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Salivary Melatonin at Night: Responding to the Night Lighting and Cow's Milk Consumption at Breakfast in Japanese Junior High and University Students

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Abstract

(1) In junior high school students aged 14–15 years old, the salivary melatonin level increased rapidly from 3.00 pg/ml at 21:45 to 9.18 pg/ml at 23:40 under orange light from light bulb, whereas it remained at less than 1.3 pg/ml under white light from fluorescent lamp. (2) In 3 weeks of intervention on university athlete students, the salivary melatonin concentration at 23:00 of G3 (protein-rich breakfast and following sunlight exposure and orange light from the light bulb at night) after intervention was significantly higher than that of G1 (protein-poor breakfast and not following exposure to sunlight and white light from the fluorescent lamp at night) and G2 (protein-rich breakfast and following exposure to sunlight and white lights from the fluorescent lamp at night). (3) This study evaluates the effects of cow's milk intake (Group 1: G1) for 20 days at breakfast on saliva melatonin concentration at 22:00 and 23:00 on 0, 10, and 21 days of the intervention period in Japanese university male athletes attending a university soccer club. In the intervention group (G1), salivary melatonin concentration increased at 22:00 in comparison with that before intervention, but there was no significant change in the control group (Group 2: G2). On the other hand, there were no significant differences in the melatonin at 23:00 between the both groups just after 21 days of intervention. Intake of cow's milk at breakfast might make the circadian phase in advance in the soccer athletes.

Keywords: junior high-school students, university soccer club students, salivary melatonin concentration in 22:00–23:00, orange or white lighting at night, cows' milk at breakfast

1. Background

Night sleep duration of Japanese children aged 10–18 years has become shorter by 1 hour during 1970–2000 in Japan [1]. In 2016, sleep hours in Japanese infants were reported to be only 9 hours (ideal hours, 12–13 hours) on average [2], while it ranged mainly in 7–10 hours for 8–11 years old of Japanese children (ideal hours, 10–11 hours) [3]. The so-called 24-hour society, which is currently in progress in Japan, seems to change environmental conditions surrounding the children. For example, mobile phones are used by more than 90% in 2000 and more than 98% in 2017 of university students, and more than 30% in 2000 and 95% in 2017 of junior high school students living in the urban area of Kochi city (33°N) had their own mobile phone (currently, most ones have smart phones) [4, 5]. Students can communicate with their colleagues even in the middle of the night with mobile phones. Frequent or long-time (more than 30 min) usage of the mobile phone makes university and junior high school students more evening-typed [4, 5]. At least for Japanese students in junior and senior high schools and universities, convenience stores have critical items for usual life, and these provide many kinds of foods and other goods for usual life. The total number of convenience stores by the main eight Japanese companies is 55,395 in 24 April 2018 all over Japan (<http://mitok.info/?p=75099>). Convenience stores are very bright places with luminance of 2000 Lux or more at the level of the eye height. Such bright light inside convenience stores may function as a merchandising technology which has been used all over the world for at least 75 years. Unconscious effects of using bright lights with higher color temperature in the evening or at night could be very serious for phase-delaying the circadian clock of students who had been exposed to bright lights [6].

Honma and Honma [6] reported an evidence that phase-delay effects were shown by light pulse exposure with higher color temperature from fluorescent lamp made only in the first half of the subjective night (about 19:00–24:00) in the circadian phase. On the other hand, phase advance of the circadian clock could be caused by the light exposure during 3 hours from the bottom point (about 4:00–5:00) of core body temperature rhythm. The effects of the night or evening use of convenience store was studied and showed that it was possible to make the diurnal rhythms of Japanese children mainly aged 12–15 years old become more evening-typed and take shorter sleep [7, 8]. Younger children attending kinder gardens and students attending elementary school were more sensitive to “light conditions” in normal life than university students, according to an epidemiological study [4].

Melatonin is synthesized in the pineal body of the hypothalamic area and secreted at night. It is well known as a key substance which may be effective in promoting the falling into night sleep by humans [9]. During daytime, the concentration of plasma melatonin was reported to be extremely low, whereas it increased rapidly during 22:00–23:00 as much as 10 or 20 times of daytime values [10, 11]. The melatonin intake caused higher EEG power density in the range of relatively low frequency of 5.25–9.0 Hz rather than that of placebo [12]. The melatonin level in the serum can be well and positively correlated with that in the saliva [13–16]. Secretion of melatonin exhibits circadian rhythms and is suppressed by bright light at night [17, 18]. Moreover, the plasma melatonin level at night was suppressed by the exposure of lights with 400 Lux for more than 4 min (or 300 Lux for more than 2 hours), whereas such suppression

