



# Sleep Promoting Effect of Luteolin in Mice via Adenosine A1 and A2A Receptors

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## Abstract

Luteolin, a widespread flavonoid, has been known to have neuroprotective activity against various neurologic diseases such as epilepsy, and Alzheimer's disease. However, little information is available regarding the hypnotic effect of luteolin. In this study, we evaluated the hypnotic effect of luteolin and its underlying mechanism. In pentobarbital-induced sleeping mice model, luteolin (1, and 3 mg/kg, p.o.) decreased sleep latency and increased the total sleep time. Through electroencephalogram (EEG) and electromyogram (EMG) recording, we demonstrated that luteolin increased non-rapid eye movement (NREM) sleep time and decreased wake time. To evaluate the underlying mechanism, we examined the effects of various pharmacological antagonists on the hypnotic effect of luteolin. The hypnotic effect of 3 mg/kg of luteolin was not affected by flumazenil, a GABA<sub>A</sub> receptor-benzodiazepine (GABAAR-BDZ) binding site antagonist, and bicuculine, a GABAAR-GABA binding site antagonist. On the other hand, the hypnotic effect of 3 mg/kg of luteolin was almost completely blocked by caffeine, an antagonist for both adenosine A1 and A2A receptor (A1R and A2AR), 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), an A1R antagonist, and SCH-58261, an A2AR antagonist. From the binding affinity assay, we have found that luteolin significantly binds to not only A1R but also A2AR with IC<sub>50</sub> of 1.19, 0.84 μg/kg, respectively. However, luteolin did not bind to either BDZ-receptor or GABAAR. From these results, it has been suggested that luteolin has hypnotic efficacy through A1R and A2AR binding.

**Key Words:** Luteolin, Sleep, Electroencephalogram, Adenosine A1 receptor, Adenosine A2A receptor, Insomnia

## INTRODUCTION

Sleep is essential to maintain human health and well-being, and sleep disturbances can affect negatively both physical and psychological health (Krueger *et al.*, 2008). Studies have shown that the prevalence of sleep disorders increase with age so that almost up to 50% of elderly people (>65 yr) suffer from sleep disturbances (Rashid *et al.*, 2012). Thus, improvement of sleep quality, especially among elderly people, is considered to be essential care issues. The most common case among sleep disturbances that affect elderly people is insomnia worldwide (Neikrug and Ancoli-Israel, 2010). To improve sleep quality, most elderly people with insomnia are known to take hypnotic agents such as benzodiazepines, antidepressants, and antihistamines (Bloom *et al.*, 2009). How-

ever, long-term use of these drugs have been demonstrated to often result in tolerance and dependence, thus leading to withdrawal syndrome following their discontinuation (Rudolph and Knoflach, 2011).

To overcome the adverse effects of sleep medications, special attention has been recently focused on alternative sleep aids such as natural and herbal therapies (Cho *et al.*, 2012). According to the recent report, over 1.6 million American people have used alternative medications to manage insomnia (Pearson *et al.*, 2006). In addition, there has been increasing needs for a new class of alternative natural products with sleep improving effects (Adib-Hajbaghery and Mousavi, 2017). Phytochemicals are known as plant-derived biologically active ingredients, and they are responsible for the pharmacological roles of plant extracts (Nabavi *et al.*, 2015a). Among

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them, especially flavonoids, a large group of plant secondary metabolites, are the most effective chemical classes with various pharmacological effects, such as antioxidant, anti-inflammatory, anticancer and neuroprotective effects (Nabavi *et al.*, 2015b). Flavonoids are ubiquitously found in the human diet and often found abundantly in various medicinal plants (Wasowski and Marder, 2012).

Among various flavonoids, apigenin and chrysin have been known to have hypnotic effects with benzodiazepine (BDZ)-binding mechanisms (Wolfman *et al.*, 1994; Zanolini *et al.*, 2000; Wasowski and Marder, 2012). While luteolin is also a well-known bioactive flavonoid which is structurally very close to apigenin and chrysin, there is little information about its hypnotic effect. In the present study, therefore, we evaluated the hypnotic effect of luteolin in pentobarbital-induced sleep model in mice. We also investigated the effect of luteolin on sleep architectures [wake, non-rapid eye movement (NREM), REM] by recording electroencephalogram (EEG) and electromyogram (EMG). Furthermore, as we were interested whether luteolin can interact with the GABA<sub>A</sub> receptor BDZ binding site (GABA<sub>A</sub>-BDZ), we performed binding assay using [<sup>3</sup>H] flunitrazepam.

