Sleep-Promoting Effect of *Ecklonia cava*: Ethanol Extract Promotes Non-rapid Eye Movement Sleep in C57BL/6N Mice

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Abstract

We investigated the effects of *Ecklonia cava* ethanol extract (ECE) on sleep architecture and sleep profiles. ECE was orally administered at a dose of 100, 250, or 500 mg/kg to C57BL/6N mice and its effects were measured by recording electroencephalogram (EEG) and electromyogram. Administration of ECE (250 and 500 mg/kg) significantly induced non-rapid eye movement sleep (NREMS) without affecting rapid eye movement sleep. The increase in NREMS by ECE (500 mg/kg) was significant ($P < 0.05$) during the first 2 h after administration. In addition, ECE had no effect on EEG power density (an indicator of sleep quality) in NREMS. These results suggest that ECE induces NREMS in a manner similar to physiological sleep.

Key words: *Ecklonia cava*, Electroencephalogram, Electromyogram, Non-rapid eye movement sleep, Delta power

Introduction

Ecklonia cava is a brown alga distributed in the coastal areas of Jeju Island, Korea. It has been used as an ingredient in functional foods and traditional medicines (Cho et al., 2012b). Phlorotannins, fucoidans, fucoxanthins, and carotenoids have been identified as its major bioactive compounds (Heo et al., 2005; Kim and Bae, 2010). Extracts of this alga show various biological properties, including antioxidative (Li et al., 2009; Kim and Kim, 2010), immune-enhancing (Ahn et al., 2008, 2011), anti-allergic (Le et al., 2009; Shim et al., 2009), anticancer (Kong et al., 2009; Lee et al., 2011), and anti-inflammatory (Kim and Bae, 2010) effects.

Recently, the sedative-hypnotic effect of *E. cava* ethanol extract (ECE) was demonstrated using pentobarbital-induced sleep tests in mice (Cho et al., 2012a, 2012b). In addition, it has been reported that ECE induces sleep via the benzodiazepine (BZD) site of the gamma-aminobutyric acid (GABA$_A$) receptor. Eckol, eckstolonol, dieckol, and triphlorethol A have

been characterized as ligands of the GABA$_A$-BZD receptor (Cho et al., 2012b).

In humans, sleep is essential to maintain health due to its primary function of providing rest and restoring the body's energy levels (Krueger et al., 2008). Sleep is important for physical and cognitive performance, the immune system, mood stability, productivity, and quality of life (Krueger et al., 2008; Imeri and Opp, 2009). Disorders and deprivation of sleep impair cognitive and psychological functioning and worsen physical health (Brand and Kirov, 2011). Although sleep is fundamental to human health, insomnia is currently a widespread health complaint and has become a prevalent and disruptive problem in modern society (Borja and Daniel, 2006; Doghramji, 2006; Erman, 2008). Sleep aids that contain constituents or extracts from foods and plants have become popular as alternatives to prescription sleep drugs to improve sleep quality and avoid adverse side effects (Meletis and Za-
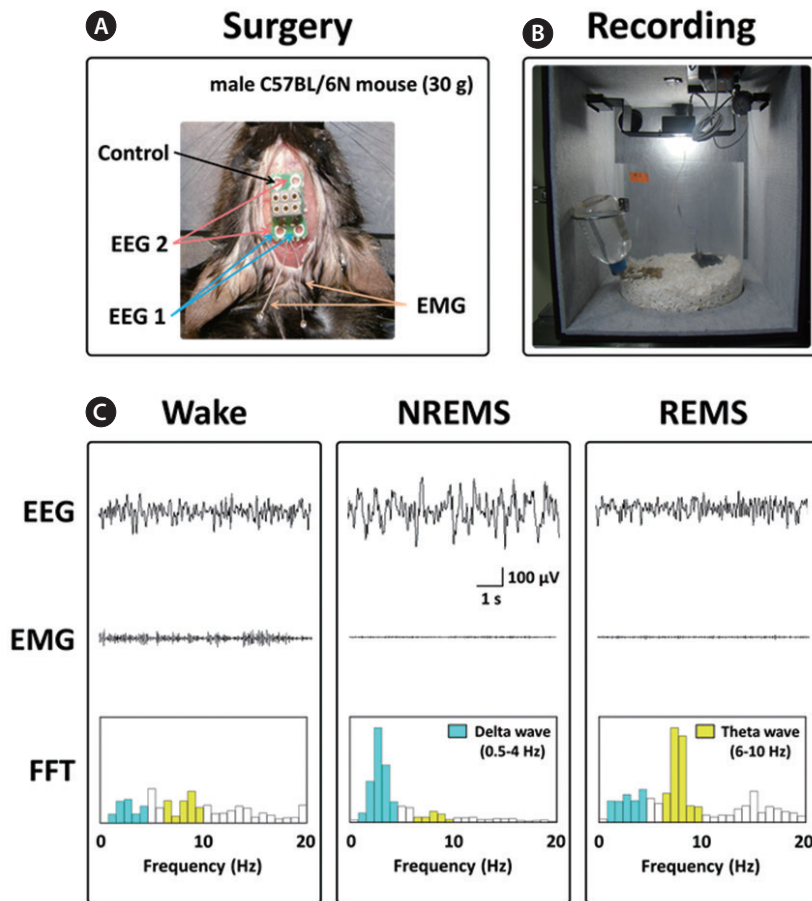
Fig. 1. Surgery for EEG and EMG recordings (A). An inside view of the recording chamber (B). Typical EEG, EMG and FFT spectra in mice (C). The EEG and EMG signals and FFT spectra were collected from a vehicle control mouse in this study. EEG, electroencephalogram; EMG, electromyogram; FFT, fast Fourier transform; NREMS, non-rapid eye movement sleep; REMS, rapid eye movement sleep; Wake, wakefulness.

