Supplementation with *Eurycoma longifolia* Extract Modulates Diurnal Body Temperature Fluctuation and Sleep Rhythm in Mice

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(Received January 6, 2022)

Summary *Eurycoma longifolia* (Tongkat Ali; TA) is a traditional medicinal herb, commonly known as Malaysian ginseng. The root tea has been traditionally applied to treat fevers, aches, sexual dysfunction and other ailments. We evaluated the effects of TA extract supplementation on diurnal core body temperature (BT) and sleep architecture in model mice. Dietary supplementation with TA extract for 4 wk resulted in significantly and moderately reduced BT during the rest and active phases, respectively. A high dose delayed the onset of BT elevation at the start of the active phase, indicating that the effect was dose-dependent. Electroencephalography findings revealed that dietary supplementation with TA extract changed sleep rhythms and delta power during the inactive phase of NREM sleep, indicating improved sleep quality. Our findings suggested that dietary TA extract could be a promising natural aid that alleviates sleep problems via thermoregulation.

Key Words *Eurycoma longifolia*, supplement, body temperature, sleep rhythm, circadian rhythm

Biological clocks regulate the diurnal rhythms of core body temperature (BT) via the reciprocal control of sympathetic and parasympathetic activities (1). Sleep propensity is closely related to core BT; sleepiness is typically initiated on the falling limb of the BT rhythm, and circadian sleepiness is maximal when core BT is minimal. Conversely, controlling a robust diurnal rhythm of the core BT improves sleep quality by causing a rapid descent into being asleep and maintaining deep sleep (1). Glycine induces hypothermia, induces sleep (2), and improves sleep quality (3).

*Eurycoma longifolia* (also known as Tongkat Ali [TA] and Malaysian ginseng), is a traditional medicinal herb in southeast Asia. It is used as tea to cure malaria, fight fatigue, and enhance male testosterone levels (4, 5). Tongkat Ali extract has antioxidant, anti-inflammatory (6), anti-tumor, and cytotoxic (7) properties. It also regulates anxiolytic activities (8), and hence can serve a psychopharmacological therapy (9).

To develop novel functional supplement for improvement of sleep quality, we carried out screening of natural materials which can modulate core body temperature. The roots of TA are used to treat high blood pressure and fever (5). Thus, we considered that TA extract would modulate thermoregulation and sleep by controlling autonomic nerve and vascular mechanisms. Here, we assessed whether dietary supplementation with TA extract for 2–4 wk can modulate the diurnal rhythms of core BT and sleep architecture in mice.

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MATERIALS AND METHODS

Animals. All experimental procedures with animals were conducted according to the guidelines for the Care and Use of Laboratory Animals at the National Institute of Advanced Industrial Science and Technology (AIST), and the Animal Care and Use Committee at AIST approved the study protocol (Approval no. 2020–054). Male C57BL/6N mice (Japan SLC Inc., Hamamatsu, Japan) were selected to exclude the effects of core BT changes caused by menstrual cycles. The mice were housed under a 12-h light:12-h dark cycle (lights on at 08:00 and off at 20:00) at a controlled ambient temperature of 24±1°C, with constant relative humidity (55±3%) and provided with food and water ad libitum throughout the study. Four mice per cage were fed with each diet as described below. The mice and the amount of food intake were weighed weekly, and then weekly food intake per mouse was determined by dividing the amount of food consumed by the number of mice.

Preparation of diet containing TA extract. Dried root chips of TA (Selangor, Malaysia) were powdered in a laboratory grinder (Wonder Blender, Osaka Chemical, Osaka, Japan), then extracted at 90°C for 1 h in 10 volumes of distilled water. The extract was passed through No. 2 filter paper (Advantec, Tokyo, Japan) and lyophilized using an FDU-1100 freeze-dryer (EYELA, Tokyo, Japan) to yield 58.5 g of TA extract from 500 g of the powdered root. The extract was thoroughly mixed with AIN-93M (Oriental Yeast Co. Ltd., Tokyo, Japan) to final ratios of 0.25%, 0.5%, and 0.75% (w/w),
and the control diet was AIN-93M. Based on the age of mice, the AIN-93G diet should be started between the ages of 8–10 wk, then changed to AIN-93M after the mice reach 10-wk of age. However, we consistently fed the mice with AIN-93M to avoid the effect of changing the diet during the study. The mixture was pelleted and dried according to the conventional protocol for the industrial scale manufacture of AIN-93M at the factory control chow (Oriental Yeast Co. Ltd.).

