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RESEARCH ARTICLE

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Association of salivary testosterone levels during the post-awakening period with age and symptoms suggestive of late-onset hypogonadism in men

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ABSTRACT

Background: The lack of association between serum testosterone levels and symptoms suggestive of hypogonadism is a significant barrier in the determination of late-onset hypogonadism (LOH) in men. This study explored whether testosterone levels increase after morning awakening, likewise the cortisol awakening response (CAR) in the hypothalamic–pituitary–adrenal (HPA) axis, and whether testosterone levels during the post-awakening period are associated with age and symptoms suggestive of late-onset hypogonadism (LOH) in men.

Methods: Testosterone and cortisol levels were determined in saliva samples collected immediately upon awakening and 30 and 60 min after awakening, and scores of the Aging Males' Symptoms (AMS) questionnaire were obtained from 225 healthy adult men.

Results: A typical CAR (an increase in cortisol level ≥ 2.5 nmol/L above individual baseline) was observed in 155 participants (the subgroup exhibiting typical CAR). In the subgroup exhibiting CAR, testosterone levels sharply increased during the post-awakening period, showing a significant negative correlation with age, total AMS score, and the scores of 11 items on the somatic, psychological, and sexual AMS subscales. Of these items, three sexual items (AMS items #15–17) were correlated with age. Meanwhile, there was no notable increase in testosterone levels and no significant correlation of testosterone levels with age and AMS score in the subgroup exhibiting no typical CAR ($n = 70$).

Conclusions: The results indicate that the hypothalamus-pituitary-gonad (HPG) axis responds to morning awakening, and determining testosterone levels during the post-awakening period in men with typical CAR may be useful for assessing HPG axis function and LOH.

KEY MESSAGES

- The present study found that the HPG axis in healthy adult men responds to the morning awakening, characterized by increased salivary testosterone levels after the awakening period.
- The levels of salivary testosterone during the first hour after awakening are negatively associated with age and the severity of symptoms suggestive of LOH in adult men with typical CAR.

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Ageing men; late-onset-hypogonadism; salivary testosterone; post-awakening period; CAR

1. Introduction

Testosterone is the principal hormone of the hypothalamic–pituitary–gonadal (HPG) axis in men and exerts a pleiotropic effect on reproductive and non-reproductive organs [1]. A distinctive characteristic of HPG axis function in men ≥40 years is the gradual decline in circulating testosterone levels [2], which is related to an increase in the prevalence of testosterone deficiency (TD) and symptomatic hypogonadism [3,4]. The concomitant presence of age-related low testosterone levels with signs and symptoms suggestive of TD in men has various names, including male climacterium, andropause, and late-onset hypogonadism (LOH) [5].

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The association between circulating testosterone levels and the severity of signs and symptoms suggestive of LOH in adult men has been examined in previous studies; however, the results were inconsistent. Several studies found no significant association between total, bioavailable, or calculated-free testosterone levels and the severity of signs and symptoms measured using the Aging Male's Symptoms (AMS) scale or Androgen Deficiency in the Aging Male (ADAM) [6,7]. Moreover, no significant differences in total or bioavailable testosterone levels have been observed between ADAM-positive and ADAM-negative men [8]. In contrast, other studies demonstrated a negative association between calculated-free and bioavailable testosterone levels and total AMS [9] and AMS sexual symptom scores [10,11]. Significant differences have also been detected in calculated-free and bioavailable testosterone levels between ADAM-positive and ADAM-negative men [9].

Circulating testosterone levels exhibit a pronounced circadian rhythm in men, with the zenith in the early morning and the nadir in the late afternoon [12]. The circadian testosterone rhythm is less pronounced in older men than in younger men due to reduced testosterone levels at the circadian acrophase that occurs near morning awakening from nocturnal sleep [13]. However, total testosterone levels measured in a blood sample taken between 0700 and 1100 h are used to evaluate the presence of hypogonadism in men [14]. Therefore, it is conceivable that determining testosterone levels in a single blood sample taken in the wider time window, without reference to the individual's time of awakening from nocturnal sleep, could be a potential reason for the inconsistent findings of previous studies on LOH.

