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Anticonvulsant and anti-apoptosis effects of salvianolic acid B on pentylenetetrazole-kindled rats via AKT/CREB/BDNF signaling

Xiaoxiang Yu, Qiaobing Guan, Yanping Wang, Heping Shen, Liping Zhai, Xudong Lu, Yuhua Jin*

Department of Neurology, The Second Affiliated Hospital of Jiaxing University, Jiaxing, Zhejiang, 314000, China

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ABSTRACT

Neuronal apoptosis is a regulated intrinsic cell mechanism and common pathological phenomenon after seizures, which involves the protein kinase B/cAMP response element binding protein / brain derived neurotrophic factor (AKT/CREB/ BDNF) signaling pathway. In this study, we aimed to identify the effects of salvianolic acid B (Sal B), a major water-soluble component of the Chinese herb, Danshen, on rats in which seizures had been induced by pentylenetetrazole (PTZ) and the underlying molecular mechanisms mediating these effects. For this, 60 adult male Sprague-Dawley rats were divided into a control group, a 'PTZ' group and a 'PTZ + Sal B' group. The animals in the control group received an intraperitoneal (i.p.) injection of saline on alternate days for a total of 15 injections and saline orally once a day for 29 days. The animals in the 'PTZ' group received PTZ (40 mg/kg, i.p.) on alternate days for a total of 15 injections and saline orally once a day for 29 days. Similarly, the animals in the 'PTZ + Sal B' group received PTZ (40 mg/kg, i.p.) on alternate days and Sal B (20 mg/kg) orally once a day for 29 days. Neural density was then evaluated using immunofluorescence (IF) staining of microtubule-associated protein 2 (MAP2). Neuronal apoptosis was detected using terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining. In addition, the expression of several proteins related to AKT/CREB/BDNF signaling was measured using Western blotting. The results indicated that more severe seizures, decreased neural density, decreased expression of Bcl-2, increased expression of Bax and cleaved caspase-3, and inactivation of AKT/CREB/BDNF signaling occurred in the 'PTZ' group in comparison with the control group. However, those changes were suppressed by Sal B. Thus, these data suggest that Sal B has anticonvulsant and anti-apoptotic effects in a PTZ-induced seizure model through activation of the AKT/CREB/BDNF signaling pathways.

1. Introduction

Epilepsy is a common chronic neurological disorder that affects 50–70 million individuals, accounting for 0.75% of the global disease burden (Trinka et al., 2018). Although, there are a myriad of antiepileptic drugs (AEDs) available, approximately one third of epileptic patients fail to achieve complete remission from the occurrence of seizures (Franco et al., 2016). In addition, some patients suffer from serious side-effects when receiving AED treatment, such as paraesthesia, teratogenicity, agranulocytosis and glaucoma (Schmidt, 2002). Therefore, the development of novel antiepileptic agents that suppress the epileptic process effectively and safely is urgently required.

The genes of the B-cell lymphoma-2 (*Bcl-2*) family encode various proteins that positively (pro-apoptotic proteins, such as Bax and Bad) or negatively (anti-apoptotic proteins, such as Bcl-2) regulate cytochrome c release and caspase activation (Breitschopf et al., 2000; Kroemer et al., 2007). In the early 1990s, Gillardon et al. identified a decrease of approximately 45% in the intensity of Bcl-2 immunoreactivity and an increase of nearly 3-fold in Bax levels in the brains of kainate-treated mice (Gillardon et al., 1995). An increasing number of researchers have subsequently focused their attention on the relationship between epilepsy and neuronal apoptosis, with studies demonstrating that neuronal apoptosis might be a cause or consequence of epilepsy, possibly offering alternative potential targets for epilepsy treatment (Henshall and Simon, 2005; Méndez-Armenta et al., 2014). Moreover, accumulating evidence has indicated that protein kinase B (AKT) is able to activate the cAMP response element binding protein (CREB) and thereby induce the transcription of target genes, such as brain-derived neurotrophic factor (*Bdnf*), which promotes neuronal survival by inhibiting neuronal apoptosis (Bonni et al., 1999; Mayr and Montminy, 2001; Chen et al., 2017). Thus, activation of the AKT/CREB/BDNF signaling pathways

* Corresponding author at: No. 1518, Huancheng north road, Jiaxing, China.
E-mail address: yuhuajin1228@163.com (Y. Jin).

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appears to be a promising strategy to inhibit neuronal apoptosis in order to suppress epilepsy.

Salvianolic acid B (Sal B), a major, water-soluble component of the Chinese herb Danshen, displays beneficial effects in neurological disorders, such as vascular dementia (Ma et al., 2017), cerebral ischemia / reperfusion injury (Wang et al., 2016a), Alzheimer's disease (Lee et al., 2013), ischemic stroke (Lv et al., 2015) and traumatic brain injury (Chen et al., 2011). In addition, it has been shown to inhibit apoptosis in non-tumor cells, such as cardiomyocytes (Chen et al., 2016), Schwann cells (Sun et al., 2012) and hepatocytes (Yan et al., 2010), and also induce apoptosis in tumor cells, such as ovarian cancer cells (Yan, 2016), retinoblastoma cells (Liu, 2012) and glioma cells (Wang et al., 2013). Nevertheless, whether Sal B displays beneficial effects in epilepsy through regulation of apoptosis remains unknown. Therefore, this study aimed to determine the anticonvulsant and neuroprotective effects of Sal B in pentylenetetrazole (PTZ)-kindled rats via regulation of the AKT/CREB/BDNF signaling pathways and whether these effects would cause a reduction in neuronal apoptosis.