Recording core BT and locomotor activity. Locomotor activity and core BT were measured every 15 min for 24 h, then averaged using a Nano-Tag device (Kissei Comtec Co., Nagano, Japan) that was implanted intraperitoneally into 8-wk-old mice (10, 11). The mice recovered in cages and were fed with the control diet for 2 wk after implantation. Thereafter, they received the diet supplemented with TA extract or not (control) (Fig. 1A). We compared the amplitude and rhythm of core BT by estimating acrophase using the least squares method to fit the BT data over 24 h to a single cosine curve as described (12).

Sleep analysis. A TL11M2-F20-EET telemeter (Data Sciences International, St. Paul, MN, USA) for recording sleep was subcutaneously implanted into the backs of the mice under 4% isoflurane anesthesia as described by the manufacturer. Two electroencephalographic (EEG) electrodes were implanted into the skull of each mouse and fixed with dental cement. Two stainless steel wires were implanted into the neck muscles to collect electromyographic (EMG) signals as described (13, 14). Nano-tag devices were also implanted in each mouse as described above. After 2 wk of recovery, polygraphic EEG and EMG data were continuously collected for 72 h at 0 and 4 wk after feeding with chow containing 0.5% TA extract (Fig. 1B).

Cortical EEG and EMG signals were digitized at a sampling rate of 500 Hz and recorded using a Dataquest A.R.T.™ (Data Sciences International). Polygraphic records were automatically scored offline in 10-s epochs into wake, REM and NREM sleep stages using SLEEP-SIGN (Kissei Comtec Co.) according to the standard criteria described below. Wakefulness was characterized by low amplitude EEG signals with mixed frequency components and relatively high, often irregular, EMG activity. The high amplitude of EEG activity during NREM sleep was dominated by 1–4-Hz slow-frequency waves and low EMG activity. Low-amplitude EEG signals dominated by 6–9-Hz theta waves and low EMG activity characterized REM sleep. Defined sleep-wake stages were visually assessed and corrected if necessary.

Statistical analysis. All values are expressed as means±SE. Differences in daily body temperature and wake/sleep length values between groups were statistically analyzed using one-way or two-way repeated measures analysis of variance (ANOVA). The homogeneity of variances was analyzed using F-tests or Levene tests before ANOVA. Variances of all data were equal. Statistical interactions between factors were compared in all groups using Tukey post hoc tests. Statistical significance was established at p<0.05.

RESULTS

**Tongkat ali extract supplementation induced lower core BT during the rest phase (Experiment 1)**

Supplementation with 0.5% TA extract reduced diet consumption to 59.5% of that control amount and increased water intake to 154% compared with the controls during the first week of the study (Fig. 2A and B). This might have been due to the bitterness of the extract. However, consumption recovered during the second week when food intake per body weight did not significantly differ. Coincident with the change in food
intake, body weight loss was 90% of the control value during week 1. However, it improved during the second week. Overall, the tendency to gain 2–3% gain per week did not significantly differ (Fig. 2C), and supplementary TA did not significantly alter total daily locomotor activities of the mice (Fig. 2D).

The diurnal fluctuation of core BT was stable and the average core BT was not altered in control mice throughout the study (Fig. 3A). In contrast, chow containing 0.5% TA extract reduced core BT at 3 and 4 wk after supplementation (Fig. 3B). The average core BT was significantly lower than pre-supplementation values in mice fed with TA extract for 4 wk during the light and dark phases (34.81 ± 0.19°C and 36.49 ± 0.18°C, respectively, vs. 35.52 ± 0.08°C and 36.90 ± 0.11°C, respectively *p<0.01). The lower core BT persisted throughout the day (Fig. 3A), whereas the effect was greater during the rest (light), than the active (dark) period (Fig. 3B). The core BT data fit to a single cosine curve, and the amplitude was significantly higher for BT after, than before supplementation (1.38 ± 0.021°C vs. 1.03 ± 0.052°C, *p<0.01). The core BT decreased for 2 wk after supplementation and became significantly remarkable at 3 wk. This effect was not evident in mice orally administered with the same dose of TA extract suspension at 10 a.m. for 5 d (data not shown). These results suggested that the continuous intake of chow containing TA extract for >3 wk, decreased core BT.