## MATERIALS AND METHODS

### Chemicals

Pentobarbital and diazepam (DZP, a reference hypnotic drug) was purchased from Hanlim Pharm. Co. Ltd. (Seoul, Korea) and Myungin Pharm. Co. Ltd. (Seoul, Korea), respectively. Luteolin, flumazenil, bicuculline and caffeine were purchased from Sigma–Aldrich Inc (St. Louis, USA). 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), and SCH58261 were purchased from Tocris Biosciences (Avonmouth, UK). In GABA<sub>A</sub> binding assay, the radioligand [<sup>3</sup>H] flunitrazepam (NEN Perkin-Elmer, Ontario, CA, USA) used for GABA<sub>A</sub>-BDZ agonist, and [<sup>3</sup>H] muscimol (NEN Perkin-Elmer) were used for GABA<sub>A</sub>-GABA binding site (GABA<sub>A</sub>-GABA) agonist. For the adenosine 1 receptor (A1R), and adenosine 2A recep-

tor (A2AR) binding assay, the radioligand [<sup>3</sup>H] CCPA and [<sup>3</sup>H] CGS21680, respectively, were purchased from NEN Life Science Products (Boston, MA, USA).

### Animals

All animals were purchased from Orient Bio Inc (Seongnam, Korea). Male Imprinting Control Region (ICR) mice of 8 weeks old (weigh 25-30 g) were used in pentobarbital-induced sleep.

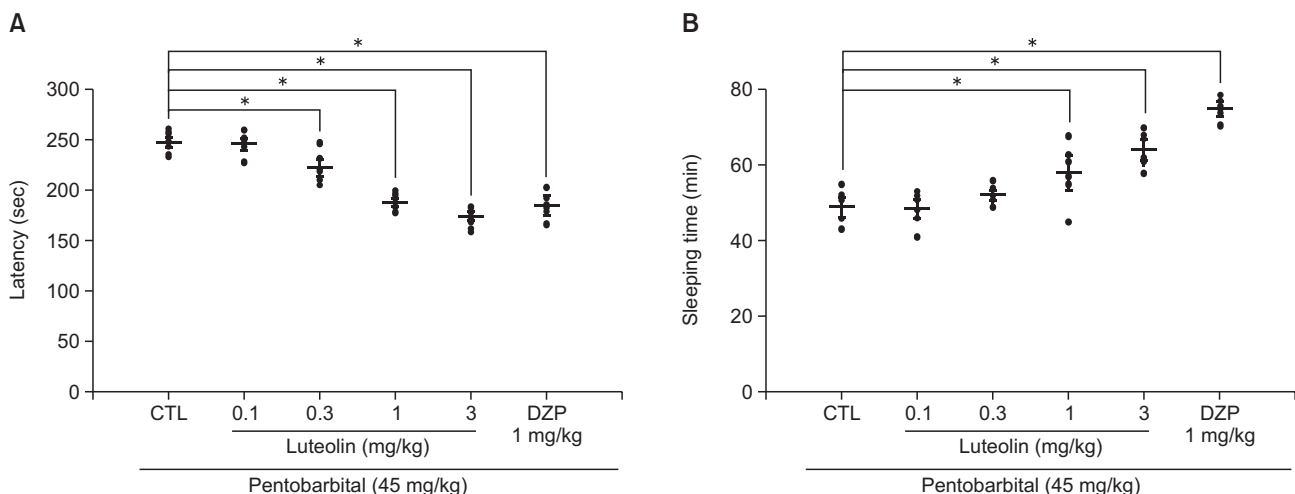
Male C57BL/6 mice of 4 weeks old (weigh 20-25 g) were used for measurements of EEG architectures. Mice were housed at 24°C and 55% humidity, with food and water were ad libitum. The room maintained 12 h light/dark cycle (light on at 8:00 and off at 20:00). Animals were acclimated for over 1 week before experiments. All animals related study protocols were approved by the Committee on Animal Research at Ajou University (Permission No. 2018-0051).

