

## Repeated Administration of Korea Red Ginseng Extract Increases Non-Rapid Eye Movement Sleep via GABA<sub>A</sub>ergic Systems

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The current inquiry was conducted to assess the change in sleep architecture after long periods of administration to determine whether ginseng can be used in the therapy of sleeplessness. Following post-surgical recovery, red ginseng extract (RGE, 200 mg/kg) was orally administered to rats for 9 d. Data were gathered on the 1st, 5th, and 9th day, and an electroencephalogram was recorded 24 h after RGE administration. Polygraphic signs of unobstructed sleep-wake activities were simultaneously recorded with sleep-wake recording electrodes from 11:00 a.m. to 5:00 p.m. for 6 h. Rodents were generally tamed to freely moving polygraphic recording conditions. Although the 1st and 5th day of RGE treatment showed no effect on power densities in non-rapid eye movement (NREM) and rapid eye movement (REM) sleep, the 9th day of RGE administration showed augmented  $\alpha$ -wave (8.0 to 13.0 Hz) power densities in NREM and REM sleep. RGE increased total sleep and NREM sleep. The total percentage of wakefulness was only decreased on the 9th day, and the number of sleep-wake cycles was reduced after the repeated administration of RGE. Thus, the repeated administration of RGE increased NREM sleep in rats. The  $\alpha$ -wave activities in the cortical electroencephalograms were increased in sleep architecture by RGE. Moreover, the levels of both  $\alpha$ - and  $\beta$ -subunits of the  $\gamma$ -aminobutyric acid (GABA)<sub>A</sub> receptor were reduced in the hypothalamus of the RGE-treated groups. The level of glutamic acid decarboxylase was over-expressed in the hypothalamus. These results demonstrate that RGE increases NREM sleep via GABA<sub>A</sub>ergic systems.

**Keywords:** *Panax ginseng*, Electroencephalogram, Non-rapid eye movement, Power density, Rapid eye movement

### INTRODUCTION

The modulation of sleep is a homeostatic course in which control mechanisms are revitalized to indemnify for deficiency and superabundant sleep [1]. In mammals, sleep is composed of two principal phases, rapid eye movement (REM) sleep and non-REM (NREM) sleep. REM sleep takes turns with cases of NREM sleep, and the voluntary NREM-REM sleep cycle takes sketchy

12 to 20 min in rats [2]. For the past forty years, mainly sleep research places emphasis on identifying referential brain structures, neuronal networks, and transmitters included in the genesis and modulation of NREM and REM sleep [3]. Some is known about the mechanisms and regulation of the rhythmed appearance of NREM-REM sleep. Insomnia is a prevalent problem in late life.

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Received 16 Jul. 2012, Revised 21 Aug. 2012, Accepted 30 Aug. 2012

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The reported prevalence of insomnia ranges between 30% and 60%. Pharmacological accesses have been performed in customary medical treatment for sleeplessness. Recently, some herbs have been charming medicines for considerable sufferers with sleep disabilities or insomnia [4,5].

Ginseng has been used as a conventional medicine during thousands of years. People take ginseng to expend vitality and keen the mind. Ginseng has a complicated activity that is occasionally difficult to symmetrize neurochemical reports. Ginseng tranquillizes and equalizes physiology and may help sustentation normal sleep and wakefulness [6]. Ginseng has been clinically used for the remedy of sleeplessness [7]. The greater part of the advantages of ginseng is immediately originated from ginsenosides, the major active components in ginseng root. Ginsenosides influence metabolism, incorporating exploding vigor, encouraging mental efficacy, and furthering larger stamina and patience, through protecting exhaustion and the debilitating efficiencies of old age. Ginsenosides affect as tonics to assist the human organism adapt to biological strain in a natural method. Together with its fitness-increasing effects, ginseng improves brain activity and favors psychological stability by exciting and staying the mind. Korean red ginseng extract (RGE) contains more ginsenosides than other ginseng, making it the most efficient of all ginsengs [8]. So, Korean RGE is an incomparable natural resource of nutrients for a fine body and mind.