briskie, 2008). Therefore, there is a growing demand for a novel class of hypnotic food constituents.

In the present study, to better understand the hypnotic effects of ECE, changes in the sleep-wake profiles of animals after oral administration of ECE were studied by recording electroencephalogram (EEG) and electromyogram (EMG). C57BL/6N mice were used because their sleep-wake cycle and delta activity are more stable than other strains (Huber et al., 2000).

Materials and Methods

Materials

To prepare ECE, dried *E. cava* from Jeju Island, Korea, was washed with water to remove salt, sand, and epiphytes attached to the surface. Then the samples were extracted with 70% (v/v) ethanol at 75°C for 16 h. The extract solution was filtered and lyophilized. Diazepam (DZP; Myungin Pharm.

Co. Ltd., Seoul, Korea), a $GABA_A$-BZD agonist, was used as a reference hypnotic drug. All other chemicals and reagents were of the highest grade available.

Animals

All procedures involving animals were conducted in accordance with the animal care and use guidelines of the Korea Food Research Institutional Animal Care and Use Committee (permission number: KFRI-M-12027). C57BL/6N mice (male; 27-30 g; 12 weeks old) were purchased from Koatech Animal Inc. (Pyeongtaek, Korea). Animals were housed in an insulated, sound-proof recording room maintained at an ambient temperatures of $23 \pm 0.5°C$, with a constant relative humidity ($55 \pm 2\%$) on an automatically controlled 12 h light/12 h dark cycle (lights on at 09:00). Mice had free access to food and water. All efforts were made to minimize animal suffering and to use only the number of animals required for the production of reliable scientific data.

Pharmacological treatments

ECE was dissolved in sterile saline containing 0.5% carboxymethyl cellulose immediately before use, and was administered orally (p.o.) to mice ($n = 8$) at 09:00 on the experimental day at doses of 100, 250, or 500 mg/kg. The positive control DZP (2 mg/kg) was administered in the same manner as ECE.

Polygraphic recordings and vigilance state analysis

Under pentobarbital anesthesia (50 mg/kg, i.p.), mice were chronically implanted with a head-mount (#8201; Pinnacle Technology Inc., Lawrence, KS, USA) installed with EEG and EMG electrodes for polysomnographic recordings. The front edge of the head-mount was placed 3.0 mm anterior to the bregma, and four electrode screws for EEG recordings were positioned in holes perforating the skull (Fig. 1A). Two EMG wire electrodes were inserted into the nuchal muscles. The head-mount was fixed to the skull with dental cement. After surgery, mice were allowed to recover in individual cages for 1 week and to acclimate to the recording conditions for 3-4 days before the experiment.

