The Effects of Nocturnal Life on the Circadian Patterns of Sex Hormones in Young Healthy Men

Li-Qiang Qin,*[,a] Takashi Kaneko,[b] Jue Li,[b] Pei-Yu Wang,[c] Jia-Ying Xu,[a] Jing Wang,[b] and Kazuhiko Hoshi[a]

[a]Department of Obstetrics and Gynecology, [b]Department of Environmental Health, School of Medicine, University of Yamanashi, 1110 Shimokato, Tamaho, Yamanashi 409–3898, Japan, and [c]Department of Social Medicine & Health Education, School of Public Health, Peking University, 38, Xueyuan Road, Haidian District, Beijing, 100083, P.R. China

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Irregular meals (mainly the skipping of breakfast) and inadequate sleep has become prevalent in young people. However, there has been inadequate attention on the adverse effects of this nocturnal life. We observed the 24-hr patterns of sex hormones and other relative hormones in ten medical students, with the randomized cross-over design, on either a diurnal life or a nocturnal life. The subjects on a diurnal life ate three meals (07:00, 13:00 and 19:00) and slept from 22:30 to 06:30. Nocturnal life was designed by skipping their breakfast but consuming a lot (> 50% of their daily food intake) in the evening and at night, sleeping from 01:30 to 08:30 the next morning. After three weeks in the experimental life, the 24-hr plasma concentrations of testosterone, free testosterone, dihydrotestosterone (DHT), androstenedione, prolactin, estrone, luteinizing hormone, cortisol and sex hormone binding globulin (SHBG) were measured every three hours. Plasma free testosterone and prolactin decreased, but cortisol increased when subjects followed the nocturnal life. In the diurnal lifestyle group, circadian rhythms were observed in the androgens (testosterone, free testosterone, DHT, androstenedione), prolactin, cortisol and SHBG in the cosinor analysis; however, circadian rhythms were almost extinguished in the nocturnal lifestyle group. Since sex hormones play important roles in the normal physiological condition, the effects of these changes in hormone concentration and its circadian rhythm should be considered and studied intensively.

Key words —— nocturnal life, circadian rhythm, testosterone, sleep

INTRODUCTION

Recently, much attention has been paid to the dietary and sleep habits in adolescents who are in a critical developmental period.[1,2] Irregular meals (mainly the skipping of breakfast) and inadequate sleep has become prevalent in adolescents and is thought to result in problems with school study and be a risk factor for health. However, few studies about lifestyle have been conducted on young people, although their dietary and sleep habits are no better than those of adolescents. According to the national nutritional survey of Japan, the percentage of non-breakfast young people (20–29 years old) was about twice as many as that in the adolescents (15–19 years old), and it showed an increasing trend (Table 1).

We carried out a nutritional survey in medical students during 1997–2002 and found 26.7% of students failed to eat breakfast. However, the daily energy intake of students without breakfast was comparable to that of students with breakfast (Kaneko *et al*., unpublished observation). We analyzed the questionnaire and found the students without breakfast ate a lot at night. They usually stayed up later and got up too late to allow time for breakfast the next morning. We defined this lifestyle as a nocturnal lifestyle and observed its effects on the circadian patterns of melatonin, leptin and insulin in healthy adults. We found that the nocturnal lifestyle is likely to be a risk factor to health in modern human.[3]

Previously, we found that a Western diet (*e.g.* high-fat diets and milk/ milk products), which was introduced to Japan after World War II, could be responsible for male reproductive disorders, cancers and diabetes.[4–8] However, we considered that dietary changes could not explain this completely. Since the

*To whom correspondence should be addressed: Department of Obstetrics and Gynecology, School of Medicine, University of Yamanashi, 1110 Shimokato, Tamaho, Yamanashi 409–3898, Japan. Tel.: +81-55-273-9632; Fax: +81-55-273-6746; E-mail: shinr@res.yamanashi-med.ac.jp

Table 1. The Percentage of Adolescents and Young People without Breakfast in Japan

	Age	1998	1999	2000
Male	15–19	13.7	12.3	13.3
	20–29	27.4	26.3	30.5
Female	15–19	9.0	7.5	9.2
	20–29	13.8	14.0	16.3

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nocturnal lifestyle was accepted by more and more young people, we tried to observe the differences in the circadian patterns of sex hormones and other relative hormones between subjects with diurnal and nocturnal lifestyles. Like melatonin and leptin, some sex hormones,[9–19] cortisol[15–19] and prolactin[19,20] also showed a circadian rhythm in normal conditions. These circadain rhythms are necessary for physiological roles and are affected by environmental input.[21] If the circadian rhythms of hormones really change after the nocturnal lifestyle, its effects on health should be considered.