However, sleep disabilities are the greater part generally experienced unfavourable efficiencies of ginseng [9]. To evaluate the role of ginseng in adjusting sleep-wake changes and sleep architecture and to conclude the conceivable mechanisms of NREM-REM sleep regulation, we indagated its impact on the amount of whole sleep and wakefulness, examined power density fluctuations in recorded electroencephalograms (EEG) of concrete sleep-wake stages, and discovered variations in  $\gamma$ -aminobutyric acid (GABA)<sub>A</sub>ergic receptors of the hypothalamus. Previously, we had been experimented that administering RGE 200 mg/kg for 5 d increased pentobarbital-induced sleep behaviors [10]. Therefore, the current investigation was conducted to assess the change in sleep architecture after long time of administration to determine whether RGE can be used in the remedy of insomnia.

## MATERIALS AND METHODS

### Ginseng and animals

Red ginseng water extract was kindly provided by Korea Ginseng Corporation (Seoul, Korea). Experiments

were performed on 14 adult male SD rats (Samtako, Osan, Korea) weighing between 250 and 350 g. The rats were housed individually with food and water provided *ad libitum* under an artificial 12 h light/dark cycle (lights on at 7:00 a.m.) and at a constant temperature ( $22\pm 2^\circ\text{C}$ ). The rats were housed in the departmental holding room for one week before testing. All of the rats were maintained in accordance with the National Institute of Toxicological Research and the Korea Food and Drug Administration guidelines for the care and use of laboratory animals.

### Surgery

After a minimum 7 d acclimation period, each rat was anesthetized with pentobarbital (50 mg/kg, ip) and implanted with a transmitter (TL10M3-F50-EEE; Data Sciences International, St. Paul, MN, USA) for recording EEG and activity via telemetry as described previously [11]. The body of the transmitter was subcutaneously implanted off the midline and posterior to the scapula. It was attached to the skin with three sutures for stabilization. The transmitters led subcutaneously to the skull, and the bare ends were placed in contact with the dura through holes in the skull (A: 2.0 [Bregma], L: 1.5; P: 7.0 [Bregma], L: 1.5 contra-lateral). The electrodes were anchored to the skull with screws and dental cement. All of the surgical procedures were performed stereotaxically under aseptic conditions.

### Red ginseng extract administration

After 7 d of recovery post-surgery, the animals were divided into two groups (control and 200 mg/kg RGE groups) with fourteen rats each. RGE was dissolved in distilled water and orally administrated at 11:00 a.m. once per day for 9 d at a dose of 200 mg/kg. At the end of RGE treatment, the animals were allowed to habituate to a polygraphic recording environment in which they could move freely. Then, polygraphic signs of sleep-wake activities were recorded for 6 h.

### Data collection

Telemetric recording of cortical EEG and activity was conducted using procedures similar to previous reports [11]. For the EEG signal, the gain of transmitters was set at  $-0.5/+0.5$  volts per/units  $\times 2$ , and raw signals generated from the transmitter were in the range of 0.5 to 0.0 Hz. The signals were processed by a Data Sciences analog converter and routed to an analog-to-digital (AD) converter (Eagle PC30, Data Sciences International). The AD converter digitized the EEG and activity signals at

128 Hz. The digitized data were transferred to a computer and graphically displayed by the program. An on-line fast Fourier transformation (FFT) was performed on EEG data. The FFT analysis generated power density values from 0.0 to 20.0 Hz at a resolution of 0.5 Hz. The FFT data were further averaged in the range of 0 to 20 Hz at 10-s intervals. The sleep data and FFT results were saved to the hard disk every 10 s for additional off-line analysis. Movements of the animal in relation to the telemetry receiver generated transistor-transistor logic pulses that were collected and counted as a measure of activity. Data were gathered on the 1st, 5th and 9th days, and oral administration of RGE was performed 24 h before EEG recording. Data were recorded from 11:00 a.m. to 5:00 p.m. EEG and activity were recorded simultaneously.

