1 **Ferulic acid ameliorates pentylenetetrazol-induced seizures behaviour by reducing neuron cell**
2 **death**
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Abstract: To investigate the neuroprotective effect of ferulic acid (FA) in a pentylenetetrazol (PTZ)-induced seizures rat model, the seizures behaviour, 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 seizures behaviour 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 bisbenzimide 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 rate, 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 seizures behaviour 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.

Keywords: Ferulic acid; Epilepsy; Spatial learning; Memory capacity; Apoptosis; Oxidative stress

38 **1. Introduction**

39 Epilepsy is a chronic recurrent brain dysfunctional syndrome with multiple causes. Epilepsy is
40 characterized by the abnormal discharge of brain neurons and has been listed by the WHO as one of the
41 five major neuropsychiatric diseases that need to be prevented and controlled. Epilepsy seriously affects
42 both health and the quality of life. At present, the pathogenesis of epilepsy has not yet been fully
43 elucidated. Epilepsy has been linked to neuron cell death, dysfunction, neuronal degeneration, and
44 neuroinflammation, among other effects (Mc Namara et al., 2006). One of the causes of epilepsy is
45 oxidative damage to neurons. Currently, among commonly used drugs for the control of epileptic seizures
46 include carbamazepine phenobarbital, phenytoin sodium, and sodium valproate, but approximately one-
47 third of epileptic patients still cannot control their disease using these treatments (Kwan and Brodie,
48 2000). Despite the introduction of a new generation of antiepileptic drugs, drug resistance is still one of
49 the biggest challenges in the treatment of epilepsy (Tang et al., 2017). Therefore, there is an urgent need
50 to find new therapeutic targets and to develop new antiepileptic drugs.

51 Ferulic acid (4-hydroxy-3-methoxycinnamic acid, FA) is one of the derivatives of cinnamic acid.
52 FA belongs to the hydroxycinnamic acid family and is extracted from *Ferula asafoetida*, where it is
53 highly abundant in the leaves and seeds and its dry weight can reach approximately 2 g/kg in its richest
54 granular form (Sosulski et al., 1982; Lempereur et al, 1997). FA also exists in grains such as brown rice,
55 whole wheat and oats (Lempereur et al, 1997). FA is widely used as a medicinal material, and as a natural
56 antioxidant, FA can prevent substrates such as lipids, proteins and DNA from being oxidated (Rice-
57 Evans et al., 1996; Kroon and Williamson, 1999). In recent years, FA has been used in the prevention of
58 cardiovascular, cerebrovascular and neurological diseases (Salazar-López et al., 2017; Hong et al., 2016;
59 Barone et al., 2009; Sgarbossa et al., 2015). FA shows neuroprotective effects against
60 ischaemia/reperfusion-induced brain injury and attenuates memory impairment in rats (Ren et al., 2017),
61 as well as protecting against rotenone-induced degenerative changes in a rat model of Parkinson's disease
62 (Ojha et al, 2015). FA has shown to have a strong cytoprotective ability by scavenging free radicals
63 (Ogiwara et al., 2002), activating cellular anti-oxidative stress responses and acting as an anti-
64 inflammatory in hypertensive rats (Alam et al., 2013; Mancuso and Santangelo, 2014; Hassanzadeh et
65 al, 2017). FA improves cognitive function (Hassanzadeh et al, 2017; Deepa et al., 2012) in response to
66 pentylenetetrazol (PTZ), a γ-aminobutyric acid type A (GABA(A)) receptor blocker that induces
67 seizure. Its ester, isopentyl ferulate, showed the anti-seizures effect in mice induced by both pilocarpine
68 and pentylenetetrazole, but can be blocked by flumazenil, which suggested the effect may be through the
69 benzodiazepine-binding site of the GABAA (Machado et al., 2015). However, there is no significantly
70 affect locomotor activity and motor coordination. Pre and post treatment with FA improve seizures
71 behavioural in rats induced by pentylenetetrazole (PTZ), attenuate of oxidative stress, e.g. reduced the
72 MDA levels and increased GSH levels, and upregulate of neuroprotective heat shock protein 70,
73 connexin 43, and monoamines in the hippocampus (Hussein et al., 2017). FA also ameliorated comorbid
74 depression caused by neuroinflammation by restoring circulating corticosterone levels, decreased
75 proinflammatory cytokines (IL-1β, TNF-α) and indoleamine 2,3-dioxygenase activity in mice brain
76 (Singh et al., 2017). FA improves odour-taste reward associative memory scores in larval Drosophila and
77 prevents the age-related decline of this appetitive memory in adult flies. FA increases excitability in

78 mouse hippocampal CA1 neurons, which leads to more stable context-shock aversive associative
79 memory in 3-month-old mice and increases memory scores in >2-year-old mice (Michels et al., 2018).
80 Seizures increased the risk of memory loss, e.g., the IQ score in children with seizures is lower than that
81 in the normal population (Rayner et al., 2016), or of having learning, cognitive and behavioural problems
82 (Bell, et al., 2011). However, there are no reports about the effect of FA on these abilities thus far. The
83 Morris water maze test is often used to assess spatially related forms of learning and memory in rodents
84 (Vorhees and Williams, 2015). Therefore, the Morris water maze test was used to assess FA pre-
85 treatment on these abilities in a PTZ-induced seizure mouse model in this study.

