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Original Research Article

Anticonvulsant and sedative effect of *Fufang Changniu* pills and probable mechanism of action in mice

Huiqin Zhang, Juanjuan Lu, Yi Zhang*, Yumei Zhao, Jia Wei and Liya Zhou

Gansu Provincial Hospital, Lanzhou 730000, PR China

*For correspondence: **Email:** yizhang207@126.com; **Tel/Fax:** +86-0931-8281857

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Abstract

Purpose: To investigate the anticonvulsant and sedative effects of *Fufang Changniu Pills* (FCP) and its probable mechanism of action in mice.

Methods: The water decoction of FCP was prepared and the main constituents were determined by high performance liquid chromatography (HPLC). The anticonvulsant activities of FCP were evaluated by maximal electroshock (MES) and pentylenetetrazole (PTZ)-induced seizures in mice. Pentobarbital sodium-induced sleeping time and locomotor activity measurements were performed to evaluate the sedative effects of FCP in mice. Finally, PTZ-induced chronic seizures were established, and expressions of gamma-aminobutyric acid A receptor (GABA-A) and glutamic acid decarboxylase 65 (GAD65) in the brains of the mice were assayed by western blot in order to explore the probable mechanisms of action of the drug.

Results: Gallic acid, liquiritin, cinnamyl alcohol, cinnamic acid and glycyrrhizic acid were detected in FCP decoction. FCP (50, 100 and 200 mg/kg) showed significant anticonvulsant and sedative effects on epileptic mice induced by MES ($p < 0.05$) and PTZ ($p < 0.05$). Moreover, pentobarbital sodium-induced sleeping time and locomotor activity tests showed that FCP possesses sedative effect ($p < 0.05$). Western blot data indicate that FCP significantly up-regulated GABA-A and GAD 65 in the brains of chronic epileptic rats ($p < 0.05$).

Conclusion: FCP has significant anticonvulsant and sedative effects, and the mechanism of its action may be related to the up-regulation of GABA-A and GAD 65 in mice brain.

Keywords: Epilepsy, *Fufang Changniu* pills, Anticonvulsant, Sedative effect, Gamma-aminobutyric acid, Glutamate dehydrogenase

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INTRODUCTION

Epilepsy is a common chronic neurological disorder worldwide, and it affects almost 50 million people according to the World Health Organization [1]. Epilepsy is caused by multiple factors such as genes, trauma, neurodegeneration and intoxication [2]. Previous investigations have proved that individuals with epilepsy have higher mortality rates than the general population [3-4]. Although a large

number of antiepileptic drugs (AEDs) were used to suppress or prevent seizures, there are still 25 – 40 % of the patients remaining with uncontrolled seizures and who have experienced severe side effects induced by these drugs [5,6]. Thus, it is necessary to discover new therapeutic drugs with fewer side-effects for the treatment of epilepsy.

Traditional Chinese Medicine (TCM) has been widely used in East and Southeast Asia, and it

still retains mainstream medicine in China today. Over thousands of years, TCMs including a variety of botanicals and herbs were used to treat epilepsy [7]. *Fufang Changniu Pills* (FCP) is a commonly used TCM formula which consists of *Acori Tatarinowii Rhizoma*, *Pharbitidis Semen*, *Cinnamomi Ramulus*, *Polygalae Radix*, *Uncariae Ramulus Cum Uncis*, and *Glycyrrhizae Radix Et Rhizoma* (Table 1). It is commonly used in Chinese folk medicine for treating amnesia, epilepsy, deafness and tinnitus, etc.

However, to the best of our knowledge, there were few reports regarding the antiepileptic effect of FCP. The present study was designed to investigate the anticonvulsant and sedative effect of FCP and its potential mechanisms.

EXPERIMENTAL

Chemicals and reagents

Pentylentetrazole (PTZ) was purchased from the R&D Systems Inc. (Shanghai, China). Phenytoin sodium and pentobarbital sodium were purchased from Shanghai Westang Biotech Co. (Shanghai, China). Diazepam was obtained from the China's National Institutes for Food and Drug Control (Beijing, China). GABA-A and GAD-65 monoclonal antibody were obtained from Santa Cruz Biotechnology, Inc. (Shanghai, China). All other chemicals used in present study were of analytical grade.

Sample preparation

The formula composition of FCP are shown in Table 1. All the crude drugs were powdered and soaked in water for 1 h before decocting extraction for 3 times and 1 h each time. The water decoction were filtered and concentrated under reduced pressure at 50 °C. As a result, the concentration of FCP (w/w) was 8.7 %. The water extract of FCP was dissolved by water and administered orally (ig).