### Determination of sleep behaviors and analysis of electroencephalograms power

The amount of time spent in wakefulness, NREM sleep, and REM sleep was determined from digitized data in 10 s intervals using the animal sleep analysis software Sleep-Sign 2.1 (Kissei Comtec, Matsumoto, Japan). Briefly, the software identifies wakefulness as a high-frequency, low-amplitude EEG and NREM by the presence of spindles interspersed with slow waves. EEG power during REM shows significantly reduced lower frequency  $\delta$ -waves (0.75 to 4.0 Hz) and increased  $\theta$ -wave activity (5.0 to 9.0 Hz, peak at 7.5 Hz).

### Data analysis

The time spent (min) in NREM, REM, and total sleep (NREM + REM) and the number of sleep-wake cycles were processed to obtain 6 h periods for each rat. We further calculated the time spent in each sleep-wake state (wake, NREM, and REM) in each recording. The absolute EEG power was calculated during wakefulness, NREM, and REM. Data were calculated in 0.5 Hz bins from 0.5 to 20 Hz for the entire 6 h recording. Subsequently, EEG power density was evaluated in three selected frequency bands for wakefulness, NREM, and REM ( $\delta$ -wave,  $\theta$ -wave, and  $\alpha$ -wave [8.0 to 13.0 Hz]).

### Western blotting of GABA<sub>A</sub> receptors and glutamic acid decarboxylase

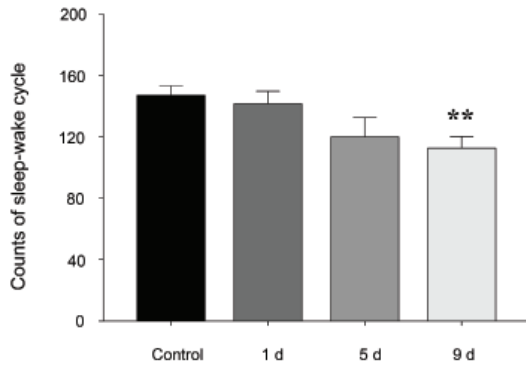
Under deep anesthesia (induced by diethyl ether), the animals were decapitated, and the brain was quickly removed and chilled in ice-cold saline. Coronal sections were made using a rodent brain matrix (ASI Instruments, Warren, MI, USA). The hypothalamus was dissected, and samples were immediately frozen on dry ice and stored

at  $-80^{\circ}\text{C}$ . Frozen tissue samples were homogenized in PRO-PREP protein-extraction solution (Intron Biotechnology Inc., Seongnam, Korea). The homogenate was centrifuged at 13,000 rpm at  $4^{\circ}\text{C}$  for 20 min, and the supernatant was recovered. The concentration of protein in the supernatant was determined, and the supernatant was then used for Western blot analysis. The concentration of total protein was determined by the modified Lowry method using bovine serum albumin as a standard. The samples were stored at  $-20^{\circ}\text{C}$ .

