The effects of antiepileptic drug valproic acid on apoptosis of hippocampal neurons in epileptic rats

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Abstract: This study activated chronic epilepsy rat model with PTZ (Pentetrazole) and controlled epilepsy with VPA (valproate). By doing that, we detected the apoptotic cells of hippocampus and figured out the expression variation of hippocampal neurons applying immunohistochemical methods. We selected 30 adolescent male SD (Sprague-Dawley) rats (201±29g), which were clean and healthy. The rats were divided into three groups and 10 were in each. Then we detected the apoptotic cells of hippocampus using TUNEL (Terminal Deoxynucleotidyl Transferase Mediated Biotinylated Deoxyuridine Triphosphate Nickel End Labeling Technique) and Bcl-2 and Bax positive cells applying immunohistochemical methods. The results showed Bcl-2 positive cells of hippocampal neuron in VPA group had no statistical variation as compared with PTZ group; Bax positive cells of hippocampal neuron in VPA group induced as compared with PTZ group; dye and density of the positive cells were both decreased, whereas there was no statistical variation when compared with normal control group. Based on the experiment, we reached the conclusion that, VPA showed no harm to epileptic rats and the hippocampal neuronal apoptosis after epilepsy and anti-epileptic drug was probably resulted from the decrease of Bcl-2 expression and increase of Bax expression.

Keywords: Epilepsy; sodium valproate; hippocampal neurons; apoptosis.

INTRODUCTION

Epilepsy (EP) is a kind of chronic brain disease caused by abnormal discharge of cerebral neurons and characterized by chronic brain disease and neural dysfunction (Sen, 2014). There are more than forty million epileptic patients all over the world, in China, nearly 6 million; there are about 400 thousand new onset patients per year. Epilepsy poses a great threat on human health and economic development. As the study of epilepsy goes deeper, the influence of epilepsy on cognitive function has attracted more and more attention, many domestic and foreign scholars have done lots of clinical and basic studies on it, the reasons are as following: the basic underlying neuropathology, the type of seizure, the age of onset, social psychological problems and treatment, the side effects etc, but the exact mechanism has not been clarified (Chanyi *et al*., 2014). In clinic, it is difficult to tell whether cognitive function damage is caused by epilepsy itself or anti-epileptic drugs. But the animal models of epilepsy can explore the possible causes of cognitive function damage of epilepsy and relative mechanism by controlling the factors of seizure so as to obtain detailed information, which cannot be identified in clinical research.

The research for the pathogenesis of epilepsy seizures, cognitive dysfunction and anti- epileptic drug mainly relies on animal experiment, that is, animal kindling model of epilepsy. Kindling model is an ideal animal model of epilepsy, which has the advantages of spontaneity and acute seizures inducing. It is the most widely used animal model of epilepsy, especially for its pathogenesis, which is considered similar to the pathogenesis of human epilepsy (Songqing, 2013). At present, kindling model has been used to study various aspects of epilepsy and provides an ideal animal model for the research on pathogenesis, the efficacy and side effects of anti-epileptic drugs etc. PTZ is a chemical convulsive agent and meanwhile a relative ideal animal model of epilepsy.

At present, epilepsy therapy mainly relies on drug treatment, and a considerable part of patients need long-term treatment, so research on the effect of antiepileptic drugs on cognitive function is of clinical significance. Foreign researches on the relationship between antiepileptic drugs and hippocampal neuronal apoptosis were restricted to the expression of sodium valporate on Bax and Bcl-2 of rat's hippocampus. Bax is an apoptosis inducing factor, and Bcl-2 is an apoptosis inhibitor, The Bcl-2 protein is encoded by the bcl-2 gene and can prevent apoptosis.

Based on a large number of related literatures at home and abroad, this paper successfully kindled the animal model of chronic epilepsy and treated the epilepsy animal with VPA as the antiepileptic drugs, which provides theoretical basis for guiding clinical work.

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MATERIALS AND METHODS

The experimental animal

A total of 30 male SD (Sprague-Dawley) rats (provided by the animal center of Hebei Medical University) of 10 weeks old which were healthy and clean were selected and been fed for a week before the experiment. The initial weight was 155～235 (201±29)g.

