Journal of Food and Drug Analysis

Volume 31 | Issue 2 Article 6

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ORIGINAL ARTICLE

Sleep-promoting activity of amylase-treated Ashwagandha (*Withania somnifera* L. Dunal) root extract via GABA receptors

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Abstract

Ashwagandha (*Withania somnifera* L. Dunal), an Indian medicinal plant that has been used for centuries to treat insomnia, exhibits a variety of biological activities, such as improving cognitive function, immunity and anxiety. In this study, the effect of enzyme-treated Ashwagandha root extract (EA) and on sleep was evaluated using rodent models. Starch contained in the Ashwagandha root extract was removed by amylase treatment to prepare EA. To evaluate the sleep-promoting activity of EA, a pentobarbital-induced sleep test and electroencephalogram analysis were performed. In addition, the sleep-promoting mechanism of EA was elucidated by analyzing the expression of sleep-related receptors. In the pentobarbital-induced sleep test, EA dose-dependently increased sleep duration. Additionally, electroencephalogram analysis revealed that EA significantly increased δ-wave and non-rapid eye movement sleep times, which are involved in deep sleep, thereby improving sleep quality and quantity. EA also effectively relieved caffeine-induced insomnia symptoms. Furthermore, the γ-aminobutyric acid (GABA) content in the brain and mRNA and protein expression of $GABA_A$, $GABA_{B1}$, and serotonin receptors were significantly increased by EA compared to the normal group. In particular, EA showed sleep-promoting activity by binding to various GABAA receptor sites. Collectively, EA exhibited sleep-promoting activity through the GABAergic system and may be used as a functional material to improve sleep deprivation.

Keywords: Antagonist, Electroencephalogram, $GABA_A$ receptor, NREM sleep, *Withania somnifera*

1. Introduction

Stress and sleep disorders are very common among modern people, and most patients tend to overcome them with alcohol or over-the-counter drugs instead of prescription drugs [1]. Drugs, such as benzodiazepine (BDZ), zolpidem, and zopiclone, are often used to treat insomnia [2]. However, long-term use of these drugs induce drug dependence and side effects, such as headache, nightmares, daytime fatigue, nausea, dizziness, and falls [3,4]. Therefore, it is necessary to identify alternative functional foods derived from natural products that have few side effects and are effective for insomnia [5]. As substitutes and supplements for sleep medicines, natural plant extracts, such as *Valeriana officinalis* L. (valerian) [6], *Humulus lupulus* L. (hop) [7], *Ziziphus jujuba* Mill. (jujube) [8], *Withania somnifera* L. Dunal (Ashwagandha) [9], and *Matricaria chamomilla* L. (chamomile) [10], have been actively examined for their sleep enhancing function.

Ashwagandha, also known as Indian ginseng, has been used for centuries in the traditional Indian medicine Ayurveda as a medicinal herb to improve physical and mental health [11]. All parts of Ashwagandha such as roots, leaves and flowers can be used medicinally, of which the root is mainly used. While the roots of Ashwagandha are rich in the active ingredients withanolides and withaferin A, the leaves and stems contain various bioactive

Received 2 November 2022; accepted 16 March 2023.
Available online 15 June 2023

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https://doi.org/10.38212/2224-6614.3456

molecules such as steroidal lactones and alkaloids [12]. Ashwagandha root is a safe herb used in traditional medicine primarily as a treatment for stress and insomnia [13], and as a natural tonic, it has been used to treat a variety of ailments by improving metabolism and reducing inflammation [14]. In addition, various neuropharmacological effects such as memory improvement [15], anxiety relief [16], and neuroprotection [17] have been reported from ashwagandha extract, which are attributed to increased catecholamine levels and inhibition of oxidative stress. In particular, various studies on sleep-promoting activities of Ashwagandha have been conducted [18]. Ashwagandha-mediated sleep induction is presumed to involve γ-aminobutyric acidergic (GABAergic) activity [19]; however, the exact mechanism remains obscure. It has been reported that the increase in non-rapid eye movement (NREM) sleep by Ashwagandha extract contributes to the increase in sleep time [20]. However, administration of an extract with a high withanolides content [21], which is known to relief stress, did not induce sleep [20], indicating that substances other than withanolides contribute to the sleep-inducing activity.

Currently, studies on the sleep-inducing activity of Ashwagandha extract are limited. Therefore, further studies confirming the sleep-inducing activity of Ashwagandha are warranted. In this study, improvement in sleep quantity and quality was verified using a caffeine-induced insomnia model and by analyzing electroencephalograms (EEGs) of a pentobarbital-induced sleep mouse model and rat model. The research results aim to provide scientific data related to the elucidation of sleep promoting mechanisms based on GABAergic action for the development of Ashwagandha extract for improving insomnia.

2. Materials and methods

2.1. Reagents

Caffeine, GABA, and antagonists (picrotoxin [PIX], bicuculline [BIC], and flumazenil [FMZ]) were purchased from Sigma-Aldrich (St Louis, MO, USA). $GABA_A$ receptors were purchased from Sigma-Aldrich. All high-performance liquid chromatography (HPLC) grade solvents were purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA).

2.2. Sample preparation

Ashwagandha root water extract (AW) and enzyme-treated AW (EA) were both provided by n-Biotech Co., Ltd. (Hwaseong, Korea). AW was treated with α- and β-amylase to remove starch, and the supernatant was collected by centrifugation and dried to prepare EA. EA contained total sugars (808.17 ± 2.32 μg/mg), reducing sugars (443.67 ± 1.40 μg/mg), proteins (32.18 ± 0.21 μg/mg), polyphenols (13.83 ± 0.08 μg/mg gallic acid equivalent), and flavonoids (0.063 ± 0.002 μg/mg catechin equivalent).