### Pentobarbital-induced sleep test

Pentobarbital-induced sleep test were performed between 13:00 and 17:00. Luteolin, flumazenil, bicuculline, caffeine, and pentobarbital were suspended in saline. DPCPX and SCH58261 were suspended in 1% aqueous solution of Tween 80. Luteolin or saline was orally administered to mice 30 min before pentobarbital injection. Antagonists for GABA<sub>A</sub>, A1R and A2AR were orally administered 15 min before luteolin administration. Control and vehicle mice were administered with saline or 1% Tween 80 at the same time for compared to the sample administration group. After pentobarbital injection (45 mg/kg i.p.), mice were placed in individual cages for the test. For sleep latency, the time was recorded from pentobarbital injection to the time when righting reflex disappeared. For sleep duration, the time was recorded until the righting reflex recovered.

### Measurement of EEG and EMG

EEG and EMG were recorded from 09:00 to 15:00 according the method described previously (Dela Pena *et al.*, 2016). Briefly, mice were anesthetized with pentobarbital sodium (50



**Fig. 1.** Effects of luteolin on sleep latency (A) and sleep duration (B) in mice administered a hypnotic dose (45 mg/kg, i.p.) of pentobarbital. Mice injected pentobarbital (i.p.) 30 min after oral administration (p.o.) of CTL (saline), DZP (1 mg/kg), or luteolin (0.1, 0.3, 1, and 3 mg/kg). Each column represents mean  $\pm$  SEM (n=6). \* $p$ <0.05, significant as compared to the control group.

mg/kg, i.p.). Hair was removed, and the skin was disinfected with betadine and alcohol. Head mount (Pinnacle Technology, Inc., Lawrence, KS, USA) was implanted with EEG and EMG electrodes for polysomnographic recordings. The front part of the head mount was placed 3 mm anterior to bregma. After that, four screws were perforated into the skull for EEG recording. Two wire electrodes were inserted into the nuchal muscle for EMG recording. 1 week after surgery for recovery, EEG and EMG recordings were performed in the cage which designed to allow mice to move freely. Recording was performed for 2 days in same animal. Data collected in first day served as a vehicle to compare with second experimental day. Luteolin or saline was administered at 9:00.

The EEG and EMG signals were routed to an 8401 conditioning/acquisition system (Pinnacle Technology, Inc.) via a tether and low-torque commutator (Part #8408, Pinnacle Technology, Inc.). Continuous sections of recorded EEG and EMG data were divided to 10 s (1 epoch). Each epoch was scored as wake, NREM or REM sleep based on which type of waveform occupied >50% of that time. The absolute EEG power recorded wakefulness, NREM and REM in the range of from 0.5 to 20 Hz. NREM, REM and wakefulness were calculated in  $\delta$  (0.5-4.0 Hz),  $\theta$  (5.0-9.0 Hz),  $\alpha$  (8.0-13.0 Hz). Values measured were calculated in Microsoft Excel (WA, USA).

**Binding assay for GABAAR**

The radioligand GABAAR binding assay was performed with Eurofins (St. Charles, IL, USA) Pharmacology Services. The cell membrane pellet from brain tissues of mice was obtained by the method described previously (Risa *et al.*, 2004). The final membrane suspensions concentration was 2.5  $\mu$ g protein per 100  $\mu$ l binding buffer. Various concentration of luteolin (1, 3, 10, and 30  $\mu$ M) and 1 nM [<sup>3</sup>H] flunitrazepam and [<sup>3</sup>H] muscimol (10  $\mu$ l) was added in suspension (180  $\mu$ l) and incubated on the ice for 40 min. For terminating the binding reaction, rapid filtration onto a glass fiber filter (Whatman GF/C) with ice-cold Tris-HCl buffer (30 mM) were performed to remove unbound [<sup>3</sup>H] flunitrazepam or [<sup>3</sup>H] msucimol. The amount of filter-bound radioactivity was counted using a liquid scintillation counter (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA).

