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Sinomenine, an Alkaloid Derived from *Sinomenium acutum* Potentiates Pentobarbital-Induced Sleep Behaviors and Non-Rapid Eye Movement (NREM) Sleep in Rodents

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Abstract

Sinomenium acutum has been long used in the preparations of traditional medicine in Japan, China and Korea for the treatment of various disorders including rheumatism, fever, pulmonary diseases and mood disorders. Recently, it was reported that *Sinomenium acutum*, has sedative and anxiolytic effects mediated by GABA-ergic systems. These experiments were performed to investigate whether sinomenine (SIN), an alkaloid derived from *Sinomenium acutum* enhances pentobarbital-induced sleep via γ-aminobutyric acid (GABA)-ergic systems, and modulates sleep architecture in mice. Oral administration of SIN (40 mg/kg) markedly reduced spontaneous locomotor activity, similar to diazepam (a benzodiazepine agonist) in mice. SIN shortened sleep latency, and increased total sleep time in a dose-dependent manner when co-administrated with pentobarbital (42 mg/kg, i.p.). SIN also increased the number of sleeping mice and total sleep time by concomitant administration with the sub-hypnotic dosage of pentobarbital (28 mg/kg, i.p.). SIN reduced the number of sleep-wake cycles, and increased total sleep time and non-rapid eye movement (NREM) sleep. In addition, SIN also increased chloride influx in the primary cultured hypothalamic neuronal cells. Furthermore, protein overexpression of glutamic acid decarboxylase ($GAD_{65/67}$) and $GABA_A$ receptor subunits by western blot were found, being activated by SIN. In conclusion, SIN augments pentobarbital-induced sleeping behaviors through $GABA_A$-ergic systems, and increased NREM sleep. It could be a candidate for the treatment of insomnia.

Key Words: *Sinomenium acutum*, Sinomenine, Pentobarbital, $GABA_A$-ergic systems, Electroencephalogram (EEG), Insomnia

INTRODUCTION

Insomnia can be defined as the inability to initiate or maintain sleep. It is also one of the most common health problems in modern society. There are individual differences in the degree of insomnia. So, it is not easy to indicate the severity of insomnia by the absolute amount of sleep time. The research has been performed to recognize sleep status or insomnia of the elder and young peoples. Over the last decade, neuroscientists have more interests in herbal medicines, which contain phytochemicals for the treatment of insomnia. The alternative herbs for the treatment of insomnia has progressed in the past decades. Many herbs such as St, John's wort, kava kava, valerian, and passion flower have been introduced in European countries (Kim *et al*., 2011). Herbs as sleep aids have been becoming more popular as alternative medicines.

γ-Aminobutyric acid (GABA), the main inhibitory neurotransmitter of the central nervous system (CNS), is the most prevalent target for treating insomnia. It is well established that activation of $GABA_A$-ergic neurons plays an important role in sleep. Glutamic acid decarboxylase ($GAD_{65/67}$), an enzyme responsible for the synthesis of GABA also plays a crucial role in sleep. On the other hand, The $GABA_A$ receptors complex consists of a Cl^- ionophore principally coupled to GABA, barbiturate, benzodiazepine (BZ), steroid, and picrotoxin binding sites (Macdonald and Olsen, 1994; Sieghart, 1995). Basic subunits of $GABA_A$ receptors are composed to α (1~6), β (1~3) and γ (1~3) (Seifi *et al*., 2014). These binding sites trigger the chloride channel's opening with resulting membrane hyperpolarization (Wang and Xu, 2006). $GABA_A$-ergic drugs have induced sedative–hypnotic effects in animals and humans (Abourashed *et al*., 2004). Depending on the configuration of the structural subunits, it determines the pharmacological properties of the $GABA_A$ receptors. α1−Subunits

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in $GABA_A$ receptors mediate sedation, amnesia, and ataxic effects of benzodiazepine (BZ), whereas α2−and α3-subunits are involved in their anxiolytic-like and muscle-relaxing actions. α5−-Subunits are involved in at least some of the memory impairment caused by BZ (Macdonald and Olsen, 1994).