2.3. Experimental animals

Institute of Cancer Research (ICR) mouse mice (28–30 g, 6 weeks old, male) and Sprague Dawley (SD) rats (180–200 g, 6 weeks old, male) were purchased from Orient Bio (Seongnam, Korea) and bred at the Laboratory Animal Resource Center, College of Health Sciences, Korea University. The temperature of the animal breeding room was maintained at 20–22 °C, the relative humidity was 50–55%, the light and dark conditions were controlled every 12 h, and water and feed were provided ad libitum. All animal experiments were approved by the Korea University Institutional Animal Care and Use Committee (KUIACUC-2021-0056).

2.4. Pentobarbital-induced sleep test in mice

After an adaptation period of one week, ICR mice were fasted for 24 h before starting the experiment. Samples (AW and EA) were administered orally 40 min before intraperitoneal pentobarbital administration (42 mg/kg). Then, all mice were moved to an independent space to measure sleep latency and duration. Sleep duration was recorded until the recovery of the upright reflex. Mice that did not sleep within 15 min of pentobarbital administration were excluded from the experiment. The administered dose of the sample was determined according to previous studies [18,22].

To evaluate the insomnia relief effect of EA in a mouse model of caffeine-induced insomnia, a pentobarbital-induced sleep experiment was performed. Caffeine (40 mg/kg) was orally administered to all experimental groups except for the NOR group, and saline was administered to the NOR group. Along with caffeine administration, the EA treatment group was orally administered EA by concentration, and the NOR and CON groups were administered saline. After 40 min of administration of EA and caffeine, pentobarbital (42 mg/kg) was intraperitoneally injected, and sleep latency and duration time were measured.

2.5. EEG measurement

SD rats were acclimatized for one week, and then, under inhalation anesthesia with isoflurane (Terrell; Piramal Critical Care, Bethlehem, PA, USA), EEG electrodes (Emka Technologies, Paris, France) were implanted into the brain as described previously [23], and after a recovery time of one week, an EEG transmitter (Emka Technologies) was attached. EEG recording was performed in a double-wall sound-proof chamber to accurately analyze the sleep enhancement effect of EA administration. All EEG analyzes in SD rats were performed under normal conditions without pentobarbital administration. Under normal conditions, rats have been reported to sleep between approximately 62–70% of their total daylight hours during a 12-h period [24].

The experimental group was divided into four groups (n = 6/group): normal control group (NOR), AW administration group (80 mg/kg), and EA administration groups (80 and 100 mg/kg). After orally administering the sample to the rats, EEG was performed for 7 h from 10:00 am to 5:00 pm for a total of 6 days. EEG data were recorded at 15 mm/s during sleep, setting the 0.1–25 Hz filter region. For the analysis of sleep architecture, brain wave (wake, sleep, REM, NREM, δ wave, and θ wave) was analyzed with fast Fourier transform (FFT) algorithm using ecgAUTO3 program (Emka Technologies).

In the caffeine-induced insomnia SD rat model, EEG was analyzed after EA administration. The experimental group was divided into four groups (n = 6/group): normal control group (NOR), negative control group (CON, caffeine treatment), positive control group (BDZ 0.2 mg/kg), and EA administration group (100 mg/kg). Caffeine (40 mg/kg) was co-administered with the sample in all groups except for the NOR group. In the NOR and CON groups, physiological saline was orally administered, and in the EA group, EA was orally administered by concentration. In the insomnia model, after oral administration of the sample and caffeine once a day, EEG analysis was performed for 4 days (7 h/day), in the same manner as in the previous method.

2.6. Binding mode analysis of antagonists to the $GABA_A$ receptor

The receptor binding type by antagonists was evaluated using a pentobarbital-induced sleep model in the normal control (NOR), antagonist alone, and antagonist and EA co-administration groups. The antagonists PIX, BIC, and FMZ were dissolved in edible oil at concentrations of 4, 6, and 10 mg/kg, respectively, and then intraperitoneally injected into the left abdomen. In the NOR and EA administration groups, only edible oil (soybean oil) was intraperitoneally injected. Sample was orally administered 15 min after antagonist administration. The NOR and antagonist alone groups (PIX, BIC, and FMZ) were orally administered with saline, and the EA and antagonist and EA co-administration groups (PIX + EA, BIC + EA, FMZ + EA) were administered 100 mg/kg EA. Forty minutes after oral administration of sample, pentobarbital was injected intraperitoneally at a concentration of 42 mg/kg and sleep latency and duration were measured.

2.7. Analysis of the GABA content and mRNA and protein expression of sleep-related receptors in the brain

After oral administration of EA once a day for four weeks, ICR mice were sacrificed, and brain tissues were collected. Total RNA was extracted from brain tissues (100 mg) using TRI reagent (Invitrogen, Carlsbad, CA, USA). To analyze the mRNA expression of sleep-related receptors ($GABA_A$, $GABA_B$ and $5HT_{1A}$), quantitative real-time polymerase chain reaction was performed as previously described [25].

Protein was extracted from the brain tissues using RIPA buffer (Abcam, Cambridge, MA, USA), and western blotting was performed to determine the protein expression of sleep-related receptors as described previously [26]. The primary antibodies used were anti-$GABA_A$ (ab72445, 1:1000; Abcam), anti-$GABA_{BR1}$ (3835, 1:1000; Cell signaling technology, Beverly, MA, USA), anti-5HT1A (ab85615, 1:1000; Abcam), and GAPDH (5174, 1:1000; Cell signaling technology), and anti-rabbit IgG (7074, 1:1000; Cell signaling technology) was used as the secondary antibody.