MATERIALS AND METHODS

Subjects —— Ten male volunteers were recruited from the university campus at the beginning of February in 2004. They studied in the same classroom and lived nearby in order to be supervised by researchers. Their ages ranged from 20 to 22 years (21.3 ± 0.9). Body mass index (BMI) before the study was 21.9 ± 5.8 kg/m^2, and showed no significant change after the study. All subjects were nonsmokers without a history of endocrine, liver or other metabolic disorders. None took any medication one month before or during study. Violent sports were forbidden for 3 days before the day of blood collecting. Informed consent was obtained from each subject. The protocol was approved by the ethical committee of University of Yamanashi.

Protocol —— Since this study was conducted to observe the characteristics in daily life, we did not attempt to change the subjects' environmental conditions. Subjects were divided equally into two groups at random. One group (diurnal lifestyle) lived an ordinary student life on campus and ate three meals at 07:00, 13:00 and 19:00. They slept from 22:30 to 06:30 without any sleep during the daytime. The subjects in the other group (nocturnal lifestyle) stayed up until just after 01:30 and got up at 08:30 in the morning. Like the diurnal lifestyle group, they studied in classrooms in the daytime and spent their spare waking time mostly in their rooms with sufficient light. They were deprived of breakfast, but had lunch (13:00) and dinner (19:00) and ate at night freely without any restriction of frequency before sleep. The foods at night were bought from a convenience store within five minutes' walk. Although the meals were not identical in composition, the consumption of energy in the evening and at night was greater than 50% of their daily food intake. All subjects in the two groups were asked to maintain their usual foods as stated in the questionnaire before the study. Before the participation, they were taught how to calculate calories in cooked and commercial foods. The subjects in both groups consumed energy amounts between 2200 and 2600 kcal every day. During the week-end, they were also asked to keep the experimental regimen.

These processes lasted for 3 weeks. On the 22nd day, blood samples were drawn from the forearm every three hours from 09:00 to 06:00 the next morning. During the dark (sleep) phase, the subjects covered their eyes with brief awakening when sampling. Blood was collected within five minutes by five trained researchers. Blood samples were immediately centrifuged at 4°C and the plasma was stored at –80°C until analyses.

A randomized cross-over design was performed with a one-month interval. In other words, the subjects in both groups were exchanged after one month and experienced the same processes described above. Plasma samples collected in both tests were used to measure the concentrations of testosterone (T), free testosterone (fT), dihydrotestosterone (DHT), androstenedione (δ), prolactin (PRL), estrone, luteinizing hormone (LH), cortisol (F) and sex hormone binding globulin (SHBG).

Hormone Assays —— Plasma hormones and SHBG were measured using commercial ELISA kits, all of which were obtained from the American Laboratory Production Company (Windham, NH, U.S.A.). All samples were analyzed in duplicate in the same assay. The detection limit, intra- and inter-assay coefficients of variation (C.V.) for these kits were listed in Table 2. The results of detective limit, inter-assay and intra-assay suggest the validation of these kits to determine the plasma hormone concentrations.

Statistical Analysis —— Values are expressed as means ± S.D. The differences in plasma concentra-

Table 2. The Detection Limit, Intra- and Inter-Assay C.V. for these Kits

	Unit	Detective limit	Intra-assay		Inter-assay	
			Mean	C.V. (%)	Mean	C.V. (%)
T	ng/ml	0.07	0.68	5.82	0.67	3.71
fT	pg/ml	0.15	2.57	6.2	2.91	3.9
DHT	pg/ml	6.0	236.7	11.4	280.9	12.1
δ	ng/ml	0.02	0.91	6.7	1.23	14.6
F	ng/ml	3.0	10	6.9	10	5.9
PRL	ng/ml	2.0	5.26	13.2	4.82	10.9
E1	pg/ml	4.5	24.3	7.7	32.1	7.1
LH	MIU/ml	1.0	4.9	8.5	4.3	12.8
SHBG	nmol/l	0.2	4.5	8.6	3.8	11.6

$n = 8$.