Sinomenine (SIN; Fig. 1), an alkaloid derived from *Sinomenium acutum*, is a chief ingredient which has been reported to have a variety of pharmacological effects including anti-rheumatism, immunomodulation and anxiolytic-like effects (Chen *et al*., 1997; Li *et al*., 2003; Rao *et al*., 2017). However, it has not been studied whether SIN is sedative or affects sleep behaviors. Form these experiments, we focused on whether SIN enhances pentobarbital-induced sleeping behaviors and modulate sleep architecture via $GABA_A$-ergic systems in rodents.

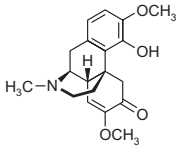

Fig. 1. Chemical structure of sinomenine (SIN).

MATERIALS AND METHODS

Animals

The animals used for experiments were 4-week-old ICR male mice and 8-week-old male Sprague Dawley (SD) rats weighing 20-25 g and 300-320 g, respectively. They were purchased from Samtako (Osan, Korea). All rodents were housed in acrylic cages (45×60×23 cm), and were kept at least 1 week for acclimation time. The room condition was maintained at 22 ± 2°C, relative humidity (50-52%), and a 12-h light/dark cycle with *ad libitum* feeding. The behavioral experiments were performed between 10:00 and 17:00 and were carried out in accordance with the Principle of Laboratory Animal Care (NIH publication No. 85-23, revised 1985). This experiment was performed in accordance with the Animal Care and Use Guidelines of Chungbuk National University, Korea.

Drugs and reagents

SIN was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Diazepam (ample), pentobarbital (ample), and muscimol were acquired, respectively, from the following companies: Samjin Pharm. (Seoul, Korea), Hanlim Pharm. Co., Ltd. (Seoul, Korea), and Tocris (Cookson, Avonmouth, UK or Ellisville, MO, USA). Fetal bovine serum and DMEM were obtained from GIBCO (Grand Island, NY, USA). The Cl−sensitive fluorescence probe N-(ethoxycarbonyl-methyl)-6-methoxyquinolinium bromide (MQAE) was purchased from Sigma-Aldrich (St Louis, MO, USA). The specific rabbit polyclonal antibodies against $GABA_A$ receptors subunits, $GAD_{65/67}$ and the corresponding conjugated anti-rabbit immunoglobulin G-horseradish peroxidase, were obtained from Abcam Inc (Cambridge, UK).

Locomotor activity measurement

Spontaneous locomotor activity was measured by a tilting-type ambulometer (AMB-10, O'Hara, Tokyo, Japan) for 1 h (Morton *et al*., 2011). The mice in each group had 10 min of adaptation time in the activity cages (20 cm in diameter and 18 cm in height). Diazepam (2 mg/kg, p.o.) and SIN (20 and 40 mg/kg, p.o.) dissolved in distilled water and 0.01% DMSO, respectively, were administered 30 min and 60 min prior to the experiment, respectively.

Pentobarbital-induced sleeping behaviors measurement

All mice were fasted for a day, and all experiments were carried out between 1:00 and 5:00 p.m. Pentobarbital was diluted in 0.9% physiological saline. Muscimol (0.2 mg/kg, i.p.) and SIN (20 and 40 mg/kg, p.o.) were orally administered before 15 min and 60 min, respectively, and then pentobarbital (42 mg/kg) was injected intraperitoneally (i.p.). After the pentobarbital, the mice were moved to another cage. Sleep latency was recorded as time elapsed after the pentobarbital injection. Sleep was recorded as the time between the elapse and the righting of animals. The mice that failed sleep within 15 min were excluded from the experiments (Wolfman *et al*., 1996; Hu, 2012).