In addition, the GABA content in the brain tissues was analyzed by HPLC using an AccQ-Tag column (Waters Corp., Milford, MA, USA) as previously described [27].

2.8. Statistical analysis

Experimental data are expressed as the mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was performed using SPSS (SPSS Inc., Chicago, IL, USA) to test the significance between each group followed by Tukey's multiple comparison test at a significance level of $p < 0.05$.

3. Results

3.1. Sleep-promoting activity of AW in pentobarbital-treated mice

The sleep-promoting activity of AW in pentobarbital-treated mice was measured (Fig. 1). Administration of AW tended to decrease sleep latency time compared to the NOR group, but no significant difference was observed (Fig. 1A). In sleep duration time, AW administration of 60 mg/kg significantly increased sleep duration compared to the NOR group ($p < 0.001$; Fig. 1B). However, sleep duration time tended to decrease when the dose concentration was higher than 60 mg/kg. The 80–300 mg/kg AW showed a slight increase in sleep duration compared to the NOR group, but the difference was not significant. Based on the results obtained after increasing the AW dose, the starch contained in AW is suspected to interfere with the sleep-promoting activity of AW.

3.2. Sleep-promoting activity of EA in pentobarbital-treated mice

To evaluate the sleep-promoting activity of EA, sleep latency and duration of pentobarbital-treated mice were measured (Fig. 2). The EA-administered group showed a tendency to decrease sleep latency compared to the NOR group, but no significant difference was observed (Fig. 2A). In addition, there was no significant difference in sleep latency between the AW-administered group and the EA-administered group at the same concentration (60 mg/kg).

Similar to the previous results, 60 mg/kg of AW significantly increased sleep duration compared to the NOR group ($p < 0.05$; Fig. 2B). The EA-administered group showed significantly higher sleep duration than the NOR group at all concentrations ($p < 0.001$; Fig. 2B). Unlike AW, EA effectively promoted sleep even at concentrations higher than 60 mg/kg. Also, EA increased sleep time more effectively than AW at the same concentration (60 mg/kg). This result may be due to the reduction of starch in which EA interferes with the sleep-promoting activity by amylase-mediated degradation. As shown in Supplementary Fig. 1 (https://www.jfda-online.com/journal/vol31/iss2/6/), enzymatic treatment of AW with amylase increased the reducing sugar content.

3.3. Sleep-promoting activity of EA in caffeine-induced insomnia mice

The sleep latency and duration of caffeine-induced insomnia mice were measured after EA administration (Fig. 3). The sleep latency and duration were significantly different between the CON and NOR groups, demonstrating successful development of the caffeine-induced insomnia model. EA decreased and increased sleep latency and duration, respectively, in a dose-dependent manner. In particular, EA significantly decreased sleep latency in the insomnia model compared to the normal model. EA administration at 100 and 150 mg/kg also significantly increased sleep time compared to CON group (Figs. 2 and 3).

3.4. EEG changes in SD rats by oral EA administration

REM and NREM sleep times were measured using EEG analysis following oral EA administration (Fig. 4). Sleep time was significantly longer in the

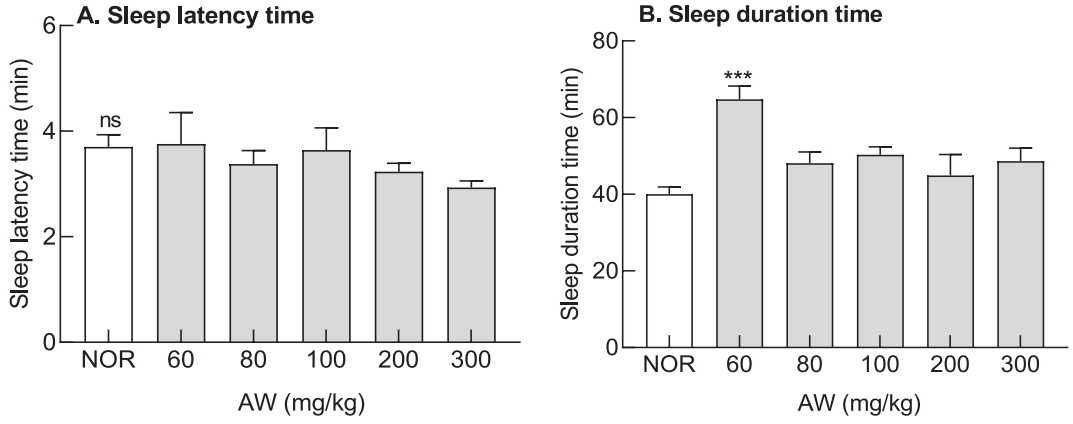

*Fig. 1. **Sleep latency (A) and duration (B) of pentobarbital-treated mice after Ashwagandha root water extract (AW) administration.** Values are presented as the mean ± standard error of the mean (SEM) for each group ($n = 7$). ***$p < 0.001$ versus NOR group by Tukey's multiple comparison test. ns: not significant.*

