The Effects of Sansoninto on the Insomnia in Socially Isolated Mice

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Running title

Sansoninto improved insomnia in isolated mice.

Abstract

Sansoninto (SAT) is a hypnotic herbal medicine used for individuals with a sleeping disorder caused by exhaustion. We have evaluated the effects of SAT on socially isolated mice with insomnia since there has been no report of such testing conducted. Four-week old, male ICR mice were used for this study. They were kept either in an independent, isolated rearing or in a group rearing for nine weeks. Administering 730 mg/kg of SAT in their drinking water each day for seven days, then we investigated their electroencephalogram and electromyogram, the locomotor activities for 24 hours, and the corticosterone concentration in plasma for evaluating insomnia and behavioral changes. Socially isolated mice showed higher locomotor activities during the lighted period than the group-housed mice. Proceeding SAT use, they showed a sign of effect in the amount of activity. Awake-time of the subjects under the isolated rearing increased for both lighted and unlighted periods. NREM sleep time shortened, but REM sleep time showed no changes. SAT showed improvements in awake-time and NREM sleep time. Plasma corticosterone concentration increased for the isolated, and was showed improvements after the SAT treatment. Socially isolated mice showed higher locomotor activities during the day, and their amount of sleep time were shorter than those of group rearing. Stress load seems to be the culprit for the difference found. SAT reduced the motor activity on this test and prolonged the length of sleeping period; thus, it suggests that SAT has positive effects on insomnia from surrounding environments.

Keywords: Herbal Medicine, Sansoninto, Isolated stress, Insomnia, Mice

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Introduction

Sansoninto (SAT) is a hypnotic herbal medicine used for individuals with sleeping disorder caused by exhaustion [1]. It is prescribed for patients feeling weakness and fatigue, annoyance, insomnia, amnesia, and/or neurotic symptoms [2]. SAT is comprised of five herbal medicines: Sansohnin, Licorice, Chimo, Senkyu and Bukuryo. Sansohnin is the principal ingredient of SAT and has hypnosis sedation. Spinosin [3-5], sanjoinine A [6-8], jujubosaides [9] are considered as active ingredients within it.

There have been clinical reports of sleeping disorders where SAT had been prescribed to 60 patients with insomnia. It showed improvements in quality of sleep when compared with the likes of placebo [10]. On the other hand, there are almost no previous reports of this particular medicine being tested on animals with insomnia. It has been reported that pentobarbital-induced sleep time shortened because of repeated cold stress, 45 min-restraint stress, social isolation stress [11-13]. SAT reversed shortened pentobarbital-induced sleep time in repeated cold stress or 45 min-restraint stress [11]. It means SAT is effective for the kinds with hyper-awakening state. However, there are no previous reports of this medicine being tested on animals with insomnia caused by environmental stress.

For people, a lot of diseases causing sleep disorder have stress: adjustment disorders, posttraumatic stress disorder, stress disorder, panic disorder, and generalized anxiety disorder. Sleep disorder with adjustment disorders is the representative [14]. According to Comprehensive Survey of Living Conditions (2010) of Ministry of Health, Labour and Welfare, the ratio of Japanese people who replied that there were a trouble and stress in the everyday life was 46.5%. Stress is classified roughly in physical stress and psychosocial stress. If it is said that the modern society is full of stress, it means psychosocial stress [15]. So, we must consider the method of stress load when we evaluate the effect of SAT in mice.

Isolation stress is a method to give a kind of psychosocial stress by the isolation breeding for a long term. It cause anxiety, depression, and behavioral change such as the aggressiveness that is not appears of group breeding [16, 17]. This is called "Isolation Syndrome", and characterized by the excitement of the sympathetic nervous system such as a change of the emotional behavior including the increase of a fright reaction, aggressiveness [18]. Therefore, we have evaluated the effects of SAT on socially isolated mice in this study.

