

# Protective effects of tetrandrine on brain cells in phenobarbital-dependent and -withdrawn rats

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**Abstract.** The aim of this study was to investigate the effects of tetrandrine (Tet) on the brain cells of phenobarbital-dependent and -withdrawn rats, and to explore the underlying mechanisms. A total of 100 rats were randomly divided into five groups: The control group, the phenobarbital-dependent model group, and Tet-treated groups of low-, mid- and high-dosages. Following drug withdrawal, the morphological changes of the frontal lobe cells were examined by hematoxylin and eosin (H&E) staining. Immunohistochemical staining was applied to detect the expression of apoptosis-related proteins Bcl-2 and Bax. Reverse transcription-polymerase chain reaction (RT-PCR) and western blotting methods were applied to detect the mRNA and protein expression levels of Bcl-2 and Bax, respectively, in the frontal lobe. The results indicated that Tet effectively reduced the withdrawal symptoms, particularly the weight loss, in phenobarbital-dependent and -withdrawn rats. H&E staining revealed that Tet significantly restored the histopathological changes in the addicted rats in a dose-dependent manner. The immunohistochemical, RT-PCR, and western blot analyses indicated that Tet treatment significantly increased the Bcl-2<sup>+</sup> brain cells and the mRNA and protein expression levels of Bcl-2, and decreased the Bax<sup>+</sup> cells and the mRNA and protein expression levels of Bax, as well as elevated the ratio of Bcl-2/Bax, in phenobarbital-dependent and -withdrawn rats. Tet may inhibit apoptosis in these addicted rats, in a dose-dependent manner. Tet alleviates the phenobarbital withdrawal symptoms and protects the brain cells against apoptosis, which may be a result of the regulation of the mRNA and protein expression levels of Bcl-2 and Bax.

## Introduction

Substance dependence, or drug addiction, as a consequence of drug abuse, refers to a state of dependence produced by the repeated consumption of a drug. When such substances are no longer available, addicted individuals suffer from withdrawal symptoms. Drug abuse has been reported to result in brain tissue damage (1). Drug addiction is a type of brain disease, which is induced by the activation of apoptosis-regulating molecules (2). Common addictive drugs include opioids, barbiturates and benzodiazepines. Phenobarbital, the most widely used antiepileptic drug in clinic (e.g., for controlling status epilepticus) (3), can also easily induce drug addiction and subsequent diseases, such as neonatal abstinence syndrome (4). It has been reported that long-term use of phenobarbital results in increased apoptosis in rat brains (5). Recent studies have found that Bcl-2 family proteins, including the pro-apoptotic proteins Bax and anti-apoptotic protein Bcl-2 are important in regulating intracellular Ca<sup>2+</sup> homeostasis and mitochondrial functions. Bax can increase intracellular Ca<sup>2+</sup> levels to promote apoptosis, while Bcl-2 reduces Ca<sup>2+</sup> contents to inhibit apoptosis (6-9).

Tetrandrine (Tet), a bisbenzylisoquinoline alkaloid isolated from a number of Chinese herbs, is a nonselective voltage-gated calcium channel blocker. Tet induces cardiovascular changes, including decreases in total peripheral vascular resistance and blood pressure (10). In addition, Tet may exert muscle relaxant, antipyretic, analgesic, anti-inflammatory and even antitumor effects under physiological and pathological conditions (11-13). As such, Tet has been used for the treatment of silicosis, anthracosilicosis, mild hypertension, rheumatism, arthralgia, and neuralgia (14). Luo *et al* (15) found that Tet modulates the expression of apoptosis-related genes, promoting Bcl-2 expression and decreasing Bax expression, to further inhibit neuronal apoptosis and effectively protect the functions of the spinal cord in acute injuries. However, there are few reports regarding the effects of Tet on brain dysfunctions in drug addiction.

In the current study, the effects of Tet on brain cells were investigated in phenobarbital-dependent and -withdrawn rats, to determine whether Tet alleviates the withdrawal symptoms induced by phenobarbital-dependency and to explore the underlying mechanisms.