EEG telemetry transmitter implantation and data collection

After the pentobarbital was administered (50 mg/kg, i.p.), the SD rats were placed on a pad in the stereotaxic apparatus under aseptic conditions. Transmitters (TA11CTA-F40, Data Sciences International, St. Paul, MN, USA) were implanted under the skin after the scalp incision. In detail, the skull periosteum was removed, and then two holes for were drilled to insert electric lines (A: 2.0 [Bregma], L: 1.5; P: 7.0 [Bregma], L: 1.5 contra-lateral) (Paxinos *et al*., 1985). The transmitter lines were subcutaneously connected to the skull, and dental cement was used to fix the electric lines to the skull. The incisions were sewn up with a silk suture. An antibiotic was given to all rats after surgery (5 million unit potassium penicillin-G Injection, Keunwha, Seoul, Korea). After the transmitters were implanted, the rats were given a week of recovery time. SIN (40 mg/kg, p.o.) were orally administered before 15 min of experiment. All signals were transmitted by AD converter (Eagle PC300, Los Gatos, CA, USA) and stored in the computer, and the computer could also graphically display the results. Fast Fourier transform (FFT) analysis generated power density values from 0 to 20.0 Hz with a resolution of 0.5 Hz. Mean FFT was also in the range of 0 and 20.0 Hz for every 10 sec. EEG data in all rats were recorded for 4 h from 11:00 a.m. to 5:00 p.m. (Sanford *et al*., 2006).

Data analysis

Sleep cycles were graphically recorded and saved in Sleep-Sign 2.1 software (KISSEI Comtec Co Ltd, Matsumoto, Japan). Data were classified into wakefulness, non-rapid eye movement (NREM), and rapid eye movement (REM) for every 10 sec (Tokunaga *et al*., 2007). Wakefulness and NREM states were found in high-frequency and slow waves, respectively. δ-wave (0.75-4.0 Hz) and θ-wave (5.0-9.0 Hz, peak at 7.5 Hz) increased in the low EEG waves during REM sleep. Wakefulness, NREM, REM, and total sleep time (NREM+REM) were recorded for each rat for 6 h. The EEG power was set up at 0.5-20.0 Hz in 0.5 Hz bins. Sleep architecture was evaluated in three waves in the range of 8.0-13.0 Hz (Ma, 2009). Data were calculated as relative values in Microsoft Excel.

Cell culture

Primary cultures of the SD rats' hypothalamus cells were

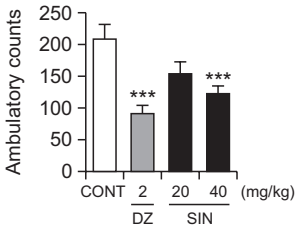

Fig. 2. Effect of SIN on locomotor activity test. Ambulation activity was measured for 1 h, 30 min after oral administration of diazepam and 1 h after administration of SIN. Each column shows the mean ± SEM. The significance of the compound's effects was assessed using ANOVA. Where there was significant variability, the individual values were compared using Student's *t*-test. ***p<0.005, compared with the control.

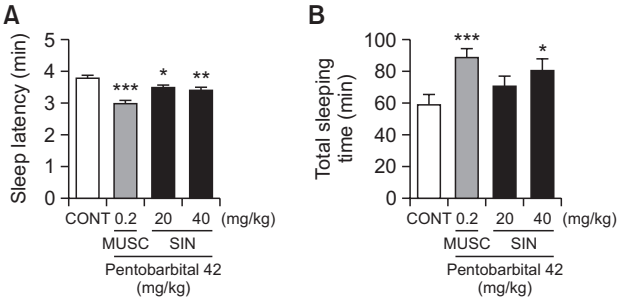

Fig. 3. Effects of SIN on sleep onset and duration in pentobarbital-treated mice. Mice were deprived of sleep for 24 h prior to the experiment. Pentobarbital (42 mg/kg, i.p.) was administered to mice following administration of muscimol or SIN, and sleep latency (A) and total sleep time (B) were measured. Each column shows the mean ± SEM. The significance of the compounds' effects was assessed using ANOVA. Where there was significant variability, the individual values were compared using Student's *t*-test. *p<0.05, **p<0.01, ***p<0.005, compared with that of the control.

