

# Effect of edaravone on apoptosis of hippocampus neuron in seizures rats kindled by pentylenetetrazole

X.-L. CHENG, J.J. ZHANG

Department of Neurology, Zhongnan Hospital of Wuhan University; Department of Neurology, The First Affiliated Hospital of Yangtze University, China

**Abstract. – OBJECTIVES:** To explore the effect of edaravone (ED) on apoptosis of hippocampus neurons in seizures rats induced by pentylenetetrazole (PTZ).

**MATERIALS AND METHODS:** Forty-eight adult Wistar rats were randomly divided into normal control (NC) group, PTZ group, and ED group. A dose of PTZ [35 mg/(kg·day)] was intraperitoneally (i.p.) injected into the rats of PTZ group and ED group until the kindling criterion was reached. After kindling, the rats of ED group were administered i.p. with ED [0.8 mg/(kg·day)]; the rats of PTZ group were administered i.p. with normal saline. After 30 min, seizures were induced by administering PTZ i.p. The influence of ED on Fas, Caspase-3, and Survivin protein immunoreactivity on hippocampus neurons was studied by immunohistochemistry method.

**RESULTS:** Fas and Caspase-3 positive cells and optical density in hippocampus in PTZ group were more than that of ED group and NC group (all  $p < 0.01$ ), but Survivin-positive cells and optical density in hippocampus in PTZ group were less than that of ED group ( $p < 0.01$ ) and were more than that of NC group ( $p < 0.05$ ); Fas- and Caspase-3-positive cells and optical density in hippocampus in ED group were more than that of NC group (all  $p < 0.05$ ), but Survivin-positive cells and optical density in hippocampus in ED group were more than that of NC group ( $p < 0.01$ ).

**CONCLUSIONS:** Seizure can induce apoptosis of hippocampus neurons on seizures rats, but ED can resist the apoptosis of hippocampus neurons by increased expression of Survivin and decreased expression of Fas and Caspase-3.

*Key Words:*

Seizure, Edaravone, Pentylenetetrazole, Fas, Caspase-3, Survivin

## Introduction

Chronic seizures cause lasting brain damage and impaired cognitive function. Pathological

changes after seizures include hippocampal sclerosis, neuronal loss, and gliosis<sup>1</sup>. Neuronal loss includes necrosis and apoptosis<sup>2</sup>, and neuronal apoptosis after seizures is an important form of neurons' loss<sup>3</sup>. Apoptosis affects in two ways: one is the mitochondrial pathway (also known as intracellular pathways): the BID → Bax → cytochrome *c* → Caspase-9 → Caspase-3; another extracellular pathways: Fas → FasL → FRADD → Caspase-8 → Caspase-3. Caspase-3 is the common pathway of the two pathways and is the executor of apoptosis; meanwhile there is a spontaneous anti-apoptotic mechanisms such as the inhibitor of apoptosis protein Survivin. At present, there are different types of the apoptosis mechanism after seizures; Oxidative stress is one of possible mechanisms in the pathogenesis of epilepsy. Firstly, oxidative stress is the initiator in the pathogenesis of epilepsy. The oxidative stress induces impaired mitochondrial respiratory chain, which can cause excess free radicals and the deficient anti-oxidant system (SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH/GSSG, reduced glutathione/glutathione disulfide), resulting in a continuous vicious cycle and progressive cell death in the epileptic area of the brain<sup>4-6</sup>. Chuang et al<sup>7</sup> demonstrated that NO<sup>-</sup>, O<sub>2</sub><sup>-</sup>, and peroxy nitrite depressed the neuronal mitochondrial respiratory enzyme complex I activity in a time-dependent manner, and induced cytosol-bound release of cytochrome *c* from the mitochondria, accompanied with activation of Caspase-3 and increasing generation of ROS (reactive oxygen species) that function as second messengers in signal transduction and mediators of oxidative damage and inflammation, which leads to apoptotic cell death in the hippocampal CA3 subfield<sup>8,9</sup>. Secondly, decreased levels of glutamine synthetase (GS) and the enzyme responsible for converting glutamate into glutamine have been report-

