# Repeated febrile convulsions impair hippocampal neurons and cause synaptic damage in immature rats: neuroprotective effect of fructose-1,6-diphosphate

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## Abstract

Fructose-1,6-diphosphate is a metabolic intermediate that promotes cell metabolism. We hypothesize that fructose-1,6-diphosphate can protect against neuronal damage induced by febrile convulsions. Hot-water bathing was used to establish a repetitive febrile convulsion model in rats aged 21 days, equivalent to 3–5 years in humans. Ninety minutes before each seizure induction, rats received an intraperitoneal injection of low- or high-dose fructose-1,6-diphosphate (500 or 1,000 mg/kg, respectively). Low- and high-dose fructose-1,6-diphosphate prolonged the latency and shortened the duration of seizures. Furthermore, high-dose fructose-1,6-diphosphate effectively reduced seizure severity. Transmission electron microscopy revealed that 24 hours after the last seizure, high-dose fructose-1,6-diphosphate reduced mitochondrial swelling, rough endoplasmic reticulum degranulation, Golgi dilation and synaptic cleft size, and increased synaptic active zone length, postsynaptic density thickness, and synaptic interface curvature in the hippocampal CA1 area. The present findings suggest that fructose-1,6-diphosphate is a neuroprotectant against hippocampal neuron and synapse damage induced by repeated febrile convulsion in immature rats.

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## Introduction

Febrile convulsions (FC) are a fever-associated, early-life neurological problem and the most common seizure disorders in childhood, affecting 2–5% of children by 6 years of age (Nelson and Ellenberg, 1976). FC are benign for most children, but repetitive or prolonged FC may directly lead to acute hippocampal damage in animals (Sloviter, 1999; Han et al., 2005; Sobaniec-Lotowska and Lotowska, 2011) and hippocampal edema in patients (Natsume et al., 2007; Orosz et al., 2011). Previous studies showed that prolonged FC could impair hippocampus-mediated learning and memory function (Chang et al., 2001; Dube et al., 2009; Martinos et al., 2012) and induce hippocampal pathological changes (Orosz et al., 2011; Kipp et al., 2012).

Fructose-1,6-diphosphate (FDP) is a cellular metabolic intermediate that can facilitate cell metabolism and has a significant protective effect against hypoxic-ischemic diseases (Antunes et al., 2006; Cohen et al., 2006; Lin et al., 2012). A number of studies have elucidated the neuroprotective mechanisms of FDP. Exogenously administered FDP stimulates the process of glycolysis, provides sufficient adenosine triphosphate levels, and sustains intracellular calcium levels (Bickler and Kelleher, 1992). A study of rat forebrain slices under oxygen-glucose deprivation revealed that FDP suppressed excitotoxic amino acid (glutamate) release and reduced the levels of neurotoxicity-associated inducible nitric oxide synthase (Cardenas et al., 2000). In dying neurons, FDP is neuroprotective, reducing levels of intracellular reactive oxygen species; this was confirmed by evaluation of cell signal transduction in the p38 mitogen-activated protein kinase/extracellular sig-

nal-regulated kinase pathway (Park et al., 2004). Pathological glycolytic changes lead to outer mitochondrial membrane dysfunction, and FDP shows protective effects in the mitochondrial membrane in animal experiments (Chi et al., 2002; de Fraga et al., 2011). Exogenous FDP administration can also elicit significant dendrite outgrowth and cell survival in cortical neurons by increasing brain-derived neurotrophic factor mRNA expression and cellular ATP levels (Lin et al., 2012).

It is possible that learning and memory impairments induced by frequent repetitive FC in immature rats are due to pathological hippocampal changes. The present study was undertaken to verify whether intraperitoneal injections of FDP could alleviate brain damage in a rat model of FC, in an effort to reduce the neuronal and synaptic impairments caused by FC, and to provide a theoretical basis for neuroprotection against FC-induced brain damage.

## Materials and Methods

### Experimental animals

Thirty 21-day-old male Sprague Dawley rats (Laboratory Medicine Center, Xi'an Jiaotong University, China), weighing $50 \pm 5$ g, were randomly divided into three groups (10 rats per group): model control (FC), low-dose FDP (LD), and high-dose FDP (HD). The rats were housed in a pathogen-free facility and kept under controlled light and temperature conditions (12-hour light/dark cycle, $20 \pm 3°C$) with free access to water and food. All experimental procedures were approved by the Subcommittee on Animal Studies of Xi'an Jiaotong University in China. Maximal care was taken to minimize the number of animals used and their suffering.