tested for 7-8 days (Ma *et al*., 2007). The cells were seeded at 1.0×10^5 cells in 96-well microplates coated with poly-L-lysine (50 μg/mL; Sigma, St. Louis, MO, USA). The DMEM used for cell cultures contained 10% fetal bovine serum, glutamine (2.0 mM), gentamicin (100 μg/mL), antibiotic-antimycotic solution (10 μg/mL; Sigma), and potassium chloride (25 mM). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% O_2 air. After 16 h, the 96-well plates were added into cytosine arabinofuranoside (final concentration 10 μM; Sigma) to inhibit non-neuronal cell growth.

Measurement of intracellular Cl^- influx

MQAE (a sensitive fluorescent substance for Cl^-) was used to measure Cl^- influx in the rats' cerebellum cells following the method of West and Molloy (1996). After overnight MQAE treatment, the cells were washed three times in a buffer (pH 7.4) that contained 2.4 mM HPO_4^{2-}, 0.6 mM $H_2PO_4^-$, 10 mM HEPES, 10 mM D-glucose, and 1.0 mM $MgSO_4$. The fluorescence data were measured according to excitation wavelength 320 nm and emission wavelength 460 nm by Elisa Reader (SpectraMax M2e Multi-mode, PA, USA) (Wagner *et al*., 2010). The data were calculated as F/F_0 based on the Cl^- data ratios. F is the fluorescence of each sample, and F_0 is the fluorescence without Cl- ions.

Western blotting

Protein samples were extracted from the rat's hypothalamus cell cultures. SIN (final concentration 40 mg/ml) was dissolved in 0.01% DMSO. The control sample was treated in the same solvent as that used in the SIN treatment. After diazepam or SIN administration, the cells were extracted and treated with a cold lysis buffer [25 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1.0 mM $CaCl_2$, 1% Triton X-100, 1.0 mM PMSF, 10 μl/mL aprotinin, 1.0 mM NaF, and 2.0 mM sodium ortho-vanadate]. Supernatant extracts were recovered after centrifugation at 13,000×g at 4°C for 20 minutes. Protein concentration was measured using Bradford protein analysis and stored at -20°C. The same amounts of protein were placed in 10% SDS-polyacrylamide gel, and then the electrophoresis was loaded. The protein was transferred to PVDF membranes (Hybond-P, GE Healthcare, Amersham, UK) using semidry transfer. The blots were blocked for 1 h at room temperature with 5.0% (w/v) BSA [applied to all primary antibodies except for glyceraldehyde 3-phosphate dehydrogenase (GAPDH)], and 5.0% (w/v) skim milk (only applied to GAPDH) in tris-buffered saline solution (TBS) containing 0.1% Tween-20. Both specific rabbit polyclonal antibodies against $GABA_A$ receptor subunits and rabbit anti-$GAD_{65/67}$ polyclonal antibody at the appropriate dilution in TBST and 5.0% BSA (1:2,500 for all the primary antibodies used) were incubated overnight at 4°C. After washing with TBST, the blots were treated 1:3,000 dilution of a secondary antibody at room temperature for 4 h (goat anti-rabbit, IgG). A the secondary antibody was detected using ECL light-emitting substrate (Roche Diagnostics, Mannheim, Germany) (Han *et al*., 2010).

Statistical analysis

All statistical analysis was performed with SigmaStat software (SPSS Inc., Chicago, USA). Experimental results are shown as mean ± SEM, and significance was measured with analysis of variance (ANOVA). When there were significant differences, values were compared with Student's *t*-test. However, in sub-hypnotic pentobarbital-induced sleep, the falling asleep/total was compared using Chi-square test. *p* was considered significant at less than 0.05.

