Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

Palmatine plays a role in sedation and hypnosis by increasing 5-hydroxytryptamine

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Received January 24, 2019; **Accepted** April 18, 2019; **Published** April 30, 2019

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Doi: http://dx.doi.org/10.14715/cmb/2019.65.4.11

Abstract: Objective of this study was to investigate the sedative and hypnotic effects of palmatine and to observe whether its mechanism is related to 5-hydroxytryptamine (5-HT) and GABA. The sedative and hypnotic effects of palmatine on mice were observed with mouse autonomic activity test, direct sleep test, pentobarbital sodium in suprathreshold and subthreshold dose sleep test. The content of GABA and 5-HT in brain homogenate was determined by ELISA method. Mouse brain specimens were observed by immunohistochemistry for 5-HT expression in the nucleus of mouse brain. Palmatine could reduce spontaneous activities of mice, prolong the sleep time of mice induced by pentobarbital sodium in suprathreshold dose and shorten the sleep latency. And it could increase the number of mice falling asleep induced by pentobarbital sodium in subthreshold dose and the incidence of falling asleep, but with no direct sleep effect. In addition, it enhanced the 5-HT content in brain, but had no effect on GABA content, and had no toxicity to PC12 cells. Palmatine plays a significant role in sedation and hypnosis, which may be associated with the increase of intra-cerebral 5-HT.

Key words: Palmatine; Sedation; Sleeping improvement; 5-HT; GABA.

Introduction

Huangteng is a dry radix of fibraurea recisa, a plant of menispermaceae family, also known as Tianxianteng, Tuhuanglian, Jingsuoshi, Tenghuanglian, Zhangyefangjijian, Bamading, etc. It is mainly distributed in Yunnan, Guangxi, Guangdong and other places in China, as well as Vietnam, Cambodia, and Myanmar. It is a traditional Chinese herb and natural dye commonly used by local ethnic minorities. It is in short supply due to large collection, so that artificial cultivation is necessary. Huangteng grows in the damp and fertile tropical and subtropical jungles of 180~1000 metres above sea level, often along tall vines (1, 2). Huangteng was first recorded in the "Compendium of Materia Medica". "Huangteng grows in Lingnan, southern of China, and looks like Stephaniae Tetrandrae, and monks often take it. Even if the food is toxic, it will not hurt the person due to detoxification of Huangteng. It is sweet, bitter and neutral in taste with no non-toxic." Chinese Pharmacopoeia (2015 edition) records that Huangteng is bitter in taste and cold in property, belongs to heart and liver meridians, and has the effect of clearing away heat and detoxification, purging fire and laxative. And it can prevent and cure various infectious diseases such as gynecological inflammation, surgical infection, bacteria Hemorrhoids, enteritis, respiratory infections, conjunctivitis, etc. (3, 4).

Palmatine, an alkaloid extracted and purified from Huangteng, has broad-spectrum antibacterial, anti-viral, anti-inflammatory and anti-tumor effects and can enhance the phagocytosis function of white blood cells, which is crude drug of some medicines such as palmatine tablets and palmatine injection (5-8). In recent years, the studies about its chemical composition has stagnated due to the increasing scarcity of Huangteng resources, and the researches on pharmacological effects focus the anti-inflammatory and antibacterial effects of palmatine. What is of interest to us is that studies have shown that tetrahydropalmatine plays a role in sedative, hypnotic and stabilizing effects (9, 10). However, it has not been reported the research on the sedative and hypnotic effects of palmatine at home and abroad. They are similar in structure so that it is worth digging whether they are in common in pharmacological properties. Therefore, we conducted the study focusing on the sedative and hypnotic effects of palmatine. Insomnia shows many symptoms in clinic due to multiple causes and complex mechanisms, involving many endogenous substances. The previous studies have revealed that among those substances, 5-hydroxytryptamine (5-HT) and gamma amino butyric acid (GABA) which are important neurotransmitters in the brain play a key role in the occurrence and maintenance of sleep (11, 12). In order to explore the sedative and hypnotic mechanism of palmatine, we further analyzed the relationship between palmatine and 5-HT as well as GABA, so as to provide scientific basis for its further development and utilization.

