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Herb pair of *Polygala tenuifolia* Willd and *Acorus tatarinowii* Schott decoction attenuates seizures and alleviates anxiety in mice: Evidence for modulating inflammation, alleviating oxidative stress and mediating GABA pathway

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Abstract: Currently, prolong use of standard anti-epileptics may cause tolerance and ineffective for about 30% of epileptic patients. Medicinal plants provide an attractive therapeutic effect in preventing and treating seizures in traditional and folk medicine. In this study, we investigate the antiepileptic effects of PTAT decoction on acute and chronic seizure models in mice and explore the potential mechanisms. PTAT decoction dose-dependently protected mice against MES and PTZ induced seizure. Meanwhile, it decreased the seizure severity and reduced seizure-caused anxious behavior in the PTZ-kindling mice, suggesting a significant antiepileptic activity and anxiolytic/anxiogenic potential. PTAT decoction dose-dependently increased the levels of GSH and the activity SOD and CAT, while decreased the level of MDA in the hippocampi of treated mice. Furthermore, a significant decrease in the proinflammatory cytokine levels, including TNF-α, IL-1β, IL-6 and MCP-1 was found in treated mice compared with the mice in the vehicle + PTZ group. Moreover, PTAT decoction dose-dependently reversed the alterations induced by PTZ in GABA, GABA-T, L-GAD and glutamate levels in kindling mice, showing an effect on the modulation of the GABA neurotransmission. Thus, PTAT decoction has a promising anticonvulsant activity mediated via multiple mechanisms, which might be used as an up-and-coming phytotherapy strategy in the management of epilepsy and its complications.

Keywords: *Polygala tenuifolia* Willd, *acorus tatarinowii* schott, anticonvulsant, oxidative stress, inflammatory, neurotransmission, seizures.

INTRODUCTION

Epilepsy, a chronic brain dysfunction disease that has many causes, which is characterized by sudden, repeated and transient dysfunction of the central nervous system caused by recurrent and unprovoked seizures (Amlerova *et al*., 2021; Thijs *et al*., 2019). At present, over 70 million people in the world suffer from epilepsy, of which about 90% of the patients come from middle to low-income countries and there are about 9 million patients with epilepsy in China, with an increase of 20,0000-40,0000 patients per year (Thijs *et al*., 2019; Löscher *et al*., 2020). The occurrence and development of epilepsy have a serious impact on the quality of life and health of these patients, especially long-term seizures that will lead to a series of behavioral and emotional disorders, including anxiety, depression, schizophrenia, autism and cognitive impairments (Modi *et al*., 2021). The etiology and pathogenesis of epilepsy are complex and are involved in multiple pathophysiological processes, such as imbalance of neurotransmitters, inflammatory response, ion channels, receptors, immune system, oxidative stress, etc. (He *et al*., 2021). Up to date, there are limited management options to improve or prevent disease progression. Clinical methods for the treatment or control of epilepsy are mainly medicine therapy. Although there are about 30 anti-epileptic drugs (AEDs) now clinically available, the mainstream AEDs exert only symptomatic relief and fail to control seizures for 30% of patients, leading to severe drug-resistant epilepsy (Hoda *et al*., 2021). What's more, a majority of the AEDs have certain side effects and may even aggravate seizures or provoke neurological disorders (Mishra *et al*., 2021). For example, studies have shown that the long-term use of topiramate and sodium valproate will affect patients' cognitive function (Strzelczyk and Schubert-Bast, 2022). Therefore, there is an urgent need to develop more effective therapeutic agents with low side effects for the prevention and management of epilepsy.

Medicinal plants with fewer side effects and accessible effects are largely used in traditional or alternative medicine for treating epilepsy with a long history (Lin and Hsieh, 2021). In recent years, the research of medicinal herbs with antiepileptic potential has attracted widespread concern and attention. It was found that most natural drugs possess therapeutic effects on controlling and improving seizures through multiple pathways including balance neurotransmitters, regulation of ion channels, reduction of inflammation and oxidative stress pathways, etc (He *et al*., 2021). In China, Compound Chinese medicine (CCM) plays a synergistic role with better therapeutic effects that have been used for thousands of years. Today, in clinical practice in China, more than 14 CCM, such as Zhenxian tablet, Dianxianping tablet, Dianxianning tablet, Xianyu capsule

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and Yixian pill, etc. have been approved by the China Food and Drug Administration for treating multiple seizures (Chinese Pharmacopoeia Committee, 2020). The effectiveness of these CCM made from medicinal plants has must been demonstrated in extensive clinical practice (Yuan *et al*., 2019; Zhao *et al*., 2018; Liu *et al*., 2018). Thus, as reasonable and effective therapeutic choices, medicinal plants and natural medicine have the great potential to offer viable complementary market-to-market AEDs.

The herb pair of *Polygala tenuifolia* Willd-*Acorus tatarinowii* (PTAT) was first recorded in medical classics Sheng Ji Zong Lu in 1117. It clearly states that PTAT can tranquilize the mind and promote intelligence, as well as eliminate irritability and remove phlegm to dredge the orifices. Clinically, as one of the most accepted herb pairs in traditional Chinese medicine (TCM), it is widely used for treating epilepsy, convulsion, febrile convulsion, syncope, stroke, dizziness, etc. *P. tenuifolia* (Yuanzhi) was the most common herb in TCM and was classified as a top-grade herb in the Chinese medicine classic *Shen Nong's Material and Medica* in 200 AD. It has shown that Yuanzhi has the functions of dispersing blood stasis, removing phlegm, relieving depression, calming the mind and benefiting intelligence (Chinese Pharmacopoeia Committee, 2020; Bai *et al*., 2019). *A. tatarinowii* (Shichangpu) was also recorded in *Shen Nong's Material and Medica* and it has the effects of inducing resuscitation, removing dampness, benefiting intelligence, etc (Chinese Pharmacopoeia Committee, 2020; Editorial Board of Chinese Materia Medica, 1999). PTAT, as a famous herb pair, was combined and most frequently used in many traditional Chinese medicines including the *Dianxian Kang* capsule, *Zhenxian* tablet, *Xianyu* capsule, etc. for treating epilepsy. Based on the compatibility theory of TCM, Yuanzhi as a sovereign drug and Shichangpu as an envoy drug, the combination of the two herbs have shown synergistic interaction and thus achieved better curative effects than using them alone. The clinically recommended dosage of *P. tenuifolia* and *A. tatarinowii* in Chinese pharmacopeia is 6-9 g/60 kg. The acute toxicity study showed that PTAT is nontoxic when the dose is up to an oral dose of 1.75 g/kg for mice.

A dramatic synergy and compatibility of two or more medicinal herbs play a key role in of preventing and treating a wide range of diseases in the clinical practice of TCM. Despite a few previously reported studies that have indicated that major bioactive constituents in both *P. tenuifolia* and *A. tatarinowii* have anticonvulsant effects (Bai *et al*., 2019; Liu *et al*., 2015), the anticonvulsant potential of this herb pair remains unclear. Therefore, this study aimed to evaluate the therapeutic effects of PTAT through seizure severity assessment and various behavioral tests in acute and chronic epileptic animal models. Furthermore, the effect of PTAT on the state of oxidative stress, inflammatory response, as well as neurotransmitter release in the brain was also evaluated in the PTZ-induced seizures. The present study provides pharmacological evidence for the use of PTAT decoction against epileptic convulsions, which will be beneficial for the complementary and alternative drug discovery and development of PTAT in the future.

MATERIALS AND METHODS

Reagents and chemicals

Pentylenetetrazol (PTZ) was purchased from Alfa Aesar, Shanghai, China. Lot: 10180463, reference drug sodium valproate (SVA) was purchased from J&K Scientific Ltd., Beijing, China. Both PTAT decoction and SVA were dissolved in saline. Test kits for superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA) and glutathione (GSH) were obtained from Nanjing Jiancheng Bioengineering Research Institute Co., Ltd. ELISA Kit for interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and Monocyte chemoattractant protein-1 (MCP-1) purchased from Elabscience Biotechnology Co., Ltd. The reagents used for the determination of GABA, glutamate, GABA-transaminase (GABA-T) and L-glutamate decarboxylase (GAD) were purchased from Shanghai Yuanxin Biotechnology Co., Ltd. PTZ was dissolved in saline. The saline, SVA and PTAT decoction were administered orally at a dose volume of 10ml/kg per body weight.

Animals and ethics

SPF adult Kunming mice (Scxk (Guangdong) 2020-0051) weighing between 24 and 28 g were obtained from the BesTest Bio-Tech Co., Ltd. They were housed in a regulated environment (23±2ºC; 50±10% humidity, 12h light/dark cycle) with free access to pellet food and water. All experiments complied following the guidance of management regulations of Guangdong Medical Laboratory Animal Center (Guangdong, China) and were carried out following the NIH guidelines. All experimental protocols were approved on 30 July 2020 by the Animal Care Committee of Zunyi Medical University (Zhuhai, China) (Permit NO: ZYLS-[2020] No. 2-081).