The main equipment and reagent

The main experimental methods

The 30 SD rats were randomly divided into 3 groups and each group consists 10. We randomly choose one group as the normal saline, NS (normal saline) group and the other 2 groups (experimental group) were kindled by PTZ. Then we randomly selected one group as epilepsy PTZ group and the other group (VPA group) was administered with VPA so as to control the attack of epilepsy.

Perform intraperitoneal injection to the 20 SD rats of experimental groups with PTZ (the concentration was 10mg/ml and diluted with NS) according to the standard 35mg/ (kg•d); observe the rats for 60mins after each injection and record their behaviors. The process lasted for 2 weeks. Two weeks later, the epileptic seizure of SD rats in experimental groups reached our expectation, the molding succeed. Among, one rat of PTZ group died from status epilepticus. Rats of control group received injection of NS for the same times, in the same way and with the same dose. When the experimental group was totally kindled, we recorded and detected the kindling effect with electroencephalogram.

Fig. 1: Apoptotic neurons in CA1 of hippocampus in the rats of NS group (200×)

Tracing of electroencephalogram

Fix the rats on stereotaxic apparatus, reference electrode was fixed to nose, forehead and temporal lobe or parietal lobe, respectively; the ground electrode was fixed on the back. The electroencephalograph was set as, paper speed 30mm/s, sensitivity 20uv/mm, time constant 0.1 and smoothing 15HZ. After the molding of experimental groups, we performed electroencephalogram tracing to each of the SD rats during seizures. The control group received electroencephalogram tracing at the same as experimental groups.

Detection of apoptosis in hippocampus neurons

Normal saline and 4% poly formaldehyde solution were poured through aortic of the left ventricular of rats in condition of deep anesthesia. Take hippocampus tissue and place it in 4% poly formaldehyde solution, then fix them and make paraffin specimen. We detected apoptosis cells of hippocampus using TUNEL and Bcl-2 and Bax positive cells using immunohistochemical method.

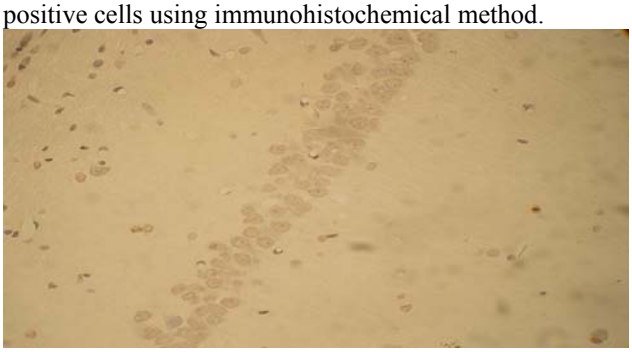

Fig. 2: Apoptotic neurons in CA1 of hippocampus in the rats of PTZ group (200×)

The judgment of results and statistical processing

We took 6 sections from each rat, and observed and counted the positive cells applying grid-counting method. Five representative high power fields were randomly selected from each of the sections and the positive cells were counted. We calculated the rate of cell apoptosis according to the following formula, the rate of cell apoptosis (%)=the number of apoptotic cells/the number of counted cells. Detection results of all the groups were expressed as Mean ± SD; ANOVA analysis on all the data was performed using SPSS 17.0 software and all results were considered statistically significant as $P<0.05$.

RESULTS

The episodes of PTZ induced epileptic rats

PTZ kindling group: epileptic seizures occurred to those 20 rats which were induced by intraperitoneal injection of PTZ for 6 to 9 times; at first, Racine I~II, which reflected as actions such as moustache move, chewing, facial twitches, repeated wash, then the symptoms of rat epilepsy exacerbated. After12-15 times of intraperitoneal injection of PTZ, the convulsions intensified, and the attack times gradually increased, then it entered the grand attack period, forelimb clonus, hind legs running jump, standing with the head to one side and systemic paroxysmal twitching appeared one by one. When the attack reaches Racine V level, the animal is shown as the fall, roll and other generalized tonic clonic seizures, the former hindlimb shrank intensively, accompanied by laryngeal stridor, drooling, acrocyanosis; the attack continued for a few minutes, then repeated after a temporal remission. The attack gradually remits after a duration of 30~70min. When the attack stopped, the animals were free. One death occurred in PTZ group because of the constant status epilepticus.