Materials and Methods

Experimental drugs and reagents

Artificially planted Huangteng was provided by Guangdong Yanguilai Health Preserving Production Co., Ltd. and identified by professionals; Pentobarbital sodium (Shanghai Merida Bio-products Co., Ltd.), batch number: 091015; Diazepam tablets (The Shenyang First Pharmaceutical Company of Northeast Pharm Group, GYZZ H21022887, specifications: 2.5 mg / tablet). 5-HT and GABA kits were purchased from Wuhan Elitet Biotechnology Co., Ltd,dulbecco's modification of eagle's medium Dulbecco (DMEM) from Thermo Fisher Biochemicals (Beijing) Ltd. (NZM1301), fetal bovine serum from Zhejiang Tianhang Biotechnology Co., Ltd. (20150918), methylthiazolyldiphenyl-tetrazolium bromide (MTT) from Beijing Dingguo Changsheng Biotechnology Co., Ltd. (51L10156), 5- HT and GABA antibodies from Cell Signal Biotech, USA, and other reagents were commercially available and analytically pure.

Experimental animals

KM mice, SPF grade, males, with 18 ~ 22 g of body weight, were provided by the Experimental Animal Center of Southern Medical University, production certificate number: SCXK (Yue) 2011-0015. The experimental conditions were 20 to 25 °C in temperature with 40% to 70% of relative humidity. The animals were prefed for three days before test. All animal experiments were complied with the ARRIVE guidelines and carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Main instruments

Instrument Enzyme - labelled meter (Thermo Electron Corporation Muhiskan Mk3); KQ-50B Ultrasonic Cleaner (Kunshan Ultrasonic Instrument Co., Ltd.); Electronic Balance (Beijing Sartorius Instrument System Co., Ltd.); YLS-1A Multifunctional Mouse Autonomous Activity Instrument (Jinan Yiyan Technology Development Co., Ltd.); high-speed refrigerated centrifuge KDC-160HR (Keda Innovation Co., Ltd.); electric heating incubator (DHG-9140A, Shanghai).

Methods

Preparation of palmatine

1) The Huangteng powder was extracted by ethanol 2 to 5 times at 30 to 70 °C, and the filtrates was mixed and cooled, which were re-filtered to remove insoluble impurities and obtain a filtrate.

2) The filtrate was evaporated to get a dry solid residue, and the solid residue was eluted with acid (hydrochloric acid) to get an eluent.

3) The eluate, whose pH value was adjusted to 9-10 with sodium hydrogencarbonate after filtered, was re-filtered when insoluble matters separated in rest. The filtrate was subjected to salting out to get crude product by filtration.

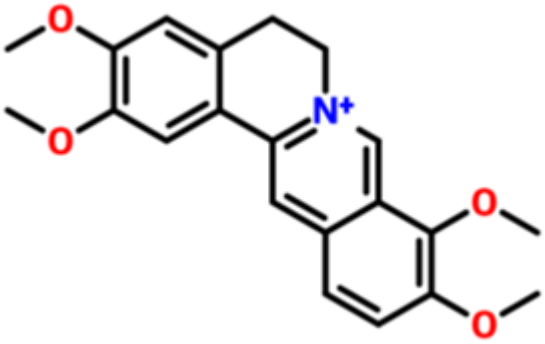

Figure 1. Palmatine structure.

4) The crude product was dissolved by heating with ethanol, and decolorized by activated carbon for 0.5 to 2 hours during dissolution. And the insoluble impurities were removed by filtration when it was hot. After the filtrate is cooled, concentrated hydrochloric acid was slowly added to precipitate sulphate palmatine, and palmatine was obtained from filtrating and drying sulphate palmatine and used for subsequent analysis (13, 14).

Effect on spontaneous activities of mice

Sixty male KM mice were randomly divided into 5 groups according to their body weight, namely, saline group, diazepam-positive group, and low-, medium-, and high-dose palmatine groups (119, 238, 476 mg/kg), 12 mice per group. Each animal in each palmatine group was given a corresponding dose of the drug daily, and the volume of gastric perfusion was 20 mL/kg, and the normal saline group was given an equal volume of normal saline for 30 days. Apart from administered an equal volume of normal saline continuously for 29 days, the diazepam-positive group was given 2.5 mg / kg of diazepam at day 30, and the administration volume was 20 mL/kg. Each group of mice was placed in a multifunctional mouse autonomous activity recorder 45 minutes after the last administration. The number of spontaneous activities of those mice was recorded within 5 min after adapting for 2 min (15-17).

