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Flavonoid in Clover Honey Exerts a Hypnotic Effect via Positive Allosteric Modulation of the $GABA_A$-BZD Receptor in Mice

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Abstract

There is a growing demand for natural sleep aids due to various side effects of long-term administration of pharmacological treatments for insomnia. Honey has been reported to exhibit numerous potential health benefits, and it is hypothesized that honey may favorably affect insomnia treatment. Therefore, this study was performed to investigate the possible hypnotic effect of clover honey (CH) and to determine its *in vivo* mechanism. The total flavonoid content (TFC) of CH and fractions extracted with ethylacetate (EtOAc) and H_2O was measured. The pentobarbital-induced sleep test using $GABA_A$-benzodiazepine (BZD) agonists and antagonists was conducted to evaluate the potential mechanism of action behind the sedative-hypnotic activity of CH in mice. The results showed that administration of 500 and 1,000 mg/kg of CH significantly ($p<0.01$) reduced the sleep latency to a level similar to that of diazepam (DZP, 2 mg/kg), and 1,000 mg/kg of CH significantly ($p<0.01$) prolonged the sleep duration, which was comparable to that of DZP (2 mg/kg). Administration of the EtOAc fraction with a higher TFC significantly reduced the sleep latency at 50 to 200 mg/kg and prolonged the sleep duration at 100 to 200 mg/kg, which were comparable to those after administration of DZP (2 mg/kg). However, co-administration of CH and EtOAc with flumazenil, a specific $GABA_A$-BZD receptor antagonist, blocked the hypnotic effect. Our findings suggest that the hypnotic activity of CH may be attributed to allosteric modulation of $GABA_A$-BZD receptors. The TFC of CH is expected to be a key factor that contributes to its hypnotic effect.

Key words: clover honey, flavonoid, hypnotic activity, insomnia, $GABA_A$-BZD receptor

Introduction

Honey is a natural sweetener that has been traditionally used in the treatment of asthma, burns, tension, colitis, and infected wounds (Al-Mamary et al. 2002; Bilsel et al. 2002). Furthermore, recent reports have demonstrated the nutraceutical effects of honey, based on the presence of antioxidant compounds and flavonoids, as well as its immunity-enhancing and weight loss-stimulating effects (Paul et al. 2007; Atanda et al. 2011).

Sleep is indispensable to human health and quality of life, but estimates on the percentage of the adult population worldwide that currently suffers from chronic or occasional insomnia range from 10~15% to 25~35% (Doghramji 2006; Krueger et al. 2008). As pharmacological treatments for insomnia induce various side effects after long-term administration, there has been a growing demand for natural sleep aids with hypnotic effects (Meletis & Zabriskie 2008; Fang et al. 2010; Cho et al. 2012). These include a range of plants and food constituents, including honey, which have been traditionally used to improve sleep worldwide.

It is believed that the bioactivities of honey vary depending on the floral source (Lieu et al. 2013). Manuka honey has been reported to improve chronic wounds and confer antibacterial activity (Gethin et al. 2008; Mavric et al. 2008). Previous studies reported that clover honey (CH) exhibits strong antimicrobial activity against various pathogenic bacteria (Elbanna et al. 2014).

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Factors responsible for this antimicrobial activity are mainly H_2O_2 and non-peroxide factors such as flavonoids, lysozymes, and phenolic acids (Taormina et al. 2001; Ahmed & Othman, 2013). Consumption of CH results in improved weight regulation and reduced triglyceride levels compared with a sucrose-based diet (Nemoseck et al. 2011). However, few studies have described the hypnotic effect of honey, and the underlying mechanism remains unknown. Therefore, this study aimed to investigate the hypnotic effect of CH, as well as its *in vivo* mechanism, to gain knowledge on the use of honey as a natural sleep aid source.

Materials and Methods

1. Materials and drugs

CH (Arataki Honey Ltd., NZ) was purchased from a domestic market in New Zealand. All chemicals and reagents were of the highest grade available. Pentobarbital was purchased from Hanlim Pharm. Co. Ltd. (Korea). Diazepam (DZP; Myungin Pharm. Co. Ltd., Korea) and zolpidem (ZPD; Korea Food & Drug Administration, Korea) were used as sedative-hypnotic reference drugs ($GABA_A$-BZD agonists). Flumazenil (FLU), a $GABA_A$-BZD receptor antagonist, was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

2. Preparation of honey fractions

For preparation of the ethylacetate (EtOAc) fraction, CH (220 g) was suspended in distilled water (1 L), and then extracted with EtOAc (1 L). The organic layer was concentrated using an evaporator to obtain the EtOAc fraction (244.7 mg; yield 0.11%). The yield of the residue (H_2O) fraction was 99.89% (219.76 g).