2. Materials and methods

2.1. Animals

60 adult male Sprague-Dawley rats, weighting 230 ± 20 g were housed in standard conditions of temperature and humidity with a 12 -h light-dark cycle. All animals were provided *ad libitum* access to food and water. All procedures were approved by the guidelines of the Animal Care and Use Committee at the Second Affiliated Hospital of Jiaxing University. Animals were randomly divided into a control group (n = 20), a 'PTZ' group (n = 20) and a 'PTZ + Sal B' group (n = 20).

2.2. PTZ-induced seizures

PTZ (Sigma-Aldrich; St. Louis, MO, USA) was diluted in saline and used to induce seizures as described in a previous study (Kaur et al., 2015). Briefly, a sub-convulsive dose (40 mg/kg) of PTZ was administered to the animals by intraperitoneal (i.p.) injection on alternate days for a total of 15 injections over a 29 day period. After each injection, the animals were observed for 30 min and scores graded according to the Racine's criterion: stage 0: no response; stage 1: chewing and face twitching; stage 2: neck spasms and head nodding; stage 3: unilateral forelimb clonus and twitching; stage 4: rearing with bilateral forelimb clonus; and stage 5: generalized clonic-tonic seizures with loss of postural control (Racine, 1972). Animals with seizures at stage 4 (starting with raising their bilateral forelimbs and subsequently lowering them) after three consecutive PTZ injections were considered to be fully kindled. The control group received a saline (i.p.) injection on alternate days for a total of 15 injections and saline orally once a day for 29 days. The 'PTZ' group received PTZ (40 mg/kg, i.p.) on alternate days for a total of 15 injections and saline orally once a day for 29 days. The 'PTZ + Sal B' group received PTZ (40 mg/kg, i.p.) on alternate days and an oral dose of Sal B (20 mg/kg in saline, National Institute for the Control of Pharmaceutical and Biological Products; Beijing, China) once a day for 29 days. Twenty four hours after the last injection of PTZ, all animals were sacrificed while under anesthesia through i.p. injection of 3.5% chloral hydrate (10 ml/kg). Brains were retrieved from the animals and the cortical and hippocampal tissues separated. Samples were stored at -80°C for Western blotting analysis (control (n = 10), 'PTZ' (n = 8) and 'PTZ + Sal B' (n = 10) groups) and as 10-μm frozen sections at −80°C for immunofluorescence (IF) and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining (control (n = 10), 'PTZ' (n = 8) and 'PTZ + Sal B' (n = 9) groups).

2.3. Evaluation of neural density

Microtubule-associated protein 2 (MAP2), a neural marker, was used to identify neurons by IF staining in order to evaluate neuronal density in the cortices and hippocampi. Briefly, after blocking with 10% goat serum, brain sections were incubated with a rabbit MAP2 antibody (1:100, Abcam; San Francisco, CA, USA) at 4°C overnight. On the following day, sections were washed three times with phosphate buffered saline (PBS) and incubated with a goat rabbit IgG heavy and light chain (H&L) antibody (FITC, 1:100, Abcam) at 4°C for 30 min. After washing three times with PBS, the sections were incubated for 5 min with 4′, 6-diamidino-2-phenylindole (DAPI) to stain the cell nuclei. Laser scanning confocal microscopy (Olympus IX71) was used to obtain images of sections. The number of MAP2-positive cells was calculated using ImageJ software.

2.4. TUNEL staining

A TUNEL staining kit (Boster Biological Engineering Co. Ltd; Wuhan, China) was used to determine the degree of cell apoptosis, in accordance with the manufacturer's instructions. Sections were stained for MAP2 protein in neurons, with cell nuclei stained with DAPI. A total of 3 individual fields of each section were randomly observed in both the CA1 and cortex regions using a laser scanning confocal microscope (Olympus IX71). ImageJ software was used to calculate the ratio of TUNEL and MAP2-positive cells (neuronal apoptosis rate).

2.5. Western blotting

After mincing the tissue with dissecting scissors on ice, the cortical and hippocampal extracted brain regions were homogenized in cold radioimmunoprecipitation assay (RIPA) lysis solution (Boster Biological Engineering Co. Ltd) containing protease and phosphatase inhibitors for 30 min at 4°C. The tissues were then centrifuged at 12,000 *g* for 20 min at 4°C and the supernatant collected. Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Boster Biological Engineering Co. Ltd) in accordance with the manufacturer's instructions. A total of 30 μg of extracted protein were separated using a sodium dodecyl sulfate (SDS)-polyacrylamide gel which were then transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% fat-free dry milk for 2 h at room temperature, each PVDF membrane was incubated overnight at 4°C with one of the following primary antibodies: rabbit anti-neuronal nuclei antigen (NeuN, 1:1000, Abcam), rabbit anti-AKT (1:1000, Abcam), rabbit anti-phospho (p)-AKT (1:1000, Abcam), rabbit anti-CREB (1:1000, Abcam), rabbit anti-p-CREB (1:1000, Abcam), rabbit anti-BDNF (1:1000, Abcam), rabbit anti-cleaved caspase-3 (1:1000, Abcam), rabbit anti-Bcl-2 (1:1000, Abcam), rabbit anti-Bax (1:1000, Abcam), or rabbit anti-GAPDH (1:4000, Abcam). The PVDF membrane was washed three times the following day with Tris-buffered saline containing Tween-20 (TBST) then incubated with horseradish peroxidase (HRP) goat anti-rabbit IgG secondary antibody (1:2000, Boster Biological Engineering Co. Ltd) for 1 h at room temperature. Protein bands were then visualized using an enhanced chemiluminescent (ECL) reagent (Boster Biological Engineering Co. Ltd) then analyzed using ImageJ 1.41 software (National Institutes of Health [NIH]; Bethesda, MD, USA).

