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Oxcarbazepine causes neurocyte apoptosis and developing brain damage by triggering Bax/Bcl-2 signaling pathway mediated caspase 3 activation in neonatal rats

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Abstract. – OBJECTIVE: Anti-epileptic drugs (AEDs) are the main methods for treatment of neonatal seizures; however, a few AEDs may cause developing brain damage of neonate. This study aims to investigate effects of oxcarbazepine (OXC) on developing brain damage of neonatal rats.

MATERIALS AND METHODS: Both of neonatal and adult rats were divided into 6 groups, including Control, OXC 187.5 mg/kg, OXC 281.25 mg/kg, OXC 375 mg/kg group, LEV and PHT group. Body weight and brain weight were evaluated. Hematoxylin and eosin (HE) and Nissl staining were used to observe neurocyte morphology and Nissl bodies, respectively. Apoptosis was examined using TUNEL assay, and caspase 8 activity was evaluated using spectrophotometer method. Cytochrome C-release was evaluated using flow cytometry. Western blot was used to examine Bax and Bcl-2 expression.

RESULTS: OXC 375 mg/kg treatment significantly decreased brain weight compared to Control group in neonatal rats (P5 rats) ($p<0.05$). OXC administration causes histological changes of neurocytes. OXC 281.25 mg/kg or more concentration significantly decreased neurocytes counts and increased TUNEL-staining positive neurocytes compared to Control group ($p<0.05$). OXC 281.25 mg/kg and OXC 375 mg/kg significantly increased caspase 3 activity compared to Control group in P5 rats ($p<0.05$). OXC 281.25 mg/kg and OXC 375 mg/kg significantly increased Bax, Bax/Bcl-2 ratio and cytochrome C release in frontal lobes compared to Control group in P5 rats ($p<0.05$).

CONCLUSIONS: Oxcarbazepine at a concentration of 281.25 mg/kg or more causes neurocyte apoptosis and developing brain damage by triggering Bax/Bcl-2 signaling pathway mediated caspase 3 activation in neonatal rats.

Key Words:
Antiepileptic drugs, Oxcarbazepine, Neonate, Developing brain, Bax/Bcl-2.

Introduction

The seizure is a sudden change in behavior caused by synchronous, rhythmic firing of neurons in the brain. The incidence of seizure in term of newborn was 1.5/1000-3.5/1000, and the incidence was higher in the premature infants, about 10/1000-130/1000[1,2]. Epilepsy poses a special challenge for development regulation because both the seizure and many of the current drug therapies used to treat it.

Antiepileptic drugs (AEDs) are still the main methods for treatment of neonatal seizures. Clinical and experimental evidence indicated that the recurrent seizure in the neonates could have some long-lasting adverse consequences, such as cognitive impairment, microcephaly[3,4]. On the other hand, recent experimental data also raised the concerns about the potential unfavorable affects of AEDs in the critical maturational periods[5,6]. Not all AEDs have (lead to) detrimental impact on the immature brain. The apoptosis in neonate rats has been confirmed to be induced by several old AEDs, such as phenobarbital (PB), phenytoin (PHT) and nitrazepam (NP)[7]. Moreover, there was no side effect of some drugs on the brain developing, such as levetiracetam (LEV) and topiramate (TPM)[8], even they play a protective effect on the neurons.

Oxcarbazepine (OXC) is the new second generation of AEDs, which showed an improved tolerability, a reduced propensity to cause liver enzyme induction and auto-induction, compared with traditional antiepileptic carbamazepine, oxcarbazepine[9]. The OXC has been listed as one of the first-line AEDs, which treated for partial epilepsy[10]. Many countries have limited the AEDs application for the epilepsy therapy and preven-

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tion of the children under 2 years old[11,12]. However, in the recent years, OXC has been relatively well studied in pediatric seizure patients, such as the antiepileptic application, and mono-therapy usage[13,14]. These studies have demonstrated that OXC illustrates good efficacy, safety and tolerability for patients as young as 1 month old[15,16].

There is little research to investigate the effect of OXC on the neonate brain. This work aims to discuss the effect of different concentrations of OXC on the developing brain of the neonatal rat brains. The second drug, PHT, which was proved to have side effect on the brain development, and the third drug, LEV, preliminarily proved to be safety, have been used as controls. The present study would provided valuable basis and data for the rational use of OXC in the clinical.

Materials and Methods

Rats

Total of 48 clean Wistar neonatal rats (P5 rats, 5 days post-birth, weight 9.5-13.5 g, selecting male and female randomly) and 48 clean adult rats (P60 rats, 60 days post-birth, weight 220-250 g, selecting male and female randomly) were purchased from the Experimental Animal Center of Daping Hospital, Third Military Medical University, Chongqing, China. The rats were housed in the environment of the ligh/dark cycle of 12 h/12 h at the room temperature. All of experiments were approved by the Ethics Committee of Daping Hospital, Third Military Medical University, Chongqing, China.

Trial Grouping

Both neonatal and adult rats were divided into 6 groups, including Control group (was intragastricly administrated with normal saline), OXC 187.5 mg/kg group (was intragastricly administrated with OXC at the final concentration of 187.5 mg/kg), OXC 281.25 mg/kg group (was intragastricly administrated with OXC at the final concentration of 281.25 mg/kg), OXC 375 mg/kg group (was intragastricly administrated with OXC at the final concentration of 375 mg/kg), LEV group (was intragastricly administrated with LEV at final concentration of 375 mg/kg) and PHT group (was intragastricly administrated with PHT at final concentration of 100 mg/kg). All of the above dosages were calculated as follows: human dosage (mg/kg) × rat body weight (kg) × 6.25. The equivalence calculation of human dosage in rats is based on the body surface area[17].