was not seen under dim lights [19, 20]. Even room lights such as fluorescent lamps can attenuate melatonin excretion duration at night [21]. Especially, blue lights with 460 nm as peak wave length in the evening can be well absorbed by melanopsin in the neuro-ganglia cells in the retina [22, 23]. Evening lighting conditions are also said to affect circadian rhythms [24, 25] and mental health in mice [26]. Tryptophan intake at breakfast is effective for the onset and offset of sleep in young children [27]. Moreover, questionnaire surveys showed that young children exposed to sunlight for more than 30 min after having sources of protein at breakfast are more morning-typed than those exposed for less than 30 min [28] and that the more young children take in vitamin B6 at breakfast, the more they exhibit morning typology [29]. These findings can lead to appear the following hypothesis. It would be that morning tryptophan and vitamin B6 intake and following exposure to sunlight in the morning can induce serotonin synthesis in the daytime especially in the morning at pineal, and the following metabolism from serotonin to melatonin synthesis at night also at pineal can be promoted. However, it is impossible to test this hypothesis by a simple questionnaire study. Moreover, this melatonin synthesis could be inhibited by the night exposure to shortwave length (blue light, high color temperature) lights emitted from fluorescent lamps through melanopsin [30]. This hypothesis can be tested by an intervention field experiment which was done [31] and introduced here.

In the case of adolescents and children, exposure to lights of 300 Lux or more during the first half of subjective night in the normal life might decrease their melatonin level and prevent the falling into sleep [32]. Japanese junior and senior students were known to study at home mainly at night and also in the private preparatory school (for upper schools). In such cases, they are exposed to bright lights in most cases from fluorescent light bulbs. Many blue or blue-green lights with 470–500 nm wave lengths which were included in the bright lights were powerful to suppress melatonin concentration [33].

In the epidemiological studies made in 2003–2013 on university students in Kochi Prefecture (33°N), 21.4% of the students used frequently (more than 4–5 days or more per week) convenience stores [34]. Twenty-two percent of convenience store users went there after sunset. Among convenience store users, 30.2 and 6.5% of junior high school students stayed there for 15–30 min and more than 30 min per one use, respectively [8]. In Japan, many junior high school students were faced to the entrance examination for the upper senior high school and went to a private preliminary school after schools. About 62.4 and 18% of the students in preparation went and studied there for 2 or 3 hours till 21:00 and 22:00, respectively [8]. Generally, in Japanese education scene, some junior and senior students use convenience stores after sunset and were exposed to bright light with high color temperature with more than 2000 Lux from the fluorescent lamp. These exposures seem to suppress the plasma melatonin level [31, 35] and also are possible to make the circadian phase delayed in junior high school and university students.

Tryptophan is one kind of amino-acid of 21 kinds and cannot be made up from another substance but only can be absorbed exclusively from meals in humans. After absorption of tryptophan, it was transported from alimentary canal through brain-vessel barrier into the pineal in the brain. There, it was metabolized first to 5-hydroxytryptamine (serotonin) in the morning time mainly, at first, by two kinds of enzymes. Serotonin was again metabolized into melatonin by another two kinds of enzymes again in the pineal at night [36, 37]. What is the

function of serotonin as a precursor of melatonin? The shortage of serotonin in the human brain has been known to induce eating disorders, sleep disorders, obsessive compulsive disorder, panic disorder, and depression [38]. The lack of serotonin also seems to cause impulsive behavior and suicidal attempts, anxiety/aggression-driven depression, and aggression [39, 40]. Thus, serotonin is called the “key” substance in the psychiatry field. For example, serotonin reuptake inhibitors (SSRIs) were widely and commonly used for the treatment of affective disorders like as depression [41].

For keeping mental health, serotonin in the brain, one kind of catecholamine, would be a key substance. Medical doctors in the field of psychiatry have used serotonin reuptake inhibitors (SSRIs) widely in the past four decades for the treatment of affective disorders including depression [41]. However, mixed opinions have been expressed to whether SSRIs are effective for the treatment of depression in children and adolescents because there has been the shortage of coincident scientific evidence of SSRIs for young human beings. Synthesis of serotonin seems to be promoted by sunlight exposure after consuming protein-rich foods at breakfast [42]. This synthesis is hypothesized to occur mainly in the morning time. Another study on young Japanese children showed that the amount of tryptophan which was consumed at supper had neither related with morningness-eveningness (M-E) scores nor with sleep habits [43].

How do cow's milk proteins promote human health? Eight ways to promote human health were demonstrated by Nongonierma and FitzGerald [44]: (1) improving satiety and weight management, (2) reducing risk of heart disease, (3) having an antimicrobial role, (4) having anti-inflammatory effects, (5) showing anticancer effects, (6) exerting antioxidant effects, (7) affecting insulin secretion and serum glucose regulation, and (8) an action upon muscle protein synthesis.

