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Methanol Extract of Zizyphi Spinosi Semen Augments Pentobarbital-Induced Sleep through the Modification of GABAergic Systems

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Abstract – Zizyphi Spinosi Semen (ZSS) have been widely used for the treatment of insomnia in Asia. This experiment was performed to investigate whether methanol extract of ZSS (MEZSS) has hypnotic effects through the γ-amino butyric acid (GABA)ergic systems. MEZSS inhibited the locomotor activity. MEZSS enhanced pentobarbital-induced sleep behaviors. However, MEZSS itself did not induce sleep at higher dose, similar to muscimol. On the other hand, both pentobarbital and MEZSS increased the non rapid eye move (NREM) sleep, especially reducing the -wave electroencephalogram (EEG) activity in REM sleep. MEZSS showed similar effects with muscimol on potentiating chloride influx induced by pentobarbital. MEZSS significantly increased $GABA_A$ receptors γ-subunit expression and slightly decreased β-subunit expression in hypothalamus and thalamus, showing that subunit-expression was similar to diazepam. In addition, MEZSS enhanced the expression of glutamic acid decarboxylase (GAD). In conclusion, it is suggested that MEZSS might augment pentobarbital-induced sleep behaviors through the modification of GABAergic systems.
Keywords – Zizyphi spinosi semen, Pentobarbital, GABA, GAD, Chloride influx

Introduction

Insomnia is highly prevalent. Pharmacological approach plays an important role in insomnia therapy, targeting the γ-amino butyric acid (GABA) ergic system, one of the major inhibitory neurotransmitter systems of the central nervous system (CNS). Past and current treatments have been dominated by sedative-hypnotic drugs acting on $GABA_A$ receptors, including the GABA, benzodiazepines, barbiturates, steroids and alcohol (Johnston, 1996; Winsky-Sommerer, 2009). These compounds act on different binding sites of the $GABA_A$ receptors to rapidly increase in permeability for chloride ion channel. In this way, they increase chloride currents through the $GABA_A$ receptors, and enhance inhibitory synaptic transmission. $GABA_A$ receptors have been shown to be major modulation site for barbiturates and benzodiazepines drugs-induced sleep through the interaction with GABAergic systems (Doghramji, 2006).

Zizyphi Spinosi Semen (ZSS), the dried seed of *Zizyphus jujube Mill var. Spinosa* (Rhamnaceae), has been used as a tranquilizer, an analgesic and an anticonvulsant in oriental countries such as China, Japan and Korea for centuries, and also has been prescribed for the treatment of insomnia and anxiety in Asia (Park *et al*., 2004; Liu *et al*., 2007; Han *et al*., 2008). In modern pharmacological studies, ZSS is known to contain many pharmacologically active components such as alkaloids, triterpenes, flavones and fatty oils (Le Croueour *et al*., 2002; Lee *et al*., 2004; Suksamrarn *et al*., 2006; Zhang *et al*., 2008). In recent years, these compounds have been studied extensively in central inhibitory activity. The oral administration of sanjoine A and spinosin prolonged pentobarbital-induced sleeping time in mice and rats by the modulation of GABAergic and serotonergic systems (Ma *et al*., 2007; Wang *et al*., 2008; Fang *et al*., 2010; Wang *et al*., 2010). Jujuboside A produced its hypnotic effects through activating the anti-calcium-binding proteins and inhibiting the glutamate-mediated excitatory signaling pathway in the hippocampus (Zhang *et al*., 2003). The high dose of suanzaorentang (ZSS) significantly increased non-rapid eye movement (NREM) sleep by the regulation of $GABA_A$ receptors (Yi *et al*., 2007) and serotonin receptors (Yi *et al*., 2007). Methanol extract from ZSS (MEZSS) was demonstrated to be the major hypnotic components of ZSS. Therefore, we were interested to

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investigate whether methanol extract exerted hypnotic effect and/or enhances pentobarbital-induced sleeping behaviors through GABAergic systems.

Experimental

Animal and chemicals – Groups of 10 - 13 adult male ICR mice (Samtako, Osan, Korea) weighting 22 - 25 g were used in all experiments. They were housed, 10 - 16 animals per cage with water and food available *ad libitum* under an artificial 12:12 (h) light/dark cycle (light at 07:00) and constant temperature (22 ± 2 °C). Chemicals used were muscimol (Sigma, Louis, MO, USA), diazepam (Samjin Pharm. Co., Seoul, Korea), pentobarbital sodium (Hanlim Pharm. Co., Seoul, Korea). All chemicals were freshly dissolved in physiological saline. Test drugs were dissolved in vehicle in saline supplemented tween 20 (1.6%) and ethanol (10%). Test drugs or muscimol (0.2 mg/kg) was treated orally (p.o.) or intraperitoneally (i.p.) 15-30min prior to pentobarbital (28 mg/kg or 42 mg/kg, i.p.) administration. All of the experiments involving animals were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1985), and the Institutional Animal Care and Use Committee of Chungbuk National University approved the protocol (CBNUR-258-10).