Meanwhile, cortisol secretion also follows a circadian rhythm [15]; cortisol levels in serum and saliva robustly increase and reach a circadian peak within the first hour after morning awakening from nocturnal sleep in men and women of all ages [16,17]. This phenomenon is called the cortisol awakening response (CAR), resulting from the suprachiasmatic nucleus (SCN)-mediated activation of the hypothalamic-pituitary-adrenal (HPA) axis [18,19]. The levels and patterns of cortisol secretion in the CAR are confounded by various types of perceived physical, psychological, and upcoming anticipatory stress and are nowadays used as a biomarker for the function of the HPA axis [18].

As with the CAR in the HPA axis, estradiol-17β and progesterone levels in saliva also sharply increase within the first hour after awakening in women with regular menstrual cycles [20]; however, this phenomenon is absent in premenarcheal girls [21] and menopausal women [20]. The findings suggest that a sharp increase in sex steroid levels after morning awakening is considered a phenomenon analogous to the CAR in the HPA axis, yet it is not a generalized occurrence across all ages because it is only seen in women with regular menstrual cycles.

Considering the difference between younger and older men in the circadian peak of testosterone levels in the early morning hours around the time of awakening [13] and the absence of increasing sex steroid levels during the post-awakening period in menopausal women [20], we hypothesized that testosterone levels would increase after morning awakening from nocturnal sleep in healthy adult men. Furthermore, we hypothesized that testosterone levels during the post-awakening period would reduce with aging, which might be associated with signs and symptoms suggestive of LOH in men. To test these hypotheses, testosterone levels were determined in the set of saliva samples collected during the post-awakening period from healthy adult men, and the associations between testosterone levels and age and AMS scores were analyzed.

2. Participants and methods

2.1. Study population

Koreans aged 19 and over are entitled to bi-annual health examinations, which are financially supported by the National Health Insurance Service of Korea. Participants (men aged more than 20 years) with the results from the periodic health examinations were initially enrolled in a study conducted by the Seoul National University Bundang Hospital and Yonsei University Gangnam Severance Hospital between May 2020 and March 2022. The initially enrolled participants were excluded if they met any of the following criteria: (1) any acute/chronic illness such as cancer, heart failure, neoplastic disease, brain injury, cerebrovascular accident, severe osteoarthritis, or coronavirus disease 2019; (2) history of consuming oral or transdermal glucocorticoid-containing drugs, analgesics other than acetaminophen, or testosterone therapy within the previous month and treatment with injectable testosterone undecanoate within the previous three months; (3) medical history of sexual disorders; or (4) current prescription of antihypertensive medications, antihyperlipidemic drugs, or antidiabetics. Subsequently, clinical data, including age, height, and body weight, were obtained from 225 participants (Figure 1). All participants completed the Korean

version of the AMS questionnaire, which is comprised of 17 items to evaluate somatic, psychological, and sexual signs and symptoms suggestive of LOH in aging men, and each item is answered on a scale of 1 to 5 [22]. This study conformed to the Declaration of Helsinki. This study was approved by the institutional review boards of Seoul National University Bundang Hospital (SNUBH) (B-1911-577-302) and Yonsei University Gangnam Severance Hospital (3-2020-0533). Written informed consent was obtained from all individual participants included in the study.

2.2. Saliva collection

The levels of unconjugated steroids in saliva reflect those of free- and total-form steroids in the serum [23]. Collecting saliva is non-invasive, allowing for repeated sampling at home without the help of medical staff. In order to determine testosterone levels in saliva samples collected during the post-awakening period, saliva samples were collected by following the procedures for measuring the CAR. Saliva collection procedures were adopted from previous studies [21,24,25]. In brief, participants were instructed to collect their saliva samples immediately upon awakening (0 min) and then 30 and 60 min after awakening, on a working day, with a minimum volume of 1.5 ml of saliva at each time point and to note each collection time on the marking area of the collection tube (Simport Inc., QC, Canada). Saliva was collected without external stimulation and only with muscle movement and expectoration into a collection tube [21,24]. In order to reduce the situational factors that influence the day-to-day stability of the CAR and cortisol levels during the post-awakening period [25], participants were asked not to alter their routine sleep-wake cycle and not to drink alcohol the night before sample collection. Participants were also asked to refrain from eating, drinking, smoking, or brushing their teeth during the entire sample collection period. Steroid