The PHT was purchased from Sigma-Aldrich. (St. Louis, MO, USA), the OXC was purchased from Novartis Pharma S.A.S. (Boston, MA, USA) and the LEV was purchased from UCB Pharma (Brussels, Belgium).

Sample Preparation

Twenty-four hours post drug administration, the mice were anesthetized by using the intraperitoneal injections of pentobarbital, and euthanized. The bilateral intact brains were quickly isolated, and were washed with phosphate-buffered saline (PBS) for three times (5 min per time). The water outside the brains was removed by using the filter paper (Whatman, London, UK). The above brains were divided into 3 parts, one part was fixed with 4% paraformaldehyde for 24 h, and used for hematoxylin and eosin (HE) staining and Nissl staining, one part (fresh brain tissue) was used to prepare the single cell suspension, and another part was used to exact the protein (for Western blot assay).

The hippocampus and frontal lobes isolation, the hippocampal slices and frontal lobes slices were prepared, maintained and treated according to the previous study described[18].

Body weight and Brain Weight Evaluation

For the brain weight, the water outside the brains was removed and weighted by using the A-120-CSI electronic balance (Castilla y Leon, Spain), which could accurate to 0.01 g. The body weight was weighted by using electronic balance 24 h post the drug administration.

Hematoxylin and eosin (HE) staining

Briefly, the hippocampus and frontal lobes were fixed with the 4% paraformaldehyde and were paraffin-embedded. Then, the tissues were cut at the thickness of 4 μm of slices (sections), and stained by using the hematoxylin and eosin (HE) by employing the standard processes and the images were captured by using the inverted microscope, according to the previous study described[19].

Nissl Staining

The hippocampus and frontal lobes were fixed with 10% paraformaldehyde and stained for the Nissl bodies by using the thionin dependent protocol, and cover-slipped with the distyrene plasticizer xylene (DPX, Merck, Poole, UK) according to the previous published study[20]. The hippocampus and frontal lobes were briefly dehydrated in

different grades of the ethanol (50%, 75% and 90% for 3 min, respectively), and cover-slipped by using the DPX. The slices were washed by using distilled water and incubated with 0.3% thionine from 50°C to 60°C for 20-30 min, and washed with distilled water. Then, the slices were differentiated by using the 95% ethanol for 30 s, made to be transparent by using xylene, and were cover-slipped by using neutral gum. Finally, the neuronal morphology in every region of hippocampus and cortex of frontal lobes were observed by inverted microscope.

TUNEL Assay

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) staining was used to evaluate the apoptosis of neurons. The hippocampus and frontal lobes were fixed with 4% paraformaldehyde overnight, paraffin-embedded and cut into sections for the TUNEL staining by using the *in situ* Apoptosis Detection Kit (Roche Diagnostics, Indianapolis, IN, USA). Then, the cxylene was used to de-paraffinize the paraffin-embedded brain tissues for 20 min, and the ethanol (75%, 85%, 95% and 100% for 3 min, respectively) series were used to rehydrate the brain tissues. The brain tissues were incubated with the proteinase K (at final concentration of 20 μg/ml in 10 mM Tris/HCl) for 30 min. The endogenous peroxidase activity was blocked with the 0.3% H_2O_2 in methanol for 10 min. The hippocampus and frontal lobes slices were permeabilized by using 0.1% sodium citrate and 0.1% Triton-X-100 for 5 min. Then, the slices were washed with the phosphate buffered saline (PBS) for 10 min and three times, and were incubated by using TUNEL reaction mixture at 37°C for 60 min. The slices were incubated by using a convertor-POD in humidity chamber for 30 min at 37°C. The slices were washed with PBS again for three times, and the color was developed by using a diaminobenzidine (DAB) substrate solution for 15 min. Finally, the slices were observed by using the light microscopy, and the cells illustrating the apoptotic morphology and TUNEL-staining positive cells were identified as apoptotic cells[21].

Cytochrome C Release Evaluation

In this study, the cytochrome C released from the mitochondria[22] was evaluated by using the Cytochrome C release apoptosis assay kit (Catalogue No. QIA87, Calbiochem., Merck, KGaA, Darmstadt, Germany) according to the instruction of manufacturer. The cell acquisition was conducted by using the FACS Calibur flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) and the Cell Quest Pro. software (BD Biosciences, Franklin Lakes, NJ, USA).

Caspase 3 and Caspase 8 Activity Determination

In this study, the activity of caspase 3 and caspase 8 was determined by using the spectrophotometer method according to the instructions of caspase 3 detection kit and caspase 8 detection kit (Jiangsu Keygentec. Co. Ltd., Nanjing, China). Briefly, a total of 50 μl cell lysate (5×10^4 cells) were dissolved in 50 μl reaction buffer (1 mM EDTA, 40 mmol/L HEPES, 20% glycerol and 4 mmol/L DTT, 400 μmol/L DEVD-pNA substrate, Sigma-Aldrich, St. Louis, MO, USA). The above mixture was treated for 4 h at 37°C, and the caspase 3 and caspase 8 activities were examined by measuring optical density (OD) values by using spectrophotometer at wavelength of 405 nm.