Plant material and preparation of extraction – ZSS was purchased from the Hwalim natural Drug Co, Ltd. Busan, Korea. ZSS (300 g). They were identified by the herbarium of College of Pharmacy at Chungbuk National University, where a voucher specimen was deposited (CBNU2001011-CM).

ZSS was extracted three times with hexane in reflux condenser for 24 h. The residues were extracted with 75% methanol for 24 h, and this methanol extract was filtered and concentrated using a rotary vacuum evaporator followed by lyophilization. The yield was about 10% (w/w).

Locomotor activity – The locomotor activity of mice was measured using a tilting-type ambulometer (AMB-10, O'Hara, Tokyo, Japan). Each mouse was placed in the activity cage (20 cm in diameter, 18 cm in height). Chemicals were administered after an adaptation period of 10 min. MEZSS (25, 50 and 100 mg/kg) was administered according to our previous methods (Eun *et al*., 2006). The vehicle mice were treated under the same condition. The mice were first allowed to perambulate for 10 min in the activity cages followed by a 1-h test period immediately after the test drugs administration. The locomotor activity was recorded during the 1-h test period.

Pentobarbital-induced sleep – All experiments were carried out between 13:00 and 17:00 on daytime. Animals were fasted 24 h prior to the experiment. After the administration of MEZSS (25, 50 and 100 mg/kg) and muscimol, mice were injected with pentobarbital and then placed in a box. All mice that stopped moving in the box within 15 min after pentobarbital injection were immediately transferred to another box. Those individuals that stayed immobile for more than 3 min were judged to be asleep. The time that elapsed from receiving pentobarbital until an animal, positioned delicately on its back, lost it righting reflex represented the latency to onset of sleep. The animals were observed constantly, and the time of awakening, characterized by righting of the animal, was noted. The sleeping time was defined as the time taken for the animal to regain spontaneous movements after having been transferred to the second box. Animals that failed to all asleep within 15 min after pentobarbital administration were excluded from the experiments.

Electroencephalogram (EEG) – Sprague Dawley (SD) male rats (Samtako, Osan, Korea) weighing between 250 and 350 g were used. Under deep anesthesia rats were implanted with a transmitter (Data Sciences International, Paul, MN, USA) for recording EEG and activity via telemetry, as described previously (Park *et al*., 2010). Briefly, EEG electrode was implanted over the parietal hemisphere (A: 2.0 [Bregma], L: 1.5; P: 7.0 [Bregma], L: 1.5 contra-lateral). Following a 7-day post-surgical recovery, rats were randomly divided into one of the following groups. MEZSS were orally administrated at 11:00 a.m. once per day for 1 day at a dose of 100 and 200 mg/kg. The vehicle mice were treated under the same condition. After the end of the MEZSS treatment, animals were allowed to habituate to a polygraphic recording environment in which they could move freely. For drug absorption, animals habituated on receiver for 1 h. Then, Polygraphic signs of sleep-wake activities were recorded for the 6 h (12:00-18:00) on daytime. The signals were gained (–0.5/ +0.5 volts per/units ×2), and generated (EEG: 0.5- 20.0 Hz) by a Data Sciences analog converter and digitized (sampling rate 128 Hz) in 10 sec. epochs and automatically stored on hard disk by an analog-to-digital (AD) converter (Data Sciences International, Paul, MN, USA). An on-line Fast Fourier transformation (FFT) with a 10-s domain was performed off-line for the computation of EEG power density values for 0.5 Hz within the frequency range 0.0-20.0 Hz. The amount of elapse time in wake, non rapid eye movement (NREM), and rapid eye movement (REM) sleep in 10-sec epochs were determined using

SleepSign 2.1 software (Kissei Comtec Co., Nagano, Japan), using sleep-scoring procedure defined previously (Guevara *et al*., 2003). Briefly, the software discriminates wake as high-frequency activity in the EEG; EEG during the NREM and REM sleep was scored on the specific frequency of δ-wave (0.75 - 4.0 Hz), θ-wave (5.0 - 9.0 Hz, peak at 7.5 Hz) and α-wave (7-12 Hz). Furthermore, the elapse time (min) in wake, NREM and REM for 6-h-period was calculated.

