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ORIGINAL RESEARCH

The Anticonvulsant and Neuroprotective Effects of Oxysophocarpine on Pilocarpine-Induced Convulsions in Adult Male Mice

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Abstract Epilepsy is one of the prevalent and major neurological disorders, and approximately one-third of the individuals with epilepsy experience seizures that do not respond well to available medications. We investigated whether oxysophocarpine (OSC) had anticonvulsant and neuroprotective property in the pilocarpine (PILO)-treated mice. Thirty minutes prior to the PILO injection, the mice were administrated with OSC (20, 40, and 80 mg/kg) once. Seizures and electroencephalography (EEG) were observed, and then the mice were killed for Nissl and Fluoro-jade B (FJB) staining. The oxidative stress was measured at 24 h after convulsion. Western blot analysis was used to examine the expressions of the Bax, Bcl-2, and Caspase-3. In this study, we found that pretreatment with OSC (40, 80 mg/kg) significantly delayed the onset of the first convulsion and status epilepticus (SE) and reduced the incidence of SE and mortality. Analysis of EEG recordings revealed that OSC (40, 80 mg/kg) significantly reduced epileptiform discharges. Furthermore, Nissl and FJB staining showed that OSC (40, 80 mg/kg) attenuated the neuronal cell loss and degeneration in hippocampus. In addition, OSC (40, 80 mg/kg) attenuated the changes in the levels of Malondialdehyde (MDA) and strengthened glutathione peroxidase and catalase activity in the hippocampus. Western blot analysis showed that OSC (40, 80 mg/kg) significantly decreased the expressions of Bax, Caspase-3 and increased the expression of Bcl-2. Collectively, the findings of this study indicated that OSC exerted anticonvulsant and neuroprotective effects on PILO-treated mice. The beneficial effects should encourage further studies to investigate OSC as an adjuvant in epilepsy, both to prevent seizures and to protect neurons in brain.

Keywords Pilocarpine · Convulsion · Oxysophocarpine · Anticonvulsant · Neuroprotection · Neuronal damage

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Introduction

Epilepsy is one of the prevalent and major neurological disorders, which is triggered by hyper neuronal excitability and excessive hyper-synchronous discharges in the brain, characterized by recurrent unprovoked seizures, behavioral and electroencephalographic (EEG) changes (DeGiorgio et al. 1992; Manuela et al. 2012; Zeng et al. 2007). Epilepsy affects approximately 70 million people in the world (Katchanov and Birbeck 2012). It is widely recognized that pathogenesis of epilepsy is the imbalance between GABA-mediated inhibitory and glutamate-mediated excitatory

neurotransmission (Ullah et al. 2015). Furthermore, seizure can subsequently cause significant cerebral damage manifested by a particular pattern of neuronal cell loss, and apoptosis occurred preferentially in the hippocampus, and it is thought to be accompanied by a cascade of the related event, including glutamate excitotoxicity, calcium overload, and oxidative stress, eventually leading to cell death (Mikati et al. 2003; Zhao et al. 2015). Meanwhile, the activation of proteins, such as Bax, Bcl-2, and Caspase-3, contribute to neuronal apoptosis, which may play an important role in epileptic injury (Ullah et al. 2015).

In recent years, many researchers have clearly suggested that oxidative stress is involved in the pathogenesis of a number of neurologic conditions and neurodegenerative disorders including epilepsy (Folbergrova 2013; Rowley and Patel 2013). There is a constant balance between the production of reactive oxygen species and their destruction by antioxidant systems under normal circumstances; this balance has been broken in the pilocarpine (PILO) epilepsy model resulting in excessive accumulation of the reactive oxygen species. Therefore, it has been showed that oxidative stress may be one of the mechanisms of epileptic activity (Sudha et al. 2001). Previous studies have also suggested that if the damage of oxidative stress induced by seizure is ameliorated, there will be beneficial effects on epilepsy (Kamida et al. 2009; Shin et al. 2011). Some studies have showed that the increase in the level of reactive oxygen species (ROS) is the major cause of neuronal injury induced by seizures (Xie et al. 2014).

The model of epilepsy with systemic treatment PILO (a muscarinic receptor agonist) is the most important and widely used in epilepsy, which induces repeated acute seizures and status epilepticus (SE), and researches the mechanisms underlying epileptogenesis (Cavalheiro et al. 1987). It reproduces the behavioral, electrographic, and histological alterations of seizure and causes initial neuronal damage after administration of PILO in mice and rats (Covolan and Mello 2000; Fujikawa 2005; Weise et al. 2005).

Although about one-third of individuals with epilepsy still experience seizures that do not respond well to the available medications, pharmacologic therapy remains the best remedy to treat the conditions (Perucca et al. 2007). Accumulating evidence from the clinical practice has showed that many antiepileptic drugs (AEDs) have some side effects, such as cognitive impairment, psychiatric problems, and recurring seizures (Schmitz 2006). Accordingly, there is an unmet medical need for safer and more efficacious AEDs to treat this neurologic disorder.

Oxysophocarpine (OSC), one of the major quinolizidine alkaloid isolated from *Sophora flavescens* Ait. (Leguminosae) and *S. alopecuroides* Linn. (approved by the State Food and Drug Administration of China, SFDA) and other leguminous plants of the *Robinia* (approved by the SFDA) (Yang et al. 2006), is widely used in the traditional herbal medicine for treatment of various diseases (Fig. 1). OSC has high solubility in lipids and the potential ability to permeate the blood–brain barrier (BBB) (Wang et al. 2000). Previous researches have verified that OSC has multiple pharmacological effects, including anti-inflammatory, immunosuppressive, antidepressant, antinociception, and neuroprotective effects (Zhao and Li 2009; Zhu et al. 2014). However, the anticonvulsant and neuroprotective effects of OSC have not been confirmed in the PILO-induced epilepsy model yet. Findings of a pharmacological study demonstrated that OSC can induce antinociception and increase the expression of GABAAα1 receptors in mice (Xu et al. 2013). We hypothesized that OSC was an anticonvulsant drug with greater efficacy, lower toxicity and fewer side effects. Therefore, the present study was designed to evaluate anticonvulsant activity of OSC in PILO-treated mice. We also evaluated the effects of OSC on oxidative stress and neuronal degeneration in this process.