RESULTS

Effect of SIN on locomotor activity in mice

SIN (20 and 40 mg/kg, p.o.) was administered to the mice. Locomotor activity was significantly inhibited by SIN at 40 mg/kg. The locomotor activity by diazepam (2.0 mg/kg, i.p.) as a positive control was also decreased compared with that of the control (Fig. 2). The value of locomotor activity to oral administration with SIN at 40 mg/kg were decreased by approximately 41.2%. Positive control DZ also decreased by approximately 56.3%, compared with that of the control. From these preliminary experiments, we suggest that SIN might be sedative.

Effects of SIN on pentobarbital-induced sleeping behaviors in mice

SIN (20 and 40 mg/kg, p.o.) reduced the sleep latency of sleep and induced dose-dependently total sleep time in pentobarbital induced sleeping mice. Pretreatment with mucsimol (0.2 mg/kg, i.p.) as a positive control 30 min before the pentobarbital (42 mg/kg, i.p.) also increased sleeping time and

Table 1. Effects of SIN on number of sleep mice and total sleep time treated by sub-hypnotic dose of pentobarbital (28 mg/kg, i.p.)

Group	Dose (mg/kg)	No. of animals	Total Sleep time (min)
Control	0	6/12	30.8 ± 2.6
Muscimol	0.2	12/12*	47.4 ± 1.8***
SIN	20	8/13	34.7 ± 2.0
	40	10/13	40.5 ± 2.3**

Each value reflects the mean ± SEM. Where there was significant variability, the individual values were compared using Chi-square and Student's *t*-test. *p<0.05, **p<0.01 ***p<0.005, compared with the control.

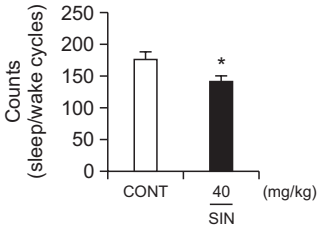

Fig. 4. Effect of SIN on numbers of sleep-wake cycles. Where there was significant variability, the individual values were compared using Student's *t*-test. *p<0.05, compared with that of the control.

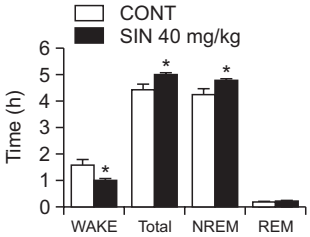

Fig. 5. Effect of SIN on rat sleep architecture. The data show the mean ± SEM of time spent, which separated the wakefulness and sleep (NREM and REM) states. The significance of the compounds' effects was assessed using ANOVA. Where there was significant variability, the individual values were compared using Student's *t*-test. *p<0.05, compared with that of the naïve control.

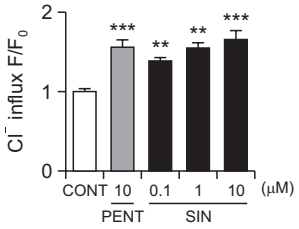

Fig. 6. Effect of SIN on Cl- influx in primary cultured cerebellar granule cells. After the hypothalamic neuronal cells were cultured for 8 days, the cells were incubated with MQAE overnight, and then SIN (0.1, 1, and 10 μM) and pentobarbital (10 μM) were added 1 h prior to measurement. Each column shows the mean ± SEM. The significance of the compounds' effects was assessed using ANOVA. Where there was significant variability, the individual values were compared using Student's *t*-test. **p<0.01, ***p<0.005, compared with that of the control.

decreased sleep latency (Fig. 3). The time of sleep latency to oral administration with SIN 40 mg/kg were decreased by approximately 10.1%. Positive control (muscimol) also decreased by approximately 24.8%, compared with that of the control. Total sleep time SIN 40 mg/kg group were increased by approximately 36.7%. Positive control MUSC also increased by approximately 50.6%, compared with that of the control. We suggest that SIN could reduce sleep latency and increase total sleep.