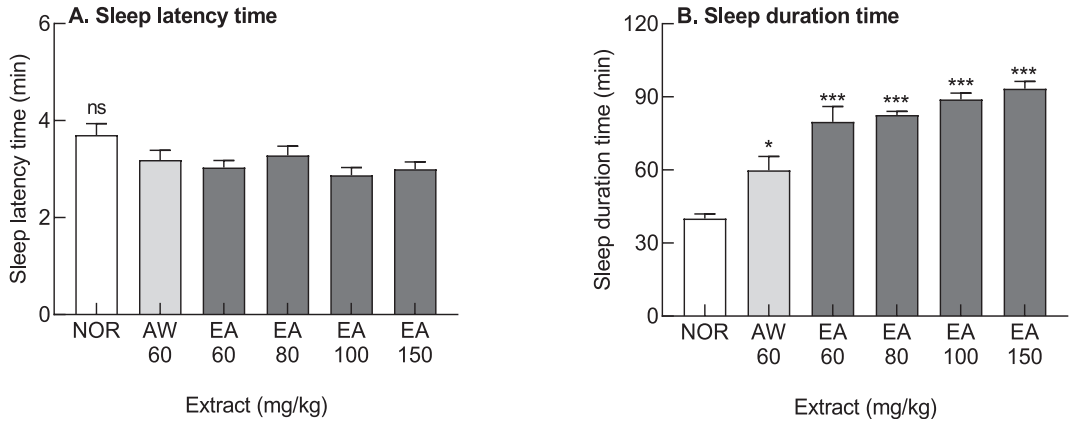

Fig. 2. ***Sleep latency (A) and duration (B) of pentobarbital-treated mice after enzyme-treated Ashwagandha root water extract (EA) administration.*** *AW60: 60 mg/kg Ashwagandha root water extract group, EA60, 80, 100, and 150: 60, 80, 100, and 150 mg/kg EA, respectively, groups. Values are presented as the mean ± standard error of the mean (SEM) for each group (n = 7). *p < 0.01 and ***p < 0.001 versus NOR group by Tukey's multiple comparison test. ns: not significant.*

80 mg/kg AW and 80 mg/kg EA administration groups than in the NOR group ($p < 0.001$; Fig. 4B); increased sleep time was attributed to increased NREM sleep time (Fig. 4D). In particular, increased NREM sleep time in the 80 mg/kg AW administration group was owing to increased θ-waves, whereas that in the 80 mg/kg EA administration group was owing to increased δ-waves. In the 80 mg/kg AW and 100 mg/kg EA administration groups, NREM and δ-wave sleep times increased in a dose-dependent manner. However, EA showed a slightly better quality of sleep than AW.

EEG changes induced by EA were measured in SD rats with caffeine-induced insomnia (Fig. 5). Sleep and awake times, excluding REM sleep time, were significantly different between the NOR and CON groups, indicating induction of insomnia. However, 100 mg/kg EA, which showed sleep-promoting activity in the insomnia model (Fig. 3), significantly increased the sleep time compared to the CON group ($p < 0.001$; Fig. 5). As shown in Fig. 4, the increase in sleep time was owing to an increase in NREM sleep time. Similarly, an increase in δ-wave sleep contributed to an increase in NREM sleep.

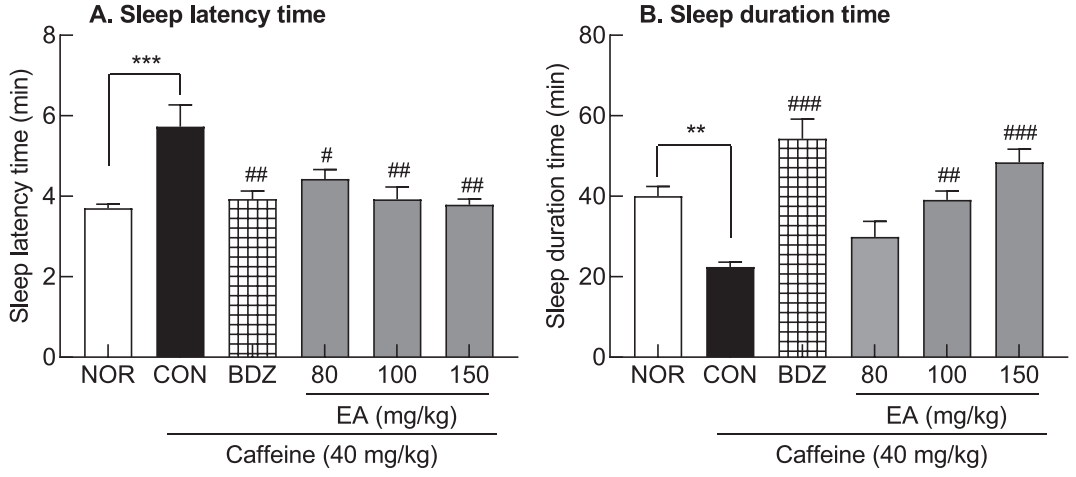

Fig. 3. ***Sleep latency (A) and duration (B) of caffeine-induced insomnia mice after enzyme-treated Ashwagandha root water extract (EA) administration.*** *NOR: normal group, CON: caffeine control group, BDZ: 0.2 mg/kg benzodiazepine and caffeine group, EA80, 100, and 150: EA (80, 100 and 150 mg/kg, respectively) and caffeine mixed treatment groups. Values are presented as the mean ± standard error of the mean (SEM) for each group (n = 7). **p < 0.01 and ***p < 0.001 versus NOR group, #p < 0.05, ##p < 0.01, and ###p < 0.001 vs CON group by Tukey's multiple comparison test.*

