Milk Collected at Night Induces Sedative and Anxiolytic-Like Effects and Augments Pentobarbital-Induced Sleeping Behavior in Mice

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ABSTRACT Milk has long been known and used to promote sleep. The sleep-promoting effect of milk has been attributed to its psychological associations (*i.e.*, the memory of a mother giving milk at bedtime) and its rich store of sleep-promoting constituents (*e.g.*, tryptophan). Studies have shown that milk harvested at night (Night milk) contains exceptionally high amounts of tryptophan and melatonin. In the present study, we evaluated the psychopharmacological properties of Night milk, particularly its probable sleep-promoting/enhancing, and anxiolytic effects. Night milk was orally administered to ICR mice at various concentrations (100, 200, or 300 mg/kg). An hour after administration, assessment of its sedative (open-field and rotarod tests) and sedative sleep-potentiating effects (pentobarbital-induced sleeping test) was conducted. For comparison, the effects of Day milk (daytime milking) were also assessed. In addition, the effects of Night milk on anxiety behavior (elevated plus maze [EPM] test) and electroencephalographic (EEG) waves were evaluated. Night milk-treated animals exhibited decreased spontaneous locomotion (open-field test) and impaired motor balance and coordination (rotarod test). Furthermore, Night milk shortened the sleep onset and prolonged the sleep duration induced by pentobarbital sodium. These effects were comparable to that of diazepam. In addition, Night milk significantly increased the percentage of time spent and entries into the open arms of the EPM, indicating that it also has anxiolytic effects. No significant changes in EEG waves were observed. Altogether, these findings suggest that Night milk is a promising natural aid for sleep- and anxiety-related disturbances.

KEY WORDS: • *anxiolytic* • *electroencephalography* • *Night milk* • *pentobarbital* • *sedative* • *sleep*

INTRODUCTION

MILK HAS LONG been known and used to promote sleep. The sleep-promoting effect of milk has been attributed to its psychological associations (*i.e.*, the memory of a mother giving milk at bedtime) and its rich store of sleep-promoting components. One of these components is the essential amino acid tryptophan.¹ Tryptophan can be converted to serotonin and melatonin, consequently inducing relaxation and sleepiness.² Melatonin is a naturally occurring hormone, secreted by the pineal gland, which helps regulate the sleep and wake cycle (circadian rhythm).^{3,4} Accordingly, in humans and animals alike, melatonin is known to be at its highest concentration at night and during sleep.^{5,6} However, melatonin levels decrease with age leading to impairment in the quality of sleep and other sleep disturbances.^{7–9} Exogenous administration of tryptophan

and melatonin aids in inducing drowsiness and sleep and may ameliorate sleep disturbances and disorders.¹⁰

Recent studies have shown that milk collected during night time (Night milk) may have unique advantages over milk harvested during daytime (Day milk). It was found that Night milk has exceptionally rich amounts of tryptophan and melatonin, and that rats given Night milk showed an increased circulating melatonin.⁴ Furthermore, a study conducted in elderly patients showed that Night milk improved sleep quality that resulted in a better daytime activity.⁹ In view of these reports, the present study evaluated the psychopharmacological effects of Night milk, particularly its probable sedative and sleep-promoting/enhancing properties. For comparison, the effects of Day milk were also assessed. In addition, its effects on anxiety-like behavior and electroencephalographic (EEG) waves were also evaluated.

MATERIALS AND METHODS

Animals

Male ICR mice (for behavioral tests [25–30 g]) and Sprague-Dawley rats (for EEG experiment [250–300 g]) were obtained from the Hanlim Laboratory Animals Co.

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(Hwasung, Korea). They were housed in a temperature-controlled ($22^{\circ}\text{C} \pm 2^{\circ}\text{C}$) and humidity-controlled ($55\% \pm 5\%$) animal room on a 12-h light/12-h dark (07:00–19:00 h light) schedule. They were given a 7-day acclimatization period before the commencement of any experiment. Food (Lab Rodent Chow, 38057; Purina Korea, Inc., Seoul, Korea) and water were freely available, except on the night before and during the experiments. Rodents were divided into groups (eight groups for behavioral test and four groups for EEG recording) with 10–12 animals per group. All experiments were performed between 10:00 and 16:00. Animal treatment and maintenance were carried out in accordance with the Principles of Laboratory Animal Care (NIH publication No. 85–23 revised 1985) and the Animal Care and Use Guidelines of Sahmyook University, Korea.