Cell culture – Primary cultures of cerebellar neurons enriched in granule cells were prepared from cerebella of 8d old SD rats (Samtako, Osan, Korea) as previously described (Houston and Smart, 2006). Briefly, cells were plated (5×10^6 cells per ml) in 96 microplates. The cells were cultured in DMEM/F-12 medium (Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum, penicillin-streptomycin (100 unit/ml; Gibco-BRL, Carlsbad, CA, USA), B-27 (Gibco-BRL, Carlsbad, CA, USA) and 25 mM KCl. This high concentration of potassium was necessary to induce persistent depolarization, which promotes the survival of granule cells. Cytosine arabinofuranoside (10 μM final concentrations, Sigma, Louis, MO, USA) was added to culture 18 - 24 h after plating to inhibit the proliferation of non-neuronal cells. Cells were maintained in culture and experiments were performed after 7 days, when cells express functional $GABA_A$ receptors, with an expression pattern similar to that apparent in the cerebellum during postnatal development but different from that observed in the adult rat cerebellum.

Measurement of intracellular Cl^- influx – The intracellular Cl^- concentration of cerebellar granule cells was estimated using the Cl^- sensitive fluorescence probe N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE) according to the method of West and Molloy with a slight modification (West and Molloy, 1996). The buffer (pH 7.4) used contained the following: 2.4 mM HPO_4^{2-}, 0.6 mM $H_2PO_4^-$, 10 mM HEPES, 10 mM D-glucose and 1.0 mM $MgSO_4$. A variety of MQAE-loading conditions were assessed. The cells were incubated overnight in a medium containing 10 mM MQAE (Dojindo, Kumamoto, Japan). After loading, the cells were washed three times in the relevant Cl^- containing buffer. The buffer was replaced with buffer with or without the MEZSS (2, 4 and 8 μg/ml), muscimol (20 and 40 μM) and/or pentobarbital (2.5, 5.0 and 10 μM) or vehicle. Repetitive fluorescence measurements were initiated immediately using a FLUOstar (excitation wavelength: 320 nm; emission wavelength: 460 nm; BMG LabTechnology, Ortenberg, Germany). The data is represented as the relative fluorescence F_0/F, where F_0 is the fluorescence without Cl^- and F is the fluorescence as a function of time. The F_0/F values were directly proportional to $[Cl^-]i$.

$GABA_A$ receptor subunits and glutamic acid decarboxylase (GAD) 65/67 expression – On the 5th day of repeated treatments with MEZSS (50 and 100 mg/kg, p.o), diazepam (4 mg/kg, i.p.) or pentobarbital (42 mg/kg, i.p.) in mice, hypothalamus and thalamus were dissected in ice-cold saline and homogenized in total protein extraction solution (Millipore, Bedford, MA, USA). Supernatants were collected and stored in frozen. The protein concentration of the supernatant was determined using Bradford method with bovine serum albumin as the standard. Equal amount of proteins were separated on a SDS/10%-polyacrylamide gel, and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membrane was blocked in 0.5% not-fat milk with TBS-T [10 mM Tris (pH 8.0) containing 0.05% tween-20], followed by three washes in TBS-T. Membranes were bond with specific antibodies using SNAP i.d. system (Millipore, Bedford, MA, USA). The rabbit polyclonal antibody against $GABA_A$ receptor subunits or GAD65/67 (1 : 150, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added. The membrane was then incubated with the corresponding conjugated anti-rabbit immunoglobulin G-horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The immunoreactivity was revealed with incubation with the ECL-Plus chemiluminescent substrate (Roche, Mannheim, Germany). Chemiluminescence was detected by imaging systems FUSION-FX7 (Vilber Lourmat, Cedex, France). Quantification of bands was performed by densitometry (OD) using analysis software FUSION-CAPI.

Statistical analysis – The results are presented as the mean ± S.E.M. The significance of the effects was assessed using analysis of variance (ANOVA). In case of significant variation, the individual values were compared with Dunnett's test. For the sub-hypnotic dose of pentobarbital-treated experiment, a Chi-square test was used to compare the proportions of sleep onset between sub-hypnotic pentobarbital alone-treated group and each of the other groups.

Results

Hypnotic effects of pentobarbital, muscimol and MEZSS – MEZSS decreased locomotor activity in a dose dependant manner. We investigated the hypnotic effects during the 1h-text period by the higher dose of MEZSS

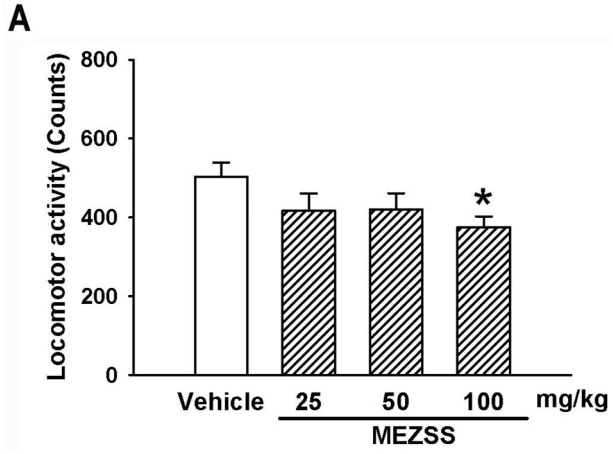

Fig. 1. Effects of methanol extract of Zizyphi Spinosi Semen (MEZSS) on locomotor activity in mice. After the oral administration of MEZSS (25, 50 and 100 mg/kg), the locomotor activity was recorded during the 1-h test period. Each column represents the mean ± S.E.M. $^{*}P < .05$, compared to that of the vehicle group.