**Binding assay for A1R and A2AR**

The radioligand binding assay for A1R and A2AR was performed with Eurofins Pharmacology Services as described by Rivkees *et al.* (1995). A1R overexpressed CHO cells and A2R overexpressed HEK293 cells were used. Cell were harvested, pelleted and resuspended to a final protein concentration of 0.5 mg/ml for binding assay. Radioligands, [<sup>3</sup>H] CCPA (33 Ci/mmol) and [<sup>3</sup>H] CGS21680 (30 Ci/mmol) were used. The concentration of final cell suspension was 75-150  $\mu$ g protein per 50  $\mu$ l. Various concentrations of luteolin (0.3, 1, 3, 10, and 30  $\mu$ M) and 1 nM [<sup>3</sup>H] CCPA or 6 nM [<sup>3</sup>H] CGS21680 was added in Multiscreen plates (Millipore, Bedford, MA, USA). After incubated for 1 h in room temperature, vacuum filtration was performed to separate bound radioactivity. Amount of bound radioactivity was counted using liquid scintillation counting.

**Statistical analysis**

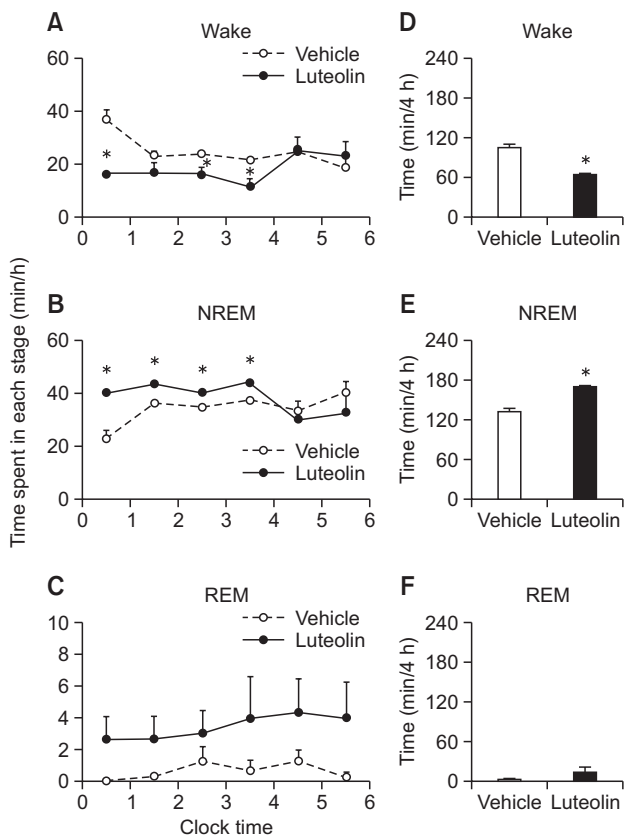
All data are expressed as mean  $\pm$  SEM. Significance was evaluated by analysis of variance (ANOVA). Differences between two-group with  $p < 0.05$  were considered to be statis-

tically significant. For the significance analysis we used the Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA).

**RESULTS**

**Luteolin improved pentobarbital-induced sleeping behaviors**

To evaluate hypnotic activity of luteolin, we examined its effect on pentobarbital-induced sleep in mouse. After injection of a hypnotic dose of pentobarbital (45 mg/kg, i.p.), sleep latency and duration of the control group were 248.0  $\pm$  5.8 sec and 49.6  $\pm$  2.7 min, respectively (Fig. 1). As expected, a well-known GABAAR-BDZ receptor agonist, DZP (1 mg/kg, p.o.) significantly promoted sleep latency and duration ( $p < 0.01$ ) relative to the control group. Luteolin (0.1-3 mg/kg, p.o.) also decreased sleep latency (Fig. 1A) and increased sleep duration in a dose-dependent manner (Fig. 1B). In particularly, oral administration of 3 mg/kg of luteolin was found to increase sleep duration up to 64.8  $\pm$  1.2 min. This result suggests that luteolin has hypnotic effect on pentobarbital-induced sleep in mice.



**Fig. 2.** Time courses of changes of Wake (A), NREMS (B), and REMS (C) after the administration of luteolin. Open circles indicate the vehicle and filled circles indicate the luteolin (3 mg/kg). Total time spent in Wake (D), NREMS (E) and REMS (F) for 4 h after administration of luteolin. Each circle represents the hourly mean  $\pm$  SEM (n=5) of Wake, NREMS, and REMS. Each column represents the mean  $\pm$  SEM (n=5). \* $p < 0.05$  compared with vehicle.