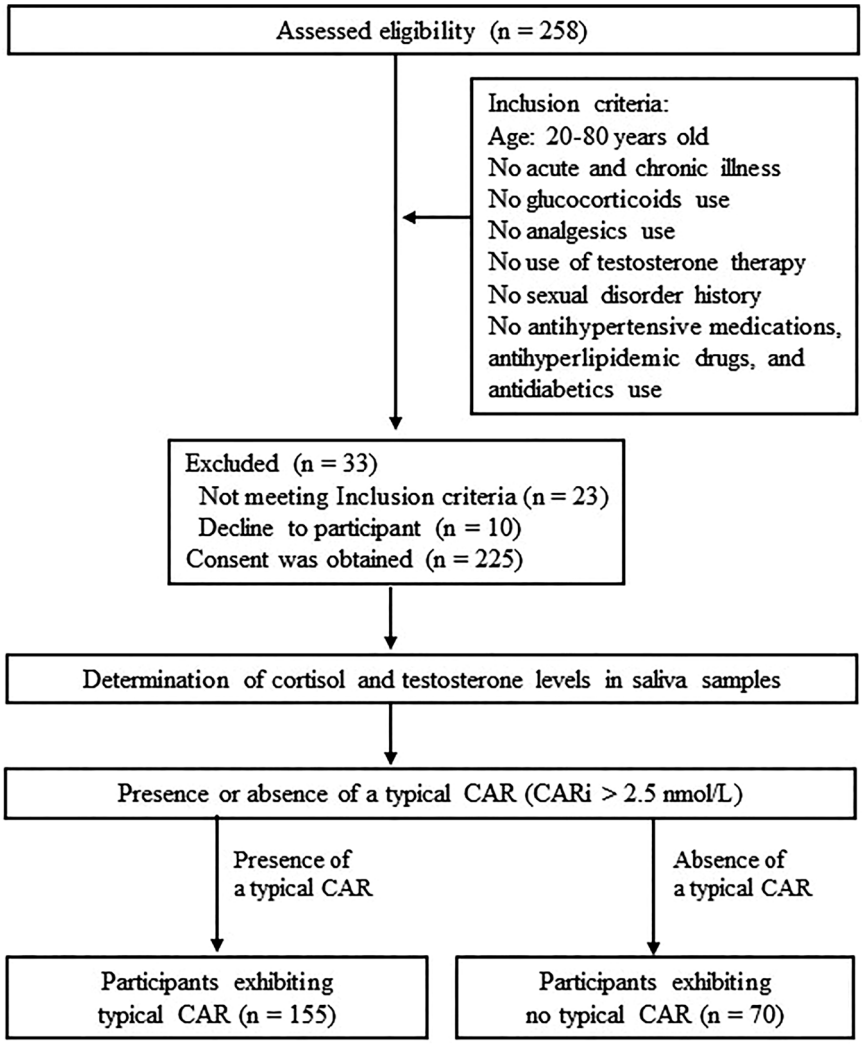

Figure 1. Flow diagram of the study population.

concentrations in saliva are stable after storage at −20 or −80 °C for up to 1 year, and the freezing and thawing of samples up to four times before analysis does not affect the measured concentrations [26]. The collected saliva samples were centrifuged (10,000 *g*, 15 min, 4 °C) to remove debris, and then supernatants were collected and stored at −70 °C until the assay was performed. The participants woke up at 05:59 *h* ± 01:03 h (range 03:35–08:10 h). According to Korean astronomy and space informatics, the average sunrise in Seoul occurred at 06:18–06:26 h during the saliva sample collection period.

2.3. Steroid assay

Saliva samples were analysed in the Hormone Research Center of the Chonnam National University. Steroid levels in the saliva samples were determined by radioimmunoassay using a liquid phase and double antibody methods, as previously described [20,21,24]. The reference standards for cortisol and testosterone were obtained from Sigma-Aldrich (MO, USA). Iodine-125-labelled cortisol and testosterone were prepared by modifying a previously described protocol for steroid radioiodination [27]. Cortisol and testosterone antiserum were purchased from Aviva Systems Biology (San Diego, CA, USA) and Bio-Rad Laboratories (Hercules, CA, USA), respectively. As described by the suppliers, cortisol antiserum cross-reacted with progesterone, deoxycortisol, estrone, and testosterone with cross-reaction levels of 9%, <2.0%, < 0.01%, and < 0.1%, respectively. Testosterone antiserum cross-reacted with 11β-hydroxy testosterone, 17α-methyltestosterone, 5α-dihydrotestosterone, oestradiol, and progesterone, with cross-reaction levels of 3.3%, <0.1%, 0.8%, < 0.1%, and < 0.1% respectively.

