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The involvement of serotonin receptors in suanzaorentang-induced sleep alteration

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Summary

Sedative-hypnotic medications, including benzodiazepines and non-benzodiazepines, are usually prescribed for the insomniac patients; however, the addiction, dependence and adverse effects of those medications have drawn much attention. In contrast, suanzaorentang, a traditional Chinese herb remedy, has been efficiently used for insomnia relief in China, although its mechanism remains unclear. This study was designed to further elucidate the underlying mechanism of suanzaorentang on sleep regulation. One ingredient of suanzaorentang, zizyphi spinosi semen, exhibits binding affinity for serotonin (5-hydroxytryptamine, 5-HT) receptors, 5-HT_{1A} and 5-HT_2, and for GABA receptors. Our previous results have implicated that $GABA_A$ receptors, but not $GABA_B$, mediate suanzaorentang-induced sleep alteration. In current study we further elucidated the involvement of serotonin. We found that high dose of suanzaorentang (4 g/kg/2 ml) significantly increased non-rapid eye movement sleep (NREMS) when comparing to that obtained after administering starch placebo, although placebo at dose of 4 g/kg also enhanced NREMS comparing with that obtained from baseline recording. Rapid eye movement sleep (REMS) was not altered. Administration of either 5-HT_{1A} antagonist (NAN-190), 5-HT_2 antagonist (ketanserin) or 5-HT_3 antagonist (3-(4-Allylpiperazin-1-yl)-2-quinoxalinecarbonitrile) blocked suanzaorentang-induced NREMS increase. These results implicate the hypnotic effect of suanzaorentang and its effects may be mediated through serotonergic activation, in addition to GABAergic system.

Insomnia is one of the most common sleep disturbances among elderly people. Psychiatric illness, medical problem, poor sleep habits or a primary sleep disorder may cause insomnia. Epidemiological surveys have shown that about 20% of adults have suffered from the moderate to severe insomnia [1]. Sleep disturbance or even insomnia has been ignored from further studying since the vigorous development of sedative-hypnotic medications. Sedative-hypnotic medications, including benzodiazepines and non-benzodiazepine benzodiazepine receptor agonists, are the

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most common sleeping pills for treating people suffered from insomnia. However, addiction, dependence and adverse effects, such as impairment of memory or movement, and next-day "hangover", have drawn caution for physician to prescribe benzodiazepines. A traditional Chinese remedy, suanzaorentang was originally stated in the Kin-Kue-Yao-Leuh, a Chinese medicine and pharmacy literature written in 1700 years ago, for patients who suffer insomnia from overloading work and irritability. Suanzaorentang comprises zizyphi spinosi semen, poria cocos, ligusticum wallichii, anemarrhenae rhizoma and glycyrrhizae in ratio of 4:2:2:2:1. Although suanzaorentang is a very common over-the-counter sleep pills in China, Hong Kong and Taiwan, there is a lack of evidence-based data of its hypnotic efficacy and safety to support its clinical use [2]. There are few studies showing that suanzaorentang statistically improved all ratings of sleep quality in insomniac patients when compared with placebo given [3] and exhibited the same anxiolytic effects as that of diazepam [4]. Although the sedative-hypnotic effect of suanzaorentang is very significant in clinic, there is a lack of scientifically mechanistic evidence to support suanzaorentang's efficacy on management of insomnia. Our previous results pioneered indicate that suanzaorentang may increase spontaneous sleep activity partially through the activation of γ-aminobutyric acid (GABA) type A receptor, but not the $GABA_B$ receptor in the brain [5].

The main composition of suanzaorentang, zizyphi spinosi semen, exhibits the central nervous system (CNS) calming effect. Pretreatment of zizyphi spinosi semen inhibits elevated cytosolic calcium concentrations induced by n-methyl-D-aspartate (NMDA) and prevents NMDA-induced neuronal cell damage [6]. Zizyphi spinosi semen also has ability to inhibit caffeine-induced excitation and to prolong hexobarbital-induced sleep [7]. Furthermore, it has been reported that the hypnotic effect of zizyphi spinosi semen may be mediated by the anticholinergic and antihistamine action of betulic acid, an active compound of zizyphi spinosi semen [8]. The second ingredient of suanzaorentang, poria cocos has long been used as a sedative and diuretic medicine in China. One study has elucidated that the 50% hot ethanol extraction of poria cocos significantly enhances the secretion of immune stimulators, such as interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α in human peripheral blood monocytes, but decreases the secretion of an immune suppressor, transforming growth factor (TGF)-β [9]. This result indicates the potentiation of immune responses by poria cocos. It has been shown that tetramethylpyrazine, one of the most active ingredients of ligusticum wallichii, significantly suppresses cerebral ischemia/reperfusion-induced inflammatory cell activation and proinflammatory mediator secretion, implicating the anti-inflammatory potential [10]. The extraction from the fourth ingredient of zuanzaorentang, anemarrhenae rhizome, possesses neurotrophic activity and proteasome inhibitory effect in rat pheochromocytoma cells [11]. Studies of glycyrrhizae radix in combination with other Chinese herbs in treating insomnia are favorable. For example, "Kanbaku-taiso-to" (KT), a mixture of glycyrrhizae radix, triticii semen and zizyphi fructus, exhibits the sedative effect by an inhibitory effect of hyperexcitability on the neuronal membrane [12]. Although few studies have shown the effect of individual ingredient of suanzaorentang, there is little evidence showing the underlying mechanism of suanzaorentang on sleep regulation. Our previous results have indicated that suanzaorentang increases spontaneous NREMS during the dark (active) period in rats, with no change in REMS [5]. We herein further designed a study to determine which ingredient is the majority ingredient for suanzaorentang to exhibit sedative-hypnotic effect, and the other possible mechanisms of sleep regulation in addition to the involvement of GABAergic system activation by suanzaorentang.