### Establishment of the rat model of FC

A hot-water bath (Jiang et al., 1999) was employed to establish FC in all rats. Hot-water immersion was carried out in a transparent glass cylinder (diameter 10 cm × height 50 cm) with several small holes at the bottom through which the hot water could flow. This cylinder was placed in a large homoiothermal ($45.0 \pm 0.25°C$) hot-water tank (100 cm × 70 cm × 50 cm). A rat was put in the cylinder, and then rubber pads were placed under the cylinder to regulate the water level until the rat could stand upright while supported by the wall of the cylinder, with only its head above water level. In general, seizures would occur if a rat was immersed in hot water for about 3–6 minutes. At the very beginning of seizure onset, the cylinder was lifted to allow the water to flow back into the tank to prevent drowning, and the rat was immediately removed from the cylinder to an observation basin. Convulsions were induced in each rat once in the morning for 10 consecutive days (10 convulsions in total for each rat).

Seizure severity was described as follows: class 0, no seizure behavior; class I, facial clonus; class II, head nodding; class III, forelimb clonus; class IV, rearing; class V, rearing and falling back (Jiang et al., 1999). In addition, the latency (from placement into the hot-water bath to the onset of each seizure) and duration (time to recovery from seizure onset) were recorded.

At 90 minutes before each hyperthermia session, rats in the

LD and HD groups received an intraperitoneal injection of FDP (guaranteed reagent grade; Hainan Changan International Pharmaceutical Co., Ltd., Haikou, Hainan Province, China) dissolved in sterile water for injection (100 mg/mL) at a dose of 500 or 1,000 mg/kg, respectively. Rats in the FC group received an injection of an equivalent volume of sterile water.

### Transmission electron microscopy (TEM) sample preparation

Twenty-four hours after the last seizure, four rats were randomly selected in each group and anesthetized with an intraperitoneal injection of 20% urethane. The heart was exposed, a tube was inserted through the aorta and left ventricle, and the rats were perfused first with 30–50 mL of 0.9% sodium chloride, followed by 2% paraformaldehyde at 4°C, and 2.5% glutaraldehyde (pH 7.4) at a volume of 100–150 mL (fast at first and then slowly, for a total of about 30 minutes). Brains were then removed and immersed in 2% paraformaldehyde and 2.5% glutaraldehyde liquid at 0°C. The hippocampi were dissected out and sectioned. About 1 mm$^3$ of the hippocampal CA1 region was quickly prepared for conventional TEM as follows: six blocks of brain tissue were selected for embedding, two embedded blocks were cut into 6–10 smaller blocks for TEM sections, and two slices were selected for observation with an H-600 microscope (Hitachi, Tokyo, Japan). The target numbers for each slice were 50 neurons or organelles and 25 synapses under a random field of view. All TEM experiments were conducted blind to the experimental groups, to eliminate the possibility of human bias.

### Observation of neurons and organelles

100 neurons and organelles (mitochondria, rough endoplasmic reticulum, and Golgi complex) were randomly selected and observed using TEM at 6,000 or 20,000 × magnification (H-600, Hitachi). Based on methods described in similar ultrastructural studies (Lopez-Meraz et al., 2010; Sobaniec-Lotowska and Lotowska, 2011), we recorded the following: number of neurons exhibiting signs of degeneration (nuclear swelling, chromatin enrichment and margination, nucleolus shrinkage or deformation) or necrosis (nuclear condensation, fragmentation, dissolved or missing nucleolus); number of swollen mitochondria (large and balloon-shaped with swollen, shortened and disordered cristae); number of mitochondria with vacuolar degeneration; extent of rough endoplasmic reticulum degranulation (mild, moderate or severe, in which the number of ribosomes was reduced by less than one-third, between one- and two-thirds, or over two-thirds, respectively); and extent of Golgi complex dilation (using a similar scale of mild, moderate, or severe dilation).

