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Sedative Effect of *Sophora flavescens* and Matrine

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Abstract

The present study investigated the sedative effects of *Sophora flavescens* (*SF*) and its bioactive compound, matrine through performing locomotor activity test and the electroencephalography (EEG) analysis in the rat. The underlying neural mechanism of their beneficial effects was determined by assessing c-Fos immunoreactivity and serotonin (5-HT) in the brain utilizing immunohistochemical method and enzyme-linked immunosorbent assay. The results showed that *SF* and matrine administration had an effect on normalization of caffeine-induced hyperactivity and promoting a shift toward non-rapid eye movement (NREM) sleep. c-Fos-immunoreactivity and 5-HT level in the ventrolateral preoptic nucleus (VLPO), a sleep promoting region, were increased in the both *SF* and matrine-injected groups. In conclusion, *SF* and its bioactive compound, matrine alleviated caffeine-induced hyperactivity and promoted NREM sleep by activating VLPO neurons and modulating serotonergic transmission. It is suggested that *SF* might be a useful natural alternatives for hypnotic medicine.

Key Words: *Sophora flavescens*, Matrine, Serotonin, Electroencephalography, Locomotor, Sedative effect

INTRODUCTION

Sleep is an essential component for assessing health-related quality of life. Sleep disturbance causes dysregulation of homeostasis and increases vulnerability to inflammation-related diseases (Irwin *et al*., 2016) and chronic diseases (Lima *et al*., 2012). Benzodiazepines are the most commonly prescribed medications for treatment of insomnia and have been reported to be associated with adverse outcomes such as mood disorder, lung disease (Kroll *et al*., 2016) and cognitive deficit (Pariente *et al*., 2016). Regarding these side effects, importance of developing alternative natural medicines has been growing.

Sophora is a genus in the Fabaceae family and the root of *Sophora flavescens* (*SF*), which is known as Kushen, has been used in traditional remedy to treat fever, dysentery and pain (He *et al*., 2015). *SF* has been reported to have an anti-infection (Yanju *et al*., 2014), an anti-rotaviral (Alfajaro *et al*., 2014), a recovery from injury (Tanabe *et al*., 2016) and whitening effect (Shin *et al*., 2013). Matrine is alkaloids which is one of the bioactive components in *SF* and is reported to have pharmacological effects on cardiac diseases (He *et al*., 2015), inflammation (Shao *et al*., 2013) and tumor (Liu *et al*., 2014). Neither *SF* nor matrine have been reported to have sedative effect yet, however, accumulating evidence of correlation between sleep disturbance and inflammatory disease (Irwin *et al*., 2016) suggests that anti-inflammatory efficacy of *SF* and matrine may contribute to sedative effect.

The ventrolateral preoptic nucleus (VLPO) located in the anterior hypothalamus acts as a sleep-promoting center and molecular targets of hypnotic medication (Ferre, 2008). Sleep active neurons have been identified by the immunohistochemical detection of the Fos protein during sleeping. Therefore, it is possible that activation of the VLPO region indicates initiation of sleep-state.

It has been known that neurons in the VLPO contain serotonin (5-HT) and its receptors, which is one of the most important neurotransmitters for its role in regulating sleep-wake homeostasis (Melancon *et al*., 2014; Whitney *et al*., 2016).

Sleep-wake state is assessed by evaluating shifts of the electroencephalogram (EEG) frequencies. A continuous transition from wake period to non-rapid eye movement (NREM) followed by rapid eye movement (REM) occurred (Irwin, 2015). Wakefulness is identified by the predominance of high

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frequency and low amplitude EEG. NREM is scored based on the presence of spindles interspersed with slow waves in the EEG. EEG power during REM was significantly reduced in lower frequency δ-wave and increased in the range of θ-wave activity.

The purpose of this study was to investigate the sedative effects of *SF* and its bioactive compound, matrine through performing locomotor activity test and EEG analysis. The underlying neural mechanism of the beneficial effect was determined by measuring c-Fos immunoreactivity and 5-HT in the brain utilizing immunohistochemical method and enzyme linked immunosorbent assay.