86 Oxidative stress in neurons is generated by the persistent imbalance between the generation of toxic
87 reactive oxygen species (ROS) and the antioxidative defences of aerobic metabolism (Shin et al., 2011).
88 The massive production of ROS can lead to the significant destruction of cell structure and function,
89 which leads to the progression of epileptic seizures (Azam et al., 2010; Sun et al., 2017). Therefore, ROS
90 reduction can improve mitochondrial function in the hippocampus (Simeone et al., 2014). Mitochondria
91 are involved in both ROS production and neuron apoptosis (Balaban et al., 2005). Approximately 90%
92 of ROS are generated by the mitochondria (Yang et al., 1997). Oxidative stress can trigger apoptosis
93 through the mitochondrial pathway. Increasing the ratio of Bax/Bcl-2 expression in the mitochondrion
94 leads to a decrease in mitochondrial membrane potential, the release of cytochrome c, and the activation
95 of caspase-induced apoptosis (Galluzzi et al., 2012a). Apoptosis can be divided into either the intrinsic
96 apoptotic pathway or the receptor-mediated extrinsic pathway. The intrinsic apoptotic pathway is caused
97 by internal stimuli such as DNA injury, oxidative stress, hypoxia, cytoplasmic Ca^{2+} overload and
98 endoplasmic reticulum stress. These signals trigger a critical event called mitochondrial outer membrane
99 permeabilization (MOMP), which promotes the release of apoptotic cytochrome c into the cytoplasm
100 and eventually leads to cell death due to caspase activation (Galluzzi et al., 2012b). The interaction
101 between Bcl-2 family proteins, indicated by the Bcl-2 homology 3 (BH3) region, regulates MOMP and
102 thereby converts cellular stress into apoptosis (Kale et al., 2018; Kalkavan and Green, 2018). Even
103 though a study showed the antioxidant and antiepileptic effect of FA pre-treatment in rats with PTZ-
104 induced seizure (Hassanzadeh et al., 2017), there have been no studies on its effect on the apoptosis
105 pathway. Thus, the oxidation and the changes in the apoptotic indexes, together with the expression of
106 Apaf-1, caspase-9, caspase-3, Bid, and cleaved caspase-3 were thoroughly investigated here. This study
107 focuses on the hippocampus because 1 day after PTZ injection, the hippocampal neurons became more
108 excitable (Postnikova et al., 2019), and 4 days after PTZ injection, rats' electroencephalography was
109 affected, γ-Aminobutyric acid B receptor (GABABR) expression was decreased and neuronal apoptosis
110 was induced in the hippocampal part of the brain (Naseer et al., 2013). Furthermore, prolonged and
111 repeated seizures affected the viability and morphological changes of neurons in the hippocampus up to
112 1 week after PTZ treatment (Vasilev et al., 2018). The number of cells with structural and functional
113 abnormalities in the hippocampus after PTZ-induced seizures decreased in the following order:
114 CA1 > CA3b, c >hilus > dentate gyrus (DG) (Vasilev et al., 2018).

115 In this study, rats were given an FA pre-treatment 20 min before PTZ injection to investigate the
116 neuroprotective effects of FA by assessing their seizures behaviour, spatial learning ability and memory

117 capacity; the potential mechanisms underline above changes were further explored by analysis of FA's
118 anti-oxidation and anti-apoptosis pathways.
119

120 **2. Methods**

121 2.1. Chemicals and reagents

122 The reagents and suppliers included the following: PTZ and isoflurane (Sigma, USA); FA
123 (Shanghai Yuanye Biotechnology Co., Ltd. China); total SOD kit, Hoechst 33258 staining kit and BCA
124 protein assay kit (Shanghai Beyotime Biotechnology Co. Ltd.China); Bid antibody, Bax antibody, Bcl-
125 2 antibody, Apaf-1 antibody, caspase-3 antibody and caspase-9 antibody (Affinity, USA); MDA kit
126 (Nanjing Institute of Biotechnology China); β-actin, goat anti-mouse IgG, goat anti-rabbit IgG (Beijing
127 Zhongshan Golden Bridge Biotechnology Co. Ltd., China); and TUNEL cell apoptosis detection kit
128 (Shenyang Wanlei Institute of Biotechnology, China)