It has been demonstrated that the water extract of zizyphi spinosi semen, the main ingredient of suanzaorentang, exhibits binding affinity for serotonin (5-hydroxytryptamine, 5-HT) receptors, 5-HT_{1A} and 5-HT_2, and for GABA receptors [13]. Serotonin has been well known to play an important role in the sleep regulation [14, 15]. Jouvet has shown that destruction of serotonergic neurons in the raphe nucleus or the inhibition of serotonin synthesis by administration of *p*-chlorophenylalanine (PCPA) decreases spontaneous sleep in cats [16, 17], which was reversed by restoring serotonin synthesis [18]. These results implicated the sleep hypothesis of serotonin, although evidence, such as waking increase by 5-HT_{1A} agonist, flesinoxan [19], and the results from 5-HT_{2A} knock-out mice [20], demonstrates the wake promoting properties

of serotonergic system. The complicate effects of serotonin on sleep regulation may be due to that serotonin is able to act at different brain regions which have been implicated in sleep-wake modulation and that different distributions of the 5-HT receptor subtypes in the brain. Nevertheless, we herein examined whether the serotonergic system mediates the effects of suanzaorentang on physiological sleep modulation by pharmacological blockade.

Methods

Pharmacological agents

The Chinese herb mixture suanzaorentang, comprising zizyphi spinosi semen, poria cocos, ligusticum wallichii, anemarrhenae rhizoma and glycyrrhizae in ratio of 4:2:2:2:1, and the placebo starch were manufactured and provided by the Sun-Ten Pharmaceutical Co., Ltd (Taipei, Taiwan). Suanzaorentang and starch dissolved in water were given by oral administration in the concentrations of 0.5, 1, 2 and 4 g/kg/2 ml for suanzaorentang and 4 g/kg/2 ml for starch. Combination I of suanzaorentang (C-I) misses ligusticum wallichii, C-II lacks of poria cocos, C-III is without anemarrhenae rhizoma, glycyrrhizae is not included in C-IV, and C-V excludes zizyphi spinosi semen. Dose used for these combinations was 4 g/kg/2 ml. Stock solutions of 5-HT_{1A} antagonist (NAN-190; Sigma, St. Louis, Mo., USA), 5-HT_2 antagonist (ketanserin; Sigma) or 5-HT_3 antagonist (3-(4-Allylpiperazin-1-yl)-2-quinoxalinecarbonitrile; Sigma) were dissolved in pyrogen-free saline (PFS). The stock solution was stored at 4 °C until use. The doses of NAN-190 and ketanserin used for intracerebroventricular (ICV) administration in this experiment were 1, 5 and 10 nmol/3 μl, and dose for 3-(4-Allylpiperazin-1-yl)-2-quinoxalinecarbonitrile was 10 nmol/3 μl.

Animals

Male Sprague–Dawley rats (250–300 g; National Laboratory Animal Breeding and Research Center, Taiwan) were used in these experiments. These animals were anesthetized (ketamine/xylazine; 87/13 mg/kg), and injected with an analgesic (morphine) and an antibiotic (penicillin G benzathine). The rats were surgically implanted with electroencephalogram (EEG) screw electrodes, a guide cannulae directed into the lateral ventricle, and a calibrated 30-kΩ thermistor (Model #44008; Omega Engineering, Stamford, CT) to monitor brain temperature at the surface of the cortex as previously described [21]. Insulated leads from EEG electrodes and thermistor were routed to a Teflon pedestal (Plastics One, Roanoke, VA). The Teflon pedestal was then cemented to the skull with dental acrylic (Cranioplastic cement and Cyanoacrylate gel, Plastics One, Roanoke, VA). The incision was treated topically with polysporin (polymixin B sulfate – bacitracin zinc) and the animals were allowed to recover for 7 days prior to the initiation of experiments. The rats were housed in individual recording cages, two cages in each environmentally controlled chamber (COCONO model # LE-539; Ron-Fong Technology Corporation, HsinChu, Taiwan). The chambers were maintained at 23 ± 1 °C with a 12:12 h light:dark cycle (20 W × 6 tubes illumination), and food and water were available *ad libitum*. All procedures performed in these studies were approved by the China Medical University Animal Care and Use Committee.

On the second postsurgical day, the rats were connected to the recording apparatus (see later) via a flexible tether. Three days after surgery, the patency and free drainage of the ICV cannulae was assessed by administering 200–400 ng angiotensin II [human angiotensin II octapeptide; Tocris, Inc.]; angiotensin elicited a drinking response mediated by structures in the preoptic area [22]. At the end of each experimental protocol, all rats were again injected with angiotensin; only data from those rats that exhibited a positive drinking response were included in the subsequent analyses. The animals were habituated by daily handling and ICV injections of PFS timed to coincide with scheduled experimental administrations.

Apparatus and recording

Signals from the EEG electrodes were fed into an amplifier (Colbourn Instruments, Lehigh Valley, PA; model V75-01). The EEG was amplified (factor of 5,000) and analog bandpass filtered between 0.1 and 40 Hz (frequency response: ±3 dB; filter frequency roll off: 12 dB/octave). Signals from the thermistors were fed into an amplifier (Axon Instruments, Union City, CA;

model: CyberAmp 380). Gross body movements were detected by custom-made infrared-based motion detectors (Biobserve GmbH, Germany), and the movement activity was converted to a voltage output. These conditioned signals (EEGs, brain temperature, gross body movements) were subjected to analog-to-digital conversion with 16-bit precision at a sampling rate of 128 Hz (NI PCI-6033E; National Instruments, Austin, TX). The digitized EEG waveform and integrated values for body movement were stored as binary computer files until subsequent analyses.

Postacquisition determination of vigilance state were done by visual scoring of 12-s epochs using custom software (ICELUS, M. R. Opp) written in LabView for Windows (National Instruments). The animal's behavior was classified as either non-rapid eye movement sleep (NREMS), rapid eye movement sleep (REMS), or waking based on previously defined criteria [23]. Briefly, NREMS is characterized by large-amplitude EEG slow waves, high power density values in the delta frequency band (0.5–4.0 Hz), lack of gross body movements, and declining brain temperature before and during entry. During REMS, the amplitude of the EEG is reduced, the predominant EEG power density occurs within the theta frequency (6.0–9.0 Hz), brain temperature increases rapidly at onset, and there are phasic body twitches. During waking, the rats are generally active, there are protracted body movements, brain temperature gradually increases, the amplitude of the EEG is similar to that observed during REMS, but power density values in the delta frequency band are generally greater than those in theta frequency band.