Preparation of the PTAT decoction

The decoction pieces of *P. tenuifolia* and *A. tatarinowii* were purchased from Beijing Tong Ren Tang Chinese Medicine Co., Ltd. Accurately weigh a sample containing 10g of *P. tenuifolia* and 10g of *A. tatarinowii* to decoction vessel and preparation of PTAT decoction as follows: add 12 times water for the first time, soak for 30 min and decoct for 120 min; add 6 times water for the second time, decoct for 60 minutes (Xu and Wu, 2018). The filtrate is combined and then the mixture is concentrated under a rotary evaporator to obtain a dry solid residue. On the day of the experiment in this study, less concentrated solutions (200, 100 and 50mg/kg) were diluted with normal saline, respectively.

Experimental design

In the acute seizures test, PTAT decoction was given every day for a period of 7 days. On the seventh day, 30 minutes after the end of drug administration, the MES experiment and PTZ (85mg/kg)-induced mice seizures test were carried out. In the chronic mice model of epilepsy, seizures were induced in the mice by subcutaneous injection of 35mg/kg PTZ in alternative days for a period of 14 days. Treatments were administered orally every day for a period of 29 days. Oncesub-convulsive injections of PTZ, the mice were placed individually and observed continually for 30 min and the behavioral seizure activity was measured the adapted Racine scale following as 0, no seizures response; 1, jerks of short duration; 2, unilateral or bilateral limb clonus; 3, clonic forelimb seizures lasting more than 3 s; 4, tonic-clonic seizures; 5, death. The experimental design is shown in fig. 1.

MES test in the mice

MES test was performed as described by He *et al*. (2018). Sixty mice were randomly divided into five groups, 12 in each group. Mice in the control group were given orally 0.9% NaCl solution only. Mice in the positive control group received SVA (100mg/kg, *p.o.*). Mice in the test groups received PTAT decoction by oral gavage at a dose of 50, 100 and 200mg/kg, respectively. All treatments lasted for 7 days and 30 min, 1h, 2h and 4h after administration on the seventh day before the stimulus (giving an electrical stimulus 64 Hz, 50 mA for 0.25 s). The mice were considered protected when no tonic hind-limb extension was displayed in mice.

PTZ-induced tonic-clonic seizures in mice

PTZ-induced mice seizures test was performed as described by He *et al*. (2018). Sixty mice were randomly divided into five groups of 12 mice each and treated as described above. After 60 min of the vehicle, positive drugs and PTAT decoction treatment, PTZ (85mg/kg) was administered subcutaneouslyto induce seizures. After each PTZ injection, each mouse was placed in a separate box to record the latency time of the 1st seizures and observe several mice with generalized seizures (seizures last no less than 5s), clonic seizures and death induced by PTZ for a period of 20 min.

PTZ-induced status-epilepticus in mice

Mice were randomly divided into six groups of twelve mice each as follows: Group I (normal group) and Group II (control group) receiving normal saline (10ml/kg, *p.o.*); Group III (positive control) receiving SVA (100mg/kg, *p.o.*). Groups IV, V and VI received PTAT decoction (50, 100 and 200mg/kg, *p.o.*, respectively). In PTZ-kindled test, 60 min after the different treatments, mice in all groups, except for those of the group received subcutaneous injections with a total of 14 injections of sub convulsive dose of PTZ (35mg/kg) on alternate days over 29 days.

Kindling procedure and behavioral seizures monitoring

After each PTZ injection, each mouse was observed for a duration of 30 minutes for recording latency to first myoclonic jerks and evaluating the severity of the seizure according to Racine stages with minor modifications as described earlier (Van Erum, Van Dam and De Deyn, 2019; Li *et al*., 2021). The cumulative kindling score was plotted against the duration of treatment.

Functional tests

Forty-eight hours after the challenge with PTZ on the 30th day, a set of assays including an elevated plus-maze (EPM) test and open field test (OPT) were undertaken on each experimental group to assess cognition impairment and exploratory behavior induced by PTZ kindling in mice. These tests were all carried out using Shanghai XR Instruments under temperature and humidity-controlled conditions. The test apparatus, once used, was cleaned and disinfected using 70 % ethanol to remove olfactory cues.

EPM test

EPM test was performed as per previously described by Mishra *et al*. (2021). The maze (Shanghai xinruan Information Technology Co., Ltd, XR-XG201) consists of a plus-shaped platform 50cm above the floor with two open (35 cm long ×5 cm wide) arms, a central square (5 cm long×5 wide) and two closed (35cm long × 5cm wide ×15 cm height) arms. In this study, the pretesting of the EPT test was performed after PTZ-kindling test at 30th days. The retention trial was performed similarly on the 31st day. In the acquisition session, each mouse was placed in the central area of the maze and monitored for 10 min and the times and residence time of mice entering the open arm within 10 min were recorded by software monitored during the test.

Open field test

OPT was performed to evaluate the free locomotion and exploratory behavior of the mice as per previously described by Kavaye Kandeda *et al*. (2021). The opening box inner with the floor divided into 9 equal quadrants (Shanghai, XR-XZ301) is 50 cm in diameter and 40 cm in height. In this study, the pretesting of OPT was performed after PTZ- kindling test at 32nd days. Retention trial was performed similarly on the 33rd day. In the acquisition session, the mice were placed in the opening box inner and the video analysis system was used to record the total distance and movement time of mice entries into the central area within 5 min.

Biochemical assays

24 h after functional tests, mice were sacrificed and their brains were removed quickly and cleaned with ice-cold buffer. The hippocampus was separated and stored at -80ºC for further measurement of the levels of oxidative stress markers, pro-inflammatory factors and GABA metabolism in the hippocampus. Before the biochemical test, the hippocampi were weighted. 10% (w/v) homogenates prepared with 0.1 M phosphate buffer (ice-cold, pH 7.4) were centrifuged by refrigerated centrifuge according to different protocols and the supernatants were collected and used to measure the determination of the level or activity of indicators selected in the study.

Determination of oxidative stress status in the hippocampus

Quantitative assessment of SOD, CAT, MDA and GSH was carried out using commercially available assays kit purchased from Nanjing Jiancheng Bioengineering Research Institute Co., Ltd, including SOD assay kit (WST-1 method), CAT) assay kit (Ultraviolet), MDA content detection kit (TBA method) and GSH assay kit (Spectrophotometric method). The protein content of each sample was measured using the BCA method (Elabscience). The measurement procedures were carried out in strict accordance with the instructions of the kit. SOD and CAT activity were calculated as U/mg protein. MDA and GSH levels were calculated as nmol/g tissue and μmol/mg tissue.

Determination of pro-inflammatory factors in the hippocampus

Quantitative assessment of TNF-α, IL-1β, IL-6 and MCP-1 was carried out using a commercially available ELISA kit (Elabscience Biotechnology Co., Ltd). The protein content of each sample was measured using BCA method (Elabscience). The measurement procedures were carried out following the manufacturer's protocols. TNF-α, IL-1β, IL-6 and MCP-1 levels were calculated as pg/mg protein.

Determination of GABA metabolism in the hippocampus

Quantitative assessment of GABA-AT, GAD, GABA and glutamate was carried out using a commercially available assay kit purchased from Shanghai Yuanxin Biotechnology Co., Ltd. The protein content of each sample was measured using the BCA method (Elabscience). The measurement procedures were carried out in strict accordance with the instructions of the kit. GABA-AT and GAD activity was calculated as U/mg protein. GABA and glutamate levels were expressed as ng/mg of tissue.

STATISTICAL ANALYSIS

Data in this study were represented as mean ± SD. One-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test was performed to analyze the data. The chi-square test was used for ratio statistics. Values of $p<0.05$ were considered statistically significant. The statistical analyses were conducted using Prism 5 software.

RESULTS

Antiepileptic effect of PTAT decoction in mice

MES test

As shown in fig. 2, PTAT decoction dose and time-dependently protected against mice hind-limb tonic extension (HLTE) in the MES model. It was showed that PTAT decoction at 50, 100 and 200mg/kg protected mice from HLTE to 50%, 58.33% and 83.33% respectively compared to the control group in 1 hour after the last administration, while SVA at 100mg/kg used as the positive drug also showed 66.67 % protection in MES test. Especially, PTAT decoction at 100 and 200mg/kg protected mice from HLTE to 16.67% and 25.00% respectively compared to the control group in 4 hours after the last administration, showing an enduring effect on the prevention seizures. It indicated that both the active components and their metabolites in PTAT decoction contributed to its antiepileptic activity.