Materials and Methods

Experiment Animals

Adult male Institute of Cancer Research (ICR) mice (Experimental Animal Center of Ningxia Medical University, certificate NO.SYXK Ningxia 2015-0001) weighing between 21 and 25 g were used in all experiments. All animals were housed in the standard plastic cages with free access to food and water, and kept in a room with control environment (23 ± 2 °C) under a 12:12 h light/dark cycle. All animal procedures were carried out in accordance with

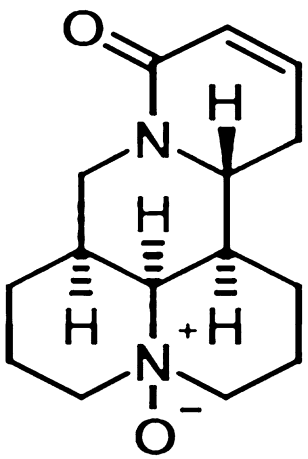

Fig. 1 Structure of oxysophocarpine (OSC). The molecular formula for OSC is $C_{15}H_{22}N_2O_2$ and the molecular weight is 262.35

the National Institute of Health Guide for the care and use of Laboratory Animals, and were approved by the Animal Ethics Committee of Ningxia Medical University.

Induction of Convulsion

In order to induce convulsion, the ICR mice received an injection of PILO (280 mg/kg, i.p, Sigma, USA). All mice were administered with methylscopolamine (1 mg/kg, i.p.) 15 min before the administration of PILO injection to minimize the peripheral effects of PILO. After each PILO administration, all mice were continuously observed for any behaviors indicative of activity for 90 min. When mice had experienced stages 4–5 seizures or SE (continuous generalized seizure activity) for 90 min, convulsions were terminated by intraperitoneal injection of 10 mg/kg diazepam to reduce the mortality rate (Xie et al. 2014).

Seizure activity was graded according to the Racine scale of grading of convulsion with some modifications (Racine 1972). The observed parameters were latency to the first convulsion (stages 4–5 seizures) and time to the SE. Besides, the percentage of survival within 24 h as well as duration of SE was also evaluated.

Drugs and Groups

In this study, a total of 120 mice were randomly divided into six groups (20 mice in each group). Thirty minutes prior to the PILO injection, the mice in each groups was administered intraperitoneally either OSC (dissolve in 0.9 % saline, Zi Jin Hua Pharmaceutical Co., purity >98 %) or 0.9 % saline or phenobarbital: (1) CON group, 0.9 % saline alone; (2) PILO group, 0.9 % saline and PILO; (3) PB group, Phenobarbital (30 mg/kg, i.p.) and PILO; (4) OSC 20 group, OSC (20 mg/kg, i.p.) and PILO; (5) OSC 40 group, OSC (40 mg/kg, i.p.) and PILO; and (6) OSC 80 group, OSC (80 mg/kg, i.p.) and PILO.

Cortical EEG Recording and Analysis

After anesthetizing by a 3.5 % chloral hydrate, the animals ($n = 8$ per group) were fixed into the stereotaxic apparatus to surgically implant the monopolar electrodes, which consisted of a polyurethane-coated stainless steel wire (100 um in diameter). Briefly, the recording electrode was implanted into the left frontal cortex (1.8 mm lateral to the midline, 1.5 mm anterior to the bregma), and the reference electrode was implanted over cerebellum (1.5 mm posterior to the lambda, on midline) for EEG recording (Zheng et al. 2016); then the dental acrylic (Dental acrylic is a mixture of numerous alloys using for dental restoration) was used to fix the electrodes. A week after the surgery, OSC and Phenobarbital (PB) were administered by intraperitoneal injection once. The EEG seizures in the cortical were recorded for 30 min using biological signal processing system (SMUP-U4) after the final injection OSC and PB. The signals were amplified 2000 times and filtered between 1 and 50 Hz. The data were examined by two individuals to quantify the number of spike wave discharges in 10 min after injection of pilocarpine, and the mean amplitude during EEG recording session.

Oxidative Stress

At 24 h after convulsion, the mice ($n = 8$ per group) were deeply anesthetized with 3.5 % chloral hydrate and killed by decapitation. The brains were quickly removed and placed on ice, and hippocampus were dissected from brain for the valuation of malondialdehyde (MDA) content, glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT) activities. For all of the experimental procedures, each hippocampus sample was homogenized to be 10 % (w/v) homogenates with ice-cold 0.9 % saline (9 volumes) using a glass homogenate. The homogenate was centrifuged at 3500 rpm at 4 °C for 15 min; then the supernatant was examined using a microplate reader (1510; Thermo Fisher Scientific, Waltham, MA, USA) according to the instructions provided with the assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Lipid Peroxidation Levels

Lipid peroxidation levels were analyzed by measuring the thiobarbituric reactive species (TBARS) in homogenates. in which the thiobarbituric acid reactive substances react with thiobarbituric acid to produce a red colored complex having a peak absorbance at 535 nm. The experimental results above were expressed as nmol of malondialdehyde (MDA)/mg protein.

Glutathione Peroxidase Activities

GSH-Px level was assayed spectrophotometrically and expressed as u/mg of protein. It depended on the reduction of 5,50-dithiobis(2-nitrobenzoic acid) with glutathione to produce a yellow color whose absorbance is measured at 405 nm.