Materials and Methods

Materials and Animals

Male ICR mice aged four weeks and weighing 30- 35 g, were obtained from Kyudo (Saga, Japan). Animals were housed either in social isolation (1 mouse per cage) or in social groups (10-12 mice per cage) for 9 weeks prior to testing, under standardized lighting conditions (lights on 07:00-19:00) at a constant temperature (23 ± 2°C) with food (CE-2; CLEA Japan, Inc., Tokyo) and water available ad libitum.

SAT was provided by TAIHO PHARMACEUTICAL CO., LTD. (Tokyo, Japan) and dissolved in distilled water. After isolated, SAT was administered in their drinking water for seven days. The doses were carried out in 730 mg/kg. Everyday, we measured amount of drinking and calculated the dose of SAT. Then,

we confirmed each dose to give it at a designated dose. Controlled animals received their vehicle (water only) via the same exact method everyday. After given this medication, we investigated their electroencephalogram (EEG) and electromyogram (EMG), the locomotor activities for 24 hours, and the corticosterone concentration in plasma in order to evaluate Awake/sleep pattern and a behavioral change.

All procedures regarding animal care and use were carried out based on the regulations established by the Experimental Animal Care and Use Committee at Fukuoka University, Japan.

Measurement of locomotor activity

We use a release type infrared detection sensor (NS−AS01, Neuroscience Inc. Tokyo, Japan) in upper part 15 cm of the measurement cage (30×36×17 cm). We recorded output counts to a computer through DIGITAL ACQUISITION SYSTEM interface (NS−DAS−8, Neuroscience Inc.) and analyzed it with analysis software (32 multi-digital port count systems 1.05.0002, NMR Co., Ltd., Tokyo, Japan). The data expressed by the momentum of light period from 7:00 to 19:00 and dark period from 19:00 to 7:00.

Polygraphic Recordings in Mice

Under pentobarbital anesthesia (50 mg/kg, i.p.), mice were chronically implanted with EEG and EMG electrodes for polysomnographic recordings [19]. Pentobarbital sodium salt was provided by TOKYO CHEMICAL INDUSTRY CO., LTD. (Tokyo, Japan).

The implant consisted of two stainless-steel screws (AN-3; EiCOM, Kyoto, Japan) serving as EEG electrodes, inserted, according to the atlas of Franklin and Paxinos [20], through the skull into the cortex (anteror, -2.0 mm and left, +1.5 mm from bregma); and two insulated, stainless-steel, Teflon-coated wires, serving as EMG electrodes (UNIQUE MEDICAL CO., LTD., Tokyo, Japan), which were placed into left trapezius muscles. All electrodes were attached to a microconnector and fixed to the skull with dental cement (SHOFU INC., Kyoto, Japan) [21, 22].

Postoperatively, the animals were administrated SAT in their drinking water for seven days. After given SAT, sleep–wakefulness states were monitored for a period of 24 hours.

Cortical EEG and EMG signals were digitized at a sampling rate of 256 Hz, a sensitive 100 μV. And they were recorded by using Vital Recorder (Kissei Comtec, Nagano, Japan). Polygraphic recordings were automatically scored offline by 4 seconds epochs as wakefulness or NREM, REM sleep including fast Fourier transform (FFT)-power spectra by SLEEPSIGN according to standard criteria [21]. As a final step, defined sleep–wake stages were examined visually, and corrected, if necessary.

Corticosterone concentration in plasma

We collected blood by decapitation after the measurement of the motor activity. This was specifically conducted at 10:00 am. The blood was centrifuged at 3,000×g and we got plasma. After that, we measured plasma corticosterone concentration by ELESA kit (AssayPro, MO, USA).

Statistical analysis

Results were analyzed by one-way analysis of variance (ANOVA), followed by the Dunnett's post-hoc test to determine differences among the groups. Values are expressed as the mean ± SEM. The criterion for statistical significance was considered to be $P < 0.05$.

Results

Locomotor activity for 24 hours

To evaluate their behavioral changes through isolation and stress, we investigated locomotor activities. Figure 1A showed the progress of every hour. All groups showed consistent increase in the quantity of locomotor activity during the dark phase. Figure 1B shows the level of locomotor activity of during the Light phase; Figure 1C shows that of the dark phase. Socially isolated mice showed significantly higher quantities of motor activity during their lighted period compared to the group-housed mice. Preceding the SAT administration, the subjects have shown a significantly noticeable sign of the effect from the medication during the lighted period (Fig. 1B). There were no significantly changes seen from the isolated group during the dark phase. There were also no major found for that of the dark phase following the SAT administration (Fig. 1C).