3. Determination of total flavonoid content (TFC)

Measurement of TFC was performed according to the method of Moreno et al. (2000). Methanol (1 mL) containing 1 mg of sample was added to test tubes containing 0.1 mL of 1 M potassium acetate, 0.1 mL of 10% aluminium nitrate, and 3.8 mL of methanol. The absorbance of the sample solution was determined at 415 nm after incubation at room temperature for 40 min. TFC was calculated based on a calibration curve constructed using quercetin (Sigma-Aldrich Inc.) as a standard. Data are expressed as μg of quercetin equivalents (QE) per 1 g of the sample (μg QE/g).

4. Animals

Male ICR mice (18~22 g) were purchased from Koatech Animal Inc. (Pyeongtaek, Korea), and were housed in a room maintained at 24℃ with a 12 h light/dark cycle. They consumed a basal diet *ad libitum* with free access to water. All procedures complied with the guidelines of the Korea Food Research Institutional Animal Care and Use Committee (permission number: KFRI-M-09118).

5. Pentobarbital-induced sleep test

All tests were performed between 1:00 PM and 5:00 PM, based on the method of Cho et al. (2012). The mice (n=10 per treatment) were fasted for 24 h before the experiment. For oral administration, all samples were suspended in 0.5% (w/v) carboxymethyl cellulose (CMC)-saline. Test solutions (10 mL/kg) were administered orally (p.o.) to mice 45 min before pentobarbital injection. An aliquot of 0.5% CMC-saline solution was administered as a control. For co-administration with FLU, 8 mg/kg of FLU was administered by intraperitoneal injection (i.p.) 15 min before oral administration of samples. After pentobarbital injection (45 mg/kg, i.p.), mice were observed to measure sleep latency and duration in individual cages. The observers were blinded to the individual treatments. Sleep latency was recorded from the time of pentobarbital injection to the time of sleep onset, and sleep duration was described as the difference in time between loss and recovery of the righting reflex.

6. Statistical analysis

All data are expressed as the mean±standard error of the mean (SEM) and were analyzed using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA). For multiple comparisons, one-way analysis of variance with Dunnett's test was used. We evaluated comparisons between two-group data using an unpaired Student's *t*-test. All differences were considered to be statistically significant at the 5% level.

Results

1. Effect of CH on sleep latency and duration

As expected, the sedative-hypnotic $GABA_A$-BZD receptor agonist DZP (2 mg/kg, p.o.) significantly ($p<0.01$) potentiated pentobarbital-induced sleep compared to the control (Fig. 1). Administration of 500 and 1,000 mg/kg of CH significantly ($p<0.01$) and dose-dependently reduced sleep latency to a level similar to that of DZP (Fig 1A), and 1,000 mg/kg of CH sig-

nificantly (p<0.01) prolonged sleep duration comparably to DZP (Fig. 1B).

2. Effect of the EtOAc fraction on sleep latency and duration

To identify sedative-hypnotic compounds, CH was extracted with EtOAc and H_2O. Total flavonoid content of CH, EtOAc and H_2O fractions was 20.8±0.5, 5,561±50.0, and 12.7±2.0 μg QE/g, respectively. The EtOAc fraction was shown to contain a significantly (p<0.001) higher TFC than CH, whereas the H_2O fraction contained a significantly (p<0.001) lower TFC than CH. The EtOAc fraction significantly (p<0.01) potentiated pentobarbital-induced sleep at a lower dosage compared to CH: administration of the EtOAc fraction reduced sleep latency at 50 to 200 mg/kg (Fig. 2A) and prolonged sleep duration at 100 to 200 mg/kg (Fig. 2B), comparable to 2 mg/kg DZP. However, the H_2O fraction did not show significant hypnotic activity compared to the control group (Fig. 2).

3. Effect of FLU on changes in sleep latency and sleep duration

To elucidate the possible *in vivo* mechanism behind the hypnotic effects of CH and the EtOAc fraction, we co-administered 8 mg/kg of FLU, a specific BZD antagonist. While sleep latency and sleep duration were potentiated in the absence of FLU treatment, injection of FLU significantly (p<0.01) inhibited the sedative-