Table 3. Mean Concentrations of Plasma Hormones in the Diurnal and Nocturnal Lifestyle Groups

	Diurnal lifestyle group	Nocturnal lifestyle group
T (ng/ml)	5.70 ± 0.71	5.51 ± 0.67
fT (pg/ml)	10.66 ± 2.30	8.53 ± 1.22*
DHT (pg/ml)	670.74 ± 74.43	710.65 ± 64.96
δ (ng/ml)	1.40 ± 0.21	1.47 ± 0.23
F (ng/ml)	59.0 ± 26.9	88.3 ± 38.3*
PRL (ng/ml)	18.62 ± 4.89	13.70 ± 3.32*
E1 (pg/ml)	29.55 ± 6.57	27.45 ± 4.17
LH (mIU/ml)	11.38 ± 1.54	12.49 ± 1.23
SHBG (nmol/l)	28.54 ± 2.30	29.08 ± 1.37
T/SHBG	0.22 ± 0.02	0.21 ± 0.02

Values are mean ± S.D. *$p < 0.05$, compared with the nocturnal lifestyle group.

tions of hormones, SHBG and the ratio between testosterone and SHBG (T/SHBG index) in the mean values and at the same time points between the diurnal and nocturnal lifestyle groups were assessed by student's *t*-test using StatView 5.0 (SAS Campus Drive, Cary, NC, U.S.A.). Although SHBG is a plasma protein, it is not noted, along with the T/SHBG index from hormones, until indicated.

To identify the circadian rhythm that might be present in plasma hormone concentrations, 24-hr cosine analysis was performed with a cosinor method.[22] Data were analyzed by the least square method, in order to find the best-fitting cosine function throughout the 24-hr cycle. It can quantify rhythm parameters, such as mesor M (24-hr rhythm adjusted mean), amplitude A (one half of the crest-though difference of a variable throughout a 24-hr cycle), and acrophase ϕ (the crest-time of the cosine curve in a 24-hr scale). Therefore, the circadian rhythm can be described with the equation of $Y_t = M + A\cos(t + \phi)$ if it exist, where Y_t is the value at time t.

The comparisons of the number of individual patterns with or without circadian rhythms between the diurnal and nocturnal lifestyle groups were carried out by Fisher's exact probability test. $p < 0.05$ was considered significant.

RESULTS

Table 3 shows the mean plasma hormone concentrations in the diurnal and nocturnal lifestyle groups. Although no significant difference in testosterone concentration was found between the two groups, free testosterone concentration was significantly lower in the nocturnal lifestyle group than in the diurnal lifestyle group. Plasma prolactin concentration was also significantly lower in the nocturnal lifestyle group. However, plasma cortisol concentration was significantly higher in the nocturnal lifestyle group than in the diurnal lifestyle group.