Brezinova and Oswald [45] showed, using electroencephalography, that sleep was significantly improved (longer and uninterrupted night sleeps) in older people when they ate a combination of cow's milk and cereal before going to bed. Laird and Drexel [46] reported that a meal of cornflakes and milk strongly improved sleep quality (as judged from an uninterrupted night sleep), with regard to the relationship between sleep health and the intake of cow's milk. On the effects of morning-drinking cow's milk, only a few studies have been performed for any improvement in sleep health. The two results of a questionnaire study have been reported on Japanese infants aged 2–6 years old [47]. Infants who added protein-rich foods at breakfast to the usual breakfast by other infants were more morning-typed and slept with significantly better quality than the other infants. Moreover, infants who drank milk at breakfast were less frequently depressed than those who did not.

Protein intake at breakfast would mean the consumption of tryptophan from the alimentary canal to the blood. Such tryptophan could be transferred to the pineal where serotonin can be synthesized and promote mental health in the daytime as an antidepressive agent. Serotonin can be further synthesized into melatonin as a sleep-onset agent at night [27–29, 31, 48]. For Japanese children, drinking cow's milk at breakfast is an important source of tryptophan and could, on the basis of a questionnaire study [47], be supposed to promote mental and sleep health. Recently, an intervention of drinking cow's milk (200 ml) at breakfast was reported to make Japanese university soccer team athletes who were originally more evening-typed than evening-typed, and this intervention also made

their soccer performances in advance [49]. Another intervention study was performed for 1 month on the Japanese university soccer team members using a leaflet entitled: "Three benefits: Go to bed early! Get up early! Eat a nutritionally rich breakfast!" [48]. As the result of this intervention, all of their soccer performance, sleep health, and mental health were improved.

An increased intake of cow's milk at breakfast, as a source of tryptophan, is hypothesized to promote the amount of serotonin in the morning and the following synthesis to melatonin at night in the Japanese university soccer team athletes. However, this hypothesis has not been tested. This study will try to test this hypothesis.

2. Effects of evening light conditions on salivary melatonin of Japanese junior high school students

2.1. Methods

2.1.1. Participants

Japanese junior high school students (four females and six males) as participants in this study were aged 14–15 years old [35]. They were Motoyama junior high school students in the third grade. This junior high school was located in the mountain area of Reihoku district (33.5°N) in Kochi Prefecture, Japan. Seven days were holidays for the participants before this study as intervention experiment. During the holidays before the experiment, they were recommended to keep usual diurnal rhythm (like as bedtime and wake-up times).

The two groups "bright light experimental group (BLEG)" and "dim light control group (DLCG)" were set up for the intervention experiment for junior high school students. For the BLEG and DLCG, participants were selected to have similar circadian typology scores as the diurnal-type scale (DTS) of the morningness-eveningness (M-E) questionnaire of Torsvall and Åkerstedt [50] (mean $\pm$ SD: 15.00 $\pm$ 4.30 by BLEG and 14.80 $\pm$ 4.09 by DLCG). Participants of BLEG showed 23.0 $\pm$ 4.2 hours (mean $\pm$ SD, bedtime), 8.4 $\pm$ 1.9 hours (wake-up time), and 9.1 $\pm$ 1.4 hours (sleep hours) just before the experiment. On the other hand, the values were 23.8 $\pm$ 1.3 hours (bedtime), 8.9 $\pm$ 1.3 hours (wake-up time), and 9.5 $\pm$ 1.5 hours (sleep hours) for the DLCG participants. Each group has two females and three males. All participants collected their own saliva using "Salivette" by collecting tubes (SARSTEDT Aktiengesellschaft & Co., Numbrecht, Germany) at 22:30–23:00 under the 200–300 Lux light from fluorescent light bulbs in their home on the day before the experimental day.

Most of the Japanese citizens are enjoying evening time under fluorescent light bulbs during the first half of the subjective night (about 19:00–24:00) based on a long-term epidemiological study [48] on 3700 small children aged 2–6 years and their mothers in 2003–2017 in Kochi.

Eighty-five percent or more of 3700 families of Japanese infants used fluorescent light bulbs as night lighting at home usually. When the illumination was measured at the level of 1 m above floor under a usual type of round-shaped fluorescent light bulb in a typical one-room apartment for university students, it was 340 Lux [31].

2.1.2. Procedure

We collected all 10 participants in front of Motoyama junior high school at 8:00 in 4 January 2003. We moved the participants by a wagon car to the experimental place which was a Japanese-style hotel located at a mountain area, Yusuhara town in Kochi Prefecture, Japan. The Yusuhara town was located 126 km west from the Motoyama town. During driving, illumination inside the car was 350–500 Lux. The car arrived at the hotel around noon on 4 January 2004. It was heavily snowing through the day around the hotel. Behavior of all participants was controlled during the experimental day in the hotel till the next morning of the experimental day. In the experimental daytime, all participants played outside and were exposed to the lights with 6000–7500 Lux at the eye level during 12:30–13:30 and 14:50.

