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Alterations of Spontaneous Sleep Architecture and Cortical Electroencephalogram Power Spectra by Red Ginseng Extract via $GABA_A$ergic Systems

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This study was undertaken to discover the effects and possible mechanisms of the effect of red ginseng extract (RGE) on spontaneous sleep. The effects of a low dose (10 mg/kg) and a high dose (200 mg/kg) of RGE were compared in rats. After recovery from a surgical operation enabling electroencephalograms recordings, rats were administered RGE orally. RGE was administered orally for 1 day or once per day for 5 days in either 10 or 200 mg/kg doses. Polygraphic signs were recorded for 12 h after oral administration of RGE. Both treatment with a large dose (200 mg/kg) of RGE for one day and treatment with either a large or a small dose for 5 days reduced the number of sleep–wake cycles. Daily treatment with RGE (either 10 or 200 mg/kg) for 5 days augmented NREM and total sleep, but reduced wakefulness. Delta wave activity recorded during non-REM (NREM) sleep and REM sleep was increased after one treatment with RGE (either 10 or 200 mg/kg). Delta wave activity during NREM was enhanced after daily treatment with RGE (either 10 or 200 mg/kg) for 5 days. Both alpha and beta subunits of the γ-aminobutyric acid $(GABA)_A$ receptor were significantly over-expressed in the hypothalamus of the RGE-treated groups. Moreover, the expression of glutamic acid decarboxylase was also increased in the hypothalamus. These results demonstrate that RGE may regulate spontaneous sleep via $GABA_A$ergic systems.

Keywords: Red ginseng extract, Sleep architecture, Electroencephalography, $GABA_A$ receptors subunits, Hypothalamus

INTRODUCTION

Sleep is critical in the recovery from tiredness and thereby has potent restorative value. Poor-quality sleep, typical of insomnia, has many adverse effects on daily life. Insomnia is defined by complaints of disturbed sleep in the presence of adequate opportunity for sleep [1,2]. It has been proposed that insomnia is a hyperarousal disorder that could reflect a deficit in sleep homeostasis [3]. Some evidence indicates that γ-aminobutyric acid (GABA) plays a major role in sleep regulation [4,5]. $GABA_A$, but not $GABA_B$, receptors are important for desynchronized sleep modulation [5].

Panax ginseng root powder has been used extensively in the Far East for a wide variety of clinical ailments and to improve general physical and mental well being for several millennia [6]. Animal studies have shown that ginseng and its constituent ginsenosides can modulate indices of stress, fatigue, and learning [7-9]. In humans, ginseng improves performance on a memory task [10],

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suppresses granulocytopoiesis [11], and regulates the erythropoiesis after paradoxical sleep deprivation [12]. Moreover, ginseng is a useful therapeutic adjunct in the management of non-insulin-dependent diabetes mellitus. It can elevate mood, improve psychophysical performance, reduce fasting blood glucose and body weight, and improve glycated hemoglobin and physical activity [13].

Many years of research has revealed that a variety of ginseng treatments, including the use of its constituent components and the combination of multiple strains of ginseng, are able to modulate sleep–wake disturbance in human and animals. Ginseng extract may exert a stabilizing effect on sleep–wake cycles, which possibly accounts for its outstanding health-improving activities [14]. *Panax quinquefolium* one strain of ginseng, can improve cognitive deficiency induced by forced sleep deprivation. When given orally for 7 days, this strain of ginseng increases spontaneous brain activity, antagonizes the effects of valium, and lengthens sodium pentobarbital-induced sleep in stressed animals [15].

Previous research has suggested that ginseng may modulate the $GABA_A$ergic system. Kimura *et al*. [16] reported that ginsenosides interact with ligand binding of the $GABA_A$ and $GABA_B$ receptors. Yobimoto *et al*. [17] have reported that the Vietnamese ginseng saponin and its major component MR2 have preventive effects on psychological stress-induced brain cell membrane damage and that the effect of MR2 is partly due to the enhancement of the $GABA_A$ergic systems in the brain. Ginsenosides, the major active ingredients of *Panax ginseng*, have been shown to modulate $GABA_A$ receptors expressed in *Xenopus* oocytes, suggesting that this regulation might be one of the pharmacological actions of *Panax ginseng* [18]. Despite these observations, the mechanism of action and resulting effect of ginseng on sleep regulation are not yet fully understood. Korean ginseng contains three times more ginsenosides than any other ginseng, making it the most effective of all ginsengs [19]. Therefore, the ethanol extract of Korea red ginseng is an unparalleled natural source of nutrients for a healthy body and mind. However, sleep disorders are the most commonly experienced adverse effects of ginseng [20].