Experimental protocol

Five groups, total of 40 Sprague-Dawley rats were used in present study. About 24 h baseline EEGs and sleep-wake activities were recorded from rats without disturbance. Rats in group 1 (n = 8) were orally administered 2 ml PFS 20 min prior to the beginning of dark period and subsequently recorded 24-h sleep-wake activity at the first experimental day. After 1-day break, 4 g/kg/2 ml starch was given and 24-h sleep-wake EEGs were recorded at the third experimental day. The same manipulation was given at the fifth, seventh, ninth and eleventh experimental days, except 0.5, 1, 2 and 4 g/kg/2 ml suanzaorentang were administered orally. The 1-day break without manipulation was to avoid the accumulated effect of stomach vagal stimulation as previous described [5]. Rats in group 2 (n = 8) received seven manipulations with 1-day break interval between each manipulation. The seven manipulations were as follow: (1) oral administration of 2 ml PFS 20 min prior to the beginning of dark period at the first day; (2) oral administration of 4 g/kg/2 ml starch at the 3rd day; (3) oral administration of 4 g/kg/2 ml C-I at the 5th day; (4) oral administration of 4 g/kg/2 ml C-II at the 7th day; (5) oral administration of 4 g/kg/2 ml C-III at the 9th day; (6) oral administration of 4 g/kg/2 ml C-IV at the 11th day; (7) oral administration of 4 g/kg/2 ml C-V at the 13th day. A 24-h EEG sleep-wake activity was recorded at the beginning of dark period after manipulation. Rats in group 3 (n = 8) have similar protocol as those in group 2, except they received different substances as follow: (1) ICV PFS + oral 4 g/kg/2 ml starch, (2) ICV PFS + oral 4 g/kg/2 ml suanzaorentang, (3) ICV 1 nmol NAN-190 + oral 4 g/kg/2 ml suanzaorentang, (4) ICV 5 nmol NAN-190 + oral 4 g/kg/2 ml suanzaorentang, (5) ICV 10 nmol NAN-190 + oral 4 g/kg/2 ml suanzaorentang. Rats in group 4 (n = 8) have exactly same protocol as those in group 3, except they received ketanserin instead of NAN-190. Rats in group 5 (n = 8) received similar protocol as those in group 3, except they received different substances as follow: (1) ICV PFS + oral 4 g/kg/2 ml starch, (2) ICV PFS + oral 4 g/kg/2 ml suanzaorentang, (3) ICV 10 nmol NAN-190 + oral 4 g/kg/2 ml suanzaorentang, (4) ICV 10 nmol ketanserin + oral 4 g/kg/2 ml suanzaorentang, (5) ICV 10 nmol 3-(4-Allylpiperazin-1-yl)-2-quinoxalinecarbonitrile + oral 4 g/kg/2 ml suanzaorentang.

Statistical analyses

All values are presented as the mean ± SEM for the indicated sample sizes. One-way analyses of variance (one-way ANOVA) for the duration of each vigilance state (NREMS, REMS, WAKE) and for sleep architecture parameters are performed across the two 12-h time blocks. If statistically significant differences are detected, *post hoc* comparisons are made to determine which hourly intervals during experimental conditions deviated from values obtained from the same

animals during control conditions. An α level of $p \leq 0.05$ is taken as indicating a statistically significant difference between manipulations.

Results

Effects of oral administration of starch and suanzaorentang on sleep-wake activity

Our results demonstrated that oral administration of 2 ml PFS did not change the amount of rats spent in three distinct vigilance states, NREMS, REMS and wakefulness, when compared with those recorded from undisturbed baseline, which is consistence with our previous observations [5]. The time spent in NREMS and REMS obtained from undisturbed rats was 21.6 ± 1.7% and 3.3 ± 0.7%, respectively. The total amounts of NREMS and REMS obtained from rats treated with PFS were 21.4 ± 1.5% and 3.6 ± 0.9%, respectively (n = 8). Oral administration of 4 g/kg/2 ml starch 20 min prior to the beginning of dark period significantly increased the amount of NREMS at the expense of wakefulness during the dark period as shown in the Figure 1A and C (n = 8, $p < 0.05$, one-way ANOVA). Oral administration of 4 g/kg/2 ml suanzaorentang further