Drugs and materials

Night and Day milk (lactose-hydrolyzed skim milk powder), obtained from Synlait Milk Ltd. (Rakaia, New Zealand), was kindly provided by CheilJedang Nutraceutical & Functional Foods Center (Seoul, Korea). Briefly, milk was collected from cows during night or day time and stored in a refrigerator at 7°C . The milk was pasteurized at 72°C for 15 sec and then skimmed. Lactose in the milk was hydrolyzed by lactase and evaporated until 50% of total solid. The concentrated milk solution was filtered and spray dried producing a readily soluble milk powder. Analysis of the milk powders revealed that Night milk contains higher amounts of tryptophan and melatonin (465.88 mg/100 g and 85.5 pg/g, respectively) than Day milk (375.16 mg/100 g and 8.8 pg/g, respectively).

Diazepam was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and pentobarbital sodium was obtained from Hanlim Pharm. Co., Ltd. (Seoul, Korea). Night and Day milk was freshly prepared in sterile distilled water and diazepam and pentobarbital in physiological saline. Night milk was orally *per orem* (p.o.) administered to mice in dosages of 100, 200, or 300 mg/kg (Night milk-treated group), and Day milk was also administered orally in the same dosages (Day milk-treated group). The milk treatment did not produce any observable side effects in rodents. Diazepam was intraperitoneally (i.p.) injected at 1 mg/kg (positive-control group). Animals in the control group received the vehicle (distilled water, p.o.).

Psychopharmacological evaluation

Open-field test. The general locomotor activity was evaluated in a square Plexiglas open-field arena measuring $42 \times 42 \times 42$ cm. Night milk, Day milk, diazepam, or vehicle was administered to mice. One hour after the administration, mice were placed in the open-field arena. To remove the bias of novelty, a 2-min habituation period was given. Then, the distance moved (cm) and movement duration (sec) of each mouse were recorded for 10 min by an automated system (Ethovision, Noldus, Netherlands).¹¹

Rotarod test. Motor balance and coordination were assessed in a rotating rod (Ugo Basile, Varese, Italy) at a fixed speed of 36 rpm. The experiment was patterned from Choi *et*

al., with slight modifications.¹² A day before the actual experiments, mice were habituated and trained to run on the rotating rod for 3 min. On the day of the experiment, mice were randomly grouped and were given Night milk, Day milk, diazepam, or vehicle. After one hour, they were placed on the rotating rod for 20 min. Recorded parameters were latency to first fall (sec) and falling frequency.

Pentobarbital-induced sleeping test. Onset of sleep and sleep duration were gauged after the administration of pentobarbital sodium (42 mg/kg, i.p.). Mice were given a single dose of Night milk, Day milk, diazepam, or vehicle 30 min before the injection of pentobarbital. The experiments were executed as previously described by Ma *et al.*, with slight modification.¹³ The disappearance (onset of sleep) and reappearance of the righting reflex were observed and recorded. The time interval between disappearance and reappearance of righting reflex was considered as the duration of sleep.

Elevated plus-maze test. The elevated plus-maze (EPM) apparatus was made of plastic. The plus maze consisted of four arms; two open arms (30×6 cm) and two closed arms (30×6 cm) enclosed by 20-cm-high walls. Each arm had a delimited central area of 6×6 cm. The entire maze was elevated to a height of 50 cm above the floor. Mice were pretreated with vehicle, Night milk, or diazepam (i.p.) 60 min before placement on the EPM. To begin a test session, mice were placed in the center of the maze facing one of the open arms. The entry into an arm was defined as the animal placing all four paws over the line marking that area. The number of entries and the time spent in the open arms were recorded during a 5-min test period.¹⁴ The Ethovision system was used for observation and recording. The percentage of open-arm entries ($100 \times \text{open}/\text{total entries}$) and the percentage of time spent in the open arm of the maze were calculated for each animal.