ed following the hippocampal sclerosis<sup>10</sup>, and an increase in glutamine and glutamate in the thalamus in epileptic patients has been reported<sup>11</sup>. In the KA (Kainic acid) model, a transient increase in GS expression during the “latent period” and a decrease in the phase of epilepsy have been reported, which suggest a decreased capacity for glutamate metabolism as spontaneous and recurrent seizures become evident<sup>12</sup>. Thus, recent evidence supports the role of mitochondrial oxidative stress not merely as a consequence of seizures, but an active contributor to seizures and epileptogenesis. Meanwhile, more results suggest that the lower oxidative stress (ROS and malondialdehyde MDA) and high anti-oxidant activity (superoxide dismutase, SOD) are the predictors for a better outcome of epileptic surgery. Hence, anti-oxidant therapy plays a crucial role in the prevention of oxidative damage in mitochondrial dysfunction and recurrence of epileptic seizure. In addition, the detection of brain oxidative stress is essential for evaluating the prognosis of epileptic patients. Edaravone (ED) (MCI-186) is a novel radical scavenger that protects neurons by inhibiting vascular endothelial cell injury and ameliorating neuronal damage caused by acute brain ischemia. ED is found to have anti-oxidant effects, quenching OH and inhibiting both OH-dependent and OH-independent lipid peroxidation, and to exert inhibitory effects on both water-soluble and lipid-soluble peroxy radical-induced peroxidation systems (including not only OH but also other free radical species such as superoxide and NO radicals). Recent results indicate that ED exerted neuroprotective effects by reducing both neuronal NOS (nNOS) and inducible NOS (iNOS) and increasing endothelial NOS (eNOS)<sup>13</sup>. However, whether ED has a protective effect on neuronal apoptosis after seizure, at present, has not been reported. So, we aim to explore the effect of ED on Fas and Caspase-3, and Survivin of hippocampus neuron in seizures rats kindled by pentylenetetrazol (PTZ) finds out the protective effect of ED on neuronal apoptosis after seizure.

## Materials and Methods

Forty-eight adult male Wistar rats (Hubei University of Medicine, Shiyan, China) weighing between 220 and 250 g were used for the study. They were housed individually with regular feeding of food and water and kept on a 12-h light/dark cycle, at controlled temperature, and in

controlled humidity. All animals were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Animal Ethics Committee of the Yangtze University Clinic Medical College (Project #0131). All rats were randomly divided into three groups: PTZ groups (induced epilepsy by PTZ,  $n = 16$ ), ED groups (intervention with ED,  $n = 16$ ), and NC groups (normal control [NC] groups,  $n = 16$ ). PTZ and paraformaldehyde were obtained from Sigma (Shanghai, China); rabbit anti-mouse Fas, Caspase-3, and Survivin antibody were obtained from Jingmei (Wuhan, China); Goat anti-rabbit antibody and DAB stain were obtained from Zhongshan (Wuhan, China), and ED was obtained from Pioneer Pharmaceutical of Jiangsu (Nanjing, China).

## Modeling and Processing

The PTZ and ED group animals were administered an injection of PTZ [35 mg/(kg·day)]<sup>14</sup> that was dissolved in 0.9% NaCl solution intraperitoneally (i.p.), and their behavioral changes were observed (e.g., seizure latency, average seizure time, and average seizure scores). The kindled seizures were classified according to Racine's five stages<sup>15</sup>: stage 1, facial twitching, head version, or eye closure ipsilateral to stimulation, 1 score; stage 2, head nodding accompanying mastication, 2 score; stage 3, clonic forelimb convulsion, 3 score; stage 4, rearing to a kangaroo-like posture or rearing with clonic forelimb clonus, 4 score; and stage 5, generalized convulsion, including falling down, 5 score. The igniting standard was three consecutive episodes attack above stage 4 in each rat. The PTZ [35 mg/(kg·day)] was injected every 3 day after the rats were ignited and maintained with the kindling effect. The ED group rats were administered an injection of ED [0.8 mg/(kg·d)] that was dissolved in 0.9% NaCl solution i.p. after all rats were ignited (31 days), and the PTZ (35 mg/kg) was injected after injecting ED 30 min. The PTZ groups rats were administered an injection of saline i.p. after all rats were ignited (31 days), and the PTZ (35 mg/kg) was injected after injecting saline 30 min. NC group were administered daily an injection of saline with PTZ same volume i.p. Three groups are continuous injection of 2 weeks.