Standards, quality control materials, and samples were assayed in duplicate. The inter-assay coefficients of variation (CVs) assessed using quality controls with mean cortisol concentrations of 3.6 and 10.9 nmol/L were 7.4% and 8.5%, respectively ($n = 26$), and those with mean testosterone concentrations of 34.7 and 173.3 pmol/L were 8.9% and 7.6%, respectively ($n = 22$). The analytical sensitivity for cortisol and testosterone was 0.4 nmol/L and 0.3 pmol/L, respectively.

2.4. Classification of participants

In healthy individuals, the CAR emerges within the first hour after the morning awakening from nocturnal sleep [16]; however, it is not observed after waking in the night or after a nap in the early evening [28,29]. Typical CAR is defined as an increase in cortisol level to at least 2.5 nmol/L above an individual's baseline in healthy individuals, that is, a net increase of cortisol levels (CARi): cortisol levels at 30 min post-awakening − cortisol levels immediately upon awakening) ≥ 2.5 nmol/L [30]. A delay of the first saliva sample more than 15 min after awakening is known to be a significant contributing factor to the absence of the CAR [31], leading to flattened or negative CAR in healthy individuals [32]. Meanwhile, studies performed on utilizing electric devices for monitoring waking and sampling times found that among healthy individuals who followed the recommended saliva collection protocols, a percent range from 14.7% to 19.7% had either no or a negligible CAR [33,34]; however, it is still uncertain whether there are authentic cases of individuals who do not exhibit typical CAR.

No information is available regarding the minimal increase in testosterone levels after awakening from nocturnal sleep in men, and the use of electronic devices, such as polysomnography and actigraphy, for the objective recording of waking time was not possible in the present study. For these reasons, the presence or absence of typical CAR was employed as an objective criterion (CARi ≥ 2.5 nmol/L) to ensure saliva samples were collected at the designated time after the awakening period. Typical CAR was observed in 155 out of the 225 participants in the present study. The participants were categorized into the subgroup of participants exhibiting typical CAR. It can be deduced that a set of salivary samples was collected during the post-awakening period, adhering to the provided protocols, in participants exhibiting typical CAR. Meanwhile, there was no typical CAR in 70 participants. The individuals were assigned to the subgroup of participants exhibiting no typical CAR, and they were suspected of conducting inaccurate sampling or genuine CAR non-responders (Figure 1). Participants woke up at varying times (range: 03:35 h–08:10 h); however, there was no difference in wake-up time between participants exhibiting typical CAR (05:56 *h* ± 58.2 min) and participants exhibiting no typical CAR (06:05 *h* ± 73.2 min) ($p > 0.05$ by Mann Whitney test), indicating the time of wake-up was not associated with the presence or absence of typical CAR in the present study.

2.5. Statistical analyses

To evaluate the secretion of testosterone and cortisol during the post-awakening period, the area under the curve with respect to ground ($Tauc_G$ and $CARauc_G$,

respectively) and the area under the curve increase with respect to ground ($Tauc_I$ and $CARauc_I$, respectively) were calculated using GraphPad Prism (GraphPad Software, CA, USA). Nonparametric tests were performed if the data were not normally distributed even after log transformation. Log-transformed data were used in the statistical analyses. Untransformed means are presented in Figures 2 and 3 for interpretational clarity. The Student's *t*-test or Mann–Whitney's U-test was applied according to the data distribution to evaluate the differences between two groups. Differences between three groups were determined using a parametric or nonparametric one-way analysis of variance (ANOVA) test.

Additionally, differences in the levels and patterns of cortisol and testosterone secretion during the post-awakening period were analysed using two-way ANOVA. The chi-squared test was used to analyse differences in categorical demographic data. Correlations between variables were analysed using Spearman's rho correlation test. The *post hoc* power calculation was performed using the G*Power 3.1.3 statistical program to determine whether the sample size of the present study was sufficiently large to attain statistical power in analysis. NCSS 11 statistical software (NCSS, Kaysville, UT, USA) was used for data analysis. The results were expressed as the mean ± standard error of the mean (SEM); a p-value < 0.05 was considered statistically significant.