Effects of SIN on sleep onset by sub-hypnotic dosage of pentobarbital in mice

SIN (40 mg/kg, p.o.) reduced the sleep latency and prolonged total sleep time induced by a sub-hypnotic pentobarbital dose (28 mg/kg, i.p.). Similarly, muscimol (a GABA receptor agonist) significantly is affected pentobarbital-induced sleep (Table 1). Total sleep time to sub-hypnotic dosage with at SIN 40 mg/kg were increased by approximately 31.5%. Positive control MUSC also increased by approximately 53.9%, compared with that of the control. We suggest that SIN would interact with $GABA_A$ receptors.

Effects of SIN on sleep-wake cycles

SIN (40 mg/kg, p.o.) significantly reduced sleep-wake cycles. The count of sleep-wake cycle were decreased by approximately 19.8%, compared with that of the control (Fig. 4). The sleep structure shows that the sleep time is shorter than the total sleep time (Fig. 5); In other words, SIN reduced wakefulness.

Effects of SIN on sleep architectures

After EEG analysis, we found that SIN (40 mg/kg, p.o.) significantly prolonged total sleep time, especially NREM sleep. SIN also decreased wakefulness (Fig. 5). The wake time of sleep architectures at SIN 40 mg/kg were decreased by approximately 36.5%, then increased total sleep and NREM sleep by approximately 13.0% and 12.7% respectively, compared with that of the control.

Effects of SIN on intracellular Cl- influx in primary cultured hypothalamus cells

SIN (10 μM) significantly increased intracellular Cl^- influx, resulting in the hyperpolarization of the neuronal cell membrane. In addition, Pentobarbital (10 μM) also significantly increased intracellular Cl^- influx in primary cultured hypothalamus cells. The intensities of Cl^- influx in hypothalmus cells treared with SIN 0.1, 1 and 10 μM were approximately 38.7%, 54.9% and 65.6% respectively. Positive control PENT also increased by approximately 55.9%, compared with that of the control (Fig. 6).

Effects of SIN on expression of $GAD_{65/67}$

$GAD_{65/67}$ expression was induced by SIN (40 mg/kg, p.o.) in the rats' primary hypothalamic neuron cells. The intensities of $GAD_{65/67}$ expression translocated to the plasma membrane in hypothalamic tissue treated with SIN was increased by approximately 85.8% compared with that of the control tissue (Fig. 7). We suggest that SIN activates $GAD_{65/67}$.

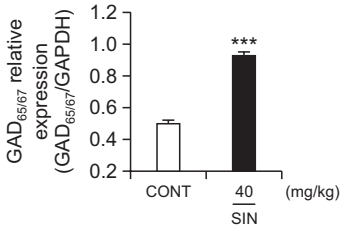

Fig. 7. Effect of SIN on the expression of GAD; the $GAD_{65/67}$ expression was induced by SIN (40 mg/kg) in the hypothalamic neuronal cells of the mice. GAPDH levels were needed in order to normalize the protein expression. Each column shows the mean ± SEM. The significance of the compounds' effects was assessed using ANOVA. Where there was significant variability, the individual values were compared using Student's *t*-test. ****p*<0.005 compared with that of the control.

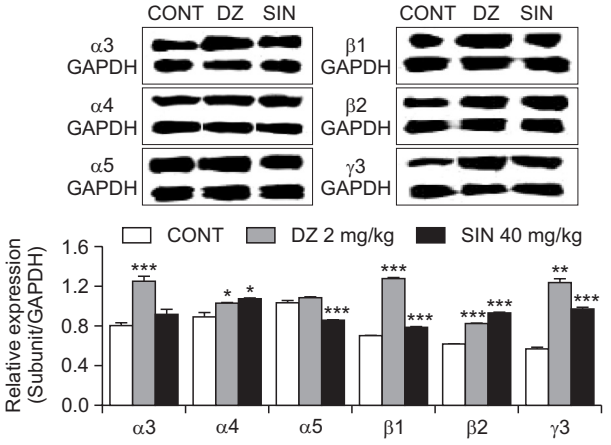

Fig. 8. Effects of SIN on expression of $GABA_A$ receptor subunits. Immunoblots are shown of lysed hypothalamic neuronal cells that were treated for 1 h following SIN. GAPDH levels were needed in order to normalize the protein expression. Each column shows the mean ± SEM. The significance of the effects of the compounds was assessed using ANOVA. Where there was significant variability, the individual values were compared using Student's *t*-test. *p<0.05, **p<0.01, ***p<0.005, compared with that of the control.