129 2.2. Animal groups and treatments

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

136 Rats were randomly divided into 3 groups (12 in each group): (1) normal saline (NS group), treated
137 intraperitoneally (*i.p.*) with saline alone; (2) PTZ (seizures) group, treated with PTZ (40 mg/kg, *i.p.,*
138 dissolved at a concentration of 0.36 mol/L) to induce seizures; (3) FA treatment (PTZ+FA) group, before
139 the injection PTZ, mice were treated with FA (*i.p.,* 60 mg/kg, dissolved at a concentration of 0.31 mol/L),
140 and 20 min later the rats were treated with PTZ (40 mg/kg, *i.p.*). Drug treatments were administered once
141 daily for 28 days. The dose of FA (60 mg/kg) was based on the following studies (Mancuso et al., 2014;
142 Hassanzadeh et al., 2017; Zhang et al., 2018)with a modification. There was no effect of pre-treatment
143 with ferulic acid (50 mg/kg), but there were significant effects at doses of 75 mg/kg and 100 mg/kg, and
144 there were no differences between these two doses (Mancuso et al., 2014; Hassanzadeh et al., 2017).
145 Fifty mg/kg FA caused the attenuation of inflammation in lipopolysaccharide-induced rat acute
146 respiratory distress syndrome (Zhang et al., 2018). The time of FA pre-treatment and the treatment
147 duration were based on a previous study (Hassanzadeh et al., 2017), but our treatment period was 10
148 days longer than their 18-day treatment, as they failed to show an improvement of locomotive activities.
149 The dose of PTZ (40 mg/kg, *i.p.*) has been routinely used in many studies (Ogiwara et al 2002; Alam et
150 al., 2013; Zhang et al., 2018).

151 The seizure intensity was scored based on the Racine scale (Racine, 1972) (Table 1). The behaviour
152 was recorded every 4 days, and the seizure latency analysis was based on data recorded at day 28. On
153 day 29, 24 h after the final drug administration, all rats underwent Morris water maze tests to assess
154 learning and memory capacity for 5 days. On day 35, rats were anaesthetized with inhaled isoflurane
155 (5% for induction and 2% for maintenance). Six rats from each group were subjected to transcardiac

156 perfusion, and their cerebral hemispheres were removed and fixed in 4% paraformaldehyde solution. The
157 remaining 6 rats were decapitated, and their hippocampus was removed and stored in liquid nitrogen for
158 further analysis.

159

160

161 **Table 1.** Racine scale.

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

162

163 2.3. Morris water maze test

164 All rats were subjected to the Morris water maze test to evaluate spatial learning and memory
165 capacity (Vorhees et al., 2006). The water maze is a circular pool with a diameter of 130 cm, a height of
166 50 cm and a depth of 30 cm. The water maze is divided into four quadrants, denoted as SW, NW, SE and
167 NE. A circular transparent platform with a diameter of 9 cm and a height of 29 cm is placed in the middle
168 of the SE quadrant. The top of the platform was 1 cm below the water surface (Fig 2). A video camera
169 with a display system was placed above the maze to record the motion trajectory of the rat. The position
170 of the circular transparent platform remained unchanged during training.

171 The Morris test was conducted in two phases. Phase 1 was the place navigation, a test to measure
172 the learning and memory of rats. The experiment lasted for 5 days. On the first day, rats were placed on
173 the platform for 15 seconds, so that they were familiar with the environment. The rats were allowed to
174 swim for 2 min. Starting on day 2, the rats were divided into a morning and an afternoon session daily
175 for 3 days. In each session, the rat was trained twice, for 2 min each time. During training, a rat was faced
176 towards the pool wall at one of the 4 quadrant points and was then placed into the water. The rat was
177 then allowed to swim towards the underwater platform from each of the four different quadrants. If the
178 rats could reach the platform and stay for 2 seconds, it was regarded as a success in finding the hidden
179 platform. The route map of the rats climbing onto the platform was observed, and the escape latency (the
180 time it takes to find the platform) was recorded. Each rat was trained 4 times from each of the four
181 quadrants. If a rat did not find the platform at the end of 120 seconds, it would be guided to the platform
182 and placed on the platform for 15 seconds. Escape latency was recorded as 120 seconds, and the training
183 interval was 60 seconds. Phase 2, the spatial probe test, was used to measure the ability of rats to learn
184 the spatial position of the platform after memorizing the position of the platform. After the rat completed
185 the 16th training session, the platform was removed on the 5th day. For the 17th test, the rat was placed
186 in the water at any one of the quadrant points. The number of times that a rat crossed the original platform

187 location (target area) within 120 seconds was recorded. Milk powder (8%) was added into water to make
188 the water opaque, and the water temperature was controlled at 20-21°C.
189

