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Spinosin, a *C*-glycoside flavonoid from semen *Zizhiphi Spinozae*, potentiated pentobarbital-induced sleep via the serotonergic system

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ABSTRACT

Semen *Zizhiphi Spinozae* has been used extensively for the treatment of insomnia. This study investigated the effect and possible mechanism of action of spinosin (also known as 2″-β-*o*-glucopyranosyl swertisin), a major constituent of semen *Zizhiphi Spinozae*, on sleep in mice. The present results showed that spinosin significantly and dose-dependently augmented pentobarbital (45 mg/kg, i.p.)-induced sleep, reflected by increased sleep time and reduced sleep latency assessed with the loss-of-righting reflex, and these effects were potentiated by the 5-hydroxytryptamine (serotonin) precursor 5-hydroxytryptophan (5-HTP, 2.5 mg/kg, i.p.). With a subhypnotic dose of pentobarbital (28 mg/kg, i.p.), spinosin significantly increased the rate of sleep onset and exhibited a synergistic effect with 5-HTP (2.5 mg/kg, i.p.). Pretreatment with *p*-chlorophenylalanine (PCPA, 300 mg/kg, s.c.), an inhibitor of tryptophan hydroxylase, significantly decreased pentobarbital-induced sleep time, and spinosin significantly reversed this effect. The dopamine precursor L-3-(3, 4-dihydroxyphenylalanine (L-DOPA) reduced pentobarbital-induced sleep, an effect not significantly affected by spinosin. These results suggest that spinosin potentiated pentobarbital-induced sleep *via* a serotonergic mechanism.

1. Introduction

Semen *Zizhiphi Spinozae*, a traditional tranquilizing medicine frequently used in China, has been used extensively for the treatment of a variety of syndromes and diseases, including insomnia, neurasthenia, and climacteric period syndrome (Peng and Zhu, 2001). Spinosin, also known as 2″-β-*o*-glucopyranosyl swertisin, is one of the major flavonoids of semen *Zizhiphi Spinozae* (Yuan et al., 1987).

Abundant literature has demonstrated the hypnotic effects of semen *Zizhiphi Spinozae* (Cui, 1987; Li et al., 2002; Wang et al., 2006; Sui et al., 2007) and its total flavonoids (Yuan et al., 1987; Guo et al., 1998). Animal studies indicated that both the decoction of semen *Zizhiphi Spinozae* and its total flavonoids prolonged barbiturate-induced sleep time. The hypnotic effect of spinosin, one the major flavonoids, also has been assessed (Shin et al., 1978; Kawashima et al., 1997), but its mechanism of action has not yet been determined. Our preliminary research demonstrated that spinosin augmented pentobarbital-induced sleep in mice. The present study further investigated the hypnotic effect and possible mechanism of action of spinosin on pentobarbital-induced sleep assessed by the loss-of-righting reflex.

2. Materials and methods

2.1. Animals

Male ICR mice (Grade I, Peking University Animal Center, Beijing, China), weighing 20–24 g were used. Each mouse was used for only one experiment. Mice were housed 6–10 per acrylfiber cage (440×270×178 mm) in a temperature- (22±2°C) and humidity- (50±10%) controlled room and were kept on a 12 h light/dark cycle. They were fed with standard diet and water *ad libitum* and were acclimated for 7 days before testing. In the case of oral administration, mice were fasted for 12 h before testing. The experiments were performed from 0800 to 1130 h in a quiet room maintained at 22–24°C. All procedures involving animals were conducted in accordance with the European Community guidelines for the use of experimental animals and were approved by the Peking University Committee on Animal Care and Use.

2.2. Drugs and drug administration

Spinosin was provided by Prof. Y.Y. Zhao, Department of Natural Medicines, School of Pharmaceutical Sciences, Peking University, Beijing, China. Spinosin was identified by comparing its physical and chemical properties with a standard specimen (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China). Other drugs used in this study were pentobarbital, 5-hydroxytryptophan (5-HTP), *p*-chlorophenylalanine (PCPA; Sigma-Aldrich, St. Louis,

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MO), L-3-(3, 4-dihydroxyphenylalanine (L-DOPA) (Alfa Aesar China Ltd, Beijing, China), and diazepam injection (10 mg/2 ml, batch number: 0611112, manufactured by People's Pharmaceutical Manufacturer, Tianjin, China).