Superoxide Dismutase (SOD) Activities

SOD activity was assayed by xanthine and xanthine oxidase to generate superoxide radicals. This method was based on the capacity of SOD in inhibiting autoxidation of adrenaline to adrenochrome. The results were expressed as u/mg of protein.

Catalase (CAT) Activities

Catalase activity was measured by the method that uses H_2O_2 to generate H_2O and O_2. The activity was measured by the degree of this reaction. Catalase reacted with a known quantity of hydrogen peroxide, and the reaction was stopped after 1 min with catalase inhibitor. Results were expressed as u/mg of protein.

Tissue Preparation for Histological Staining

For tissue analysis($n = 3$ animals per group), the animals were deeply anesthetized (3.5 % chloral hydrate, i.p.) and then transcardially perfused with 0.9 % saline solution, followed by 4 % ice-cold paraformaldehyde (PFA) after 72 h of SE onset. The brains were removed and postfixed for 12 h in 4 % PFA, then dehydrated and embedded with paraffin. The brain tissues were cut into 4-um-thick coronal sections and deparaffined in xylene, then rehydrated in gradient ethanol from 100 to 70 % for Nissl and FJB staining. The histopathological changes of the brains were observed using a microscope (Olympus, Tokyo, Japan) with high magnification (×400).

Nissl Staining

After being deparaffinized and rehydrated, coronal sections were stained in cresyl violet solution for 60 min at 56 °C, then finished quickly in distilled water, differentiated in 95 °C alcohol twice for 2 min and checked microscopically for the best result, dehydrated in 100 % alcohol for 5 min, cleared in xylene for 5 min, and mounted with neutral gum solution. Finally, for every tenth section (six sections per animal), we counted in a blinded manner the number of surviving pyramidal cells per 1 mm length in the hippocampal CA1 and CA3 region. (Hui et al. 2005).

Fluoro-Jade B Staining

FJB staining of brain slices is an established technique for revealing neurons undergoing degeneration. The slides were transferred to a solution of 0.06 % potassium permanganate for 10 min and then rinsed in distilled water for 2 min. The sections were immersed in a solution of 0.1 % acetic acid and 0.0004 % FJB (Chemicon International, USA) on a shaker. After 20 min in the staining solution, the slides were rinsed for one minute in each of the three distilled water washes and allowed to dry for 5–10 min. The dry slides were cleared by immersion in xylene for at least a minute before cover slipping with a neutral gum solution. For every tenth section (six sections per animal), FJB-positive cells in the CA1 and CA3 regions of each section per 1 mm length were counted by observers who were blind to the treatment condition (Ullah et al. 2015).

Western Blot Analysis

After 24 h of EEG record, the mice ($n = 8$ from each group) were decapitated after injection of 3.5 % chloral hydrate and were followed by a rapid removal of brains. The hippocampi were than rapidly dissected, placed on ice, and kept at −20 °C. The hippocampus tissues were homogenized in ice-cold lysis buffer (Nanjing Key Gen Biotech Co., Ltd., Nanjing, China). Total protein concentrations were determined by a BCA protein assay reagent kit (Beijing TransGen Biotech Co., Ltd.). The protein (50 ug per sample) were electrophoresed by sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE) and transferred to nitrocellulose membrane. The membrane was blocked with PBST containing 5 % (w/v) skimmed milk for 1 h and incubated with primary rabbit polyclonal antibody against Bax, Bcl-2, and Caspase-3 (Proteintech Group) at 4 °C overnight, and β-actin (20536-1-AP; Proteintech Group) served as the control. After washing three times with PBST containing 20 % Tween-20, the membrane was incubated with a secondary antibody (anti-rabbit IgG, SA00001-2; Proteintech Group). After washing with PBST, the signals were visualized using an enhanced chemiluminescence (ECL) kit in a dark chamber, and the optical densities of the bands were scanned and analyzed by densitometry using western blotting detection system (Quantity One software; Bio-Rad Laboratories, Hercules, CA, USA).

Statistical Analyses

All experimental data were analyzed by SPSS 18.0 statistical software (SPSS Inc., Chicago, IL, USA). The results were expressed as the mean ± standard error of the mean (SEM). The number of animals that SE and survived were calculated as percentages (percentage SE and percentage survival, respectively), and compared with a nonparametric test (χ^2). Other data were statistically analyzed using one-way analysis of variance (ANOVA) followed by the LSD post hoc test (compared more than two groups) or using student's t test (compared two groups), and statistical significance was set at $p < 0.05$.

Results

Behavioral Observations and EEG Findings

Except CON group and PB group, all mice in other groups exhibited high-grade seizure (stage 4–5), generalized tonic, clomic convulsion of four limbs while lying down, rearing,

and even death. The first episode of stage 4 seizures occurred at 526.40 ± 140.26 s after injection in the PILO group. PB group mice showed low-grade seizure behaviors (seizure grades 1–3), such as head bobbing, scratching, and forelimb clonus. As expected, none of the mice showed seizures in control group. The latencies to the first seizure in high-dose OSC groups were significantly prolonged ($p < 0.01$) compared to the PILO group (764.00 ± 246.68 and 1002.10 ± 389.78 s for 40 and 80 mg/kg, respectively). However, OSC at the dose of 20 mg/kg had no significant effect on the latency to the first seizure (598.50 ± 156.33 s) compared to PILO group ($p > 0.05$). Seizure developed into SE in 812.40 ± 293.31 s after PILO injection in the PILO group. When mice were pretreated with higher doses of OSC (40 and 80 mg/kg), the incidence of SE was significantly reduced; meanwhile, the time to SE was significantly increased. 58.3 % (7 of 12) mice survived in the PILO group, while 83.3 % (10 of 12) mice were still alive in each of the three groups pretreated with OSC. Apparently, all mice in the control group and PB group survived. Taken all the results together, it is apparent that pretreatment with OSC relieved PILO-induced seizures and reduced mortality rate in mice (Table 1).