PTZ-induced tonic-clonic seizures

In the acute PTZ-induced seizures model, the subcutaneous injection of 85mg/kg PTZ induced tonic seizures, clonic seizures and mortality in 100 % of the mice. As shown in fig. 3A and 3B, PTAT decoction at 50, 100 and 200mg/kg reduced the incidence of tonic seizures and mortality significantly and dose-dependently in PTZ tests. In addition, PTAT decoction has dose-dependent effects on the latency time of the 1st seizures. It has been observed that PTAT decoction at 200mg/kg prolonged the latency of seizures to 216.93 s from 114.85 s (PTZ treated group), while in 50 and 100mg/kg tested groups the latency time of the 1st seizures was prolonged to 138.3s and 203.65s respectively in comparison to the PTZ treated group. Similarly, SVA (100mg/kg) which was used as positive drug prolonged the latency of seizures in the PTZ test. However, both PTAT decoction and SVA failed to control clonic seizures, although they reduced mortality in mice caused by PTZ to varying degrees.

Effect of PTAT decoction on behavioral seizures in PTZ-kindling mice

As shown in fig.4 A, sub convulsive doses (35mg/kg) of repetitive PTZ notably and shorten the latency to the first myoclonic seizures in the last PTZ injection in 28th days. While, effects of 29-day PTAT decoction treatment with doses of 50, 100 and 200mg/kg prolonged onset latency of seizures to 118.81, 155.34 and 184.39 s from 83.88 s (PTZ treated group), respectively, Similarly, SVA (100mg/kg) which was used as reference drug also increased the latency to first myoclonic jerk as compared to the PTZ treated group ($p<0.001$). In addition, as shown in fig.4 B, the consecutive injection of PTZ (35mg/kg) induced a gradual increase in the seizure severity evaluated according to Racine's scale. The 29-day PTAT decoction treatment dramatically hindered the kindling process and alleviated seizure severity. The protection offered to the mice in the PTAT decoction group (100mg/kg) was comparable to that in the SVA group at the same dose compared to the PTZ-pretreated group. In particular, the final mean seizure score in the high-dose of PTAT decoction group was 2.61±0.12 at a dose of 200mg/kg, while in the model group of mice, it was 3.44±0.13. Pre-treatment with PTAT decoction attenuated seizures with dose-dependently manner PTZ induced kindling in mice, showing that PTAT decoction has a potential anti-epileptogenic effect.

Effect of PTAT decoction on anxious behavior in mice on EPM test

As shown in fig. 5A, the mean percentage of time spent in the open arm of the EMP in the non-kindled group was 66.8±8.2, while in the PTZ-kindled group, it was reduced to 18.0±4.4 ($p<0.001$). The concurrent administration of PTZ and PTAT decoction (50, 100 and 200mg/kg) dramatically increased the time spent on the open arm as compared to the PTZ-treated group, showing PTAT decoction improved cognition impairment caused by PTZ. Similarly, repeated oral administration of PTAT decoction at doses of 100 and 200mg/kg increased the time spent and entries into the open arm, but the effects were not significant at test doses of 50mg/kg ($p>0.05$) (fig.5B). Furthermore, treatment with VPA (100mg/kg) did not significantly increase the percentage of time spent and entries into the open arm in the EPM test in comparison to that of mice in the PTZ-kindled group.

Effect of PTAT decoction on anxious behavior in mice on OPT

The effects of PTAT decoction on the free locomotion of mice in the OPT are presented in fig. 5C and fig. 5D. The study found that PTZ injections significantly decreased the time spent and movement distance in the central zone in OFT, showing that PTZ inhibited the exploratory behavior and free locomotion in mice as compared with that of mice in the normal group. While PTAT decoction dose-dependently attenuated the PTZ kindling-induced decline in exploration and free locomotion. PTAT decoction increased time spent and movement distance in the central zone with an increase of 95.5 and 44.2% at the highest dose of 200mg/kg, respectively. The PTAT decoction-treated mice in the doses of 50 ($p<0.05$) and 100mg/kg ($p<0.01$) showed significantly decreased immobility time and increased innate exploratory behavior compared with the saline+ PTZ kindled group. Similarly, animals treated with SVA also enhanced free locomotion in OPT, showing that the anxiety-like behavior was relieved.

Effects of PTAT decoction on oxidative stress markers

The effect of the PTAT decoction on some oxidative stress markers is presented in fig.6. As shown in fig.6A, the SOD activity in the hippocampus in the PTZ group was significantly lower than the normal control group. PTAT decoction pretreatment dose-dependently induced an increasing activity of SOD with activity increased by 57.5, 106.7 and 105.0% at 50, 100 and 200mg/kg compared to the PTZ kindled group. Similarly, a significant reduction in CAT activity was found in the PTZ kindled group ($p<0.01$). Pre-treatment with PTAT decoction seemed to cause a significant increase in this enzyme activity (fig.6B). fig. 6C demonstrated that MDA levels were significantly increased in PTZ kindling mice compared to that of the normal control group ($p<0.001$). Pretreatment with PTAT decoction significantly reversed the increase of MDA levels in each treated group when compared with saline+PTZ- kindled group in the hippocampus of the mice. However, SVA did not significantly change the level of MDA ($p>0.05$). As shown in fig.6D, PTZ-induced kindling in the control group resulted in a decrease in the GSH levels compared to the normal group ($p<0.01$). After PTAT decoction and SVA pretreatment, the GSH level was effectively increased compared to PTZ kindling group. Therefore, PTZ kindling resulted in the overproduction of reactive oxygen species and caused oxidative stress, while PTAT decoction reduced oxidative damage in epileptic mice, showing an antioxidant mechanism.

Effects of PTAT decoction on pro-inflammatory markers

The effect of the PTAT decoction on the level of TNF-α, IL-1β, IL-6 and MCP-1 in the hippocampus is presented in fig.7. The data showed that the TNF-α, IL-1β, IL-6 and MCP-1 level significantly increased in PTZ kindling group with an increase of 92.5, 227.0, 72.8 and 92.3% as compared to the normal group, respectively. Administration of PTAT decoction remarkably decreased TNF-α, IL-1β, IL-6 and MCP-1 levels at doses of 100 and 200mg/kg. While TNF-α was found to be decreased remarkably in the PTAT decoction 50mg/kg group. SVA at a dose of 100mg/kg decreased only the IL-1β level, by 69.4% when compared to PTZ kindling group.

Effect of PTAT decoction on GABA metabolism

The effect of PTAT decoction on the level of GABA and GAD, as well as the activity of GABA-T and GAD in the hippocampal tissue of the kindled mice are shown in fig.8. PTZ-induced kindling group resulted in an increase in the activity of GABA-T, while resulted in a decline in GAD activity compared to the normal group (fig. 8A and 8C). The PTAT decoction decreased GABA-T activity at all test doses compared to the PTZ kindling group. In addition, PTAT decoction at doses of 100 and 200mg/kg increased GAD activity by 69.1 and 78.1%, respectively. Similarly, it was found that glutamate levels were significantly increased, while GABA levels were significantly decreased in the hippocampal of the PTZ-induced kindling mice as compared with the normal group ($P<0.001$). Interestingly, GABA levels were found to be increased significantly in PTAT decoction (100 and 200mg/kg) with an increase of 83.6 and 94.8% (fig.8B), whereas the levels of glutamate were significantly decreased in all the PTAT decoction-treated groups (50, 100 and 200mg/kg) with a decline of 13.2, 25.5 and 34.3% (fig.8D) as compared with PTZ kindling group.

DISCUSSION

Based on a traditional clinical practice regarding the effectiveness and therapeutic value of PTAT decoction on epilepsy, the present study aimed to assess the antiepileptic effect of PTAT decoction on MES, PTZ-induced acute seizure model, as well as the PTZ-kindling model of epilepsy and clarify possible action mechanisms. The MES model is recognized as a classic and commonly used experimental model for antiepileptic drug screening and it is also often used to screen effective candidate drugs for generalized tonic-clonic seizures (Fisseha, Hammeso and Nureye, 2022).