190 2.4. Measurement of SOD activity and MDA content in the hippocampus

191 Both the activity of SOD (U/mg protein) and MDA content (nmol/g protein) in the hippocampus of
192 rats was measured with commercial kits. The protein concentration was determined by using a BCA
193 protein assay kit (Biagini et al., 1995).
194

195 2.5. Hoechst 33258 measurement of the apoptosis of hippocampal neurons

196 The apoptosis of hippocampal neurons was measured with the method described by Huang et al.
197 (2016). Briefly, the hippocampus was fixed with 4% paraformaldehyde and was dehydrated, embedded
198 in paraffin and was then sectioned (5 μm) and baked (56°C, 24 h). The sections were deparaffinized with
199 xylene and an alcohol gradient, incubated in citrate buffer ($pH = 6.0$) for 15 min at 97°C, and then washed
200 twice with PBS, for 3 min each. Then, the slides were incubated in a solution of Hoechst 33258 stain for
201 5 min and washed twice with PBS. Finally, a drop of mounting solution and a clean coverslip was added
202 onto the specimen slide, while avoiding air bubbles. The fluorescence intensity was detected with an
203 excitation wavelength of 350 nm and an emission wavelength of 460 nm. After Hoechst staining,
204 apoptotic nuclei appear condensed and light blue (blue-white). The Ipp6.0 software was used to assess
205 the number of positive cells and total cells in the same area, and the number of positive cells/total
206 cells×100% was used to calculate the rate of apoptosis.

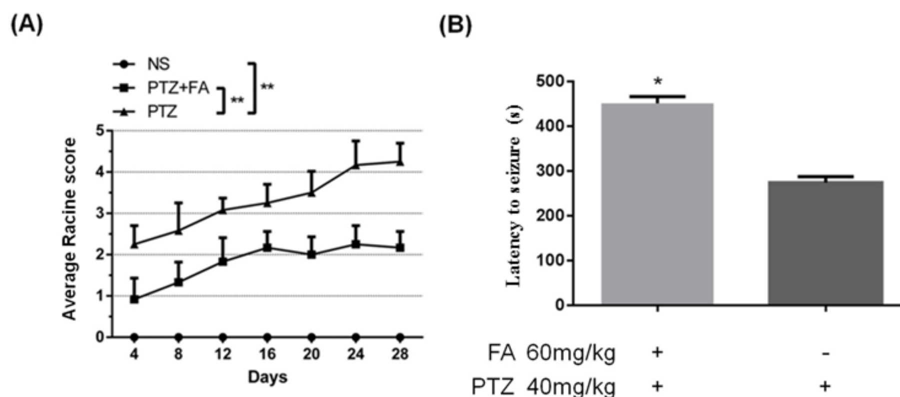
207 2.6. TUNEL assay to assess the apoptosis of hippocampal neurons

208 The paraffin sections of hippocampal tissues were deparaffinized with xylene and an alcohol
209 gradient and then hydrated with citrate buffer (pH=6.0) for 8 min at 97°C, followed by washing twice
210 with PBS for 3 min each. Proteinase K was added and incubated for 20 min at room temperature,
211 followed by washing five times with PBS for 3 min each. A drop of 3% H_2O_2 in methanol was added
212 and incubated for 15 min at room temperature. Then, TUNEL solution was added and incubated at 37°C
213 for 70 min in the dark, followed by washing three times with PBS for 3 min each. The nuclei were
214 counterstained with DAPI solution and incubated for 5 min at room temperature, followed by washing
215 three times with PBS for 3 min each. Then, a drop of anti-fluorescent quencher was applied and covered
216 with a clean cover slip. Slides were scanned using an OLYMPUS laser confocal microscope (400x
217 magnification; DAPI excitation at 358 nm; blue light for TUNEL at 488 nm). The Ipp6.0 software was
218 used to assess the number of positive cells and total cells in the same area, and the number of positive
219 cells/total cells×100% was used to calculate the rate of apoptosis.

220 2.7. Western blot assay for the protein expression of Apaf-1, caspase-9, caspase-3, Bax, Bcl-2, Bid
221 and cytochrome c

222 The hippocampal tissue was removed from liquid nitrogen and placed in a 2 ml Eppendorf tube.
223 Magnetic beads and Western blot cell lysis buffer were added. The tissue was crushed and homogenized
224 with a tissue grinder, allowed to stand at 4°C for 30 min and then centrifuged at 12,000 rpm for 10 min
225 at 4°C. The supernatant was collected. The protein concentration was measured by a BCA assay, 5×