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Table I. Primer sequences for reverse transcription-polymerase chain reaction.

Primer	Sequence	Product length (bp)
Rattus Bcl-2	Forward: 5'-GTGGCCTTCTTTGAGTTCG-3' Reverse: 5'-TCCCAGCCTCCGTTATCC-3'	146
Rattus Bax	Forward: 5'-TGTGCACTAAAGTGCCCGAG-3' Reverse: 5'-GGAAGCCTCAGCCCATCTTCTT-3'	213
Rattus GAPDH	Forward: 5'-TGATGCCCCCATGTTTGTGA-3' Reverse: 5'-AGCCCTTCCACGATGCCAAA-3'	144

## Materials and methods

**Reagents and animals.** Phenobarbital was provided by Shanghai Sine Pharmaceuticals Co., Ltd. (Shanghai, China). Tet was provided by Beihai Sunshine Pharmaceuticals Co., Ltd. (Guangxi, China). Sodium carboxymethyl cellulose (CMC-Na) was purchased from Chongqing Lihong Fine Chemicals Co., Ltd. (Chongqing, China).

A total of 100 Sprague-Dawley rats (50 males and 50 females), weighing 150-200 g, were provided by Chongqing Tengxin Biotechnology Co., Ltd. (Chongqing, China). All animal experiments were conducted according to the ethical guidelines of Luzhou Medical College (Luzhou, China).

**Animal modeling and drug administration.** Drugs were dissolved in 0.5% CMC-Na for gastric administration. Animals were kept in separate cages for one week, and then randomly divided into five groups: A normal control group, a model group and Tet-treated groups of low- (6.25 mg/kg/day), mid- (18.75 mg/kg/day), and high-dosages (31.25 mg/kg/day), with 20 rats (half male and half female) per group. Rats in the normal control group were fed with normal powdered food, while the other four groups were administrated phenobarbital tablets in increasing doses, which were mixed into their diet. The rats were kept in standard conditions with free access to food and water. In the first 10 days, phenobarbital was administered at a dose of 75 mg/kg/day, and the dosage levels for days 11-20, 21-30 and 31-45 were 120, 165 and 210 mg/kg/day, respectively (16).

Rats in the treatment groups were subjected to Tet (Beihai Sunshine Pharmaceutical Co., Ltd., Beihai, China) administration via an intragastric tube, at low-, mid- and high-dosages, while the rats in the control and model groups were gavaged with saline of the same volume. Tet administration was performed on the same day as phenobarbital administration. At day 46, the rats were fed with normal food, in the absence of phenobarbital. The withdrawal symptoms were then observed and scored, and the rat weights were recorded every 8 h (17). The percentage weight change was calculated accordingly. The withdrawal symptoms were scored as previously described (16). Briefly, no obvious symptoms was defined as score 0. Score 1 was defined as the presence of symptoms of excitement and anorexia. Score 2 was defined as when sweating, intense tremor and weight loss (< 10%) were observed. Score 3 was defined when clonic convulsions and weight loss (> 10%) were observed. Score 4 was defined as symptoms of tonic convulsions and death.

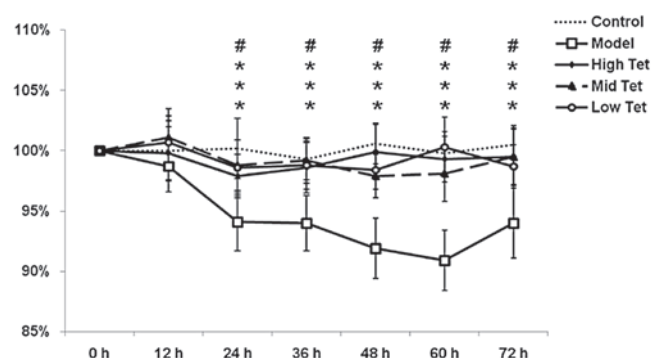


Figure 1 Weight changes in the phenobarbital-dependent and -withdrawn rats. Weights were recorded in 12-h intervals following the phenobarbital withdrawal, and the percentage change in weight was calculated accordingly. \* $P < 0.05$  compared with the normal control group, # $P < 0.05$  compared with the model group.