Effect on the direct sleep of mice

The animal grouping and drug administration methods were the same as previously. The sleep of mice in each group was observed after continuous gavage with the corresponding drugs for 30 days. It would be considered as falling asleep if the mice's righting reflex disappeared for more than 60 s. If their righting reflex recovered, it would be considered as wakening up. And the duration between righting reflex disappearance and recovery was the animal's sleep time. The number of sleeping animals and the sleep time in the saline group and the administration group were recorded (18, 19).

Effect on the sleep time of the mice with pentobarbital sodium in suprathreshold dose

The animal grouping and drug administration methods were the same as previously. Each group of mice was intragastrically administrated for 30 days with the corresponding drugs. Each group was intraperitoneally injected with pentobarbital sodium in threshold hypnotic dose of 50 mg/kg 45 min after the last administration, and the injection volume was 10 mL/kg (the dose was determined by the pre-test which could make

100% of the animals fall asleep, but did not make them sleep too much). Righting reflex disappearance of the mice over 1 min was regarded as the sleep judgment index. The sleep time and latency of each mouse were recorded (20, 21).

Effect on the sleep time of the mice with pentobarbital sodium in subthreshold dose

The animal grouping and drug administration methods were the same as previously. Each group was intragastrically administered for 30 days with the corresponding drugs. Each mouse was intraperitoneally injected with sodium pentobarbital for a maximum subthreshold hypnotic dose of 30 mg/kg 45 min after the last administration, and the injection volume was 10 mL/kg (the dose was determined by the pre-test which could make 80%-90% of righting reflex not disappear, namely, the maximum subthreshold hypnotic dose of sodium pentobarbital). The sleep state of the mice within 30 min was observed, Disappearance of the righting reflex over 1 min was regarded as the criteria of falling asleep. And the number of sleeping mice in each group was recorded, and the incidence of sleeping mice was calculated (20).

Determination of 5-HT and GABA in the mice brain

The animal grouping and drug administration methods was the same way as previously. After drug administration for 30 minutes, each mouse was anesthetized with 20% urethane. Their hearts were perfused with 40 mL saline with thoracic cavity opened. The mice were immediately decapitated with craniotomy. The whole brain was taken out on ice bath, weighed with a balance, and then were mixed with normal saline based on mass-to-volume ratio to prepare a whole brain homogenate. The homogenate was centrifuged at 8 000 r pm, 4 ℃ to get supernatant, which was placed in a refrigerator at -70 °C for use.

Establishment of 5-HT and GABA standard curves: The standard solution of the system was configured, and the content of 5-HT was determined by the mouse 5-HT kit. The linear regression equation of the standard curve was calculated by the concentration of the standard and its corresponding OD value: Y= 0.0068 X + 0.3209 (R2 = 0.9932, n=5). It showed that 5-HT had a good linear relationship in the range of 25~400 nmol.L. The lowest quantitative concentration was 25 nmol.L-1. The standard curve was shown in Figure 2. The linear regression equation of GABA standard curve was calculated by the same method: Y= 0.0071X - 0.133 (R2 = 0.9988, n=5). It was indicated that the linear relationship of GABA was good in the range of 25~400 nmol.L-1, and the lowest quantitative concentration was 25 nmol.L-1, and the standard curve was shown in Figure 3 (22, 23).

After 30 days of drug administration, the whole brain was taken out after the mice were killed by cervical vertebra dislocation according to the ethical laws. The shredded brain tissue was weight 0.5g accurately and homogenized with 4.5 mL of PBS pH=7.4 for 2 min, and centrifuged at 4 °C, 3 000 r pm , in a high-speed refrigerated centrifuge for 20 min. The supernatant was collected and placed in a EP tube for use. According to the instructions of the enzyme-linked immunosorbent assay kit, the content of 5-HT and GABA in mouse brain tissue was determined by ELISA method in Infinite M200 light absorption microplate reader (TECAN, Switzerland) (21).