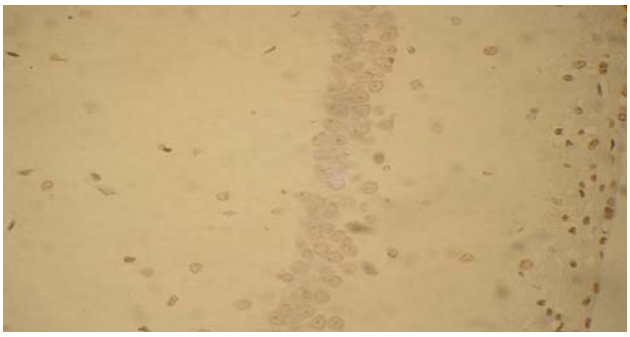

Fig. 3: Apoptotic neurons in CA1 of hippocampus in the rats of VPA group (200×)

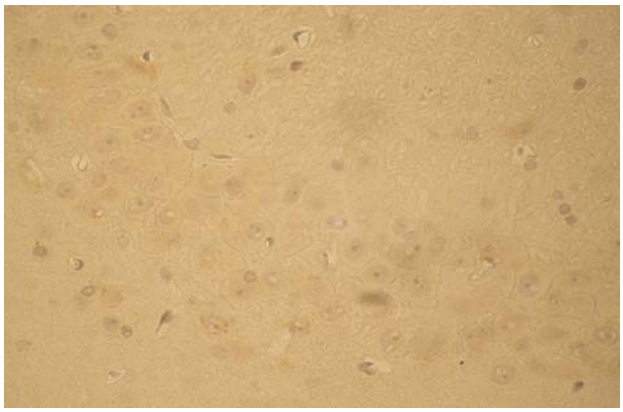

Fig. 4: Bcl-2 positive neurons in CA3 of hippocampus in the rats of NS group (200×)

Normal control group: No epilepsy seizure occurred after the intraperitoneal injection of normal saline. There was no obvious change and the behavior was the same as normal rats.

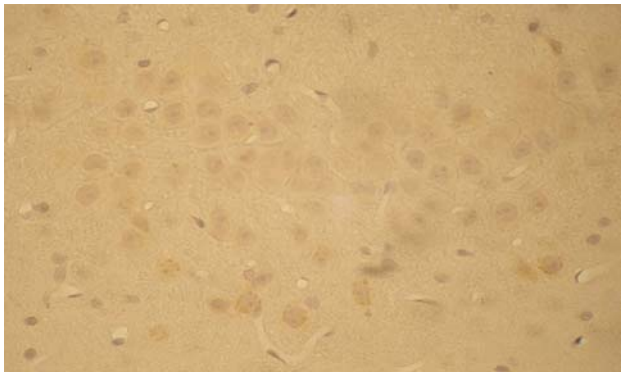

Fig. 5: Bcl-2 positive neurons in CA3 of hippocampus in the rats of PTZ group (200×)

EEG results

PTZ kindling group: Electroencephalography showed typical high amplitude spike slow wave, sharp slow wave, spike wave and sharp wave issue. Normal control group: EEG alpha wave was mainly 9-11Hz, and scattered in the theta, with bilateral symmetry no epileptiform discharges.

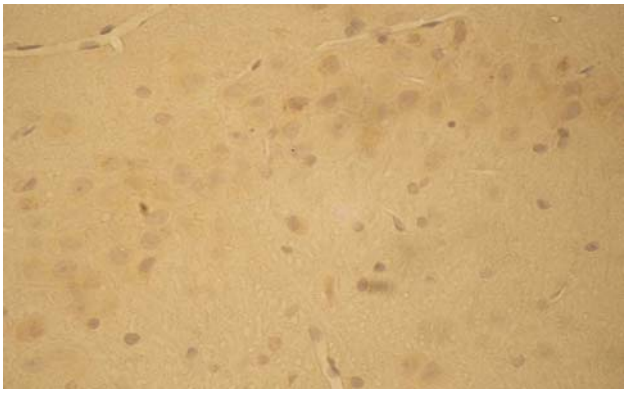

Fig. 6: Bcl-2 positive neurons in CA3 of hippocampus in the rats of VPA group (200×)