MATERIALS AND METHODS

Animals

All the experimental procedures performed on the animals were conducted with the approval of the Ethics Committee of the Kyung Hee University (KHUAP(SE)-13-041; Seoul, Korea) and in accordance with the US National Institutes of Health (Guide for the Care and Use of Laboratory Animals, 8th edition, revised 2011). Male Sprague-Dawley rats (Samtaco Animal Corp., Gyeonggi, Korea) aged 7 weeks (280-320 g) were housed in an air-conditioned room kept at a temperature of 20-25°C, and a humidity of 45-65%, with a 12:12 h light/dark cycle (lights-on at 8 a.m.). Animals had access to water and food *ad libitum* and were acclimated for 7 days prior to experiments.

Preparations of methanol extract of *SF* and matrine

SF was purchased from Dongwoodang Pharmacy Co., Ltd (Gyungbuk, Korea). A voucher specimen of *SF* has been deposited at the herbarium located at the College of Korean Medicine, Kyung Hee University (Reference No. KHSFLM01). The dried *SF* (200 g) was extracted with 10-fold volume of 70% of methanol in the ultrasound bath and filtered through Whatman filter paper (Maidstone, UK). The extracts were concentrated with a rotary evaporator (Eyela, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) under reduced pressure (-760 mmHg) yielding 22.8 w/w%. Matrine was purchased from EOS MED CHEM Co., Ltd (Jinan, China).

Administration of *SF* and matrine

Methanol extract of *SF* was dissolved in 0.9% saline at a concentration of 400 mg/ml (*SF*400) or 800 mg/ml (*SF*800) then administered per os (p.o.) in a volume of 10 ml/1 kg of body weight. Matrine was dissolved in 0.9% saline at a concentration 30 mg/ml (M30) or 100 mg/ml (M100) then injected intraperitoneally (i.p.) in a volume of 1 ml/1 kg of body weight.

Measurement of locomotor activity

Locomotor activity was measured to assess sedative effect of *SF* and matrine in the caffeine-induced hyperactive animal model. Caffeine was purchased from Sigma-Aldrich Co (St. Louis, MO, USA). Caffeine was dissolved in 0.9% saline at a concentration of 10 mg/ml then was injected once intraperitoneally (i.p.) in a volume of 1 ml/1 kg. *SF*400, *SF*800, M30 or M100 was administered 30 min prior to caffeine injection and locomotor activity was measured in a black polyethylene cuboid (30×30×45 cm). The distance of movements was monitored by computerized video-tracking system using the S-MART program (Panlab Co., Barcelona, Spain) for 2 hours. The first 1 hour was regarded as an adaptation period and data during latter 1 hour were collected and analyzed for locomotor activity.

Surgery

The animals were divided into 5 groups (control; *SF*400 and 800 mg/kg groups; matrine 30 and 100 mg/kg groups, N=6 in each group). EEG electrodes were implanted for polygraphic recording as described in stereotaxic atlas (Paxinos *et al*., 1985). Surgical anesthesia was achieved with pentobarbital (40 mg/kg, i.p.), and rats were chronically implanted with the head mount. Three stainless steel screws were implanted through the head mount into predrilled holes of the skull, and the device was secured with dental cement. The leads from all the electrodes were then fixed to the skull with dental cement and additional screws were inserted in the skull to aid fixation of the connector to the skull. After surgery, each rat was allowed 7 days in an individual transparent barrel for recovery.

Electroencephalography (EEG)

After recovery, rats were habituated to the recording conditions before the test. Oral administrations of *SF* and matrine were loaded 10 min before EEG recording. Recording began at 8:00 pm and 12-h EEG and activity were recorded in all rats. The amount of time in wakefulness, NREM and REM sleep were determined from the digitized data on 10 s epochs using professional animal sleep analysis software SleepSign Ver. 3 software (Kissei Comtec, Nagano, Japan). The software discriminates wakefulness as high-frequency low-amplitude EEG, and NREM was scored on the basis of the presence of spindles interspersed with slow waves in the EEG. EEG power during REM was significantly reduced in lower frequency δ-wave (0.75-4 Hz) and increased in the range of θ-wave activity (5.0-9.0 Hz, peak at 7.5 Hz).