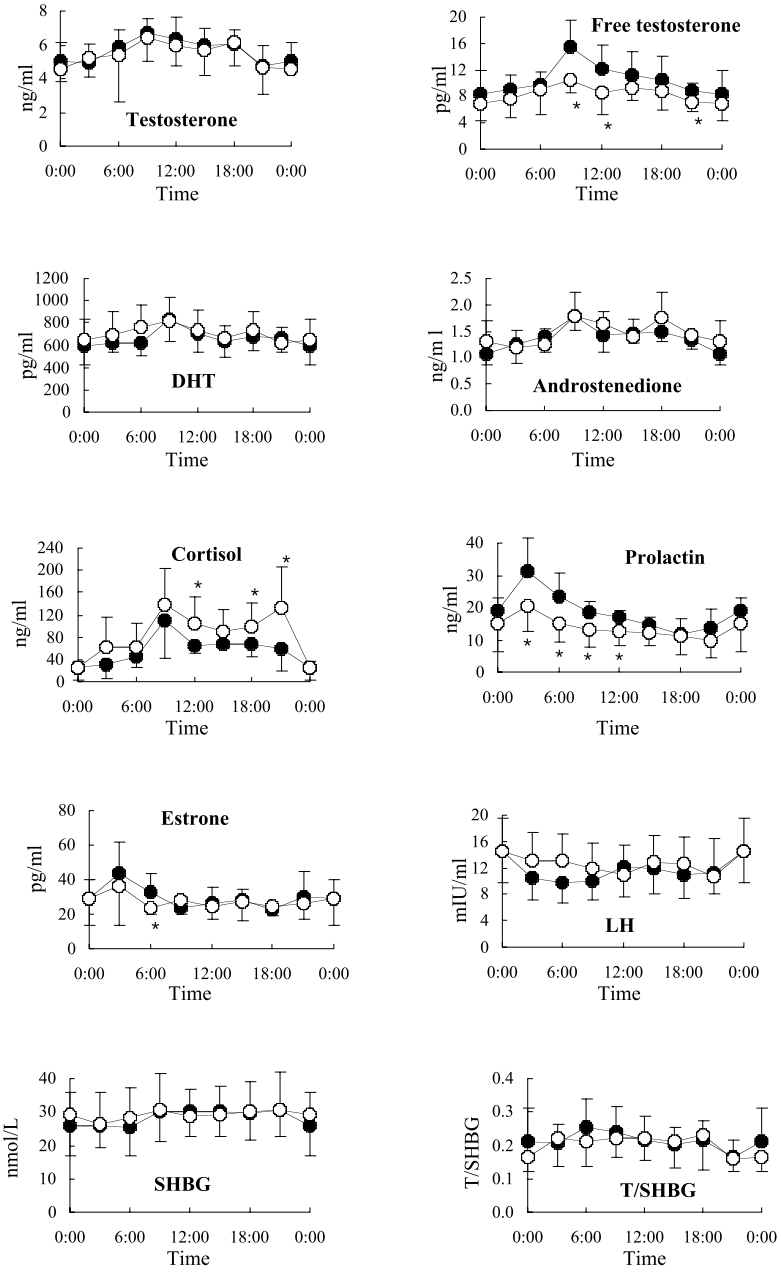

Fig. 1. The Patterns of Plasma Hormone Concentrations in the Diurnal (●) and Nocturnal (○) Lifestyle Groups
*$p < 0.05$, compared with the diurnal lifestyle group.

Similar to the comparisons of mean values, the differences in concentration at the same time point between the two groups mainly presented in free testosterone, prolactin and cortisol (Fig. 1). Plasma concentrations of free testosterone were significantly lower at 09:00, 12:00 and 21:00 in the nocturnal lifestyle group than in the diurnal lifestyle group. Plasma prolactin concentrations from 03:00 to 12:00 were significantly lower in the nocturnal lifestyle group. However, plasma cortisol concentrations were significantly higher at 12:00, 18:00 and 21:00 in the nocturnal lifestyle group. At 21:00, plasma cortisol concentration in the nocturnal lifestyle group increased 2.3-fold that in the diurnal lifestyle group. In general, the peak levels of androgens (testosterone, free testosterone, dihydrotestosterone, androstenedione) and cortisol appeared at the time point of 09:00, and reached a nadir at midnight. The patterns of plasma cortisol and androstenedione were biphasic, with two peaks at 09:00 and 21:00 in the nocturnal lifestyle group. Female sex hormone (estrone) and prolactin had peaks at night at 03:00. Al-

Table 4. Number of Individual Patterns with or without Circadian Rhythm in the Diurnal and Nocturnal Lifestyle Groups

	Diurnal lifestyle group		Nocturnal lifestyle group	
	Rhythm	Non-rhythm	Rhythm	Non-rhythm
T	7	3	3	7
fT	8	2	2	8*
DHT	2	8	1	9
δ	5	5	2	8
F	8	2	2	8*
PRL	7	3	1	9*
E1	2	8	2	8
LH	2	8	2	8
SHBG	5	5	0	10*
T/SHBG	3	7	2	8

*$p < 0.05$ compared with the diurnal lifestyle group.