3. Results

3.1. Demographic characteristics

The 225 participants in the present study were composed of 45 participants aged 20–39, 141 participants aged 40–59, and 39 participants aged ≥ 60 years. Table 1 shows the difference in demographic characteristics between the two subgroups of participants. Demographic variables (age and BMI) and AMS scores were comparable between the two subgroups ($p > 0.05$ by Student's t-test). The relative frequency distributions of age and the total AMS score did not differ between the two subgroups ($X^2 < 1.37$, $p > 0.05$ by Chi-squared test).

3.2. Differences in post-awakening period testosterone and cortisol levels between the two subgroups

Figure 2 shows the differences in cortisol and testosterone levels during the post-awakening period between the two subgroups. Two-way ANOVA revealed that testosterone levels were significantly higher in

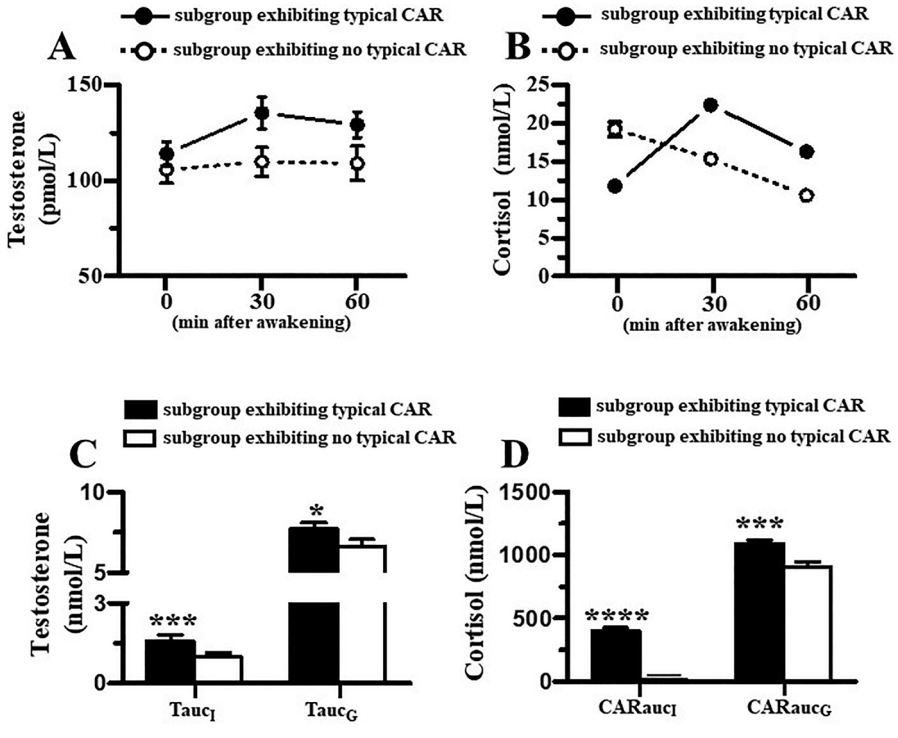

Figure 2. Salivary testosterone and cortisol levels during the post-awakening period in each subgroup. Salivary testosterone and cortisol levels during the post-awakening period in each subgroup are presented in Figure 2A and 2B, respectively. The levels of $Tauc_I$ and $Tauc_G$ are presented in Figure 2C, and the levels of $CARauc_I$ and $CARauc_G$ are presented in Figure 2D. Each data point represents the mean ± standard error of the mean. Different lowercase letters on the bar indicate a significant difference at $p < 0.05$..