226 SDS-PAGE protein loading buffer was added and the samples were placed in a 95°C water bath for 10
227 min and then cooled at room temperature. Ten milligrams of protein sample was loaded onto the SDS
228 gel. Proteins were separated by SDS gel electrophoresis. After the separation was completed, the proteins
229 were transferred onto PVDF membranes. Then, the PVDF membrane was blocked at 37°C in a 5% skim
230 milk powder TBS solution for 1.5 h. Incubation was performed with the primary antibody (β-actin
231 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
232 cytochrome c 1:500) overnight at 4°C. On the second day, the membrane was washed 3 times with TBST
233 (5 min/wash). An additional incubation was performed with a secondary antibody (HRP-labelled goat
234 anti-mouse IgG 1:1000 and HRP-labelled goat anti-rabbit IgG 1:1000) for 1 h at room temperature. The
235 membranes were then washed 3 times with TBST (5 min/wash). A drop of ECL were placed on the
236 PVDF membrane, and the protein bands were visualized by using an electrochemiluminescent system.
237
238 2.8. Statistical analysis
239 SPSS 24.0 software was used for the data analysis. The experimental data are expressed as the mean
240 ± SD. For the variance homogeneity test, when the variance was homogenous, a one-factor variance
241 homogeneity test was used; when the variance was not uniform, Welch's method was used for
242 comparison, and Fisher's least significant difference was used for multiple comparison post hoc tests.
243 Differences were considered statistically significant when $p < 0.01$.
244
245 **3. Results**
246 3.1. Racine score and seizure latency
247 In our study, rats treated with PTZ (40 mg/kg) daily had increased seizure scores compared to those of
248 the controls (Fig. 1A), which subsequently led to systemic clonic seizures. However, when rats were pre-
249 treated with FA (60 mg/kg) 20 min before PTZ injection, the Racine score was significantly reduced,
250 and the latency to seizure was significantly increased, compared to those of the PTZ group ($p < 0.01$).



251
252 **Figure 1.** Racine scores (A) during the experimental period, data are represented as the mean
253 ± SD, n=12/group; and latency to seizures (B) at day 28, each column represents the mean ±
254 SD, n=12/group, *, $p < 0.05$ and **, $p < 0.01$ compared all data points of PZ + FA group to
255 the PTZ group.

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 PTZ-induced seizures rats.

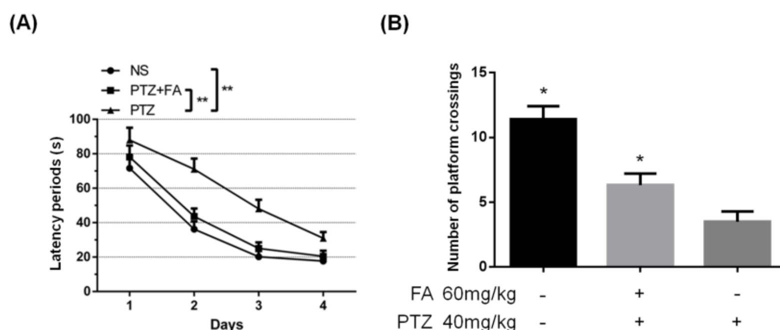


Figure 2. The escape latency (the time to find the platform, in seconds) (A) data of the rats are represented as the mean ± SD, n=12/group; the number of platform crossings were measured (B), and each column represents the mean ± SD, n=12/group, *, $p < 0.05$ and **, $p < 0.01$ compared all data points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

3.3. Superoxide dismutase (SOD) activity and malondialdeyde (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 seizures rats.

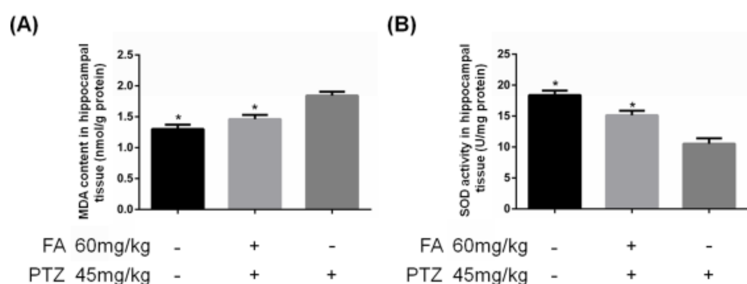
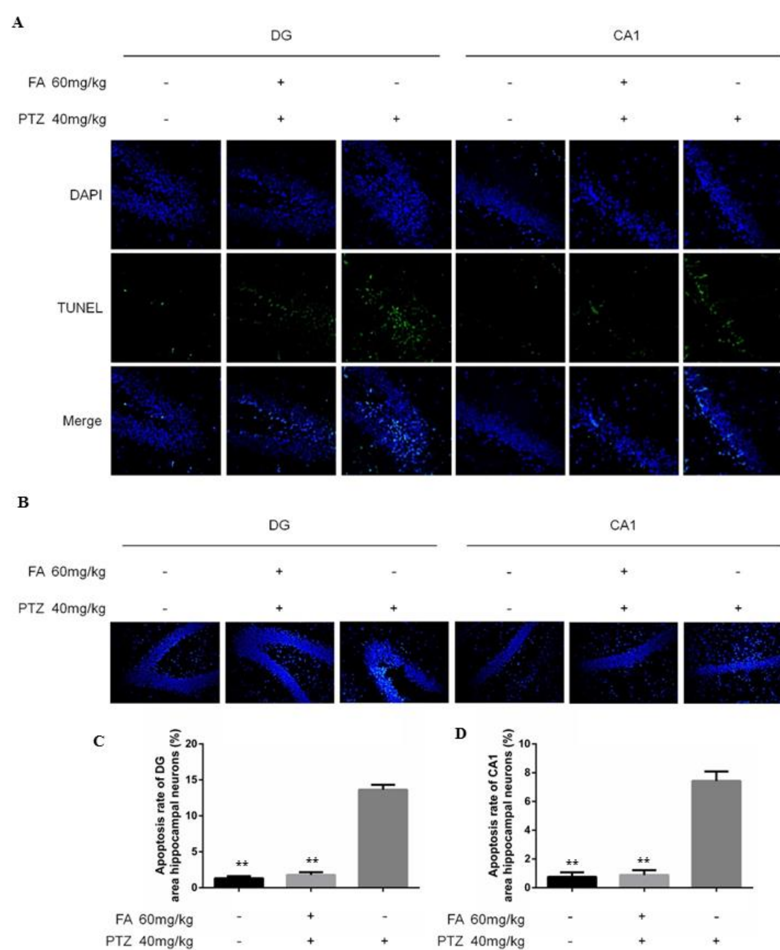