Several studies have demonstrated that the discharge rate of the neurons of the basal forebrain and preoptic area, in which GABA is the main inhibitory neurotransmitter, is higher during sleep than during wakefulness [21]. Additionally, two important nuclei related to sleep–wake regulation are located in the hypothalamus [22]. Therefore, to further evaluate the action of red ginseng extract (RGE) on the regulation of sleep–wake fluctuations and sleep architecture in freely moving rats administered RGE, we measured the amount of total sleep and wakefulness, investigated power density changes in recorded electroencephalograms (EEG) of specific sleep–wake stages, and then detected alterations in $GABA_A$ergic receptors of the hypothalamus. Our data suggest that RGE modulates sleep architecture and sleep–wake cycles in a dose-dependent manner during acute and chronic treatment, and these effects may involve the $GABA_A$ergic systems of the hypothalamus.

MATERIALS AND METHODS

Animals and red ginseng extract

Sprague Dawley male rats (Samtako, Osan, Korea) weighing between 250 and 350 g were used. Food and water were available *ad libitum* under an artificial 12 h light/dark cycle (light on at a.m. 7:00) and at a constant temperature (22±2°C). All rats were maintained and all experiments were conducted according to the guide for the Care and Use of Laboratory Animals (National Academic Press, Washington DC, 1996). RGE was kindly supplied by the Korea Ginseng Corporation (Daejeon, Korea). The RGE yielded 4.37% saponin; the other main components were ginsenoside-Rb_1 (12.59%), -Rb_2 (6.18%), -Rc (6.86%), -Rd (3.43%), -Re (6.64%), -Rf (2.06%), -Rg_1 (15.79%), and -Rg_3 (1.37%).

Surgery

After a minimum 7-day 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 [23]. The body of the transmitter was implanted subcutaneously off the midline and posterior to the scapula. It was attached to the skin with several sutures for stabilization. Leads from the transmitter were passed 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 dental cement. All surgical procedures were performed stereotaxically under aseptic conditions.

Red ginseng extract administration

Following a 7-days post-surgical recovery, rats were randomly divided into one of the following groups. RGE was dissolved in distilled water and orally admin-

istrated at 7:00 a.m. once per day for either 1 or 5 days at a dose of either 10 or 200 mg/kg. After the end of the RGE treatment, 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 12 h. In similar, parallel experiments, animals were sacrificed for Western blot experiments.

Data collection

Telemetric recordings of cortical activity were conducted using procedures similar to those reported previously [23]. For the EEG signal, the gain of the transmitters was set at -0.5/+0.5 volts per/units ×2, and raw signals generated from the transmitters were in the range of 0.5 to 20.0 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 the computer and displayed graphically. An online fast Fourier transformation (FFT) was performed on EEG data. The FFT analysis generated power density values from 0.0 Hz to 20.0 Hz at a resolution of 0.5 Hz. The FFT data were further averaged in the time domain every 10 sec. The sleep data and FFT results were saved to a hard disk every 10 sec for additional off-line analyses. Movement of the animal in relation to the telemetry receiver generated transistor–transistor-logic pulses that were collected and counted as a measure of activity.

Analysis of sleep

The amount of time in wakefulness, non rapid eye movement (NREM), and rapid eye movement (REM) sleep were determined from the digitized data in 10-sec epochs using sleep analysis software, SleepSign 2.1 (Kissei Comtec Co., Matsumoto, Japan). Briefly, the software discriminates wakefulness as high-frequency low-amplitude activity in the EEG; NREM was scored based on the presence of spindles interspersed with slow waves in the EEG. EEG power during REM is significantly reduced in lower frequency δ-wave (0.75–4.0 Hz) and enhanced in the range of θ-wave activity (5.0–9.0 Hz, peak at 7.5 Hz). The time spent (min) in NREM, REM, and total sleep time (NREM+REM) and the number of sleep–wake cycles were processed to obtain 12 h-period totals for each rat. We further calculated the time spent in each sleep–wake state (wakefulness, NREM, and REM).

Western blot of $GABA_A$ receptors and glutamic acid decarboxylase

After deep anesthesia (induced by diethyl ether), 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°C. Frozen tissue samples were homogenized in PRO-PREP protein-extraction solution (Intron Biotechnology Inc., Seongnam, Korea). The homogenate was centrifuged at 15,000x *g* at 4°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°C.