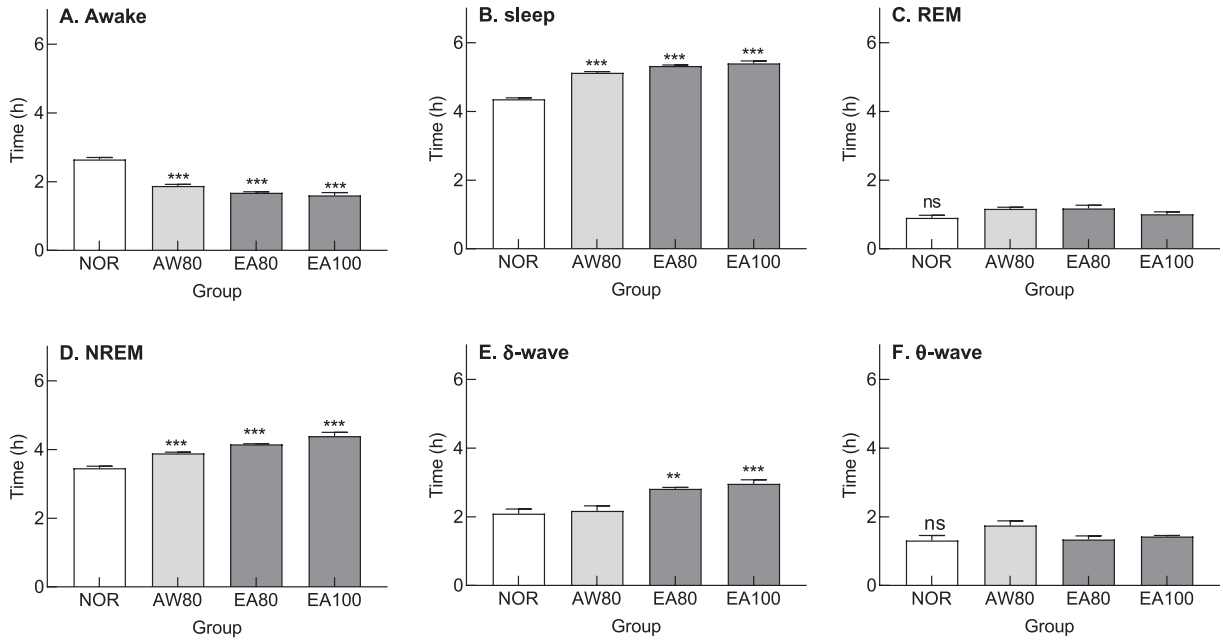

Fig. 4. ***Electroencephalogram (EEG) changes in SD rats upon enzyme-treated Ashwagandha root water extract (EA) and Ashwagandha root water extract (AW) administration.*** *AW80: 80 mg/kg AW group, EA80 and 100: 80 and 100 mg/kg EA, respectively, groups, REM: rapid eye movement sleep, NREM: non-rapid eye movement sleep. Values are presented as the mean ± standard error of the mean (SEM) for each group (n = 6). **p < 0.01 and ***p < 0.001 versus NOR group by Tukey's multiple comparison test. ns: not significant.*

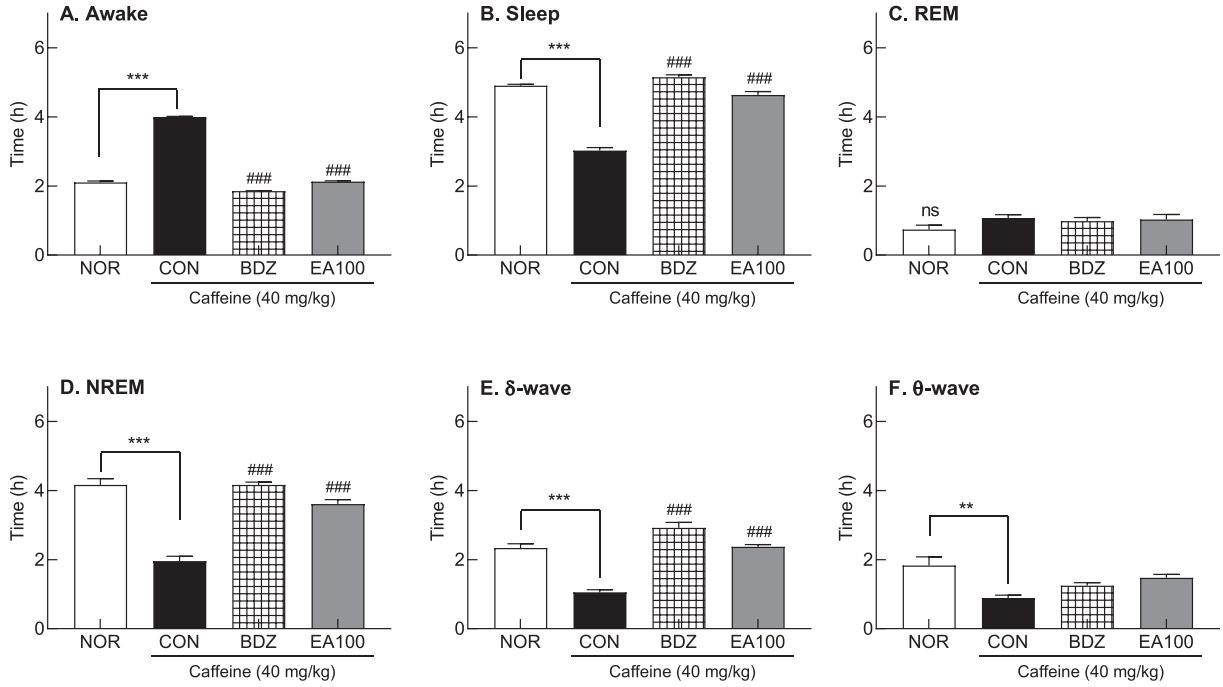

Fig. 5. ***Electroencephalogram (EEG) changes in caffeine-induced insomnia SD rats upon enzyme-treated Ashwagandha root water extract (EA) and Ashwagandha root water extract (AW) administration.*** *NOR: saline group, CON: saline and caffeine group, BDZ: 0.2 mg/kg benzodiazepine and caffeine group, EA100: 100 mg/kg EA group, REM: rapid eye movement sleep, NREM: non-rapid eye movement sleep. Values are presented as the mean ± standard error of the mean (SEM) for each group (n = 6). **p < 0.01 and ***p < 0.001 versus NOR group, ###p < 0.001 vs CON group by Tukey's multiple comparison test. ns: not significant.*