After the PILO injection, EEG was monitored for 30 min to confirm the seizure. The EEG recordings from all mice except those in the control and PB groups showed abnormal activities, such as high-amplitude spikes, frequent polyspikes, and spike wave complexes. However, in the mice pretreated with OSC (40 and 80 mg/kg), extent of the abnormality in EEG was significantly reduced, and these include a decreased number of spike wave discharges and decreased amplitude of spikes (Fig. 2).

OSC Pretreatment Reversed the Degree of Oxidative Stress in Hippocampus

The MDA level of hippocampus significantly increased in PILO group compared to control group ($p < 0.05$). Pretreatment with OSC (40 and 80 mg/kg) could remarkably reduce the MDA level in the hippocampus compared to PILO group ($p < 0.05$, $p < 0.05$, respectively). There was a remarkable fall in CAT and GSH-Px activity in hippocampal tissue in PILO group compared to control group ($p < 0.05$). In the OSC 40 and 80 groups, CAT and GSH-Px levels were increased significantly compared to PILO group (both $p < 0.05$). In the PILO group, the SOD activities in the hippocampal tissue had no markedly change compared to the control group. Three dose of OSC pretreatment also had no effect on SOD activity in hippocampus (Fig. 3).

OSC Pretreatment Rescued the Neuronal Loss Induced by Convulsion in the Hippocampus

Nissl Staining

At 3 days after convulsion, Nissl staining was used to examine the histopathological changes of hippocampal CA1 and CA3 regions. In the control group, neurons in CA1 and CA3 regions of hippocampus displayed large number of dense granule cells, pyramidal cells, and Nissl Body. In the PILO group, most neurons disappeared, and the remaining neurons are irregular, and with swelling or even crack, and Nissl Body decreased in the cytoplasm, nuclear pyknosis, or lack of cellular structure. Pretreatment

Table 1 Effect of pretreatment with OSC on pilocarpine-induced convulsions and lethality in adult male mice

Groups	Number of animals/group	Latency to first convulsion (second)	Percentage convulsion (%)	Time to SE (second)	Percentage SE (%)	Percentage survival (%)
CON	12	NS	0	NS	0	100
PILO	12	526.40 ± 140.26##	100	812.40 ± 293.31##	100 (12/12)	58.3 (7/12)
PB	12	NS	0	NS	0	100
OSC 20	12	598.50 ± 156.33	100	907.63 ± 399.96	66.7 (8/12)[a]	83.3 (10/12)[a]
OSC 40	12	764.00 ± 246.68**	100	1158.29 ± 472.30*	58.3 (7/12)[a]	83.3 (10/12)[a]
OSC 80	12	1002.10 ± 389.78**	100	1296.67 ± 174.53*	35 (3/12)[b]	83.3 (10/12)[a]

Results for latency to first convulsion and Time to SE are expressed as mean ± SEM

Data on survivors and the number of animals that SE were calculated as percentages

* $p < 0.05$ as compared to the PILO group

** $p < 0.01$ as compared to the PILO group (ANOVA and LSD post hoc test)

$p < 0.01$ as compared to the control group

[a] $p < 0.05$ as compared to the PILO group

[b] $p < 0.01$ as compared to the PILO group (χ^2 method and Fischer's exact probability test)

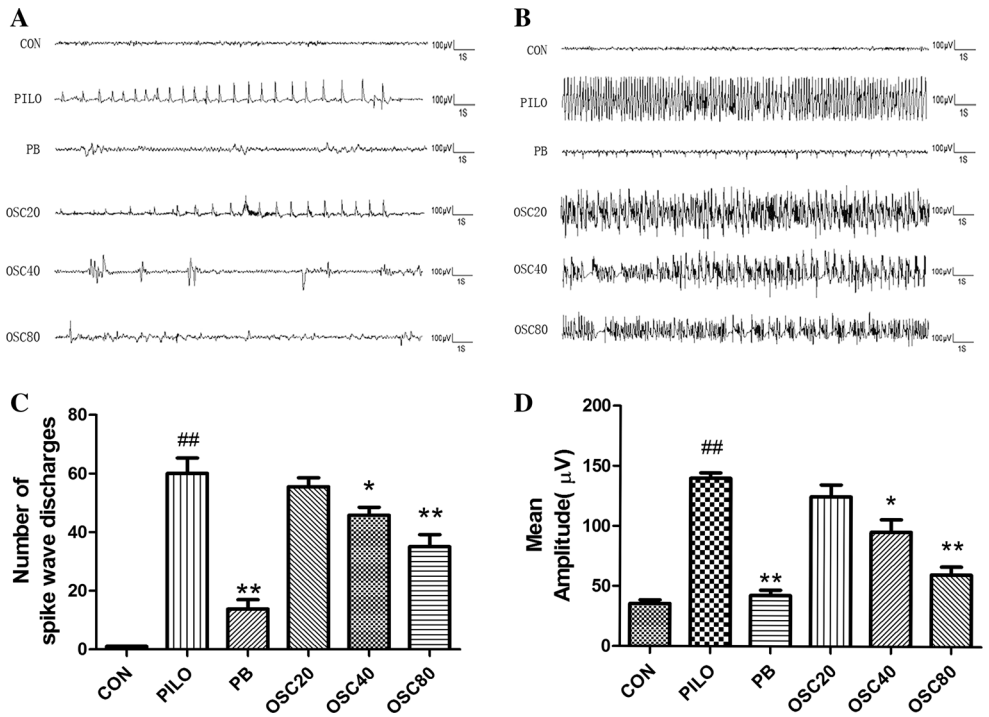

Fig. 2 Electroencephalographic (EEG) recordings from adult mice after pilocarpine treatment ($n = 8$ in each group). **a** Mice were analyzed for EEG, and representative samples were recorded 10 min after pilocarpine injection. **b** Mice were analyzed for EEG and representative samples recorded SE. **c** The number of spike wave discharges was quantified in 10 min after pilocarpine injection. **d** The mean amplitude of seizure EEG was quantified for comparison. Results are expressed as mean ± SEM. $^{\#\#}p < 0.01$ as compared to the control group, * $p < 0.05$ as compared to the PILO group, and ** $p < 0.01$ as compared to the PILO group