Western Blot Assay

The extracted hippocampus and frontal lobes proteins were separated by using 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Sigma-Aldrich, St. Louis, MO, USA) and electro-transferred onto the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The PVDF membranes were blocked by using the 5% defatted milk for 2 h at 4°C overnight. Then, the PVDF membranes were incubated with mouse anti-rat Bcl-2 monoclonal antibody (Catalogue No: sc-23960, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-rat Bax polyclonal antibody (Catalogue No. ab32503, Epitomics Biotech., Co. Ltd., Burlingame, CA, USA) and mouse anti-rat GAPDH monoclonal antibody (Catalogue No. sc-166545, Santa Cruz Biotechnology, Santa Cruz, CA, USA.) for 2 h at room temperature. The PVDF membranes were then incubated with horse-radish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Catalogue No. ZB-2301, ZSGB Bio. Tech. Co. Ltd., Beijing, China) and goat anti-mouse IgG (Catalogue No. ZB-2305, ZSGB Biotech., Co., Ltd., Beijing, China) at 37°C for 1 h. Finally, the Western blot bands were visualized by employing the enhanced chemiluminescent (ECL) kit (Millipore, Billerica, MA, USA).

Statistical Analysis

The data were described as mean ± standard deviation (SD) and analyzed by utilizing SPSS

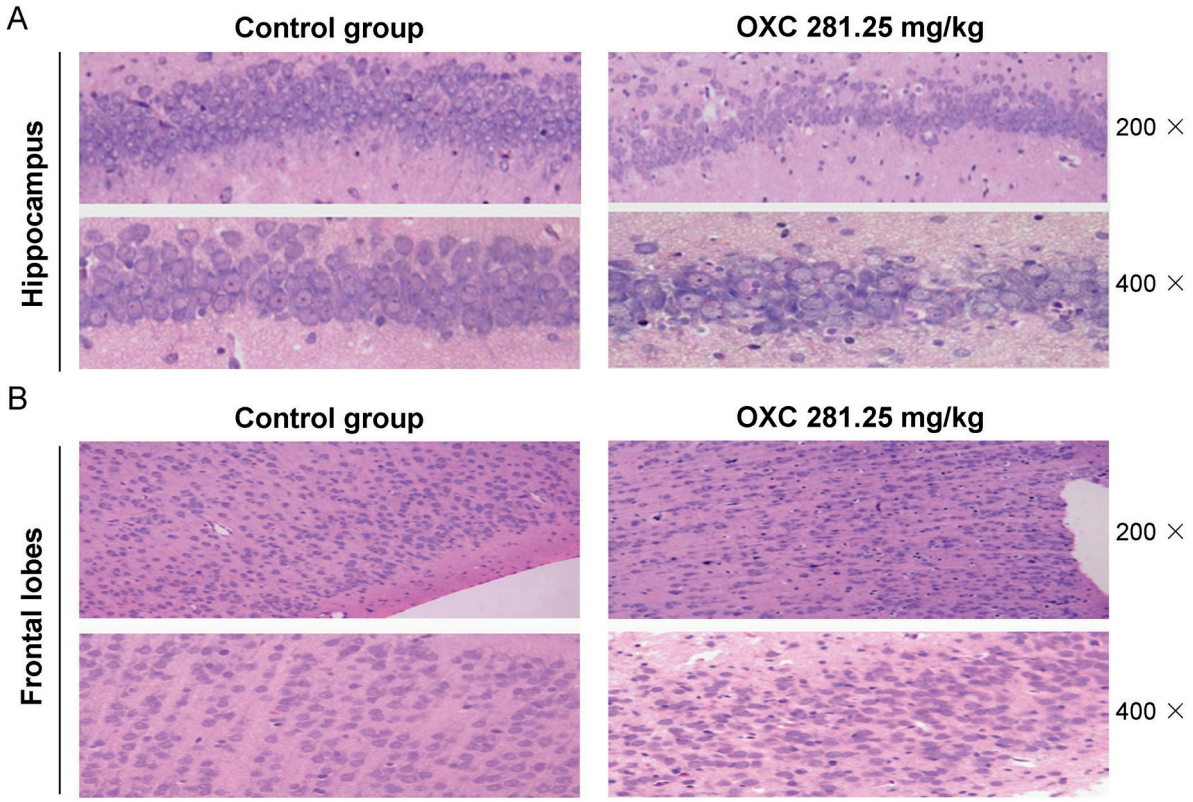

Figure 1. The HE staining for the hippocampus and frontal lobes in normal control rats and OXC 281.25 mg/kg treated neonatal rats (P5 rats). ***A,*** HE staining for the hippocampus in both Control group and OXC 281.25 mg/kg group in P5 rats. ***B,*** HE staining for the frontal lobes in both Control group and OXC 281.25 mg/kg group in P5 rats (×200 and ×400).

software 16.0 (SPSS Inc., Chicago, IL, USA). All of the data were obtained from at least six independent experiments. Student's *t*-test was used for statistical analysis between two groups. A statistical significance was defined when less than 0.05.

Results

OXC Administration Decreases Brain Weight

Three dosage of OXC treatment resulted in the decrease of brain weight and body weight both in P5 rats and P60 rats; however, only the brain weight in OXC 375 mg/kg group was significantly decreased compared to Control group in P5 rats (Table I, $p<0.05$). Meanwhile, the LEV and PHT treatment had no effect on the brain weight and body weight in both P5 rats and P60 rats (Table I, $p>0.05$).

OXC Administration Causes Histological Changes of Neurocytes

The HE staining is always used to observe the histological changes of the neurocytes of brains. The results indicated that in the Control group, the morphology of neurocytes in hippocampus (Figure 1A) and frontal lobes (Figure 1B) was oval and round in the shape and the nucleus were clearly to be observed. A large number of the apoptotic neurocytes with the cell gaps, karyopyknosis, and cell debris, were observed in the OXC 281.25 group both in hippocampus and frontal lobes of P5 rats (Figure 1).