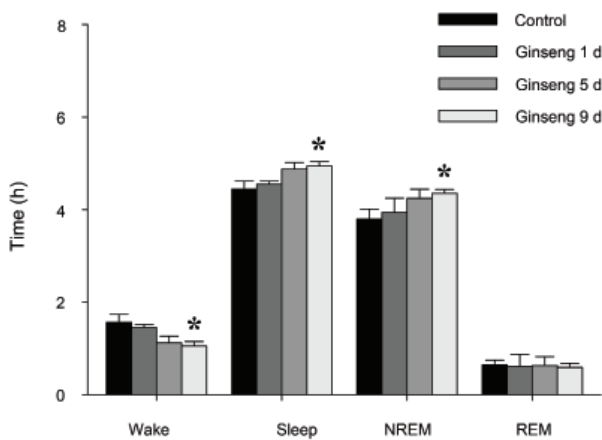
Forty micrograms of protein was added to each lane, and sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed using 10% polyacrylamide gels. Proteins were transferred to polyvinylidene fluoride membranes (Hybond-P; GE Healthcare, Amersham, UK) using a wet transfer system. Immunoblots were incubated with one of the following primary antibodies: rabbit anti-GABA<sub>A</sub>  $\alpha$ 1 polyclonal antibody (diluted 1:2,000 in TBS containing 0.5% Tween20; Abcamplc, Cambridge, UK), rabbit anti-GABA<sub>A</sub>  $\beta$ 1 polyclonal antibody (diluted 1:2,500 in TBS containing 0.5% Tween20), rabbit anti-GABA<sub>A</sub>  $\gamma$ 3 polyclonal antibody (diluted 1:2,500 in TBS containing 0.5% Tween20) and rabbit anti-glutamic acid decarboxylase (GAD) polyclonal antibody (diluted 1:2,000 in TBS containing 0.5% Tween20). Blots were then washed and incubated with the horseradish peroxidase-conjugated secondary antibody: goat anti-rabbit IgG (diluted 1:3,000 in TBS containing 0.5% Tween20). Immunoreactive bands were developed with a BM chemiluminescence detection kit (Roche Diagnostics, Mannheim, Germany). The quantitative analysis of detected bands was performed with densitometric scanning, and all of the values were normalized to the amount of glyceraldehyde 3-phosphate dehydrogenase (GADPH) in the sample, which was measured as follows. All of the immunoblots were stripped, incubated with sheep anti-GADPH (diluted 1:2,000 in TBS containing 0.5% Tween20, Abcamplc), subsequently incubated with rabbit anti-sheep IgG (diluted 1:2,000 in TBS containing 0.5% Tween20), and developed to confirm equal protein loading.

### Statistical analysis

All statistical analyses were conducted using Sigma-Stat software (SPSS Inc., Chicago, IL, USA). Measures of analysis of variance (ANOVA) were used in the data analysis. After extensive ANOVA testing, *post hoc* comparisons of means were performed using Tukey's *t*-tests. A *p*-value of less than 0.05 was considered to be significant. The values are expressed as the mean $\pm$ SEM.



**Fig. 1.** The effects of 1, 5, and 9 d of red ginseng extract treatment on sleep-wake cycles. Values are expressed as the mean±SEM. \*\* $p<0.01$  compared with the control.



**Fig. 2.** The effects of 1, 5 and 9 d of red ginseng extract treatment on rat sleep architecture. The data for 200 mg/kg doses are shown. Values are expressed as the mean±SEM. NREM, non-rapid eye movement; REM, rapid eye movement. \* $p<0.05$  compared with the control.