### Measurement of neural synapses in morphologic parameters

We randomly selected 50 Gray type I synapses (Guillery, 2000) and observed them under 30,000 × magnification. A Q550 Information Collection and Analysis System (Leica, Wetzlar, Germany) was used to analyze images of the synapses to measure the following parameters (Jiang et al., 2003): synaptic cleft width (mean of three values comprising the

**Table 1 Effects of intraperitoneal injection of fructose-1,6-diphosphate on seizure latency, duration, and severity in rats with repeated febrile convulsions**

| Group | Latency (second) | Duration (second) | Severity (%) | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| | | | I | II | III | IV | V |
| FC | 233.9±66.9 | 75.5±29.6 | 8[b] | 6[b] | 12[b] | 26[b] | 48[b] |
| LD | 266.1±67.0[a] | 59.2±26.2[a] | 13[b] | 12[b] | 11[b] | 22[b] | 42[b] |
| HD | 274.2±68.2[a] | 48.5±25.6[a] | 11 | 25 | 14 | 19 | 31 |

Latency and duration are presented as mean ± SD. Seizure severity is given as percentage of all 100 seizures per group (10 rats per group, 10 seizures per rat). [a]$P < 0.05$, *vs.* FC group; [b]$P < 0.05$, *vs.* HD group. One-way analysis of variance with least significant difference *post-hoc* test was used to analyze seizure latency and duration; the chi-square test was used to analyze seizure severity. FC: Febrile convulsion group (control); LD: low-dose (500 mg/kg) fructose-1,6-diphosphate; HD: high-dose (1,000 mg/kg) fructose-1,6-diphosphate.

**Table 2 Effects of intraperitoneal injection of fructose-1,6-diphosphate on the frequency distribution (%) of pathological ultrastructural changes of neurons and organelles in the hippocampal CA1 region in rats with repeated febrile convulsions**

| Group | Neurons | | | Mitochondria | | | Rough endoplasmic reticulum degranulation | | | | Golgi dilation | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | Nor. | Deg. | Nec. | Nor. | Swe. | Vac. | Nor. | Mild | Mod | Sev. | Nor. | Mild | Mod | Sev. |
| FC | 65 | 29 | 6 | 49 | 31 | 20 | 33 | 29 | 21 | 17 | 45 | 23 | 17 | 15 |
| LD | 67 | 26 | 7 | 52 | 25 | 23 | 56 | 17 | 13 | 14 | 60 | 17 | 14 | 9 |
| HD | 73 | 22 | 5 | 72[a] | 20[a] | 8[a] | 59[a] | 19[a] | 15[a] | 7[a] | 71[a] | 14[a] | 10[a] | 5[a] |

$n = 2 \times 50$ fields of view. [a]$P < 0.05$, *vs.* FC group (chi-square test). FC: Febrile convulsion group (control); LD: low-dose (500 mg/kg) fructose-1,6-diphosphate group; HD: high-dose (1,000 mg/kg) fructose-1,6-diphosphate group; Nor: normal; Deg: degeneration; Nec: necrosis; Swe: swelling; Vac: vacuolation; Mod: moderate; Sev: severe.

**Table 3 Effects of intraperitoneal injection of fructose-1,6-diphosphate on neuronal synapse ultrastructure in the hippocampal CA1 region of rats with repeated FC**

| Group | Cleft (nm) | PSD (nm) | Active zone (nm) | Synaptic interface curvature |
| --- | --- | --- | --- | --- |
| FC | 45.68±8.46 | 68.16±15.33 | 330.27±111.74 | 1.04±0.25 |
| LD | 40.17±7.72[a] | 81.41±17.91[ab] | 368.41±107.26 | 1.15±0.28[a] |
| HD | 37.05±7.64[a] | 89.06±18.36[a] | 395.07±120.51[a] | 1.19±0.24[a] |

Data are presented as mean ± SD and analyzed using one-way analysis of variance with least significant difference *post hoc* test. FC: Febrile convulsions; LD: low-dose fructose-1,6-diphosphate group; HD: high-dose fructose-1,6-diphosphate group; Cleft: width of synaptic cleft (mean of the largest, middle, and smallest distances); PSD: thickness of the postsynaptic density at the thickest part; active zone: the length of the active zone. $n = 2 \times 25$ synapses. [a]$P < 0.05$, *vs* FC group; [b]$P < 0.05$, *vs.* HD group.

largest, intermediate, and smallest parts); postsynaptic density thickness (at the thickest part); active zone width; and the synaptic interface curvature.

### Statistical analysis
Enumeration data are presented as frequencies and constituent ratio, and measurement data are described as mean ± SD. SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA) was used to perform the chi-square test or one-way analysis of variance with least significant difference or Student-Newman-Keuls *post hoc* tests, according to the nature of the data. Significance was set at $P < 0.05$.