OXC Administration Decreases Neurocytes Counts in Frontal Lobes

The counts of the neurocytes in the frontal lobes exhibit the developing of the brain; therefore, we evaluated the neurocytes in the frontal lobes. The results indicated that the OXC 281.25 mg/kg group and OXC 375 mg/kg significantly decreased the counts of neurocytes compared to the Control group in P5 rats (Figure 2, Table II, $p<0.05$). However, there were even no effects of LEV and PHT on the neurocytes in frontal lobes in both P5 rats and P60 rats (Table II, $p>0.05$).

Table I. The body weight and brain weight of the rats after AEDs application.

Group	P5 rats		P60 rats	
	Body weight (g)	Brain weight (g)	Body weight (g)	Brain weight (g)
Control group	12.50±0.95	0.51±0.03	233.70±9.58	2.30±0.11
OXC 187.5 mg/kg	12.34±1.78	0.51±0.06	235.68±11.59	2.38±0.18
OXC 281.25 mg/kg	11.70±0.82	0.48±0.03	232.23±19.18	2.28±0.12
OXC 375 mg/kg	11.17±1.09*	0.47±0.02	232.50±17.86	2.25±0.18
LEV	12.61±1.53	0.51±0.05	233.68±16.29	2.34±0.17
PHT	10.60±0.89**	0.43±0.03*	231.05±8.92	2.28±0.12

*$p<0.05$, **$p<0.01$ *vs.* NS group.

Table II. Cell counting of the neurocytes in frontal lobes of neonate and adult rats by Nissl staining after AEDs application.

Group	P5 rats	P60 rats
Control group	136.33±8.50	84.00±7.55
OXC 187.5 mg/kg	124.50±7.94	80.25±7.72
OXC 281.25 mg/kg	113.00±6.27*	76.50±8.39
OXC 375 mg/kg	105.25±8.73*	64.75±7.27*
LEV	124.75±10.81	83.50±7.33
PHT	103.25±5.38*	77.00±4.69

*$p<0.05$ *vs.* Control group.

OXC Increases Counts of TUNEL-Positive Neurocytes in Both Hippocampus and Frontal Lobes

In order to observe the apoptosis of the neurocytes of hippocampus and frontal lobes, the TUNEL staining was applied in this experiment. The results showed that both of OXC 281.25 mg/kg and OXC 375 mg/kg significantly increased the TUNEL staining neurocytes compared to Control group in both hippocampus and frontal lobes of P5 rats (Figure 3, Table III, $p<0.05$). Meanwhile, only the OXC 375 mg/kg treatment significantly increased TUNEL staining neurocytes compared to Control group in P60 rats (Figure 3, Table III, $p<0.05$). The PHT administration also significantly increased the TUNEL staining neurocytes compared to Control group (Table III, $p<0.05$) in P5 rats. However, there were no effects of LEV treatment on the TUNEL staining neurocytes in both P5 rats and P60 rats (Table III).

OXC Activates Caspase 3 activity in Hippocampus and Frontal Lobes

The spectrophotometer determination results showed that both of OXC 281.25 mg/kg and OXC 375 mg/kg significantly increased the caspase 3 (Table IV) activity compared to Control group in P5 rats; however, only OXC 375 mg/kg group significantly increased caspase 3 compared to Control group in P60 rats. Meanwhile, the PHT also triggered significantly increased caspase 3 (Table IV) activity compared to Control group in P5 rats ($p<0.01$).

However, there are no effects of the OXC at different concentrations on the caspase 8 activity in the hippocampus and frontal lobes in both P5 rats and P60 rats (Table V).

Table III. Cell counting of TUNEL positive neurocytes in hippocampus and frontal lobes of neonatal and adult rats after administration with AEDs.

Group	P5 rats		P60 rats	
	hippocampus	Frontal lobes	hippocampus	Frontal lobes
Control group	36.00±6.04	35.75±5.91	25.50±2.88	23.67±3.93
OXC 187.5 mg/kg	41.20±6.30	39.00±5.48	25.17±4.07	26.33±4.55
OXC 281.25 mg/kg	47.60±5.18*	62.50±5.32**	27.17±4.70	25.83±1.47
OXC375 mg/kg	51.40±5.77**	72.75±7.50**	31.17±3.54*	31.67±2.94**
LEV	34.20±4.09	37.00±3.16	25.50±2.88	27.83±1.94
PHT	51.20±3.49**	67.50±8.23**	28.17±1.94	27.50±4.04

*$p<0.05$, **$p<0.01$ *vs.* Control group.