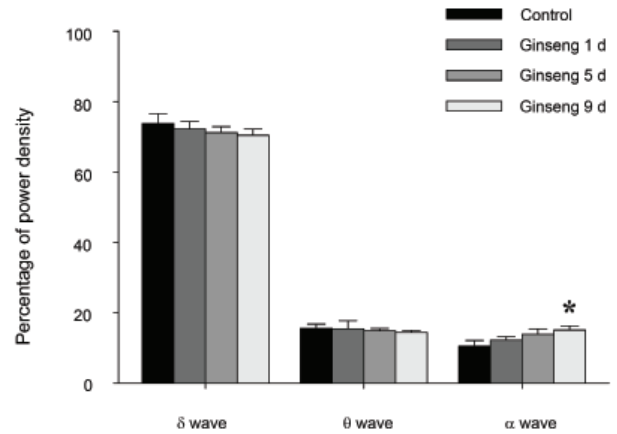
## RESULTS

### Effects of red ginseng extract on the number of sleep-wake cycles

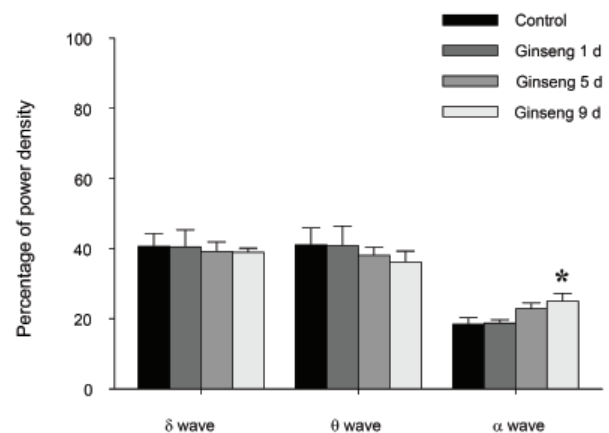
As shown in Fig. 1, on the 1st and 5th day of RGE treatment, no significant changes in sleep-wake cycles were exhibited during a 6 h recording. However, after administering RGE for 9 d, 200 mg/kg doses reduced sleep-wake cycles ( $p<0.01$ ).

### Effects of red ginseng extract on sleep architecture

Although no significant changes in sleep architecture were shown on the 1st and 5th day of RGE treatment during a 6 h recording, treatment with RGE for 9 d significantly increased NREM and total sleep but decreased wakefulness ( $p<0.05$ ) (Fig. 2).



**Fig. 3.** The effects of 1, 5, and 9 d of red ginseng extract treatment on electroencephalograms (EEG) power density during non-rapid eye movement (NREM) sleep. EEG power densities in  $\delta$ -wave,  $\theta$ -wave and  $\alpha$ -wave spectral bandwidths were evaluated. The values are expressed as the mean±SEM of EEG power densities in three selected frequency bands for NREM sleep. \* $p<0.05$  compared with the control.



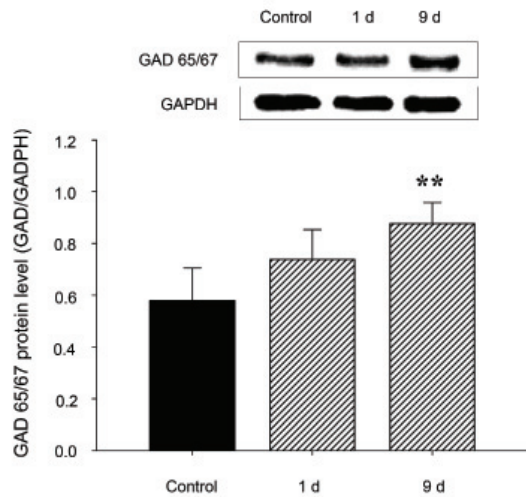
**Fig. 4.** The effects of 1, 5, and 9 d of red ginseng extract treatment on electroencephalograms (EEG) power density during rapid eye movement (REM) sleep. EEG power densities in  $\delta$ -wave,  $\theta$ -wave and  $\alpha$ -wave spectral bandwidths were evaluated. The values are expressed as the mean±SEM of EEG power densities in three selected frequency bands for the REM sleep. \* $p<0.05$  compared with the control.

### Effects of 1 or 5 d of RGE administration on EEG power density during NREM sleep, REM sleep, and total sleep time

On the 1st and 5th day of treatment with RGE, there were no changes in  $\delta$ -wave,  $\theta$ -wave, or  $\alpha$ -wave power density during NREM and REM sleep (Fig. 3).

### Effects of 9 d of RGE treatment on EEG power density during NREM sleep, REM sleep, and total sleep time

RGE treatment (200 mg/kg) for 9 d showed no significant changes in  $\delta$ -wave or  $\theta$ -wave power density during



**Fig. 5.** The expression of glutamic acid decarboxylase (GAD) protein in the rat hypothalamus after 1, 5, and 9 d of red ginseng extract (RGE) treatment. Hypothalamus membrane extracts from rats treated as controls or with RGE were analyzed by Western blotting. A representative image is shown in each case. The intensity of the immunoreactive bands of 3 to 4 independent experiments was measured by densitometry scanning and normalized using glyceraldehyde 3-phosphate dehydrogenase (GADPH) as a standard (bar graph). The results are presented as the percentage immunoreactivity detected in the hypothalamus with respect to the GADPH protein-loading control. Values are expressed as the mean±SEM. \*\* $p < 0.01$  compared with the control.