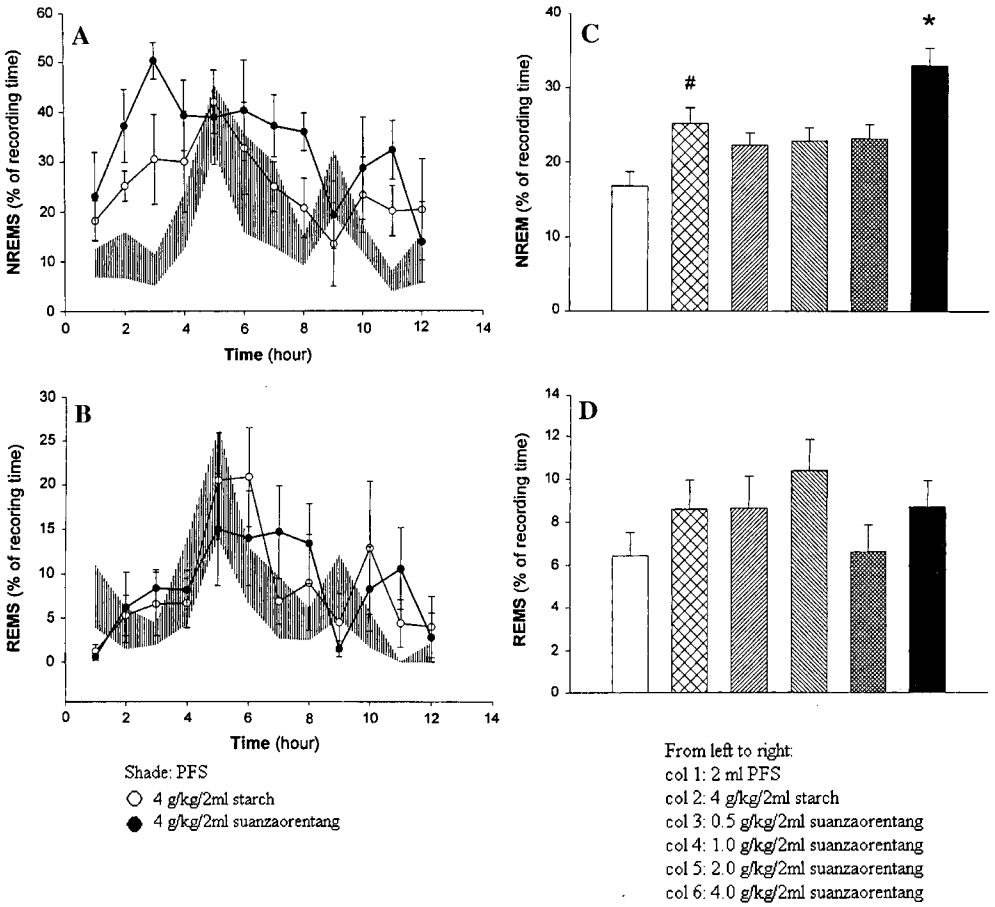

Figure 1. Oral administrations of high dose of suanzaorentang and placebo starch, 4 g/kg/2 ml, increase NREMS during the dark period in a 12:12 h light:dark cycle. The values represent as mean ± SEM. (A & B) Shade areas represent the values obtained after oral administration of 2 ml PFS during the dark period. Open circles indicate the data collected after oral administration of 4 g/kg/2 ml starch. Close circles represent the values obtained after oral administration of 4 g/kg/2 ml suanzaorentang. (C & D) The bars from left to right represent the values obtained after oral administration of 2 ml PFS, 4 g/kg/2 ml starch, 0.5, 1, 2 and 4 g/kg/2 ml suanzaorentang, respectively. * Depicts that the values obtained after oral administration of 4 g/kg/2 ml suanzaorentang achieve statistically significant difference comparing with those after oral administration of starch. # Depicts that the values obtained after oral administration of 4 g/kg/2 ml starch achieve statistically significant difference comparing with those after oral administration of PFS. NREMS: non-rapid eye movement sleep, REMS: rapid eye movement sleep.

increased the NREMS and the increase of NREMS achieved statistical significance when comparing with the results obtained after placebo starch (Figure 1A, C, $p < 0.05$, one-way ANOVA). Neither 4 g/kg/2 ml starch nor 4 g/kg/2 ml suanzaorentang altered REMS as shown in Figure 1B and D. Suanzaorentang at doses of 0.5, 1.0 and 2.0 g/kg/2 ml did not alter both NREMS and REMS (Figure 1C, D) when compared with placebo. The high dose of starch may mask the effects of relative low-dose of suanzaorentang on sleep alteration.

Effects of five different combinations with only four ingredients of suanzaorentang on sleep alteration

Our result indicated that the five different combinations of suanzaorentang with any one-missing ingredient could not exhibit its ability to enhance NREMS. The total amounts of NREMS obtained after treated with PFS, placebo starch, C-I, C-II, C-III, C-IV and C-V were 21.4 ± 1.5%, 26.3 ± 1.5%, 25.6 ± 2.1%, 21.0 ± 1.6%, 20.6 ± 1.5%, 23.2 ± 1.9% and 21.9 ± 2.2%, respectively (Figure 2A, $n = 8$). We found that NREMS obtained after administration of C-II and C-III was statistically significant less than that obtained after giving placebo starch (Figure 2A, $n = 8$, $p < 0.05$, one-way ANOVA). This result implicated that poria cocos and anemarrhenae rhizoma may play an important role for the suanzaorentang's action on sleep regulation, since the combination of C-II lacks of poria cocos and C-III excludes anemarrhenae rhizoma. There was no statistical significant alteration on REMS obtained after oral administration of those five combinations of suanzaorentang (Figure 2B, $n = 8$). The total amounts of REMS obtained after placebo starch, C-I, C-II, C-III, C-IV and C-V were 4.4 ± 0.7%, 3.5 ± 0.6%, 4.3 ± 0.6%, 3.1 ± 0.5%, 6.9 ± 1.8% and 5.6 ± 1.2%, respectively.

Effects of 5-HT_{1A} and 5-HT_2 receptor antagonists, NAN-190 and ketanserin, on suanzaorentang-induced sleep alteration

ICV administration of 5-HT_{1A} receptor antagonist, NAN-190, 20 min prior to the beginning of the dark period suppressed suanzaorentang-induced NREMS increase during the dark period. Total amount of NREMS was increased from 20.5 ± 1.5% obtained after ICV PFS + oral 4 g/kg/2 ml starch to 30.4 ± 1.4% after manipulation of ICV PFS + oral 4 g/kg/2 ml suanzaorentang in this group of rats ($n = 8$, $p < 0.05$, one-way ANOVA). ICV administrations of NAN-190 in doses of 1.0, 5.0 and 10.0 nmol significantly decreased the NREMS to 24.1 ± 2.4%, 25.8 ± 2.3% and 23.9 ± 2.2%, respectively (Figure 3A, C, $n = 8$, $p < 0.05$, one-way ANOVA), with a mirror enhancement in wakefulness. We found that the suppression of suanzaorentang-induced NREMS