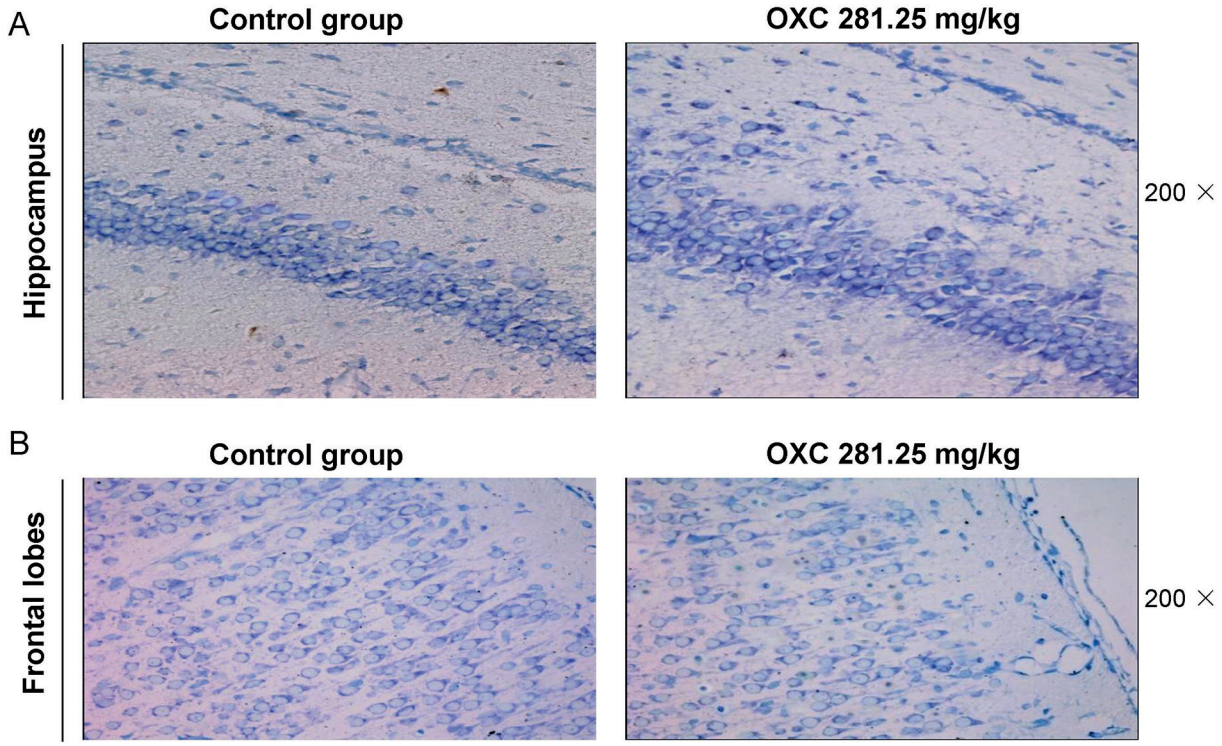

Figure 2. The Nissl staining for the hippocampus and frontal lobes in normal control rats and OXC 281.25 mg/kg treated neonatal rats (P5 rats). ***A,*** Nissl staining for the hippocampus in both Control group and OXC 281.25 mg/kg group in P5 rats. ***B,*** Nissl staining for the frontal lobes in both Control group and OXC 281.25 mg/kg group in P5 rats (×200).

Table IV. OD values of caspase-3 in hippocampus and frontal lobes of the rats after AEDs application (λ=405 nm).

	P5 rats		P60 rats	
Group	**Hippocampus**	**Frontal lobes**	**Hippocampus**	**Frontal lobes**
Control group	0.66±0.12	0.71±0.06	0.36±0.05	0.35±0.02
OXC 187.5 mg/kg	0.74±0.11	0.77±0.13	0.39±0.04	0.37±0.02
OXC 281.25 mg/kg	0.93±0.18*	1.28±0.14*	0.41±0.04	0.41±0.05
OXC 375 mg/kg	1.09±0.13**	1.47±0.11**	0.45±0.03*	0.47±0.03**
LEV	0.84±0.10	0.80±0.05	0.40±0.04	0.42±0.01
PHT	1.03±0.12**	1.50±0.11**	0.41±0.04	0.34±0.04

*$p<0.05$, **$p<0.01$ *vs.* Control group.

OXC Administration Enhances Bax Levels and Bax/Bcl-2 ratio in Frontal Lobes

The Western blot assay results showed that the OXC 281.25 mg/kg and OXC 375 mg/kg significantly increased the Bax levels and Bax/Bcl-2 ratio in happocampus and frontal lobes compared to the Control group in both P5 rats (Figure 4, Table VI, $p<0.05$). However, only the OXC 375 mg/kg group significantly increased Bax levels and Bax/Bcl-2 ratio in P50 rats compared to Control group (Figure 4, Table VI, $p<0.05$).

OXC Administration Increases Cytochrome C Release in Hippocampus and Frontal Lobes

According to the previous published study [23], the cytochrome C release was considered as the biomarker for the apoptosis. The results showed that the cytochrome C release in OXC 281.25 mg/kg and OXC 375 mg/kg group were significantly increased compared to Control group in hippocampus and frontal lobes in both P5 rats and P60 rats (Table VII, $p<0.001$). The PHT significantly

Table V. OD values of caspase-8 in hippocampus and frontal lobes of the rats after AEDs application (λ=405 nm).

	P5 rats		P60 rats	
Group	**Hippocampus**	**Frontal lobes**	**Hippocampus**	**Frontal lobes**
Control group	0.75±0.10	0.83±0.01	0.43±0.06	0.47±0.06
OXC 187.5 mg/kg	0.85±0.08	0.94±0.06	0.36±0.06	0.45±0.05
OXC 281.25 mg/kg	0.83±0.07	0.93±0.10	0.43±0.06	0.41±0.04
OXC 375 mg/kg	0.79±0.11	0.91±0.10	0.47±0.06	0.48±0.04
LEV	0.78±0.11	0.96±0.13	0.47±0.12	0.48±0.04
PHT	0.78±0.08	0.91±0.12	0.45±0.04	0.49±0.03

$^{*}p<0.05$, $^{**}p<0.01$ *vs.* Control group.

Table VI. Expression of Bax and Bcl-2 protein in frontal lobe of the rats after single dose AEDs application.