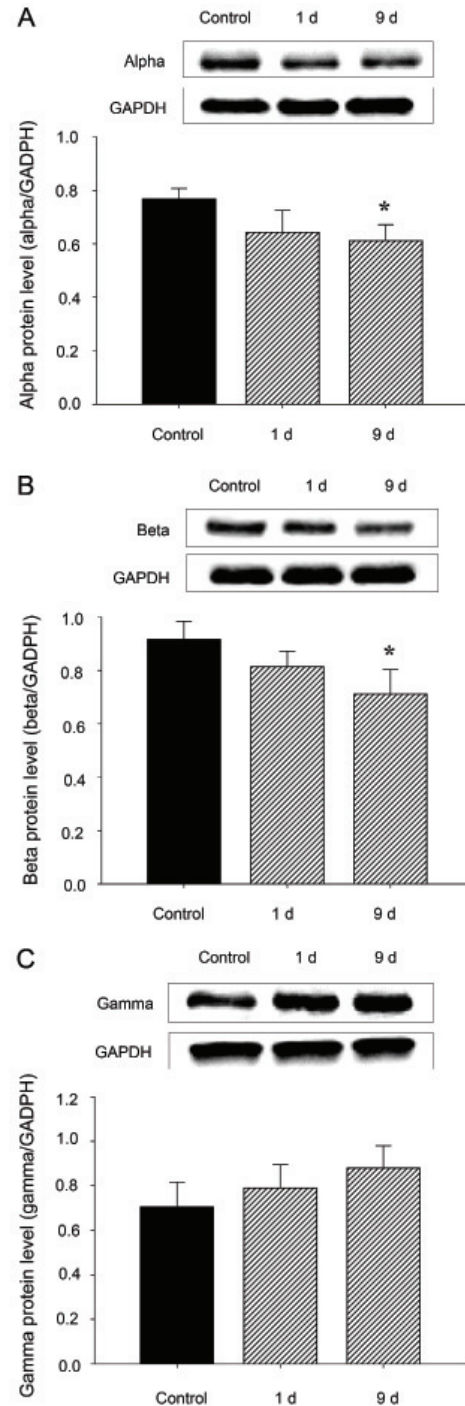
NREM and REM sleep, whereas  $\alpha$ -wave power density was increased during NREM sleep and REM sleep ( $p < 0.05$ ) (Fig. 4).

### Effects of RGE administration on GABA<sub>A</sub> receptors and GAD protein expression in the hypothalamus

We examined the protein levels of GAD and the GABA<sub>A</sub> receptor  $\alpha$ -,  $\beta$ - and  $\gamma$ -subunits in rats. As indicated in Figs. 5 and 6, 9 d of treatment with 200 mg/kg RGE reduced the expression of GABA<sub>A</sub>  $\alpha$ - and  $\beta$ -subunits (Fig. 6A, B) but not  $\gamma$ -subunits (Fig. 6C) in the rat hypothalamus compared with controls. RGE treatment with 200 mg/kg for 9 d significantly augmented the protein levels of GAD 65/67 (Fig. 5).