## Results
### Effects of FDP on seizures of rats with repeated FC
Seizures occurred in all rats in the three groups. Seizure latencies increased and durations, while severity of seizures decreased between the groups from FC to LD to HD. Rats that had received either low- or high-dose FDP had longer seizure latencies and shorter durations ($P < 0.05$), with the HD group having the most effective reduction in seizure severity. The seizure latency, duration, and severity are shown in **Table 1**.

### Effects of FDP on pathological changes of neurons in the hippocampal CA1 region in rats with repeated FC
The percentage of neuronal degeneration and necrosis in the hippocampal CA1 was 35%, 33% and 27% for the FC, LD and HD groups, respectively, but this did not reach statistical significance ($P > 0.05$). The results demonstrate that FDP does not protect neurons from degeneration and necrosis (**Table 2, Figure 1A**).

### FDP protected mitochondrial structure in the hippocampal CA1 region in rats with repeated FC
The percentage of mitochondrial swelling and vacuolation
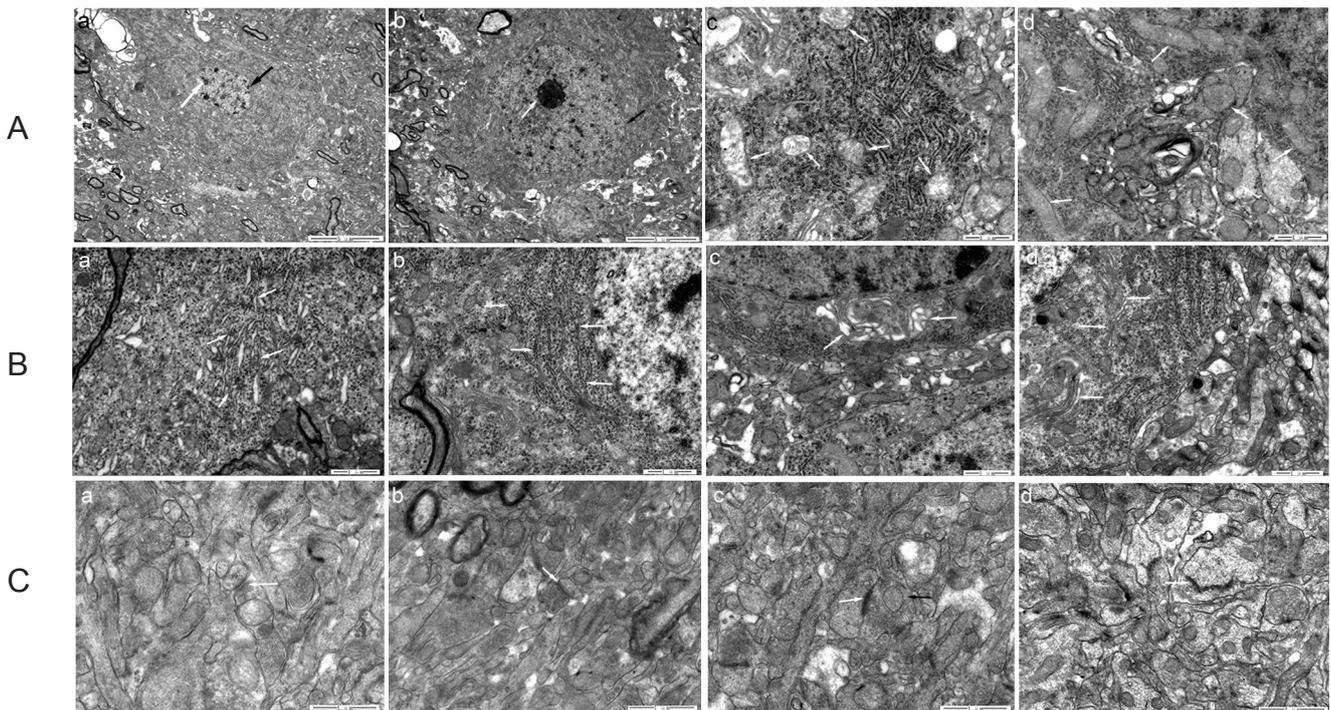
**Figure 1 Effects of intraperitoneal injection of low-dose or high-dose fructose-1,6-diphosphate on ultrastructure of neurons and organelles in the hippocampal CA1 in rats with repeated febrile convulsions (osmium tetroxide staining).**
(A) Neurons (a, b) and mitochondria (c, d): (a) neuronal necrosis in FC group (white arrow indicates fragmented nucleolus; black arrow indicates disintegrating nuclear membrane), scale bar: 5 μm; (b) normal neurons in HD group (white arrow indicates nucleolus, black arrow indicates intact nuclear membrane), scale bar: 5 μm; (c) mitochondrial swelling and vacuolation in FC group (white arrow indicates expansion, cristae disappearance, and structureless vacuoles), scale bar: 1 μm; (d) normal mitochondria in HD group (white arrow indicates elliptic mitochondria with clear, parallel inner cristae), scale bar: 1 μm. (B) Endoplasmic reticulum (a, b) and Golgi complex (c, d) (scale bars: 1 μm): (a) rough endoplasmic reticulum degranulation in FC group (white arrow indicates disappearing ribosomes separated from the rough endoplasmic reticulum; (b) normal rough endoplasmic reticulum in HD group (white arrow); (c) Golgi complex dilation in FC group (white arrow indicates structural disorder and sack expansion); (d) normal Golgi complex in HD group (white arrow). (C) Altered (a, b) and normal (c, d) synapses (scale bars: 1 μm): (a) altered synapses in FC group (wide synaptic cleft, thinned postsynaptic density, and a shortened active zone, white arrow); (b) altered synapses in FC group (small synaptic interface curvature and thinner postsynaptic density, white arrow); (c) normal synapses in HD group (narrow synaptic cleft, large synaptic interface curvature, thicker postsynaptic density, long active zone, white arrow; black arrow indicates a normal mitochondrion); (d) normal synapses in HD group (thicker postsynaptic density and long active zone, white arrow). FC: Febrile convulsion group (control); LD: low-dose (500 mg/kg) fructose-1,6-diphosphate; HD: high-dose (1,000 mg/kg) fructose-1,6-diphosphate.