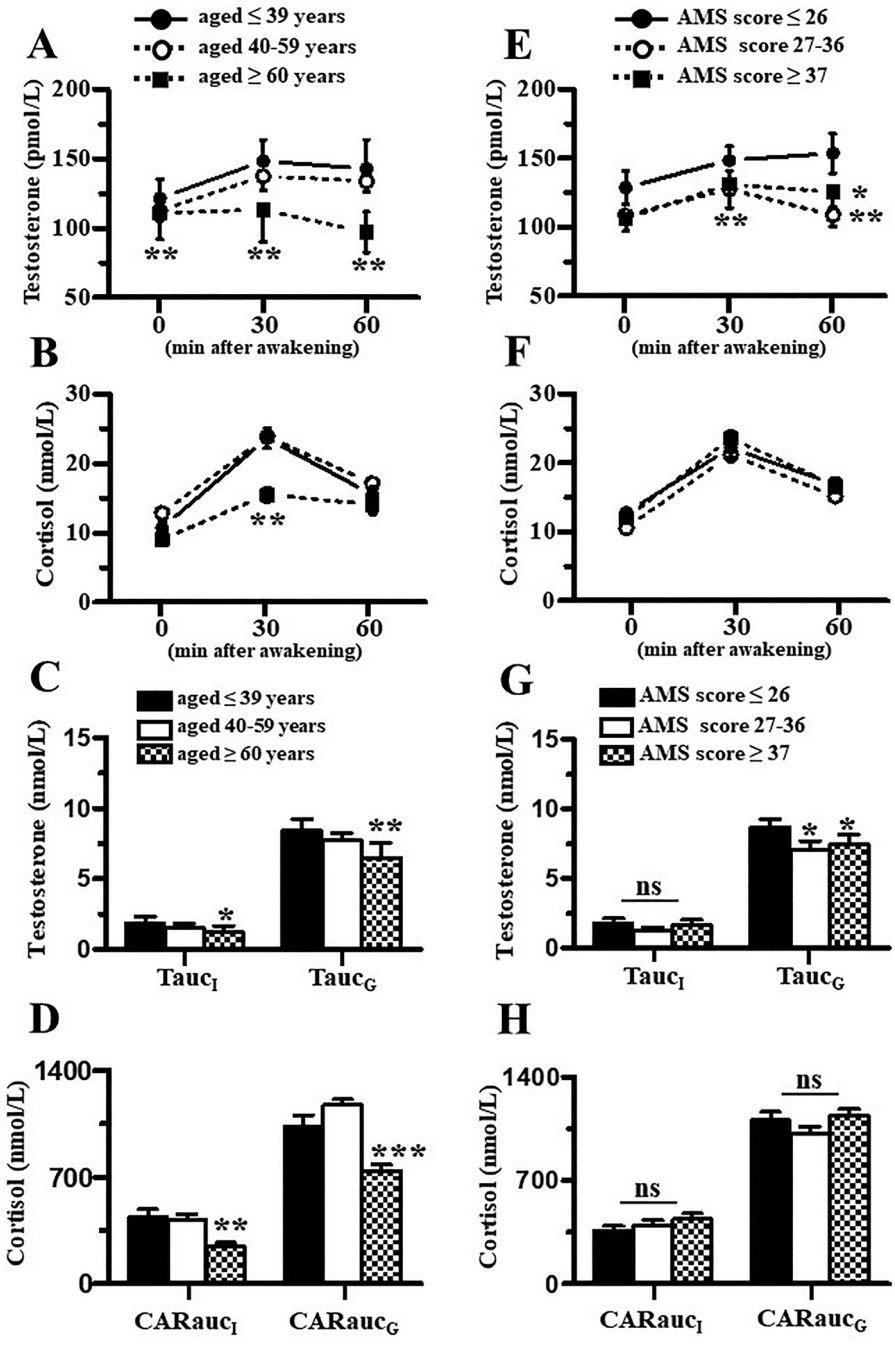

Figure 3. Differences in the salivary testosterone and cortisol levels during the post-awakening period between subgroups classified by age or AMS scores. The differences in salivary testosterone and cortisol levels after the awakening period and the levels of $Tauc_I$, $Tauc_G$, $CARauc_I$, and $CARauc_G$ between the three subgroups, classified by age, are presented in Figure 3A, 3B, 3C, and 3D, respectively, and those classified by total AMS scores are presented in Figure 3E, 3F, 3G, and 3H, respectively. Each data point represents the mean ± standard error of the mean. The asterisks *, **, and *** in Figure 3A, 3B, 3C, and 3D represent a significant difference at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, between subgroups aged ≤ 39 years and ≥ 60 years. The asterisks * and ** in Figure 3E and 3G represent a significant difference at $p < 0.05$ and $p < 0.01$, respectively, between subgroups with total AMS scores between ≤ 26 and ≥ 37.

participants exhibiting typical CAR than in those exhibiting no typical CAR (group effect: $F_{1,669} = 6.89.26$, $p > 0.05$); however, the patterns of testosterone secretion were similar between the two groups (grouped-by-time interaction: $F_{2,669} = 0.55$, $p > 0.05$) (Figure 2(A)). Testosterone levels at 30 and 60 min after awakening (AT-30 and AT-60 levels, respectively) were significantly higher than those immediately upon awakening (AT-0) ($t > 2.24$, $df = 154$, $p < 0.01$ by paired t-test) in participants exhibiting typical CAR; however, AT-0, AT-30,

Table 1. Demographic characteristics of the participants.