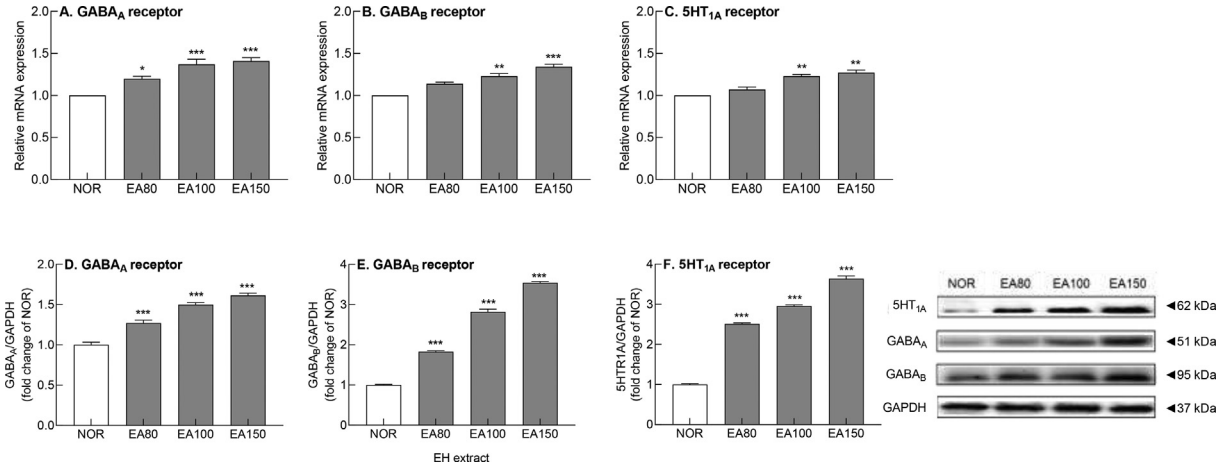

*Fig. 6. Effect of EA on the mRNA and protein expression of GABA$_A$, GABA$_B$, and 5HT$_{1A}$ receptors in the mouse brain. Values are presented as the mean ± standard error of the mean (SEM) for each group (n = 7). *p < 0.05, **p < 0.01, and ***p < 0.001 versus NOR group by Tukey's multiple comparison test. GABA, γ-aminobutyric acid; 5HTR1A, serotonin 1A receptor.*

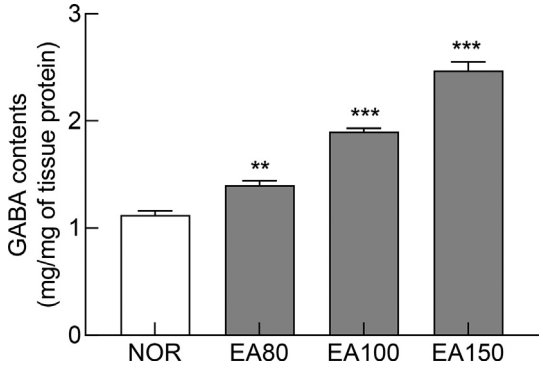

*Fig. 7. Effect of EA on the GABA content in the mouse brain. Values are presented as the mean ± standard error of the mean (SEM) for each group (n = 7). **p < 0.01 and ***p < 0.001 versus NOR group by Tukey's multiple comparison test. GABA: γ-aminobutyric acid.*

3.5. Effects of EA on the mRNA and protein expression of receptors and the GABA content in the brain

Analysis of the mRNA expression of receptors after EA administration for four weeks (Fig. 6A–C) revealed that the expression of GABA$_A$ and GABA$_B$ receptors was increased. Expression of sleep-related receptors, GABA$_A$, GABA$_B$ and 5HT$_{1A}$ receptor, significantly higher in the 100 mg/kg and 150 mg/kg EA administration groups than in the NOR group ($p < 0.01$ and $p < 0.001$, respectively).

In addition, western blotting revealed that EA significantly increased the expression of the GABA$_A$, GABA$_B$, and 5HT$_{1A}$ receptors in a dose-dependent manner ($p < 0.01$ and $p < 0.001$, respectively; Fig. 6D–F). Collectively, EA administration increased not only the mRNA expression of GABA$_A$, GABA$_B$, and 5HT$_{1A}$ receptors but also their protein expression. These findings suggest that these three receptors are likely to be involved in the sleep-promoting activity of EA.

Furthermore, the GABA content in mouse brains after four weeks of oral EA administration was measured. EA dose-dependently and significantly increased the GABA content in the brain compared to the NOR group ($p < 0.01$ and $p < 0.001$, respectively; Fig. 7).

