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Associations of urinary 6-sulfatoxymelatonin with biomarkers related to cardiovascular disease in Japanese women

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ARTICLE INFO

Article history:
Received 7 December 2010
Accepted 25 May 2011

ABSTRACT

Melatonin's potential preventive effect against cardiovascular disease (CVD) remains hypothetical. No study has evaluated the relationships between endogenous melatonin and the established blood biomarkers related to CVD. The objective of the present study is to examine the association between the endogenous melatonin level and various established blood biomarkers of risk of CVD, including white blood cell (WBC) count and plasma concentrations of lipids, homocysteine, uric acid (UA), and high-sensitivity C-reactive protein (hs-CRP). This cross-sectional study included 181 Japanese women who attended a health checkup program provided by a general hospital between March 2005 and March 2006. All study subjects responded to a self-administered questionnaire and were measured for weight, height, and blood pressure. Venous fasting blood and first-void morning urine were obtained from all subjects. Statistically significant inverse correlations were observed between urinary 6-sulfatoxymelatonin (aMT6s), the major metabolite of melatonin in urine, and WBC count, UA, and hs-CRP after controlling for age, body mass index, menopausal status, smoking status, diet, sleeping habits, and exercise (r = –0.19, –0.21, and –0.24, respectively). There were no significant correlations between urinary aMT6s and plasma lipids and homocysteine. These data suggested that the urinary aMT6s level was inversely associated with established independent risk factors for CVD, such as WBC, UA, and hs-CRP. Endogenous melatonin may have implications for the risk of CVD.

1. Introduction

Cardiovascular diseases (CVDs) are currently the leading cause of mortality in Japan as well as in other industrialized countries. A recent survey in Japan has shown that heart disease ranks as the second cause of mortality after cancer. As well as lifestyle factors such as cigarette smoking, obesity, hypertension, and metabolic disorders, some blood biomarkers have been recognized to be associated with CVD. For example, white blood cell (WBC) count, lipids, homocysteine, uric acid (UA), and high-sensitivity C-reactive protein (hs-CRP) are independent established risk factors of CVD [1-6].

Author contributions: TM: analyzing and interpreting the data, and writing the manuscript. KW: analyzing the data and editing the manuscript. MH, NT, and KY: supervising the field activity and interpreting the data. TD: editing the paper and supervising the study. CN: initiating and organizing the study.

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0026-0495/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2011.05.021

Melatonin, a hormone mostly secreted by the pineal gland at night, has received attention worldwide since its identification by Lerner et al [7] in 1958. It has gradually become clear that melatonin contributes to the regulation of many biological systems. In addition to being a direct powerful scavenger of free radicals, melatonin has potential beneficial effects on blood pressure (BP), cardiac ischemia/reperfusion, and myocardial contractility [8-10]. Dominguez-Rodriguez et al [9] updated the biological role of melatonin from a broad point of view. For example, several studies have demonstrated that the treatment with melatonin decreased BP in spontaneously hypertensive rats. The degree of cardiac arrhythmia after occlusion of coronary artery and following reperfusion was significantly greater in pinealectomized rats and reduced in the melatonin-infused rats. Reiter et al [10] also summarized experimental data from both animals and human, and pointed out the possibility of inclusion of melatonin in clinical trials for CVD.

Most of previous reports about melatonin, however, dealt with animal experimental data. In humans, some studies suggested melatonin's beneficial influence on hypertension and acute myocardial infarction [11-13]. However, subjects in those reports were restricted to patients; and melatonin was administered to the subjects for treatment purpose. Several studies have assessed the association between endogenous melatonin level and lifestyle related to the risk of CVD, such as body mass index (BMI), smoking, and menopausal status in healthy subjects [14,15]. For example, Schernhammer et al [14] reported that greater BMI and smoking were associated with a lesser level of melatonin. Furthermore, a significant inverse association between endogenous melatonin level and incident hypertension was reported from the Nurses' Health Study II [16]. However, few studies have assessed the association between the endogenous melatonin level and blood biomarkers related to CVD among healthy subjects. To our knowledge, one study assessed this association; but only serum lipid was dealt with in the study [17]. Therefore, in the present study, we examined the relationship between the endogenous melatonin level and WBC count, and plasma levels of lipids, homocysteine, UA, and hs-CRP.