Analysis of the sleep cycle

In order to identify the quantity of their sleep, we checked the sleep cycle to distinguish between the states of their sleep versus the state of repose. This is imperative since their level of locomotor activity alone would not be able to determine whether they are sleeping or resting. Socially isolated mice showed longer awake-time for both lighted and unlighted phases compared to those of the group-housed mice. SAT resulted in a significantly decrease of their awake-time, which was also similar for those of the group-housed mice as well (Fig. 2A). NREM sleep time of the subjects under the isolated rearing was also significantly shorter for both periods. SAT showed significantly prolonged NREM sleep time, and this was true to the levels similar to those of the group-housed mice also (Fig. 2B). The Grouped and the Isolated disclosed no change in their REM sleep time. The Isolated also showed no change for both lighted and dark periods after SAT was administered to them (Fig. 2C).

For the purpose of identifying the quality of their sleep, we estimated the depth of their sleep by analyzing the EEG, which may be instrumental in spotting the characteristics of SAT. The details from this research found for NREM sleep period show no changes in the δ wave rates between the regular and the isolated rearings. The same was found true for the Isolated and the Isolated medicated with SAT (Fig. 3A, 3B).

Corticosterone concentration in plasma

In order to confirm whether or not the stress was the cause of changes detected in specific areas such as their motor activity levels and their sleep cycles, we measured their glucocorticoid. Glucocorticoid acting on predominance in mice in particular is corticosterone; thus, we showed the result that we discovered through ELESA kit in Figure 4. Plasma corticosterone concentration increased for the Isolated. After SAT use, it showed improvements.

Discussion

In this study, we measured locomotor activity and the sleep analysis by the EEG/ EMG and plasma

corticosterone concentration for group breeding group, isolation breeding group, and SAT administrated group in order to evaluated efficacy of SAT. Socially isolated mice showed longer awake-time and shorter NREM sleep time for both lighted and dark periods. In other words, this study showed the isolated mice had a sleep disorder. As mentioned above, isolation breeding causes anxiety, depression, behavioral changes such as excessive aggression [16, 17], and pentobarbital-induced sleeping time is shorted [11-13]. Miyashita et, al. also reported that adrenal enlargement and corticosterone concentration rise in a social isolated stress mouse [23]. In this study, we detected behavioral change of the increase in locomotor activity. And they increased corticosterone concentration in plasma. These results do not contradict a previous report. The report of sleep disorder in the stress load animal does not exist until now. In this study, we detected that a sleep disorder occurred in the stress load animal by analyzing EEG / EMG. It is important that we established a stressful sleeplessness animal model when we examine the effectiveness of the therapeutic drug of sleep disorder.

To detect a physiological sleep disorder, we adopted the EEG / EMG measurement, not the measurement of the righting reflex disappearance time by pentobarbital which had been performed conventionally [11-13]. As a result of EEG / EMG analysis, the NREM sleep that decreased with isolation breeding increased by the dosage of the SAT. Because the share of each power spectra in NREM sleep did not have a change, it was thought that sleep disorder by the isolation breeding was an obstacle of the quantity rather than the quality of the sleep. Therefore, the increase of locomotor activity is more likely to depend on the WAKE time. It is said that surplus of the central noradrenaline nervous system, corticotropin releasing hormone nervous system, or the drop of the gamma-aminobutyric acid A ($GABA_A$) receptor function are regarded as the cause that the pentobarbital-induced sleep shorten in isolated mice [12, 13]. When we gave SAT to this stress model mouse, they showed less quantity of motor activity during the lighted period, improvements in awake-time and prolonged the NREM sleep time. And plasma corticosterone concentration decreased to levels similar to those of the group-housed mice. The active components of SAT and their pharmacological mechanism of action have not been clarified yet. Many reports have demonstrated the hypnotic effects of Sansohnin and its total flavonoids [24, 25]. Spinosin, also known as 2-β-o-glucopyranosyl swertisin, is one of the major flavonoids of Sansohnin, and reported the effect for insomnia [2]. It is thought that serotonergic mechanism involved in the effect. We should elucidate the mechanism of SAT in future.