**Hematoxylin and eosin (H&E) staining.** Ten rats (including five male and five female rats) were anesthetized with 1% pentobarbital (Jing Rong Biological Technology Co., Ltd, Shanghai, China). When the rats were deeply anesthetized, 4% paraformaldehyde (Central Laboratory, Luzhou Medical College, Luzhou, Sichuan, China) was then perfused intracardially for ~1 h. The rats were sacrificed by perfusion. Following perfusion, the frontal lobes were removed and fixed overnight with 4% paraformaldehyde. Once dehydrated and embedded in paraffin, the tissues were cut into serial sections, and then stained with H&E. The morphology and structure of the cells in the frontal lobe were observed under an optical microscope (Olympus BX53; Olympus Corp., Tokyo, Japan).

**Immunohistochemical staining.** Paraffin-embedded sections were subjected to immunohistochemical staining to detect the expression of apoptosis-related factors Bcl-2 and Bax. A brown or tan color was considered to indicate positive staining. Immunostaining of Bcl-2 and Bax was primarily localized to the cell membrane or cytoplasm. A high-resolution optic microscope (Olympus BX53; Olympus Corp.) was used to observe the brain tissues. Cells positive for Bax and Bcl-2 in each section were then counted and compared (5).

**Western blot analysis.** Total protein was extracted from the frontal lobe tissues, and subjected to 10% SDS-PAGE. The proteins were then electrotransferred onto nitrocellulose membranes (Shanghai Jieyi Biotechnology Co., Ltd., Shanghai, China). Rabbit anti-mouse monoclonal anti-Bcl-2 (1:5,000;