	Participants exhibiting typical CAR ($n=155$)	Participants exhibiting no typical CAR ($n=70$)	p-values
Age (years)	49.7 ± 12.6 (22–80)*[1]	49.1 ± 13.2 (24–80)	0.75
	20–30s (18.7%)	20–30s (22.9%)	0.51
	40–50s (63.9%)	40–50s (60.0%)	
	≥ 60s (17.4%)	≥ 60s (17.1%)	
BMI (kg/m^2)	25.2 ± 2.8 (18.1–32.1)	25.2 ± 2.7 (20.3–31.4)	0.95
Somatic AMS score*[2]	14.1 ± 5.6 (7–32)	15.3 ± 5.1 (7–27)	0.31
Psychological AMS score*[3]	9.4 ± 4.4 (5–25)	10.0 ± 4.0 (5–25)	0.24
Sexual AMS score*[4]	10.5 ± 4.3 (5–24)	10.1 ± 3.7 (5–18)	0.58
Total AMS score*[5]	33.9 ± 12.4 (17–81)	35.4 ± 11.2 (17–66)	0.41
Total AMS scores 17–26	31.6%	27.1%	0.50
Total AMS scores 27–36	31.6%	28.6%	
Total AMS scores ≥ 37	36.8%	44.3%	

*[1]represents mean ± standard deviation (range), *[2]somatic AMS score represents the sum of the seven individual AMS item (item numbers 1–5, 9, and 10) scores, *[3]psychological AMS score represents the sum of the five individual AMS item (item numbers 6–8, 12, and 13), *[4]sexual AMS score represents the sum of the five individual AMS item (item numbers 12 and 14–17), and *[5]total AMS score represents the sum of all AMS item (item numbers 1–17) scores.

and AT-60 levels were not different from one another in participants exhibiting no typical CAR ($F_{2,138}$ = 0.33, $p>0.05$ by repeated measures one-way ANOVA) (Figure 2(A)). The within-group CVs for three testosterone measures were 28.60 ± 20.68% and 32.30 ± 19.49% in participants exhibiting typical CAR and those no typical CAR, respectively.

The levels and patterns of cortisol secretion during the post-awakening period were significantly different between the two subgroups (group effect: $F_{2,669}$ = 9.76, $p<0.01$; grouped-by-time interaction: $F_{4,666}$ = 65.79, $p<0.0001$) (Figure 2(B)). Cortisol levels at 30 and 60 min after awakening (AC-30 and AC-60 levels, respectively) were significantly higher than those immediately upon awakening (AC-0) in participants exhibiting typical CAR (all $p<0.0001$ by paired t-test), but AC-0 levels were higher than AC-30 and AC-60 levels in participants exhibiting no typical CAR (all $p<0.005$ by paired t-test) (Figure 2(B)). The within-group CVs for three cortisol measures were 39.73 ± 19.42% and 32.72 ± 18.95% in participants exhibiting typical CAR and those no typical CAR, respectively.

Meanwhile, the levels of $Tauc_I$ and $Tauc_G$ were significantly higher in participants exhibiting typical CAR than in those exhibiting no typical CAR ($t>2.0$, $df=223$, $p<0.05$) (Figure 2(C)). The $CARauc_I$ and $CARauc_G$ levels in participants exhibiting typical CAR were higher than in those exhibiting no typical CAR ($t>3.7$, $df=223$, $p<0.001$) (Figure 2(D)).