Forty micrograms of protein was added to each lane, and sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed using 12% polyacrylamide gels. Proteins were transferred to PVDF membranes (Hybond-P, GE Healthcare, Amersham, UK) using a semidry transfer system. Immunoblots were incubated with one of the following primary antibodies: rabbit anti-$GABA_A$ α1 polyclonal antibody (diluted 1:1,000 in PBS containing 0.5% Tween20; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-$GABA_A$ β1 polyclonal antibody (diluted 1:1,000 in PBS containing 0.5% Tween20), goat anti-$GABA_A$ γ3 polyclonal antibody (diluted 1:1,000 in PBS containing 0.5% Tween20) and anti-glutamic acid decarboxylase (GAD) polyclonal antibody (diluted 1:1,000 in PBS containing 0.5% Tween20). Blots were then washed and incubated with one of the following horseradish peroxidase secondary antibodies: goat anti-rabbit IgG (diluted 1:5,000), donkey anti-goat IgG (1:3,000). Immunoreactive bands were developed with a BM chemiluminescence detection kit (Roche Diagnostics, Mannheim, Germany). Quantitative analysis of detected bands was performed with densitometric scanning, and all values were normalized to the amount of glyceraldehyde 3-phosphate dehydrogenase (GADPH) in the sample, measured as follows. All immunoblots were stripped, incubated with rabbit anti-GADPH (1:1,000, Santa Cruz Biotechnology), followed by goat anti-rabbit IgG, and then developed to confirm equal protein loading.

Statistical analysis

All data were analyzed using SPSS ver. 17.0 (SPSS

Inc., Chicago, IL, USA). Group differences were assessed using a one-way ANOVA. Post hoc analyses 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 mean±SEM.

RESULTS

Effects of red ginseng extract on the number of sleep–wake cycles

As shown in Fig. 1, after one RGE treatment, no significant changes in sleep–wake cycles were exhibited during a 12 h recording ($p<0.05$). However, after administering RGE for 5 days, both 10 and 200 mg/kg doses reduced sleep–wake cycles ($p<0.05$ and $p<0.01$, respectively).

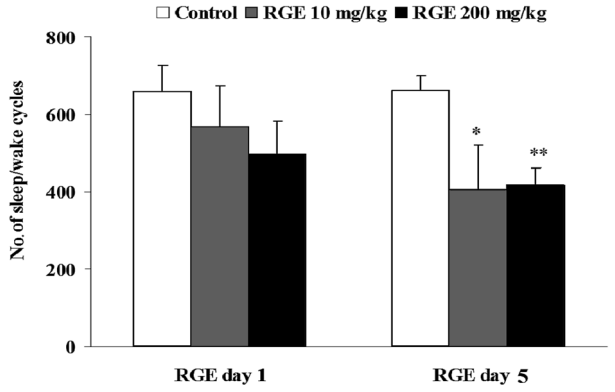

Fig. 1. The effects of acute (1-day) and chronic (5-days) red ginseng extract (RGE) treatments on sleep–wake cycles (10 or 200 mg/kg doses). Values are expressed as mean±SEM. *$p<0.05$, **$p<0.01$ compared with the control.

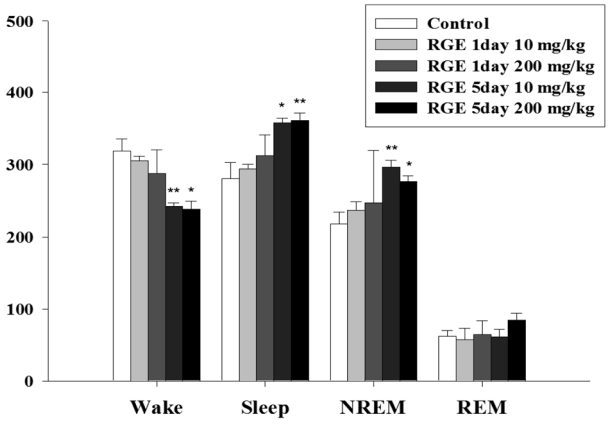

Fig. 2. The effects of acute (1-day) and chronic (5-days) red ginseng extract (RGE) treatments on rat sleep architecture. The data for 10 and 200 mg/kg doses are shown, respectively. Values are expressed as mean±SEM of time spent in the sleep–wake state (awake, total sleep, non rapid eye movement [NREM] sleep, and rapid eye movement [REM] sleep). (mean±SEM). *$p<0.05$, **$p<0.01$.