For oral administration (0.1 ml/10 g, volume/body weight, intragastric [i.g.]), spinosin was suspended, and diazepam was dissolved in distilled water. For intraperitoneal (i.p.) injection (0.1 ml/10 g), 5-HTP and pentobarbital were dissolved in physiological saline. For subcutaneous (s.c.) injection (0.1 ml/10 g), PCPA was suspended in 0.5% gum acacia/physiological saline, and L-DOPA was suspended in 0.5% carboxymethyl cellulose solution.

The present study used 45 mg/kg (i.p.) as the hypnotic dose of pentobarbital (rate of sleep onset: 100%) and 28 mg/kg (i.p.) as the subhypnotic dose (rate of sleep onset <10%). Spinosin and diazepam were administered (i.g.) 60 min before pentobarbital administration (i.p.). 5-HTP was injected (i.p.) 15 min before pentobarbital administration (i.p.). PCPA-pretreated mice received an injection of PCPA (300 mg/kg, s.c.) between 0800 and 0900 h, 24 h prior to the injection of pentobarbital (i.p.). L-DOPA-pretreated mice were divided into six groups. Each group received 0 (vehicle), 6.25, 12.5, 25, 50, and 100 mg/kg of L-DOPA (s.c.), respectively, for 3 days between 0800 and 0900 h. On the third day, mice received L-DOPA 60 min prior to pentobarbital.

2.3. Evaluation of sleep onset and sleep time

Observers were blind to drug treatment. Following pentobarbital injection, each mouse was observed for onset of sleep. A mouse reached the criterion for sleep if it was placed on its back and exhibited a loss-of-righting reflex for 5 min. Mice that righted themselves in less than 5 min were considered to be awake. Sleep latency time was recorded from the time of pentobarbital injection until 1 min after mice exhibited a loss-of-righting reflex. Sleep time was recorded from 1 min after exhibiting a loss-of-righting reflex until regaining the righting reflex (Vogel, 2002). In the test with a subhypnotic dose of pentobarbital, the percentage of sleep onset was calculated as: (number of animals falling asleep/total number of animals)×100.

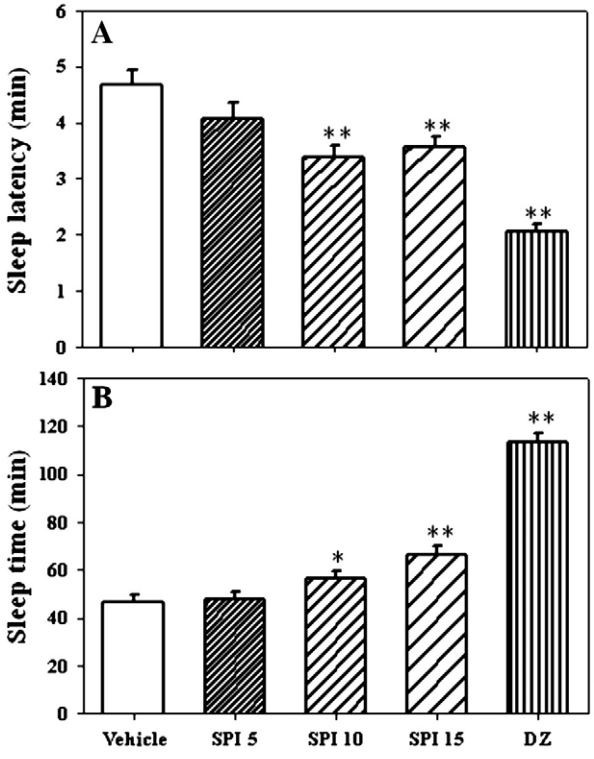

Fig. 1. Effect of spinosin on the hypnotic response to pentobarbital-induced sleep in mice. The sleep latency (A) [$F(4,78)=17.146$, $P<0.000$] and the sleep time (B) [$F(4,78)=59.319$, $P<0.000$] were assessed. All values are presented as mean±S.E.M. ($n=17$ for vehicle; $n=15$ for SPI5; $n=18$ for SPI10; $n=19$ for SPI15 and $n=14$ for DZ2). SPI: spinosin and DZ: diazepam. *$P<0.05$ and **$P<0.01$ vs vehicle (Student–Newman–Keuls test).