Table 1. Sleep-inducing effects of pentobarbital, methanol extract of Zizyphi Spinosi Semen (MEZSS) and muscinol in mice

	Dose (mg/kg)	No. falling asleep/total	Sleeping time (min)
Pentobarbital	28	7/16	28.1 ± 2.6
	42	14/15	98.5 ± 6.3
Muscimol	10	0/13	0
MEZSS	1000	0/13	0

Each value represents the mean ± S.E.M. (n = 13 - 16)

(100 mg/kg) (Fig. 1) and found that MEZSS alone could not induce sleep even at a very high dose (1 g/kg). These results were similar to those of muscimol, a $GABA_A$ agonist (Table 1). On the base of EEG signature, MEZSS and pentobarbital markedly increased the NREM sleep during the total sleep, especially reducing the δ-wave activity in the REM sleep. However, MEZSS might increase the REM sleep and the δ-wave activity in the REM sleep in dose dependant manner (Fig. 2).

Effects of MEZSS on sleeping onset of mice treated by sub-hypnotic dose of pentobarbital – MEZSS increased the rate of sleep onset and the duration of sleeping time induced by a sub-hypnotic dose of pentobarbital (28 mg/kg, i.p.). Pretreatment with muscimol and treatment with pentobarbital (28 mg/kg, i.p.) also increased the rate of sleep onset and prolonged the sleep time (Table 2).

Enhancement of MEZSS of the latency and duration of sleeping in pentobarbital-treated mice – MEZSS decreased the latency of sleep and increased

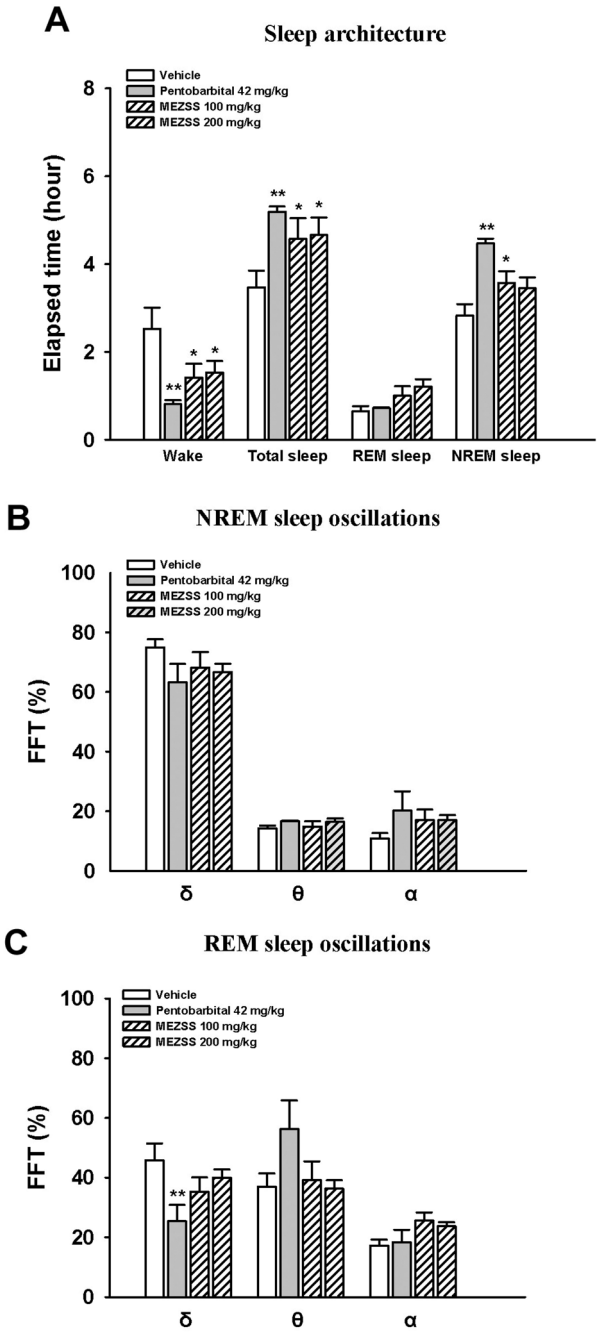

Fig. 2. Effects of MEZSS and pentobarbital on electroencephalogram (EEG) activity in rat. The elapse time of wake, total sleep, rapid eye movement (REM) sleep and non rapid eye movement (NREM) sleep was calculated during daytime of 6-h test period (A). Under the range of specific frequency (δ, θ and α), EEG activity was recorded in NREM (B) and REM sleep (C). FFT: Fast Fourier Transformation. Each column represents the mean ± S.E.M (n = 3-6). $P < .05$ and $^{**}P < .01$, compared to that of the vehicle group.

sleeping time induced by pentobarbital (42 mg/kg) in a dose-dependent manner. Pretreatment with muscimol as a

Table 2. Effects of MEZSS on sleeping onset of mice treated by sub-hypnotic dose of pentobarbital (28 mg/kg, i.p.)