Effects of SIN on expression of $GABA_A$ receptors subunits

From these experiments, the $GABA_A$ receptor subtype activation was measured by western blotting. All subtypes of $GABA_A$ receptors except α5 subtype were overexpressed with the SIN (40 mg/kg, p.o.). DZ as a positive control also showed similar patterns. The expression of GABA subunit (α4, β1, β2, γ3) increased in hypothalamic sample treated with SIN were by approximately 20.6%, 11.9%, 50.7% and 70.4% respectively. Positive control DZ (α3, α4, β1, β2, γ3) also increased by approximately 55.4%, 15.6%, 81.9%, 33.3% and 116.9% respectively, compared with that of the control (Fig. 8).

DISCUSSION

Many alkaloids such as SIN, disinoimenine, sinactine, sinoactine, acutumine, and magnoflorine, as well as the lignan syringaresinol have been isolated from *Sinomenium acutum* (Bao *et al*., 2005; Wang *et al*., 2007; Jin *et al*., 2008). Among them, SIN has been reported to have a variety of pharmacological effects including anti-rheumatism, immunomodulation and sedative effects (Chen *et al*., 1997; Li *et al*., 2003; Rao *et al*., 2017). Recently, it was reported that *Sinomenium acutum*, has sedative and anxiolytic effects mediated by GABA-ergic systems. In addition, they reported that SIN exerts considerable antinociceptive property for neuropathic pain via $GABA_A$-mediated mechanism, and it could be useful for the management of chronic painful conditions such as neuropathic pain (Zhu *et al*., 2014). Based on previous studies, we focused on the hypnotic effect of SIN as the ultimate goal of the experiment. The preliminary experiment results demonstrate that SIN inhibited locomotor activity, showing sedative effects in mice. We investigated the effects of different dosages of SIN and muscimol in rodents with pentobarbital treatment and found that SIN enhanced pentobarbital-induced sleep, similar to muscimol, It is suggested that potentiation of SIN's hypnotic effect can interact with $GABA_A$-ergic systems.

The sleep architectures of rat after oral SIN administration were also analyzed. The spontaneous electrical activity from the rat brain can be recorded by SSG over a short period of time, and sleep/wake cycles can be measured using EEG frequency analysis. Spectral EEG analysis technique of sleep/wake cycles can be used as a tool for insomnia treatment. We found that SIN itself modulated sleep architectures; SIN reduced the counts of sleep/wake cycles in rats, which is important in treating insomnia. Sleep can be divided three stages, wake, non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep. REM Sleep is also composed of rapid eye movement (REM) sleep and non-REM (NREM) sleep. REM sleep is the major source of dreams, whereas synchronous cortical oscillations, called slow waves, are observed during NREM sleep. Both stages are unique to certain mammalian species, and therefore, REM and NREM sleep are thought to be involved in higher-order brain functionssleep is most often associated with vivid dreaming and a high level of brain activity. The other phase of sleep, NREM sleep or slow wave sleep (SWS), is usually associated with reduced neuronal activity. As one goes to sleep, the low voltage fast EEG of waking gradually gives way to a slowing of frequency and, as sleep moves toward the deepest stages, there is an abundance of delta waves, EEG waves with a frequency of 0.5 to <4 Hz and of high amplitude (McCarley, 2007). According to the sleep research, REM sleep was characterized by fast-wave sleep along with muscle atonia, brain activation, and eye movement. NREM sleep was discovered to play a role in restoring physiological functions (Siegel, 2005). REM sleep is a distinctive sleep stage that alternates with episodes of NREM sleep (Trachsel *et al*., 1991; Gottesmann, 1996; Datta and Hobson, 2000). About 25 percent of sleep is spent in an REM sleep and the remaining 75 percent is for the NREM sleep. We especially focused on determining whether SIN increased REM, NREM, and total sleep time and alter sleep architectures. From these experiments, it is suggested that SIN could enhance total sleep and NREM sleep in rats, reducing sleep/wake cycles.