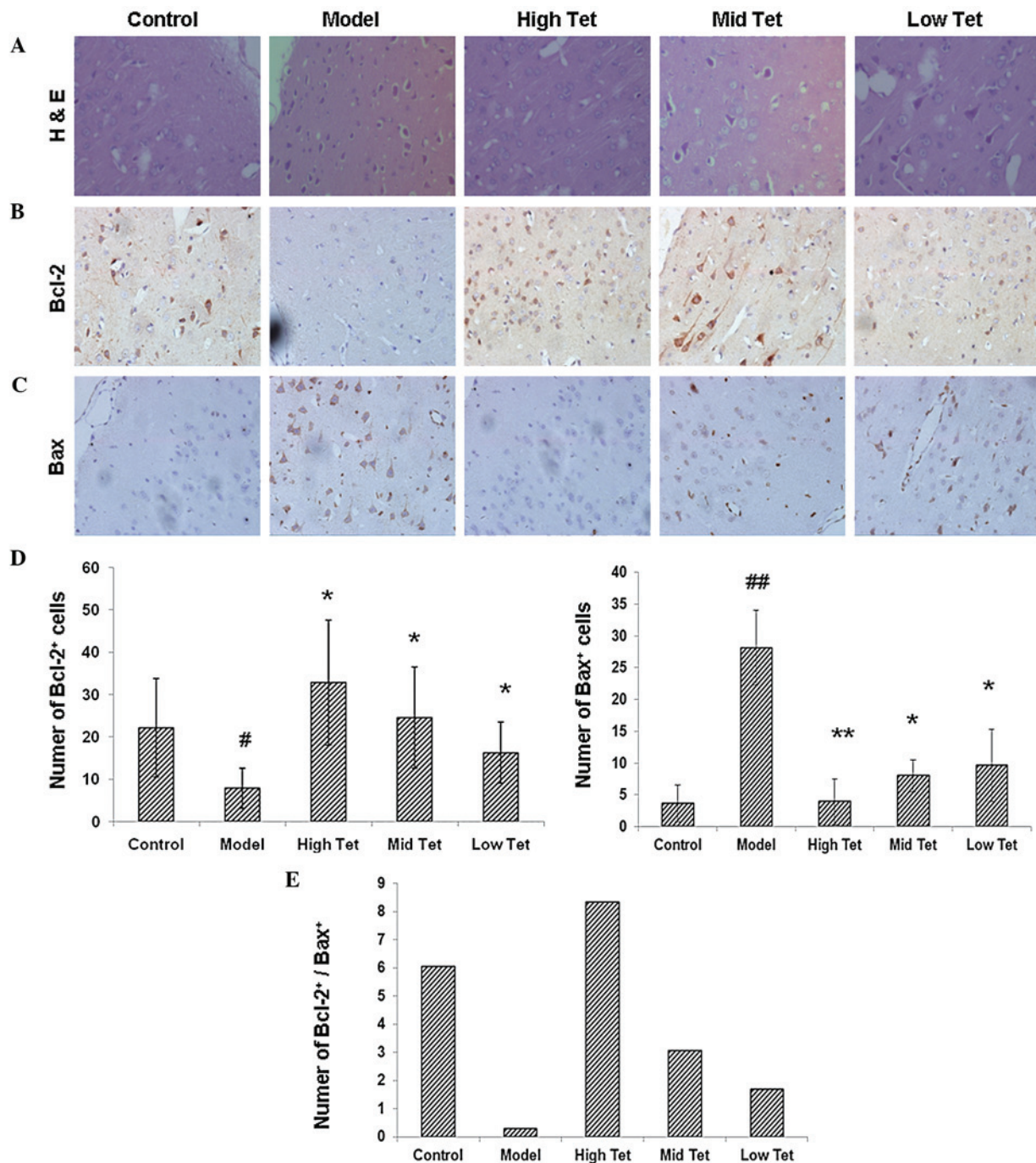


Figure 2. Hematoxylin and eosin (H&E) and immunohistochemistry staining of the brain tissues in phenobarbital-addicted rats (magnification, x400). (A) Images of H&E staining of the rat brain tissues in the normal control group, model group, and Tet-treated groups of low-, mid-, and high-dosages. (B) Immunohistochemistry staining to detect Bcl-2 positive cells in the brain sections from the control group, model group, and Tet-treated groups of low-, mid-, and high-dosages. (C) Immunohistochemistry staining to detect Bax positive cells in the brain sections from the control group, model group, and Tet-treated groups of low-, mid-, and high-dosages. Statistical analysis of the numbers of (D) Bcl-2<sup>+</sup> and (E) Bax<sup>+</sup> cells. <sup>#</sup>P<0.05, <sup>##</sup>P<0.01 compared with the normal control group; <sup>\*</sup>P<0.05, <sup>\*\*</sup>P<0.01 compared with the model group.

ab7973), rabbit anti-mouse monoclonal anti-Bax (1:5,000; ab32503) and rabbit anti-mouse monoclonal anti- $\beta$ -actin (1:5,000; ab156302; all from Abcam, Cambridge, MA, USA) primary antibodies were incubated with the membranes overnight at room temperature. Secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibody; 1:10,000; ab97047; Abcam) was added and the membranes incubated for 1 h at room temperature. Subsequently, the membrane was placed in Enhanced Chemiluminescence

reagent (Tiangen Biotech Co., Ltd., Beijing, China). The protein bands were imaged and analyzed with Image Lab software version 3.0 (Bio-Rad Laboratories, Hercules, CA, USA).  $\beta$ -actin was used as a loading control, and the relative contents of the target proteins were calculated accordingly.

**Reverse transcription polymerase chain reaction amplification RT-PCR analysis.** Total RNA was extracted with TRIzol reagent, using the RNA Extraction kit from Tiangen (Beijing,