Groups	Dose (mg/kg)	No. falling asleep/total	Sleeping time (min)
Vehicle	--	7/16	28.1 ± 2.6
Muscimol	0.2	15/15**	66.5 ± 3.2***
MEZSS	25	8/14	33.4 ± 2.7
	50	8/14	47.6 ± 3.8***
	100	13/15*	58.8 ± 4.7

Each value represents the mean ± S.E.M. (n = 14 - 16). $^{*}P < .05$, $^{**}P < .01$ and $^{***}P < .001$, compared to that of the vehicle group

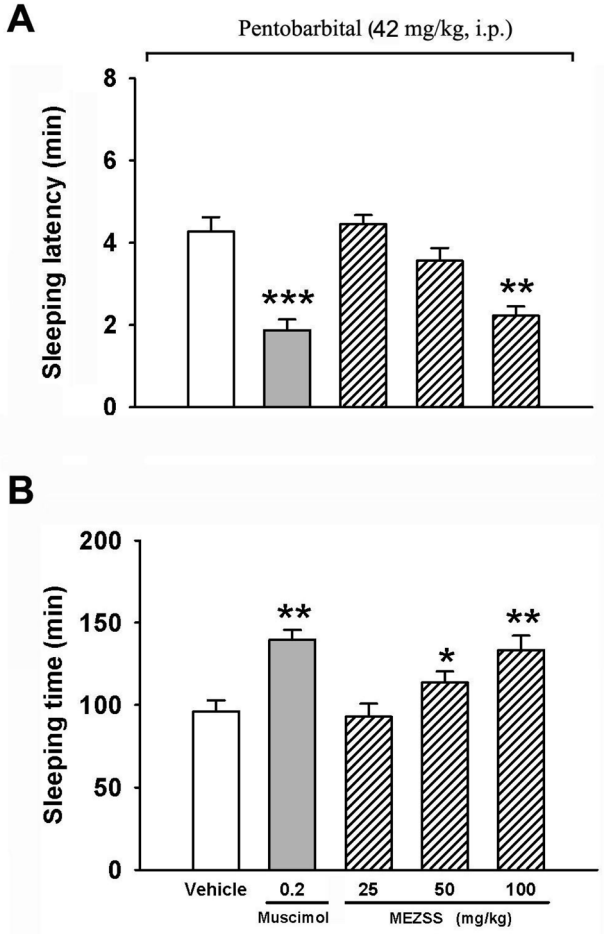

Fig. 3. Effects of MEZSS on onset and duration of sleeping in pentobarbital-treated mice. Mice were fasted for 24 h before the experiment. After the administration of muscimol and MEZSS, pentobarbital (42 mg/kg) was given intraperitoneally to mice. The sleep latency (A) and sleeping time (B) were recorded. MUS: muscimol. Each column represents the mean ± S.E.M. $^{*}P < .05$, $^{**}P < .01$ and $^{***}P < .001$, compared to that of the vehicle group.

positive control 15 min before the administration of pentobarbital also decreased the latency of sleep and increased the total sleeping time (Fig. 3).