Figure 3. The SOD activities (A) and MDA content (B) in the hippocampus of the three groups are indicated. Each column represents the mean ± SD, n=6/group, *, $p < 0.05$ compared to the PTZ group.

280 3.4. Apoptosis rate in hippocampal neurons

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

289



290

291 **Figure 4.** Apoptosis in the hippocampus of rats with seizures. Effect of FA on the levels of
292 the apoptosis of the neurons in the hippocampus of rats with seizures based on the TUNEL
293 assay (A). The apoptosis rate of the neurons in the DG area and CA1 area of the hippocampus.
294 The apoptosis in the neurons in the hippocampus of rats with seizures based on Hoechst 33258
295 (B). The apoptosis rate of neurons in the DG area (C) and in the CA1 area (D) of the
296 hippocampus. Each column represents the mean ± SD, n = 6/group; **, $p < 0.01$ compared to
297 the PTZ group).

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-5J. 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 ($p < 0.05$).

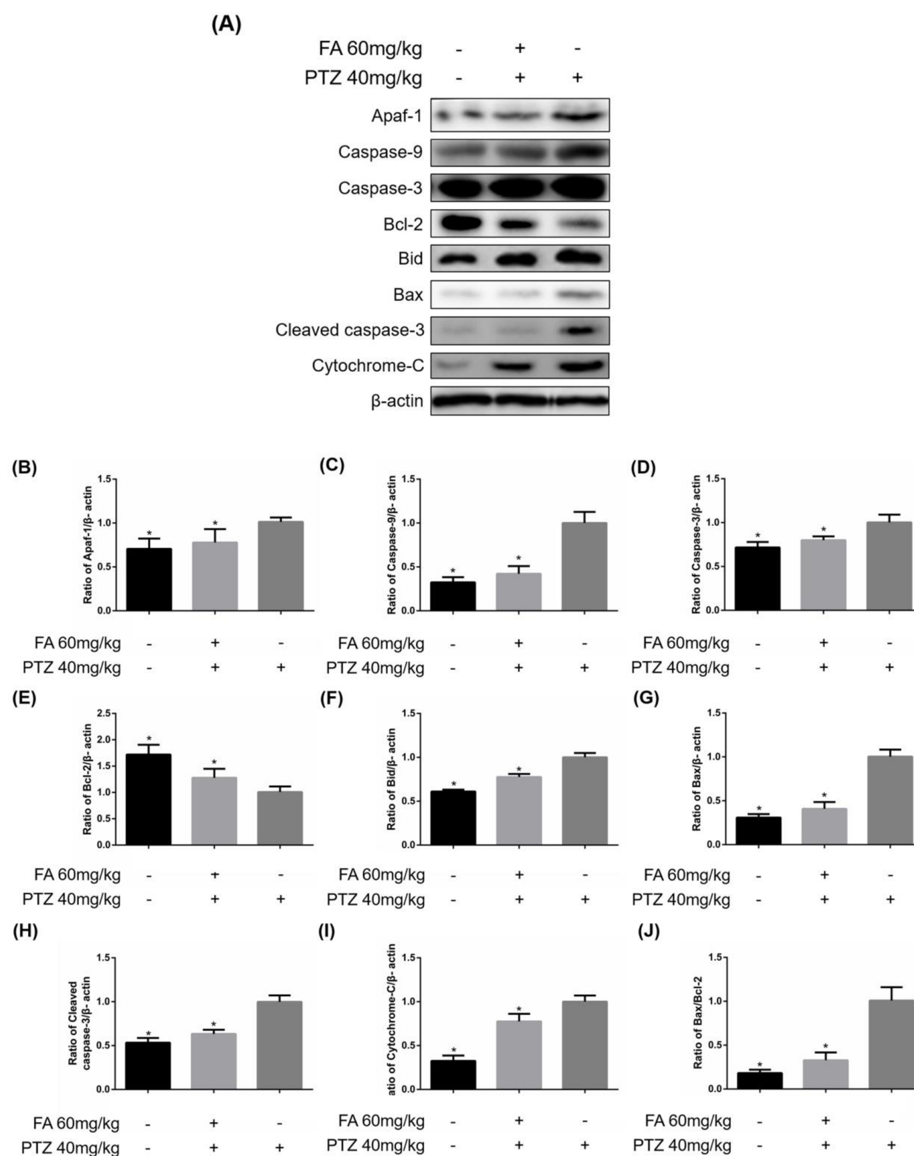


Figure 5. Western blot results showing the effect of FA on Apaf-1, caspase-9, caspase-3, Bcl-2, Bid, Bax, cleaved caspase-3 and cytochrome C expression in the hippocampus of rats with seizures. Western blot results show the expression of all of the measured proteins (A). The bar graph (B-J) showing

313 the ratio of the density of the individual proteins to the density of beta-actin, including Apaf-1, caspase-
314 9, caspase-3, Bcl-2, Bid, Bax, cleaved caspase-3, cytochrome c and Bax/Bcl-2. In the bar graph, each
315 column represents the mean ± SD, n = 6/group, *, $p < 0.05$ compared to the PTZ group).
316

317 ## 4. Discussion

318 This study investigated the preventive effect of FA in seizures, especially focusing on apoptosis in
319 the rat hippocampus when the rats were treated with FA 20 min before being treated with PTZ. The
320 results showed that FA pretreatments (1) significantly reduced the seizures behaviour from day 4; (2)
321 significantly increased the seizures latency at day 28; and (3) significantly reduced the escape latency
322 period from day 2 and increased the frequency of crossing the platform location, which suggests a
323 significant improvement of spatial cognition and memory. All of these effects may have occurred via the
324 following mechanisms: (a) FA exhibited strong antioxidant ability via a significant increase in the SOD