Table 5. Circadian Rhythm of Mean Plasma Hormone Concentrations in the Diurnal and Nocturnal Lifestyle Groups

	Unit	Diurnal lifestyle group	Nocturnal lifestyle group
T	ng/ml	$Y_t = 5.7 + 0.8\cos(t + 172^\circ)$	$Y_t = 5.4 + 0.7\cos(t + 176^\circ)$
fT	pg/ml	$Y_t = 10.6 + 2.4\cos(t + 167^\circ)$	$Y_t = 8.5 + 1.4\cos(t + 163^\circ)$
DHT	pg/ml	$Y_t = 669.4 + 64.6\cos(t + 166^\circ)$	
F	ng/ml	$Y_t = 58.4 + 25.0\cos(t + 185^\circ)$	
PRL	ng/ml	$Y_t = 18.6 + 0.8\cos(t + 69^\circ)$	

M: mesor (the rhythm adjusted mean); A: amplitude (half of the 24 hr variability due to the rhythm); ϕ: acrophase (the estimated peak time); *$p < 0.05$ from the zero-amplitude assumption.

though there was no significant difference in mean concentration, Plasma estrone concentration at 06:00 was significantly lower in the nocturnal lifestyle group. Plasma SHBG concentration in the diurnal lifestyle group showed two plateaus: a low plateau at late night/early morning (00:00–06:00), increasing to a high plateau during the remainder of period. The pattern of T/SHBG index in both groups showed little 24-hr circadian variation based only on the analysis of time points.

When the individual pattern was analyzed using the cosinor method, there were no hormones whose circadian rhythm was significant in all the subjects (Table 4). Hormones whose circadian rhythm was significant in half or more than half of the subjects included testosterone, free testosterone, androstenedione, prolactin, cortisol and SHBG in the diurnal lifestyle group. Of them, eight subjects demonstrated circadian rhythm in free testosterone and cortisol. However, we found no hormones whose circadian rhythm surpassed our arbitrary criterion in the nocturnal lifestyle groups. Significant differences between the two groups were found in free testosterone, prolactin, cortisol and SHBG.

As for the patterns of mean plasma hormone concentrations, testosterone and free testosterone maintained significant circadian rhythm in the diurnal and nocturnal lifestyle groups and DHT, prolactin and cortisol in the diurnal lifestyle group (Table 5). We did not find any circadian rhythm in the other hormones. Because only two subjects showed circadian rhythm, the rhythm of mean DHT was inconclusive in the diurnal lifestyle group. Similarly, although rhythms of mean plasma testosterone and free testosterone were significant, few subjects (three subjects for testosterone and two for free testosterone) showed significant circadian rhythms in the nocturnal lifestyle group. It is noteworthy that in spite of no circadian rhythm, 5 subjects had significant circadian rhythm in SHBG in the diurnal lifestyle group (Table 4). In general, the peak concentrations of androgens appeared at 11:00–11:30 in the cosine analysis. The peak concentration of prolactin and cortisol appeared at about 04:30 and 12:30, respectively, in the diurnal lifestyle group. The peaks of these hormones, which can be explained by the cosinor

method, were delayed by some hours compared with the analysis of time points. Overall, the circadian rhythm appeared in androgens (especially in free testosterone), prolactin, cortisol and SHBG in the diurnal lifestyle group; however, all of these rhythms were almost undetectable in the nocturnal lifestyle group.

DISCUSSION

In the present study, seven hormones and SHBG were measured. Testosterone (including testosterone and free testosterone), DHT and androstenedione are important androgens. Cortisol has a well definded circadian rhythm[15–19] and is related to androstenedione, which is produced by both the adrenals and testes.[18] LH and prolactin, which are secreted by the pituitary, are considered to regulate testis function through the pituitary-testis axis.[23] As important female sex hormone, estrone in males is synthesized partly by testosterone through enzymatic aromatization.[24] SHBG has a high affinity with testosterone and DHT, and influences the circadian rhythms of these hormones.[13] Therefore, in addition to the respective role of each hormone, the 24 hr patterns of these hormones reflect the milieu of sex hormones in males to a large degree.

The circadian rhythm of testosterone in normal men was detected several decades ago.[9] Because more than 90% of testosterone was bound to plasma protein (mainly to SHBG), the T/SHBG index has been thought to reflect the biologically active fraction of testosterone.[15] In the present study, the pattern of SHBG concentration in the diurnal lifestyle group was consistent with previous studies.[12,13] However, the pattern of the T/SHBG index did not show any circadian rhythm in the time point figure and after the cosine analysis. Although SHBG is the most important protein bound with testosterone, the effects of other plasma proteins should not be neglected. In the present study, the amplitude of circadian rhythm in free testosterone was three-fold (2.4/0.8) that in testosterone in the diurnal lifestyle group. This suggested that free testosterone is a better parameter to estimate the circadian rhythm than the T/SHBG index and testosterone. In order to investigate the testosterone with biological activity in the circulation, the best way is to measure the concentration of free testosterone directly.