	P5 rats			P60 rats		
Group	**Bax**	**Bcl-2**	**Bax/bcl-2**	**Bax**	**Bcl-2**	**Bax/bcl-2**
Control group	18.13±2.92	5.78±0.78	3.23±0.38	17.93±1.29	5.09±0.65	3.54±0.25
OXC 187.5 mg/kg	21.19±3.66	5.71±0.33	3.70±0.52	19.73±2.24	5.06±0.56	3.91±0.27
OXC 281.25 mg/kg	23.85±1.88*	5.73±0.32	4.23±0.39*	20.36±2.52	5.09±0.55	3.99±0.13
OXC 375 mg/kg	26.99±1.46*	5.60±0.56	4.87±0.69*	22.26±1.81*	5.04±0.65	4.43±0.24*
LEV	18.43±1.96	5.77±0.32	3.19±0.18	18.63±1.70	5.11±0.75	3.67±0.24
PHT	28.12±2.01*	5.68±0.51	5.00±0.77*	19.06±1.89	5.08±0.04	3.75±0.39

$^{*}p<0.05$, $^{*}p<0.01$ *vs.* Control group.

increased the cytochrome C release compared to Control group in both hippocampus and frontal lobes in P5 rats (Table VII, $p<0.001$). However, there were even no effects of OXC at different concentrations on cytochrome C release in hippocampus and frontal lobes in both P5 rats and P60 rats (Table VII).

Discussion

Oxcarbazepine is a molecular derived from carbamazepine (CBZ), which is rapidly and extensively metabolized due to its pharmacologically active component, 10-monohydroxy (MHD)[24]. OXC is indicated for use as monotherapy or adjuctive therapy in the treatment of partical seizure with or without secondarily generalized seizure in adults and children older than 6 years. In Taiwan and many areas it has been approved for use in infants[25]. Kwong et al[26] investigated the prescribing patterns of antiepileptic drugs in Hong Kong; the use of OXC increased 15-fold several years, especially for the young and children. Blume et al[27] identified 6099 infants with neonate seizure and summarized the treatment regime in 2009. Among them, 28 cases treated with the combination of PB and OXC, and with seizure, could not be controlled by a variety of other antiepileptic drugs[27].

The major mechanism of the brain damage on AEDs was the apoptosis of the neurons. The excessive apoptosis in the neonatal rats after the AEDs was through several mechanisms[28]. Firstly, the activator of GABA-A, such as PB. Secondly, the blocker of sodium channel, such as PHT or VPA. Thirdly, the antagonist of NMDA receptor, such as MK801. OXC is an analogue of CBZ, with comparable anticonvulsant efficacy. Due to the advantage of a lower occurrence of allergic reactions and enzyme induction, the OXC is usually better tolerated than CBZ in clinical[29]. However, several recent studies have reported that OXC causes edema and apoptosis in retinal ganglion cells, and ovarian and endometrial tissues[30,31]. Moreover, Araújo et al[32] also suggested that brain damage was still present in OXC pretreated animals.

Therefore, in this study, we investigated the effects of the different concentrations of OXC on the body/brain weight, neurocytes morphology,

Table VII. Activity of the intracellular cytochrome C in hippocampus and frontal lobe of the neonatal and adult rats after AEDs application.

	P5 rats		P60 rats	
Group	**Hippocampus**	**Frontal lobes**	**Hippocampus**	**Frontal lobes**
Control group	11.07±1.28	11.87±1.50	12.19±0.79	12.47±0.80
OXC 187.5 mg/kg	12.03±1.44	13.36±1.02	13.08±1.45	13.28±0.53
OXC 281.25 mg/kg	16.27±1.01***	19.79±1.15***	15.08±1.00**	15.45±0.60***
OXC 375 mg/kg	19.96±1.82***	21.71±2.16***	19.23±1.15***	19.96±1.76***
LEV	11.46±1.91	12.09±1.58	12.62±0.99	13.16±0.73
PHT	21.05±1.28***	22.29±3.10***	13.12±1.07	13.28±1.02