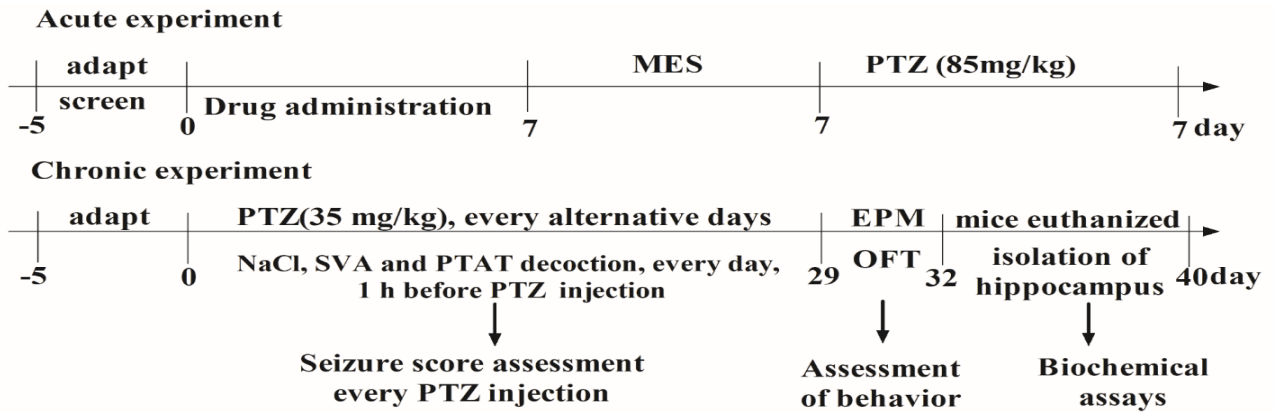

Fig. 1: Graph depicting the experimental protocol of this study. Behavioral seizure evaluation was initiated immediately after the MES or PTZ injection for mice in acute experiments. The anxiety-like behavior evaluation for post-kindling mice was initiated 24 h after the last PTZ injection. Mice used in the chronic experiment were then killed for biochemical analysis after completing all the rat behavioral tests.

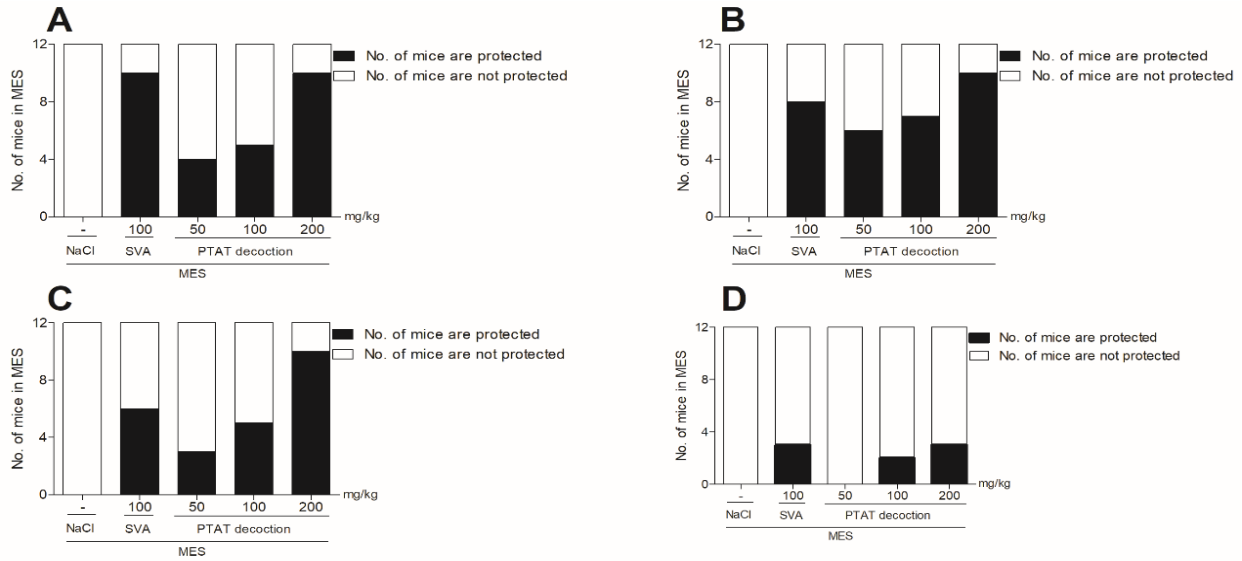

Fig. 2: Effect of PTAT decoction pretreatment for 7 days on maximal electroshock seizures (MES) in mice (n=12). A. 0.5 h after the last drug administration; B. 1.0 h after the last drug administration; C. 2.0 h after the last drug administration; D. 4.0 h after the last drug administration.

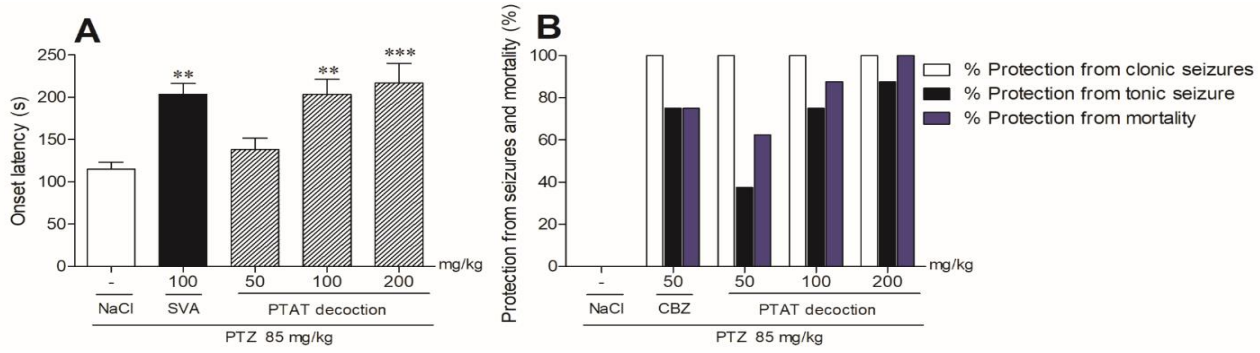

Fig. 3: Effect of PTAT decoction pretreatment for 7 days on subcutaneous PTZ (85mg/kg) induced acute seizures in mice (n=12). A, the onset latency time of the 1st seizures in the PTZ injection; B, the % protection from tonic seizures, clonic seizures and mortality. $^{**}p<0.01$, $^{***}p<0.001$ vs. model group (NaCl+PTZ). NaCl, normal saline; PTZ, pentylenetetrazole; SVA: sodium valproate;

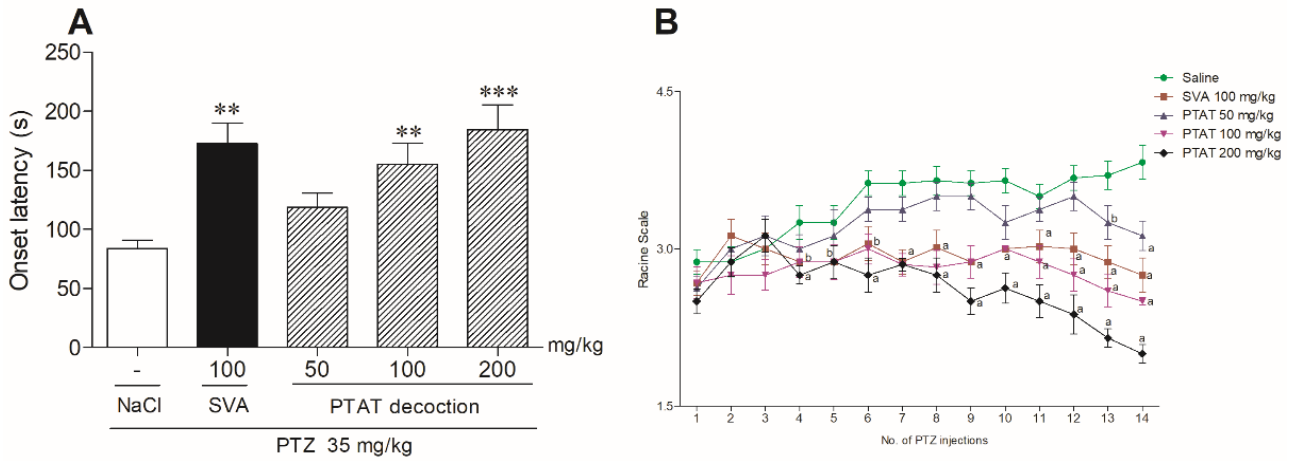

Fig. 4: Effect of PTAT decoction pretreatment for 28 days on the severity of seizures in PTZ (35mg/kg)-kindled in mice. A total of 14 injections of sub-convulsive dose of PTZ (35mg/kg) were administered on alternate days during 29 days period. Data are means ± SEM, n=8 per group. A, the onset latency time of the 1st seizures in the last PTZ injection; B, the Racine scale (kindling score) in 14 days from PTZ injection. $^{**}p<0.01$, $^{***}p<0.001$ vs. model group (NaCl+PTZ). a, significantly different from model control ($p<0.001$); b, significantly different from model control ($p<0.01$).