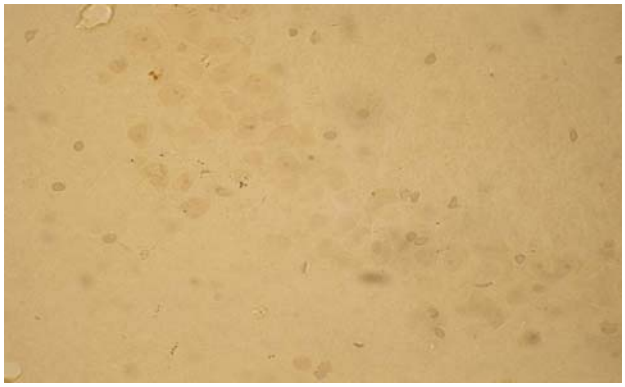

Fig. 7: Bax positive neurons in CA3 of hippocampus in the rats of NS group (×200)

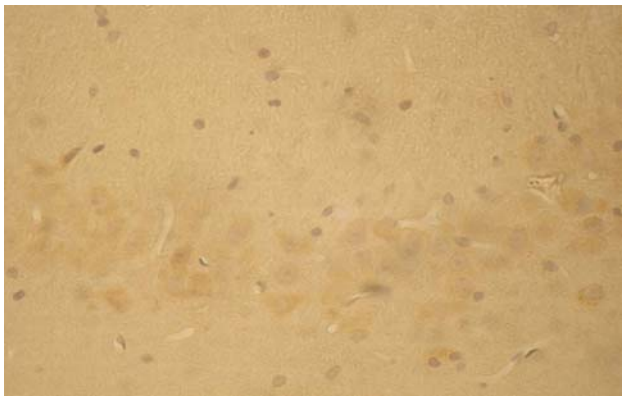

Fig. 8: Bax positive neurons in CA3 of hippocampus in the rats of PTZ group (×200)

Detection results of hippocampal neurons apoptosis

The positive hippocampus nerve cells marked by TUNEL, mainly distributed in CA1 and CA3 area, the nuclei were dyed brown or tan with shape irregular and inconsistent size. Some were in conglobation; and some gathered in a membrane surrounding, which formed a typical "ring like" and "half moon like" characteristics of apoptosis; some parts gathers a number of small clumps aggregation; and some parts split into 3 to 4 bodies. The characteristics were consistent with the apoptosis. The count of

hippocampal neuronal apoptosis, which is shown in the following table 2.

Detection of the expression of Bax and Bcl-2 in hippocampal neurons

Bcl-2 and Bax positive cells mainly distributed in CA1 and CA3 areas. The dyes of Bcl-2, Bax positive cells were mainly distributed in the cytoplasm, protuberance and nuclear membrane of neurons. The dye was brown and presented as fine particles or diffuse and homogeneous. Nucleus was not dyed and the forms of the positive cells were regular.

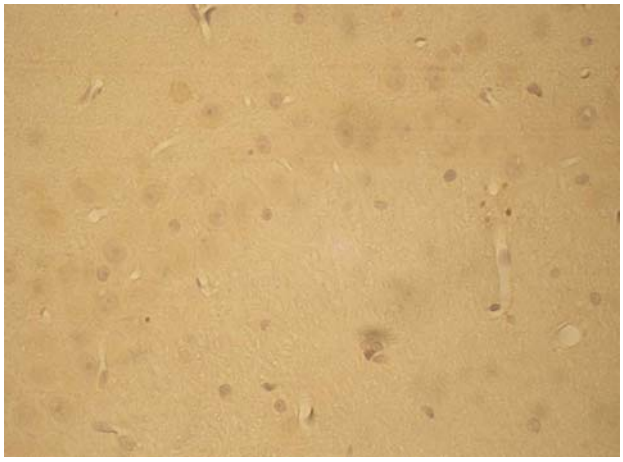

Fig. 9: Bax positive neurons in CA3 of hippocampus in the rats of VPA group (×200)

Bcl-2 expression of hippocampal neurons in each group

In normal control group, the Bcl-2 positive cells of hippocampal neurons were weakly dyed, and scattered (fig. 4). In PTZ model group, hippocampal neurons contains lots of Bcl-2 positive cells, and the dye and density of them were enhanced (fig. 5); In VPA group, Bcl-2 positive cells in hippocampal neurons had no statistical change as compared with the PTZ group (fig. 6).

Expression of hippocampal neurons in the case of bax

In the normal control group, the hippocampal neurons of Bax positive cells were weakly stained and scattered in the cell (fig. 7). In PTZ model group, the Bax positive cells of hippocampal neurons were dense, and the dye of which was deep and density enhanced (fig. 8); In VPA group, The Bax positive cells in hippocampal neurons reduced as compared with PTZ group, of which the dye and density of them were both reduced; and there was no statistical change when compared with normal control group (fig. 9).