2. Materials

2.1. Study subjects

This study is part of a study designed to assess the relationships of lifestyles, environmental factors, and women's health. The study subjects were participants in a medical health checkup program provided by a general hospital in Gifu, Japan, between October 2003 and March 2006. A total of 2073 women, including return visitors to the program during the study period, were invited to join the study; and 1536 agreed to participate (the acceptance rate was 74.1%). When calculated for only new visitors to the program during the study period, the acceptance rate was 83.0% (1100 of 1325 individuals). The details of this study are presented elsewhere [18].

The objective of the present study was to examine the associations of various biomarkers of CVD with the endogenous melatonin level. Therefore, this component study included subpopulations who participated in the study between March 2005 and March 2006 by providing blood and morning urine specimens. Written informed consent was obtained from each subject. This study was approved by the ethical board of the Gifu University Graduate School of Medicine.

During the study period, a total of 526 women were invited to the present study. Some agreed to participate in the questionnaire survey and biomarker measurements but not to donate the morning urine specimens. Thus, 192 women completed all the study items (the response rate was 36.5%). The characteristics and laboratory results of these women were similar to those of women who participated in the study but did not provide urine samples. Of the 192 women, 11 were excluded from the analysis, including those whose urine samples had been left unfrozen for a long time (n = 2), those who had had ischemic heart disease (n = 2), and those who were currently using antihypertensive agents (n = 4) and oral contraceptives (n = 3). Thus, a total of 181 women were included in the analysis.

3. Methods

3.1. Data collection and determination of biomarker levels

All study subjects responded to a self-administered questionnaire that included questions on basic demographic characteristics, including smoking and drinking habits; exercise; comorbidities; past medical, medication, and reproductive histories; mental status; sleeping habits; and diet. Each subject was measured by authorized personnel for weight (in kilograms), height (in centimeters), BP, and BMI. Exercise was assessed on the basis of the average number of hours spent weekly performing various activities over the past year. The details are described elsewhere [19]. To obtain information on sleeping habits, we referred to the questions used in a study on light at night and breast cancer risk reported by Davis et al [20]. Questions regarding the time that a subject usually turned off the lights before going to sleep and their wake-up times on weekdays and weekends were included. Six response categories adopted from the study by Davis et al [20] were provided for the question asking about the ambient light level in the bedroom while sleeping (from level 1, the subject wore a mask to keep out light, to level 6, she could read comfortably). Diet was assessed with a validated 169-item semiquantitative food frequency questionnaire. The questionnaire asked participants how often on average they consumed each of the food items listed and what was the usual serving size of each item during the year before the study. Intake of nutrients and food groups was estimated from the frequency of ingestion and portion size using the fourth and fifth editions of the *Standard Tables of Food Composition in Japan* [21]. Detailed information about the questionnaire, including its validity and reproducibility, has been described elsewhere [22].

Venous fasting blood and first-void morning urine were obtained from all subjects. Blood samples obtained at approximately 10:00 AM were placed in plastic tubes containing ethylenediaminetetraacetic acid and centrifuged. The urine samples had been obtained by the participants at their home in