### Luteolin improved sleep architectures and changed power density

After oral administration of luteolin (3 mg/kg, p.o.) to the mice, the sleep architectures (NREM sleep, REM sleep, wake) were recorded. As shown in Fig. 2, compared to control group (Vehicle), luteolin significantly increased NREM sleep (approximately 16.7%). In addition, wake time was significantly decreased by luteolin approximately 40.9%. Luteolin (3 mg/kg) also changed EEG power density so that in NREM sleep, the power density of  $\delta$ -wave significantly increased about 9.7% (Fig. 3).

### Hypnotic mechanism of luteolin did not involve GABAAR

In order to investigate whether GABAAR is involved in the hypnotic mechanisms of luteolin, we used pharmacological antagonists for GABAAR-BDZ (flumazenil), and GABAAR-GABA (bicuculline). As shown in Fig. 4, the GABAAR-BDZ agonist, DZP (1 mg/kg), significantly decreased sleep latency and increased sleep duration—an effect that was blocked by administration of flumazenil. Unlike DZP, the hypnotic effect of luteolin (3 mg/kg) was not affected by either flumazenil or bicuculline. We also evaluated the binding affinity of luteolin for GABAAR-BDZ or GABAAR-GABA. As expected, luteolin was not displaced by both [ $^3$ H] flunitrazepam, and [ $^3$ H] muscimol binding (Table 1). These results suggested that, hypnotic effect of

luteolin were not related with GABAAR-BDZ, and GABAAR-GABA.

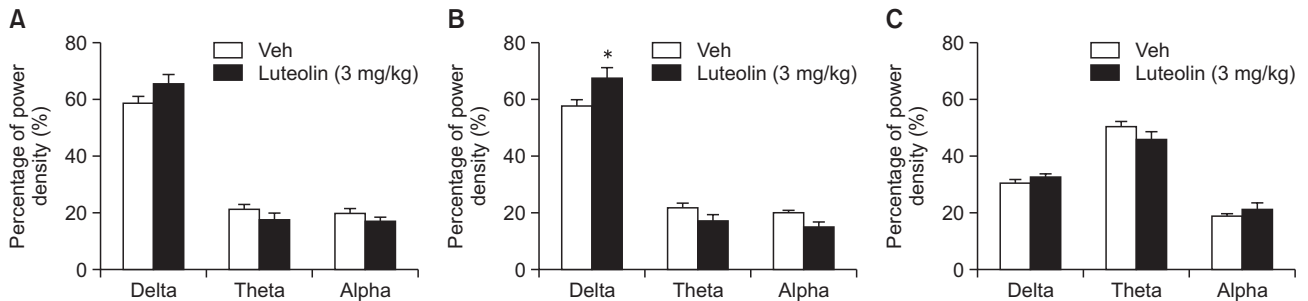
### Hypnotic mechanism of luteolin involved both A1R and A2AR

To test whether the hypnotic effects of luteolin were related with AR, we used caffeine (an A1R, A2AR antagonist), DP-CPX (an A1R antagonist), and SCH58261 (an A2AR antagonist). As shown in Fig. 5, the hypnotic effect of luteolin (3 mg/kg) was antagonized by caffeine, DP-CPX and SCH58261. We also evaluated the binding affinity of luteolin for A1R, and A2AR. Luteolin was found to displace over 90% of both [ $^3$ H] CCPA, and [ $^3$ H] CGS21680 binding at 10  $\mu$ g/ml. Its  $IC_{50}$  values for A1R and A2AR were 1.19 and 0.84  $\mu$ g/ml, respectively (Table 1). These results suggest that hypnotic effect of luteolin involves both A1R and A2AR.