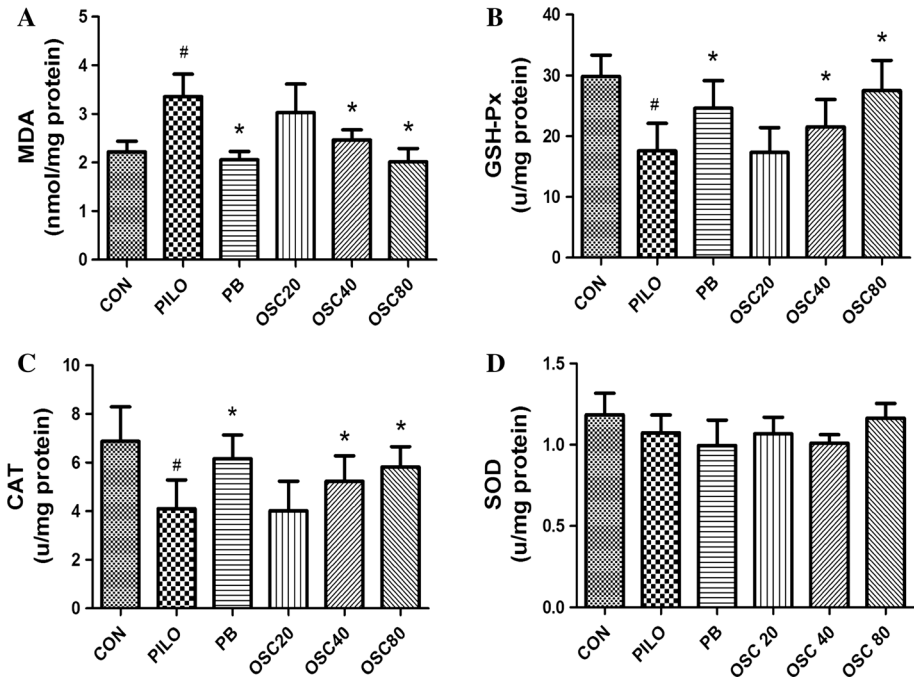

Fig. 3 Effects of OSC on lipid peroxidation level (**a**), GSH-Px level (**b**), catalase (**c**), and superoxide dismutase activities (**d**) in mice hippocampus after 24 h of pilocarpine-induced seizures ($n = 8$ in each group). Results are expressed as mean ± SEM. $^{\#}p < 0.05$ as compared to control group and * $p < 0.05$ as compared to PILO group

with OSC reduced the degenerated neurons in the CA1 and CA3 regions of hippocampus. The number of surviving cells was quantitatively analyzed in each group and statistically shown in histogram (Fig. 4).

FJB Staining

FJB staining was performed to reliable marker for degenerating neurons. At 72 h after convulsion, no FJB-positive

cells were detected in hippocampus CA1 and CA3 regions in control group, while abundant FJB-positive cells were observed in PILO group. In mice pretreated with OSC prior to PILO injection, the hippocampus had a decreased number of FJB-positive cells in both CA1 and CA3 regions. The number of FJB-positive cells was quantitatively analyzed in each group and statistically shown in histogram (Fig. 5).

Effects of OSC on Apoptosis-Associated Proteins Expression Induced by Convulsion

The expression of the apoptotic proteins Bcl-2, Bax, and Caspase-3 in the hippocampus of the mice was examined by western blot analysis. The protein expression levels of Bcl-2 in PILO group significantly decreased compared to control. In contrast, expression of caspase-3 and proapoptotic protein Bax increased at 24 h after seizures compared to control. However, in comparison with the PILO group, pretreatment with OSC at 80 mg/kg or PB markedly increased Bcl-2 levels, while decreasing Bax and Caspase-3 levels ($p < 0.05$) (Fig. 6).

Discussion

In this study, we investigated the following: (1) pretreatment with OSC in adult mice decreased the percentage of seizures, mortality rate, and increased the latency to the onset of seizure induced by PILO when compared to the PILO group. (2) Administration of OSC markedly reduced the abnormality in EEG in the form of high amplitude and high frequency induced by PILO. (3) OSC inhibited the increase in lipid peroxidation level and the decrease of GSH activities induced by administration of PILO to some extent. (4) OSC protected hippocampus against neuronal loss and degeneration induced by PILO. (5) Pretreatment with OSC reversed the apoptosis-associated proteins expression induced by seizures. The above results demonstrated that OSC exerted dose-dependent anticonvulsive activity in the PILO-induced epilepsy mice model.