## DISCUSSION

Our inquiry implied that RGE increased total and NREM sleep in rodent and declined wakefulness. In the current study, we indicated that after 5 or 9 d of continuous RGE treatment, only 9 d of treatment preeminently augmented NREM and total sleep and diminished wakefulness. These data indicate that RGE modulates sleep architecture, notably NREM sleep. Analogously, RGE regulated sleep-wake cycles during long periods of ad-



**Fig. 6.** The expression of  $\gamma$ -aminobutyric acid (GABA)<sub>A</sub> receptor alpha (A), beta (B), and gamma (C) subunits in the rat hypothalamus after 1, 5, and 9 d of red ginseng extract (RGE) treatment. GABA<sub>A</sub> receptor subunits in the hypothalamus of RGE-treated rats after 1, 5, and 9 d of treatment were analyzed by Western blotting. The intensity of the immunoreactive bands of 3 to 4 independent experiments was measured by densitometry scanning and normalized using glyceraldehyde 3-phosphate dehydrogenase (GADPH) as a standard (bar graph). The results are presented as the percentage immunoreactivity detected in the hypothalamus with respect to the GADPH protein-loading control. Values are expressed as the mean±SEM. \* $p < 0.05$  compared with the control.

ministration. Furthermore, on the 1st and 5th days, the oral administration of RGE failed to alter the number of sleep-wake cycles for a 12 h recording period. On the other hand, RGE treatment for 9 d reduced the sleep-wake cycle. The above findings are somewhat coincide with a past report [12], where the amount of wakefulness and slow-wave sleep (SWS) for a 12 h light period was reduced and enhanced, respectively, by RGE administration, while REM sleep was scarcely influenced.

In addition, even though RGE administration had alike effects on total sleep time and sleep efficacy in our investigations, it had dissimilar effects on sleep architecture. The dissimilarity among these inquiries may be due to differences in administration plans. In addition, a previous report by Kitaoka et al. [13] indicated that dissimilar species of ginseng normally have different ginsenoside constituent. Supplementarily the ginsenoside constituent of ginseng can vary according to the species, the age and section of the plant, the conservation method, the period of harvest, and the extraction process [14,15]. The total amount and the frequency of sleep rely on the activity figures of cortical EEG waves [12]. Standardized ginseng extract G115 has been indicated to create a desynchronizing effect on an EEG [16]. Using a double-blind, placebo-controlled, balanced crossover test in 15 lusty volunteers, Kennedy et al. [17] described that ginseng resulted in considerable declines in frontal 'eyes closed' theta and beta activity, with an extra decline in the alpha wave bandwidth, which illustrated that *Panax ginseng* can immediately regulate cerebroelectrical activity. Several innocent saponins segregated from *P. ginseng* Meyer likewise had regular delirifacients on EEG and the action and EEG stimulus reaction induced by electrical irritation in the midbrain in cats [18]. Few papers have examined the role of RGE in EEG power density in mammals. Hence, we analyzed the influence of RGE on brain waves in rats. Even though 1 and 5 d of RGE treatment did not affect any wave power density, 9 d of RGE treatment enhanced alpha activity during NREM sleep and REM sleep. The amount of EEG analysis allowed the extraction of significant functional parameters, like as slow-wave activity (or delta activity) for NREM sleep, which includes constituents of the EEG signal in the frequency range of 0.5 to 4.5 Hz [19]. REM sleep and waking are dominated in animals (i.e., rodents) by a noticeable activity in the theta (6 to 9 Hz) frequency range, although the functional importance of slow-wave and REM sleep modulation is not well-known. Some studies in humans indicated that the EEG activity of the  $\alpha$ -wave activity (frequency range of 8 to 13 Hz) might be a indicator of

REM sleep homeostasis [20]. Nevertheless, it is indicated that  $\alpha$ -wave activity may not be related to the REM sleep homeostasis of rats [21].

GABA<sub>A</sub> receptors function as sleep modulation. Some compounds interfering with the sleep-wake cycle, containing barbiturates and benzodiazepines, are agonists acting at various combining sites on the GABA<sub>A</sub> receptors [22]. The mechanism proposed suggests that GABAergic neurons from the basal forebrain and preoptic area project to the posterior lateral hypothalamus [23]. Moreover, evidence gained using microdialysis detection ways indicates that extracellular levels of GABA are increased for sleep [24]. Pharmacological tests have proved that enhancing GABA<sub>A</sub> receptor activity improves SWS [25].