## Material and Immunohistochemistry

The rats were deeply anesthetized with a 10% chloral hydrate (350 mg/kg i.p.) and sequentially perfused transcardially with 4% paraformaldehyde.

hyde. Brains were removed and cut en bloc in the coronal plane, and embedded in paraffin. The 4-mm thick coronal sections through the hippocampus were stained immunohistochemically using the labeled peroxidase-conjugated method at designated times. The following primary antibodies were used: rabbit anti-mouse Fas, Caspase-3 and Survivin antibody, and Goat anti-rabbit antibody.

Control experiment: (1) Displacement experiments: substituting level 1 antibody with normal rabbit serum, incubating slice; (2) Blank experiment substituting level 1 antibody with phosphate buffered saline (PBS), incubating slice.

**Picture Analyzing and Data Processing**

Twelve pieces of brain slice out of each rat were taken, and the pictures were analyzed by PIPS-2011 color pathology graphic analysis and management system. All the brain slices were analyzed under the same magnification factor (×200) and light intensity. The first step is to analyze Fas-, Caspase-3-, and Survivin-positive cells in hippocampus, then detect the average optical density of positive cells. The degree of immune reaction is indicated by optical density value; the darker it is, the larger the value is.

**Statistical Analysis**

Data is indicated by mean ± standard deviation (mean ± SD). After all rats were ignited, the seizure latency, average seizure time, and average seizure scores on PTZ group and the ED group animals were used by the analysis of *t*-test. The average positive cell number and average optical density of positive cells were used by analysis of variance (ANOVA) and the pairwise comparisons were handled by *q*-test. *p* < 0.05 was considered statistically significant.

**Results**

**The Behavior Change**

The PTZ group and ED group rats had seizure on the sixth day after injecting PTZ [35

mg/(kg·d)]. The time of ignition was from 18 to 31 days, that is an average of 24 days. After all rats were ignited, the seizure latency of PTZ group rats was shorter than that of ED group rats; the average seizure time of PTZ group rats was prolonged than that of ED group rats, and the average seizure scores of PTZ group rats were higher than that of ED group rats (*p* < 0.01) (Table I).

**Immunohistochemistry Results**

The comparison of positive cells and optical density with Fas, Caspase-3, and Survivin in the hippocampus of rats in each group (Tables II to IV): Fas- and Caspase-3-positive cells and optical density in the hippocampus of rats in PTZ group were higher than those of rats in ED group and NC group (*p* < 0.01); Survivin-positive cells and optical density in the hippocampus of rats in PTZ group were less than those of rats in ED group (*p* < 0.01), but higher than those of rats in NC group (*p* < 0.05); Fas- and Caspase-3-positive cells and optical density in the hippocampus of rats in ED group were higher than those of rats in NC group (*p* < 0.05); Survivin-positive cells and optical density in the hippocampus of rats in ED group were higher than those of rats in NC group (*p* < 0.01).

**The Results of Control Experimental**

With normal rabbit serum and PBS instead of first antibodies were incubated slices, the results were negative.

**Discussion**

At present, apoptosis is the major cause of hippocampal neuronal loss during seizures based on the clinical and basic research<sup>16,17</sup>. The apoptosis mechanism after seizures is more types; oxidative stress is one of possible mechanisms in the pathogenesis of epilepsy. ROS cause damage to cells by reacting with various cellular components such as lipids, proteins, and nucleic acids.