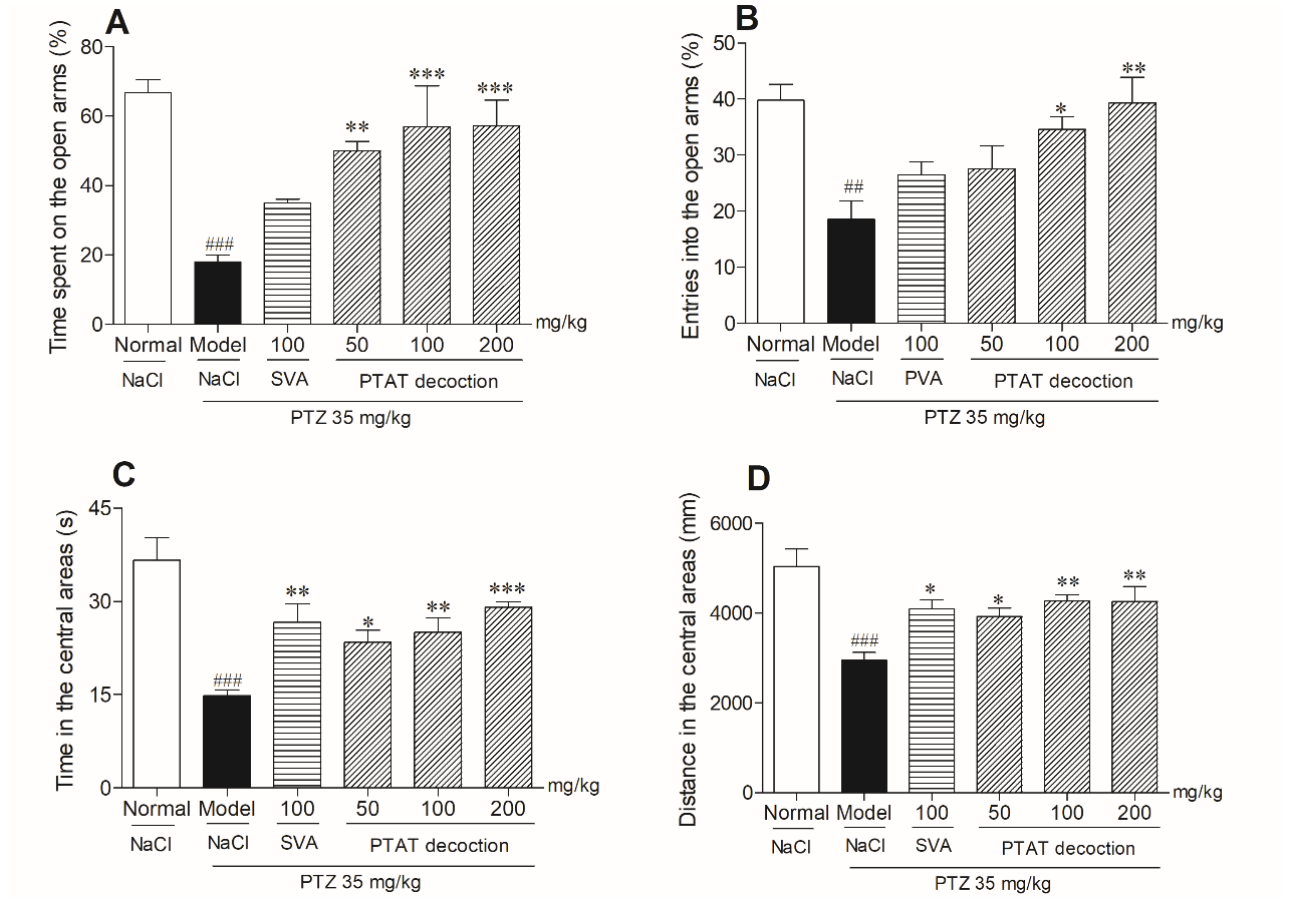

Fig. 5: Effect of PTAT decoction pretreatment on anxiety-like behavior in PTZ-kindled mice in an elevated plus maze and open field tests. A, the percent time spent on the open arms in the EPM test; B, the percentage of the number of mice entries into the open arms in the EPM test; C, time spent at the central areas in OFT; D, walking distance in the central areas in OFT. Data are expressed as means ± SEM, n = 5. Dunnett *post hoc* test and one-way ANOVA test and Chi-square test: vs. normal group (NaCl), $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; vs. model group (NaCl+PTZ), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p <$

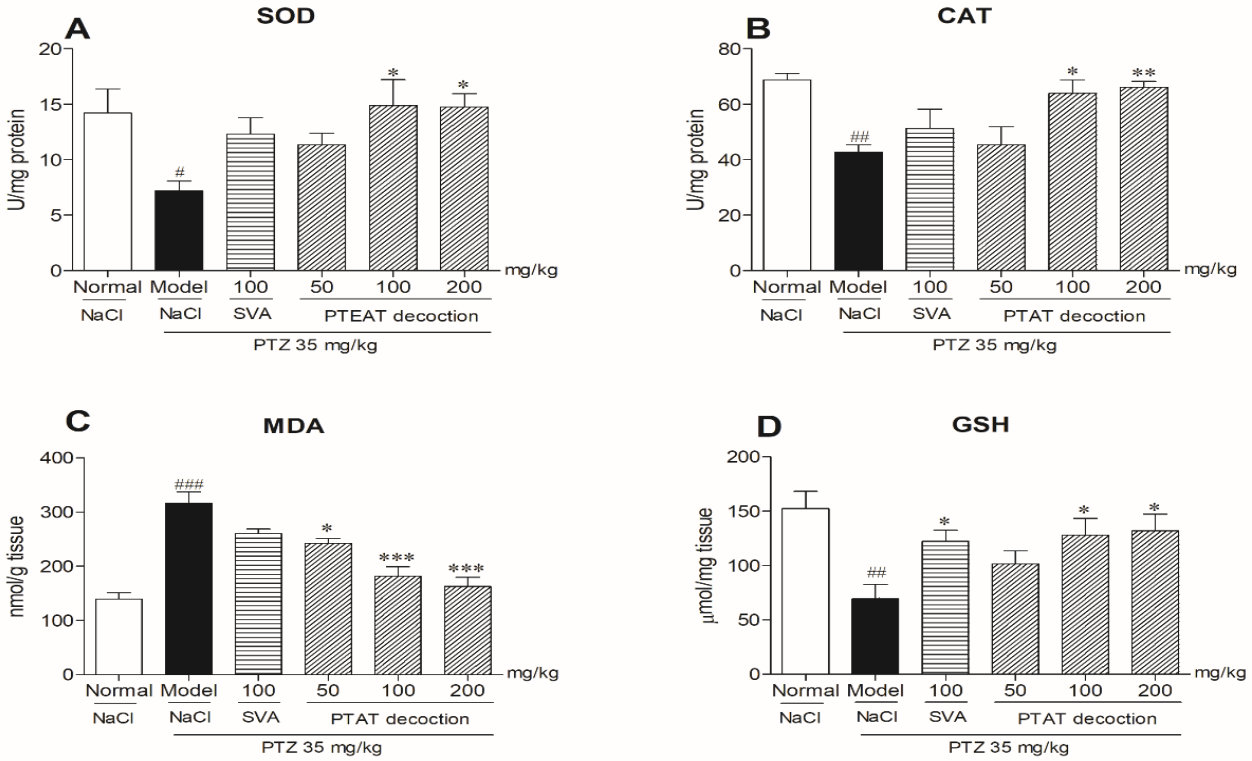

Fig. 6: Effect of PTAT decoction pretreatment on oxidative stress markers after PTZ-induced seizures of mice. Data are expressed as means ± SEM, n = 5. Dunnett *post hoc* test: vs. normal group (NaCl), $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; vs. model group (NaCl+PTZ), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