2.6. Statistical analysis

All data are presented as means ± standard deviation after analysis by researchers blind to the experimental conditions, using SPSS 16.0 statistical software (IBM; Armonk, NY, USA). Significance between groups was ascertained using one-way analysis of variance (ANOVA) followed by a *post hoc* Tukey's test for multiple-group comparisons. Differences with a p value < 0.05 were considered statistically significant.

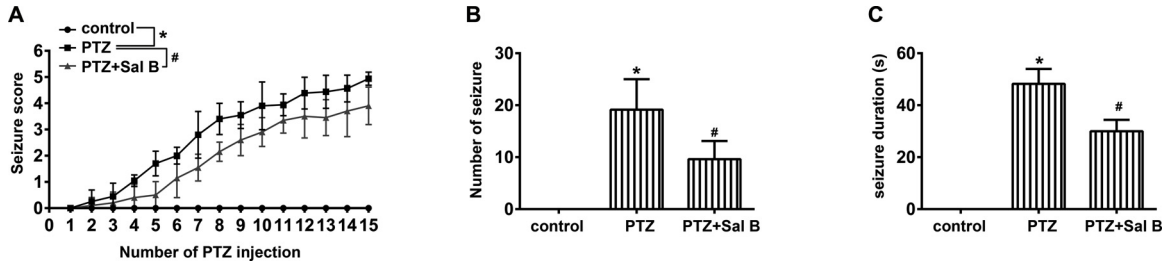

Fig. 1. Effects of Sal B treatment on pentylenetetrazole (PTZ)-induced seizures. **(A)** Sal B treatment decreased seizure scores in rats in which seizures were induced by PTZ. **(B)** Sal B treatment decreased the number of seizures (Stage 4 or greater) in rats in which seizures had been induced by PTZ (PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; F (2, 52) = 112.8, $p < 0.0001$). **(C)** Sal B decreased the duration of seizures (Stage 4 or greater) in rats in which seizures had been induced by PTZ (PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; F (2, 52) = 668.3, $p < 0.0001$). * $P < 0.05$ compared with the control group; $^{\#}$ $P < 0.05$ compared with the 'PTZ' group.

3. Results

3.1. Effects of Sal B on PTZ-induced seizures

After 15 PTZ injections, four animals in the 'PTZ' group and one in the 'PTZ + Sal B' group died due to serious generalized clonic-tonic seizures. However, no animals died in the control group. The incidence of full kindling was 90% and 55% in the 'PTZ' and 'PTZ + Sal B' groups, respectively. We analyzed the seizure score, number of seizure (Stage 4 or greater) and duration of seizures (Stage 4 or greater). As shown in Fig. 1A, with increasing numbers of PTZ injections, the animals in the 'PTZ group' showed a high Stage of seizure, and after 15 injections, the seizure Stage in the 'PTZ group' reached 4.938 ± 0.250. However, animals in the 'PTZ + Sal B' group in comparison showed a lower Stage of seizure, which after 15 injections, reached 3.900 ± 0.718 (Fig. 1A, PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; F (2, 52) = 670.5, $p < 0.0001$). Importantly, Sal B treatment ('PTZ + Sal B' group) caused a significant decrease in the total number of seizures across the entire experiment, from 19.150 ± 5.851–9.600 ± 3.515 (Fig. 1B, PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; F (2, 52) = 112.8, $p < 0.0001$). Moreover, Sal B treatment ('PTZ+ Sal B' group) caused a significant decrease in the duration of seizures from 48.200 ± 5.782s to 29.950 ± 4.442s (Fig. 1C, PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; F (2, 52) = 668.3, $p < 0.0001$).

3.2. Effects of Sal B on neuronal density in rats in which seizure was induced by PTZ

The number of neurons in the cortices decreased significantly in the 'PTZ' group compared with the control group. However, Sal B treatment caused a significant increase in neuronal density in the cortices compared with rats that had received only saline injections (Fig. 2A, PTZ vs control, $p = 0.0003$; PTZ + Sal B vs PTZ, $p = 0.0028$; F (2, 24) = 11.65, $p = 0.0003$). Furthermore, Western blotting was used to measure the expression of NeuN, a neuron specific marker of neuronal nuclei. As shown in Fig. 2B, NeuN expression in the cortices decreased in the 'PTZ' group compared with the control group, and increased in the 'PTZ + Sal B' group compared with the 'PTZ' group (Fig. 2B, PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; F (2, 25) = 80.53, $p < 0.0001$). Similarly, neuronal density in the hippocampal CA1 region, decreased significantly in the PTZ group, compared with the control group, and increased significantly in the 'PTZ + Sal B' group, compared with the PTZ group (Fig. 3A, PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p = 0.0002$; F (2, 24) = 17.35, $p < 0.0001$). In addition, expression of NeuN in the hippocampi decreased in the 'PTZ' group, compared with the control group, and increased in the 'PTZ + Sal B' group, compared with 'PTZ' group (Fig. 3B, PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; F (2, 25) = 27.46, $p < 0.0001$).