Table 1
Effect of spinosin on sleep onset of mice induced by subhypnotic dosage of pentobarbital (28 mg/kg, i.p.)

Groups	No. falling asleep/total no.	Sleep onset (%)
Vehicle	1/20	5.0
Spinosin 2.5 mg/kg	2/20	10.0
Spinosin 5 mg/kg	10/20	50.0[a]
Spinosin 10 mg/kg	9/20	45.0[a]
Spinosin 15 mg/kg	12/20	60.0[a]
Diazepam 2 mg/kg	10/10	100.0[a]

[a] $P<0.01$ vs vehicle (Chi-square test).

2.4. Statistical analysis

All values are expressed as mean±SEM. For multiple comparisons, data were analyzed by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test. For the test with a subhypnotic dose of pentobarbital, the χ^2 test was used to compare the percentages of sleep onset between the group that received a subhypnotic dose of pentobarbital alone and each of the other groups. $P<0.05$ was considered statistically significant.

3. Results

3.1. Effect of spinosin on the onset and duration of sleep in pentobarbital-treated mice

Spinosin pretreatment significantly potentiated the hypnotic effects of pentobarbital (45 mg/kg, i.p.), reflected by reduced sleep latency (Fig. 1A) and prolonged sleep time (Fig. 1B), at both 10 and 15 mg/kg. In mice treated with a subhypnotic dose of pentobarbital (28 mg/kg, i.p.), spinosin increased the rate of sleep onset, with significant effects at 5, 10, and 15 mg/kg ($P<0.01$) (Table 1). Diazepam (2 mg/kg, p.o.), the positive control for the study, also potentiated the hypnotic effect of pentobarbital. Spinosin administered alone did not induce sleep, based on the present sleep criterion.

3.2. Synergic effects of spinosin and 5-HTP on sleep induced by pentobarbital

To investigate the relationship between the hypnotic effects of spinosin and the serotonergic system, low doses of spinosin (5 mg/kg, p.o., Figs. 1B and 2) and 5-HTP (2.5 mg/kg, i.p., Fig. 2B) were used that did not affect pentobarbital-induced sleep time when administered alone. Spinosin and 5-HTP were administered 60 min and 15 min prior to pentobarbital injection (45 mg/kg, i.p.), respectively. Neither spinosin (5 mg/kg, p.o.) nor 5-HTP (2.5 mg/kg, i.p.) administered alone affected sleep time induced by the hypnotic dose of pentobarbital (45 mg/kg, i.p.). However, co-administration of spinosin (5 mg/kg, p.o.) and 5-HTP (2.5 mg/kg, i.p.) significantly decreased pentobarbital-induced sleep latency ($P<0.05$, Fig. 2A) and sleep duration ($P<0.05$, Fig. 2B). In mice treated with a subhypnotic dose of pentobarbital (28 mg/kg, i.p.), low-dose spinosin (2.5 mg/kg, p.o., Tables 1 and 2) and 5-HTP (2.5 mg/kg, i.p., Table 2) did not affect the rate of sleep onset when administered alone. However, co-administration of spinosin (2.5 mg/kg, p.o.) and 5-HTP (2.5 mg/kg, i.p.) exhibited a synergistic effect on the rate of sleep onset ($P<0.05$, Table 2).