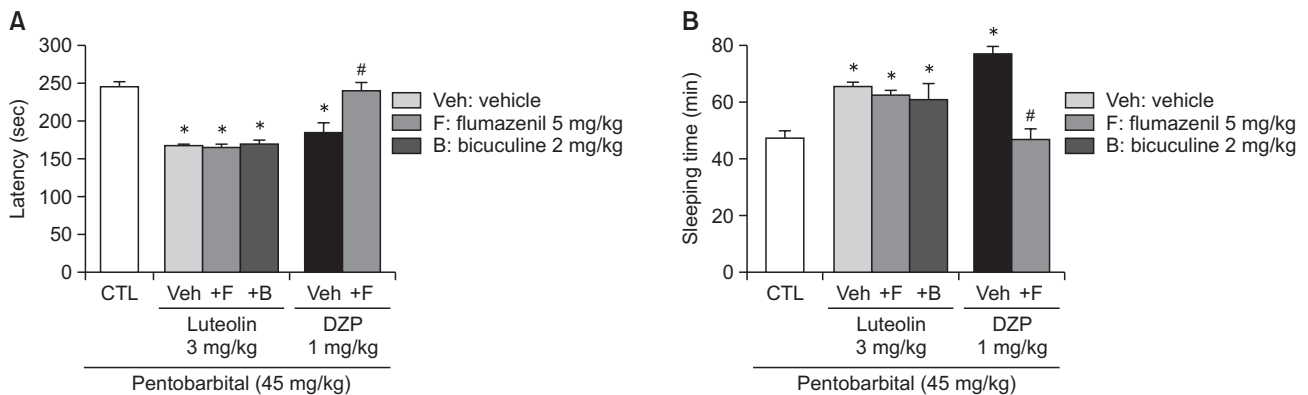
## DISCUSSION

The major finding of this study is that luteolin improves quantity and quality of sleep, especially NREM, and its underlying mechanisms may involve A1R and A2AR binding, but neither GABAAR-BDZ nor GABAAR-GABA binding.

Sleep disorders can be caused by lack of sleep, exces-



**Fig. 3.** The effects of luteolin on EEG power density of Wakefulness (A), NREM sleep (B) and REM sleep (C). The power density was classified with  $\delta$  wave (0.5-4 hz), theta-wave (4-8 hz) and alpha-wave (8-12 hz). Each bar represents mean  $\pm$  SEM (n=5). \* $p$ <0.05, significant as compared to the Vehicle.

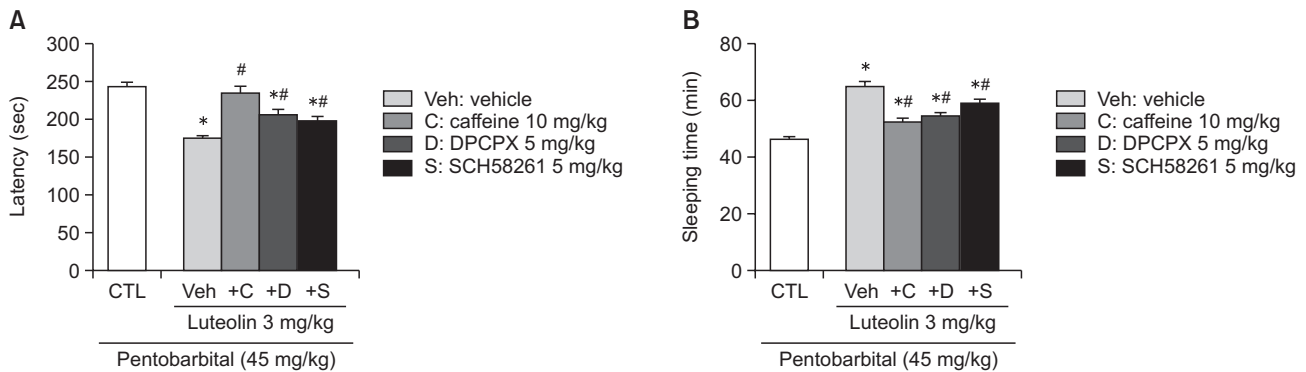


**Fig. 4.** Effects of GABAAR antagonist on the changes in sleep latency (A) and sleep duration (B) in mice treated with luteolin. Mice were injected with pentobarbital (i.p.) 30 min after oral administration (p.o.) of CTL (saline), DZP (1 mg/kg) or luteolin (3 mg/kg). Vehicle (saline), flumazenil (5 mg/kg, p.o.), or bicuculline (2 mg/kg, p.o.) was administered 15 min before luteolin administration. Each column represents mean  $\pm$  SEM (n=6). \* $p$ <0.05, significant as compared to the control group. # $p$ <0.05, significant as compared to each vehicle group. B, bicuculline; CTL, control; DZP, diazepam; F, flumazenil; Veh, vehicle.

