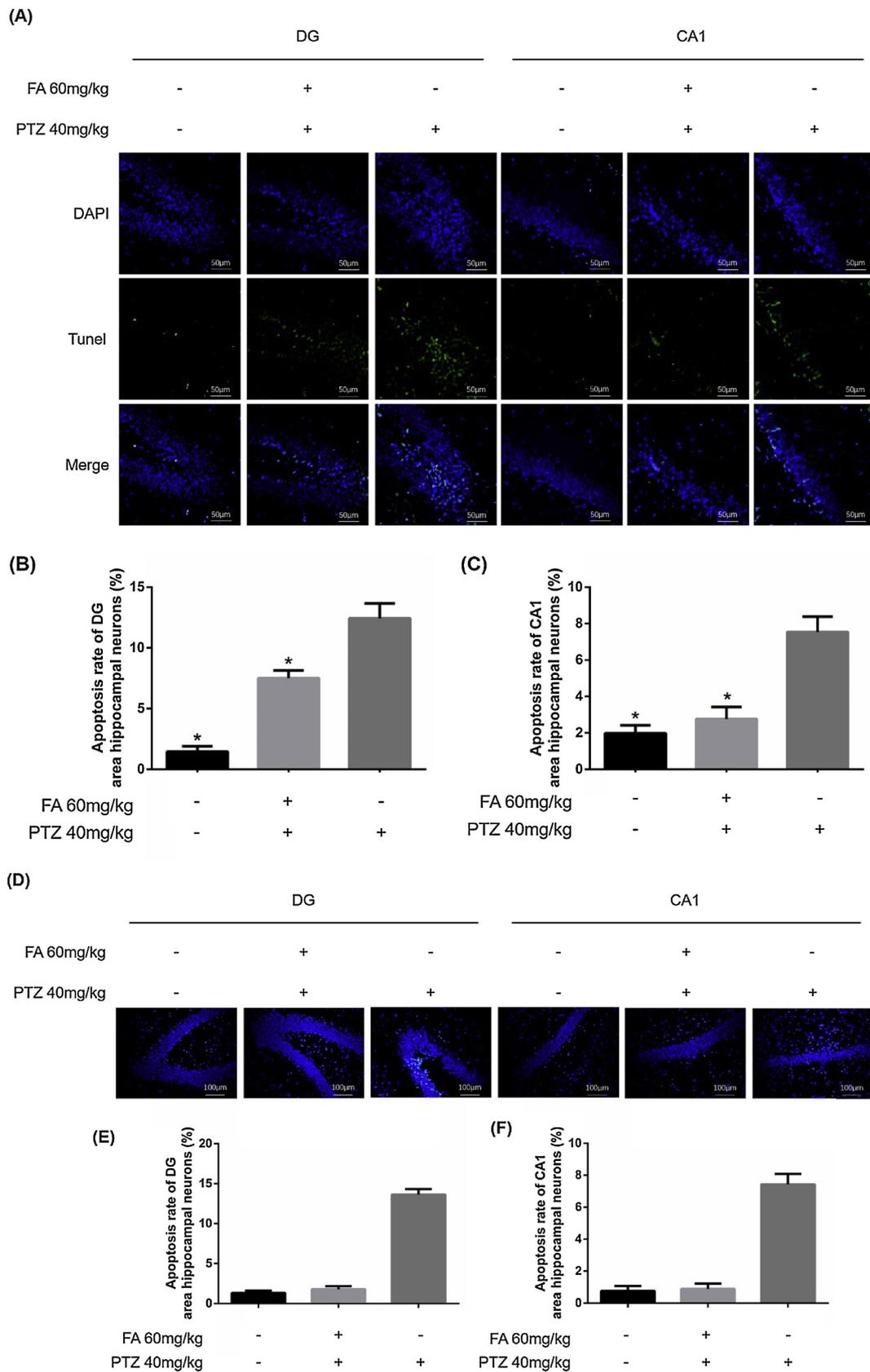


Fig. 4. Apoptosis in the hippocampus of rats with seizures. Effect of FA on the levels of the apoptosis of the neurons in the hippocampus of rats with seizures based on the TUNEL assay (A). The apoptosis percentage of the neurons in the DG area (B) and CA1 area (C) of the hippocampus. The apoptosis in the neurons in the hippocampus of rats with seizures based on Hoechst 33258 (D). The apoptosis percentage of neurons in the DG area (E) and in the CA1 area (F) of the hippocampus. Each column represents the mean \pm SD, n = 6/group; **, p < 0.01 compared to the PTZ group).