Effects of red ginseng extract on sleep architecture

Both small (10 mg/kg) and large (200mg/kg) doses treatment with RGE for 5 days significantly increased NREM and total sleep, but decreased wakefulness.

Effects of RGE acute administration on EEG power density during NREM sleep, REM sleep, and total sleep time

After one treatment with RGE, both 10 and 200 mg/kg doses enhanced δ-wave power during NREM sleep (Fig. 3A), but reduced θ-wave power during NREM sleep (Fig. 3A,B) and increased α-wave power during REM sleep stages (Fig. 3B). Moreover, both 10 and 200 mg/kg one-time doses of RGE lowered θ-wave power during REM sleep (Fig. 3B).

Effects of 5-day RGE treatments on EEG power density during NREM sleep, REM sleep, and total sleep time

RGE treatment with either 10 or 200 mg/kg doses for 5 days showed no significant changes in power during NREM sleep (Fig. 4A) and total sleep (Fig. 4C), but reduced θ-wave power during REM sleep (Fig. 4B) but increased α-wave power REM sleep stages (Fig. 4B). Low doses of RGE (10 mg/kg) enhanced δ-wave power during NREM and total sleep time (Fig. 4A,C) .

Effects of RGE administration on $GABA_A$ receptors and GAD protein expression in the hypothalamus

It has been reported that the ability of fermented ginseng (FG) to reduce the first-night effect may be related to an anxiolytic effect of FG that occurs via $GABA_A$ergic modification [24]. Here we examined the protein levels of GAD and the $GABA_A$ receptor α, β, and γ subunits in rats. As indicated in Fig. 5, an one-time treatment with 10 or 200 mg/kg RGE enhanced the expression of $GABA_A$ α and β subunits (Fig. 5A, B), but not that of γ subunits (Fig. 5C), in rat hypothalamus when compared with controls. Five days of RGE 200 mg/kg doses significantly augmented the protein levels of GAD 65/67 (Fig. 6).

DISCUSSION

Panax ginseng C. A. Meyer is a perennial herb native to Korea and China and has been used as an herbal remedy in eastern Asia for thousands of years. Traditionally, it has been used to restore and enhance normal well-being; Modern therapeutic claims refer to vitality, immune function, cancer, cardiovascular diseases, and