All the participants were exposed to the sunlight with 6000–7500 Lux at the eye level during 12:30–13:30 and 14:00–14:50 during they played in the yard of the hotel. After the playtime, they were allowed to have a rest in the living room of the hotel. The floor of the rest room was filled with 12 *tatami* mats. The illumination of the light at the eye level in the rest room was 250 Lux from fluorescent light bulbs during the resting time till 16:00. Bath was taken by the participants one by one between 16:30 and 18:00. After that, supper was together taken by all participants between 18:15 and 19:20 in the living room.

BLEG participants moved to a Japanese-style room with eight *tatami* mats at 19:25. They were exposed to the high color temperature (white-colored) light with 2000 Lux at the eye level from fluorescent light bulbs. On the other hand, the DLCG ones moved to another Japanese-style room with eight *tatami* mats at the same time. Instead, the DLCG ones were exposed to the low color temperature (orange-colored) light with 60 Lux from an electronic light bulb.

In each room for BLEG and DLCG, all the participants did homework (e.g., literatures and mathematics) or made a small wooden folk craft object which is typical in the Yusuhara district, under each light condition till 22:30. An oil heater in both rooms functioned to keep the room temperature $15 \pm 2^\circ\text{C}$.

At 22:30, they moved back to the former living room (12 *tatami* mats) and stayed there under the light of 250 Lux till 23:40. After that, experimental staffs ordered female and male participants to move to separate rooms and go to bed just before 24:00. At 21:45, 22:30, and 23:40, the participants collected their own saliva using the “Salivette” as collecting tubes. Their salivary samples were preserved in a refrigerator at less than -20°C . Melatonin concentration in the samples was analyzed by a professional-analyzing company (MSL Co. Ltd.) which was a professional company for analyzing several chemicals and microbiological organisms.

All the participants of BLEG and DLCG were ordered to get up at 7:00 in the next morning by calling out. They were enforced to get up between 7:00 and 7:15. Then, they took breakfast and left the experimental place, the hotel, at 9:00 for Motoyama junior high school. Light exposure at the eye level was measured by using a digital illuminance meter through the study.

2.1.3. Ethic treatment

Detailed explanation of the objectives and methods of the experiment was provided before the experimental performance to the participants and their parents. The research project received full and complete agreement from all of them.

2.1.4. Statistical analysis

The software used for statistical analysis was SPSS 12.0 J for Windows (SPSS Inc., Chicago, IL, USA). The Wilcoxon test was used for the pair-wise test for melatonin concentration for temporal change of melatonin concentration before, through and after the intervention day.

2.2. Results

Salivary melatonin concentration in the DLCG increased from 3.00 ± 3.34 (mean $\pm$ SD) pg/ml at 21:45 to 9.18 ± 7.66 pg/ml at 23:30 in the experimental day (t-test between values at 21:45 and 23:30; $t = 3.60$, $df = 4$, $p < 0.05$) (Figure 1). On the other hand, it remained at less than 1.3 pg/ml till 23:30 in BLEG ($t = 2.07$, $df = 4$, $p = 0.2$). On the day before the experiment, significant difference was not shown in the melatonin concentration in saliva between BLEG and DLCG (Wilcoxon test: $z = -1.163$, $p = 0.31$). In comparison with the melatonin concentration at 22:30

Figure 1. Effects of light condition on salivary melatonin concentration. Values shown are means ($n = 5$ per group) and SEM [35].

under fluorescent lamps emitting 250 Lux white lights, the value for BLEG at experimental 22:00 tended to be lower, whereas for DLEG, it was significantly higher. In the day before the experimental day, melatonin concentration under the fluorescent lamp with 200–400 Lux in each home of both BLEG and DLGG was similar and had no significant difference (Mann-Whitney U-test: $z = -1.163$, $p = 0.31$). At 22:30 of the experimental day, melatonin concentration of BLEG tended to be lower than that of BLEG on the day before the experiment (Wilcoxon test: $z = -1.604$, $p = 0.109$). On the other hand, the concentration of DLGG at 22:30 become higher than that of the previous day at home ($z = -2.023$, $p = 0.043$).

The bright light of 2000 Lux from fluorescent lamps with high color temperature seems to have suppressed the night increase of melatonin concentration of junior high school students. On the other hand, the relatively low color temperature light with 60 Lux did not. In Japan, the light condition with 2000 Lux and high color temperature can be seen usually inside all the convenience stores of Japan, which are now increasing and become common. Such bright light conditions are also common as room lightings in Japanese private schools, the so-called Juku, which are preliminary schools for preparation of going through the entrance examination, which is a very severe competition for upper schools. Therefore, exposure to bright lights in the evening “Juku” and convenience store would suppress the night increase in the plasma melatonin level as a direct effect also would make the circadian phase delayed and as a result phase of sleep wake cycle can be delayed.