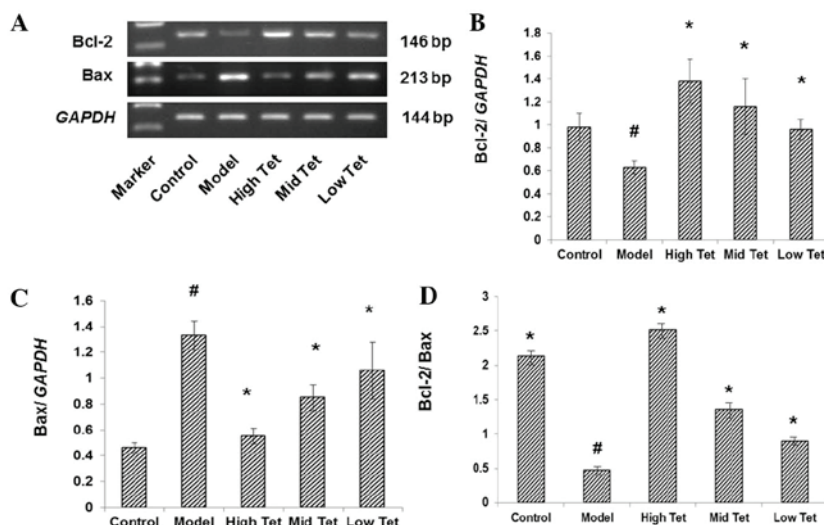


Figure 3. mRNA expression levels of Bcl-2 and Bax in the brains of phenobarbital-addicted rats. (A) Reverse transcription-polymerase chain reaction was performed to detect the mRNA expression levels of Bcl-2 and Bax in the brains of the addicted rats. Statistical analysis of the relative mRNA expression levels of (B) Bcl-2 and (C) Bax, as well as (D) the Bcl-2/Bax ratios. <sup>#</sup>P<0.05 compared with the normal control group; <sup>\*</sup>P<0.05 compared with the model group.

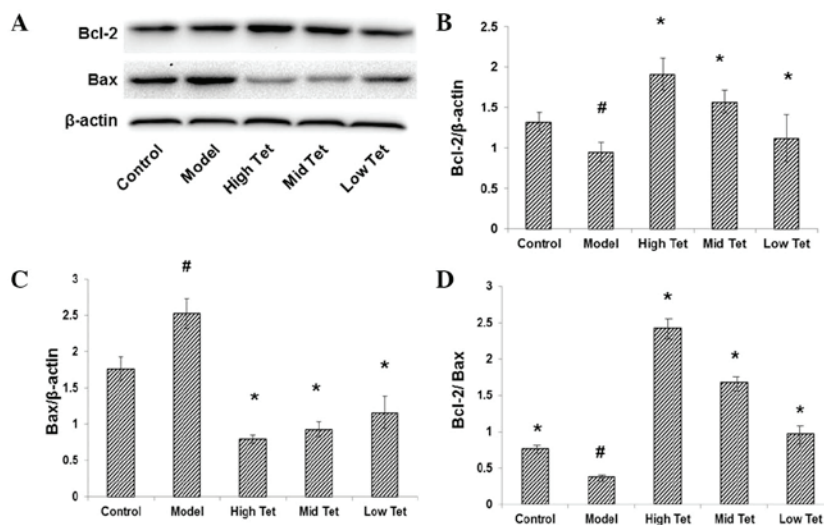


Figure 4. Protein expression levels of Bcl-2 and Bax in the brains of phenobarbital-addicted rats. (A) Western blot analysis was performed to detect the protein expression levels of Bcl-2 and Bax in the addicted rat brains. Statistical analysis of the relative protein expression levels of (B) Bcl-2 and (C) Bax, as well as (D) the Bcl-2/Bax ratios. <sup>#</sup>P<0.05 compared with the normal control group; <sup>\*</sup>P<0.05 compared with the model group.

China). RNA (2  $\mu$ g) was reverse transcribed into complementary DNA with the PrimeScript RT Regent Kit from Takara (Tokyo, Japan). The expression of Bcl-2 and Bax at mRNA levels were analyzed with RT-PCR. Primer sequences are shown in Table I. The following RT-PCR procedures were used: Bcl-2, pre-denaturation at 95°C for 10 min, 35 cycles of 94°C for 1 min, 58°C for 30 sec, 72°C for 15 sec and final extension at 72°C for 10 min; Bax, pre-denaturation at 95°C for 10 min, 40 cycles of 94°C for 1 min, 60°C for 30 sec, 72°C for 20 sec and final extension at 72°C for 10 min; GAPDH, pre-denaturation at 95°C for 10 min, 31 cycles of 94°C for 1 min, 58°C for 30 sec, 72°C for 15 sec and final extension at 72°C for 10 min. RT-PCR was performed, with GAPDH as an internal control. Amplification products were subjected to 2% agarose gel electrophoresis, and Quantity One software (Bio-Rad Laboratories) was used for image acquisition and semi-quantitative analysis.