Measurement of serotonin (5-HT) level

After EEG recordings, the animals were deeply anesthetized with sodium pentobarbital (80 mg/kg, administered i.p.) and the brains were immediately removed and coronally sectioned by using rodent brain matrix (ASI instruments Inc., MI, USA). The cortex and VLPO regions of the brain were punched out on a cold plate and stored at -70°C until the assay. The obtained tissue was homogenized and incubated in ice cold protein extraction solution (iNtRON Biotechnology, Inc., Gyeonggi, Korea) for 30 min and centrifuged (10,000×g at 4°C for 5 min). Supernatant was transferred to a fresh tube and 5-HT concentration in duplicate aliquots was assessed by enzyme linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Labor Diagnostika Nord, Inc., Minneapolis, MN, USA).

c-Fos immunohistochemistry

After 24 hours later of *SF* or matrine administration, all rats were deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.). The rat brains were removed after transcardial perfusion with 4% solution of formaldehyde (Sigma-Aldrich Co.), then post-fixed in the same fixative for 24 hours, and placed in PBS containing 20% of sucrose for 72 hours. Serial coronal sections were cut into 30 μm thickness by using a cryostat microtome (CM1850UV, Leica Microsystems Inc., Wetzlar, Germany). The sections were stored at -20°C and these slices were histochemically processed as free-floating sections.

The brain sections were washed three times in PBS containing 0.2% Triton X-100 (PBST). The primary rabbit monoclonal antibodies against c-Fos were used (diluted 1:2000 in PBST with 10%v/v normal goat serum, Santa Cruz Biotechnology, Inc., TX, USA) and the sections were incubated for 72 hours at 4°C with constant agitation. After rinsing in PBST, the sections were incubated in biotinylated goat anti-rabbit (Vector Laboratories, Inc., Burlingame, CA, USA) diluted 1:200 in PBST with 2%v/v normal goat serum for 2 hours at room temperature. The sections were then incubated in the avidin-biotin-peroxidase complex reagent (Vector Laboratories) for 2 hours at room temperature. Following further rinsing with PBST, the tissues were developed using diaminobenzadine peroxidase substrate kit (Vector Laboratories). The sections were mounted on slides, air-dried, and cover slipped for microscopic observation. The numbers of immunopositive neurons in the VLPO (AP, 0.00 to -0.36 from the bregma) was counted at 200x magnification using a microscope rectangle grid that measured 100×100 μm according to Paxinos *et al* (1985).

Statistical analysis

The data were expressed as the mean ± standard error of the mean (SEM). Comparisons among different groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. 5-HT level of *SF* in the cortex (Fig. 3A) was analyzed by ANOVA followed by LSD post-hoc test. All the statistical analyses were assessed using SPSS 23 (IBM) software (Chicago, IL, USA). *p*-values less than 0.05 were regarded as statistically significant.

RESULTS

Effects of *SF* and matrine on caffeine-induced hyperactivity

Locomotor activity was measured to investigate sedative effect of *SF* and matrine on caffeine-induced hyperactivity. Traveled distance was recorded and analyzed during latter 1 hour (Fig. 1). *SF* 800-treated group showed significant reduction on locomotor activity [F(3,18)=5.510, $p<0.05$, Fig.1A] and matrine-administered group had a dose-dependent tendency to decrease movements [F(3, 27)=1.990, $p=0.142$, Fig.1B].

Effects of *SF* and matrine on EEG

Effects of *SF* and matrine on EEG sleep architecture and profile were presented in Fig. 2. The EEG signals in rats were recorded for 12 hours after oral *SF* and matrine treatment (*SF*; 400 and 800 mg/kg, matrine; 30 and 100 mg/kg) at 08:00 PM. *SF* (800 mg/kg) significantly decreased wake time and increased NREM time [F(2,17)=4.810, $p<0.05$ and F(2,17)=4,467, $p<0.05$, Fig. 2A, 2C)]. No significant change in REM sleep was observed, but slight increase was shown in dose-dependent manner [F(2,17)=0.556, $p=0.585$, Fig. 2B]. Matrine (30 mg/kg) significantly decreased wake time