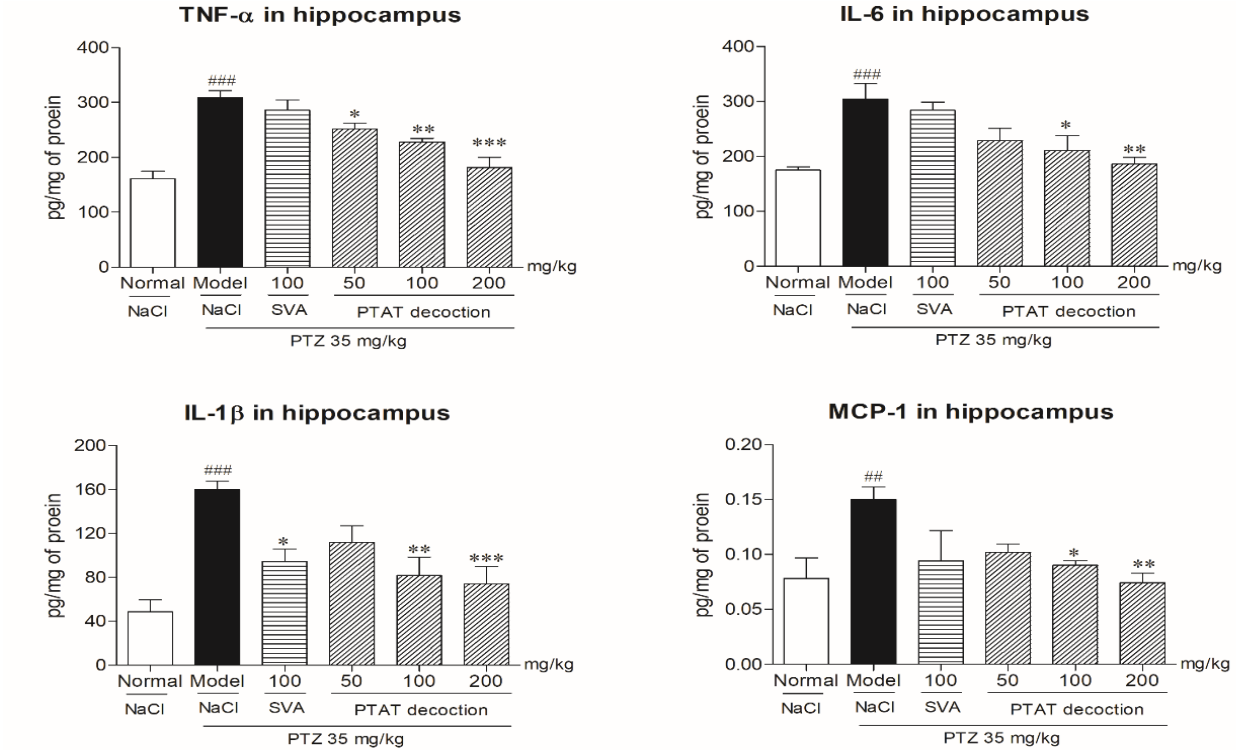

Fig. 7: Effect of PTAT decoction pretreatment on the pro-inflammatory factors after PTZ-induced seizures of rat's hippocampus. Data are expressed as means±SEM, n=5. Dunnett *post hoc* test: vs. normal group (NaCl), $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$; vs. model group (NaCl+PTZ), $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

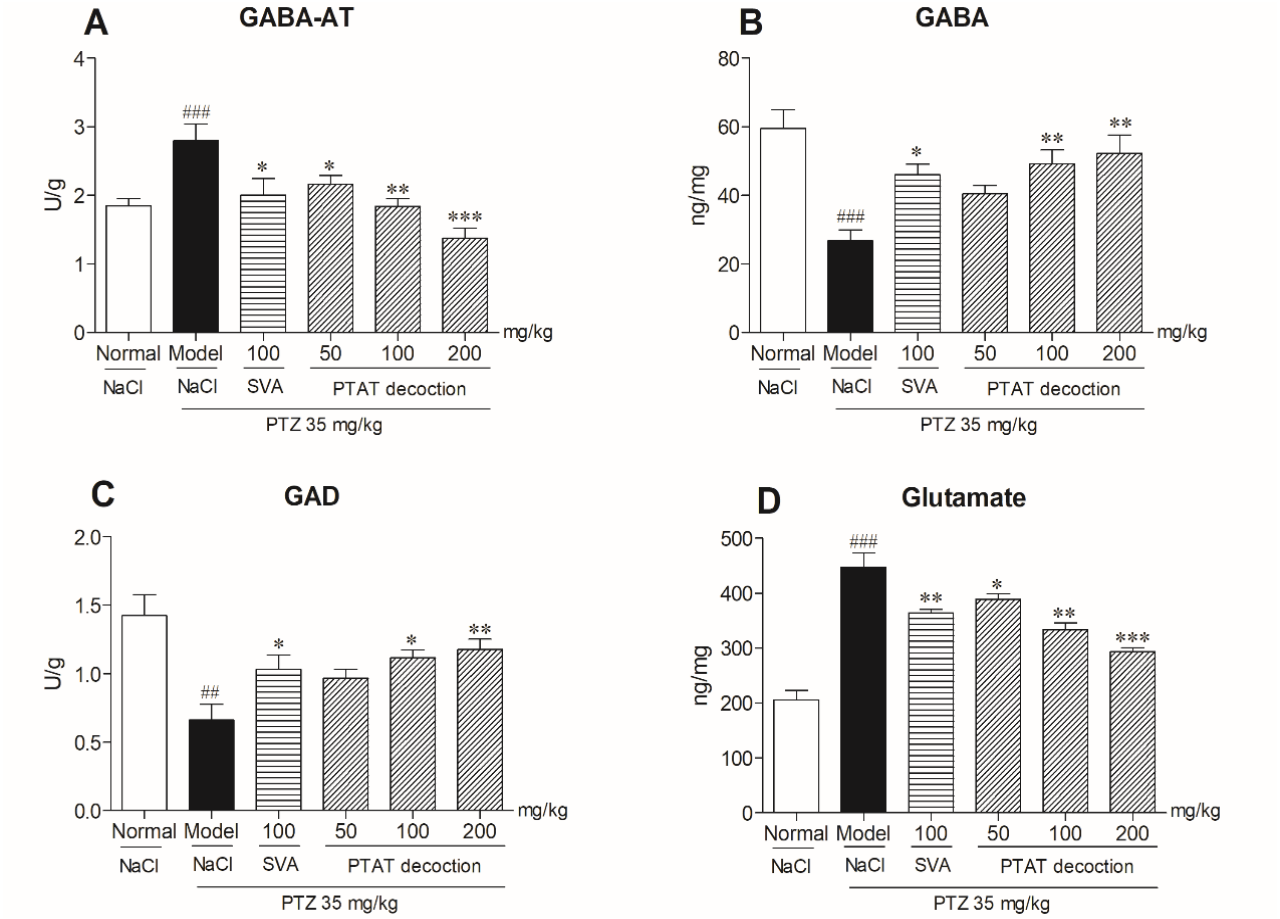

Fig. 8: Effect of PTAT decoction pretreatment on GABA metabolism after PTZ-induced seizures of rat's hippocampus. A, GABA-AT activity; C, GAD activity; B, levels of GABA; D, levels of glutamate. Data are expressed as means±SEM, n=5. Dunnett *post hoc* test: vs. normal group (NaCl), $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$; vs. model group (NaCl+PTZ), $^{*}p<0.05$, $^{**}p < 0.01$, $^{***}p<0.001$.

It is well known that the MES model enhances the influx of Na^+ and increased the level of glutamate at the synaptic terminals (Khattak *et al*., 2021). In the present study, PTAT decoction protected 50% of mice against HLTE induced by MES at 50mg/kg 1 hour after administration, showing time- and dose-dependent anti-epileptic activity. It also indicates that this protective effect may be due to the chemical components contained in the PTAT decoction, which prevent seizures by inhibiting sodium channels and/or attenuating glutamate neurotransmission in the brain. In addition, the effects of the PTAT decoction were comparable to those of SVA, a widely used antiepileptic drug that has proven broad-spectrum of antiepileptic activity observed in humans (Nevitt, Marson and Tudur Smith, 2018). As no known adverse reactions have been discovered from its traditional application, PTAT decoction may be available in the form of a natural phytotherapy remedy for seizures.

PTZ is an antagonist of the GABA receptor and has been commonly used to construct acute seizures model for rapid screening anticonvulsant agents. In addition, it is thought that repeated injection of sub-convulsant doses of PTZ produced convulsive behavior simulation of clinically resistant epilepsy by regulating GABAergic neurotransmission and thereby interacting with glutamate-aggravated seizures (Kavaye Kandeda *et al*., 2021; Seo *et al*., 2020). In the current study, we construct an acute seizure model to evaluate the anticonvulsant activity of PTAT decoction. It was found that PTAT decoction treatment for 7 consecutive days dose-dependently delays the onset of tonic-clonic seizures induced by PTZ (85mg/kg). Meanwhile, PTAT decoction also produced an obvious reduction in the percentage of generalized seizures and death in acute seizure models in mice. Furthermore, in the PTZ-kindling seizure model in mice, the chronic administration of PTZ (35mg/kg) increased prominently the seizure score. While PTAT decoction increased the latency of the 1st seizures and decreased convulsive seizures score and intensity. Especially, the protection conferred by PTAT decoction at a dose 100mg/kg was comparable to that offered by SVA at the same dose, a clinically commonly used antiepileptic drug in humans. Moreover, PTAT decoction at 200mg/kg

decreased seizure intensity and the mice in this group rarely occurred generalized seizures. The results of these data demonstrated that PTAT decoction has a promising anti-seizure activity, which could explain and support its traditional use against seizures in traditional medicine practice. Of course, these delightful results also encourage us to conduct more in-depth research.