3.6. Effect of GABA$_A$ receptor antagonists on the sleep-promoting activities of EA

A GABA$_A$ receptor antagonist was used to analyze whether EA binds to any site on the GABA$_A$ receptor to exhibit its sleep-promoting activity. Changes in the sleep activity following treatment with antagonists, namely PIX, BIC, and FMZ, were analyzed to confirm the GABA$_A$ receptor-binding site of EA (Fig. 8). Sleep latency was not significantly different among the groups. Contrarily, sleep duration was significantly different between the EA and NOR groups ($p < 0.001$). There was no significant difference in sleep duration between the NOR group and antagonist alone groups (PIX, BIC, and FMZ). On the contrary, sleep duration was significantly different between the antagonist and EA co-administration (PIX + EA, BIC + EA, and FMZ + EA) groups and the NOR group as well as the EA group. Thus, it is presumed that EA binds to the binding sites of PIX, BIC, and FMZ on the GABA$_A$ receptor to exhibit its sleep-promoting activity. In addition, various active substances contained in EA may be responsible for this result.

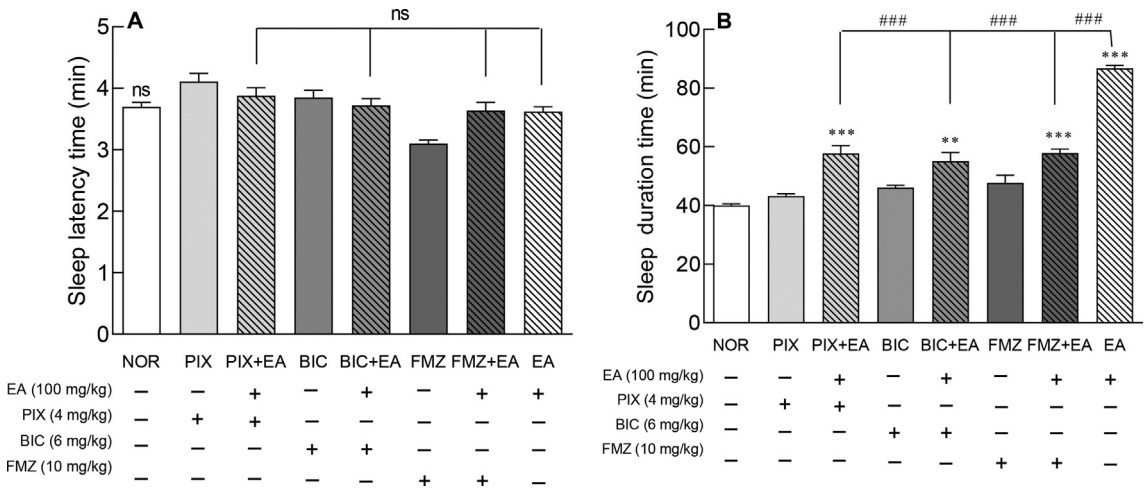

*Fig. 8. Effect of $GABA_A$ receptor antagonists on the sleep-promoting activities of enzyme-treated Ashwagandha root water extract (EA) in ICR mice. In the experimental group except for the NOR group, $GABA_A$ receptor antagonists (PIX: 4 mg/kg, BIC: 6 mg/kg, FMZ: 10 mg/kg) were intraperitoneally administered to the left side. PIX + EA, BIC + EA, FMZ + EA, and EA administration groups were orally administered 100 mg/kg of EA 15 min after antagonist administration, and saline was orally administered to the other experimental groups. After 40 min of oral administration of the sample, all experimental groups administered pentobarbital (42 mg/kg) intraperitoneally on the right side. Values are presented as the mean ± standard error of the mean (SEM) for each group (n = 7). **p < 0.01 and ***p < 0.001 versus NOR group, ###p < 0.001 vs EA group by Tukey's multiple comparison test. ns: not significant. PIX: picrotoxin, BIC: bicuculline, FMZ: flumazenil.*

4. Discussion

Sleep is a physiological process essential to maintain homeostasis; therefore, sleep disturbance acts as a stress factor and affects physical symptoms and brain functions [2]. Sleep disorders are usually treated using pharmacological and non-pharmacological approaches. Most pharmacological substances are associated with various side effects, such as dependence and tolerance; therefore, non-pharmacological treatment is preferred, and among the various non-pharmacological treatments, medicinal herbs are preferred [28]. Ashwagandha, a medicinal herb used to treat sleep disorders, has been used in India for centuries. Natural products such as Ashwagandha contain a variety of polyphenols. Polyphenols such as quercetin-3-glucuronide, kaempferol, and catechin have been reported to act on the brain by being transported through the blood-brain barrier [29]. Although the active substances of EA were not identified in this study, the polyphenols (13.88 μg/mg) of EA appear to contribute to sleep promotion. Numerous clinical studies revealed that it is effective in improving sleep disorders, and its sleep-promoting effect is presumed to be because of GABAergic activity [18], but there are limited studies demonstrating the action mechanism. Therefore, in this study, the sleep-promoting activity and action mechanism of the Ashwagandha root extract were analyzed through animal experiments.

Oral administration of AW (80 mg/kg or higher) decreased sleep duration compared to 60 mg/kg AW (Fig. 1). Sleep duration did not increase in a concentration-dependent manner owing to the entrapment capacity of the starch contained in the extract. Ashwagandha root reportedly contains 13.1–17.4% starch [30], and the extract has a starch content of 7.6–8.2 mg/g [31]. Starch has an entrapping capacity because the inside of the helical structure of amylose is hydrophobic [32]. In this study, after starch-degrading enzyme treatment of AW, the sleep duration increased in a concentration-dependent manner (Fig. 2). Administration of an ethanol extract of Ashwagandha root has been reported to have a hypoglycemic effect in a diabetic rodent model [33]. However, EA administration for 4 weeks showed a tendency to increase blood glucose compared to normal mice, but there was no significant difference (Supplementary Table 2 (https://www.jfda-online.com/journal/vol31/iss2/6/)). EA seems to slightly increase blood sugar by enzymatically breaking down the starch in Ashwagandha root to increase its reducing sugar content.