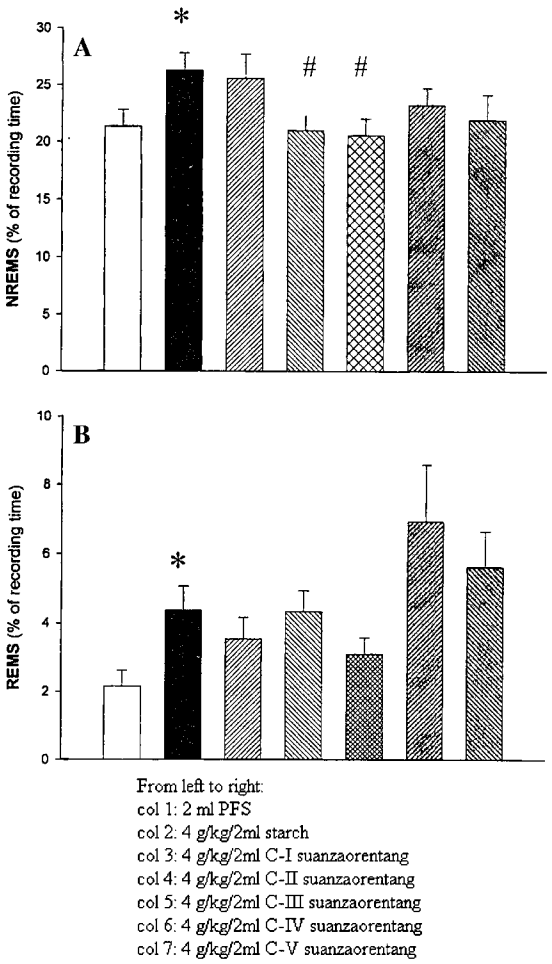

Figure 2. Effects of oral administration of five combinations of suanzaorentang with one ingredient missed during the dark period. The values represent as mean ± SEM. The bars from left to right represent the values obtained after oral administration of 2 ml PFS, 4 g/kg/2 ml starch, C-I, C-II, C-III, C-IV and C-V of suanzaorentang, respectively. * Depicts that the values obtained after oral administration of 4 g/kg/2 ml starch achieve statistically significant difference comparing with those after oral administration of PFS. # Depicts that the values obtained after oral administration of 4 g/kg/2 ml C-II and C-III achieve statistically significant difference comparing with those after oral administration of 4 g/kg/2 ml starch.