3.3. Effects of Sal B treatment on neuronal apoptosis in rats with PTZ-induced seizure

In order to detect whether Sal B treatment was able to suppress neuronal apoptosis, IF staining of DAPI/TUNEL/MAP2 was performed and the neuronal apoptosis rate calculated. The rate of neuronal apoptosis in the cortices increased significantly in the 'PTZ' group compared with that in the control group and decreased significantly in the 'PTZ + Sal B' group compared with the 'PTZ' group (Fig. 4A, PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; F (2, 24) = 50.94, $p < 0.0001$). Moreover, Bcl-2 (Fig. 4B, PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; F (2, 25) = 23.26, $p < 0.0001$), Bax (Fig. 4B, PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; F (2, 25) = 64.12, $p < 0.0001$) and cleaved caspase-3 (Fig. 4B, PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; F (2, 25) = 22.33, $p < 0.0001$) protein levels were measured using Western blotting analysis. Notably, Bcl-2 expression in the cortices decreased in the 'PTZ' group compared with that in the control group and increased in the 'PTZ + Sal B' group compared with the 'PTZ' group. Conversely, expression of Bax and cleaved caspase-3 in the cortices increased in the 'PTZ' group compared with the control group and decreased in the 'PTZ + Sal B' group compared with the 'PTZ' group. Similarly, the rate of neuronal apoptosis in the hippocampi was significantly higher in the 'PTZ' group compared with the control group, and significantly lower in the 'PTZ + Sal B' group, compared with the 'PTZ' group (Fig. 5A, PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; F (2, 24) = 249.4, $p < 0.0001$). In particular, the expression of Bcl-2 protein in the hippocampi was lower in the 'PTZ' group than the control group, and higher in the 'PTZ + Sal B' group than the 'PTZ' group (Fig. 5B, PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p = 0.0083$; F (2, 25) = 17.11, $p < 0.0001$). The expression of Bax (Fig. 5B, PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; F (2, 25) = 108.9, $p < 0.0001$) and cleaved caspase-3 (Fig. 5B, PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; F (2, 25) = 117.9, $p < 0.0001$) in the hippocampus was higher in the 'PTZ' group than in the control group, and lower in the 'PTZ + Sal B' group than in the 'PTZ' group.

3.4. Effects of Sal B on AKT/CREB/BDNF signaling in rats in which seizure was induced by PTZ

Finally, we also measured the expression of BDNF and phosphoinositide 3-kinase (PI3K) pathway-related proteins, namely AKT and CREB. In both the cortices (Fig. 6A) and hippocampi (Fig. 6B), a significant decrease in the expression levels of p-AKT (Fig. 6A, PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; F (2, 25) = 68.9, $p < 0.0001$) (Fig. 6B, PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; F (2, 25) = 82.69, $p < 0.0001$), p-CREB (Fig. 6A, PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; F (2, 25) = 74.99, $p < 0.0001$) (Fig. 6B, PTZ vs control, $p < 0.0001$; PTZ + Sal