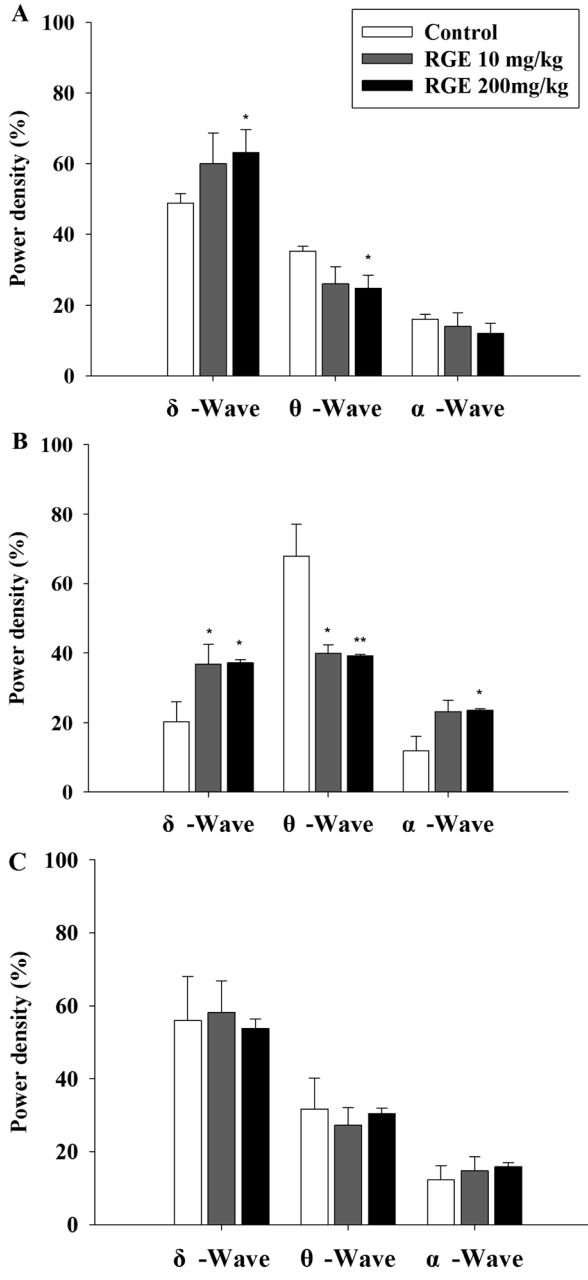

Fig. 3. The effects of acute (1-day) red ginseng extract (RGE) treatment on electroencephalograms (EEG) power density during non rapid eye movement (NREM) sleep (A), rapid eye movement (REM) sleep (B), and total sleep (C). EEG power densities in δ-wave, θ-wave, and α-wave spectral bandwidths were evaluated. The values are expressed as mean±SEM of EEG power densities in three selected frequency bands for the NREM sleep, REM sleep, and total sleep states. (mean±SEM). *p<0.05, **p<0.01.

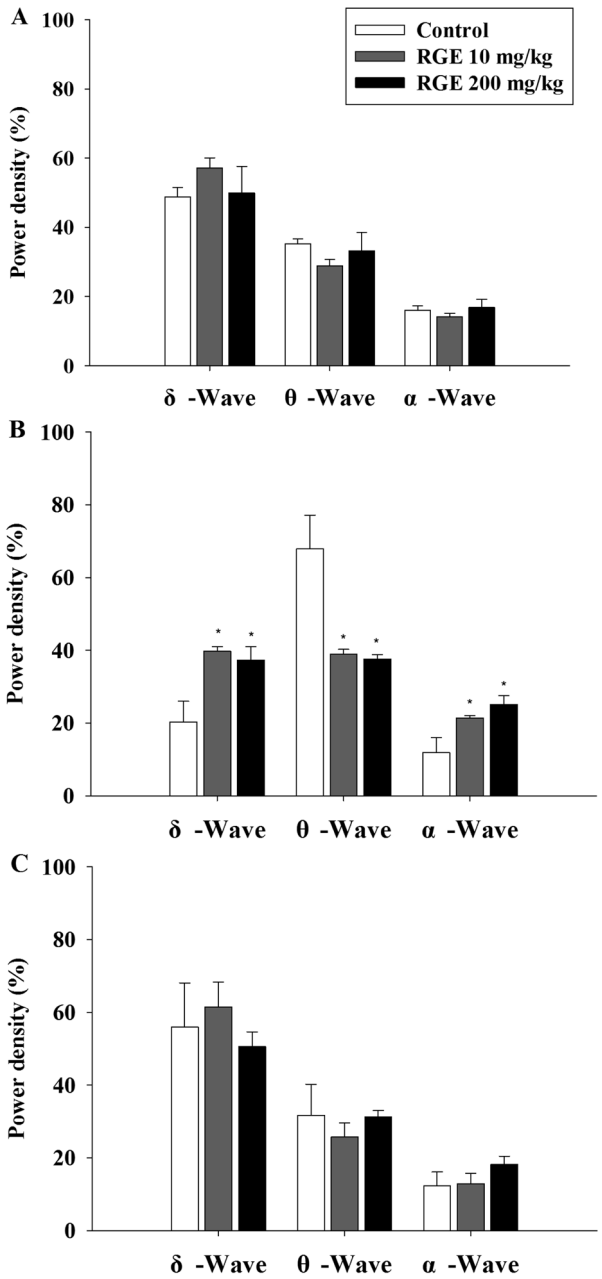

Fig. 4. The effects of chronic (5-day) red ginseng extract (RGE) treatment on electroencephalograms (EEG) power density during non rapid eye movement (NREM) sleep (A), rapid eye movement (REM) sleep (B), and total sleep (C). EEG power densities in δ-wave, θ-wave, and α-wave spectral bandwidths were evaluated. The values are expressed as mean±SEM of EEG power densities in three selected frequency bands for the NREM sleep, REM sleep, and wakefulness states. (mean±SEM). *p<0.05.

improvement of cognitive and physical performance and sexual function [25]. Growing evidence has shown that ginseng is able to regulate sleep behavior. For example, ginseng has been clinically used for the treatment of insomnia [26]. Siberian ginseng can decrease sleep latency and increase sleep duration following both acute administration and chronic administration [27]. In the present study, we have shown after 5 days of repeated RGE treatments, we found that both high and low doses markedly enhanced NREM and total sleep, but reduced wakefulness. These data indicate that RGE regulates sleep architecture, especially NREM sleep. Similarly,