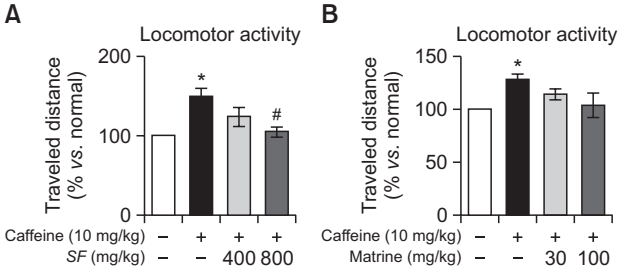

Fig. 1. The effect of (A) *SF* and (B) matrine on caffeine-induced hyperactivity. Each column represents the mean ± SEM. **p*<0.05 vs saline treated group, #*p*<0.05 vs caffeine treated group; one-way ANOVA.

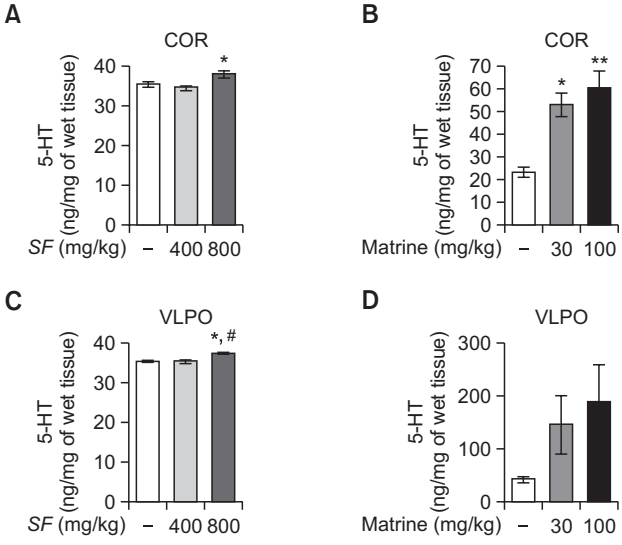

Fig. 3. The effect of *SF* and matrine on 5-HT concentration. Mean (± SEM) 5-HT level of the Cortex (A, B) and VLPO (C, D) in the *SF* and matrine treated groups **p*<0.05 and ***p*<0.01 vs saline treated group, #*p*<0.05 vs SF400; one-way ANOVA.

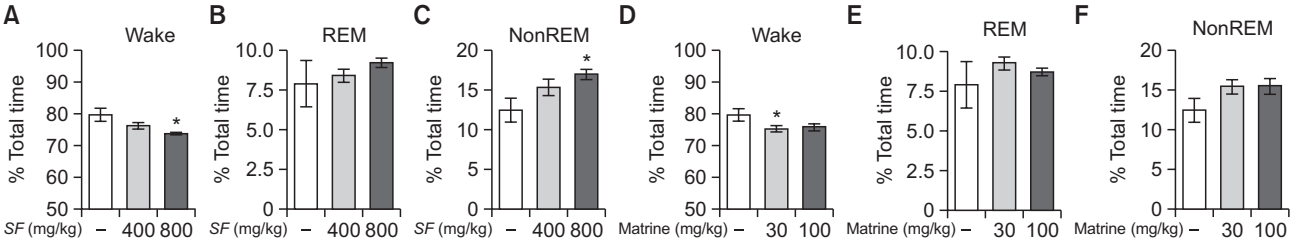

Fig. 2. The effect of *SF* and matrine on sleep architecture. Changes in the percentage of wake (A, D), REM sleep (B, E) and NREM sleep (C, F) during dark phase were shown in the *SF* and matrine treated groups. The data represent the mean ± SEM of percent time spent in sleep-wake state. **p*<0.05 vs saline treated group; one-way ANOVA.