**$p<0.01$, ***$p<0.001$ *vs.* Control group.

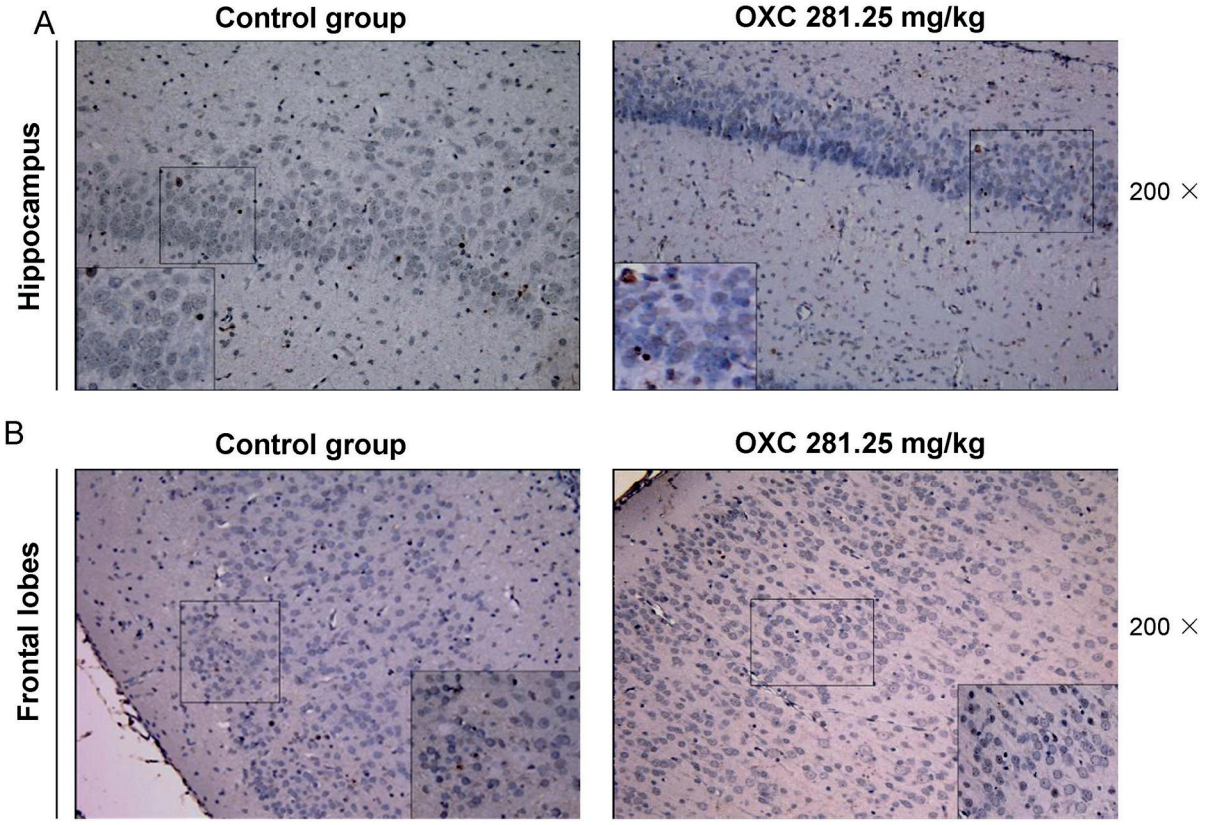

Figure 3. TUNEL positive staining neurocytes in both hippocampus and frontal lobes in neonatal rats (P5 rats). ***A,*** TUNEL staining for hippocampus in both Control and OXC 281.25 mg/kg group in P5 rats. ***B,*** TUNEL staining for frontal lobes in both Control and OXC 281.25 mg/kg group in P5 rats (×200).

neurocyte apoptosis in hippocampus and frontal lobes in both P5 rats and P60 rats. It must be considered that many anticonvulsant drugs higher dosages can even be pro-convulsant. However, the previous acute toxicity study across multiple species, reported that extremely high dosages of OXC (600-3000 mg/kg) were tolerated without any related death[33]. In the present study, we assigned the lowest dosage, medium dosage and highest dosage of 187.5 mg/kg, 281.25 mg/kg and 375 mg/kg, respectively, according to the human therapeutic dose of 30 mg/kg/d, 45 mg/kg and 60 mg/kg in clinical. However, our findings showed that the OXC concentration of 281.25 mg/kg and 375 mg/kg significantly caused the decrease of brain weight, decreased counts of neurocytes,