The benzodiazepine sleeping drug, generally used for insomnia, reduce REM sleep and a slow-wave sleep [26]. They have the side effects such as daytime sleepiness by hang over, a fall or the bone fracture by muscle relaxation effect [27, 28]. Resistant problems by the long-term use are pointed out elsewhere. Because we did not recognize a difference to a sleep EEG share of each stage in SAT, it is thought that SAT improves a sleep disorder without benzodiazepine receptor. It is suggested that SAT would have no side effect such as carry-over or muscle relaxation effect like a benzodiazepine sleeping drug. In clinical situation, it has been widely reported that SAT would show the side effects such as a fall, bone fracture, daytime sleepiness [29]. Such piece of knowledge is vital as we consider drug choices for insomnia. SAT, being used since ancient times, should become one of the clear choices when treating sleep disorders.

Socially isolated mice showed higher locomotor activities during the day in our studies, and their

sleep hours were shorter than those of the group rearing. The stress load seems to be the culprit for the difference seen in the two. SAT reduced the motor activity shown on this test and prolonged the length of the subjects' sleeping period; thus, it suggests that SAT has effects on insomnia caused by the subjects' surrounding environments.

Acknowledgments

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Legends for Figures

Fig. 1. Motor activity for 24 hours

(A) This showed the progress of every hour. All groups showed consistent increase in the quantity of locomotor activity during the dark phase. (B) This shows the level of locomotor activity of during the light phase. Socially isolated mice showed more quantity of locomotor activity during the lighted period than the group-housed mice. Proceeding SAT being given, the medication showed a sign of effect on controlling the amount of activities during the lighted period. (C) This shows the level of locomotor activity of during the dark phase. During the dark period, there were no changes. Values are expressed as the means ± S.E.M. of 10-14 mice. *, **; $P < 0.05$, $P < 0.01$ *vs*. respectively, one-way ANOVA and *post-hoc* Dunnett's test.

Fig. 2. Analysis of the sleep cycle for EEG and EMG: Each stage time

(A) Socially isolated mice showed longer awake-time for both lighted and unlighted periods than the group-housed mice. (B) NREM sleep time of the subjects under the isolated rearing shortened for both periods. After isolated, SAT was administered in their drinking water for seven days. The doses were carried out in 730 mg/kg. SAT showed improvements in awake-time and significantly prolonged the NREM sleep time in socially isolated mice to levels similar to those of the group-housed mice. (C) REM sleep time showed no change. Values are expressed as the means ± S.E.M. of 12 mice. *, **, ***; $P < 0.05$, $P < 0.01$, $P < 0.001$ *vs*. respectively, one-way ANOVA and *post-hoc* Dunnett's test.

Fig. 3. Analysis of the sleep cycle for EEG and EMG: FFT-power spectra in the NREM sleep