2.3. Discussion

Traditional lightings by ancient Japanese citizens were mainly low color temperature lights which were emitted from a traditional Japanese hearth fire or an oil lamp or candle (20–30 Lux). Such “orange” lightings might be healthy for adolescent and children, because the ancient lightings included only a low amount of the light components with 460–480 nm wave lengths (blue or high color temperature lights), which are peaks of energy consumption by melanopsin (relative to conopsin and iodopsin). Melanopsin is included in neuro-segmental cells and key substance for melatonin suppression [30]. Such orange lights do not stimulate the melanopsin, and melatonin suppression also does not occur. Therefore, junior high school children can fall in night sleep very smoothly [51]. This part 2 was already published in [35].

3. A tryptophan-rich breakfast and exposure to light with low color temperature at night improve the sleep and salivary melatonin level in Japanese students

3.1. Methods

3.1.1. Participants

Ninety-four participants were male university students aged 19–22 years old with averaged 20.33 who were belonging to a university soccer club [31]. They participated in an intervention study; 63 of them answered to the integrated questionnaire before and after the intervention period.

Group 1 (G1) consisted of 20 soccer players without intervention, Group 2 (G2) had 22 soccer club members who were asked to have protein-rich foods such as fermented soybeans and vitamin B6-rich foods such as bananas at breakfast and sunlight exposure after breakfast, and Group 3 (G3) consisted of 21 members who were asked the same breakfast contents and sunlight exposure after that and additionally were asked to use incandescent light as night lighting. All participants were males.

For the night lighting, all the participants in the three groups have used fluorescent lamps (white light). Integrated questionnaires were administered to all participants three times to estimate the effects of the 1-month intervention. Questionnaire studies were performed just before the intervention period, soon after the end of the intervention, and 1 month after the intervention. The same questionnaire was used for study before the intervention and also 1 month after that. The contents of the questionnaire were the diurnal-type scale constructed by Torsvall and Åkerstedt [50], questions on sleep habits and meal habits [52], an Irritation Index, the General Health Questionnaire (GHQ), the Sense of Coherence (SOC) questionnaire, and FFQ (Food Frequency Questionnaire). After the intervention, the questionnaire which had been administered before it was again done. Moreover, other self-assessment questions were also administered to the members of the three groups. The self-assessment questions were on how many days during the month-long intervention period they followed the three recommendations. The first one was on protein-rich foods as breakfast contents, the second one was sunlight exposure after breakfast, and the third one was the usage of orange lightings (like light bulbs which emit orange (lower color temperature)) lights at night.

3.1.2. Procedure

Based on the two questionnaire scores of FFQ (good, mild, and bad: three groups) and the diurnal-type scale (DTS) (morning-typed, middle-typed, evening-typed), nine groups (3×3) were made. Members of each of nine groups were randomly divided into the three experimental groups. As the results of the treatment, no significant differences were made also in the body height, body weight, and age, among the three groups.

Through the intervention of 30 days in October–November in 2010, we asked all participants to keep a sleep diary. The sleep diary included a question and a selection list of answer: “How was the depth of your last night’s sleep?” “(1) Deep, (2) Relatively deep, (3) Relatively shallow, (4) Shallow.” To the members of Group 3, incandescent light bulbs (emitting orange lights) were distributed one by one before the intervention period. After that, they were asked to install the light bulbs (distributed) in their bed room. The G1 and G2 members were asked to switch fluorescent lamps on as usual, whereas the G3 ones were asked to switch incandescent light bulbs (distributed) on instead, when they were asked on the lightings when they got back to their residences after sunset. Room lights for G1 and G2 (white: fluorescent lamp) and G3 (orange: incandescent lamp) were 100–400 Lux and <100 Lux, respectively. Sixty-seven percent of the participants (63 of 94) answered the first questionnaire before intervention and 81% of participants (51 of 63) wrote their own sleep diaries during the intervention of 1 month.

On “high-protein content breakfast” and following “exposure to >30 min exposure to sunlight,” the implementation scores were calculated as the sum of days when the “protein-rich

breakfast and following light exposure” was implemented. On another intervention “night exposure to low color temperature light,” it was defined as implementation score how many minutes per night they were exposed to the low color temperature light emitted from the incandescent bulb.

We asked the participants of G2 and G3 to mark (marking as 0–100 points as satisfaction score) the scores on “To what extent do they satisfy on their carryout of the two (G2) or three (G3) intervention contents for 30 days in the total.” We measured the salivary melatonin of the 10 participants of three groups. The 10 participants in each group were selected randomly because there was financial limitation for the chemical analysis. Ninety salivary samplings were needed for the experiment, because three groups prepared one sampling at 23:00 before, in the middle, and in the end of intervention ($10 \times 3 \times 3$). We asked participants to extract their own saliva at around 23:00 with cylindrical cotton (1 cm diameter, 3 cm long). If participants put them under their tongues for 3 min, full amount of saliva (1–3 ml) for analysis can be extracted outside and absorbed into the cotton. It was kept frozen at -25°C till analysis for 1–2 weeks. Melatonin concentrations in the saliva were determined using an ELISA kit (Direct Saliva Melatonin ELISA, Bulmann, Switzerland).