The EEG and EMG recordings were performed using a slip ring designed to allow the mice to move freely (Fig. 1B). Two EEG channels and one EMG channel were recorded using the PAL-8200 data acquisition system (Pinnacle Technology Inc.). The signals were amplified (100×), filtered (low-pass filter: 25 Hz EEG and 100 Hz EMG), and stored at a sampling rate of 200 Hz. Recording started at 09:00 and continued for 12 h. To evaluate sleep-promoting effects, recording was performed for 2 days. Data collected during the first day served as a baseline comparison (vehicle) for the second experimental day (test article).

The vigilance states were automatically scored by a 10 s epochs as wakefulness (Wake), rapid eye movement sleep (REMS), or non-REM sleep (NREMS) based on fast Fourier transform (FFT) analyses by SleepSign ver. 3.0 (Kissei Comtec, Nagano, Japan), according to the standard criteria (Qu et al., 2010). The FFT is a computational tool that facilitates signal analysis, such as power spectrum analysis and filter simulation using digital computers (Cochran et al., 1967). FFT analyses of EEG could be classified into two frequency bands; namely, δ (0.75-4 Hz) and θ (6-10 Hz) (Kohtoh et al., 2008). As a final step, defined sleep–wake stages were examined visually and corrected if necessary.

Data analysis

All data are expressed as the mean ± SEM ($n = 6$-8). Statistical analysis was performed using Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). The time-course of hourly amounts of sleep and Wake at each stage, histograms of the amounts of sleep and Wake, sleep latency, and mean duration of sleep and Wake were analyzed using the paired *t*-test, with each animal serving as its own control. For sleep latency and the total amount of each vigilance stage during the 3 h period immediately following drug treatment, one-way repeated measures analysis of variance (ANOVA) was performed, followed by the Dunnett's test to determine whether the differences among groups were statistically significant. The significance level was set at $P < 0.05$ for all statistical tests.

Results and Discussion

Effects of ECE on sleep latency and the amounts of NREMS and REMS

EEG and EMG signals in mice were recorded for 12 h after oral administration of ECE (100, 250, or 500 mg/kg) at 09:00, and its effects were compared to the positive control DZP (2 mg/kg). Fig. 2A shows representative EEG and EMG signals and the corresponding hypnograms for vehicle, ECE, and DZP. As shown in Fig. 2B, ECE (250 and 500 mg/kg) significantly ($P < 0.01$) decreased sleep latency compared to the vehicle. DZP also significantly ($P < 0.01$) decreased sleep latency, and was not significantly different from ECE (500 mg/kg). The short sleep latency in mice administered ECE indicates that ECE accelerated the initiation of NREMS.

The sleep structure of animals is composed of NREMS and REMS. The sleep-wake states are generally characterized as follows: Wake, low-amplitude EEG and high-voltage EMG activity; NREMS, high-amplitude slow or spindle EEG and low-voltage EMG activity; and REMS, low-voltage EEG and EMG activity (Fig. 1C) (Bastien et al., 2003; Kohotoh et al., 2008). The total time spent in NREMS and REMS for the first 3 h after ECE or DZP administration was calculated (Fig. 2C). ECE (250 and 500 mg/kg) significantly ($P < 0.05$) increased the total amount of NREMS by 47.8% and 71.4%, respectively. The rate of increase in NREMS after administering DZP (2 mg/kg) was 103.8% ($P < 0.01$). However, ECE and DZP did not produce significant changes in the total amount of REMS. BZD agents, such as DZP, are known to increase NREMS without changing REMS (Tobler et al., 2001; Qiu et al., 2009)