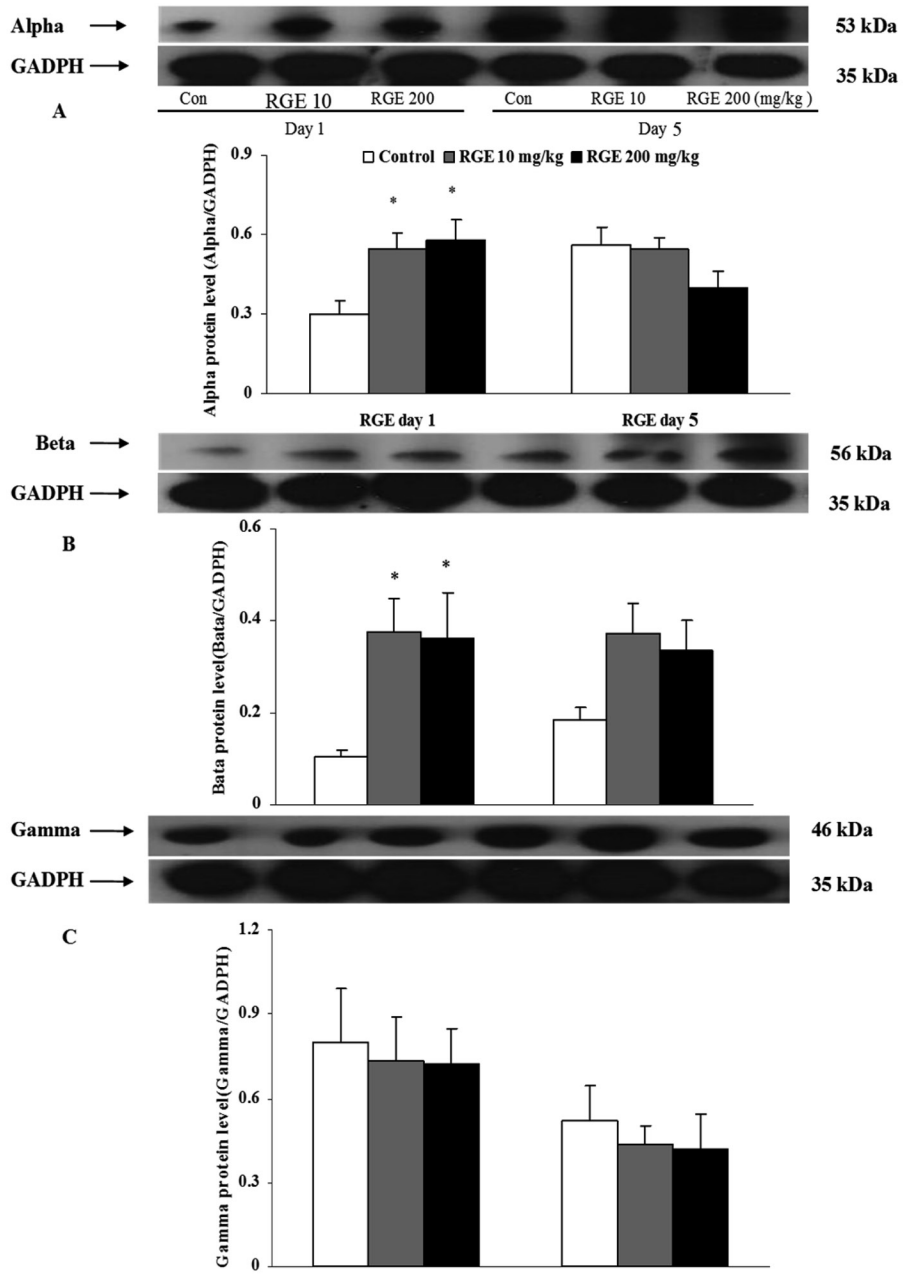

Fig. 5. The expression of γ-aminobutyric acid $(GABA)_A$ receptor alpha (A), beta (B), and gamma (C) subunits in rat hypothalamus after acute (1-day) and chronic (5-day) red ginseng extract (RGE) treatments. $GABA_A$ receptor subunits in the hypothalamus of RGE-treated rats after 1 day or 5 days of treatment were analyzed by Western blotting. The intensity of the immunoreactive bands of 3–4 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 % immunoreactivity detected in the hypothalamus with respect to the GADPH protein-loading control (mean±SEM). *p<0.05, **p<0.01.