**Statistical analysis.** Data are expressed as the mean  $\pm$  standard deviation. The SPSS software package version 18.0 (SPSS, Inc., Chicago, USA) was used for statistical analysis. One-way analysis of variance was performed to determine the differences between the groups. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Tet alleviates the withdrawal symptoms of phenobarbital-dependent and -withdrawn rats.** The effects of Tet on phenobarbital withdrawal symptoms were investigated. Compared with the normal control group, the rats in the model group developed withdrawal symptoms 16 h after phenobarbital was withdrawn, these included excitement, anxiety, piloerection and intermittent tremors. The severity of these symptoms peaked at 24-60 h

post-phenobarbital withdrawal, and was marginally alleviated in the following 12 h. As one of the most important withdrawal symptoms, weight loss was carefully monitored. These results indicated a clear weight loss in the rats of the model group, which did not begin to recover until 72 h following drug withdrawal. By contrast, rats in the Tet-treated groups demonstrated minimal withdrawal symptoms, and their weight loss was not as evident as that of the rats in the model group. The percentage weight change and the scores of the withdrawal symptoms in the Tet-treated groups were significantly less than those of the model group ( $P<0.05$ ; Fig. 1). These results suggest that Tet effectively reduces withdrawal symptoms, particularly weight loss, in phenobarbital-dependent and -withdrawn rats.

*Tet reduces the histopathological changes in phenobarbital-dependent and -withdrawn rats.* To investigate the effects of Tet on the histopathology of the addicted rats, H&E staining was performed on the brain sections for morphometric analysis. As shown in Fig. 2A, in the normal control group, the brain cells exhibited intact morphology, clear nuclei and a normal cytoplasm color. However, in the model group, the cells were smaller, with concentrated cytoplasm and condensed chromatin. In the low-dosage Tet treatment group (6.25 mg/kg), the brain cells possessed a relatively intact morphology compared with the model group, however, there were still a number of small cells with condensed chromatin in different forms. In the mid-dosage Tet group (18.75 mg/kg), a more intact brain cell morphology was observed, and there were fewer small cells with slightly concentrated cytoplasm and condensed chromatin. Furthermore, in the high-dosage group (31.25 mg/kg), the brain cells displayed an intact morphology, and there were no significant differences in the cell structure and morphology compared with the control group. These results indicate that Tet prevents the histopathological changes in these addicted rats, in a dose-dependent manner.

*Tet increases the number of Bcl-2<sup>+</sup> cells and reduces the number of Bax<sup>+</sup> cells in phenobarbital-addicted rat brains.* Apoptosis is involved in brain tissue damage as a result of drug abuse. To investigate the possible mechanisms through which Tet exerts its protective effects and whether the expression levels of apoptosis-related proteins were influenced by Tet, immunohistochemical staining, RT-PCR and western blotting were performed to detect the expression levels of Bcl-2 and Bax. The results of the immunohistochemical staining revealed that the numbers of Bcl-2 positive cells in the model group were significantly lower than those of the control group ( $P<0.05$ ) (Fig. 2B and D). Furthermore, the Bcl-2<sup>+</sup> cell numbers in the Tet-treated groups were significantly higher than those of the model group ( $P<0.05$ ) (Fig. 2B and D), increasing in the following order: Low-dosage, mid-dosage, and high-dosage, with statistically significant differences between these groups. Conversely, the numbers of cells positive for Bax were significantly increased in the model group, compared with those of the normal control group ( $P<0.05$ ) (Fig. 2C and E). In the Tet treatment groups, a low-dosage of Tet markedly reduced the number of Bax<sup>+</sup> cells, compared with that of the model group, which was further decreased by the mid-dosage and high-dosage treatments ( $P<0.05$ ) (Fig. 2C

and E), with significant differences between the high-dosage group and the low- or mid-dosage group ( $P<0.05$ ).