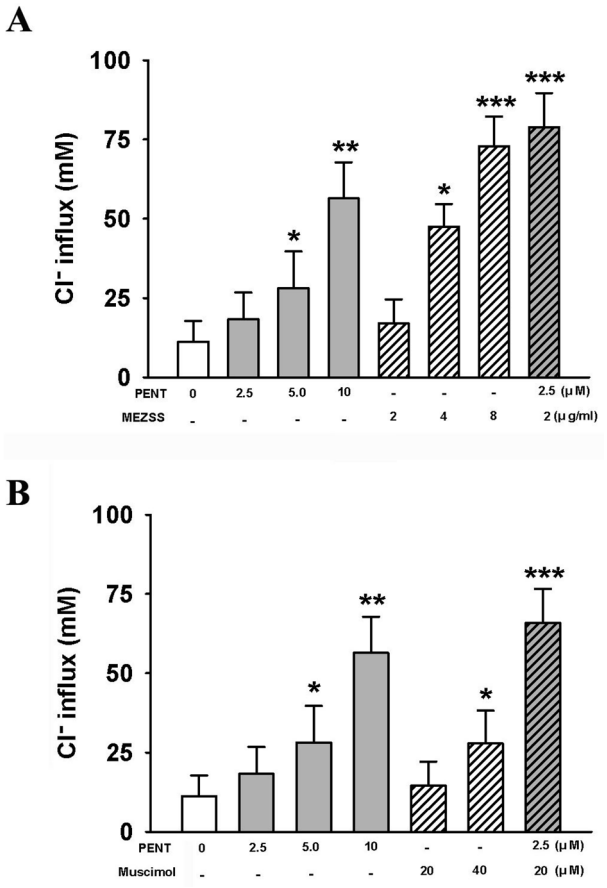

Fig. 4. Effects of MEZSS, muscimol and pentobarbital on chloride influx in primary cultured cerebellar granule cells (A: MEZSS and B: muscimol). MUS: muscimol. Each column represents the mean ± S.E.M. $^{*}P < .05$, $^{**}P < .01$ and $^{***}P < .001$, compared to that of the vehicle group.

Increase of MEZSS on chloride influx in primary cultured cerebellar granule cells – Resting intracellular Cl^- concentrations were calibrated using standard Cl^- solutions of 0, 10, 20, 40, 80 and 120 mM Cl^-, each containing 140 mM K^+. Appropriate amounts of methylsulfate were used to replace Cl^- in these solutions. Tributyltin chloride (5 μM) and nigericin (5 μM) were present to artificially facilitate the balance between intracellular Cl^- and extracellular Cl^- concentrations. Resting $[Cl^-]i$ in cultured cerebellar granule cells was 18.9 ± 5.6 mM, and treatment of granule cells with MEZSS (4 and 8 μg/ml) increased $[Cl^-]i$ to 69 and 77 mM respectively. Pentobarbital (5 and 10 μM) also increased the influx of Cl^- in primary cultured cerebellar granule cells. MEZSS (2 μg/ml) and muscimol (20 μM) treatment alone could not induce a significant Cl^- influx increase in cultured granule cells, while MEZSS (2 μg/ml) potentiated effects of 2.5 μM pentobarbital, which did

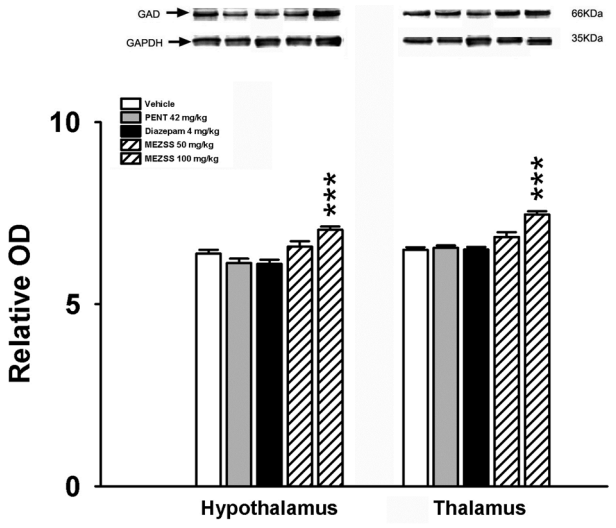

Fig. 5. Effects of MEZSS on GAD65/67 in hypothalamus and thalamus of mice. Quantifications of immunoreactivity by measuring relative densitometry (OD) compared to GAPDH are shown. PENT: pentobarbital. Each column represents the mean±S.E.M (n = 4). $^{***}P < .001$, compared to that of the vehicle group.

not induce Cl^- influx also, on increasing the influx of Cl^-. Similarly, co-treatment of muscimol (20 μM) and pentobarbital (2.5 μM) significantly increased Cl^- influx in primary cultured cerebellar granule cells (Fig. 4).

Expression of GAD65/67 by pentobarbital, diazepam and MEZSS – GAD protein expression was measured in hypothalamus and thalamus of mice after the treatment of pentobarbital (42 mg/kg), diazepam (4 mg/kg) or MEZSS (50 and 100 mg/kg), respectively, for 5 days. Pentobarbital and diazepam had no effect on the abundance of GAD, but the higher dose of MEZSS enhanced the amount of the GAD significantly in hypothalamus and thalamus (Fig. 5).

Subunits expression of $GABA_A$ receptors by pentobarbital, diazepam and MEZSS – $GABA_A$ subunits expression was measured after treatments with different doses (50 and 100 mg/kg) of MEZSS, 4 mg/kg diazepam or 42 mg/kg pentobarbital in hypothalamus and thalamus in mice for 5d. MEZSS induced a slight decrease in the amount of the β-subunit and a significant increase in the γ-subunit, but had no effect on the abundance of the α-subunit. The amounts of the α- and β-subunit were reduced by pentobarbital and the α- and γ-subunit were enhanced by diazepam (Fig. 6).