Previously, Sugiyama et al. [26] proved that ginseng radix activated intracellular chloride channel mediated by the GABA<sub>A</sub> receptor. More inquiry has verified the opinion that some of the behavioral efficiencies of ginseng at least take place through the variation of the GABA<sub>A</sub> receptor. For instance, social isolation commonly lead to a stress-evoked reduction in the duration of sleep induced by pentobarbital. The capability of majonoside-R2 (a principal ocotillol-type saponin content of Vietnamese ginseng) to reverse this effect is mediated by the neurosteroid sites on the GABA<sub>A</sub> receptors in mice [27]. Besides, Kimura et al. [28] reported that ginsenosides interact through ligand combining to the GABA<sub>A</sub> and GABA<sub>B</sub> receptors. However, Lee et al. [29] indicated that not all behavioral actions of ginseng saponins are caused by the modulation of GABA<sub>A</sub> receptor activation. To indagate whether the efficacy of RGE administration on sleep behavior related the GABA<sub>A</sub>ergic system, we indagated the protein levels of GAD 65/67 and GABA<sub>A</sub> receptor  $\alpha$ -,  $\beta$ -, and  $\gamma$ -subunits in the rat hypothalamus. Our observations indicated that 9 days of repeated RGE treatment decreased the expression level of GABA<sub>A</sub>  $\alpha$ - and  $\beta$ - but not  $\gamma$ -subunits in the rat hypothalamus; Although, in the 1 d administration groups, the expression of GABA<sub>A</sub>  $\alpha$ -,  $\beta$ - and  $\gamma$ -subunits and GAD 65/67 was not affected in rats treated with 200 mg/kg RGE. These results presumed that the modulation of sleep behavior by a long period of RGE treatment involves a decrease in the expression level of GABA<sub>A</sub> receptor  $\alpha$ - and  $\beta$ - but not  $\gamma$ -subunits. Increased GABA production may be significant for a long time RGE treatment. Commonly, our data demonstrate that RGE modulates sleep via the GABA<sub>A</sub>ergic systems [13,26].

In 1979, ginseng abuse syndrome (GAS) was coined as the result of an inquiry of people who had been using a variety of ginseng preparations for at least 1 mo. It was

reported that administration of ginseng for more than 1 mo induced GAS. Some patients underwent GAS, defined as hypertension, erethism, somniphathy, pimple and diarrhea. However, one subject underwent hypotension, feebleness and quiver when ginseng use was suddenly stopped. Ginseng was used to treat “hot syndromes”, such as stress, insomnia, palpitations, and headache. Recently, it was reported that ginseng induces sleep in mammals [4]. Ginseng would also be a good candidate for the treatment of insomnia.

In summary, RGE enhances total sleep and NREM sleep and reduces the number of sleep-wake cycles. The  $\alpha$ -wave activities in cortical EEG were increased in sleep architecture by RGE. The current inquiry illustrated that RGE regulated sleep-wake cycles and sleep architecture in a time-dependent manner. In addition, the regulation of sleep behavior by RGE administration for 9 d may relate a decline in the expression level of GABA<sub>A</sub> receptors composed mainly of  $\alpha$ - and  $\beta$ - but not  $\gamma$ -subunits. As a result, longer RGE administration may modulate sleep behavior by increasing GABA production.

Therefore, RGE administration widely modulated the power spectral densities of EEGs, even though no dose-dependent tendencies were determined. Furthermore, our data allude that the regulation of these sleep behaviors by RGE relates the GABA<sub>A</sub>ergic system. These results are assumed that the mechanism through which RGE modulates EEG and sleep behavior may be far more muddled than previous perspectives have implied. However, whether RGE influences sleep behavior by modulating EEG power densities remains to be further analyzed.

## ACKNOWLEDGEMENTS

This work was supported by the grant from the Korean Society of Ginseng funded by Korean Ginseng Corporation (2010).

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