325 activity and a decrease in the MDA content in the hippocampus; and (b) FA significantly reduced
326 apoptosis of the hippocampal neurons. Thus, the study demonstrated that FA targeted the intrinsic
327 apoptosis pathway of neurons to prevent neuronal apoptosis in the hippocampus and improved spatial
328 learning and memory in seizures rats.

329 FA has been shown to have many pharmacological properties, including proliferative, anti-
330 inflammatory, antioxidative and neuroprotective activities in neuronal progenitor cells (Yabe et al., 2010;
331 Kim et al, 2015). FA was also well tolerated in the treatment of cardiovascular and cerebrovascular
332 diseases (sang et al., 2017; Alam et al, 2013). Recently, studies have demonstrated the anti-inflammatory
333 and antioxidative effects of FA in epilepsy (Deepa et al, 2012), as well as its antiepileptic effect and its
334 ability to improve cognition but not locomotor activity (Hassanzadeh et al., 2017). PTZ is a central
335 nervous system stimulant that has an effect on the entire cerebrospinal axis. PTZ can trigger whole-body
336 tonic-clonic seizures; thus, PTZ has often been used as a model for studying seizures (Dhir, 2012; Atinga
337 et al, 2015). This study further confirmed that the repeated use of PTZ at a convulsive dosage caused
338 seizures based on the behavioural assessment (Racine, 1972), but pretreatments with FA reduced the
339 seizures behaviour. Under normal physiological conditions, the endogenous antioxidative system in the
340 rat hippocampus can scavenge the normal production of free radicals (Atinga., et al., 2015). In the
341 pathological state of PTZ-induced seizures, the overproduction of free radicals disturbs and destroys the
342 antioxidative system and causes oxidative damage (Postnikova et al., 2019). By comparing the results
343 from the PTZ+FA group and the PTZ group, it was demonstrated that FA reduced the level of seizures
344 and prolonged the latency of seizures in a PTZ-induced seizure model. These findings are consistent with
345 a previous study[17]. Mitochondria are the major source of their oxidative stress, and ROS accumulation is
346 associated with many diseases, including neurological diseases. The SOD activity and MDA content are
347 commonly used to assess oxidative stress. Our results showed that FA increased the level of SOD and
348 decreased the level of MDA in the hippocampus, further confirm the results of Hassanzadeh et al., (2017)
349 and support the FA neuron protection from free radical damage.