DISCUSSION

There is similarity between animal model of chronic epilepsy caused by kindling effect and the formation of human epilepsy. The model is considered as a relative ideal model for basic research and drug treatment with increased epileptogenic property and permanent reservation.

Kindling effect refers to give sub threshold stimulus upon the experimental animal in certain intensity and internal time, and there was no clinical change or abnormal changes of EEG in the beginning. With the stimulus accumulates, the reaction is gradually strengthening, and finally systemic spasm occurs. Once the kindling leads to occurrence of epilepsy, course of disease would last for a long time. When kindled, it has the following features: regulated epileptiform discharge of abnormal EEG lasts long, and second stimulation may lead to clonic transmission between cynapse and finally induce epilepsy. The model has the following advantages: it is easy to make and with high success rate; it is free from the influence of anesthetic and operation process which benefits the comprehensive observation of seizure attack to animals; it requires simple devices thus easy to popularize; and it does no injure or necrosis to neuronal, which simplifies the further detection of indexes of brain tissue; with the advantages mentioned above, the model is perfectly suitable for morphological observation of epileptic brain tissue.

Consequently, the study chose chronic epilepsy animal model with PTZ chemical kindling as the research object. PTZ is a tetrazole derivative and we have not fully understood about the epileptogenic mechanisms. Early studies suggested that it mainly acted on cortex and later researches found that the activation of neurons within midbrain nonspecific reticular system was prior to cortical neurons (Shiqian and Baozhen, 2014), and PTZ might interact complexly with gamma aminobutyric acid (GABA)-benzodiazepine-chloridion channel at the synaptic level, to some extent, decreasing the inhibitory voltage and promoting the onset of epilepsy. PTZ itself has no special neurotoxic effects, Therefore it is the ideal model for researching the relationship between the onset of epilepsy and neuron damage. (Chengqing and Yujun, 2012). An experiment suggested that during the process of PTZ igniting rat (the process of epilepsy formation), the hippocampal neural cells were activated to recombine the neure and the pathological neural relationship formed. As the incubation period went on, abnormal neural network formed. Once the network formed stable relationship with the encephalic region, which controls motion, clinical convulsions occurred, and the incubation period ended. It indicated that hippocampal neuronal activation was one of the important mechanisms of rat igniting and epilepsy formation (Ran and Hua, 2013).

Cell apoptosis is an active death process to main the stability of internal environment when the cells are stimulated by some kind of signal or some factors. It is closely related with many physiological and pathological

Table 1 The main equipment and reagent

EEG machine	Shanghai Photoelectric Medical Electronic Instrument Co., Ltd.
Optical microscope	Olympus, Japan
Paraffin slicing machine	LEICA Company, Germany
PTZ	Sigma Company, USA
VPA	Hunan Xiangzhong Pharmaceutical Co., Ltd.
Rabbit antimouse Bcl-2, Bax monoclonal antibody dilution	Beijing Zhongshan Biological Technology
TUNEL Kit	Beijing Zhongshan Biological Technology

Table2 Comparison of the apoptotic neurons and Bcl-2 and Bax positive neurons during goups (Mean ± SD)

Group	Cell apoptosis%	The expression of Bcl-2	The expression of Bax
NS group	4.17±0.84	18.50±8.36◆	10.26±6.59◆
PTZ group	6.91±1.02	48.54±13.61▲	59.16±13.12▲
VPA group	5.62±0.92	50.56±10.53▲	24.46±11.62◆

▲: P<0.05,compared with NS group;◆: P<0.05, compared with PTZ group; The normal control group, PTZ group and rare hippocampus cell apoptosis of rats in VPA group. Shown in the fig. 1, 2, 3.

processes such as growth, differentiation and death of cells and the occurrence and development of tumor, and it has become a hot research topic in biology and medicine. To date, many studies have confirmed the presence of neurons apoptosis in animal models of epilepsy, Clear programmed cell death was found in epilepsy model with different mechanisms, especially in hippocampus. (Wenlian, 2013). Therefore, some scholars speculated that epilepsy caused by brain damage was mainly dominated by the neuronal apoptosis, and its mechanism may be the result of neural excitotoxic cascade reaction of the onset of epilepsy, and puts forward the anti-epilepsy treatment may be efficient in preventing brain damage after epileptic occurs.