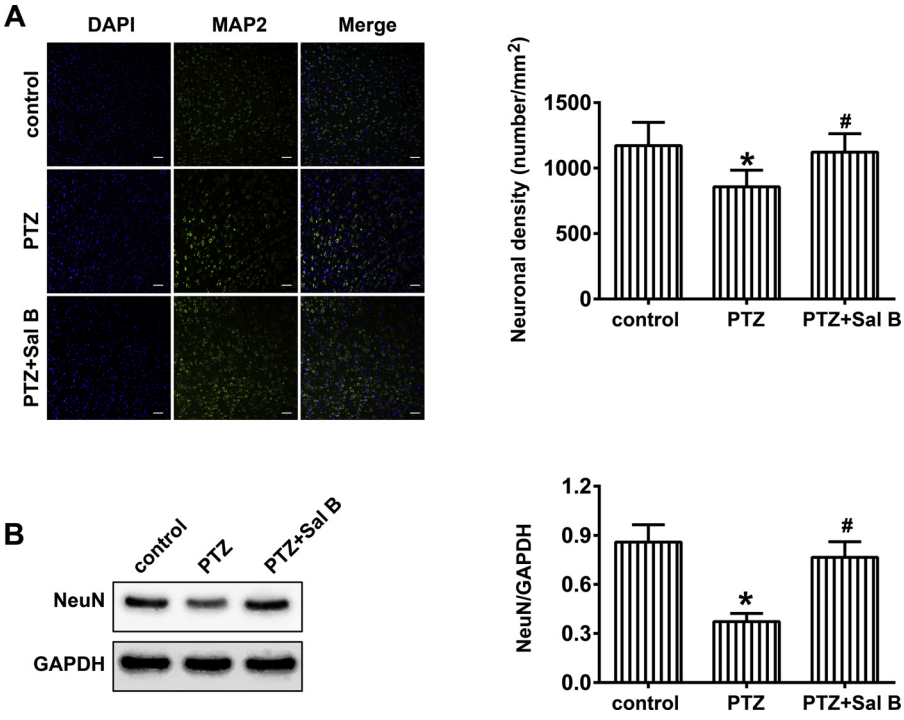

Fig. 2. Effects of Sal B on neuronal density in the cortices of rats in which seizures had been induced by PTZ. **(A)** Representative images of immunofluorescence (IF) staining of rat cortices in control (n = 10), 'PTZ' (n = 8), and 'PTZ + Sal B' (n = 9) groups. Statistical analysis indicated that Sal B increased neuronal density in the cortices of rats in which seizures had been induced by PTZ (PTZ vs control, $p = 0.0003$; PTZ + Sal B vs PTZ, $p = 0.0028$; $F(2, 24) = 11.65$, $p = 0.0003$). Scale bar: 50 μm. **(B)** Representative bands from Western blot analysis of NeuN protein in rat cortices of control (n = 10), 'PTZ' (n = 8), and 'PTZ + Sal B' (n = 9) groups. The results indicate that Sal B can upregulate NeuN protein expression in the cortices of PTZ-treated rats (PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; $F(2, 25) = 80.53$, $p < 0.0001$). * $P < 0.05$ compared with control group; # $P < 0.05$ compared with the 'PTZ' group.

B vs PTZ, $p < 0.0001$; $F(2, 25) = 54.21$, $p < 0.0001$) and BDNF (Fig. 6A, PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; $F(2, 25) = 42.01$, $p < 0.0001$) (Fig. 6B, PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; $F(2, 25) = 28.68$, $p < 0.0001$) was detected in the 'PTZ' group compared with the control group. In contrast, the expression levels of p-AKT, p-CREB and BDNF in the 'PTZ + Sal B' group were significantly higher than in the 'PTZ' group.

4. Discussion

In the present study, we found that the severity index, which determines the intensity of seizures, of PTZ-kindled rats treated with Sal B was lower than rats without Sal B treatment. Importantly, Sal B treatment suppressed neural loss through inhibition of neuronal apoptosis. Moreover, we also demonstrated that Sal B treatment activated the AKT/CREB/BDNF signaling pathway. Thus, we conclude that Sal B exhibits anticonvulsant and anti-apoptotic effects through activation of the AKT/CREB/BDNF signaling pathway in PTZ-kindled rats.

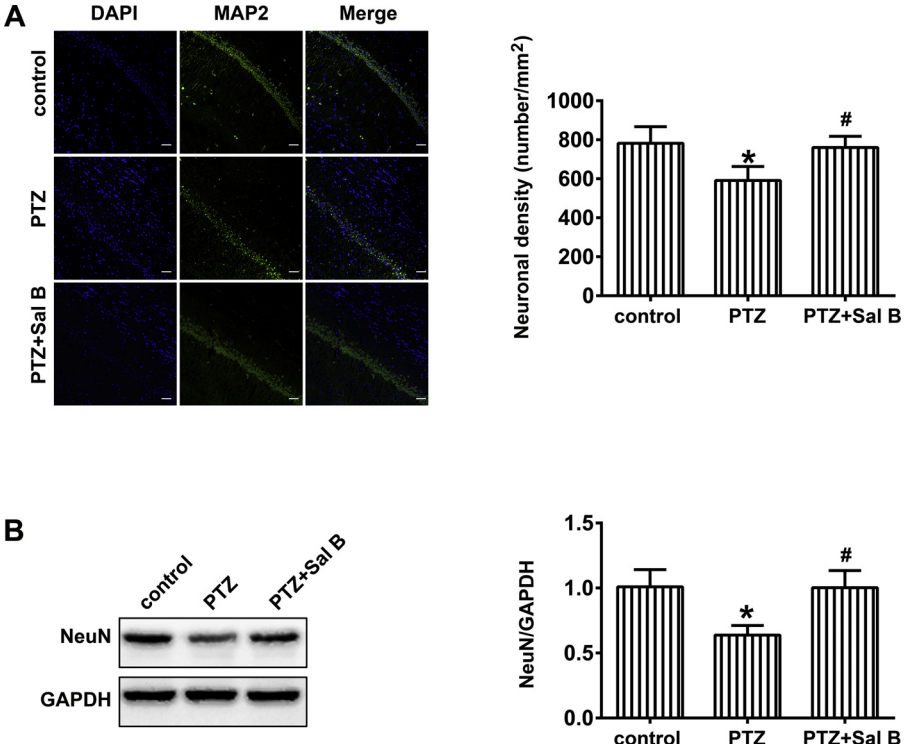

Fig. 3. Effects of Sal B treatment on neuronal density in the hippocampi of rats in which seizures had been induced by PTZ. **(A)** Representative images of IF staining in the rat CA1 hippocampal region of control (n = 10), 'PTZ' (n = 8), and 'PTZ + Sal B' (n = 9) groups. Statistical analysis indicate that Sal B increased neuronal density in the CA1 hippocampal region of PTZ-treated rats (PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p = 0.0002$; $F(2, 24) = 17.35$, $p < 0.0001$). Scale bar: 50 μm. **(B)** Representative bands from Western blot analysis of NeuN protein expression in the hippocampus of control (n = 10), 'PTZ' (n = 8), and 'PTZ + Sal B' (n = 10) groups. Statistical analysis indicates that Sal B can upregulate NeuN protein expression in the hippocampus of PTZ-treated rats (PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; $F(2, 25) = 27.46$, $p < 0.0001$). * $P < 0.05$ compared with control group; # $P < 0.05$ compared with the 'PTZ' group.