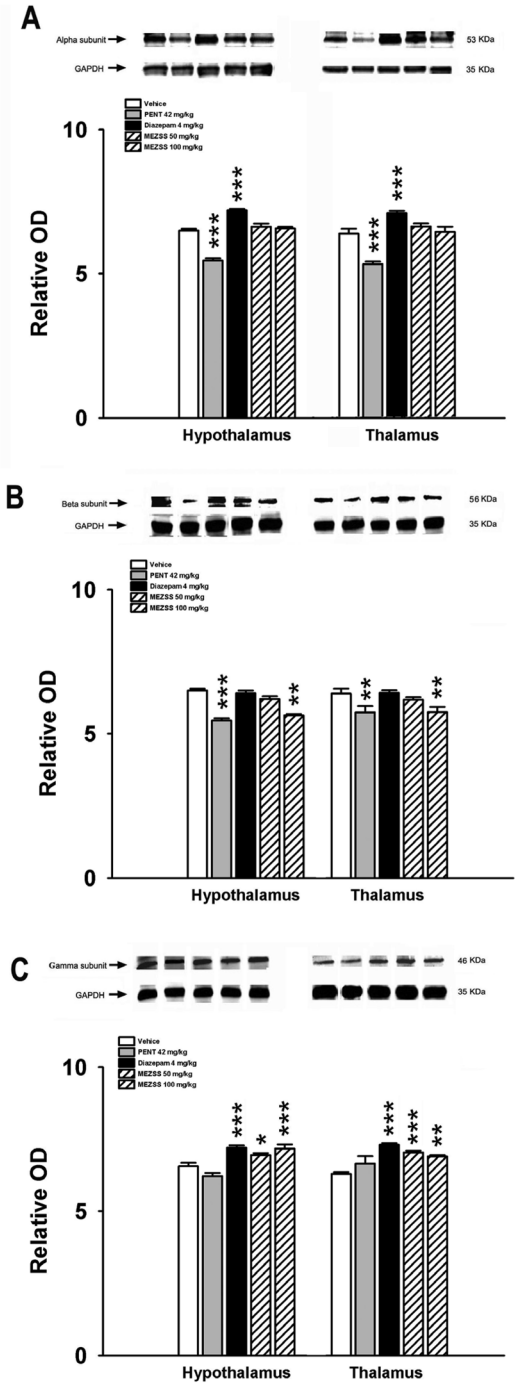

Fig. 6. Effects of MEZSS on $GABA_A$ receptors subunits in hypothalamus and thalamus of mice (A: α-subunit, B: β-subunit and C: γ-subunit). Quantifications of immunoreactivity by measuring relative densitometry (OD) compared to GAPDH are shown. PENT: pentobarbital. Each column represents the mean ± S.E.M (n = 4). $^{*}P < .05$, $^{**}P < .01$ and $^{***}P < .001$, compared to that of the vehicle group.

Discussion

Zizyphi Spinosi Semen has been used for the treatment of insomnia in combinative form in Asia since ancient times. Our previous research indicate that Sanjoinine A of

Zizyphi Spinosi Semen have hypnotic and anticonvulsant effects (Ma *et al*., 2007; Yoon *et al*., 2009). This research measured the effects of the 75% methanol extract of Zizyphi Spinosi Semen on the locomotor activity, sleeping behavior and EEG activity.

The results of present study showed that MEZSS reduced the locomotor activity of mice in dose dependent manners, but only the higher dose (100 mg/kg) group revealed significant difference compared with the vehicle group (Fig. 1). MEZSS increased the total sleep and NREM sleep (Fig. 3). According to the results, though MEZSS, similar to muscimol of $GABA_A$ receptors agonist, alone could not induce sleep even at much higher dose, MEZSS not only significantly prolonged the sleeping time in pentobarbital-pretreated mice, but also increased the number of mice falling asleep and shortened the sleeping latency (Fig. 2 and Table 2). The increase and decrease in pentobarbital-induced sleep time can be a useful tool for examining stimulatory or inhibitory effects on the CNS (Ma *et al*., 2008; Kim *et al*., 2011; Wu *et al*., 2011), in particular for investigating the influence on the GABAergic system and pentobarbital is well known to potentiate the effects of GABA by acting at GABA receptor ionophore complex. It has been reported that Sanjoinine A of Zizyphi Spinosi Semen enhanced hypnotic effects in pentobarbital-treated mice by increasing the inhibition of GABAergic neurons (Ma *et al*., 2007). Taken together, it suggested that MEZSS might generate hypnotic effects by $GABA_A$ receptors/Cl^- channel complex.