To improve sleep disorders, it is important to increase the sleep time; however, the quality of sleep is also important. Sleep is divided into REM and NREM sleep [34]. During REM sleep, mental activity is refreshed, whereas, during NREM sleep, both the brain and body are resting and physical vitality is restored. Under normal conditions, sleep begins

with NREM sleep, REM sleep occurs after latency, and NREM and REM sleep alternate periodically at regular intervals during the night. In NREM sleep, δ-waves increase; this sleep is called deep sleep or slow-wave sleep. In the present study, EA increased NREM sleep, which was attributed to increased δ-wave sleep (Fig. 4). In addition to increasing the sleep duration, EA increased δ-wave sleep, which is deep sleep.

In addition, EA decreased sleep latency and increased sleep time in the caffeine-induced insomnia model (Fig. 3). Moreover, the awakening time increased and slow-wave sleep decreased (Fig. 5). Caffeine, which is used to induce insomnia, is a methylxanthine compound that increases arousal, induces cortical activation, and reduces fatigue. Xanthine-based compounds inhibit the action of phosphodiesterase, which metabolizes intracellular cAMP and blocks adenosine receptors on cell membranes. Caffeine induces arousal by blocking adenosine receptors in the basal forebrain and decreases slow-wave activity in the cortex [35]. Here, EA showed sleep-promoting activity in the caffeine-induced insomnia model, and the increase in sleep time was attributed to increased δ-wave sleep, which is deep sleep.

Sleep drugs, which are most commonly used, increase GABA neurotransmission and bind to a specific site on $GABA_A$ receptors to induce sleep. Many medicinal plants also exhibit sleep-promoting effects by enhancing GABAergic signaling through the interaction with $GABA_A$ receptors [36]. An increase in the content of GABA, a neurotransmitter in the brain, typically results in emotional stability, antipsychotic anxiety, anticonvulsant effects, sleep induction, and sleep maintenance [37,38].

GABA acts as a ligand, binds to the $GABA_A$ receptor [39], and acts as an inhibitory neurotransmitter in the central nervous system. Moreover, because the ventrolateral preoptic nucleus is activated in the hypothalamus, a large amount of GABA is secreted by suppressing various arousal centers in the brain, leading to sleep [40]. In this study, the GABA content increased following EA administration (Fig. 7), and this sleep-promoting activity is related to the GABAergic system [19]. $GABA_A$ receptors are chlorine ion channels with binding sites for GABA, barbiturates, BDZ, steroids, and PIX [41]. The metabolic $GABA_B$ receptor is not involved in waking time, but is involved in the maintenance of deep sleep [42]. Here, EA increased the mRNA and protein expression of $GABA_A$ and $GABA_B$ receptors (Fig. 6).

Serotonin (5-HT) is also an inhibitory neurotransmitter involved in circadian rhythms [43]. In particular, $5\text{-}HT_{1A}$, a serotonin receptor, has been reported to suppress REM sleep by regulating the sleep-wake effect of serotonin [44]. EA also increased the mRNA and protein expression of $5\text{-}HT_{1A}$ receptors, which interact with serotonin, a metabolite of tryptophan [45]. EA did not have a significant effect on sleep latency of pentobarbital-treated mice (Fig. 2A), but showed a significant decrease in waking time in the caffeine-induced insomnia model (Fig. 3A). Additionally, there was a significant increase in slow-wave sleep (Fig. 4E).

The sleep-promoting activity of EA was attenuated by PIX (a non-competitive inhibitor), BIC (a competitive antagonist), and FMZ (a BDZ antagonist), which are $GABA_A$ receptor antagonists (Fig. 8). $GABA_A$ receptor antagonists act by inhibiting the binding of agonists. Previous studies have also reported that treatment with a $GABA_A$ receptor antagonist alone does not affect sleep latency and sleep time in a pentobarbital-induced mouse model [25,46]. Similarly, in this study, a single dose of a $GABA_A$ receptor antagonist did not affect sleep in mice. In a previous study, Bhattarai et al. [47] reported that the increase in ^{36}Cl uptake by Ashwagandha was blocked by BIC and PIX. In addition, docosanyl ferulate, an active ingredient in Ashwagandha extract, has an anxiolytic effect by acting on the BDZ site of the $GABA_A$ receptor [48]. The $GABA_A$ receptor, which is a target of medicinal plants and somnifacient drugs, contains a binding site for GABA, BDZ, PIX, barbiturates, and steroids [49]. The sleep-promoting activity of EA involves various sites on the $GABA_A$ receptor, which may be because of the presence of various active ingredients in the extract. Withaferin A- and withanolide A-containing Ashwagandha do not activate $GABA_A$ and $GABA_{A\rho1}$ receptors, suggesting that other components in Ashwagandha induce $GABA_A$ receptor-mediated responses [50].

5. Conclusion

Taken together, EA dose-dependently improved NREM sleep, increasing total sleep time and effectively alleviated insomnia symptoms in a caffeine-induced insomnia model. In addition, EA administration upregulated mRNA and protein expression levels of $GABA_A$, $GABA_B$ and $5HT_{1A}$ receptors and increased GABA content in the brain. In particular, EA was confirmed to effectively increase sleep time by acting as a potential agonist for various binding sites of the $GABA_A$ receptor. The above results

suggest that the sleep promoting activity of EA is due to the GABAergic action. Therefore, the active substances contained in the Ashwagandha extract should be identified in future studies.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

This work was supported by the Technology development Program (RS-2022-00167151) funded by the Ministry of SMEs and Startups (MSS, Korea).

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