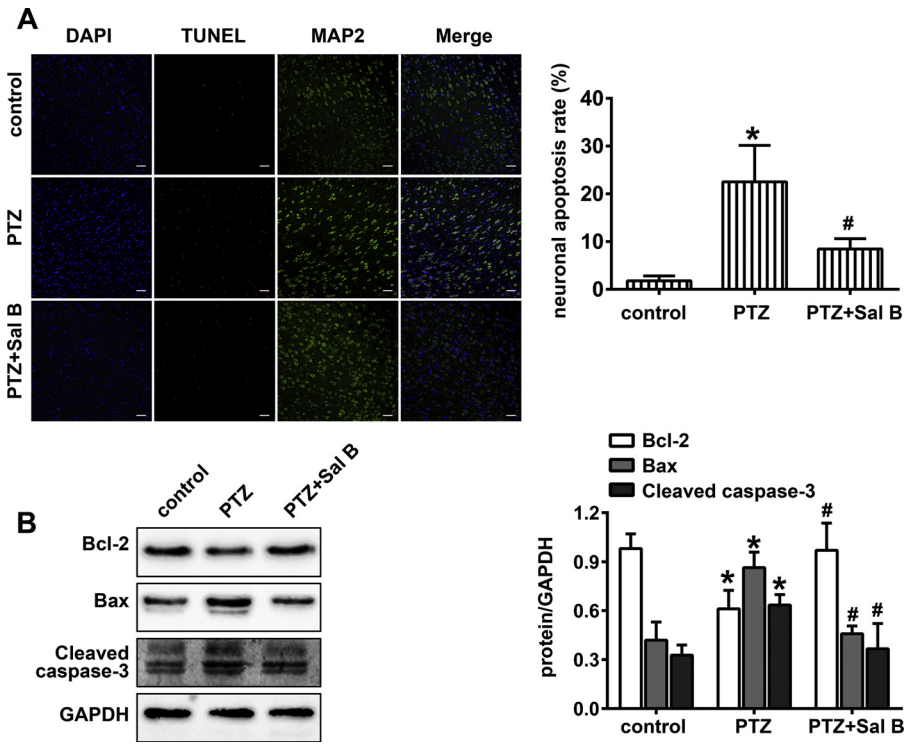

Fig. 4. Effects of Sal B treatment on neuronal apoptosis in the cortices of rats in which seizures had been induced by PTZ. **(A)** Representative images of TUNEL staining in the cortices of control (n = 10), 'PTZ' (n = 8), and 'PTZ + Sal B' (n = 9) groups. Statistical analysis indicates that Sal B could suppress neuronal apoptosis in the cortices of rats in which seizures had been induced by PTZ (PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; $F(2, 24) = 50.94$, $p < 0.0001$). Scale bar: 50 μm. **(B)** Representative bands from Western blotting of Bcl-2, Bax and cleaved caspase-3 proteins in the cortices of control (n = 10), 'PTZ' (n = 8), and 'PTZ + Sal B' (n = 10) groups. Statistical analysis indicates that Sal B can upregulate Bcl-2 expression (PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; $F(2, 25) = 23.26$, $p < 0.0001$) and downregulate Bax (PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; $F(2, 25) = 64.12$, $p < 0.0001$) and cleaved caspase-3 expression (PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; $F(2, 25) = 22.33$, $p < 0.0001$) in the cortices of the PTZ-treated rats. * $P < 0.05$ compared with control group; $^{\#}$ $P < 0.05$ compared with the 'PTZ' group.

Seizure-induced neuronal apoptosis is a well-established phenomenon that occurs in the cortices and hippocampi of patients with epilepsy and experimental seizure animal models (Henshall and Simon, 2005). Furthermore, the balance between the pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 is closely related to the release of cytochrome c from the mitochondria, which inhibits activation of caspase-9 and caspase-3 protease enzymes that mediate programmed cell death (Breitschopf et al., 2000; Kroemer et al., 2007). In this research study, we clearly detected neuronal apoptosis in the cortices and hippocampi of rats that received 15 PTZ injections, accompanied by the down-regulation of Bcl-2 and up-regulation of Bax and cleaved caspase-3, which is in agreement with a previous research study (Hao et al., 2016). Several first-line antiepileptic drugs, such as sodium valproate (Li et al., 2018), levetiracetam (Kikuyama et al., 2017) and topiramate (Chen et al., 2009), are believed to exert antiepileptic effects through regulation of the balance between pro-apoptotic and anti-apoptotic proteins in the Bcl-2 family. In addition, many non-antiepileptic drugs also show antiepileptic effects through mediation of Bcl-2 family protein