MTT Experiment

PC12 cells were cultured in DMEM containing 10% fetal bovine serum at 37 °C in a 5% CO_2 incubator, and passaged with 0.25% trypsin, and the fluid was changed every 2 d ays . The cell suspension was seeded in a 96-well cell culture plate at 1 × 104 cells per well, and cultured for several hours at 37 °C in a 5% CO_2 incubator to adhere the cells. The palmatine was grouped according to the purpose of the experiment (1 μg, 0.1 μg and 0.01 μg, respectively). Six parallel wells was set up in each group, and each well was add 200 μL of culture medium, placed in a CO_2 incubator, cultured persistently for 48 h at 37 °C. Each well was added 20 μL of MTT solution, and continues incubated for 4 h at 37 °C, and the supernatant was carefully aspirated. Each well was added 150 μL of DMSO, and shaken for 10 min to dissolve the blue crystals. The absorbance (A) value was measured on a microplate reader at a wavelength of 490 nm, and the average value was what we wanted. Each experiment was repeated at least 3 times (25).

Western blot assay

The brain tissue was chopped, homogenized in ice PIPA lysate, and centrifuged at 12000 rpm for 5 minutes at 4 °C. Based on the protein concentration determined by the BCA method, an equal amount of 5-HT, GABA, and GAPDH were respectively electrophoresed with a 10% SDS gel, and the electrophoresis was terminated until the bromophenol blue was just run out, and the film was transferred. The PVDF membrane was immer-

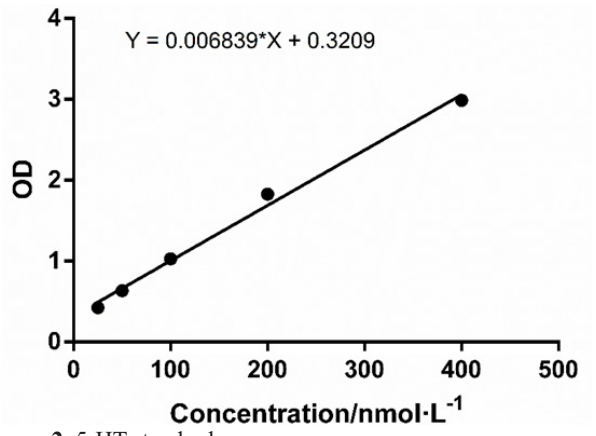

Figure 2. 5-HT standard curve.

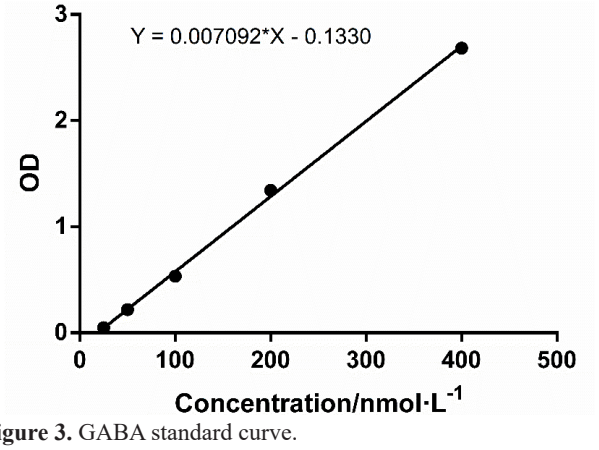

Figure 3. GABA standard curve.

sed in TBST blocking solution (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) containing skim milk powder (5%) and sealed on a shaker at room temperature for 1 h, and then the primary antibody was diluted with antibody solution to the working concentration. The PVDF membrane was immersed in the antibody, incubated on a shaker overnight at 4 °C (>12 h), and then washed three times with TBST on a decolorizing shaker at room temperature, 10 min for each time. The enzyme-labeled secondary antibody dilution was contacted with the membrane, incubated for 2 h at room temperature, and washed three times with TBST on a decolorizing shaker at room temperature, 10 min for each time, followed by a chemiluminescence reaction. The result was analyzed using a Bio-rad imaging system and the QuantityOne software.

Statistical analysis

All data were analyzed using software SPSS 21.0. The measurement data was expressed by $\overline{x} \pm s$. The difference between the saline group and each drug-administered group was compared by t test. The enumaration data were tested by X2 test, $P<0.05$ was considered statistically significant.