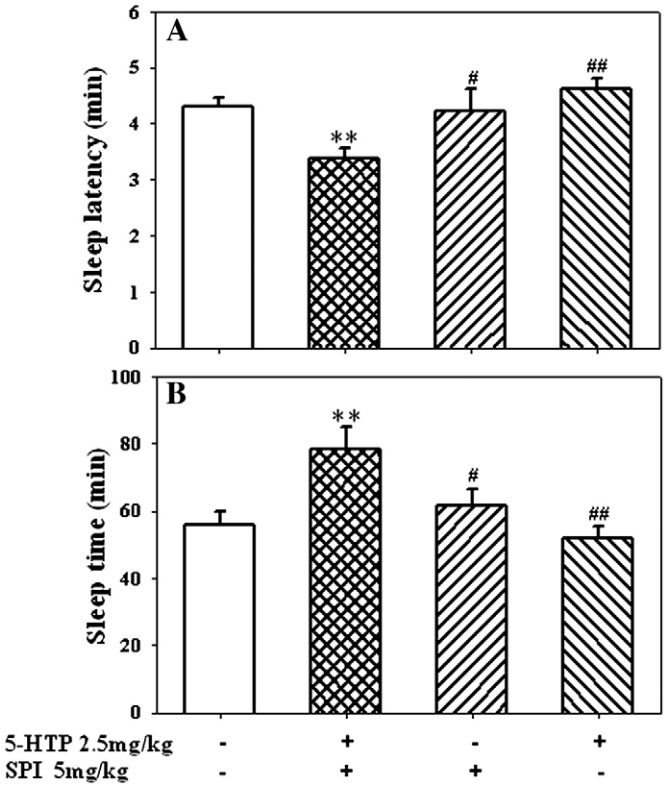

Fig. 2. Synergic effects of spinosin with 5-HTP on pentobarbital sleep in mice. The sleep latency (A) [$F(3,58)=5.153$, $P<0.003$] and the sleep time (B) [$F(3,58)=5.809$, $P<0.002$] were assessed. All values are presented as mean±S.E.M. ($n=16$ for vehicle; $n=16$ for SPI 5 mg/kg+5-HTP 2.5 mg/kg; $n=14$ for SPI 5 mg/kg and $n=16$ for 5-HTP 2.5 mg/kg). $^{**}P<0.01$ vs vehicle. $^{\#}P<0.05$ and $^{\#\#}P<0.01$ compared with 5-HTP (2.5 mg/kg)+ spinosin (15 mg/kg) group (Student–Newman–Keuls test).

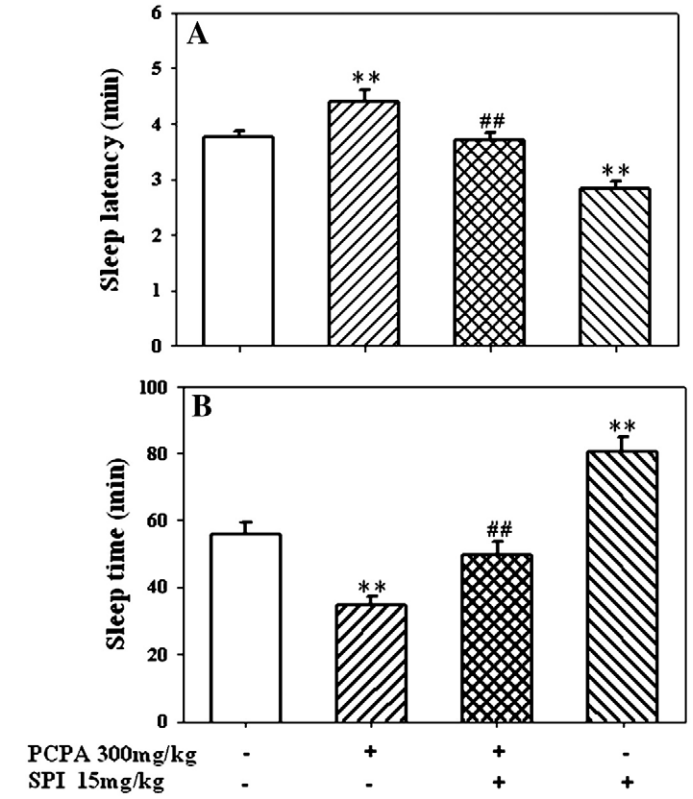

Fig. 3. Effects of spinosin on PCPA-induced insomnia in pentobarbital-treated mice. The sleep latency (A) [$F(3,75)=16.830$, $P<0.000$] and the sleep time (B) [$F(3,75)=25.054$, $P<0.000$] were assessed. All values are presented as mean±S.E.M. ($n=20$ except for SPI 15 mg/kg group $n=19$). $^{**}P<0.01$ vs vehicle and $^{\#\#}P<0.01$ vs group treated PCPA alone (Student–Newman–Keuls test).