Extract of TA dose-dependently decreased core BT (Experiment 2)

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Modulation of Core Body Temperature with Dietary Intake of Eurycoma longifolia extract for 4 wk on core BT. Figure 4A shows average core BT during the light and dark phase (12 h each) before and after 2 or 4 wk of supplementation with TA extract. Figure S1 (Supplemental Online Material) shows that the diurnal profiles of core BT in mice fed with chow containing 0.25%, 0.5%, or 0.75% TA extract for 4 wk significantly differed (p<0.05; control vs. 0.5% and 0.75% TA). Core BT dose dependently decreased as the contents of TA extract increased (Fig. 4A). Furthermore, the effect of TA intake on core BT was more evident during the light, than the dark period, which was consistent with our earlier findings. The core BT of mice fed with 0.25% TA extract significantly decreased during the light phase at 6 w after supplementation (35.25 ± 0.08°C, p<0.01; data not shown). The diurnal profiles of core BT in mice fed with chow without or with 0.75% TA extract for 4 wk significantly decreased during the light phase at 6 w after supplementation (35.25 ± 0.08°C, p<0.01; data not shown).

Supplementation with 0.75% TA extract also altered the diurnal rhythms of core BT. A wakeful state is typically initiated by a nocturnally increasing limb of the core BT rhythm at the beginning of the dark period. We compared the peak times (acrophases) between the control and 0.75% TA groups to determine differences in the timing of this increase. After 4 wk of supplementation, the peak time of core BT was significantly delayed in the 0.75% TA group, compared with the control (ZT17.33 ± 0.25 vs. ZT16.42 ± 0.23 h, p<0.01; Fig. 4B), whereas the effect was not obvious in mice fed with chow containing 0.25% and 0.5% TA extract (Supplemental Online Material, Fig. S1). These data suggested that supplementation with 0.75% TA retarded the transition from the rest, to the active phase at the time of lights off. Furthermore, the reduced core BT in the latter half of the dark phase (ZT19–21) evidenced by control mice, disappeared. These results suggested that 0.75% TA also affected the circadian rhythm of core BT. These data showed that dietary intake of TA extract significantly increased the amplitude of daily core BT and depended on the intake dose and duration.

**Tongkat ali modulated sleep rhythm and quality**

Core BT is closely associated with sleep quality. Therefore, we recorded EEG and EMG for three consecutive days in mice fed with chow containing 0.5% TA extract for 4 wk to determine whether TA extract supplementation could alter sleep architecture. We applied 0.5% TA because supplementation with 0.75% TA affected circadian rhythms (Fig. 4B). The reduction in core BT was consistent with the data shown above. Figure 5A, B, and C shows the time course of 1 h-bin ratios of wakefulness, NREM, and REM sleep. The duration of NREM and REM sleep increased and that of wakefulness significantly decreased during the latter half of the dark period in mice fed with TA extract. These data indicated prolonged wakefulness in the TA group during the active (night) phase. In contrast, TA did not alter the sleep architecture.
duration of NREM sleep and wakefulness during the rest (light) phase.

We evaluated sleep quality by comparing the EEG power density of NREM sleep in control and TA groups. Figure 5D shows that the ratio of the delta wave during NREM sleep tended to increase in the TA group for 24 h. Notably, the delta wave significantly increased during the first 3 h of light (ZT0–2) and the last 3 h of darkness (ZT21–23). These results suggested that TA supplementation ameliorated sleep quality by changing sleep duration and depth.

**DISCUSSION**

The present study aimed to determine whether dietary supplementation with TA extract would affect the diurnal rhythms of locomotion, core BT, and sleep in mice. The most remarkable effect of TA extract was the dose dependent decrease in core BT that persisted throughout the day and was more pronounced during the rest, than the active phase. In addition, TA delayed onset of the active phase and improved sleep parameters.

The effects on core BT were obvious at 2 wk after TA supplementation and persisted throughout the day. Several agents induce a lower BT followed by sleep modulation in mice. Glycine induces sleep accompanied by a decrease in BT (2, 3). Glycine (2 g/kg body weight) lowered BT sooner and more obviously than TA extract. However, the effect of glycine lasted only ~60 min, then BT quickly recovered to a value similar to that of the control.

The anti-hyperlipidemia drug bezafibrate also induces a lower core BT during latter half of the active phase, increases the daily BT amplitude, and advances the onset of the diurnal rhythms of BT (15). This effect is induced via the PPAR-FGF21 pathway. Upregulated FGF-21 expression induces day-long hypothermia (16). We found that FGF-21 gene expression in the liver did not differ between control mice and mice fed with TA extract for 4 wk (data not shown), indicating that TA exerts its effects via a different pathway.