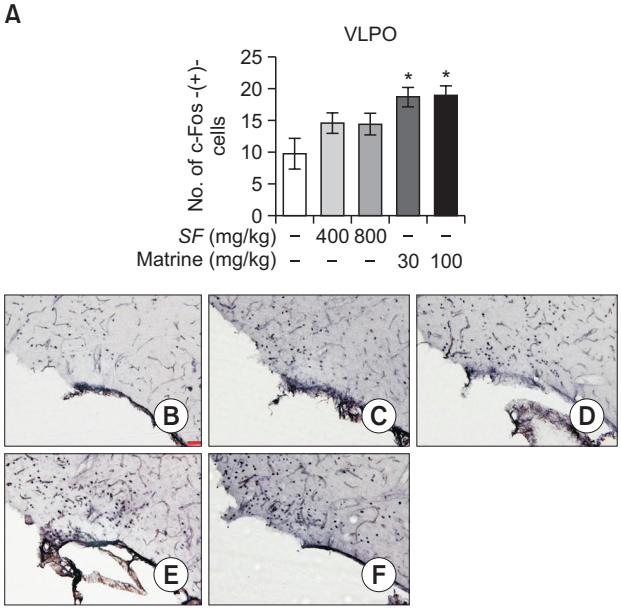

Fig. 4. The effect of *SF* and matrine on c-fos immunoreactivity. (A) Mean (± SEM) expression of c-Fos in the VLPO. Representative images showed number of c-Fos-immuno reactive neurons in the (B) normal group, (C) *SF*400 group, (D) *SF*800 group, (E) M30 group and (F) M100 group. **p*<0.05 vs normal group; one-way ANOVA. Photomicrographs were taken at x200 magnification. Scale bar =50 μm.

[F(2,17)=2.885, *p*<0.05, Fig. 2D] and tendency of increment in REM and NREM sleep was observed [F(2,17)=0.591, *p*=0.566 and F(2,17)=2.316, *p*=0.133, Fig. 2E, 2F].

Effects of *SF* and matrine on 5-HT level in the cortex and VLPO

Concentration of brain 5-HT protein was assessed in the *SF*400, *SF*800, M30 and M100-treated groups (Fig. 3). Both *SF* and matrine significantly increased 5-HT level in the cortex region of the brain [F(2,11)=5.999, *p*<0.05 and F(2,15)=10.616, *p*<0.01, Fig. 3A, 3B, respectively]. However, in the VLPO region, only SF-treated group had significantly increased 5-HT concentration [F(2,11)=9.617, *p*<0.01, Fig. 3C], whereas matrine-treated group showed tendency to increase 5-HT level in dose-dependent manner [F(2.10)=4.438, *p*=0.05, Fig. 3D].

Effects of *SF* and matrine on c-Fos expression in the VLPO

Expression of c-Fos in the VLPO was measured in the *SF*400, *SF*800, M30 and M100-treated groups (Fig. 4). c-Fos immunoreactive neurons of the VLPO were higher in the *SF* and matrine-administered groups [F(4,28)=4.243, *p*<0.05]. In particular, there was significant increment in the *SF*400 and *SF*800 groups (*p*<0.05), whereas matrine groups showed tendency to increase c-Fos expression in the VLPO.

DISCUSSION

The findings of the current research demonstrated the sedative efficacy of *SF* and its bioactive compound, matrine-type alkaloids have sedative efficacy. Pretreatment of *SF* and matrine prior to caffeine administration alleviated caffeine-induced hyperactivity. Administrsion of *SF* and matrine significantly reduced total wake time and increased NREM time. 5-HT level in the cortex and VLPO and c-Fos expression in the VLPO were considerably increased in the *SF* and matrine group. The data showed the first experimental evidence that *Sophora flavescens* has sedative efficacy suggesting *SF* and matrine as novel natural medications to enhance sleep by modulating serotonergic system and activating sleep-promoting region in the brain.

It has been reported that 1g of *SF* contains 1.21 mg of matrine (Li and Wang, 2004). In accordance with this report, it is assumed that 400 mg of *SF* extracts contain approximate 22 mg of matrine. Therefore, two matrine doses, 30 mg/ml and 100 mg/ml were chosen in this study.