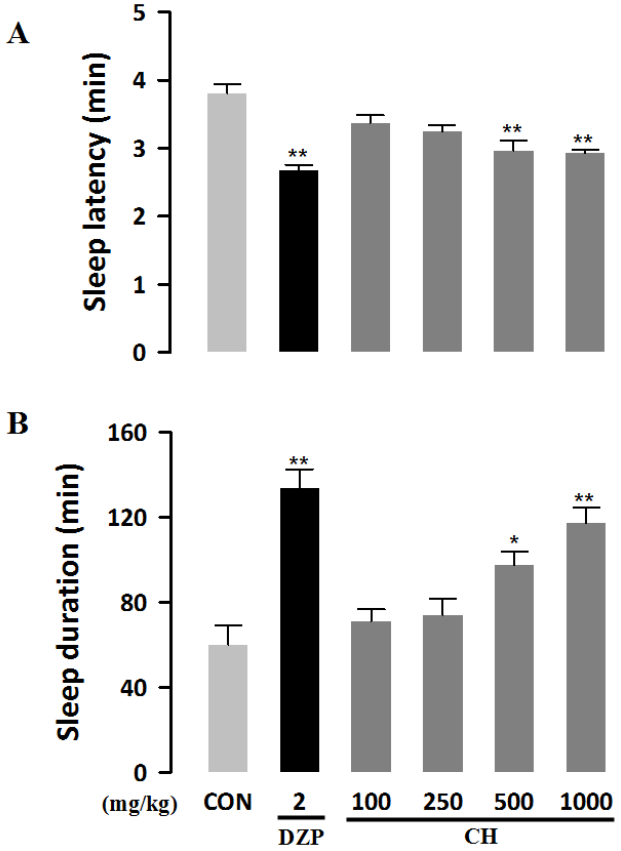

Fig. 1. Effects of CH on sleep latency (A) and sleep duration (B) in mice induced by a hypnotic dose (45 mg/kg, i.p.) of pentobarbital. Mice were injected with pentobarbital 45 min after oral administration of CH. * p<0.05, ** p<0.01, significant when compared to the control group (Dunnett's test). CH, clover honey; CON, control group (0.5% CMC-saline 10 mL/kg); DZP, diazepam. Data are shown as means ±SEM (n=10).

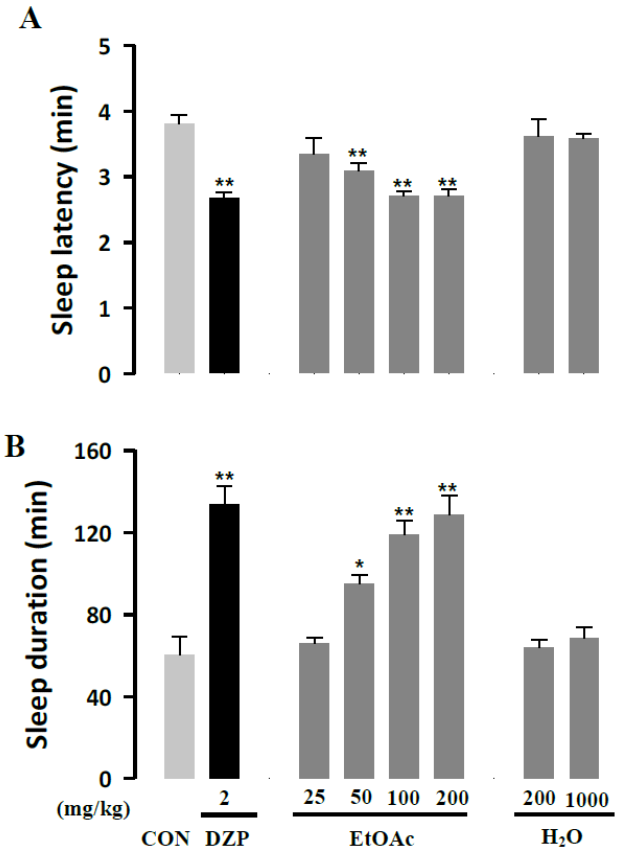

Fig. 2. Effects of EtOAc and H_2O fractions from CH on sleep latency (A) and sleep duration (B) in mice induced by pentobarbital (45 mg/kg, i.p.). Mice were injected with pentobarbital 45 min after oral administration of the fractions. * p<0.05, ** p<0.01, significant when compared to the control group (Dunnett's test). CH, clover honey; CON, control group (0.5% CMC-saline 10 mL/kg); DZP, diazepam; EtOAc, ethylacetate. Data are shown as means±SEM (n=10).

hypnotic effects of DZP, ZPD, CH, and the EtOAc fraction (Fig. 3).

Discussion

As a preliminary trial, different blends of honey, including Manuka honey, were evaluated by pentobarbital-induced sleep tests in order to determine the potential sedative-hypnotic activity of honey (data not shown). CH resulted in the shortest sleep latency and longest sleep duration, which initiated an investigation into its hypnotic effects and possible mechanism of action. The pentobarbital-induced sleep test used in this study has been reported to be effective in determining the sedative-hypnotic activities of potential pharmaceutical materials for insomnia treatment (Zhu et al. 1996). The previous studies successfully investigated the neurological activity of brown seaweed polyphenols using this method (Cho et al. 2012).