Electroencephalography. Rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and fixed in a stereotaxic apparatus (51600 Stoelting Co., Wood Dale, IL, USA). A two-channel digital transmitter (Telemetry Research, Auckland, New Zealand) was i.p. implanted and then electrodes were shunted toward the skull. Electrodes were positioned into the left frontal and right occipital cortical regions and then fixed with screws and stabilized with dental cement. Rats were given a 7-day postsurgical recovery period. Rats were given vehicle, Night milk, or diazepam, an hour before the commencement of EEG recording. EEG waves were monitored, recorded, and analyzed by the LabChart 7.3 software (ADInstruments, Colorado Springs, CO, USA). Two frequency bands, delta (0.5–4.0 Hz; slow wave) and alpha (8–13 Hz; fast wave), were quantified using the Fast Fourier-transform.

Statistical analyses

All data are expressed as mean $\pm$ standard error of the mean. Data were analyzed using one-way analysis of variance

FIG. 1. Effects of Night milk and Day milk on the locomotor activity in mice. Each bar represents the mean $\pm$ SEM of the distance moved (A) and movement duration (B) for 10 min. * $P < .05$, ** $P < .01$, and *** $P < .001$ significantly different from the control group; $n = 10-12$. Con, control; Dzp, diazepam; SEM, standard error of the mean.

(ANOVA). To compare each group versus the control group, a one-tailed unpaired t -test was employed. All statistical analyses were conducted using GraphPad Prism version 5.02 software (GraphPad Software, Inc., La Jolla, CA, USA). Differences were considered statistically significant when $P < .05$.

RESULTS

Open-field test

Figure 1 shows the distance moved (A) and the movement duration (B) of mice treated with Night milk, Day milk, diazepam, or vehicle (control group). One-way ANOVA showed significant differences in the distance moved [$F(7, 73) = 2.62$, $P < .05$] and movement duration [$F(7, 73) = 7.07$, $P < .001$] between the experimental groups. As expected, the diazepam-treated group showed a significant decrease in the distance moved [$t(20) = 3.14$, $P < .01$; Fig. 1A] and movement duration [$t(20) = 4.00$, $P < .001$; Fig. 1B] compared

with the control group, demonstrating its sedative effect. In the same manner, the Night milk also significantly decreased the distance moved and movement duration at dosages of 100 mg/kg [$t(20) = 1.75$, $P < .05$ and $t(20) = 2.14$, $P < .05$], 200 mg/kg [$t(20) = 2.28$, $P < .05$ and $t(20) = 2.08$, $P < .05$], and 300 mg/kg [$t(20) = 2.26$, $P < .05$ and $t(20) = 2.20$, $P < .05$]. Day milk failed to induce significant changes compared with the control group.

Rotarod test

Figure 2 shows the latency to first fall (A) and the falling frequency (B) of mice treated with Night milk, Day milk, diazepam, or vehicle (control) on the rotarod test. One-way ANOVA showed significant differences in latency to first fall [$F(7, 73) = 2.54$, $P < .05$] and falling frequency [$F(7, 73) = 6.98$, $P < .001$] between the experimental groups. Diazepam-treated mice showed a decreased latency to first fall [$t(19) = 5.73$, $P < .001$; Fig. 2A] and an increased falling frequency [$t(19) = 4.32$, $P < .001$; Fig. 2B] compared with

FIG. 2. Effects of Night milk and Day milk on the rotating rod performance of mice. Each bar represents the mean $\pm$ SEM of the latency time (A) and falling frequency (B) during the 20-min rotarod test. * $P < .05$, ** $P < .01$, and *** $P < .001$ significantly different from the control group; $n = 10-12$.

FIG. 3. Effects of Night milk and Day milk on pentobarbital-induced sleeping test in mice. Each bar represents the mean $\pm$ SEM of the onset of sleep (A) and total duration of sleep (B) during the 120-min sleeping test. * $P < .05$, ** $P < .01$, and *** $P < .001$ significantly different from the control group; $n = 10-12$.

the control group. Likewise, Night milk produced a statistically significant decrease in latency time and an increase in falling frequency at dosages of 100 mg/kg [$t(19) = 2.53$, $P < .05$ and $t(19) = 2.52$, $P < .05$], 200 mg/kg [$t(19) = 2.39$, $P < .05$ and $t(19) = 2.51$, $P < .05$], and 300 mg/kg [$t(19) = 2.01$, $P < .05$ and $t(19) = 3.23$, $P < .01$]. Day milk-treated mice did not show any significant change versus the control group.