3.1.3. Ethic treatment

Participants received a full explanation with the code of the guideline for a study targeting humans before the study [53]. The explanation included that the results of the study would be used only for academic purposes and that all participants completely agreed to participate in this study.

3.1.4. Statistical analysis

We used a statistical analysis software as SPSS 12.0 J for Windows (SPSS Inc., Chicago, IL, USA). As nonparametric analyses, Kruskal-Wallis test and following Bonferroni multiple comparison were used among the three groups. Wilcoxon’s signed-rank sum test was used for the pair-wise test in the same group and after the intervention (**Figure 2**).

Figure 2. Graphic schema of intervention study for university soccer club athletes.

3.2. Results

We could get significant positive correlation between the feeling of sleeping well in the last week period (LWP) of the intervention period and hours spent under incandescent light at night (Pearson's correlation test $r^2 = 0.265$, $p = 0.034$). Salivary melatonin concentration by G3 participants was significantly higher than that of G1 and G2 in the midpoint and the day before the last day of intervention period (Bonferroni multiple comparison test: G1 vs. G3, $p = 0.018$; G2 vs. G3, $p = 0.011$). On the other hand, we have got no significant differences in the salivary melatonin among the three groups on the day just before the start of the intervention period (Kruskal-Wallis test: χ^2 -value = 0.92, $df = 2$, $p = 0.63$) (**Figure 3**). In the middle period (MP) of the intervention period of G3, the "high implementation period" tended to show a higher concentration of salivary melatonin than the "low implementation group" did (Mann-Whitney U-test: $z = -2.000$, $p = -0.071$).

Group 2 participants tended to follow the morning intervention recommendations (high-protein breakfast and sunlight exposure) on less days than G3 participants did (Mann-Whitney U-test: first week period (FWP), $z = -1.952$, $p = 0.053$; MP, $z = -1.628$, $p = 0.105$;

Figure 3. Salivary melatonin level just before intervention (A) (no significant difference: Kruskal-Wallis test: χ^2 -value = 0.92, $df = 2$, $p = 0.63$). All three groups showed low salivary melatonin level of 2–5 pg/ml just before intervention. Melatonin in the saliva collected at the midpoint and on the day before the last day of the intervention (B) (Bonferroni multiple comparison test: G1 versus G3, $p = 0.018$; G2 versus G3, $p = 0.011$). Comparison of salivary melatonin concentration among the three groups. Group 1: no intervention; Group 2: recommendation of high-protein breakfast and exposure to sunlight; and Group 3: same as Group 2 plus the recommendation of exposure to orange lights from incandescent lamps.

LWP, $z = 1.253$, $p = 0.221$). The implementation rate in FWP tended to be higher than MP (Wilcoxon's signed-rank sum test: G2, $z = -1.851$, $p = 0.064$; G3, $z = -1.914$, $p = 0.056$) and LWP (G2, $z = -2.298$, $p = 0.022$, G3, $z = -2.898$, $p = 0.004$). The implementation rate in LWP in G2 and G3 tended to be lower than that in MP (G2, $z = -1.681$, $p = 0.093$; G3, $z = -2.533$, $p = 0.011$).

There was a significantly positive correlation between the regularity of time to take breakfast and supper and the implementation satisfaction index (maximum score, 100) (Kendall tau-b test: breakfast $r^2 = 0.058$, $p = 0.038$; supper $r^2 = 0.057$, $p = 0.036$). There was a significant positive correlation between the index on implementation days and the diurnal-type scale (DTS) at 1 month later after the intervention period (higher scores showing morning-typed) (Pearson's correlation test: $r^2 = 0.195$, $p = 0.006$). The index on intervention days was how many days (among intervention of 30 days) the participants satisfied the implementation on the breakfast contents as high-protein foods.

A significant positive correlation was shown between the number of nights (among 30 days) when participants were exposed to orange-colored lights emitted from incandescent lamp and the index of meal time for three meals just after the intervention (Kendall tau b-test: breakfast $r = -0.574$, $p = 0.007$; lunch $r^2 = 0.146$, $p = 0.084$; supper $r^2 = 0.215$, $p = 0.029$). In comparison with those who ate breakfast less frequently for 1 month after the intervention, participants who took breakfast more frequently took late-night snacks with less frequency (Kendall tau-b test = -142 , $p = 0.003$).

In G3, participants after the intervention showed a lower anger/irritation index than those before the intervention (Wilcoxon's signed-rank sum test: $z = -3.072$, $p = 0.002$). On the other hand, G1 showed only a tendency of reduced irritation ($z = -1.786$, $p = 0.074$), and G2 had no differences in mental health index after the intervention ($z = -0.956$, $p = 0.340$). Anger/irritation indices (the frequency to be irritated and the frequency to become angry due to small trigger) were also decreased after the intervention in G3 (Wilcoxon's signed-rank sum test: irritation $z = -2.496$, $p = 0.013$; anger $z = 2.714$, $p = 0.007$).