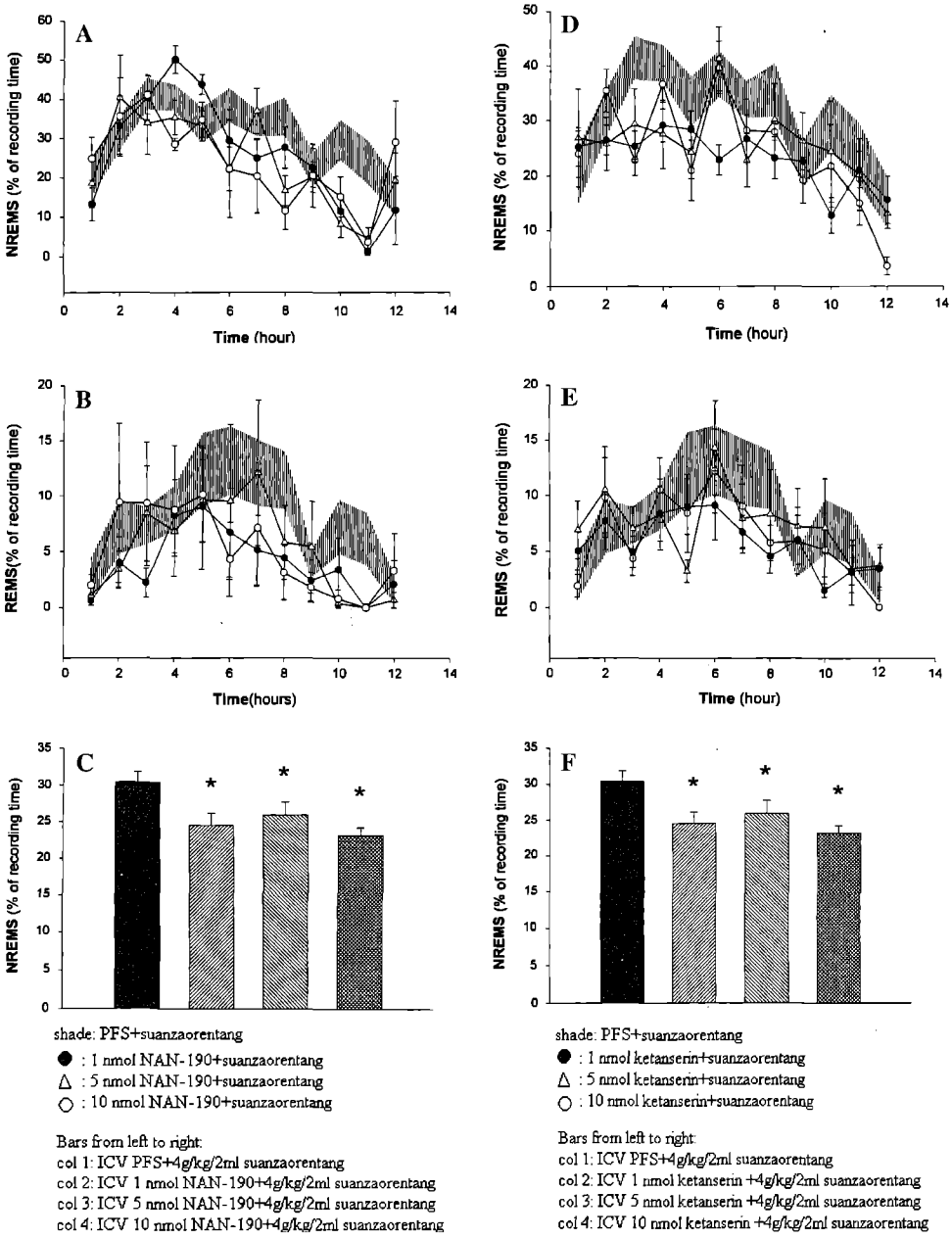

Figure 3. The effects of ICV administration of 5-HT_{1A} receptor antagonist, NAN-190, and 5-HT_2 receptor antagonist, ketanserin, on the suanzaorentang-induced sleep alteration. The values represent as mean ± SEM. (A & B) Shade areas represent the values obtained after ICV PFS + oral 4 g/kg/2 ml suanzaorentang, close circles are obtained after ICV 1 nmol NAN-190 + oral 4 g/kg/2 ml suanzaorentang, open triangles indicate the data obtained after ICV 5 nmol NAN-190 + oral 4 g/kg/2 ml suanzaorentang, and open circles indicate the values obtained after ICV 10 nmol NAN-190 + oral 4 g/kg/2 ml suanzaorentang. (C) The bars from left to right represent the values after ICV PFS + oral 4 g/kg/2 ml suanzaorentang, ICV 1 nmol NAN-190 + oral 4 g/kg/2 ml suanzaorentang, ICV 5 nmol NAN-190 + oral 4 g/kg/2 ml suanzaorentang and ICV 10 nmol NAN-190 + oral 4 g/kg/2 ml suanzaorentang. (D & E) Shade areas represent the values obtained after ICV PFS + oral 4 g/kg/2 ml suanzaorentang, close circles are obtained after ICV 1 nmol ketanserin + oral 4 g/kg/2 ml suanzaorentang, open triangles indicate the data obtained after ICV 5 nmol ketanserin + oral 4 g/kg/2 ml suanzaorentang, and open circles indicate the values obtained after ICV 10 nmol ketanserin + oral 4 g/kg/2 ml suanzaorentang. (F) The bars from left to right represent the values after ICV PFS + oral 4 g/kg/2 ml suanzaorentang, ICV 1 nmol ketanserin + oral 4 g/kg/2 ml suanzaorentang, ICV 5 nmol ketanserin + oral 4 g/kg/2 ml suanzaorentang and ICV 10 nmol ketanserin + oral 4 g/kg/2 ml suanzaorentang. * Depicts that the values achieve statistically significant difference comparing with those after ICV PFS + oral 4 g/kg/2 ml suanzaorentang.

increase occurred primarily during the second 6-h of the dark period. REMS was not significantly altered by all manipulations (Figure 3B).

ICV administration of 5-HT_2 receptor antagonist, ketanserin, 20 min prior to the beginning of the dark period also suppressed suanzaorentang-induced NREMS increase during the dark period, which is similar to the effect of 5-HT_{1A} receptor antagonist, NAN-190. Total amount of NREMS was increased from 19.6 ± 1.7% obtained after ICV PFS + oral 4 g/kg/2 ml starch to 30.6 ± 1.2% after manipulation of ICV PFS + oral 4 g/kg/2 ml suanzaorentang in this group of rats (n = 8, $p < 0.05$, one-way ANOVA). ICV administrations of ketanserin in doses of 1.0, 5.0 and 10.0 nmol significantly decreased the NREMS to 24.5 ± 1.6%, 25.9 ± 1.8% and 23.2 ± 1.0%, respectively (Figure 3D, F, n = 8, $p < 0.05$, one-way ANOVA). We found that the suppression of suanzaorentang-induced NREMS increase by ketanserin occurred primarily during the first 6-h of the dark period. REMS was not significantly altered by all manipulations (Figure 3E). Our previous result has indicated that ICV administration of NAN-190 or ketanserin has no change in the sleep-wake activity during the dark period. The amounts of time spent in NREMS were 23.3 ± 1.5%, 21.1 ± 2.3% and 22.4 ± 1.7% obtained after ICV administration of PFS, 10 nmol of NAN-190 and 10 nmol of ketanserin, respectively; and the total amounts of REMS were 6.8 ± 1.1%, 7.4 ± 1.3% and 6.6 ± 1.5% obtained after ICV administration of PFS, 10 nmol of NAN-190 and 10 nmol of ketanserin, respectively. The possible reason that ICV administration of NAN-190 or ketanserin has no alteration in the sleep-wake activity during the dark period is that the dose we used is 4–100 times less than the dosage used in other literatures [24–26]. We used this relative lower dose in current experiment to avoid nonspecific blockade of serotonin effect in rats with no suanzaorentang treatment during the dark period, but it could specifically block suanzaorentang-induced sleep effect.

Effects of 5-HT_3 receptor antagonists, 3-(4-Allylpiperazin-1-yl)-2-quinoxalinecarbonitrile, on suanzaorentang-induced sleep alteration

Our results demonstrated that ICV administration of 5-HT_3 receptor antagonists, 3-(4-Allylpiperazin-1-yl)-2-quinoxalinecarbonitrile in the dose of 10.0 nmol significantly decreased suanzaorentang-induced NREMS enhancement during the dark period. The total amount of time spent in NREMS decreased from 33.0 ± 1.4% obtained after ICV PFS + oral 4 g/kg/2 ml suanzaorentang to 22.4 ± 1.6% after ICV 10 nmol 3-(4-Allylpiperazin-1-yl)-2-quinoxalinecarbonitrile + oral 4 g/kg/2 ml suanzaorentang (Figure 4A, B, n = 8, $p < 0.05$, one-way ANOVA). The effect of 5-HT_3 receptor antagonists, 3-(4-Allylpiperazin-1-yl)-2-quinoxalinecarbonitrile, on NREMS suppression was similar to the results of NAN-190 and ketanserin (Figure 4B). REMS during the 12-h dark period was not significantly altered by NAN-190, ketanserin or 3-(4-Allylpiperazin-1-yl)-2-quinoxalinecarbonitrile (Figure 4D, n = 8). However, the time spent in REMS during the first 6-h dark period was significantly enhanced. The total amounts of time spent in REMS during the first 6-h of dark period were 8.1 ± 1.2% and 15.5 ± 2.6% obtained after ICV PFS + oral 4 g/kg/2 ml suanzaorentang and ICV 10 nmol 3-(4-Allylpiperazin-1-yl)-2-quinoxalinecarbonitrile + oral 4 g/kg/2 ml suanzaorentang, respectively (Figure 4C, n = 8, $p < 0.05$, one-way ANOVA). NREMS and REMS obtained from control rats were 23.3 ± 1.5% and 6.8 ± 1.1%. ICV administration of 3-(4-Allylpiperazin-1-yl)-2-quinoxalinecarbonitrile 20 min prior to the dark onset did not alter sleep-wake behavior during the dark period; the total amounts of time spent in NREMS and REMS were 20.7 ± 2.1% and 7.2 ± 1.2% obtained after ICV administration of 10 nmol of 3-(4-Allylpiperazin-1-yl)-2-quinoxalinecarbonitrile.