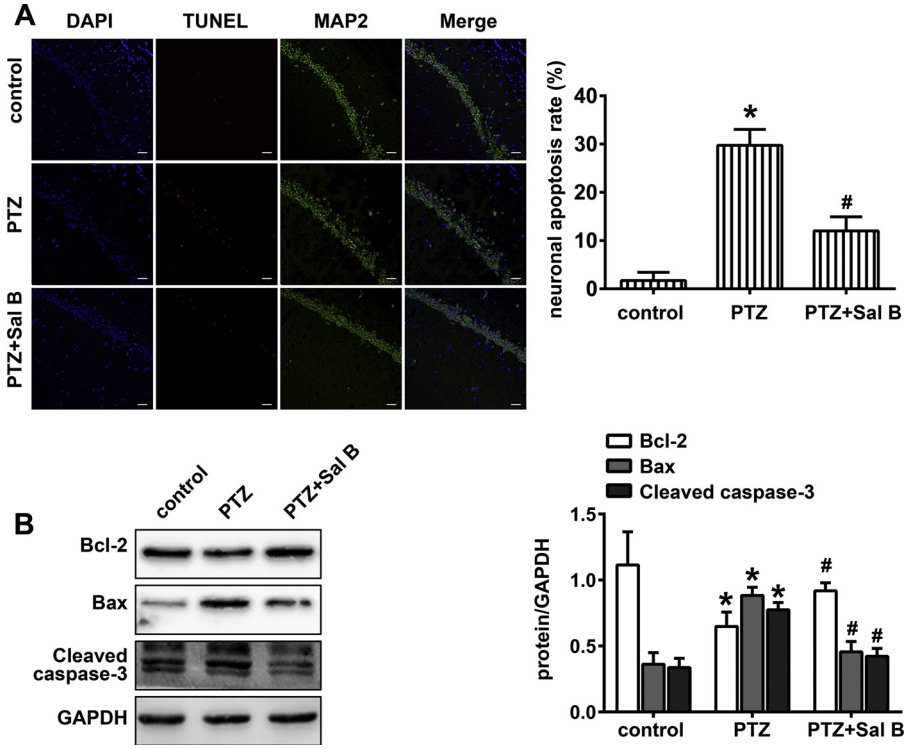

Fig. 5. Effects of Sal B treatment on neuronal apoptosis in the hippocampus of rats in which seizures had been induced by PTZ. **(A)** Representative images of TUNEL staining in control (n = 10), 'PTZ' (n = 8), and 'PTZ + Sal B' (n = 9) groups. Statistical analysis indicates that Sal B can suppress neuronal apoptosis in the hippocampus of rats in which seizures had been induced by PTZ (PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; $F(2, 24) = 249.4$, $p < 0.0001$). Scale bar: 50 μm. **(B)** Representative protein bands from Western blots showing Bcl-2, Bax and cleaved caspase-3 protein levels in the hippocampus of the control (n = 10), 'PTZ' (n = 8) and 'PTZ + Sal B' (n = 10) groups. Statistical analysis indicates that Sal B can upregulate Bcl-2 (PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p = 0.0083$; $F(2, 25) = 17.11$, $p < 0.0001$) and downregulate Bax (PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; $F(2, 25) = 108.9$, $p < 0.0001$) and cleaved caspase-3 (PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; $F(2, 25) = 117.9$, $p < 0.0001$) expression in the hippocampus of PTZ-treated rats. * $P < 0.05$ compared with control group; $^{\#}$ $P < 0.05$ compared with the 'PTZ' group.