Results

Effect of palmatine on the mice body mass

Compared with the normal saline group, there was no significant difference in the body mass and the final body mass in the palmatine groups with various doses and the diazepam-positive group, indicating that the palmatine had no significant effect on the increase of mice body mass.

Effect of palmatine on the mice spontaneous activities

As shown in Figure 4, the number of spontaneous activities of the mice was significantly reduced in the palmatine groups with various doses and the diazepam-positive group compared with the normal saline group, suggesting that palmatine can greatly inhibit the spontaneous activity of the mice.

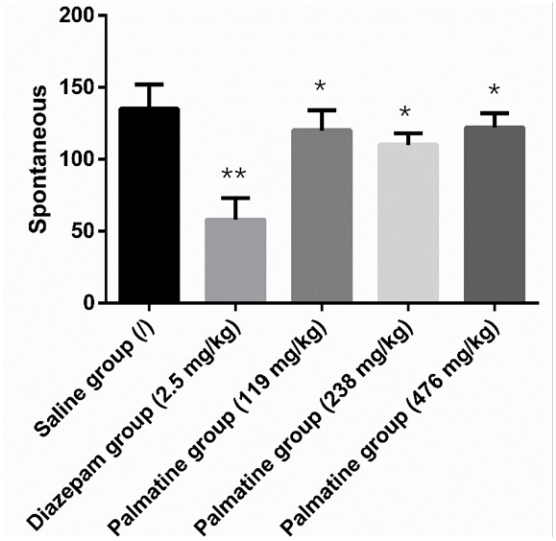

Figure 4. Effect of Palmatine on spontaneous activities in mice (n=12)**.** It was compared with the normal saline group: $^{*}P < 0.05$ and $^{**}P < 0.01$.

Effect of palmatine on direct sleep

Through experimental observation, no mice showed normal righting reflex disappearance after 60 minutes of palmatine administration, that is, no direct sleep phenomenon was noted, indicating that palmatine did not directly induce sleep in mice.

Effect of palmatine on sleep time in mice induced by the suprathreshold dose of pentobarbital sodium

As shown in Figure 5, the sleep times of mice were significantly prolonged in the low, middle and high dose palmatine groups and the diazepam-positive group compared with the normal saline group, and the difference was statistically significant ($P<0.01$). The sleep time in mice gradually prolonged along with the increase of palmatine dose. The sleep latency of mice was largely shortened in the palmatine groups with various doses and the diazepam-positive group compared with the saline group, and the difference was statistically significant ($P<0.01$). It was suggested that various doses of palmatine had a significant synergistic effect on the suprathreshold dose of pentobarbital sodium.

Effect of palmatine on mice hypnotic test induced by the subthreshold dose of pentobarbital sodium

As shown in Figure 6, compared with the normal saline group, the difference in the incidence of sleep in the

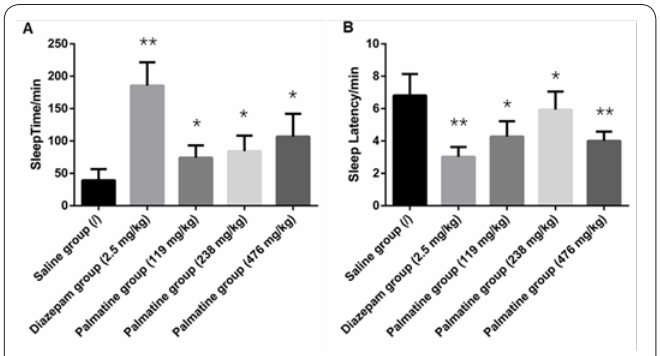

Figure 5. Effect of Palmatine on the A) sleep time and B) sleep latency in mice induced by the superthreshold dose of pentobarbital sodium (n=12)**.** It was compared with the normal saline group: $^{*}P < 0.05$ and $^{**}P < 0.01$.