The experimental results showed that in the model of PTZ epilepsy rats, hippocampal neuron had positive apoptotic cells in different amount, but there was no significant increase compared with non-epileptic rats, which might be related to the onset intensity of epilepsy in rats. Bcl-2, Bax and their corresponding proteins played an important role. The main function of Bcl-2 genes was to inhibit apoptosis and prolong cell life, while Bax gene played a role in accelerating cell death (Yuanhua *et al*., 2010).

Apoptosis is a complex active death process involving multi-gene regulations, of which Bcl-2 gene family plays an important role in the regulation process of apoptosis. Its members are divided into two categories according to their roles in apoptosis: first is pro-apoptotic genes, including Bax, Bak, Bad, etc. and the other is anti-apoptotic genes, including Bcl-2, Bcl-xL, Bcl-w, etc.; Bcl-2 gene family members themselves or between themselves have the ability of forming dimer or multimer. Protein-protein interactions of Bcl-2 family regulate the survival and apoptosis of cells and the interaction between Bax and Bcl-2 is the center. Increase of Bax promotes cell apoptosis while increase of Bcl-2 inhibits cell apoptosis.

VPA, as a broad-spectrum antiepileptic drug, can effectively control the occurrence of various types of epilepsy. Antiepileptic effect of VPA may be accomplished by multiple ways, but they are not clearly understood. It is generally believed that the role of A subunit of the GABA can increase the synthesis and release of the GABA, and improve the appetency of postsynaptic components and GABA. Recently the literature has proposed that it can cut off the internal flow of sodium of voltage gate, and reduce the T-type calcium influx of primary afferent neurons, and decline the release of γ- hydroxybutyric acid that has the effect of causing epilepsy and lower the nervous excited effect induced by glutamate receptor of N- methyl -D- asparaginic acid (NMDA) subtype. In recent years, the research on nerve protection and anti-apoptosis of VPA has broken through the scope of anti-epileptic. And the study results find that VPA has complex pharmacological effect. The drug effectiveness refers to the treatment of mania and migraine; its mechanism may be related to its regulation of the transmission of dopamine and 5-hydroxytryptamine (Rao *et al*., 2014).

The test results of TUNEL demonstrated that the number of positive cells in hippocampal neurons declined compared with the untreated group after controlling the epileptic seizure effectively using VPA, but there was no obvious difference, which might be related to different degrees of epileptic seizure in each rat. The study said that the reason might be that VPA can induce the expression of Bcl-2 protein, and inhibit the expression of Bax protein, which led to the increased ratio of Bcl-2/Bax and stable mitochondrial membrane, thereby achieving

the function of anti apoptosis. The results of immunohistochemistry showed that the expression of Bcl-2 in hippocampal neurons of rats in VPA group increased significantly, and had significant difference compared with NS group ($P<0.05$), while there was no significant difference compared with PTZ group ($P>0.05$). The Bax in hippocampal neurons of rats in VPA group had no significant change compared with NS group ($P>0.05$), and decreased significantly compared with PTZ group ($P<0.05$). It indicated that VPA can urge the expression of Bcl-2 in hippocampal neurons of rats increased significantly and inhibit the expression of Bax, which can prevent the apoptosis of hippocampus cells effectively. This result supported that VPA might have the direct anti-apoptosis function (Zhangyu and Yuanxiao, 2012).

This study confirmed that the apoptosis of hippocampal neurons had no obvious change compared with the control group after the application of VPA in the treatment of epilepsy in rats, and the expression of Bcl-2 protein increased, and the expression of Bax protein reduced, which made the ratio of Bcl-2/Bax increased, thereby inhibiting the apoptosis.

Doctors must keep a clear understanding towards cognitive impairment of epilepsy in the clinical work so as to reduce the iatrogenic damage and take effective measures for prevention and control. How to choose the most appropriate drug in antiepileptic drugs so as to ensure the best treatment for each patient becomes one of the main problems in the current treatment of epilepsy.

CONCLUSION

Anti-epileptic drugs VPA does no harm to epileptic rats, the apoptosis of hippocampal neurons after the application of antiepileptic drugs may be related to the decreased expression of Bcl-2 and increased expression of Bax.

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