Discussion

Our previous report has shown that oral administration of suanzaorentang at the beginning of the dark period dose-dependently increased NREMS during the dark period, but had no significant effect on REMS [5]. We further confirmed this result in present study by using relative higher dose, 4 g/kg/2 ml of suanzaorentang. Although the effect of high dose of suanzaorentang on sleep regulation may be masked by high dose of starch placebo, NREMS was statistically significantly enhanced after oral administration of 4 g/kg/2 ml of suanzaorentang. The non-specific NREMS enhancement after food

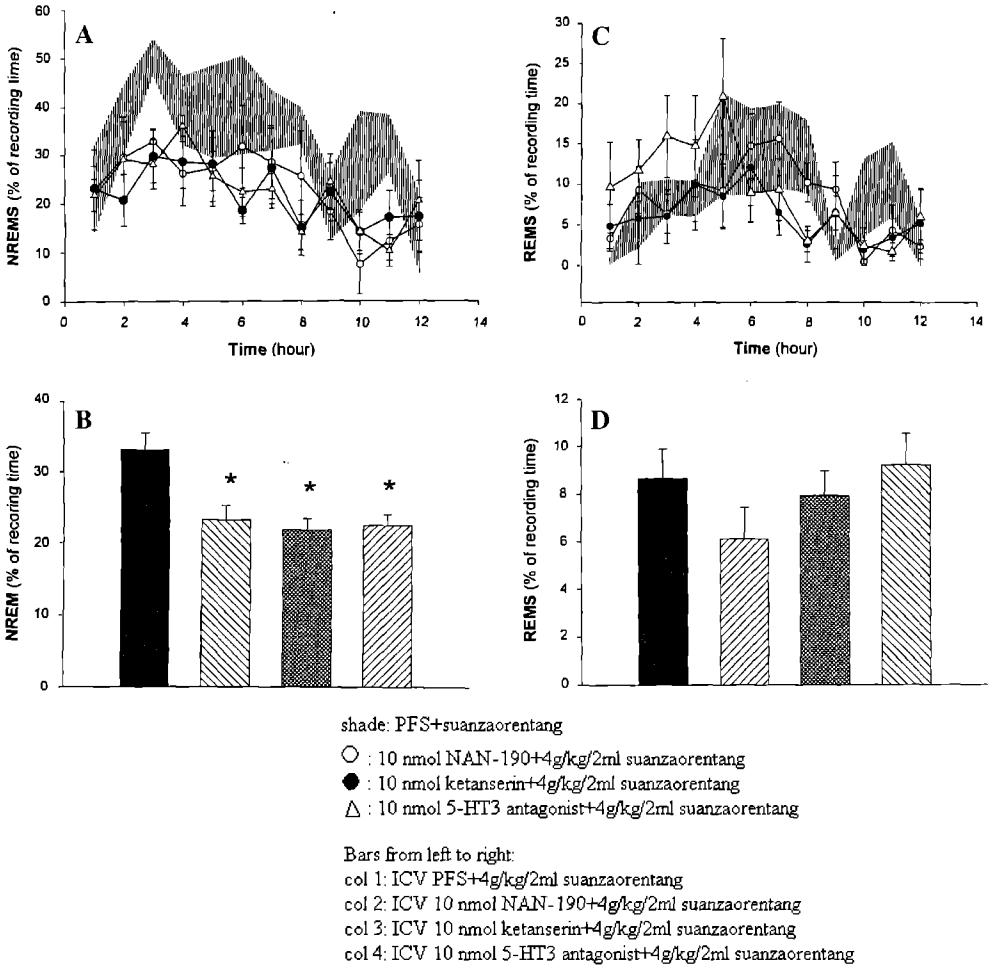

Figure 4. The effects of ICV administration of 5-HT_3 receptor antagonist, 3-(4-Allylpiperazin-1-yl)-2-quinoxalinecarbonitrile on the suanzaorentang-induced sleep alteration. The values represent as mean ± SEM. (A & C) Shade areas represent the values obtained after ICV PFS + oral 4 g/kg/2 ml suanzaorentang, open circles are obtained after ICV 10 nmol NAN-190 + oral 4 g/kg/2 ml suanzaorentang, close circles indicate the data obtained after ICV 10 nmol ketanserin + oral 4 g/kg/2 ml suanzaorentang, and open triangles depict the values obtained after ICV 10 nmol 3-(4-Allylpiperazin-1-yl)-2-quinoxalinecarbonitrile + oral 4 g/kg/2 ml suanzaorentang. (B & D) The bars from left to right represent the values after ICV PFS + oral 4 g/kg/2 ml suanzaorentang, ICV 10 nmol NAN-190 + oral 4 g/kg/2 ml suanzaorentang, ICV 10 nmol ketanserin + oral 4 g/kg/2 ml suanzaorentang and ICV 10 nmol 3-(4-Allylpiperazin-1-yl)-2-quinoxalinecarbonitrile + oral 4 g/kg/2 ml suanzaorentang. * Depicts that the values achieve statistically significant difference comparing with those after ICV PFS + oral 4 g/kg/2 ml suanzaorentang.

intake has been demonstrated [27, 28] and discussed previously [5]. The ability of sleep alteration by food may be mediated by its metabolic substances, stretching muscles because of the expansion of stomach, or both, which stimulates ascending vagus nerves and results in NREMS enhancement, since it has been reported that feeding cafeteria diet in vagotomized rats results in a suppression in NREMS, in contrast to the increase of NREMS in normal rats, suggesting that vagus nerve plays an important role in cafeteria diet-induced enhancement of NREMS [27]. There is evidence suggesting that the influence of food intake on sleep alteration, mediated through the vagal activation, is associated with enhancement of sleep-promoting cytokines, such as interleukin (IL)-1β and tumor necrosis factor. (TNF)-α. It has been observed that the plasma concentrations of TNF-α and IL-6 were elevated in obesity patients with excessive daytime sleepiness [29], and vagotomy attenuates TNF-α-induced sleep and EEG delta activity in rats [30].