HPLC analysis

A Waters e2695-2998 HPLC with the diode array detector (DAD) (Waters, USA) was used for determining the constituents of FCP. All samples separation were performed on the Kromasil C18 (250 mm × 4.6 mm, 5 μm) (AkzoNobel, Sweden) column using a gradient elution at a flow rate of 1 mL/min. The mobile phase consisted of A (0.1 % formic acid) and B (acetonitrile) with a gradient program as follows: 0 - 15 min, 5 - 20 % B; 15 - 25 min, 20 - 30 % B; 25 - 35 min, 30 - 40 % B; 35 - 50 min, 40 - 70 % B. The detection wavelength was set at 280 nm, the injection volume was 20 μL, and the column temperature was at 30 °C.

Animals

Male ICR mice (21 ± 2 g) and SD rats (190 ± 20 g) used in this study were purchased from the Shanghai laboratory animal center (Shanghai, China). Animals were housed under a 12 h light/dark cycle with free access to food and water, and the temperature was maintained at 25 °C and humidity at 50 %. Each animal was used only once in the experiment. All the experiments carried out were in accordance with the "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) [8] and approved by the Animal Ethics Committee of Gansu Provincial Hospital (Approval No. AN 201505-7).

Maximal electroshock (MES) seizure test

The MES induced seizure test was performed according to the method described previously with some modifications [9]. In the present study, electrical alternating current stimulus of 50 mA for 0.2 s was used. Mice were pretreated with FCP (50, 100 and 200 mg/kg, ig), phenytoin (20 mg/kg, intraperitoneally, ip) and normal saline (20 mL/kg, ig). The electroshock was administered after 30 min. Animal hind limb tonic extension (HLTE) was observed within 10 s after electroshock delivery. The complete abolition of HLTE was defined as anticonvulsant effect against MES-induced seizures.

Table 1: Crude materials composition of FCP

Name	Family	Original Plant	Weight (g)
Acori Tatarinowii Rhizoma	Araceae	<i>Acorus tatarinowii</i> Schott	10.0
Pharbitidis Semen	Convolvulaceae	<i>Pharbitis purpurea</i> (L.) Voigt.	5.0
Cinnamomi Ramulus	Lauraceae	<i>Cinnamomum cassia</i> Presl	10.0
Polygalae Radix	Polygalaceae	<i>Polygala tenuifolia</i> Willd.	10.0
Uncariae Ramulus Cum Uncis	Rubiaceae	<i>Uncaria rhynchophylla</i> (Miq.) Miq.ex Havil.	12.0
Glycyrrhizae Radix Et Rhizoma	Leguminosae	<i>Glycyrrhiza uralensis</i> Fisch.	6.0

Pentylentetrazole (PTZ) seizure test

PTZ-induced seizure test was carried out as described by Hosseinzadeh *et al* [10]. Mice were pretreated with FCP (50, 100 and 200 mg/kg, ig), diazepam (4 mg/kg, ip), and normal saline (20 mL/kg, ig). At 30 min after administration, mice in all groups were treated with PTZ (85 mg/kg, subcutaneously). All the mice were observed within 30 min, and the complete abolition of HLTE was defined as anticonvulsant effect against MES-induced seizures.

Pentobarbital-induced sleeping time test

The test of pentobarbital-induced sleeping time in mice was also carried out according to a previous report [11]. Mice were pretreated with FCP (50, 100 and 200 mg/kg, ig), diazepam (4 mg/kg, ip), and normal saline (20 mL/kg, ig). After 30 min, mice were administered with pentobarbital sodium (50 mg/kg, ip). Each mouse was observed for the loss of righting reflex, i.e., the mice cannot roll back when turned over. The interval time between the loss and recovery of the righting reflex was recorded as the index of hypnotic effect.

Locomotor activity measurement

Locomotor activity measurements were performed according to the method per Chindo *et al* [12]. Mice were pretreated with FCP (50, 100 and 200 mg/kg, ig), diazepam (4 mg/kg, ip), and normal saline (20 mL/kg, ig). A ZH-YLS-1C photoelectrical spontaneous locomotor activity recorder (ZhengHua Biological instrument & equipment Co., Ltd., Anhui, China) was used to record the locomotor activity of the mice after 30 min. Each mouse was placed individually in the spontaneous recorder for 5 min and the basal activity score was obtained. The activities of the mice were recorded during 5 min.