The sedative-hypnotic effects of ECE are caused by the phlorotannins found in brown seaweed. The phlorotannin fraction of ECE significantly promotes pentobarbital-induced sleep in mice; however, the residual fraction does not show any significant hypnotic effects (Cho et al., 2012a). The phlorotannin fraction induces sleep via the positive allosteric modulation of GABA$_A$-BZD receptors (Cho et al., 2012a). In addition, major phlorotannin constituents dieckol, eckol, eckstolonol, and triphlorethol A have been found to have binding affinity to the GABA$_A$-BZD receptors (Cho et al., 2012b) and potentiate the pentobarbital-induced sleep in mice (data not shown).
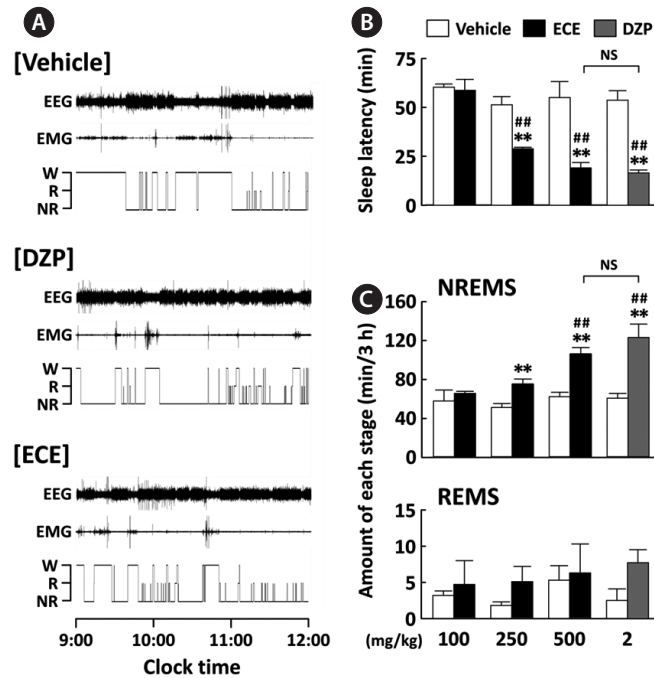
Fig. 2. (A) Representative examples of EEG and EMG signals and corresponding hypnograms in a mouse treated with vehicle, ECE, and DZP. (B) Effects of ECE and DZP on sleep latency. (C) Total time spent in NREMS and REMS for 3 h after administration. Each column represents the mean ± SEM ($n = 8$). $^{**}P < 0.01$, compared with vehicle (unpaired Student's t-test). $^{##}P < 0.01$, significant as compared with ECE (100 mg/kg; Dunnett's test). DZP, diazepam; ECE, *Ecklonia cava* ethanol extract; EEG, electroencephalogram; EMG, electromyogram; NREMS (or NR), non-rapid eye movement sleep; NS, not significant; REMS (or R), rapid eye movement sleep; Wake (or W), wakefulness.
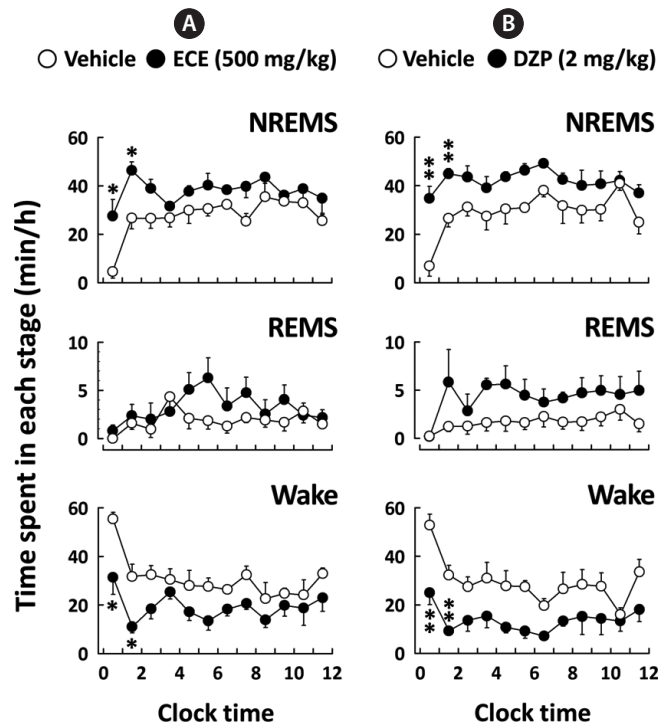