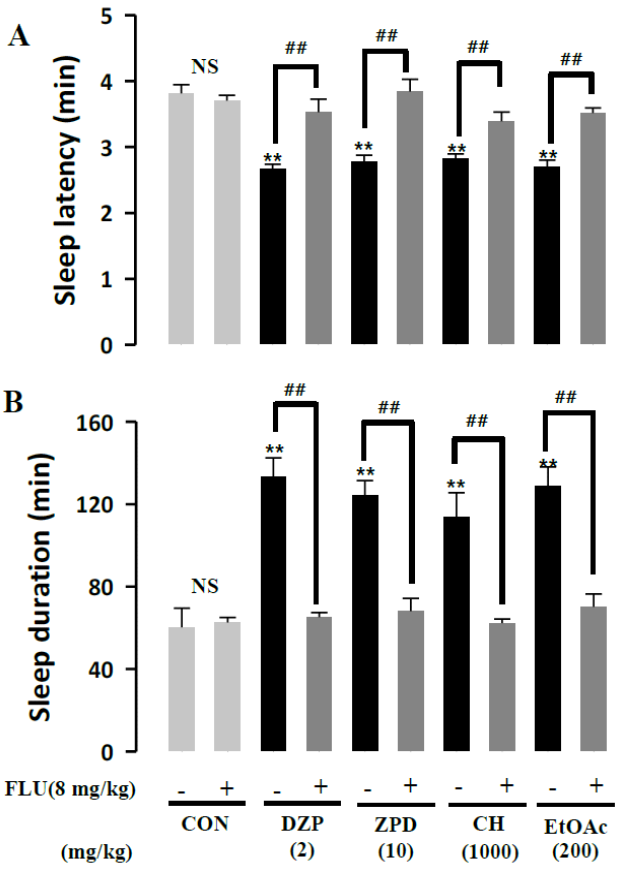

Fig. 3. Effects of FLU on sleep latency (A) and sleep duration (B) in mice administered DZP, ZPD, CH, and the EtOAc fraction. Mice were treated with FLU (i.p.) 15 min before oral administration of the samples. ** $p<0.01$, significant when compared to the control group (Dunnett's test). ## $p<0.01$, significant difference with and without FLU treatment (unpaired Student's *t*-test). CH, clover honey; CON, control group (0.5% CMC-saline, 10 mL/kg); DZP, diazepam; EtOAc, ethylacetate; FLU, flumazenil; NS, not significant; ZPD, zolpidem. Data are shown as means±SEM (n=10).

Generally, plant polyphenols (mainly flavonoids) are positive allosteric modulators of $GABA_A$ receptors (Johnston 2005). Although honey consists of various potential components such as non-digestible oligosaccharides, vitamin B group, enzymes, phenolic acids, flavonoids, minerals, and antioxidants (Taormina et al. 2001; Bogdanov et al. 2008; Akanmu et al. 2011), the flavonoids in particular are hypothesized to contribute to the observed hypnotic activity.

Flavonoids were extracted from CH with EtOAc, BuOH, hexane, and H_2O. The EtOAc fraction contained a 10-fold higher TFC than the BuOH and hexane fractions (data not shown), and was therefore used in the pentobarbital-induced sleep test. In our results, the EtOAc fraction showed a similar hypnotic effect at a lower dosage compared to CH (Figs. 1 & 2).

Numerous compounds of various structural classes have been reported to activate or inhibit $GABA_A$ receptors (Sieghart 2015). Pentobarbital and BZD interact with distinct binding sites on the $GABA_A$ receptor (Gottesmann 2002). Pentobarbital can directly activate the $GABA_A$ receptor, resulting in induction of sleep, whereas sedative-hypnotic materials acting on the BZD-binding site of $GABA_A$ receptor potentiate pentobarbital-induced sleep (Möhler 2011; Cho et al. 2012). FLU is a specific BZD antagonist that inhibits the hypnotic effects of DZP and ZPD by blocking their binding to the BZD-binding site of $GABA_A$ receptor (Johnston 2005).

Co-administration of CH and the EtOAc fraction with FLU was performed to investigate the possible mechanism of action behind the hypnotic effect of CH. The hypnotic activities of CH and the EtOAc fraction were completely inhibited by FLU, implying that the flavonoids from CH and the EtOAc fraction interacted with the BZD-binding site of the $GABA_A$ receptor, via a similar mechanism of action as DZP and ZPD. Although the hypnotic activity of CH may result from the combined effects of various bioactive compounds, our findings suggest that it may be mainly attributed to the allosteric modulation of $GABA_A$-BZD receptors by CH flavonoids. To our knowledge, this is the first report regarding the *in vivo* mechanism of action behind the hypnotic effects of CH.

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