After three weeks of the nocturnal lifestyle, the mean concentration of free testosterone decreased significantly. One possible reason for this is the decrease in testosterone and increase in SHBG on average, although neither reached a significant difference compared with the diurnal lifestyle group. Moreover, the individual circadian rhythm of free testosterone was disturbed in the nocturnal lifestyle group. Since testosterone has many biological effects, especially for male sexual function,[21,25] further study should be done to clarify the effects of the decrease in free testosterone concentration and the disappearance of its circadian rhythm on males with a nocturnal lifestyle. It has been reported that plasma testosterone concentration decreased significantly after short-term fasting[26] and its circadian rhythm was disrupted by sleep fragmentation.[27] It was suggested that both the meal and sleep regimen caused the changes in the testosterone pattern. However, we could not separate the effect of an irregular meal schedule from slight sleep debt and shift in the present study. In a study of Ramadan life, during which food intake was restricted to the night hours with a debt and shift of sleep, there was no change in the 24-hr mean concentration of testosterone, but there was a delay in the onset of its increase.[19] This reconfirms that free testosterone should be measured to observe the pattern of bioactive testosterone in plasma.

In the present study, the pattern of mean DHT showed significant circadian rhythm in the diurnal lifestyle group, but was extinguished in the nocturnal lifestyle group. Meanwhile, five subjects kept androstenedione circadian rhythm in the diurnal lifestyle group and it became two subjects in the nocturnal lifestyle group. This suggested that their circadian rhythm became worse after the nocturnal life. Among androgens, DHT is the most important hormone contributing to male sexual behavior[28] and the incidence of prostate cancer.[29] An abnormal DHT pattern in nocturnal life is likely to be a risk factor to health for modern people. Because about 70% of androstenedione is derived from adrenals, the pattern of androstenedione was similar to that of cortisol, even in the nocturnal lifestyle group, which was also supported by a previous study.[16] In the nocturnal lifestyle group, the cortisol concentration increased markedly at 21:00, possibly caused by some anxious anticipation of the subsequent nocturnal life and the continuous intake at night. On the other hand, the increase in cortisol concentration may be responsible for the decrease of free testosterone in the nocturnal lifestyle group by competing with testosterone for an SHBG binding site.[13]

It is known well that plasma prolactin concentration is relative to sleep.[30] Although the subjects in the nocturnal lifestyle group reduced only one hour of bed-time and delayed three hours, the circadian rhythm of prolactin extinguished and its concentrations decreased at late night and in the morning. The change in the prolactin pattern is also consistent with that of elderly males, who often lost their sleep time.[31] Unlike our findings, night workers almost maintained a similar, but shifted peak level, during sleep.[32] This suggested that a large intake before sleep may play an important repressive role on the night secretion of prolactin.

We were unable to establish the presence of circadian rhythm for plasma estrone and LH. This may be the result of the estrone concentration being too low in the male circulation compared with females, and the LH measurement having been too infrequently compared with their pulse secretion.

The purpose of the present study was to observe the endocrine functions in real life and obtain the basic data for the further research. So, we did not set the meal time at night for the nocturnal lifestyle except the total energy and percentage (> 50%) in the evening and at night. For the same purpose, we also did not force the subjects to eat food in a special chamber. However, they were asked not to significantly change their usual food kinds during the study period. To offset the individual difference, a randomized cross-over design was conducted with an interval of one month. It was suggested that the intakes of nutrients such as carbohydrate, fat and protein, which maybe affect the hormone levels, were balanced in a permissible range in the two groups, despite the differences in individual intake.

In conclusion, a nocturnal life has become prevalent in college students. However, there has been inadequate attention on the adverse effects of this nocturnal life. When the subjects lived a diurnal life, 24-hr hormone patterns maintained a normal circadian rhythm, which is necessary for physiological roles. Almost all of these circadian rhythms extinguished, with a decrease in free testosterone and prolactin and an increase in cortisol when they lived a nocturnal life. Although these young healthy subjects did not complain of any clinical problems during the study period, the effects of these changes of hormone concentration and its circadian rhythms after the nocturnal life should be considered and studied intensively.

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