Fig. 3. Time courses of NREMS, REMS, and Wake after the administration of ECE (A) and DZP (B). Each circle represents the hourly mean ± SEM ($n = 8$) of NREMS, REMS, and Wake. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with vehicle (unpaired Student's t-test). DZP, diazepam; ECE, *Ecklonia cava* ethanol extract; NREMS, non-rapid eye movement sleep; REMS, rapid eye movement sleep; Wake, wakefulness.
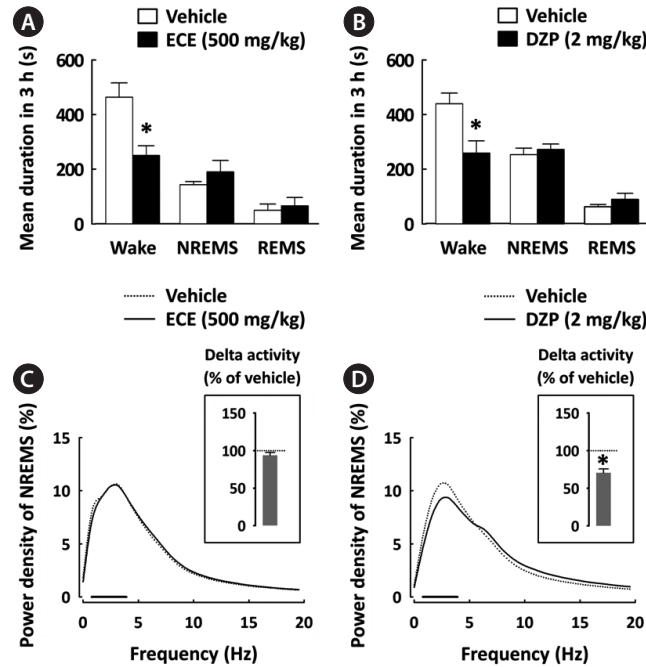
Fig. 4. Effects of ECE and DZP on changes in the mean duration of each sleep stage (A, B) and EEG power density in NREMS (C, D). Delta activity in NREMS, an index of sleep intensity, is shown in the inset histogram of (C) and (D). The bar (▬) represents the range of the delta wave (0.5–4 Hz). *$P < 0.05$, compared with vehicle (unpaired Student's *t*-test). DZP, diazepam; ECE, *Ecklonia cava* ethanol extract; EEG, electroencephalogram; NREMS, non-rapid eye movement sleep; REMS, rapid eye movement sleep; Wake, wakefulness.

Effects of ECE on the time spent in each sleep stage

Fig. 3 shows the time course changes in NREMS, REMS, and Wake for 12 h after the administration of ECE or DZP. After ECE administration, the amount of NREMS immediately increased, while the amount of Wake decreased. These effects were significant ($P < 0.05$) compared to the vehicle for the first 2 h. There was no further significant disruption of sleep architecture during subsequent periods. These results indicate that ECE induces NREMS without having adverse effects after sleep induction (Masaki et al., 2012). DZP also showed a significant difference ($P < 0.01$) for the first 2 h; however, during the subsequent period, the hourly amount of NREMS was higher than for ECE.

Effects of ECE on the mean duration of each sleep stage and EEG power density in NREMS

To better understand the sleep profile caused by ECE, the mean duration of each sleep stage and EEG power density in NREMS were calculated. ECE and DZP significantly ($P < 0.05$) decreased the mean duration of Wake by 54.0% and 58.8%, respectively; however, they did not affect the mean duration of NREMS or REMS (Fig. 4A and 4B). This suggests that ECE decreased the maintenance of Wake (Masaki et al., 2012). ECE did not affect EEG power density (0-20

Hz) in NREMS compared to the vehicle (Fig. 4C), whereas DZP produced a significant ($P < 0.05$) decrease in delta (0.5-4 Hz) activity, as shown in the inset histogram of Fig. 4D. Delta activity is an indicator of the depth or intensity of NREMS (Bastien et al., 2003; Winsky-Sommerer, 2009). A decrease in delta activity caused by DZP has been reported in humans and rodents (Tobler et al., 2001; van Lier et al., 2004). BZD agents increase sleep duration but decrease sleep quality (Bastien et al., 2003; Ishida et al., 2009). Generally, DZP increase beta activity (13–30 Hz), which is the highest EEG frequency associated with attention and arousal. Although BZD sleep drugs induce sleep, they also induce beta activity (Coenen and van Luijtelaar, 1991; van Lier et al., 2004). In the present study, DZP also increased beta activity; however, ECE did not. In summary, ECE decreased sleep latency and increased the amount of NREMS, similar to DZP; however, it did not affect EEG power density, unlike DZP. These results suggest that ECE induces NREMS in a similar manner as physiological sleep.

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