About 30%-40% of epilepsy patients have cognitive impairment and other neurological disorders. Recurrent seizures can lead to a decrease in attention, memory, planning and judgment (Allendorfer and Arida, 2018). In addition, chronic seizures are commonly accompanied by depression, anxiety and psychosis (Gruenbaum *et al*., 2021; Singh and Goel, 2021; Michelin *et al*., 2022). Experimental findings showed that the repeated administration of sub convulsant doses of PTZ increased behavioral alteration and anxiety-like behavior in mice (Jaiswal and Kumar, 2022; Rehman *et al*., 2022). Hence, there is a need to identify PTAT decoction with curative and beneficial effects on behavioral changes in PTZ kindling mice. In the present study, the EPM and OPT were undertaken to evaluate how the behavioral alterations developed. The results of the EPM and OPT indicated that pretreatment with PTAT decoction enabled long-term functional recovery. However, SVA had no significant effects on the exploration and behavioral alterations. Thus, it fully demonstrated traditional Chinese medicine has quite the characteristics of relieving the primary and secondary symptoms of epilepsy at the same time.

There is a growing body of evidence showed that neuronal hyperexcitability and oxidative damage caused by excessive generation of free radicals might closely be related to the occurrence and development of epilepsy (Geronzi, Lotti and Grosso, 2018; Singh *et al*., 2022). Studies have also certificated that targeting oxidative stress giving drugs more targeted for a limited time window starting early after injury efficiently improved long-term disease outcomes in a rat model of acquired epilepsy (Pauletti *et al*., 2019). Besides, animal studies have indicated that inhibiting the production of reactive oxygen species or reactive nitrogen species improved the survival rate and cognitive defects after status epileptics and reduced neuronal damage in chronic epilepsy (Vezzani, Balosso and Ravizza, 2019). It has also been found that the functions of superoxide dismutase and glutathione peroxidase in human progressive myoclonic epilepsy have changed. This is a serious form, which is related to progressive neurological deterioration and seizures, but that are refractory to most drugs (Vezzani, Balosso and Ravizza, 2019; Rehman *et al*., 2022). Therefore, drugs with significant antioxidant activity will have important clinical value for the management of epilepsy. Our experimental findings suggested administration of PTAT decoction significantly raised CAT and SOD activities, increased GSH level and decreased MDA level in the hippocampus of PTZ kindling mice, showing that PTAT decoction has an amelioration impact on oxidative stress injury. Therefore, PTAT decoction with multi-component has multi-target and multi-pathway effects in controlling seizures and improving symptoms, which is characteristic of a multi-level and integrated treatment for epilepsy. This is also the advantage of PTAT decoction different from the commonly used clinical drug SVA.

Neuroinflammatory pathways are known to contribute to the development and progression of seizures and epilepsy (Vezzani, Balosso and Ravizza, 2019). There is a growing body of clinical and experimental studies certificated that brain inflammation might be a cause or a consequence of seizures (Alvi *et al*., 2021). For example, the level of pro-inflammatory factors such as TNF-α, IL-1β, IL-6 and MCP-1 are increased in the brains of the PTZ-kindling model of epilepsy in mice and rats (Kavaye Kandeda *et al*., 2021; Alvi *et al*., 2021). It was found that PTZ kindling mice resulted in a significantly increased level of this pro-inflammatory factor, which is consistent with the previous literature report. In addition, we found that PTAT decoction decreased the level of each of these cytokines in PTZ-kindling mice's hippocampus. This also provides a basis for PTAT decoction which is commonly used to reduce swelling in traditional applications. Additionally, these properties could demonstrate that PTAT decoction inhibited the activation of resident glial cells and also showed a protective effect against PTZ-induced hippocampal neuron damage. However, future studies must be carried out to explore the inflammatory signaling pathway and associated neuron-inflammatory cascades related to the antiepileptic and neuroprotection activity of the PTAT decoction.

It is well established that PTZ kindling interfered with GABAergic neurotransmitters and glutamatergic neurotransmission by alteration in the level of GABA, glutamate, DA, NE, 5-HT and their metabolites in the brain of mice (Koshal and Kumar, 2016). In agreement with the previously reported findings, our study found that PTZ treatment induced a decrease in the levels of GABA and an increase in the levels of glutamate. While pretreated PTAT decoction dose-dependently reversed PTZ-caused changes in GABA and glutamate in mice hippocampal, suggesting the involvement of neurotransmission. GABA is the main inhibitory neurotransmitter in the body and GABA-T is the rate-limiting enzyme that metabolizes and regulates GABA (Al-Obaidi, Elmezayen and Yelekçi, 2021). Our data indicated that the PTZ kindling showed a significant increase in GABA-T activity. However, the mice that were pretreated with PTAT decoction inhibited the activity of this enzyme, suggesting GABA generation recovery. GAD is a key enzyme that converts glutamate into the inhibitory neurotransmitter GABA in the brain

(Dade *et al*., 2020). It was observed a decrease in GAD activity in the hippocampus of PTZ kindling mice. While PTAT decoction treatment dose-dependently reversed PTZ-induced decline in GAD activity, which could be an explanation for the increased GABA level and decreased glutamate concentration observed in the study. Our research shows that PTAT decoction has the effect of promoting the synthesis of main inhibitory neurotransmitters and increased degradation of the excitatory neurotransmitter. This effect might be mediated by its abundant bioactive phytochemicals and interaction between these compounds. These previous studies found that α-asarone isolated from *A. tatarinowii* possesses the potential to modulate GABA catabolism by inhibiting GABA-T activity, increasing GABA synthesis through up-regulating the expression of GAD67 (Miao *et al*., 2011; Yuan *et al*., 2019) though further studies of specific components that are responsible for modulating neurotransmitters from PTAT decoction are needed. In the future, if we can explore and make good use of these potential active ingredients to the greatest extent, it will help to further clarify their antiepileptic material basis, developing scientific quality evaluation as well as revealing the mechanism of action at the molecular level action mechanism for PTAT decoction.

CONCLUSION

PTAT decoction protected mice against MES and PTZ-induced acute seizures. It also ameliorated seizure severity, suppressed the development of kindling and relieved anxiolytic-like behavior in PTZ-kindled mice, showing prominently anticonvulsant properties. These effects seem to be associated with and mediated by mitigating oxidative stress, attenuating inflammation and regulating the neurotransmitters in PTZ-kindled mice. The findings in the current study both validated and illuminated ethnopharmacological application of PTAT decoction in the clinical of traditional Chinese medicine in the treatment of epilepsy in China. Therefore, PTAT decoction could be used as a valuable phytotherapy therapy in the management of seizures and related diseases. However, further studies should be conducted to explore in-depth topics of material basis, quality evaluation and systematic clinical evaluation for PTAT decoction.

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ETHICS APPROVAL

Animals studies were performed in strict accordance with the guidelines for the Care and Use of Laboratory Animals and granted by the Ethics Committee of Zunyi Medical University (Date: July 30, 2020/NO: ZYLS-[2020] No. 2-081).

REFERENCES

Allendorfer JB and Arida RM (2018). Role of physical activity and exercise in alleviating cognitive impairment in people with epilepsy. *Clin. Ther*., **40**(1): 26-34.

Al-Obaidi A, Elmezayen AD and Yelekçi K (2021). Homology modeling of human GABA-AT and devise some novel and potent inhibitors via computer-aided drug design techniques. *J. Biomol. Struct. Dyn*., **39**(11): 4100-4110.

Alvi AM, Shah FA, Muhammad AJ, Feng J and Li S (2021). 1,3,4, oxadiazole compound a3 provides robust protection against ptz-induced neuroinflammation and oxidative stress by regulating Nrf2-pathway. *J. Inflamm. Res*., **14**: 7393-7409.

Amlerova J, Šroubek J, Angelucci F and Hort J (2021). Evidences for a role of gut microbiota in pathogenesis and management of epilepsy. *Int. J. Mol. Sci*., **22**(11): 5576.

Bai YJ, He XR, Bai YJ, Sun Y, Zhao ZF, Chen XF, Li B, Xie J, Li Y, Jia P, Meng X, Zhao Y, Ding YR, Xiao CN, Wang SX, Yu J, Liao S, Zhang YJ, Zhu ZL, Zhang Q, Zhao YH, Qin FG, Zhang Y, Wei XY, Zeng M, Liang J, Cuan Y, Shan GZ, Fan TP, Wu B and Zheng XH (2019). *Polygala tenuifolia-Acori tatarinowii* herbal pair as an inspiration for substituted cinnamic α-asaronol esters: Design, synthesis, anticonvulsant activity and inhibition of lactate dehydrogenase study. *Eur. J. Med. Chem*., **183**: 111650.

Chinese Pharmacopoeia Committee (2020). Chinese Pharmacopoeia. Vol. 1. *Chemical Industry Press*, Beijing.