Pentobarbital-induced sleeping test

Figure 3 shows the onset (A) and duration (B) of sleep induced by pentobarbital sodium in mice pretreated with Night milk, Day milk, diazepam, or vehicle (control). One-way ANOVA showed significant differences in sleep onset [$F(7, 74) = 10.57$, $P < .001$] and duration [$F(7, 74) = 17.01$, $P < .001$] between the experimental groups. Diazepam treatment augments pentobarbital-induced sleeping behaviors as evidenced by a decreased sleep onset [$t(20) = 7.81$, $P < .001$; Fig. 3A] and increased sleep duration [$t(20) = 8.94$, $P < .001$;

Fig. 3B]. Similarly, Night milk at dosages of 100 mg/kg [$t(20) = 2.35$, $P < .01$ and $t(20) = 2.60$, $P < .01$], 200 mg/kg [$t(20) = 2.51$, $P < .01$ and $t(20) = 3.34$, $P < .01$], and 300 mg/kg [$t(20) = 3.26$, $P < .01$ and $t(20) = 4.01$, $P < .001$] decreased the sleep onset and increased the sleep duration of mice. The Day milk failed to produce any significant difference compared with the control group.

EPM test

Figure 4 shows the percentage of time spent (A) and entries (B) into the open arms of the EPM. One-way ANOVA revealed a significant difference between the experimental groups in the percentage of entries [$F(4, 44) = 5.29$, $P < .01$] and time spent [$F(4, 45) = 5.16344$, $P < .001$] in the open arms of the EPM. An increase in the percentage of entries and time spent in the open arms of an EPM is indicative of the anxiolytic property of a substance. Diazepam, a known anxiolytic drug, significantly increased the percentage of entries [$t(18) = 4.62$, $P < .001$; Fig. 4A]

FIG. 4. Effects of Night milk on the elevated plus-maze test in mice. Each bar represents the mean $\pm$ SEM of the percentage of time spent (A) or the percentage of entries (B) in the open arms for 5 min. * $P < .05$, ** $P < .01$, and *** $P < .001$ significantly different from the control group; $n = 10-12$.

FIG. 5. Effects of Night milk on the electroencephalogram recording in rats. Each bar represents the mean $\pm$ SEM of the total power of delta (A) or alpha (B) waves for 60 min. * $P < .05$ and ** $P < .01$ significantly different from the control group; $n = 10-12$.

and time spent [$t(18) = 6.80$, $P < .001$; Fig. 4B] in the open arm. Night milk also demonstrated its anxiolytic effect by increasing the percentage of entries and time spent in the open arms at dosages of 100 mg/kg [$t(18) = 1.78$, $P < .01$ and $t(18) = 3.08$, $P < .01$], 200 mg/kg [$t(17) = 2.06$, $P < .01$ and $t(18) = 2.58$, $P < .01$], and 300 mg/kg [$t(18) = 1.78$, $P < .01$ and $t(18) = 2.62$, $P < .01$].

EEG recordings

Figure 5 shows the total power of delta (A) and alpha (B) waves in the EEG of rats treated with Night Milk, diazepam, or vehicle. One-way ANOVA showed significant differences in total power of slow (delta) [$F(4, 49) = 3.21$, $P < .05$] and fast (alpha) [$F(4, 49) = 2.88$, $P < .05$] waves. Diazepam-treated rats displayed a significant increase [$t(19) = 2.60$, $P < .01$; Fig. 5A] in delta waves and a significant decrease [$t(19) = 2.45$, $P < .05$; Fig. 5B] in alpha waves. Night milk-treated rats showed an upward trend in delta and a downward trend in alpha waves; however, it failed to reach statistical significance compared with the vehicle-treated (control) rats.

DISCUSSION

In the present study, we have found that Night milk, but not Day milk, produces sedative and anxiolytic-like effects and potentiated pentobarbital-induced sleep in mice. The effects of Night milk were comparable to that induced by the benzodiazepine, diazepam. However, it failed to induce significant EEG changes in rats.