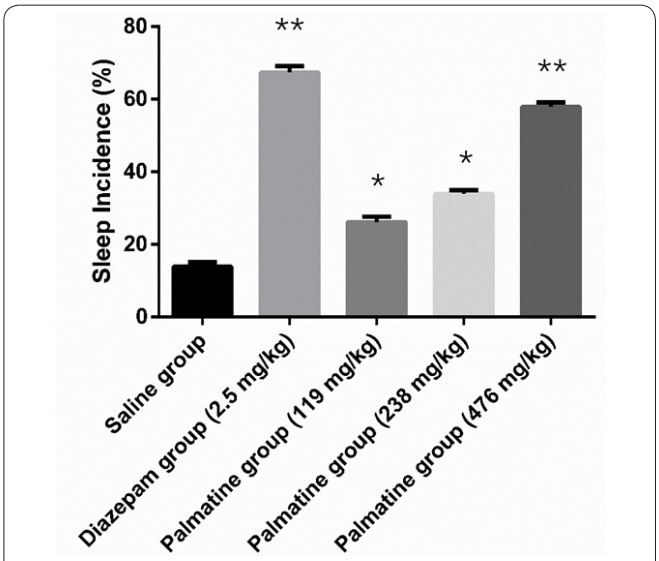

Figure 6. Effect of Palmatine on the sleep incidence in mice induced by the subthreshold dose of sodium pentobarbital (n=12)**.** It was compared with the normal saline group: $^{*}P < 0.05$ and $^{**}P < 0.01$.

high-dose palmatine group (P<0.01) and the diazepam-positive group (P<0.01) was statistically significant, and there was also an increase of the number of sleeping mice in the palmatine-low dose group and -middle dose group (P<0.05) but not so much, suggesting that various doses of palmatine had a synergistic effect on the sub-threshold dose of pentobarbital sodium.

MTT outcomes

The findings of MTT showed that the differences between the groups were statistically significant ($P < 0.01$). Compared with the blank control group (0.725 ± 0.001), the absorbance values of the 0.1 μg (0.724 ± 0.001) and 1 μg (0.727 ± 0.001) dose groups were significantly increased ($P < 0.05$). As shown in Figure 7, palmatine had no toxicity to PC12 cells.

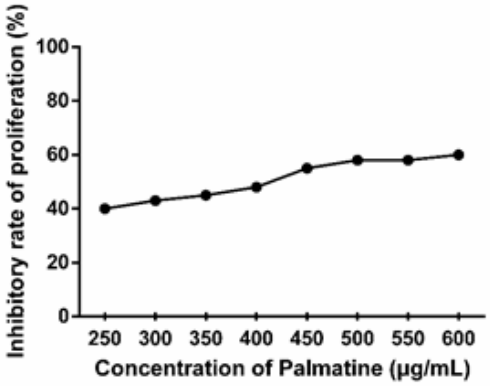

Figure 7. Effect of Palmatine on inhibitory rate of proliferation against PC12 cells.

Effect of Palmatine on the content of 5-HT and GABA in the brain of mice

The high and low doses of palmatine could increase the content of GABA in the brain of mice. The difference was significant compared with the control group ($P < 0.01$) . At the same time, the content of 5-HT in the brain of mice was decreased, but the difference was not significant, as shown in Figure 8 .

The results of Western blot showed that there was no significant difference in the expression of GABA in the lysate when the brain tissue was incubated with various concentrations of palmatine (119, 238, 476 mg/kg), which indicated that the sedative and hypnotic effect of palmatine was achieved by reducing the expression of GABA. On the contrary, the expression of 5-HT increased significantly with the raise of concentration, as shown in Figure 8. It was indicated that the sedative and hypnotic effect of palmatine was achieved by increasing the expression of 5-HT.

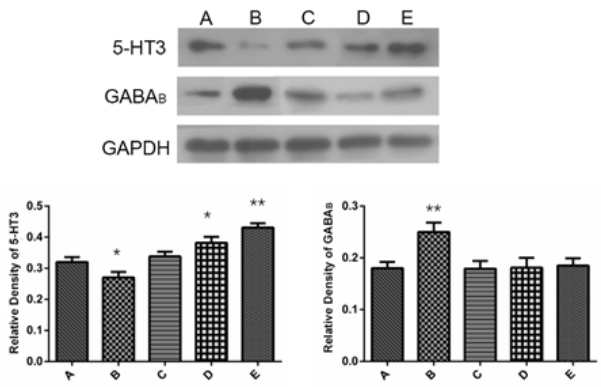

Figure 8. Effect of Palmatine on the expression of 5-HT3 and GABA$_B$ receptor according to Western Blotting. Control group (A); Diazepam (2.5 mg/kg) group (B); Palmatine (119 mg/kg) group (C); Palmatine (238 mg/kg) group (D); Palmatine (476 mg/kg) group (E). It was compared with the normal saline group: $^{*}P < 0.05$ and $^{**}P < 0.01$.