350 The Morris water maze test is considered a standard experiment for studying spatial learning and
351 memory. In both the behavioural tracking test and spatial exploration test, seizures rats showed severely
352 impaired spatial learning and memory capacity, which is consistent with the findings of Frisch (Frisch et

353 al, 2007). The comparison of the results from the PTZ+FA and PTZ groups confirm the results of
354 Hassanzadeh et al., (2017) that FA can significantly improve the spatial learning and memory capacity
355 of rats. These effects might also have been caused by the antioxidant effect of FA. As oxidative stress
356 injury caused by ROS is a key factor leading to a memory disorder (Chong et al., 2005), the increased
357 SOD activities and decreased MDA with FA pre-treatment further demonstrated that FA has strong
358 antioxidative activity and the ability to scavenge free radicals[6,15]. This finding showed the improvement
359 the learning and memory ability of seizures rats, which is consistent with the view of de Chaves (De
360 Chaves et al., 2008), however, they could not indicate that FA may promote the regeneration of neurons
361 by eliminating lipid oxidation and oxygen free radicals, to verify this, more study is required. MDA is a
362 marker of lipid peroxidation. When the MDA content is higher, the degree of oxidative stress is more
363 severe; anti-oxidative enzymes, which are responsible for eliminating free radicals such as superoxide
364 and hydrogen peroxide, play a defensive role against oxidation. SOD, an antioxidative enzyme, has a
365 strong antioxidative ability (Camkurt et al., 2016; su et al., 2014). The results of this study that showed
366 that there was increased MDA content and reduced SOD activity in PTZ-induced rats are also consistent
367 with the results of Fartseva and Xiang (Frantseva et al., 2000; Xiang et al., 2000). However, when
368 comparing the MDA content and SOD activity among the groups, the results showed that pre-treatment
369 with FA reduced the MDA generation and improved the SOD activities compared to those of the controls;
370 thus, it is possible neurons could have been protected by scavenging lipid oxidation and oxygen free
371 radicals. This effect led to the improved learning and memory ability after damage by PTZ in seizures
372 rats, which is consistent with other studies (De Chaves et al., 2008; Frantseva et al., 2000; Xiang et al.,
373 2000).

374 Increased ROS can directly or indirectly damage the mitochondrial membrane, resulting in a
375 decrease in the mitochondrial membrane potential (Carmody and Cotter, 2000). This can lead to an
376 increase in membrane permeability, in turn causing MOMP (Fernandezgomez et al., 2005), a "key event"
377 in the process of intrinsic apoptosis. In intrinsic apoptosis, MOMP promotes the release of cytochrome
378 c, a pro-apoptotic factor, eventually leading to cell death due to the activation of caspases (Galluzzi et
379 al., 2012a, b). However, the formation of MOMP requires the activation of Bax and other proteins in the
380 Bcl-2 family (Tait and Green, 2010), such as Bcl-2, which is an anti-apoptotic protein. Bcl-2 not only
381 antagonizes activated Bax but also antagonizes pro-apoptotic BH3-only proteins, e.g., BID, to prevent
382 apoptosis (Czabotar et al., 2014). Bid binds to Bax in the binding groove of the BH3 domain in Bax
383 (Moldoveanu et al., 2014; Czabotar et al., 2013) and Bid also activates apoptosis by neutralizing the Bcl-
384 2 protein (Shamas-Din et al., 2013). This study clearly showed that PTZ-induced increased Bax
385 expression and decreased Bcl-2 expression, while FA pre-treatment prevented these effects and
386 decreased the ratio of Bax/Bcl-2 expression, based on TUNEL methods (Fig. 4A) and the Hoechst 33258
387 method (Galluzzi et al., 2012a) (Fig. 4B); combined results of FA pre-treatment reduce the release of
388 cytochrome c (Fig. 5I), and reduce caspase-induced apoptosis in both the DG (Fig. 4C) and CA1 (Fig.
389 4D) areas of the hippocampus, further indicate anti-apoptosis effect of FA pre-treatment. MOMP releases
390 cytochrome c from the mitochondrial membrane compartment into the cytoplasm, allowing cytochrome
391 c to bind to Apaf-1(Liu et al., 1996; Li et al., 1997). Subsequently, dATP/ATP is substituted in Apaf-1
392 with ADP to form apoptotic bodies (Kim et al., 2005; Bao et al., 2007). Finally, apoptotic Apaf-1

393 catalyses the self-activation of procaspase-9 and forms active caspase-9, which in turn activates caspase-
394 3 (Anuradha et al., 2001). PTZ treatment increased Apaf-1, procaspase-9, caspase-3 and cleaved caspase-
395 3 expression (Fig. 5) compared to that of the controls. Activated caspases can further lead to the rupture
396 of mitochondrial membranes, causing mitochondria to release other caspases and activating factors,
397 leading to apoptosis.

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

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

421

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