PTZ induced chronic epileptic rats

The chronic epileptic rat model was established using a modified method of Yang *et al* [13]. The rats were divided into 4 groups: control group and three treatment groups of FCP (50, 100 and 200 mg/kg). Rats were administered with PTZ (40 mg/kg/d, ip) for 4 weeks to establish the chronic epileptic model.

Then, rats were pretreated with FCP (50, 100 and 200 mg/kg/d, ig) and normal saline (20 mL/kg, ig) for 21 days. After that, all rats were anesthetized with sodium pentobarbital (40 mg/kg, ip) and sacrificed, and then the whole brains were collected.

Western blot assay

Total proteins of the brain tissue were extracted, and 40 µg equal amounts of protein were separated by SDS-PAGE, blotted on polyvinylidene difluoride (PVDF) membranes. The proteins were probed with anti-GAD 65 and GABA-A rabbit polyclonal IgG, and subsequently goat anti-rabbit/HRP before detecting with chemiluminescence peroxidase reagents. Antibodies directed against GAPDH were used to measure protein loading.

Statistical analysis

The anticonvulsant effects against MES and PTZ-induced seizures among groups (inhibition and mortality) were analyzed by Chi-square exact test. Data are presented as mean ± SD, while two-tailed Student's t test was used to perform statistical analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Main constituents of FCP

The main constituents in FCP were analyzed by HPLC method and the results were showed in Figure 1. The FCP sample was well separated under the used conditions in the experiment. As a result, five main constituents were detected by comparing their retention times and UV spectra with the corresponding reference standards.

The five constituents were identified as Gallic acid, Liquiritin, Cinnamyl alcohol, Cinnamic acid and Glycyrrhizic acid, respectively.

MES seizure

The MES-induced seizure model was established to investigate anticonvulsant effects of FCP in mice. As shown in Table 2, the results of MES-induced seizure test indicated that FCP had significant anticonvulsant effects at the doses of 50, 100 and 200 mg/kg ($p < 0.05$, $p < 0.01$, $p < 0.001$, respectively), compared with the control group. Additionally, the inhibitions of FCP treated group at the doses of 50, 100 and 200 mg/kg were 0, 60 and 100 %, respectively.

PTZ-induced seizure

The PTZ-induced seizure in mice was commonly used to evaluate the anticonvulsant effect of drugs. From Table 3, the results showed that FCP had significant anticonvulsant effect against PTZ-induced seizures at the doses of 50, 100, and 200 mg/kg ($p < 0.05$, $p < 0.01$ and $p < 0.001$,

respectively), and the inhibitions were 60, 80, and 90 %, respectively.

Furthermore, FCP significantly decreased the mortality or prevented the death of the mice at the doses of 50, 100, and 200 mg/kg ($p < 0.01$, $p < 0.01$ and $p < 0.001$, respectively).

Pentobarbital-induced sleeping time

As shown in Figure 2, the mice treated with diazepam (4 mg/kg, ip) showed obvious loss of righting reflex. After treatment with FCP (50, 100 and 200 mg/kg), the duration of sleeping time was significantly prolonged ($p < 0.05$, $p < 0.01$ and $p < 0.01$, respectively). Additionally, sleep latency was also significantly shortened by treating with FCP at the doses of 50, 100 and 200 mg/kg ($p < 0.05$, $p < 0.05$ and $p < 0.05$, respectively).

Locomotor activity

As can be seen from Figure 3, the locomotor activity of the mice treated with diazepam (4 mg/kg) were significantly reduced compared with the control group ($p < 0.01$). FCP (50, 100 and 200 mg/kg) significantly reduced the locomotor activity of mice compared with control groups ($p < 0.01$, $p < 0.05$, and $p < 0.01$, respectively) with dose-dependent manner.