Discussion

Insomnia is a common disease in which you can't fall asleep or can't stay asleep with insufficient sleep. Many insomnia patients often take benzodiazepine sedative hypnotics, but long-term use of these drugs will give rise to side effect and certain dependence (26, 27). A large number of studies have shown that many ingredients extracted from Chinese medicine have good sedative and hypnotic effects but fewer adverse reactions (28). They can significantly improve the quality of sleep. Therefore, with the guidance of traditional Chinese medicine theory, the development of non-dependent sedative and hypnotic drugs with efficacy will produce good social and economic benefits. The effects of drugs on the central nervous system can be intuitively verified and measured by experiments on the effects of mouse spontaneous activity. The findings of this experiment reveal that artificially planted palmatine has obvious central inhibitory effect. It can significantly inhibit the spontaneous activity of mice, and largely reduce the number of spontaneous activities of mice. Besides, it can increase the number of mice falling asleep who are intraperitoneally administrated subthreshold dose of pentobarbital sodium and the incidence of falling asleep, with a synergistic effect. And it can greatly prolong the sleep time of mice and shorten the sleep latency, and has a significant synergistic effect on the suprathreshold dose of pentobarbital sodium. As an important part of the uplink activation system, 5-HT plays a major role in maintaining the state of awakening and alertness (29). 5-HT, also known as serotonin, is a precursor of the central inhibitory transmitter melatonin. When its content is elevated, the central melatonin content will also increase, leading to better sleep quality (30). Consequently, 5-HT can not

Table 1. Effect of Palmatine on the content of 5-HT and GABA in mice (n=12).

Group	Dose (mg/kg)	5-HT (nmol/L)	GABA (nmol/l)
Normal saline group	/	5.51 ± 1.25**	16.26 ± 3.72**
Palmatine groups			
Low dose group	119	15.71 ± 1.63*	17.03 ± 3.86**
Middle dose group	238	44.72 ± 7.52**	17.18 ± 4.15**
High dose group	476	76.19 ± 13.30**	16.48 ± 3.05**

It was compared with the normal saline group: $^{*}P < 0.05$ and $^{**}P < 0.01$.

only promote sleep by binding to self-receptors, but also have a synergistic effect with other neural pathways. The determination of 5-HT and GABA in the brain show that the content of 5-HT in the brain of mice is greatly increased in the high and low dose gentianine groups, which may be one of the mechanisms of sedative and hypnotic effects. In addition, GABA, an essential inhibitory neurotransmitter in the central nervous system, is widely distributed in the central system. About 20% ~ 40% of neurotransmitter in central synapses are mainly GABA. Studies have shown that the content of GABA in brain tissue is 15% higher during sleep than awaking (31). However, this study suggests that the effect of palmatine on GABA is not significant ($P > 0.05$). PC12 cells are rat chromaffin cell lines, and differentiated PC12 cells are very similar in morphology and function to neurons, so they are widely used in the study about neuronal apoptosis and neuronal differentiation (32). In order to study whether palmatine is cytotoxic, this study investigated the effects of palmatine at various doses on the growth of PC12 cells, and the results showed that it is not toxic to PC12 cells. Western blot analysis showed that the sedative and hypnotic effects of palmatine are associated with the increasing 5-HT but not GABA. To sum up, palmatine has obvious sedative and hypnotic effects, and increasing the expression of 5-HT is one of its mechanisms, but the complete sedative and hypnotic mechanism needs further study. The results of this study can provide a scientific basis for the research of palmatine in this area.

Acknowledgements
None.

Conflict of Interest
There are no conflicts of interest in this study.

Author's contribution
All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Liuji Qiu; Jianwei Du, Yanbo Yu, Liuji Qiu, Mizhen Qiu and Qing Zhu collected and analysed the data; Jianwei Du and Yanbo Yu wrote the text and all authors have read and approved the text prior to publication.
Jianwei Du and Yanbo Yu contributed equally to this work and should be considered as co-first authors.

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