3.3. Discussion

This study showed that a triple intervention concerning breakfast content, sunlight exposure after breakfast, and exposure to low temperature light emitted from incandescent bulbs are powerful methods for inducing secretion of high amounts of melatonin by the pineal gland in human adults. Underlying mechanisms can be hypothesized to consist of two components. The first component is that serotonin synthesis from tryptophan taken at breakfast may be enhanced by the exposure to sunlight just after taking breakfast. The second component is that the high potential of melatonin synthesis based on the high serotonin synthesis in the pineal during daytime might be available due to the night exposure to the "low-temperature light" emitted from incandescent bulbs. Many reports have shown that melatonin secretion is suppressed by evening or night light emitted from fluorescent lamps including shortwave length (with around 460 nm of wave length) components [10, 33, 35, 54–56]. Moreover, this study newly implies that the combined behaviors of (1) modifying breakfast content, (2) receiving sunlight exposure, and (3) receiving exposure to low color

temperature lighting at night can facilitate achievement of high plasma melatonin at night in humans. Melatonin, a hormone secreted from the pineal gland, has been reported to cause the core body temperature to decrease and induces sleep [9, 12, 57]. An important role of the high plasma melatonin level at night was reported as a sleep-onset agent and sleep quality promoter [58]. There was a significant and positive correlation between the duration when participants spent under incandescent lights at night and the scores which they marked to feel deep sleep, in this study.

High sleep quality would be promoted by high plasma melatonin in human beings. The principle theory of this study is promotion of serotonin synthesis in the morning and succeeding melatonin synthesis at night. The intervention of this study is composed of three issues: (1) having sources of tryptophan and vitamin B6 at breakfast, (2) following up breakfast with exposure to sunlight, and (3) the exposure to low color temperature lights as night lighting. Serotonin works as antidepressant agent, and melatonin is a natural sleep-onset pill. Moreover, these two hormones would act as a circadian clock as inner-zeitgebers. As a result, shift to morning-typed would lead to promoted mental health.

A limitation of this study of the intervention study is as follows: this intervention could not set a control group with low-tryptophan breakfast, sunlight exposure, and exposure to low-temperature light at night for finding out the importance of the intake of tryptophan at breakfast in the physiological mechanism of serotonin-melatonin synthesis. This study was not the so-called physiological experiment to set up several experimental groups and control group under controlling their usual life like as meals, lightings, and social activity in some extents. In the future, another intervention study controlling more life habits would be possible. Another limitation of this study is that this intervention study was performed only with men. In the future, women participants from sports club could add important data for making gender differences in response to breakfast modulation and the change at night lighting into orange light at night. This part 3 was already published in [31].

4. Effects of drinking cow's milk at breakfast on saliva melatonin concentration in Japanese university athletes

4.1. Methods

4.1.1. Participants

Sixty percent of 93 participants were born and grown in Shikoku island, whereas the others were from other parts of all over Japan. Participants in this study were 19–25 years old (Takeuchi et al., unpublished).

4.1.2. Procedure

Seventy-three participants of the experimental group were asked to drink 200 ml of cow's milk at breakfast each morning for a period of 21 days, from 13 November 2014 to 4 December

2014. We provided the milk for them. Twenty participants in the control group did not drink the cow's milk in the morning at breakfast. Twenty men did not like milk originally. Between the two groups, no significant differences were shown in age, diurnal-type scale [50] scores, and sleep habits (wake-up time, bedtime, and sleep hours both in the weekdays and weekends) [52]. After the questionnaires and sleep diaries had been distributed, participants were allowed to answer them at home.

4.1.3. Ethic treatment

Before administrating the intervention study, all participants were given a written explanation that detailed the concepts and purposes of the study and also stated that we would use the saliva melatonin data only for academic purpose. All participants agreed completely with the proposal and gave written consents after the explanation. The explanation stated that they could withdraw at any time and the withdrawal (canceling) had negative consequences for them. However, there were no withdrawals at fact. The intervention research was performed in accordance with the guidelines which have been established by the *Chronobiology International* journal for the conduct of research on human beings [53]. The study was also permitted by the Kochi university soccer club committee and the committee in the Laboratory of Environmental Physiology, Graduate School of Integrated Arts and Sciences, Kochi University, which carried out ethical inspections regarding the contents of the methodology in this study.

4.1.4. Statistical analysis

The software used for statistical analysis was SPSS 12.0 J for Windows (SPSS Inc., Chicago, IL, USA). ANOVA and T-test were used for the tests on spacing ratio variables between ranked variables. Friedman test was used for paired variables. We were sometimes interested in changes *during* the intervention (Friedman).