Dade M, Berzero G, Izquierdo C, Giry M, Benazra M, Delattre JY, Psimaras D and Alentorn A (2020). Neurological Syndromes Associated with Anti-GAD Antibodies. *Int. J. Mol. Sci*., **21**(10): 3701.

Editorial Board of Chinese Materia Medica (1999). Chinese Materia Medica. *Shanghai Science and Technology Press, Shanghai*. pp. 1-252.

Fisseha N, Hammeso, WW and Nureye, D (2022). Anticonvulsant activity of hydro alcoholic extract and solvent fractions of *Biophytum umbraculum* welw. Syn (Oxalidaceae) root in mice. *J. Exp. Pharmacol*., **14**: 291-299.

Geronzi U, Lotti F and Grosso S (2018). Oxidative stress in epilepsy. *Expert. Rev. Neurother.*, **18**(5): 427-434.

Gruenbaum BF, Sandhu MRS, Bertasi RAO, Bertasi TGO, Schonwald A, Kurup A, Gruenbaum SE, Freedman IG, Funaro MC, Blumenfeld H and Sanacora G (2021). Absence seizures and their relationship to depression and anxiety: Evidence for bidirectionality. *Epilepsia*, **62**(5): 1041-1056.

He LY, Hu MB, Li RL, Zhao R, Fan LH, He L, Lu F, Ye X, Huang YL and Wu CJ (2021). Natural medicines for the treatment of epilepsy: bioactive components, pharmacology and mechanism. *Front. Pharmacol*., **12**: 604040.

He XR, Bai YJ, Zeng M, Zhao ZF, Zhang Q, Xu N, Qin FG, Wei XY, Zhao MM, Wu N, Li ZH, Zhang YJ, Fan TP and Zheng XH (2018). Anticonvulsant activities of α-asaronol ((E)-3'-hydroxyasarone), an active constituent derived from α-asarone. *Pharmacol Rep*., **70**(1): 69-74.

Hoda U, Jain S, Samim M, Jain GK and Agarwal NB (2021). Embelin ameliorates cognitive dysfunction and progression of kindling in pentylenetetrazol-induced kindling in mice by attenuating brain inflammation. *Epilepsy Behav*., **116**:107788.

Jaiswal G and Kumar P (2022). Neuroprotective role of apocynin against pentylenetetrazole kindling epilepsy and associated comorbidities in mice by suppression of ROS/RNS. Behav. *Brain Res*., **419**: 113699.

Kavaye Kandeda A, Okomolo Moto FC, Mbomo Ayissi RE, Omam Omam JP, Ojong L and Ngo Bum E (2021). *Pergularia daemia* hydro-ethanolic extract protects against pentylenetetrazole kindling-induced seizures, oxidative stress and neuroinflammation in mice. *J. Ethnopharmacol*., **279**: 114338.

Khattak ZF, Ansari B, Jamal M, Awan AA, Sherkheli MA and Ul Haq R. (2021). Anticonvulsant activity of methanolic extract of *Withania cogulans* in mice. *Metab. Brain Dis*., **36**(8): 2437-2443.

Li DY, Bai XY, Jiang Y and Cheng YH (2021). Butyrate alleviates PTZ-induced mitochondrial dysfunction, oxidative stress and neuron apoptosis in mice via Keap1/Nrf2/HO-1 pathway. *Brain Res. Bull*., **168**(issue): 25-35.

Lin CH and Hsieh CL (2021). Chinese herbal medicine for treating epilepsy. *Front. Neurosci*., **15**(issue): 682821.

Liu H, Song Z, Liao DG, Zhang TY, Liu F, Zhuang K, Luo K, Yang L, He J and Lei JP (2015). Anticonvulsant and sedative effects of eudesmin isolated from *Acorus tatarinowii* on mice and rats. *Phytother. Res*., **29**(7): 996-1003.

Liu Y, Gao JL, Peng M, Meng HY, Ma HB, Cai PP, Xu Y, Zhao Q and Si GM (2018). A review on central nervous system effects of gastrodin. *Front. Pharmacol*., **9**: 24.

Löscher W, Potschka H, Sisodiya SM and Vezzani A (2020). Drug resistance in epilepsy: Clinical impact, potential mechanisms and new innovative treatment options. *Pharmacol. Rev*., **72**(3): 606-638.

Miao JK, Chen QX, Wu XM and Zhang XP (2011). Effect of alpha-asarone on regulation of GABA system in the lithium-pilocarpine model of epilepsy. *Chinese Pharmacological Bulletin*, **27**(8): 1067-1071.

Michelin AP, Maes MHJ, Supasitthumrong T, Limotai C, Matsumoto AK, de Oliveira Semeão L, de Lima Pedrão JV, Moreira EG, Kanchanatawan B and Barbosa DS (2022). Reduced paraoxonase 1 activities may explain the comorbidities between temporal lobe epilepsy and depression, anxiety and psychosis. *World J. Psychiatry*, **12**(2): 308-322.

Mishra P, Mittal AK, Rajput SK and Sinha JK (2021). Cognition and memory impairment attenuation via reduction of oxidative stress in acute and chronic mice models of epilepsy using antiepileptogenic Nux vomica. *J. Ethnopharmacol*., **267**(issue): 113509.

Modi AC, Mara CA, Schmidt M, Smith AW, Turnier L and Wade SL (2021). Pilot executive functioning intervention in epilepsy: Behavioral and quality of life outcomes. *J. Pediatr. Psychol*., **46**(4): 363-374.

Nevitt SJ, Marson AG and Tudur Smith C (2018). Carbamazepine versus phenobarbitone monotherapy for epilepsy: an individual participant data review. *Cochrane Database Syst. Rev*., **10**(10): CD001904.

Pauletti A, Terrone G, Shekh-Ahmad T, Salamone A, Ravizza T, Rizzi M, Pastore A, Pascente R, Liang LP, Villa BR, Balosso S, Abramov AY, van Vliet EA, Del Giudice E, Aronica E, Patel M, Walker MC and Vezzani A (2019). Targeting oxidative stress improves disease outcomes in a rat model of acquired epilepsy. *Brain*, **142**(7): e39.

Rehman Z, Farooq T, Javaid S, Ashraf W, Fawad Rasool M, Samad N, Tariq M, Anjum SMM, Sivandzade F, Alotaibi F, Alqahtani F and Imran I (2022). Combination of levetiracetam with sodium selenite prevents pentylenetetrazole-induced kindling and behavioral comorbidities in rats. *Saudi Pharm J*., **30**(5): 494-507.

Seo S, Song Y, Gu SM, Min HK, Hong JT, Cha HJ and Yun J. (2020). D-limonene inhibits pentylenetetrazole-induced seizure via adenosine a2a receptor modulation on gabaergic neuronal activity. *Int. J. Mol. Sci.*, **21**(23): 9277.

Singh T and Goel RK (2021). Epilepsy associated depression: an update on current scenario, suggested mechanisms and opportunities. *Neurochem. Res*., **46**(6): 1305-1321.

Singh R, Sarangi SC, Singh S and Tripathi M (2022). A review on role of metformin as a potential drug for epilepsy treatment and modulation of epileptogenesis. *Seizure*, **101**: 253-261.

Strzelczyk A and Schubert-Bast S (2022). Psychobehavioural and cognitive adverse events of anti-seizure medications for the treatment of developmental and epileptic encephalopathies. *CNS drugs*, **36**(10): 1079-1111.

Thijs RD, Surges R, O'Brien TJ and Sander JW (2019). Epilepsy in adults. *Lancet*, 2 **1393**(10172): 689-701.

Van Erum J, Van Dam D and De Deyn PP (2019). PTZ-induced seizures in mice require a revised Racine scale. *Epilepsy Behav*., **95**: 51-55.

Vezzani A, Balosso S and Ravizza T (2019). Neuroinflammatory pathways as treatment targets and biomarkers in epilepsy. *Nat. Rev. Neurol*., **15**(8): 459-472.

Xu YP and Wu XM (2018). Preliminary study on the compatibility mechanism of couplet medicines rhizoma *Acori tatarinowii* and radix polygalae. *China J. Trad. Chin. Medi. Pharm*., **33**(12): 5629-5632.

Yuan X, Li Z, Wang XT, Li XY, Hua H, Li XC, Tang RX and Liu XM (2019). Roles and mechanisms of traditional Chinese medicine and its active ingredients in treating epilepsy. *Zhongguo Zhong Yao Za Zhi*, **44**(1): 9-18.

Zhao ZF, He XR, Ma CX, Wu SP, Cuan Y, Sun Y, Bai YJ, Huang LH, Chen XF, Gao T and Zheng XH (2018). Excavating anticonvulsant compounds from prescriptions of traditional Chinese medicine in the treatment of epilepsy. *Am. J. Chin. Med*., **46**(4): 707-737.