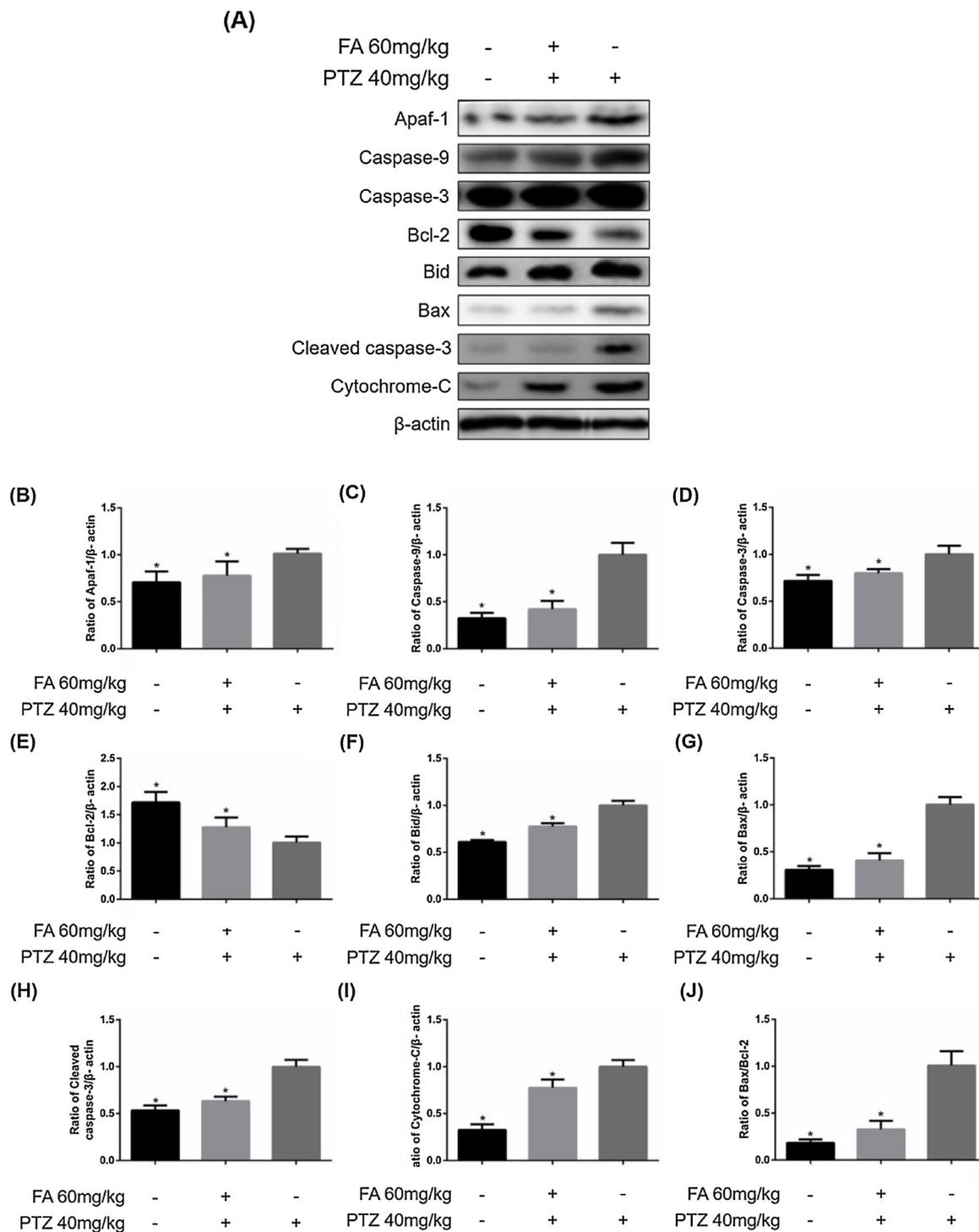


Fig. 5. Western blot results showing the effect of FA on Apaf-1, caspase-9, caspase-3, Bcl-2, Bid, Bax, cleaved caspase-3 and cytochrome C expression in the hippocampus of rats with seizures. Western blot results show the expression of all of the measured proteins (A). The bar graph (B–J) showing the ratio of the density of the individual proteins to the density of beta-actin, including Apaf-1, caspase-9, caspase-3, Bcl-2, Bid, Bax, cleaved caspase-3, cytochrome c and Bax/Bcl-2. In the bar graph, each column represents the mean \pm SD, n = 6/group, *, p < 0.05 compared to the PTZ group).

of the controls; thus, it is possible neurons could have been protected by scavenging lipid oxidation and oxygen free radicals. This effect led to the improved learning and memory ability after damage by PTZ in rats with seizures, which is consistent with other studies (De Chaves and Narayanaswami, 2008; Frantseva et al., 2000; Xiang and Jiang, 2014).