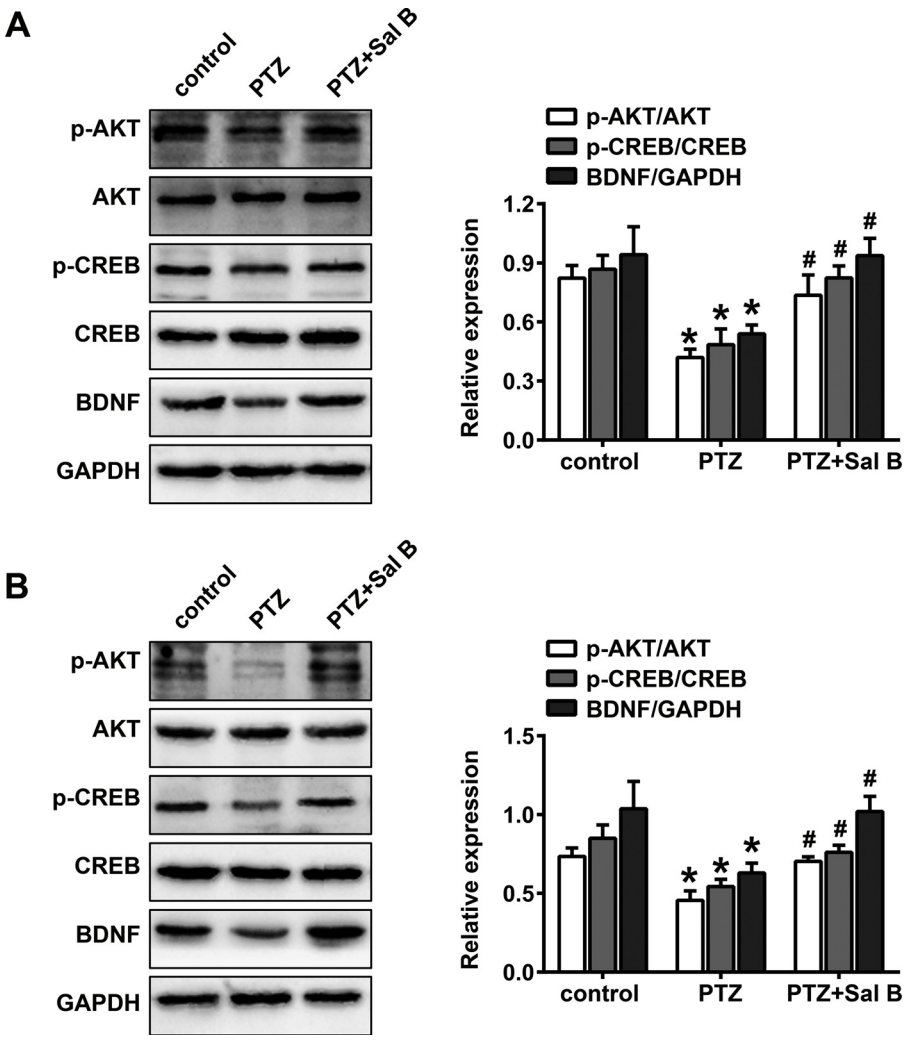

Fig. 6. Effects of Sal B treatment on AKT/CREB/BDNF signaling in the cortices and hippocampi of rats in which seizures had been induced by PTZ. **(A)** Representative protein bands from Western blot analysis for p-AKT, AKT, p-CREB, CREB and BDNF in the cortices of control (n = 10), 'PTZ' (n = 8) and 'PTZ + Sal B' (n = 10) groups. Statistical analysis indicates that Sal B can upregulate p-AKT (PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; $F(2, 25) = 68.9$, $p < 0.0001$), p-CREB (PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; $F(2, 25) = 74.99$, $p < 0.0001$), and BDNF (PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; $F(2, 25) = 42.01$, $p < 0.0001$) expression in the cortices of PTZ-treated rats. **(B)** Representative bands from Western blotting of p-AKT, AKT, p-CREB, CREB and BDNF in the rat hippocampi of control (n = 10), 'PTZ' (n = 8) and 'PTZ + Sal B' (n = 10) groups. Statistical analysis indicates that Sal B can upregulate p-AKT (PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; $F(2, 25) = 82.69$, $p < 0.0001$), p-CREB (PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; $F(2, 25) = 54.21$, $p < 0.0001$) and BDNF (PTZ vs control, $p < 0.0001$; PTZ + Sal B vs PTZ, $p < 0.0001$; $F(2, 25) = 28.68$, $p < 0.0001$) expression in the hippocampus of PTZ-treated rats. * $P < 0.05$ compared with control group; $^{\#}$ $P < 0.05$ compared with the 'PTZ' group.

expression. For example, Guo *et al.* suggested that tangeretin can alter neuronal apoptosis and ameliorate the severity of seizures in experimental epilepsy-induced rats by modulation of the expression of apoptotic proteins, such as cleaved caspase-3, Bad, Bcl-2, Bcl-xL and Bax (Guo et al., 2017). Sal B, the most abundant and bioactive member of the salvianolic acids in the Chinese herb Danshen, can cross the blood-brain barrier and exert neuroprotective effects (Zhu et al., 2013). A previous study demonstrated that SMND-309, a novel derivative of Sal B, significantly increased Bcl-2 protein levels and decreased the activity of caspase-3, thereby preventing neuronal cell death in differentiated human neuroblastoma SH-SY5Y cells exposed to oxygen-glucose deprivation (Wang et al., 2016b). Moreover, Chen *et al.* reported protective effects of Danshen dripping pills alone and in combination with carbamazepine in a kainic acid-induced temporal lobe epilepsy and cognitive impairment rat model, likely related to its anti-apoptotic effects (Jia et al., 2018). In the PTZ-kindled seizure rat model, we also found that Sal B could ameliorate the severity of seizures, decrease the rate of neuronal apoptosis, up-regulate Bcl-2 protein expression and down-regulate cleaved caspase-3 and Bax expression. These results indicate that Sal B can protect neurons against apoptosis after PTZ-induced seizures.

Furthermore, previous studies have demonstrated that BDNF is able to promote neuronal survival and growth on various central nervous system (CNS) neurons, including hippocampal and cortical neurons, and that it was also implicated in epileptogenesis (Binder, 2004). BDNF expression normally increases in the early postnatal period during development and continues to be expressed at high levels in adulthood, being regulated by a variety of molecules, such as CREB (Dong et al., 2018). After phosphorylation at the serine-133 residue by protein kinases, such as AKT, CREB becomes active and up-regulates BDNF expression (Dong et al., 2018). Therefore, activation of AKT/CREB/BDNF signals could promote, at least in part, neuronal survival and growth. Likewise, in the cortices and hippocampi of PTZ-induced rats, we found that seizure-induced neuronal apoptosis was accompanied by down-regulation of AKT/CREB/BDNF signals, which suggests that inactivation of the AKT/CREB/BDNF signaling pathway might contribute to seizure-induced neuronal apoptosis. Nevertheless, after Sal B treatment, AKT/CREB/BDNF signals were activated in the cortices and hippocampi of our PTZ-induced seizure rat model, indicating that the anti-epileptic effects of Sal B might be related to its effect on AKT/CREB/BDNF signaling.

In conclusion, we have found that Sal B treatment has neuroprotective and antiepileptic effects against a PTZ-induced seizure rat model. These effects may be attributed to activation of the AKT/CREB/BDNF signaling pathways and inhibition of neuronal apoptosis. There are some limitations to the present study. Firstly, how Sal B affects apoptosis and AKT/CREB/BDNF signaling pathways under control conditions is unknown. Additionally, whether other signaling pathways are implicated in the protective effects of Sal B on PTZ-induced seizure requires further study. Nevertheless, our findings provide a novel intervention strategy for human epilepsy.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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