Effect of FCP on expressions of GAD65 and GABA-A

The chronic epileptic rat model was established to investigate the effects of FCP on expressions of GAD65 and GABA-A in brains. As shown in the Figure 4, after treatment of FCP, GABA-A expressions in the brains of the rats were significantly up-regulated ($p < 0.01$), compared

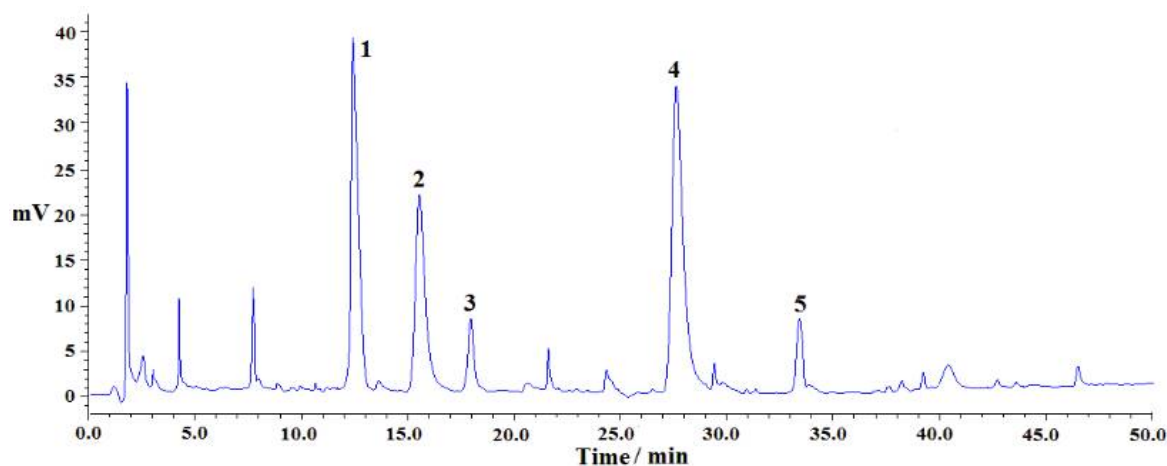


Figure 1: HPLC chromatograms of the FCP extract where (1) Gallic acid, (2) liquiritin, (3) cinnamyl alcohol, (4) cinnamic acid and (5) glycyrrhizic acid

Table 2: Effect of FCP on tonic seizures induced by maximal electroshock in male mice (n = 10)

Treatment	Dose	Convulsions	Inhibition of seizure (%)	Death	Mortality (%)
Control		10	0	4	40
Phenytoin	20 mg/kg	0	100***	0	0
	50 mg/kg	6	40*	0	0
	100 mg/kg	4	60**	0	0
	200 mg/kg	0	100***	0	0

Phenytoin was used as the positive drug; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, vs control group

Table 3: Effect of FCP on PTZ-induced seizures in male mice (n=10)

Treatment	Dose	Convulsions	Inhibition (%)	Death	Mortality (%)
Control		10	0	100	100
Diazepam	4 mg/kg	0	100***	0	0***
	50 mg/kg	4	60*	3	30**
	100 mg/kg	2	80**	1	10**
	200 mg/kg	1	90***	1	10***

Diazepam was used as the positive control drug; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, vs control group

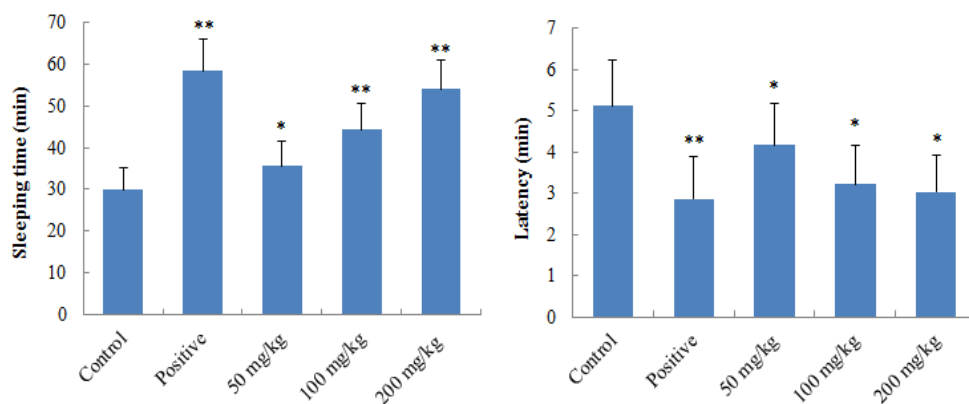


Figure 2: Effects of the FCP on pentobarbital sodium -induced sleeping time in mice (n=10). Diazepam (4 mg/kg) was treated as the positive drug; * $p < 0.05$, ** $p < 0.01$, vs control group