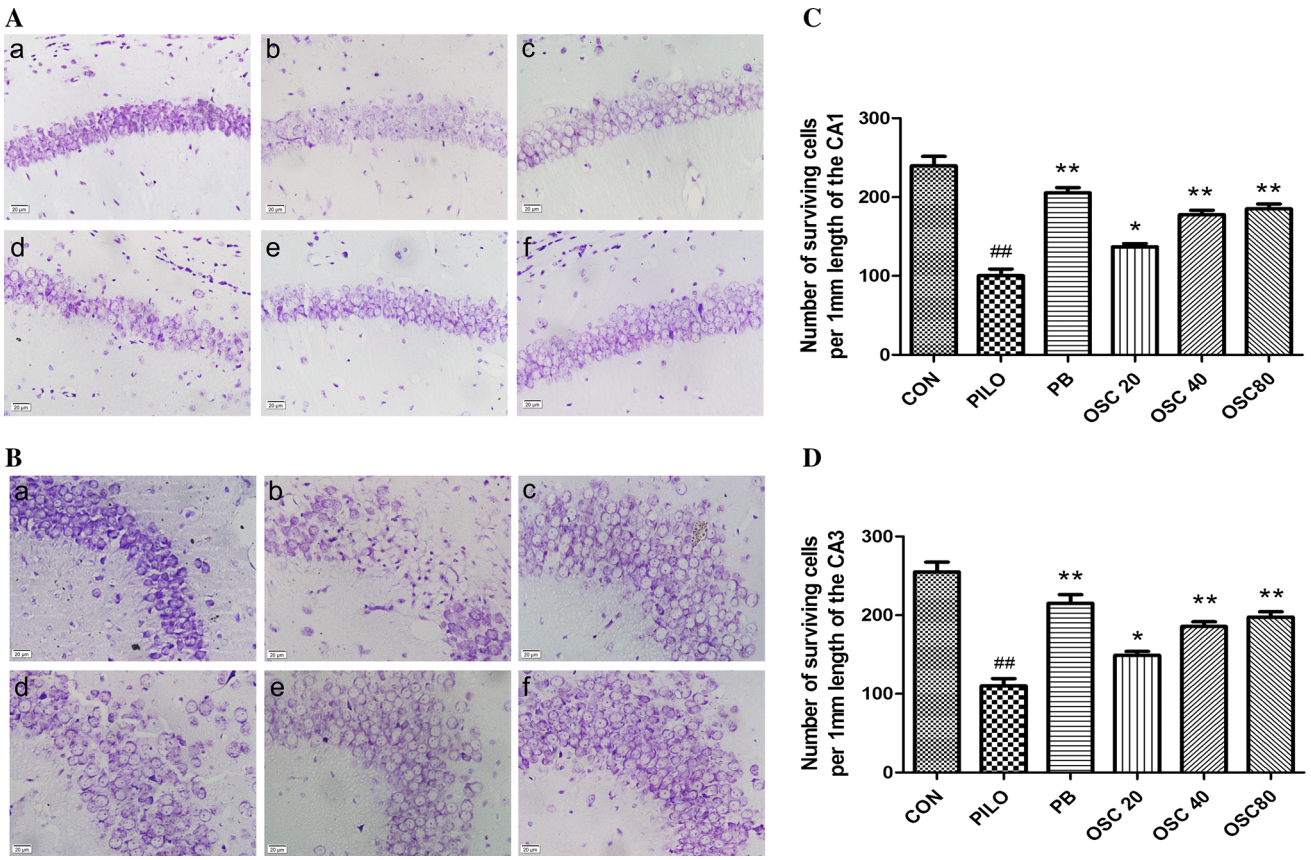

Fig. 4 Nissl staining of the hippocampal CA1 (**a**) and CA3 (**b**) pyramidal neurons with cresyl violet at 72 h after convulsion (×400 magnification). (*a*) CON group. (*b*) PILO group. (*c*) PB group. (*d*) OSC 20 group. (*e*) OSC 40 group. (*f*) OSC 80 group. *Bar* 50 um. Quantitative representation of the expression of surviving neurons in CA1 (**c**) and CA3 (**d**). ($n = 4$ animals per group, six sections per animal). Results are expressed as mean ± SEM. $^{\#\#}p < 0.05$ as compared to control group, * $p < 0.05$ as compared to PILO group, and ** $p < 0.01$ as compared to the PILO group