*Tet increases Bcl-2 expression and decreases Bax expression in phenobarbital-dependent and -withdrawn rats.* The mRNA and protein expression levels of Bcl-2 and Bax in the brain tissues were further analyzed with RT-PCR and western blot analysis, respectively. For Bcl-2 expression, the results indicated that the mRNA and protein expression levels were reduced in the model group compared with those of the normal control group ( $P<0.05$ ). Treatment with Tet markedly elevated the Bcl-2 mRNA and protein expression levels compared with those of the model group ( $P<0.05$ ) (Figs. 3A and B, and 4A and B). For Bax expression levels, the mRNA and protein expression levels were higher in the model group than those in the control group ( $P<0.05$ ), which were decreased by Tet treatment ( $P<0.05$ ) (Figs. 3A and C and 4A and C). Furthermore, within the Tet-treated groups, the changes in the mRNA and protein expression levels of Bcl-2 and Bax increased in a dose-dependent manner, with significant differences between the mRNA expression levels of each group ( $P<0.05$ ). Notably, the ratio of Bcl-2/Bax, as indicated by mRNA and protein levels, was significantly increased by Tet treatments in a dose-dependent manner (Figs. 3D and 4D). These results indicate that Tet treatment significantly increases the number of Bcl-2<sup>+</sup> brain cells and the Bcl-2 mRNA and protein expression levels, while reducing the number of Bax<sup>+</sup> cells, the Bax mRNA and protein expression levels and elevating the Bcl-2/Bax ratio, in phenobarbital-dependent and -withdrawn rats. Hence, Tet may inhibit apoptosis in phenobarbital-addicted rats in a dose-dependent manner.

## Discussion

The current study indicated that treatment with Tet significantly alleviated the phenobarbital withdrawal symptoms of excitement, anxiety, piloerection, intermittent tremor and particularly weight loss, which is consistent with the results of Guo and Wang (18). In addition, the results of the present study confirmed the hypothesis that the muscle relaxant effects of Tet can protect against the excitement and agitation following drug withdrawal. The frontal lobe is one of the most important regions during cerebral development, the damage of which may lead to disorders of voluntary movements, speech, cranial nerves, autonomic function and mental activity (19). This brain region may also be associated with the pathological changes that occur in drug addiction (20). Observation of H&E staining with a light microscope revealed that Tet could counteract the cellular apoptosis in this brain region, as indicated by the restored cell morphology.

Further results of immunohistochemistry, RT-PCR, and western blotting showed that Tet could upregulate the Bcl-2/Bax ratios at the mRNA and protein levels, which opposed the regulation of phenobarbital on these ratios. Based on these results, it can be concluded that Tet effectively counteracts neuronal apoptosis induced by phenobarbital addiction and protects neural cells. In addition, it was revealed that in the Tet-treated groups the Bcl-2/Bax ratio increased as the Tet dosages increased, which indicates that the anti-apoptotic and protective effects of Tet are exerted in a dose-dependent manner.

Brain cell damage induced by drug addiction is a complex process, which may involve multiple mechanisms. In the present study, Tet at low-, mid- and high-dosages was found to alleviate the symptoms of phenobarbital withdrawal, increase the expression level of anti-apoptotic factor Bcl-2 and decrease the expression level of pro-apoptotic factor Bax, which illustrates that the mechanism through which Tet antagonizes apoptosis and protects the brain cells from damage may involve the regulation of Bcl-2 and Bax expression levels. Further studies are required to determine a more detailed and specific mechanism of action of Tet in the protection against sedative-hypnotic dependence.

In conclusion, these results indicate that Tet alleviates phenobarbital withdrawal symptoms and protects brain cells against apoptosis in a dose-dependent manner, which may be related to the regulation of the mRNA and protein expression levels of Bcl-2 and Bax. These results may provide experimental evidence for the potential future use of Tet in the treatment of drug withdrawal.

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