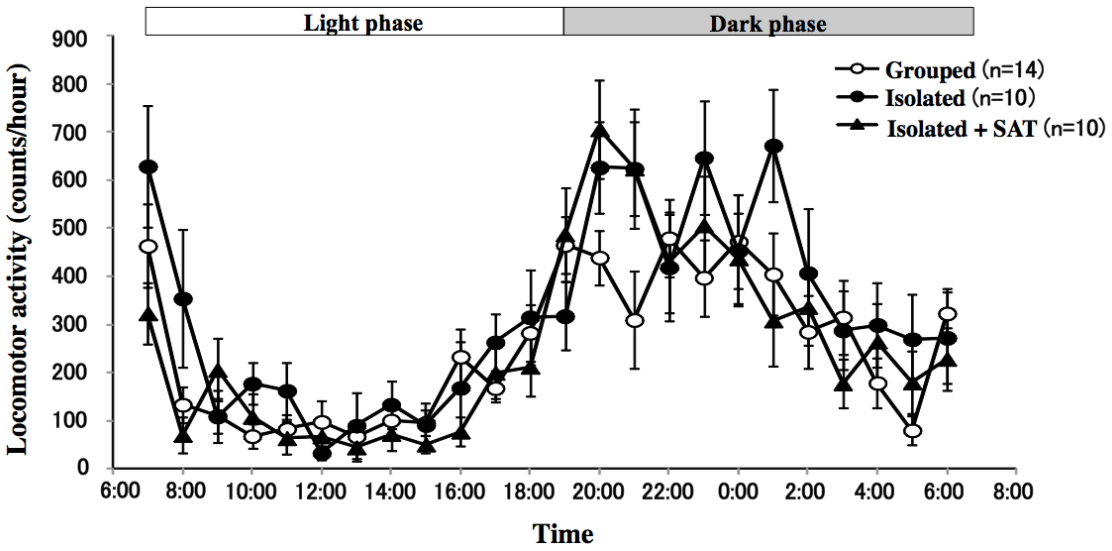
(A, B) Details on NREM sleep period, No changes seen in the δ wave rates with the isolation and the SAT.

Fig. 4. Corticosterone concentration in plasma

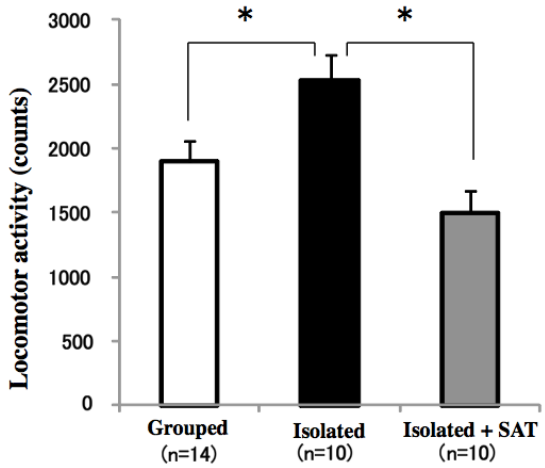
Plasma corticosterone concentration increased for the isolated. After SAT use, it showed improvements. Values are expressed as the means ± S.E.M. of 12 mice. *; $P < 0.05$ *vs*. respectively, one-way ANOVA and *post-hoc* Dunnett's test.

Figure 1. Motor activity for 24 hours

A) 24 hours



B) Light phase



C) Dark phase

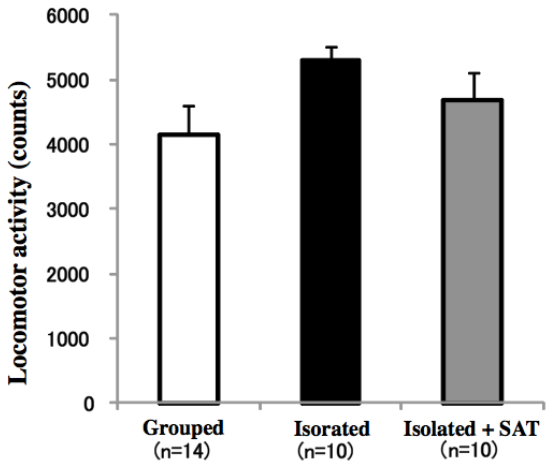
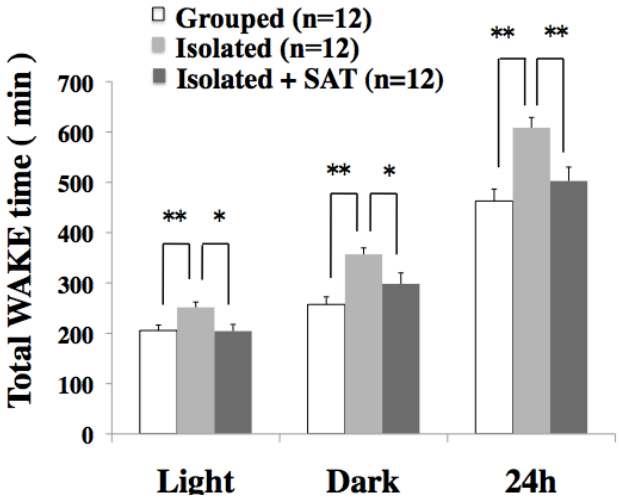
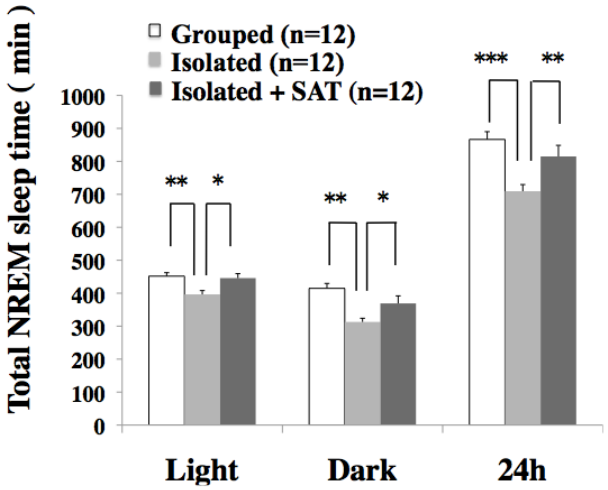