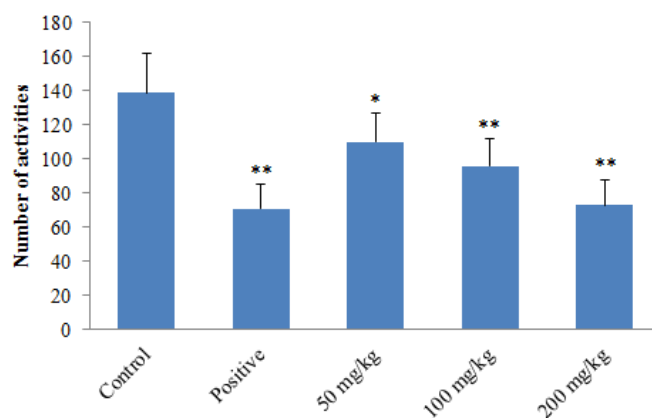


Figure 3: Effects of the FCP on motor function in mice (n=10). Diazepam (1mg/kg) was treated as the positive agents; * $p < 0.05$, ** $p < 0.01$, vs control group

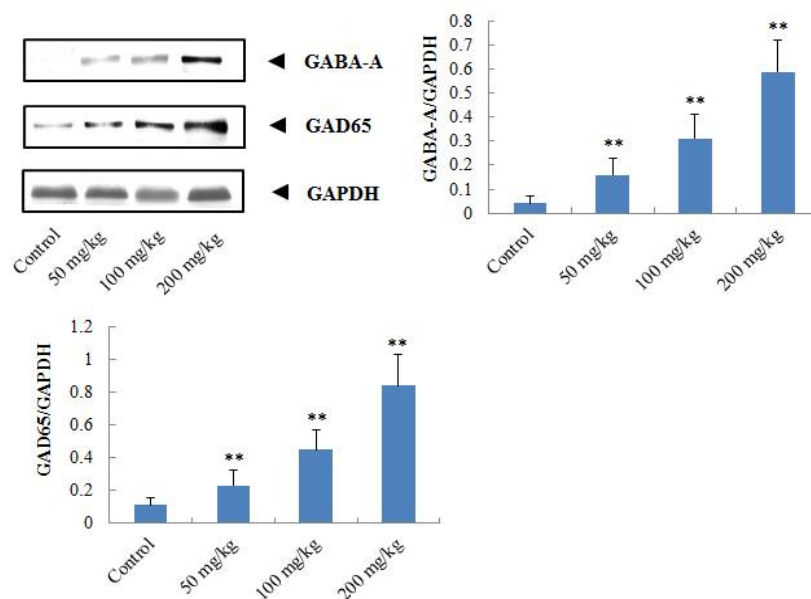


Figure 4: Effects of FCP on the expression of GABA-A and GAD 65 in the brain of chronic epileptic rats (n=10). ** $p < 0.01$, vs control group

with the control group. Furthermore, expressions of GAD65 were also significantly up-regulated by treating with FCP (50, 100, 200 mg/kg) ($p < 0.01$).

DISCUSSION

It was reported that the antiepileptic drugs could neither provide a cure nor prevent relapse, and these drugs often cause some adverse reactions, such as cognitive dysfunction, teratogenesis, and blood dyscrasias, etc. [14,15]. TCM is often considered to be a gentle and safe way to synthetically manufacture drugs, and it is the most widely practiced form of herbalism worldwide [7]. FCP is a TCM formula widely used for treating epilepsy in Chinese folk medicine. The present study proved that FCP had significant antiepileptic and sedative effect, which was conducive to find new agents for the treatment of epilepsy.

MES and PTZ models are considered the "Gold standards" in the early stages of testing epilepsy [16]. MES and PTZ tests are used to identify anticonvulsant effect against petit mal seizures and generalized tonic-clonic seizures [12,17]. The effects of FCP against PTZ-induced seizures suggested the anticonvulsant efficacy against epilepsy in humans. The effect of most anti-epileptic agents is to enhance the response to GABA, by facilitating the opening of GABA-activated chloride channels. GABA-A receptors were involved in epilepsy and their direct activation would have an anti-epileptic effect [9,18]. GAD65 is one of the major isoforms of GAD, and it comprises 70 % of total GAD protein and highly enriched in nerve terminals, where it is believed to be involved in the regulation of vesicular GABA synthesis [19]. In the present study, the results indicated that the expressions of GAD65 and GABAA can be up-regulated in the epileptic rats' brains by treatment of FCP, which might be a probable mechanism of FCP for the treatment of epilepsy.

CONCLUSION

The findings of the present study indicated that FCP had significant anticonvulsant and sedative effects, and the probable mechanism might be closely correlated with the up-regulating effect of GABA-A and GAD 65 in the brain.

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DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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