the next morning and sent by overnight mail with a frozen water bottle. The plasma and urine samples were immediately frozen and stored at –80°C until being assayed. The plasma UA and hs-CRP concentrations were measured using the uricase-peroxidase method and the nephelometry method, respectively [23,24]. The reagents used were Pure Auto S UA purchased from Daiichi Pure Chemicals, Tokyo, Japan, for UA and N-latex CRP II purchased from Siemens Healthcare Diagnostics, Tokyo, Japan, for hs-CRP. The laboratory-reported inter- and intraassay coefficients of variation were 1.41% and 0.99% for UA and 3.04% and 1.72% for hs-CRP. The concentrations of plasma total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides (TG) were determined using the enzymatic assay. The reagents used were Pure Auto S CHO-N, Cholestest N HDL, Cholestest LDL, and Pure Auto S TG-N, respectively [25-28]. These 4 reagents were all purchased from Daiichi Pure Chemicals, Tokyo, Japan. The inter- and intraassay coefficients of variation were 0.68% and 0.42% for TC, 0.45% and 0.87% for HDL-C, 0.82% and 0.44% for LDL-C, and 5.29% and 2.33% for TG. The concentration of total homocysteine was measured using high-performance liquid chromatography with a column of YMC-Pack Pro C18 purchased from YMC, Kyoto, Japan [29]. The laboratory-reported inter- and intraassay coefficients of variation were 1.45% and 2.46%, respectively.

Urinary 6-sulfatoxymelatonin (aMT6s), a typical determiner of the urinary melatonin level, was measured radioimmunologically using kits purchased from IBL Laboratories (Hamburg, Germany) [30]. The sensitivity was 1.0 ng/mL; and the inter- and intraassay coefficients of variation were 12.7% and 10.4%, respectively. To adjust for variation in the diluteness of urine, the urinary aMT6s levels were expressed as urine aMT6s per urine creatinine. Urinary creatinine was measured by the conventional enzymatic method [31]. The laboratory-reported inter- and intraassay coefficients of variation were 0.98% and 1.06%, respectively.

3.2. *Statistical analysis*

The urinary aMT6s level and plasma levels of the biomarkers were transformed into logarithmic values for statistical analyses. To assess the associations between the urinary aMT6s level and the biomarkers of CVD, partial Pearson correlation coefficients (r) were calculated after controlling for potential confounders including age, smoking status, BMI, and menopausal status. Exercise has been associated with endogenous melatonin levels in some studies [32,33]. It has been suggested that some food groups such as green vegetables, mushrooms, and meat could affect endogenous melatonin levels [34,35]. Melatonin level has also been considered to be dependent on sleeping habits such as sleep duration and lighting conditions of bedroom [36,37]. Therefore, additional adjustment with exercise; intake of green vegetables, mushrooms, and meat; and sleeping habits was performed. All statistical analyses were performed using the SAS statistical package, version 9.1 (SAS Institute, Cary, NC). A P value < .05 was considered to be statistically significant.

4. Results

Selected clinical and biological characteristics of the study subjects are shown in Table 1. The correlation coefficients

Table 1 – Characteristics of study subjects (n = 181)

Variables		Reference range
Age, mean ± SD (range), y	46.1 ± 9.4 (20-67)	
BMI, mean ± SD (range), kg/m^2	21.7 ± 2.9 (16.1-31.3)	18.5-25
SBP, mean ± SD (range), mm Hg	115.8 ± 16.8 (80-169)	<130
DBP, mean ± SD (range),mm Hg	71.9 ± 10.3 (35-101)	<85
WBC, mean ± SD (range), /μL	4798 ± 1205 (2420-8070)	3500-9100
UA, mean ± SD (range), mg/dL	4.0 ± 0.8 (2.2-6.8)	2.5-7.0
hs-CRP, mean ± SD (range), ng/mL	596.3 ± 2154.4 (50-26800)	<1500
TC, mean ± SD (range), mg/L	206.8 ± 35.6(132-310)	150-219
HDL-C, mean ± SD (range), mg/L	64.8 ± 13.0(35-114)	40-96
LDL-C, mean ± SD (range), mg/L	116.2 ± 32.6(64-197)	70-139
TG, mean ± SD (range), mg/L	84.8 ± 47.1(22-320)	50-149
Homocysteine, mean ± SD (range), nmol/mL	9.2 ± 2.2(5.2-17.9)	3.7-13.5
Urinary aMT6s, mean ± SD (range), ng/mg creatinine	53.9 ± 44 (3.2-221.7)	
Smoking (n and %)		
Never	162 (89.5)	
Former	13 (7.2)	
Current	6 (3.3)	
Exercise, mean ± SD (range), METs	26.1 ± 33.6 (0-178.5)	
Sleep duration, mean ± SD (range), h/d	6.8 ± 1.1 (3.5-10.0)	
Meat intake, mean ± SD (range), g/d	81.5 ± 51.7 (5.4-509.8)	
Green vegetable intake, mean ± SD (range), g/d	395.0 ± 207.2 (95.6-1037.9)	
Mushroom intake, mean ± SD (range), g/d	28.5 ± 24.5 (3.4-126.0)	
Postmenopausal (n and %)	48 (26.5)	