It has been demonstrated that the water extract of the main ingredient of suanzaorentang, zizyphi spinosi semen, has receptor binding affinity for

serotonin receptor (5-HT_{1A} and 5-HT_2 receptors) and for GABA receptors. Our previous results had also indicated that ICV administration of $GABA_A$ antagonist, bicuculline significantly blocked suanzaorentang-induced enhancement in NREMS during the dark period, but $GABA_B$ antagonist, 2-hydroxysaclofen had no effect, implicating the involvement of GABAergic system. We herein further investigate whether the serotonergic system mediates suanzaorentang-induced sleep alteration. Serotonin is one of the major neurotransmitter participating in the control of sleep–wake circadian rhythm, but its function is still controversial. Previous studies have suggested that serotonin is a sleep promoter due to the evidence that destruction of serotonergic neurons in the raphe nucleus decreases sleep [16, 17], intraperitoneal injection of *p*-chlorophenylalanine to inhibit serotonin synthesis induces severe insomnia [18], and the discharge of serotonin neuron in the dorsal raphe nucleus is tightly associated with the sleep–wake behavior, exhibiting the highest discharge during wakefulness, a relative lower discharge in NREMS and a near quiescence during REMS [31]. Recently a literature has elucidated that the potentiation of L-type calcium channel blockers on pentobarbital-induced hypnosis is mediated by the serotonergic system [32]. Furthermore, both NREMS and REMS were increased when orally administered L-5-hydroxytryptophan (L-5-HTP), a precursor of serotonin, into hamsters [33]. Fluoxetine, a selective serotonin reuptake inhibitor, decreases REMS; however it enhances NREMS at lower doses (5 and 10 mg/kg) and decrease NREMS at higher doses (20 and 40 mg/kg) [34]. Neckelmann et al. recently have shown that the postsynaptic 5-HT_{1A} stimulation increases slow wave activity in NREMS EEG, and that other receptor subtypes may have an opposite effect on slow wave activity in REMS EEG [35]. However, serotonin has been widely proposed as a waking promoter later. Evidence that systemic administration of 5-HT_{1A} receptor selective agonist increases wakefulness and sleep latency and reduces REMS [19], and that blockade of 5-HT_{2A} receptors induces an increase in NREMS in 5-HT_{2A} +/+ (wild-type) mice, but not in 5-HT_{2A} −/− (knock-out) mice [20], suggests the involvement of serotonin in the waking regulation. It has been proposed that 5-HT_{1A} receptor agonist may influence wakefulness, NREMS and REMS depending on the dose and agonist used [36–38]. Nevertheless, the biphasic effect of serotonin on sleep-wake regulation may be due to the fact that serotonin can act at distinct brain regions that involve the control of sleep-wake activity, and that of different distributions of different 5-HT receptor subtypes in the brain [39]. Our results of decreasing suanzaorentang-induced NREMS enhancement by administrating 5-HT_{1A} antagonist (NAN-190), 5-HT_2 antagonist (ketanserin) and 5-HT_3 antagonist (3-(4-Allylpiperazin-1-yl)-2-quinoxalinecarbonitrile) suggest the involvement of 5-HT receptors, especially 5-HT_{1A}, 5-HT_2 and 5-HT_3 receptors, although it has been hypothesized that the main receptor subtypes mediated NREMS are 5-HT_2 and 5-HT_{1A}, and 5-HT_3 seems to be involved in the regulation of REMS or wakefulness [40].

The hypocretins are a pair of neuropeptides contributing to the regulation of sleep–wake activity [41], in addition to its energy homeostasis. It has been shown that hypocretin neurons exhibit discharge associated with wakefulness and its discharge is quiescent during the NREMS and REMS [42]. Microinjection of hypocretin into various brain regions, such as basal forebrain [43] and locus coeruleus [44], promotes wakefulness. Mice lacking of the hypocretin gene have similar phenotypes to the human sleep disorder, narcolepsy [45]. Furthermore, human narcoleptics exhibit hypocretin neuron loss [46]. Immunocytochemical study has shown that the hypocretin neurons in the hypothalamus are surrounding by dense serotonergic nerve endings which originate from the raphe nuclei in the midbrain [47]. In addition, Muraki et al. demonstrated that serotonin hyperpolarizes hypocretin neurons through the 5-HT_{1A} receptor and subsequent activates the G-protein-coupled inward rectifier potassium channels [48]. Therefore, this inhibitory serotonergic input to the hypocretin neurons is likely to be important for the effect of suanzaorentang on NREMS enhancement during the dark period in rats. However, whether the sleep alteration induced by suanzaorentang is mediated through activation of serotonergic system and subsequently inhibit hypocretin activity needs to be further investigated. It has been proposed that 5-HT_2 receptor mediates IL-1, the somnogenic cytokine-induced increase of NREMS [49]. Our present result also suggests that suanzaorentang may influence 5-HT_2 activity to exhibit its sleep regulation. Although the effect of 5-HT_3 on sleep

regulation has received little attention, one literature demonstrated that ICV administration of 5-HT_3 agonist, *m*-chlorophenylbiguanide, reduces REMS [50]. This observation may explain the results of REMS increase after administering 5-HT_3 receptor antagonist, 3-(4-Allylpiperazin-1-yl)-2-quinoxalinecarbonitrile.

Suanzaorentang comprises zizyphi spinosi semen, poria cocos, ligusticum wallichii, anemarrhenae rhizoma and glycyrrhizae in ratio of 4:2:2:2:1. It would be worth to understand which component(s) is (are) essential for exhibiting the sleep–wake regulation. Our results indicate that none of those combinations with any one ingredient missed exhibits the ability of NREMS enhancement, suggesting that any one of those ingredients is important for expressing suanzaorentang's action. We also found that NREMS obtained after administration of C-II and C-III was statistically significant less than that obtained after giving placebo starch, implicating that poria cocos and anemarrhenae rhizoma may play an important role for the suanzaorentang's action on sleep regulation. This result is consistent with the concept of "sovereign, minister, assistant, and envoy" in the traditional Chinese Medicine, which means each ingredient has its own pharmaceutical effect and the interactions between ingredients may reinforce/exhibit its therapeutic effect and/or reduce the adverse effect in one mixture of Chinese remedy. To exhibit the therapeutic effects, the compound of Chinese medicine cannot lack any single original ingredient.

Collectively, our current results and previous observation [5] suggest that the effect of suanzaorentang on sleep enhancement could act as an alternative therapy for treating insomnia, and its underlying mechanisms may be mediated through the activation of serotonergic system in additional to the activation of $GABA_A$ receptors [5].

Acknowledgements

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