$GABA_A$ receptors possess different binding sites such as GABA, benzodiazepine and barbiturate. $GABA_A$ receptors formed heteromeric GABA-gated chloride channels are assembled from a large family of subunits (α, β and γ). $GABA_A$ receptor channels open after activating binding sites to give a net inward flux of negative chloride ions (outward current), hyperpolarizing the membrane and reducing neuronal firing. The pharmacological profile of a $GABA_A$ receptor depends upon subunit composition and distinct $GABA_A$ receptor subtypes. MEZSS alone could not induce sleep but enhanced pentobarbital-induced sleeping. On the base of EEG signature, REM sleep is quite distinct from NREM sleep. NREM sleep is characterized by the δ- and α-wave activity. REM sleep is characterized in rodents by a pronounced activity in the θ-frequency range. MEZSS increased the NREM sleep, but might increase the REM sleep in dose dependant manner. Consistently the increase of the REM sleep during daytime was induced by the repeated treatment with jujubosides from ZSS (Cao *et al*., 2010). The likelihood of increases in the REM sleep by MEZSS was different from the might NREM sleep increase by pentobarbital. Taken together, it suggested that there were differences between MEZSS and pentobarbital in their pharmacological properties on $GABA_A$ receptors. MEZSS increased Cl^- influx in a similar way as pentobarbital, MEZSS also showed similar effects with muscimol on potentiating Cl^- influx by the low dose pentobarbital. Because the activation of glycine receptors increase chloride ion channels open that facilitate inhibitory neurotransmission in brain in the mammalian, it is possible that MEZSS might increase Cl^- influx by glycine receptors (Vale *et al*., 2003; Wang *et al*., 2007).

The role of $GABA_A$ receptors/Cl^- channel complex is involved in the physiology and pharmacology of sleep-wake cycles in CNS, associated with a type of neuronal activation typical of sleep in hypothalamus (Murillo-Rodriguez *et al*., 2009; Sapin *et al*., 2010) and a type of neuronal inhibition typical of wake in thalamus (Winsky-Sommerer, 2009; Sapin *et al*., 2010). In hypothalamus and thalamus, sleep-wake cycles are induced either by directly activating the binding sites such as GABA, at the interface between the α- and β-subunits or, more usually, by enhancing the action of ligands on $GABA_A$ receptors such as benzodiazepines at the interface between α- and γ-subunits, and thereby specifically bind to γ-containing receptors (Winsky-Sommerer, 2009). This latter action is known as positive allosteric modulators, regarded as good targets for the development of subtype specific drugs. The EEG signature in combination with the data analysis uncovered for NERM sleep and REM sleep specific EEG frequencies (δ, θ and α-wave), which associate with different modulation mechanisms of sleep-wake cycles. Our analysis of EEG frequencies suggests that the might increases of δ-wave activity in REM sleep by MEZSS in dose dependant manner, as well as decreases of those by pentobarbital are under different modulation mechanisms, and specifically distinct pharmacological properties on $GABA_A$ receptors. So we tried to find out the typical responding subunits of MEZSS compared those of pentobarbital and diazepam, which might have at least a close relationship with the binding site by which MEZSS acts on $GABA_A$ receptors in hypothalamus and thalamus and exerts its sleep potentiating effects. We found MEZSS slightly decreased the abundance of the $GABA_A$ receptor β-subunit, similar to that of pentobarbital. Furthermore, MEZSS significantly increased the abundance of the γ-subunits, similar to that of diazepam. It is suggested that the enhancement of $GABA_A$ receptor

by MEZSS might be more strongly modulated by activating allosteric sites indirectly compared with the GABA binding site directly. In addition, we investigated the effects of MEZSS, pentobarbital and diazepam on expression of GAD 65/67 in hypothalamus and thalamus. GAD 65/67, which is necessary for GABA synthesis, plays a major role in GABA transmission in physiological condition. MEZSS increased the GAD expression significantly. It is suggested that MEZSS might increase GABA transmission in hypothalamus and thalamus by activating GAD.

Our results suggested that MEZSS might exert its sleeping potentiating effects by three pathways: ① increase of GABA synthesis by GAD activation; ② enhancement of GABA receptors to pentobarbital by influence GABA receptors subunits compositions, especially by increasing γ-subunits expression; ③ it is also possible that MEZSS activate GABA binding sites directly or allosteric sites on benzodiazepine indirectly. A considerable number of herbal constituents whose behavioral effects and pharmacological actions have been well characterized may be good candidates for further investigations that may ultimately lead to clinical use. ZSS might be another good candidate for insomnia.

In conclusion, MEZSS itself did not induce sleeping and only enhanced hypnotic effects in pentobarbital-treated mice. The $GABA_A$ receptor-chloride channel complex in hypothalamus and thalamus might be involved in the modulation mechanisms of these effects. It is also suggested that pentobarbital and MEZSS acts on $GABA_A$ receptors pharmacologically differently. Further investigation is needed in order to understand the pharmacological actions of MEZSS.

Acknowledgement

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