4.2. Results

In saliva collected at 22:00, melatonin concentration was increased during the intervention period in the cow's milk consumption group (ANOVA due to GLM repeating measurement analysis: $df = 2$, $F\text{-value} = 8.038$, $p = 0.080$; Friedman test: $p = 0.044$) (**Figure 4A**). Moreover, the differences in the individual levels in the saliva melatonin concentrations tended to be higher (more increased) in the cow's milk consumption group than the control group (T-test: $t\text{-value} = 2.05$, $p = 0.061$) (**Figure 4A**). On the other hand, at 23:00, there were no significant differences during the intervention in the melatonin level (ANOVA: $df = 2$, $F\text{-value} = 1.999$, $p = 0.172$; Friedman test: $p = 0.867$) in the experimental group (cow's milk consumption) (**Figure 4B**). On the other hand, there were no differences in the saliva melatonin concentrations through the intervention of 21 days both at 22:00 (**Figure 4A**) (ANOVA: $df = 2$, $F\text{-value} = 0.794$, $p = 0.235$; Friedman test: $p = 0.867$) and at 23:00 (**Figure 4B**) (ANOVA: $df = 2$, $F\text{-value} = 0.176$, $p = 0.841$; Friedman test: $p = 0.867$) in the control group (no cow's milk consumption).

Figure 4. Comparisons of saliva melatonin concentration in saliva taken at 22:00 (A) and 23:00 (B) before intervention, 10 days later and 20 days later (just after the intervention). Black bars (cow's milk consumption group), white bars (no cow's milk group) (mean $\pm$ 95% confidence) (Takeuchi et al., unpublished).

4.3. Discussion

The intake of cow's milk at breakfast made the evening-typed members more morning-typed, whereas the diurnal type of the morning-typed members shifted to more evening-typed [49]. Tryptophan included in the cow's milk was metabolized to serotonin in the morning and serotonin again synthesized to melatonin at night [28, 29, 31, 48, 49, 59]. Therefore, the sleep latency seems to become shorter during the intervention [49] due to the melatonin synthesis which has been known as the natural sleep inducer [60] based on cow's milk intake at breakfast. This shorter sleep latency leads to the longer sleep hours for the university soccer club members.

The melatonin onset timing especially at dim light conditions can be used as a "sign" of the circadian phase [60]. For example, this melatonin-onset timing could be used for the signs of mood disorders and accompanied phase delay of the circadian phase shown by the monopolar and bipolar depression phenotypes [61]. The promotion of the melatonin concentration level at 22:00 due to morning cow's milk consumption might show the phase advance of university soccer team members. If the participants in this study would be senior high school students, the increase timing might be 1 or 2 hour(s) in advance, because the melatonin onset timing was around 21:00 to 22:00 for the senior high school students [62]. However, the number of samples was very small, and the significant differences in the saliva melatonin at 23:00 was not shown in this study. More number of samplings of saliva will be taken in the future study.

On the 10th day and 21st day of the intervention period, a questionnaire on performance/skill was administered to all participants [49]. The group who drank cow's milk showed higher frequency of improvement of soccer performance than the control group who did not drink [49]. The improved sleep quality which might be induced and enhanced at 22:00 in this study in the melatonin group could be related to such improvement of soccer performance.

5. Integrated discussion

Melatonin secretion is very important for sleep induction at bedtime and also keeping sleep quality high [62]. Moreover, night melatonin for mothers can be related to brain development of their children. Braam et al. [63] showed a hypothesis that low parental melatonin levels could be one of the contributors to autism spectrum disorder (ASD) and possibly intellectual disability (ID) etiology. If this hypothesis is correct, this could lead to policies to detect future parents who are at risk and to treatment strategies to ASD and intellectual disability risk.

Pfeffer et al. [64] reviewed that the present contribution of melatonin confirmed the synchronizing effect of endogenous melatonin and the melatonergic system in humans and rodents. However, they discussed that these effects would be subtle. These relatively subtle effects stand in contrast with their markable, overt therapeutic successes that have been achieved using melatonin as an externally applied chronobiotic in humans [65]. Thus, melatonin does not appear as the master of internal synchronization but as one component in an integrated system of synchronizing agents. Melatonin might be one indicator of a phase point of human circadian clock. And, it can be influenced by breakfast protein consumption and also evening lighting especially modern lighting which includes "blue lights (460–480 nm)" as peak wave lengths of melanopsin [66].

Several studies including Bouwmans et al. [67] that showed the possibilities of network mapping for dynamic person-specific psychological and biological data revealed that there were not only large heterogeneity between affect and fatigue in depression and melatonin secretion which can be related to fall in sleep and sleep quality, namely, negatively and positively correlation with wide personal variety in the relationship. Meaningful generalizations can be made on the interplay of melatonin with affect and fatigue in depression.

Anyway, melanopsin is a key photoreception substance to control circadian phase shift in human beings [68, 69] and moreover to relate to sleep and mood disorders in the parents [66]. Melanopsin function might be related to melatonin synthesis via circadian phase shift indirectly. Twenty-four hours of commercialization society might be dangerous for disruption of circadian phase and normal melatonin secretion through over usage of smartphone (e.g., line and game) and watching TV program at night for children.

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