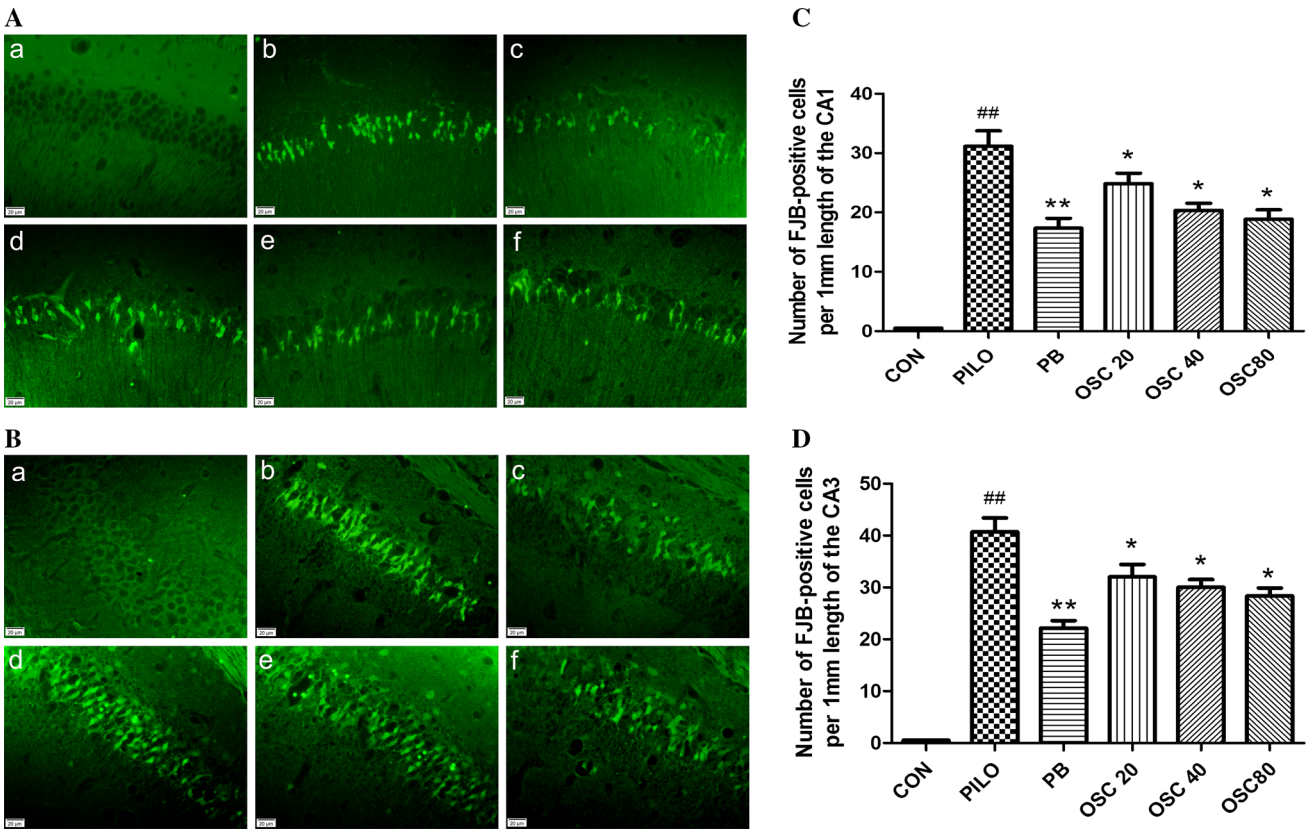

Fig. 5 The representative photomicrographs of FJB staining in hippocampal CA1 (**a**) and CA3 (**b**) for damaged and dead pyramidal neuronal at 72 h after convulsion are shown with high power (×400 magnification). (*a*) CON group. (*b*) PILO group. (*c*) PB group. (*d*) OSC 20 group. (*e*) OSC 40 group. (*f*) OSC 80 group. *Bar* 50 um. Quantitative analysis of FJB-positive cells demonstrated that OSC reduced FJB-positive cells in CA1 (**c**) and CA3 (**d**). ($n = 4$ animals per group, six sections per animal). Results are expressed as mean ± SEM. $^{\#\#}p < 0.05$ as compared to control group, * $p < 0.05$ as compared to PILO group, and ** $p < 0.01$ as compared to the PILO group

Simultaneously, OSC reduced the degree of oxidative stress by a decrease in the MDA content, and increase in GSH-Px and CAT activity in the hippocampus. Furthermore, OSC alleviated neuronal degeneration induced by PILO in hippocampal CA1 and CA3 region in mice. Moreover, our data demonstrated that the neuroprotective effects of OSC might be associated with the suppression of the cell apoptosis through the regulation of the expression of Bax, Bcl-2, and Caspase-3 proteins.

Epilepsy is a disorder of the brain characterized by recurrent seizures, behavioral changes, and abnormal electrical activity. Experimental models of seizures and epilepsy have been of a great value for understanding the basic mechanisms underlying histogenesis and epileptogenesis, and for contributing to the development of therapeutic alternatives (Marchi et al. 2009). One of the most frequently used models is the animal seizure elicited by PILO, a muscarinic cholinergic agonist, and it resembles some aspects of human temporal lobe epilepsy with electroencephalographic waves, behavioral and morphological sequelae (Manuela et al. 2012). Accumulating evidence suggests that OSC provided anti-inflammatory, antitumor, neuroprotection, and antinociceptive effects (Yang et al. 2015; Zhu et al. 2014). Our study showed that OSC delayed the latency of the first seizure, time to SE; meanwhile, it decreased the numbers of mice developing seizure, SE, and the mortality of mice. Additionally, OSC diminished the abnormality in EEG in the form of high amplitude, high frequency, and it delayed the time to polyspikes and the spike wave related to seizure behaviors changes. From Collective, these studies demonstrated that OSC possesses an anticonvulsant effect in seizure induced by PILO in mice.

In recent years, a growing number of studies suggest that oxidative stress is a contributing factor for the onset and development of epilepsy (Rowley and Patel 2013), and oxidative stress is also considered to be an index of irreversible neuronal damage. It has been suggested that oxidative stress is one of the possible mechanism of epileptic activity (Dal-Pizzol et al. 2000; Pazdernik et al. 2001). It is well known that the epileptiform activity causes excessive free radical production of reactive oxygen

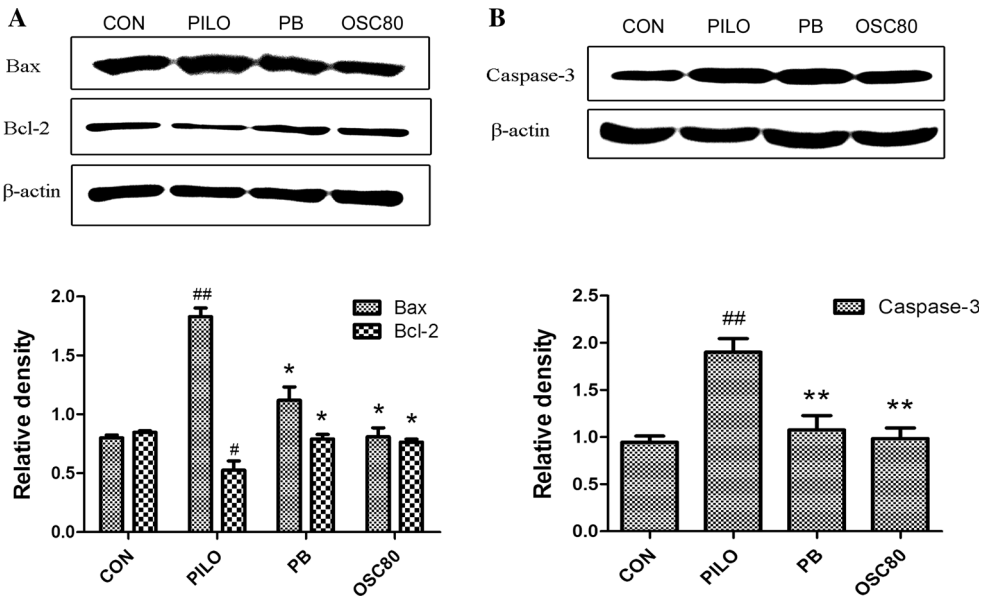

Fig. 6 Effect of OSC on the expression of Bax, Bcl-2, and Caspase-3 at 24 h after pilocarpine-induced seizures in hippocampus ($n = 8$ in each group). **a** Immunoblot and quantitative analysis of the effect of OSC on Bax and Bcl-2 expression. **b** Immunoblot and quantitative analysis of the effect of OSC on Caspase-3 expression. β-actin was used as an internal standard. The relative density are expressed as the mean ± SEM. $^{\#}p < 0.05$ as compared to control group, $^{\#\#}p < 0.01$ as compared to control group and * $p < 0.05$ as compared to PILO group, ** $p < 0.01$ as compared to the PILO group