**Table 1.** Effect of luteolin on radioligands binding to GABA and adenosine receptors

Receptor subtype	Luteolin ( $\mu\text{M}$ )					IC <sub>50</sub> ( $\mu\text{M}$ )
	0.3	1	3	10	30	
GABAAR-BZD	-	4.3 ± 1.8	3.3 ± 0.5	4.0 ± 3.1	3.9 ± 3.4	-
GABAAR-GABA	-	0.1 ± 4.2	3.2 ± 8.4	7.2 ± 0.4	3.9 ± 3.2	-
A1R	11.7 ± 2.1	24.0 ± 1.9	50.3 ± 3.1	76.5 ± 1.2	94.6 ± 0.9	1.19
A2AR	6.8 ± 3.3	22.9 ± 0.7	56.2 ± 0.2	83.8 ± 1.1	97.6 ± 2.4	0.84

The results were expressed as mean ± SD (n=3).



**Fig. 5.** Effects of adenosine receptor antagonist on the changes in sleep latency (A) and sleep duration (B) in mice treated with luteolin. Mice were injected with pentobarbital (i.p.) 30 min after oral administration (p.o.) of CTL (saline), or luteolin (3 mg/kg). Vehicle (1% Tween80), caffeine (10 mg/kg, p.o.), DPCPX (5 mg/kg, p.o.), or SCH58261 (5 mg/kg, p.o.) was administered 15 min before luteolin administration. Each column represents mean ± SEM (n=6). \**p*<0.05, significant as compared to the control group. #*p*<0.05, significant as compared to the vehicle group. C, caffeine; CTL, control; D, DPCPX; S, SCH58261; Veh, vehicle.

sive amounts of sleep, or abnormal movements during sleep. Among various sleep disorders, insomnia is that the patient reports not only dissatisfaction with sleep (sleep-onset insomnia or sleep-maintenance insomnia) but also other daytime symptoms (sleepiness, attention disorders, mood disorders) for at least 3 nights per week and last for more than 3 months (K. Pavlova and Latreille, 2019). Most epidemiological studies show that about one third (30%-36%) of adults have at least one symptom of insomnia, such as difficulty in starting or maintaining sleep (Ohayon, 2002). Insomnia increases the public health burden and health care costs, and decreased work ability in many people (Atkin *et al.*, 2018). However, this growing burden has not been successfully relieved yet because synthetic chemicals actually failed to prevent efficiently the progress of sleep disorders. Therefore, increasing attentions are focused on the use of natural products and alternative medicine for sleep promotion due to their safety and efficiency (Hu *et al.*, 2018). Indeed, several dietary and plant supplements, such as suanzaoren (*Ziziphi Spinosaes Semen*) and valerian (*Valeriana officinalis*), are already available in clinic (Choi *et al.*, 2018).

Luteolin, one of the important bioactive flavonoids, has been suggested to be a useful therapeutic constituent for neurodegenerative diseases based on not only its anti-inflammatory effects which has a crucial role in the neuroprotection, but also its lipophilic property indicating the possibility to cross blood brain barrier (Orhan *et al.*, 2015; Theoharides *et al.*, 2015; Zhang *et al.*, 2017). In addition, a recent study has reported that luteolin shows an anti-anxiolytic effect via the low affinity for the GABAAR-BDZ (Coleta *et al.*, 2008). In the