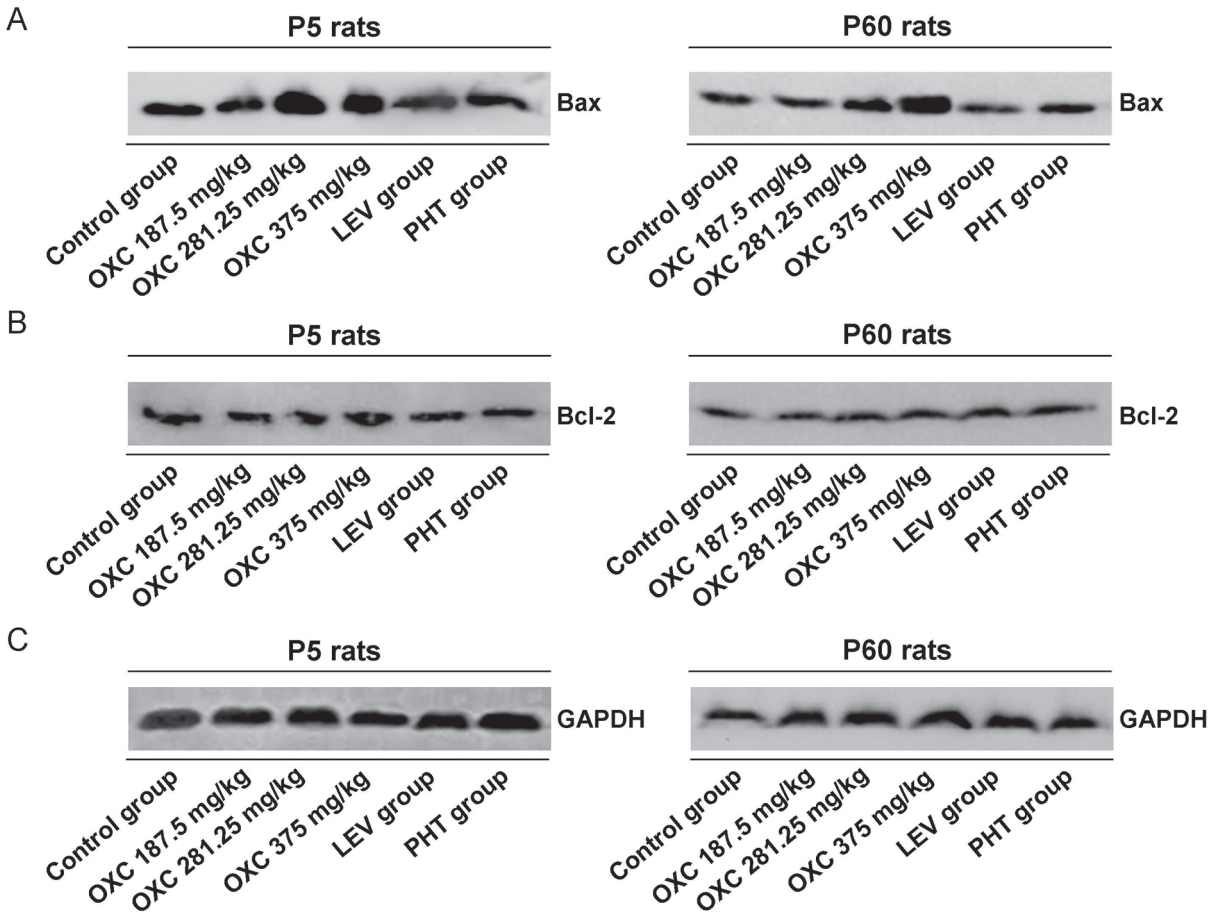

Figure 4. Western blot bands for the expression Bax, Bcl-2 and GAPDH in both hippocampus and frontal lobes in neonatal rats (P5 rats) and adult rats (P60 rats). ***A,*** Western blot bands for Bax. ***B,*** Western blot bands for Bcl-2. ***C,*** Western blot for GAPDH.

increased TUNEL-staining positive neurocytes, suggesting that the higher concentration of OXC (281.25 mg/kg and 375 mg/kg) causes the brain damage in neonatal rats (P5 rats).

In order to investigate apoptotic-signaling pathway of neurocytes, caspase 3 and caspase 8 associated apoptotic signaling pathway were evaluated. The results showed that the OXC 281.25 mg/kg and OXC 375 mg/kg significantly increased the caspase 3 activity compared to Control group in P5 rats; however, the caspase 8 signaling pathway was not involved in the apoptosis of neurocytes in P5 and P60 rats. Our results are consistent with Ambrosio et al[34] findings, which concluded that the antiepileptic drug, OXC, increased the activity of caspase 3. In this study, the mechanism causing the neurocyte apoptosis was also explored by studying the Bax/Bcla-2 signaling pathway. Bax acts as a pro-apoptotic biomarker, and Bcl-2 acts as anti-apoptotic biomarker, both of which are key molecules for the apoptosis[35]. Our results showed that the OXC 281.25 mg/kg and OXC 375 mg/kg administration significantly enhanced the Bax levels and Bax/Bcl-2 ratio in frontal lobes. Das et al[36] reported that the voltage-gated Na (+) channel blocker, OXC, also increased the Bax expression and decreased the Bcl-2 expression, not involving the research of OXC application to developing brain of rats. Therefore, this study employed the OXC for the first time in the developing brain in neonatal rats. Cytochrome C is an important biomarker for the apoptosis when it is releasing from the mitochondria to the cytoplasm[37]. Therefore, we evaluated the release of the cytochrome C in hippocampus and frontal lobes in P5 rats and P60 rats. The results indicated that OXC administration significantly increased the cytochrome C release in hippocampus and frontal lobes

in P5 rats. Moreover, the other AEDs, LEV and PHT, illustrated no effects on the brain weight, neurocyte apoptosis, Bax, Bax/Bcl-2 ratio, caspase 3 activity and cytochrome C release in hippocampus and frontal lobes in both of P5 rats and P60 rats. The PHT functioned because of the blocker of sodium channel, which shares a few similar characteristics with the OXC. OXC plays antiepileptic role by blocking the voltage-sensitive Na^+ channels. This brings brain damage on the immature brain. Therefore, the PHT also plays a few roles in causing the injury of the developing brains. However, the LEV has distinctive mechanism, which is different from OXC and PHT, and it is not involved with the other two mechanisms as before. Meanwhile, there was no evidence of damage caused by the LEV application on the developing brain. Thus, we believed that the LEV and PHT are relative safety for the application in clinical for seizure therapy, which conclusion is consistent with the previous studies[38,39].

Additionally, our results also exhibited that the effects of OXC were also age-dependent and dose-dependent. Not only in neonatal rats, but also the adult rats received the 375 mg/kg OXC, and the brain damages were also obvious. When the OXC reduced to 281.25 mg/kg, the brain damage was seen in the neonatal rats. When the rats were given OXC 187.5 mg/kg, there was no damage on the neonatal and adult rats. Therefore, associating with the clinical application, the lowest and safety dosage of OXC in human is 30 mg/kg, and higher dosages (45 mg/kg and 60 mg/kg) may induce the developing brain damage or injury. Meanwhile, in our study the AEDs were given by a single dose; if given consecutively, the brain damage may also be found with the lower OXC dosage. A previous study[40] reported that the brain damage side effects of OXC were also aggravated by coordinating with the other drugs. Therefore, in the clinical, we should consider the dosage of OXC, the age of patients and the synergistic action, and attempt to prevent the aggravated developing brain damage in clinical.

Although this study received a few interesting findings, there were also some limitations. Firstly, our experiment has not been applied the OXC and associated tests in human clinically. Secondly, the half-life of these drugs may be different in rats compared with those of human, which may cause differences on the effects of OXC in clinical. Thirdly, it is also difficult to find acute and chronic doses of antiepileptic drugs exactly in rats compared to human.

Conclusions

The present research attempted for the first time at investigating the effects of OXC on the developing brain damage in the neonatal rats. The OXC at concentration of 281.25 mg/kg (equal to 45 mg/kg in human dosage) or more causes brain weight decrease and neurocyte apoptosis by triggering the Bax/Bcl-2 signaling pathway mediated caspase 3 activation in hippocampus and frontal lobes of neonatal rats.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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