Which ingredient(s) in TA extract induced the effects that emerged after 2 wk of continuous feeding remain unknown. Moreover, daily oral administration of TA extract suspended in water for 5 d neither induced acute hypothermia nor changed the daily fluctuation of core BT. The composition of our hot water extract of TA might be similar to that of traditional TA tea. Mice generally consume >80% of their daily intake of chow during the first 3 h of the dark period, and blood levels of active components often peak with some effects during the nighttime. However, the effects of TA persisted throughout the day and were obvious during the nighttime. The content of active ingredients in TA extract might be low and difficult to adsorb or persist for long periods in blood plasma. The threshold of the compound concentration required to alter sleep quality might be high. The active ingredients need to be identified before the emergence of effects at 2 wk after continuous feeding can be explained.

Although the ingredients of TA extract and their pharmacological effects have been identified (5) none induce hypothermia. Nitric oxide is a vasorelaxation mediator in endothelial cells (17). Our preliminary findings of a partially purified fraction of TA extract revealed nitric oxide production in human and porcine vascular endothelial cells (data not shown). The vasorelaxant effect of TA extract in peripheral capillaries might induce lower core body temperature via heat dissipation.

The phosphodiesterase 5 inhibitor sildenafil is a vasodilator used to treat pulmonary arterial hypertension (18). It is contaminant of TA extract (19). However, sildenafil was undetectable in our TA extract that nevertheless had vasorelaxation activity (data not shown). We speculated that components other than sildenafil in TA extract induce nitric oxide production in vascular endothelial cells and cause vascular relaxation that results in a decrease in core BT. The components that induce nitric oxide production in endothelial cells must be identified before the mechanism can be elucidated.

We avoided changes in body temperature caused by the menstrual cycle by using male mice. The intake of TA extracts can elevate plasma testosterone and enhance spermatogenesis in rodents (20) and humans (21). The weight of testes in mice fed with and without TA extract did not differ in the present study. Furthermore, TA extracts did not enhance locomotive activity. These findings indicated that hypothermia induced by supplementation with TA extract is not associated with male fertility.

Supplementation with 0.75% TA extract delayed the transition from the rest, to the active phase at the time of lights off. To determine whether such supplementation affects the circadian clock mechanisms, clock gene expression and free-running locomotor activity should be investigated under constant darkness.

Supplementing a mouse diet with TA prolonged arousal during active phase and ameliorated sleep quality during the rest period. The effects are similar to those in mice fed with Lactobacillus brevis SBC8803 (14), although hypothermia did not occur. The amelioration of sleep in mice fed with TA might be due to enhanced arousal independently of hypothermia.

Poor sleep can affect people of all ages, including children and elderly persons, and substantially reduce the quality of life. Therefore, it should be resolved. Pharmacological intervention with sleep medications often generates side-effects such as intermittent sleep and daytime sleepiness. However, several trials have attempted to solve sleep problems using dietary supplements. The present study found that the dietary supplementation with TA extract mildly altered sleep duration and quality, indicating that TA intake might benefit persons with disordered sleep.

Core BT is less likely to decrease in elderly persons at night. This results in a smaller amplitude of daily core BT fluctuations, and advanced timing of fluctuations (16). This phenomenon in elderly has been linked to poor sleep and early morning awakening. Furthermore,
high summer temperatures and humidity disturb the reduction in nighttime rectal core BT and worsens sleep efficiency (17). The present findings indicated that TA extract supplementation lowers core body temperature and consequently might improve sleep. Furthermore, the time of increasing BT onset at the start of the active phase was delayed in mice supplemented with TA extract suggesting that TA extract could improve sleep by delaying the early awakening associated with sleep disorders.

Authorship
Conceptualization, K.M. and Y.K.; methodology, K.M. and N.I.; formal analysis, K.M. and N.I.; investigation, K.M., N.I. and P.S.; data curation, K.M. and N.I.; writing—original draft preparation, writing—review and editing, K.M.; supervision, K.M.; project administration, K.M. and Y.K. All authors critically reviewed the manuscript and approved the final version.

Disclosure of state of COI
This study was funded by Japan Tabacco Inc. Y.K. is an employee of Japan Tabacco Inc.

Acknowledgments
The authors thank Dr. Takayasu Sekine (D-LAB, Japan Tobacco Inc.) for helpful comments and discussions.

Supporting information
Supplemental online material is available on J-STAGE.

REFERENCES