Caffeine increased locomotor activity and wake time and proportionally diminished both NREM and REM time (Okuro *et al*., 2010) and caffeine at a dose of 10 mg/kg caused insomnia in the rat (Paterson *et al*., 2007). Caffeine disrupts sleep by antagonizing adenosine A_2A receptors in the VLPO thereby reduces inhibitory transmission to the tuberomammillary nucleus (TMN) which is responsible for wakefulness-promotion (Ferre, 2008). It was reported that attenuation of locomotor activity is innovative approach to assess sedative effect of herbal extracts (Klein *et al*., 2014). The present study showed that *SF* and matrine administration had an effect on normalization of caffeine (10 mg/kg)-induced hyperactivity indicating that *SF* and matrine can be candidates to treat insomnia. 5-HT depletion caused significant increment of motor activity and modulation of sleep-wake cycle (Solarewicz *et al*., 2015). Thus, it is suggested that locomotor-reducing ability of *SF* and matrine may contribute to sleep promotion through increasing 5-HT release.

Previous studies have reported that administration of extracts from natural herbal medicine, which exhibited hypnotic effect, altered total sleep time and NREM rather than REM sleep (Abdollahnejad *et al*., 2016; Hajiaghaee *et al*., 2016). In accordance with these studies, the present research showed that *SF* and matrine at a dose of 800 mg/kg and 30 mg/kg, respectively, reduced wake time considerably and *SF* (800 mg/kg) promoted a significant shift toward NREM sleep.

The central serotonergic systems have been implicated in promoting wakefulness (Monti, 2011), but the exact role on sleep-wake system is still controversial. Extracellular 5-HT release during sleep deprivation was reduced (Bjorvatn *et al*., 2002) or increased (Lopez-Rodriguez *et al*., 2003). Tryptophan, the precursor of 5-HT, was reported to improve sleep (Bravo *et al*., 2013; Melancon *et al*., 2014). Another study suggested that 5-HT depletion-induced sleep disturbance occurs only in condition under hypothermia (Murray *et al*., 2015).

In the present study, 5-HT level in the VLPO, a sleep promoting region, and in the cortex was increased in the both *SF* and matrine-injected groups. There are two types of VLPO neurons which differently respond to 5-HT. 5-HT suppresses input to type 1 neurons which have 5-HT 1, 2, 4, 7 receptors. Input to type 2 neurons containing 5-HT 2, 4, 7 receptors, however, is enhanced by 5-HT. Although 2 neurons are inversely influenced by 5-TH, they promotes sleep by involving initiation and maintenance of sleep, respectively (Sangare *et al*., 2016). It was also reported that injection of 5-HT receptor 1A agonist increases wake time (Monti and Jantos, 2003). Accumulating evidences and the present results drives that increment 5-HT

in VLPO may promote sleep through diverse 5-HT receptors located in type 1 and 2 neurons.

Fos protein, a marker for postsynaptic activation, is expressed in the neurons of the VLPO only during sleep-phase (Sherin *et al*., 1996). In agreement with this result, it was observed that treatment of matrine caused significantly increased expression of c-Fos-immunoreactivity in the VLPO and *SF*-treated group showed tendency to increase c-Fos expression.

*SF*800 group showed significant changes in locomotor activity, wake-sleep state and 5-HT concentration, whereas c-Fos immunoreactivity was slightly increased. Notable changes in 5-HT level and c-Fos expression followed by enhanced total wake time were observed in the Matrine 100 mg/kg-treated group. Taken together, it is assumed that increased release of 5-HT in the VLPO and cortex is sufficient to induce sedative behavioral changes.

It has been shown that the VLPO cells contain a variety of neurotransmitters including GABA and 5-HT. The role of these 5-HT containing cells in regulating the sleep behaviors is clearly not known, but it may be possible that *SF* and martine may activate the central serotonergic cells and increase release in the VLPO. In turn, activated c-Fos immunoreactive cells, evidenced by increased c-Fos immunoreactivity, play an important role in facilitating sleep initiation and maintenance as well as inhibiting activation of neurons which involved in arousal. Further studies examining interaction of c-Fos immunoreactive cells and 5-HT receptors in the VLPO will interpret the exact neural mechanisms involved in sleep promotion.

In conclusion, *SF* and its bioactive compound, matrine alleviated caffeine-induced hyperactivity, increased NREM and decreased wake time by activation of sleep-promoting center, VLPO, and modulating serotonergic transmission. It is suggested that *SF* might be a useful natural alternatives for hypnotic medicine.

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