RGE modulated sleep–wake cycles in dose-dependent manner. In addition, acute low and high oral doses of RGE failed to change the number of sleep–wake cycles during a 12 h recording period. However, RGE treatment of low or high doses for 5 days attenuated sleep-wake cycle. The above results are partly consistent with a previous report [28], in which the amount of wakefulness and slow-wave sleep (SWS) during a 12 h light period was decreased and increased, respectively, by ginseng treatment, whereas paradoxical sleep was affected little. Furthermore, although RGE treatment had similar effects on total sleep time and sleep efficiency in our experiments, it also had different affects on sleep architecture. The difference between these studies may be due

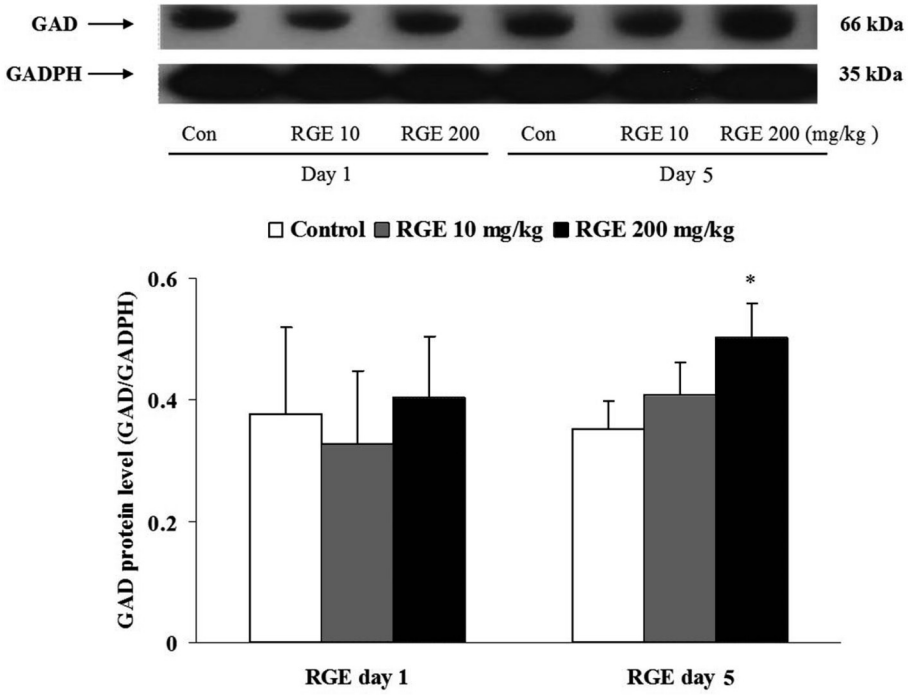

Fig. 6. The expression of glutamic acid decarboxylase (GAD) protein in rat hypothalamus after acute (1-day) and chronic (5-day) red ginseng extract (RGE) treatments. Hypothalamus membrane extracts from rats treated as controls or with RGE were analyzed by Western blotting. A representative image is given in each case. The intensity of the immunoreactive bands of 3–4 independent experiments was measured by densitometry scanning and normalized by using glyceraldehyde 3-phosphate dehydrogenase (GADPH) as a standard (bar graph). The results are presented as % immunoreactivity detected in the hypothalamus with respect to the GADPH protein-loading control (mean±SEM). *p<0.05, **p<0.01.

to differences in RGE dose sizes, treatment schedules, and ginsenoside content. A recent report by Kitaoka et al. [24] showed that different species of ginseng usually have different ginsenoside content. Also, the ginsenoside content of ginseng can vary depending on the species, the age and part of the plant, the preservation method, the season of harvest, and the extraction method [7,29].