in the CA1 was highest in the FC group, and lowest in the HD group. There was a significant difference between the FC and HD groups ($P < 0.05$). The results show that intervention with 1000 mg/kg FDP significantly reduces mitochondrial damage in the CA1 region (**Table 2, Figure 1A**).

**FDP protected endoplasmic reticulum structure in the CA1 of rats with repeated FC**
The degree of rough endoplasmic reticulum degranulation (mild, moderate, or severe) in the hippocampal CA1 region was highest in the FC group and lowest in the HD group ($P < 0.05$). The data indicate that high-dose FDP notably reduces rough endoplasmic reticulum degranulation in the hippocampal CA1 field of rats with repeated FC (**Table 2, Figure 1B**).

**FDP protected Golgi complex structure in the CA1 of rats with repeated FC**
The degree of pathological changes in the Golgi complex (mild, moderate or severe) in the CA1 region of the hippocampus was highest in the FC group and lowest in the HD

group ($P < 0.05$). These findings suggest that high-dose FDP reduces Golgi dilation in the hippocampal CA1 region of rats with repeated FC (**Table 2, Figure 1B**).

**Effects of FDP on neuronal synapse ultrastructure in the CA1 of rats with repeated FC**
FDP administration resulted in significantly greater postsynaptic density thickness ($P < 0.05$), active zone length ($P < 0.05$), and synaptic interface curvature ($P < 0.05$), as well as shorter synaptic clefts ($P < 0.05$) of neural synapses in the hippocampal CA1 area. The widest postsynaptic density, the longest active zone, the highest synaptic interface curvature and the narrowest synaptic cleft were found in the HD group. The postsynaptic density thickness was significantly different between the FC and HD groups ($P < 0.05$), between FC and LD groups ($P < 0.05$), and between HD and LD groups ($P < 0.05$) (least significant difference and Student-Newman-Keuls tests), indicating that alterations in postsynaptic density thickness were sensitive to FDP in a dose-dependent manner. Significant differences in synaptic cleft distance were found between the FC and HD groups ($P < 0.05$) and be-

tween the FC and LD groups ($P < 0.05$). Similarly, there were significant differences in the synaptic interface curvature between the FC and HD groups ($P < 0.05$), and between the FC and LD groups ($P < 0.05$) as detected by further multiple comparisons. Analysis of the active zone length revealed a significant difference between the FC and HD groups ($P < 0.05$), indicating that only high-dose FDP significantly increases the length of the active zone (**Table 3, Figure 1C**).