3.3. Effect of spinosin on PCPA-induced insomnia in pentobarbital-treated mice

Treatment with PCPA (300 mg/kg) has been shown to induce insomnia. In accordance with a previous report (Borbely et al., 1981), the present study showed that treatment with PCPA (300 mg/kg, s.c.) 24 h prior to pentobarbital injection significantly prolonged sleep latency ($P<0.01$, Fig. 3A) and decreased sleep time ($P<0.01$, Fig. 3B). Spinosin (15 mg/kg, p.o.) significantly attenuated the insomnia effect of PCPA in pentobarbital-treated mice, reflected by decreased sleep latency ($P<0.01$, Fig. 3A) and increased sleep time ($P<0.01$, Fig. 3B).

3.4. Effect of spinosin on the hypnotic-reversing action of L-DOPA in pentobarbital-treated mice

Acute treatment with L-DOPA did not affect pentobarbital-induced hypnosis (data not shown); therefore, we employed a chronic treatment regimen in which L-DOPA was administered (s.c.) for 3 consecutive days. On the third day, 60 min after the last L-DOPA injection, mice received pentobarbital (45 mg/kg, i.p.). Pentobarbital-induced sleep time was significantly decreased by L-DOPA at both 50 mg/kg ($P<0.01$) and 100 mg/kg ($P<0.05$) (Fig. 4A). Spinosin (15 mg/kg, p.o.) had no effect on L-DOPA-induced reductions in pentobarbital-induced sleep (Fig. 4B).

Table 2
Synergic effects of spinosin with 5-HTP on sleep onset of mice treated with subhypnotic dosage of pentobarbital (28 mg/kg, i.p.)

Groups	No. falling asleep/total no.	Sleep onset (%)
Vehicle	1/16	6.3
2.5 mg/kg 5-HTP	2/16	12.5[a]
2.5 mg/kg spinosin	2/16	12.5[a]
2.5 mg/kg 5-HTP+2.5mg/kg spinosin	8/16	50.0[b]

[a] $P<0.05$ vs 2.5 mg/kg 5-HTP+2.5 mg/kg spinosin (Chi-square test).
[b] $P<0.01$ vs vehicle (Chi-square test).

4. Discussion

Spinosin, a *C*-glycoside flavonoid, is one of the bioactive constituents of semen *Zizhiphi Spinozae* (Kawashima et al., 1997). The present study showed that spinosin significantly potentiated the hypnotic effect of pentobarbital by decreasing sleep latency (Fig. 1A), increasing sleep time (Fig. 1B), and increasing the rate of sleep onset induced by a subhypnotic dose of pentobarbital (Table 1).

The dopaminergic system is closely related to the modulation of sleep and wakefulness (for review, see Monti and Monti, 2007). Several experiments showed that chronic administration of the dopamine precursor L-DOPA promoted wakefulness and decreased sleep (Riederer et al., 2007; Dzirasa et al., 2006; Nishino and Mignot, 1997). In accordance with these findings, the present study also showed that pretreatment with L-DOPA for 3 days significantly decreased pentobarbital-induced sleep time (Fig. 4A), and this effect was not attenuated by spinosin (Fig. 4B). These results suggest that spinosin-induced augmentation of the hypnotic effect of pentobarbital may not be related to the dopaminergic system.

Serotonin has been considered for decades to be the predominate regulatory center for sleep (Portas et al., 2000; Jouvet, 1999). The