The total amount and frequency of sleep depend on the activity patterns of cortical EEG waves [28]. Standardized ginseng extract G115 has been shown to produce a desynchronizing effect on the electrocorticogram [30]. Using a double-blind, placebo-controlled, balanced crossover experiment in 15 healthy volunteers, Kennedy *et al*. [31] reported that ginseng led to significant reductions in frontal 'eyes closed' theta and beta activity, with an additional reduction by ginseng in the alpha wave bandwidth, which demonstrated that *Panax ginseng* can directly modulate cerebroelectrical activity. Several pure saponins isolated from *Panax ginseng* C. A. Meyer also had moderate depressant actions on the EEG as well as on the behavior and EEG arousal response induced by electrical stimulation in the midbrain in cats [32]. Up to now, few reports have examined the role of RGE in EEG power density in humans or animals. Therefore, we further analyzed the effects of RGE on brain waves in rats. Although acute dose of RGE did not alter sleep architecture or the sleep–wake cycle. Though both low and high acute doses of RGE enhanced delta activity during NREM sleep, but reduced theta activity during REM sleep. Following 5 days of RGE treatments, both low and high doses of RGE enhanced delta wave power and reduced theta wave power during REM sleep. Quantitative EEG analysis allowed the extraction of important functional parameters such as slow-wave activity (or delta activity) during NREM sleep that encompasses components of the EEG signal in the frequency range 0.5 Hz to 4.5 Hz [1]. REM sleep and waking are characterized in animals (i.e., rodents) by a pronounced activity in the theta (6 to 9 Hz) frequency range, but the functional significances of slow wave and REM sleep regulation are still unknown. Our experiments showed that RGE altered EEG power density in each of these spectral ranges, although no dose-dependent trends existed between low and high dose(s) of RGE. These findings are interesting and imply that how RGE regulates the EEG and sleep behavior may be far more complicated than previous viewpoints have suggested. However, whether and/or how RGE affects sleep behavior by regulating

EEG power densities remains to be further explored.

It is well known that $GABA_A$ receptors play a major role in sleep regulation. Several compounds interfering with the sleep–wake cycle, including barbiturates and benzodiazepines, are agonists acting at different binding sites on the $GABA_A$ receptor complex [4]. The mechanism proposed suggests that GABAergic neurons from the basal forebrain and preoptic area project to the posterior lateral hypothalamus [33]. Further evidence obtained using microdialysis detection methods suggests that extracellular levels of GABA are augmented during sleep [34]. Pharmacological experiments have demonstrated that increasing $GABA_A$ receptor activity enhances SWS [35].

A number of years ago, Sugiyama et al. [36] confirmed that ginseng radix activated I_{Cl} mediated by the $GABA_A$ receptor. Further work has supported the idea that at least some of the behavioral effects of ginseng occur through the modification of the $GABA_A$ receptor. For example, social isolation typically results in a stress-evoked decrease in the duration of sleep induced by pentobarbital. The ability of majonoside-R_2 (a major ocotillol-type saponin constituent of Vietnamese ginseng) to reverse this effect is mediated by the neurosteroid site on the $GABA_A$ receptor complex in mice [37]. More recently, FG has been shown to reduce the first-night effect, an action that has been related to a potential anxiolytic effect of FG occurring via GABAergic modification [24]. In addition, Kimura et al. [16] reported that ginsenosides interact with ligand binding to the $GABA_A$ and $GABA_B$ receptors. However, Lee et al. [38] have shown that not all behavioral actions of ginseng saponins are attributable to the regulation of $GABA_A$ receptor activation. To investigate whether the effect of RGE treatment on sleep behavior involved $GABA_A$ergic system, we examined the protein levels of GAD 65/67 and $GABA_A$ receptor α, β, and γ subunits in rat hypothalamus. Our observations showed that acute RGE treatment with either a low or a high dose caused the over-expression of $GABA_A$ α and β, but not γ, subunits in rat hypothalamus; however, in the multiple-treatment groups, the over-expression of GAD 65/67 was only seen in rats treated with high doses of RGE. These findings suggest that the modulation of sleep behavior by acute RGE treatment primarily involves the over-expression of $GABA_A$ receptor α and β, but not γ, subunits. In contrast, increased GABA production may be important during chronic RGE administration. Generally, our data support most of the aforementioned conclusions that ginseng regulates sleep via the $GABA_A$ergic system [24,36].

In general, the present study demonstrated that RGE modulated sleep–wake cycles in a manner dependent upon the number of doses, whereas sleep architecture and NREM sleep were regulated by RGE in a manner dependent upon both the number and the size of the doses. Furthermore, RGE treatment extensively regulated the power spectral densities of EEGs, although no dose-number- or -size-dependent trends were found. In addition, the modulation of sleep behavior by acute RGE treatment may primarily involve the over-expression of $GABA_A$ receptors composed mostly of α and β, but not γ, subunits. In contrast, longer RGE treatment may modulate sleep behavior by raising GABA production. Thus, our findings suggest that RGE treatments with a range of dosage numbers and sizes modulate sleep–wake cycles, sleep architecture, and power spectra of EEGs in a dose-dependent manner. Moreover, our data suggest that the modulation of these sleep behaviors by RGE involves the $GABA_A$ergic system. Nonetheless, the mechanisms underlying the modulation of EEG activity need to be explored more deeply in the future.

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