Figure 2. Analysis of the sleep cycle for EEG and EMG: Each stage time

A) Awake time



B) NREM sleep time



C) REM sleep time

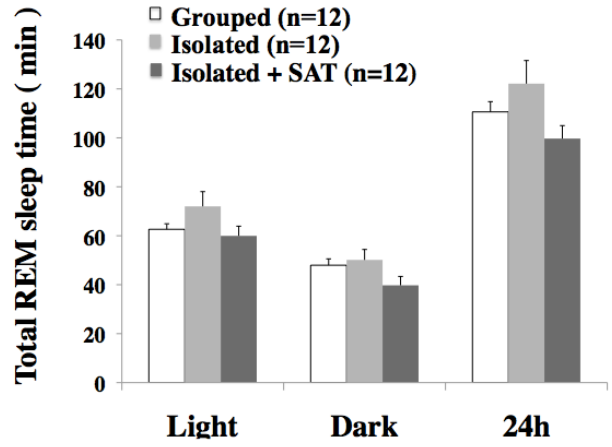
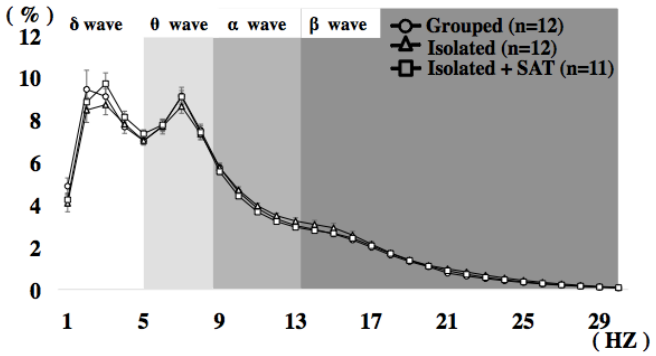


Figure 3. Analysis of the sleep cycle for EEG and EMG: FFT-power spectra in the NREM sleep

A) Light phase



B) Dark phase

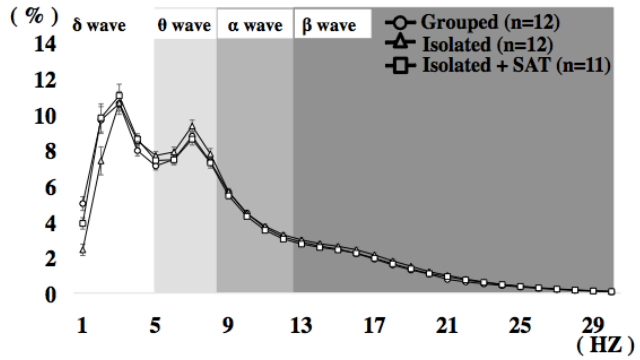


Figure 4. Corticosterone concentration in plasma