present study, we first found a remarkable hypnotic effect of luteolin through a pentobarbital-induced sleep test along with EEG/EMG analysis. Sleep cycle is well known to consist of two parts, NREM and REM. NREM is a relaxed type of sleep that takes a deeper sleep, and REM is an active type of sleep with brain waves activated (Choi *et al.*, 2018). The amount of these two sleep types is known to determine the quality of sleep so that increasing NREM sleep leads to deeper sleep and increased quality of sleep (Meerlo *et al.*, 1997; Meerlo *et al.*, 2001; Kwon *et al.*, 2017). In general, NREM sleep is considered to be the time during which the brain recovers from prior wakefulness (Benington and Heller, 1995; Kamphuis *et al.*, 2015). Among sleep waves of EEG, such as  $\theta$ , waves are known to increase in REM sleep, while  $\delta$  waves increase in NREM sleep (Hutchison and Rathore, 2015). Indeed, slow-wave sleep (SWS) refers to phase 3 sleep, which is the deepest phase of NREM sleep, and is characterized by  $\delta$  waves of EEG. However, many hypnotics acting at GABAAR-BDZ, such as diazepam, are known to reduce EEG  $\delta$  wave in NREM, which limits the use of diazepam. (Bastien *et al.*, 2003; Ishida *et al.*, 2009). Our results showed that sleep-improving effect of luteolin was not affected by either flumazenil or bicuculline, and that luteolin bound neither with GABAAR-BZD nor GABAAR-GABA, suggesting that the sleep-promoting effect of luteolin is not related with GABAAR. On the other hand, luteolin increased not only NREM sleep time but also  $\delta$  waves, and thus suggesting a beneficial role of luteolin in promoting sleep quality as well as sleep time.

Adenosine is known as an endogenous sleep-promoting substance (Zhang *et al.*, 2012). Increased adenosine firing in

specific areas of the brain during prolonged awakening has been reported to play a very important role such as increasing sleep homeostasis (Greene *et al.*, 2017). Adenosine and stable adenosine analogs were shown to induce sleep when administered to experimental animals (Basheer *et al.*, 2004). In physiological concentration of adenosine, it regulates cell function through membrane receptor binding cascade (Fredholm *et al.*, 1999). There are four subtypes of adenosine receptors expressed in the central nervous system: A1R, A2AR, A2BR and A3R. Among them, A1R and A2AR have been reported to be related to sleep regulation (Fredholm *et al.*, 2001). A1R is known to couple to  $G_{i/o}$  protein which can inhibit adenylyl cyclase (AC), whereas A2AR couples to  $G_{s/olf}$  protein which can activate AC. Following the activation of the A2AR, cAMP production increases leading to the activation of protein kinase A (PKA) and phosphorylation of the cAMP response element binding protein (CREB) (Sheth *et al.*, 2014). A1R has been shown high level in hypocretin/orexin neurons of lateral hypothalamus (LH), where is the center for the maintenance of arousal. Adenosine binding to this A1R may results in the reduction in wakefulness, because the neuropeptides orexin A and B produced in LH neurons, play a crucial role in the promotion and maintenance of wakefulness (Cun *et al.*, 2014). In consistent with these studies, a recent report has just revealed that application of A1R agonist, N6-cyclopentyladenosine (CPA), into the perifornical LH area promotes sleep in rats (Alam *et al.*, 2009). In addition, microdialysis of A1R antisense oligonucleotides into the LH has been reported to significantly decrease NREM sleep and increase wakefulness in rats (Thakkar *et al.*, 2003). As for A2AR, it is known to play an important role in sleep promotion, supported by the studies that intracerebroventricular infusion of the A2AR agonist CGS21680 significantly promoted sleep (Satoh *et al.*, 1999). It has also been reported that in urethane-anesthetized rats, CGS21680 increased GABA secretion in histaminergic tuberomammillary nucleus, and that the expression of glutamate decarboxylase 67 (GAD67) was reduced in striatum of A2AR knockout mice (Yin *et al.*, 2017). Thus, A2AR seems to be associated with GABAergic neuron that promotes sleep and plays a role in promoting sleep, although further study is needed to clarify the mechanism between A2AR and GABAergic neuron.

In this study, we found that luteolin directly bound to both A1R and A2AR and with  $IC_{50}$  value of 1.19  $\mu$ g/ml and 0.84  $\mu$ g/ml, respectively. We also found that the sleep-improving effect of luteolin was significantly inhibited by DPCPX and SCH58261, suggesting that the sleep-promoting effect of luteolin may be mediated via activation of not only A1R but A2AR. Further study remains to clarify whether the level of GABA or adenosine in brain tissue can be changed by luteolin. In conclusion, this study for the first time demonstrate that luteolin improves quantity and quality of sleep, especially for NREM in mice. As a mechanism for the effect of luteolin, the involvement of both A1R and A2AR activation, but neither GABAAR-BDZ nor GABAAR-GABA binding seems to be very convincing. From these results, it is suggested that luteolin may have a potential for sleep-promoting agent.

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