species (ROS), a factor believed to be involved in the mechanism leading to cell death and neurodegeneration (Delorenzo et al. 2005). ROS have been implicated in the development of seizures and SE induced by PILO (Freitas et al. 2004, 2005). The increased lipid peroxidation may do harm to the protein, lipids, and deoxyribonucleic acid in the brain (Liang and Patel 2006). In our study, there was an increase in lipid peroxidation in the hippocampus of mice at 24 h after treatment with PILO, which was in line with previous studies (Freitas et al. 2005; Liu et al. 2012). Further, our data indicated that administration of OSC diminished markedly the rise in brain lipid peroxidation levels. This process may be attributed to free radical scavenging properties, and thus protect the nerve cell (Kim et al. 2005; Shieh et al. 2000). At 24 h after seizures, the level of CAT significantly increases in the hippocampus compared with the control mice. The result suggests that CAT activity may play a protective role during acute period of seizures (Freitas et al. 2005; Santos et al. 2008). Our date indicated that pretreatment with OSC (40, 80 mg/kg, i.p.) significantly increased CAT level compared to the PILO group. Meanwhile, our results showed that the activity of SOD remained unaltered at 24 h after SE in all treatment groups; it suggested that these enzymes cannot be activated during their phase of seizures induced by PILO, and other antioxidant systems could be responsible for inhibition of acute epileptic activity. It probably used other scavenging systems (CAT and GSH). GSH is one of the most important antioxidant agents of the cellular antioxidant defense system in the brain (Galleano and Puntarulo 1995; Liu et al. 1997). In the present study, PILO-induced SE decreases the GSH level in the rat hippocampus, which damages the antioxidant defense system (Liu et al. 2012). Our data showed that a significant decrease in GSH level was observed in PILO-induced SE mice compared to the control group, and pretreatment with OSC significantly prevented acetaminophen-induced depletion of glutathione (GSH) contents in a dose-dependent manner compared with PILO-induced seizure mice. This demonstrated that OSC may enhance antioxidative ability in brain through increasing the level of CAT, GSH and decreasing the level of MDA.

Studies have demonstrated that seizures induce a mixed pattern of cell death that include the features consistent with both apoptosis and necrosis (Ullah et al. 2015). Although great progress has been made in elucidating exact process of the cell death after seizures, the underlying mechanisms still remain unclear. Subsequently, strategies to prevent neuronal death are still limited. The neuronal loss can be observed by the methods of Nissl and FJB staining. In addition, FJB was used as a fluorescence marker for neurodegeneration. Previous study found that the presence of FJB-positive cells in several brain regions were analyzed in the acute and latent phases of the PILO model (Voutsinos-Porche et al. 2004).

In this study, the results of Nissl staining revealed typical morphological characteristic of neurodegeneration, such as the deceased Nissl body, breakup of the nuclear membrane, pyknosis of the nucleolus, and the disruption of the mitochondrial ridge in the CA1 and CA3 regions following 72 h after seizure. Pretreatment with OSC alleviated these effects. Additionally, we found that FJB-positive neurons increased significantly in 72 h after PILO-induced seizure, and OSC alleviated hippocampal neuronal degeneration, demonstrated by decreasing CA1 and CA3 FJB-positive cells. In this study, we provide the first direct

evidence that PILO-induced seizures caused prominent neuronal loss in the hippocampus, and OSC could significantly protect neurons from such loss. Therefore, pretreatment with OSC reversed these changes and showed neuroprotective effect.

Continuous seizures can lead to neuronal apoptosis in brain regions (Liu et al. 2012). Recent study indicated that the Bcl-2 family proteins, composed of proapoptotic and antiapoptotic members, are vital to the intrinsic apoptotic pathway and control the activation of downstream Caspases (Peng et al. 2015). Bcl-2 promotes cell survival and inhibits cell death induced by various apoptotic stimuli. Bax, a proapoptotic protein, can integrate into the mitochondrial membrane in response to apoptotic stimuli that induce CytC release. CytC release ultimately activates Caspase-3, leading to DNA breaks, nuclear chromatin condensation, and apoptosis (Xie et al. 2014). In this study, PILO-treated animals showed downregulation of Bcl-2 and upregulation of Bax and caspase-3 in hippocampus. However, pretreatment with OSC inhibited the expression of Bax and caspase-3, and further increased Bcl-2 in hippocampus. These results were further supported by morphological studies. Furthermore, pretreatment with OSC reversed these changes and showed neuroprotection by reversing the expression of Bax, Bcl-2, and Caspase-3.

In conclusion, the results of this study further concluded that OSC exerted an anticonvulsant effect against PILO-treated mice, and this pharmacological property might be dose-dependent, although more studies were necessary to elucidate the complete mechanism involved in this effect. Moreover, pretreatment with OSC exerted a neuroprotective effect in the CA1 and CA3 regions of hippocampus against epileptic injury; its beneficial effect was due to its potential to mitigate the oxidative stress burden, at least to some extent, and reversed the expression of Bax, Bcl-2, and Caspase-3. From above, OSC might be a potential therapeutic for the treatment of epilepsy.

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Compliance with Ethical Standards

Conflict of interest The authors have no conflicts of interest to declare.

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