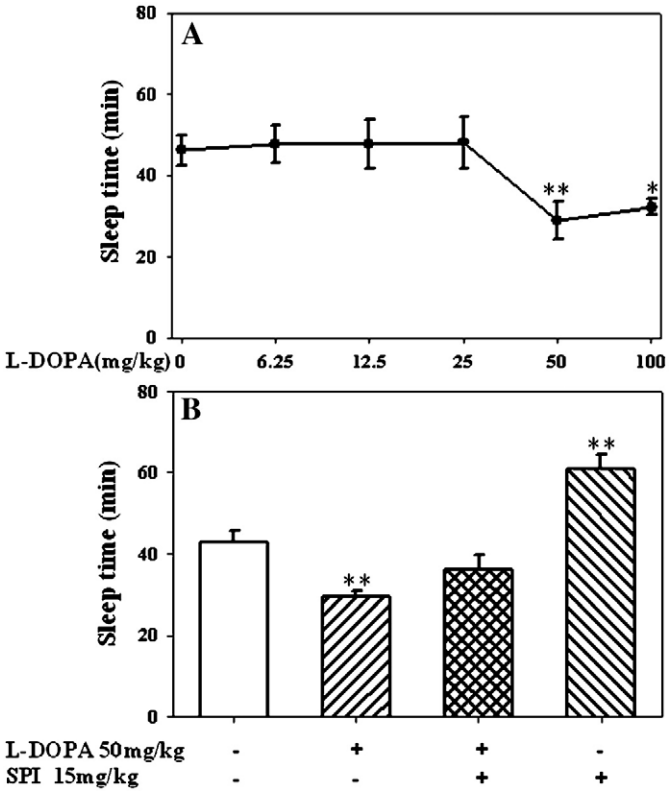

Fig. 4. Effects of spinosin on L-DOPA induced insomnia in pentobarbital-treated mice. (A) The sleep time [$F(5,70)=3.717$, $P<0.005$] were assessed. All values are presented as mean±S.E.M. ($n=18$ for vehicle; $n=11$ for L-DOPA 6.25, 12.5 and 25 mg/kg, respectively; $n=12$ for L-DOPA 50 mg/kg and $n=13$ for L-DOPA 100 mg/kg). (B) The sleep time [$F(3,59)=21.476$, $P<0.000$] were assessed. All values are presented as mean±S.E.M. ($n=15$ for vehicle; $n=15$ for L-DOPA 50 mg/kg; $n=19$ for SPI 15 mg/kg and $n=14$ for L-DOPA 50 mg/kg+SPI 15 mg/kg). *$P<0.05$ and **$P<0.01$ vs vehicle (Student–Newman–Keuls test).

complex effects of serotonin in the regulation of sleep is attributable to the actions of serotonin at different brain sites that have been associated with the control of sleep and wakefulness (Ursin, 2002; for review, see Dugovic, 2001). The immediate serotonin precursor, 5-HTP, appears to have a general deactivating effect on the waking state (Ursin, 1976) and primarily induces drowsiness (Ursin, 2002). Our previous study showed that 5-HTP dose-dependently prolonged pentobarbital-induced sleep time (Zhao et al., 2004). Early experiments demonstrated that lesions of the raphe nuclei caused reductions in both sleep and brain serotonin (Arpa and De Andres, 1993; Jouvet, 1968, 1972). In addition, complete insomnia or substantially reduced sleep was induced by chronic administration of PCPA, which blocks tryptophan hydroxylase, the rate-limiting enzyme that catalyzes serotonin biosynthesis (Koella et al., 1968; Ursin, 1972; Borbely et al., 1981). These insomnia effects were reversed by treatment with 5-HTP, which may have restored serotonin synthesis and thus restored sleep (Pujol et al., 1971; Denoyer et al., 1989; Touret et al., 1991; Zhao et al., 2004). The serotonin-sleep connection, therefore, was proposed (Jouvet, 1984), in which the serotonin system was proposed to be hypnogenic. This PCPA/5-HTP model consolidates the importance of 5-HTP and 5-HT in the control of sleep (Huitron-Resendiz et al., 1997; Smith and Kennedy, 2003). The present study showed that spinosin exerted synergic effects with 5-HTP on both sleep latency and sleep time with a hypnotic dose of pentobarbital in mice (Fig. 2) and also on the rate of sleep onset with a subhypnotic dose of pentobarbital (Table 2). In addition, spinosin inhibited PCPA-induced suppression of the hypnotic effect of pentobarbital (Fig. 3). These results suggest that the serotonergic system may be involved in the potentiating mechanism of spinosin's effects on the hypnotic effect of pentobarbital.

To our knowledge, this is the first study that explored the possible mechanism by which spinosin augments the hypnotic effect of pentobarbital. Future studies will investigate the precise mechanism of this effect and elucidate the 5-HT receptor subtypes that may be involved in the potentiating effect of spinosin on pentobarbital-induced sleep.

Acknowledgements

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