Activating $GABA_A$–ergic transmission is important for treating insomnia. From the first *in vitro* experiments, we found that SIN increased intracellular Cl- influx in primary cultured hypothalamic neuronal cells of rats, resulting in hyperpolarization of neuronal cells in the CNS. These effects can be caused by the increased levels of GABA in the neuronal cells. GABA is

synthesized from glutamate exclusively in $GABA_A$-ergic neurons by GAD, which consists of two isoforms with molecular weights of 65-kDa and 67-kDa (Bu *et al*., 1992). $GAD_{65/67}$ is the rate-limiting enzyme in GABA biosynthesis which also plays an important role in maintaining GABA level in the brain (Tillakaratne *et al*., 1995). GABA activates the ionotropic GABA receptors on the presynaptic, postsynaptic, and extrasynaptic neurons. GABA is released to the synapse which is the extracellular space existing between the neurons. The $GABA_A$ receptor Cl- channel opens after binding with GABA to give a net inward flux of negative Cl^- (outward current), hyperpolarizing the membrane and reducing neuronal firing (Macdonald and Olsen, 1994). Protein expression levels of $GAD_{65/67}$ were measured in primary cultured hypothalamic neuronal cells; SIN increased protein expression levels in these cells. It is suggested that SIN activates $GAD_{65/67}$. SIN also increased Cl- influx, similar to pentobarbital. This result shows that SIN can lead $GABA_A$ receptors to open the Cl- channel.

The expression levels of $GABA_A$ receptor α-, β- and γ-subunits were also investigated. The most abundant $GABA_A$ receptor subunit composition, $\alpha1$, $\beta2$ and $\gamma2$, including the cerebellum, is related to the hypnotic/sedative effect of $GABA_A$ receptors (Rudolph and Mohler, 2006). Previous studies have shown $\alpha1$ subunit associated with sedation (Rudolph *et al*., 1999; McKernan *et al*., 2000), the $\alpha2/3$ subunits associated with anxiety (Low *et al*., 2000; Crestani *et al*., 2001), and the $\alpha5$ subunit associated with temporal and spatial memory (Collinson *et al*., 2002; Crestani *et al*., 2002). Structural and physiological heterogeneities of $GABA_A$ receptors as well as the differential distribution of its receptor subtypes in specific brain areas provide an important basis for the development of therapeutic drugs. We examined expression patterns of α-subunits ($\alpha3$, $\alpha4$, $\alpha5$), β-subunits ($\beta1$, $\beta2$) and γ-subunit ($\gamma3$) in $GABA_A$ receptors in hypothalamic cells. SIN, non-selectively activated the subunits of $GABA_A$ receptors in these experiments. All subunits which we have tested except for $\alpha3$, $\alpha5$- subunits were over-expressed by SIN. On the other hand, diazepam increased high protein levels in the $\alpha3$, $\alpha4$, $\beta1$, $\beta2$, $\gamma3$ subunits. The activation of $GABA_A$ receptors by SIN seem unlikely with those of diazepam.

The present study provides evidence that SIN possesses not only sleep-prolonging but also sleep quality-enhancing effects when administered orally to rats. SIN itself decreases sleep/wake cycle and increases total sleep time. $GABA_A$-ergic transmissions including $GAD_{65/67}$, intracellular chloride influx, and $GABA_A$ receptor subtypes were reduced by SIN. In conclusion, SIN can be a candidate for the treatment of insomnia.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

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