Increased ROS can directly or indirectly damage the mitochondrial membrane, resulting in a decrease in the mitochondrial membrane potential (Carmody and Cotter, 2000). This can lead to an increase in

membrane permeability, in turn causing MOMP (Fernandezgomez et al., 2005), a "key event" in the process of intrinsic apoptosis. In intrinsic apoptosis, MOMP promotes the release of cytochrome c, a pro-apoptotic factor, eventually leading to cell death due to the activation of caspases (Galluzzi et al., 2012a, 2012b). However, the formation of MOMP requires the activation of Bax and other proteins in the Bcl-2 family (Tait and Green, 2010), such as Bcl-2, which is an anti-apoptotic protein. Bcl-2 not only antagonizes activated Bax but also antagonizes

pro-apoptotic BH3-only proteins, e.g., BID, to prevent apoptosis (Czabotar et al., 2014). Bid binds to Bax in the binding groove of the BH3 domain in Bax (Moldoveanu et al., 2013; Czabotar et al., 2013) and Bid also activates apoptosis by neutralizing the Bcl-2 protein (Shamas-Din et al., 2013). This study clearly showed that PTZ-induced increased Bax expression and decreased Bcl-2 expression, while FA pre-treatment prevented these effects and decreased the ratio of Bax/Bcl-2 expression, based on TUNEL methods (Fig. 4A) and the Hoechst 33258 method (Galluzzi et al., 2012a) (Fig. 4D); combined results of FA pre-treatment reduce the release of cytochrome c (Fig. 5I), and reduce caspase-induced apoptosis in both the DG (Fig. 4E) and CA1 (Fig. 4F) areas of the hippocampus, further indicate anti-apoptosis effect of FA pre-treatment. MOMP releases cytochrome c from the mitochondrial membrane compartment into the cytoplasm, allowing cytochrome c to bind to Apaf-1 (Liu et al., 1996; Li et al., 1997). Subsequently, dATP/ATP is substituted in Apaf-1 with ADP to form apoptotic bodies (Kim et al., 2005; Bao et al., 2007). Finally, apoptotic Apaf-1 catalyses the self-activation of procaspase-9 and forms active caspase-9, which in turn activates caspase-3 (Anuradha et al., 2001). PTZ treatment increased Apaf-1, procaspase-9, caspase-3 and cleaved caspase-3 expression (Fig. 5) compared to that of the controls. Activated caspases can further lead to the rupture of mitochondrial membranes, causing mitochondria to release other caspases and activating factors, leading to apoptosis.

The hypoxia and cell damage have been detected in both experimentally rodent and human epileptogenic tissues (Gualtieri et al., 2013). Oxygen availability was reduced and cell damage marker, e.g. cleaved caspase-3, in the nucleus was examined during the disease process. The results of the Hoechst apoptotic staining as well as the TUNEL results indicated that PTZ caused apoptosis of hippocampal neurons, but the PTZ-induced apoptosis could be reduced by pre-treatment with FA (Fig. 4). The results of the Western blot assay showed that the apoptosis of hippocampal neurons in rats with seizures might occur through the intrinsic apoptotic pathway. However, in this study, the comparison of the both Hoechst/TUNEL apoptotic staining and the Western blot protein expression results between the PTZ + FA group and PTZ group suggested that FA pre-treatment prevented PTZ-induced hippocampal neuronal damage via the inhibition of intrinsic apoptosis. During PTZ-induced, the reduction of the SOD activity in this study and of glutathione in another study (Hassanzadeh et al., 2017) led to lipid peroxidation that enhanced oxidative stress, e.g., increased the MDA content in both studies; however, this effect could be prevented by FA pre-treatment. FA was clearly demonstrated to be an effective antioxidant and a free radical scavenger. The results indicate that FA pre-treatment can inhibit intrinsic apoptosis by increasing antioxidant activity, reducing the apoptosis of hippocampal neurons and further improving neuron function by reducing cerebral neuropathological damage in rats with seizures. These findings suggest that interneurons are continuously endangered in epileptogenic condition, FA has showed the anti-apoptosis effect, it is worth to study whether its anti-seizure effect also caused by improvement the hypoxia condition.

In summary, this study showed that pre-treatment with FA in PTZ-induced seizures can (1) exert a strong neuroprotective effect and (2) cause strong antioxidation and anti-apoptotic effects. The neuroprotective effect was shown with a reduction in the seizure severity and an improvement of the spatial learning ability and memory capacity.

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Declaration of Competing Interest

This project was conceived by J. Z and S.W. The in vivo experiments in this paper were performed by S.H. Z and DL, and the data were analysed and interpreted by Q.H., YZ., and S.Z. The manuscript was prepared by J. Z, S.Z., E.A. and S.H.Z. All authors have read and approved the final copy. The authors report no conflicts of interest in this work.

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