Administration of Night milk decreased the exploratory activity (distance moved and movement duration) of mice in the open-field test (Fig. 1), a measure generally believed to reflect reduced central nervous system excitability and/or sedative effects.¹⁵ This was supported by the findings of the rotarod test, wherein mice given Night milk showed a significantly decreased motor balance and coordination (Fig. 2). Night milk's effects on these tests were comparable to that of diazepam, a known sedative agent. Taken together, these results suggest that Night milk has sedative effects.

Previous studies have suggested that milk produces sedative effects through its rich source of pharmacologically active components. For instance, opioid peptides released from casein, the principal protein component of cow's milk, produce sedative effects during digestion.¹⁶⁻¹⁸

Likewise, orally administered tryptophan was demonstrated to have a sedative effect in healthy individuals,¹⁹⁻²¹ which is believed to be mediated through a nonserotonin mechanism.²² Melatonin, another hormone derived from tryptophan, was repeatedly demonstrated to induce sedative effects²³⁻²⁵ and thus may likely mediate the tryptophan-induced sedative effects. Considering the fact that tryptophan and melatonin are abundant in Night milk,⁴⁻⁶ it is possible that the sedative effect of Night milk may be attributable to these substances.

Night milk also displayed anxiolytic-like effects in mice, as evidenced by a significant increase in the percentage of time spent and total entries into the open arms of the EPM (Fig. 4). The EPM test is a widely used and accepted tool to measure anxiety-related behaviors in rodents; an increase in the percentage of time spent and entries into the open arms of the EPM indicates a reduction of anxiety/anxiolytic effects.^{26,27} The anxiolytic effect of Night milk was comparable to that of diazepam, a well-known anxiolytic drug. In support of this finding are other studies that have also reported the anxiolytic property of milk and its constituents. Of note is a published study reporting that bovine α S1-casein tryptic hydrolysate, a milk protein, has anxiolytic effects similar to diazepam.²⁸ Tryptophan, serotonin, and melatonin have also been reported to have potent anxiolytic effects.²

Corroborating the findings that Night milk has sedative and anxiolytic or relaxing effects is the result of the pentobarbital-induced sleeping test, wherein the Night milk administration shortened the onset and prolonged the duration of sleep (Fig. 3). Indeed, substances that potentiate pentobarbital-induced sleep are considered to possess centrally acting sedative and/or anxiolytic effects.^{13,29} This result supports the study of Valtonen *et al.* reporting that Night milk is an effective sleep-promoting agent in elderly

individuals.⁹ Altogether, the results of the behavioral experiments strongly suggest that Night milk possesses sedative, anxiolytic, and sleep-promoting effects.

Additionally, the brain electrical activity or EEG waves were recorded in rats after the administration of Night milk. EEG is considered to be an essential and sensitive parameter during sleep examination and is used to evaluate and diagnose sleep problems.³⁰ In general, sedative-hypnotic drugs decrease the high-frequency (alpha wave) EEG activity, and as the sleep stages progress, low frequency (delta waves) appears to govern the EEG tracing.^{3,31} Indeed, diazepam-treated rats displayed a significant increase in slow (delta) waves and a significant decrease in fast (alpha) waves compared with the control, an indication of its sedative-hypnotic property. Although the Night milk treatment at dosages 100, 200, and 300 mg/kg somewhat increased the total power of the delta waves, it failed to reach statistical significance versus the control group. No other significant difference was observed. This result may seem contradictory given the aforementioned EEG profiles of sedative-hypnotic drugs. Due to the limitations of the present study, we cannot fully explicate the exact reason or mechanism behind this result. However, Rajaratnam *et al.* have found that melatonin administration does not significantly change delta and alpha activities in humans.³² They discussed that unlike classic hypnotics, melatonin facilitates, rather than induces, sleep. This might also be the case with night-time milk; thus, no changes in the brain electrical wave or sleep structure were observed. Other factors (*e.g.*, strain differences, surgical procedure, and dose) may have also accounted for this result. These explanations remain only assumptions, and further studies are necessary to validate them. Regardless, the results of the behavioral experiments strongly imply that Night milk has sleep-promoting/sedative properties.

In conclusion, the present study demonstrates that milk collected during night time (Night milk) is capable of producing sedative, anxiolytic, and sleep-promoting effects, comparable to those produced by the benzodiazepine, diazepam. These findings suggest that Night milk might be an effective natural sleep aid for managing sleep-related disturbances and a promising alternative for the treatment of anxiety disorders.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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