SBP indicates systolic blood pressure; DBP, diastolic blood pressure; MET, metabolic equivalent.

Table 2 – Pearson partial correlation coefficients between urinary melatonin and parameters related to CVD

	r [a]	P	r [b]	P	r [c]	P
BMI	−0.16	.037	-	–	–	–
WBC	−0.18	.015	−0.18	.019*	−0.19	.031*
UA	−0.21	.006	−0.22	.005†	−0.21	.019*
hs-CRP	−0.26	.001	−0.22	.005†	−0.24	.007†
TC	0.08	.31	0.10	.22	0.17	.051
HDL-C	0.07	.34	0.02	.85	−0.05	.56
LDL-C	−0.12	.49	−0.06	.74	−0.01	.97
TG	−0.10	.16	−0.04	.60	0.03	.75
Homocysteine	−0.02	.79	−0.03	.75	−0.05	.61

* <.05
† <.01
[a] Model 1: adjusted for age.
[b] Model 2: adjusted for model 1 plus BMI, menopausal status, and smoking status.
[c] Model 3: adjusted for model 2 plus intake of meat, green vegetable, and mushroom; exercise; lighting condition; and sleep duration.

between urinary aMT6s and the blood biomarkers associated with CVD are shown in Table 2. Statistically significant inverse correlations were observed between urinary aMT6s and WBC count, UA, and hs-CRP with adjustments for age, BMI, menopausal status, smoking status, exercise, diet, and sleeping habits. Urinary aMT6s was not significantly correlated with TC, HDL-C, LDL-C, TG, and homocysteine after controlling for the covariates.

It has been previously reported that urinary aMT6s is associated with alcohol intake in healthy women [38]. However, alcohol consumption was unrelated to urinary aMT6s level in the present study; and additional adjustment for alcohol consumption did not substantially alter the results (r for WBC, UA, and hs-CRP was −0.19, −0.21, and −0.25, respectively). There were 17 (9.4%) women with systolic BP of at least 140 mm Hg and diastolic BP of at least 90 mm Hg in the present study. Additional analysis excluding them did not alter the results (r for WBC, UA, and hs-CRP was −0.20, −0.20, and −0.26, respectively). One woman had a high value of hs-CRP (>10 000 ng/mL). Exclusion of this woman also did not alter the results. None of the women had a history of gout in the present study.

5. Discussion

To the extent of our knowledge, this is the first report focusing on a direct association between endogenous melatonin level and blood biomarkers related to CVD in healthy human subjects. We found that WBC, UA, and hs-CRP, the established independent risk factors for CVD, were inversely associated with the urinary aMT6s level. We took account of the various potential confounders to assess these associations.

A number of epidemiological studies have evaluated the associations between these 3 biomarkers and risk of CVD. From the Women's Health Initiative Observational Study, Margolis et al [39] described that the WBC count was an independent predictor of coronary heart event among postmenopausal women with no CVD risk factors with an adjusted hazard ratio of 1.7 (95% confidence interval, 1.28-2.27) for the highest compared with the lowest WBC count quartiles. Regarding UA, it used to be believed that the relationship between UA and CVD was paradoxical because of the dual aspect of UA as an antioxidant and a prooxidant agent [40]. However, in an additional 5-year follow-up of the first National Health and Nutrition Examination Survey epidemiologic study, greater serum UA was found to be an independent risk factor for CVD death in both sexes [5]. Rifai and Ridker [41] reviewed 10 prospective studies and indicated that hs-CRP is a powerful biomarker to predict a future first coronary event in apparently healthy people.