**Table I.** Comparison of each group in seizure latency, seizure time, and seizure scores ( $\bar{x} \pm s$ ).

Group	Seizure latency	Seizure time	Seizure scores
PTZ group	196.32 ± 73.65s	2763.8 ± 721.4s	5.0 ± 0.25
ED group	271.69 ± 86.45s*	1364.8 ± 317.6s*	3.14 ± 0.17*

Note: \**p* < 0.01 compared to PTZ group.

**Table II.** Results comparison of each group in Fas-positive cells and optical density ( $\bar{x} \pm s$ ).

Group	n	Positive cells	Optical density
PTZ group	16	136.45 $\pm$ 26.27 <sup>*,<math>\Delta</math></sup>	0.637 $\pm$ 0.082 <sup>*,<math>\Delta</math></sup>
ED group	16	63.54 $\pm$ 8.16 <sup>a</sup>	0.339 $\pm$ 0.041 <sup>a</sup>
NC group	16	54.76 $\pm$ 9.34	0.231 $\pm$ 0.034

Note: In comparison with NC group: <sup>a</sup> $p < 0.05$ ; <sup>\*</sup> $p < 0.01$ . In comparison with ED group:  <sup>$\Delta$</sup>  $p < 0.01$ .

There is an evidence that ROS are involved in the development of seizures under pathological conditions, and ROS have been implicated in seizure-induced neuronal death<sup>18</sup>. Several recent studies<sup>19</sup> have demonstrated that free radical scavengers can inhibit neuronal death induced by excitotoxins. When a sufficient dose of a free radical scavenger is given before seizure onset, a preventive effect against neuronal loss can be obtained<sup>20</sup>. ED (MCI-186) is a novel radical scavenger that protects neurons by inhibiting vascular endothelial cell injury and by ameliorating neuronal damage caused by acute brain ischemia, meanwhile studies have reported that ED can protect neurons in seizures. But the current study is less whether ED can directly inhibit apoptosis. Therefore, we observed the effect of ED on the Fas, Caspase-3, and Survivin of hippocampal neurons on PTZ-induced epileptic rat and evaluated both the anti-apoptosis and anti-epileptogenic effects of ED on the kindling rats. Furthermore we learnt about its protective effect on neuronal apoptosis.

In this experiment, we successfully copied the kindling model of rats with chronic epilepsy by applying injection i.p. subthreshold dose of PTZ. Because PTZ is an induced convulsion agent in central nervous system, and is a GABA receptor antagonist, pathology shows the model-induced hippocampal CA1 neurons apoptosis and dentate back to mossy fiber sprouting is very similar to human epilepsy in the kindling model rats by continuous injection of subthreshold dose of PTZ.<sup>21</sup> The kindling model is an ideal model to

simulate generalized tonic-clonic seizures.<sup>22</sup> And there are the main features of the hippocampal pyramidal cell apoptosis, necrosis and lack in this model. Therefore, the kindled model by PTZ is widely used for studies on epileptogenic processes and on drug targets by which epilepsy can be prevented or modified.

Firstly, we have found in these experiments that the seizure latency of PTZ group rats was shorter than that of ED groups rats; the average seizure time of PTZ groups rats was prolonged than that of ED groups rats, and the average seizure scores of PTZ groups rats was higher than that of ED groups rats. Furtherly, we confirmed the anti-epilepsy effect of ED; secondly, we have found that Fas- and Caspase-3-positive cells and optical density in the hippocampus of rats in PTZ group were higher than those of rats in NC group. That confirmed furtherly that seizures could lead to neuronal apoptosis; meanwhile, in this experiment, we have found that Fas- and Caspase-3-positive cells and optical density in the hippocampus of rats in ED group were less than those of rats in PTZ group, which shows that ED can prevent neuronal apoptosis caused by the extracellular route. Fas is also known as Apo-1 or CD95 or death receptors that belong to tumor necrosis factor and nerve growth factor receptor superfamily, a wide expression in cell surface. Caspase-3 is considered to be the only way of apoptosis protein cascade. It is the strong implementation of enzymes that kill the neurons. Various damage factors such as free radicals, excitatory amino acids, and inflammatory