## Discussion

FC are fever-associated, early-life seizures and the most common form of pathological brain activity that can cause brain damage and cognition impairments during development. Although the outcome of FC remains debated, many studies provide evidence that FC results in brain damage, emotional disturbance, behavioral disorders, and temporal epilepsy (Sloviter, 1999; Peredery et al., 2000; Han et al., 2005; Sobaniec-Lotowska and Lotowska, 2011). The objective of the present study was to examine whether FDP could reduce brain damage induced by frequent, repetitive FC. Our major findings are as follows: (a) frequent, repetitive FC can lead to pathological ultrastructural changes of neurons and organelles, such as neuronal degeneration, mitochondrial swelling, rough endoplasmic reticulum degranulation, and dilation of the Golgi complex; (b) increases in synaptic cleft distance and decreases in active zone length, postsynaptic density thickness, and synaptic interface curvature are found in rats with FC; and (c) FDP can attenuate the pathological ultrastructural changes of neurons and organelles and improve morphologic synaptic parameters. The present findings support the hypothesis that FDP can protect the rat brain from structural damage induced by frequent, repetitive FC.

The potential neuroprotective activity of FDP has become the subject of much discussion. One question that has been raised is whether exogenously administered FDP could enter the central nervous system. It was demonstrated that GLUT5, a transporter of fructose located in ependymal cells and in the epithelial cells of the choroid plexus, plays an intriguing role in the direct transportation of intravascular fructose into the brain parenchyma (Ueno et al., 2014). Although the levels of FDP fall to baseline in the liver, kidney, and muscle, and significantly decrease in the blood, FDP is able to access the brain, and peak levels are observed 12 hours after oral administration (Xu and Stringer, 2008). Kinetics data suggest that an oral formulation of FDP could be developed to exert a neuroprotective action. By decreasing fatty acyl chain interaction, FDP can significantly increase membrane permeability and diffuse into the target cell (Ehringer et al., 2002). Intraperitoneally-administered FDP plays a significant role in reducing the size of cortical plate lesions (Rogido et al., 2003). A neuroprotective study of FDP indicates that systemic administration after hypoxia-ischemia reduces central nervous system injury in neonatal rats (Sola et al., 1996).

How does FDP rescue neuronal dysfunction and promote rehabilitation under repeated FC? The present findings suggest that dysfunction of neuronal energy metabolism plays a role in the process of FC-induced brain damage in rats. When FC are induced in experimental rats, altered breathing or hypopnea exposes the brain to hypoxia and, consequently, to disrupted energy metabolism for the entire duration of the convulsions. FDP serves as a high-energy glycolytic pathway intermediate that can enhance cell metabolism and has a significant protective effect against hypoxia-ischemia (Antunes et al., 2006; Lin et al., 2012). When hypoxia-ischemia occurs, FDP reduces damage in a variety of tissues (Antunes et al., 2006; Cohen et al., 2006; de Fraga et al., 2011) and plays multiple roles in improving energy metabolism, such as suppressing $Ca^{2+}$ influx, regulating cellular signal transduction systems, and preventing accumulation of free radicals, peroxides, and electrophilic xenobiotics (Cuesta et al., 2006; Markov et al., 2007; Ding et al., 2010; Kim et al., 2012; Lin et al., 2012).

With regard to the neuroprotective activity of FDP, several lines of data suggest that higher doses and administration before brain insults can achieve the maximum effects. Intravenous injection of high doses of FDP (100 or 333 mg/kg) 15 minutes before transient cerebral ischemia induction in a rodent model ameliorated hippocampal neuronal death, whereas a lower dose (33 mg/kg) administered 15 minutes after the ischemic insult did not attenuate histopathological damage (Trimarchi et al., 1993). Intraperitoneal high-dose (1,000 mg/kg) but not low-dose (500 mg/kg) administration of FDP delayed hippocampal kindling through the brain-derived neurotrophic factor/tyrosine kinase receptor B signaling pathway (Ding et al., 2010). These results are similar to our present findings that FDP intervention with a dose of 1,000 but not 500 mg/kg could effectively reduce mitochondrial swelling, rough endoplasmic reticulum degranulation, and Golgi dilation. Recently, it has been shown that FDP did effectively ameliorate partial or generalized convulsions in rodent models (Lian et al., 2008; Stringer and Xu, 2008; Ding et al., 2010).

The present study suggests that FDP acts as a protective agent against synaptic damage in hippocampal neurons in rats with experimental frequent, repetitive FC. Whether the abovementioned processes are at work simultaneously and interactively remain to be determined in further research.

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