All 3 blood biomarkers reflect systemic inflammation [42]. The observed low levels of WBC count and UA and hs-CRP levels may be explained by the anti-inflammatory effects of melatonin. Laboratory studies have shown that melatonin and its metabolites have anti-inflammatory actions through the inhibition of cyclooxygenase 2, which plays an important role in the inflammatory response [43]. On the other hand, the important role of inflammation in the pathogenesis of coronary artery disease and atherosclerosis has been indicated. The major cause of myocardial infarction is the formation of an occluding thrombus on the surface of the plaque [44]. This occluding thrombus is developed by plaque rupture, and numerous inflammatory molecules can reduce the stability of plaque [45,46].

Melatonin is also known to have an antioxidative function. Reactive oxygen species (ROS), such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, play an important role in the pathogenesis of cardiac ischemia/reperfusion injury, coronary atherosclerosis, and its complications [47]. Melatonin downregulates ROS with its direct scavenging action [8]. Melatonin may have preventive effects on CVD through its ROS regulation, which may be reflected by the observed lesser level of biomarkers. It has been suggested that neutrophils also release ROS [48]. It is also possible that the lesser WBC count due to melatonin's effects, if true, may reduce ROS production, which may lead to the reduction of the risk of CVD.

Laboratory studies have also shown that melatonin has direct protective effects of the cardiovascular system [8]; melatonin reduced the incidence and severity of reperfusion-induced ventricular arrhythmias in the isolated rat heart [49] and the infarct size/risk area in mouse [50]. The observed associations of urinary aMT6s with some biomarkers of CVD, together with these laboratory findings, may suggest the implications of melatonin in the genesis of CVD. To confirm whether urinary melatonin level is associated with the risk of CVD and to know the magnitude of the association, epidemiological studies with a prospective design are needed. Physiological oral dose of the melatonin concentrations, which elevate serum melatonin concentrations only to levels normally occurring nocturnally, were shown to promote sleep onset and sleep maintenance [51]. Hypothetically, if our data were extrapolated into clinical settings, administration of oral melatonin may provide benefits by improving sleep status, in addition to decreasing the blood level of WBC, UA, and hs-CRP in women with CVD. However, it would be premature to refer to the importance of exogenous melatonin as a remedy or a prophylactic agent for CVD.

There are some limitations that should be considered as well. The low response rate is one of the limitations of the present study. This study required that all subjects self-collect their morning urine at home and send the samples by mail. It is possible that the inconvenience of the request resulted in a smaller number of participants. However, it is unlikely that participation behavior was directly dependent on the urinary aMT6s level and other blood biomarkers. In fact, in the present study, the measured biomarkers did not differ between women who provided morning urine and those who did not. It is unlikely that the observed associations between the urinary aMT6s level and the blood biomarkers are due to response biases. For the urinary aMT6s measurement, we collected spot morning urine but not 24-hour–pooled urine. However, it was previously validated that the urinary aMT6s level in morning urine is strongly correlated with total nocturnal plasma melatonin output and pineal nocturnal melatonin value (r = 0.89). A sufficient reproducibility of measurement of aMT6s in morning urine over 5 years (intraclass correlation coefficient = 0.56) has also been reported [52,53]. Another study also demonstrated that overnight shipping of spot morning urine was acceptable for aMT6s measurement [36]. Because the present study is cross-sectional, it is impossible to establish the causal association between the urinary aMT6s level and WBC, UA, and hs-CRP values.

In conclusion, in a cross-sectional sample of healthy women, we found that urinary aMT6s was inversely associated with CVD risk blood biomarkers such as WBC, UA, and hs-CRP. Our findings may stimulate studies investigating the role of melatonin in the development of CVD.

Funding

Financial support: The study was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

Conflict of Interest

None of the authors has any financial or other interest in the dissemination of this article.

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