**Table III.** Results comparison of each group in Caspase-3positive cells and optical density ( $\bar{x} \pm s$ ).

Group	n	Positive cells	Optical density
PTZ group	16	136.72 $\pm$ 21.43 <sup>*,<math>\Delta</math></sup>	0.516 $\pm$ 0.053 <sup>*</sup>
ED group	16	81.65 $\pm$ 14.32 <sup>a</sup>	0.249 $\pm$ 0.047 <sup>a</sup>
NC group	16	73.42 $\pm$ 11.54	0.163 $\pm$ 0.036

Note: In comparison with NC group: <sup>a</sup> $p < 0.05$ ; <sup>\*</sup> $p < 0.01$ . In comparison with ED group:  <sup>$\Delta$</sup>  $p < 0.01$ .

**Table IV.** Results comparison of each group in Survivin-positive cells and optical density ( $\bar{x} \pm s$ ).

Group	n	Positive cells	Optical density
PTZ group	16	31.43 $\pm$ 5.62 <sup>*<math>\Delta</math></sup>	0.232 $\pm$ 0.037 <sup>*<math>\Delta</math></sup>
ED group	16	58.39 $\pm$ 8.26 <sup>a</sup>	0.363 $\pm$ 0.065 <sup>a</sup>
NC group	16	24.29 $\pm$ 8.53	0.172 $\pm$ 0.061

Note: In comparison with NC group: <sup>a</sup> $p < 0.05$ ; <sup>\*</sup> $p < 0.01$ . In comparison with ED group:  <sup>$\Delta$</sup>  $p < 0.01$ .

factors trigger the Fas abundantly expressed in seizures, Fas combined with Fas-L and triggered cell apoptosis cascade reaction, leading to neuronal apoptosis, but ED can play the role of anti-apoptosis by inhibiting the expression of Fas caused Caspase-3 expression decreased; thirdly, we have found that Survivin-positive cells and optical density in the hippocampus of rats in ED group were higher than those of rats in PTZ groups and NC groups; this confirms that ED not only inhibits the expression of apoptosis-related proteins, but also can promote apoptosis inhibitory protein (Survivin) expression to further play the role of anti-neuronal apoptosis in the seizures. Due to Survivin directly impact on caspase-3 and block the common pathway of apoptosis induced by various stimuli, it may also be one of the reasons that the caspase-3 of ED group significantly was reduced in this experiment. ED anti-apoptotic mechanism is consistent with their pharmacological effects: (1) quenching OH and inhibiting lipid peroxidation<sup>13</sup>; (2) inhibiting nitric oxide level<sup>23</sup>; and (3) inhibiting the production of inflammatory cytokines.<sup>24</sup>

However, in our experiment, we have found the following results: firstly, Fas- and Caspase-3-positive cells and optical density in the hippocampus of rats in ED group were higher than those of rats in NC group. this is because we apply the ED after all models have been kindled; model application before the application has been the presence of apoptosis. Apoptosis already exists before applying ED. This shows that neuronal apoptosis has different levels of existence in each seizures regardless of the length of time. It may also be the reason of hippocampal sclerosis in chronic epilepsy patients. Although ED has good effect of anti-neuronal apoptosis, it can't against neuronal apoptosis if not as soon as possible applications. Meanwhile, we found Survivin-positive cells and optical density in the hippocampus of rats in PTZ group were higher than those of rats in NC group. In this experiment, this shows that seizures induced endogenous anti-

apoptotic mechanism, however, this protective effect is weak and cannot be completely against the epilepsy-induced neuronal apoptosis.

## Conclusions

ED should be applied early as a free radical scavenger in patients with epilepsy in order to reduce epilepsy-induced neuronal apoptosis.

## Conflict of Interest

The Authors declare that there are no conflicts of interest.

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