3.3. Correlation of steroid levels with AMS scores and demographic variables

Table 2 shows the correlations of steroid levels with AMS scores and demographic variables (age and BMI) in the participants exhibiting typical CAR and those exhibiting no typical CAR. Age showed a significant positive correlation with sexual AMS subscores ($p<0.05$) but not with somatic, psychological, or total AMS scores ($p>0.05$) in both two subgroups (Table 2(A,B)). Age showed a significant negative correlation with the levels of the indices of testosterone secretion (AT-30, AT-60, and $Tauc_G$) and those of cortisol secretion (AC-30, CARi, $CARauc_I$, and $CARauc_G$) in participants exhibiting typical CAR ($p<0.05$) but not in those exhibiting no typical CAR ($p>0.05$) (Table 2(B,C)). Meanwhile, BMI did not have an association with age or indices of testosterone or cortisol secretion in both two subgroups ($p>0.05$) (Table 2(A,B)).

The levels of AT-30, AT-60, $Tauc_I$, and $Tauc_G$ had significant negative correlations with AMS sexual subscores in participants exhibiting typical CAR ($p<0.01$) but not in those exhibiting no typical CAR ($p>0.05$) (Table 2(A,B)). The levels of AT-30 also had significant negative correlations with AMS somatic and psychological subscores and AMS total scores, and the levels of $Tauc_G$ also had significant negative correlations with AMS somatic subscores and AMS total scores in participants exhibiting typical CAR (Table 2(A)). However, the indices of testosterone secretion showed no correlation with AMS subscores and total scores in participants exhibiting no typical CAR (Table 2(B)). No correlation between the indices of cortisol secretion and AMS scores was observed in both two subgroups ($p>0.05$) (Table 2(A,B)). Moreover, there were no significant correlations between the indices of cortisol and those of testosterone secretions in both two subgroups ($p>0.05$) (Table 2(A,B)).

3.4. Age- and AMS score-related differences in testosterone and cortisol levels in participants exhibiting typical CAR

In order to examine age-related differences in testosterone and cortisol levels, the 155 individuals who

Table 2. Correlation of steroid levels with AMS scores and demographic variables in the examined subgroups.

				AMS subscore			
	Age	BMI	AT-30	Somatic	Psycho.	Sexual	Total AMS score
A. Subgroup exhibiting typical CAR ($n=155$)							
Age				−0.06	−0.06	**0.42**[d]	0.10
BMI	−0.13			0.04	−0.04	0.04	0.01
AT-0	−0.15	0.01		−0.11	−0.09	−0.11	−0.13
AT-30	**−0.21**[b]	−0.03		**−0.20**[a]	**−0.17**[a]	**−0.31**[d]	**−0.27**[b]
AT-60	**−0.16**[a]	0.02		−0.10	−0.09	**−0.21**[b]	−0.15
$Tauc_I$	−0.08	−0.04	**0.49**[c]	−0.06	−0.08	**−0.23**[b]	−0.15
$Tauc_G$	**−0.20**[a]	−0.04	**0.93**[d]	**−0.17**[a]	−0.14	**−0.26**[c]	**−0.23**[b]
AC-30	**−0.30**[c]	0.02	0.02	0.14	0.10	−0.12	0.06
CARi	**−0.24**[b]	0.07	−0.04	0.16	0.00	−0.12	0.04
$CARauc_I$	**−0.20**[a]	0.08	−0.06	**0.18**[a]	0.01	−0.0	0.07
$CARauc_G$	**−0.23**[b]	−0.01	−0.02	0.10	0.10	−0.09	0.05
B. Subgroup exhibiting no typical CAR ($n=70$)							
Age				0.05	0.03	**0.38**[b]	0.15
BMI	−0.06			0.06[a]	0.02	0.06	0.05
AT-0	0.09	−0.05		−0.14	−0.11	−0.02	−0.10
AT-30	−0.07	−0.01		−0.17	−0.11	−0.11	−0.16
AT-60	−0.15	0.10		**−0.27**[a]	−0.19	−0.11	−0.19
$Tauc_I$	**−0.28**[a]	0.07	**0.35**[b]	−0.16	−0.16	−0.26	−0.23
$Tauc_G$	−0.05	0.02	**0.94**[d]	−0.23	−0.19	−0.11	−0.19
AC-30	−0.02	0.00	0.04	−0.01	−0.01	−0.08	−0.01
CARi	0.04	0.09	0.14	0.00	−0.07	−0.03	−0.05
$CARauc_I$	0.11	0.00	0.00	−0.11	−0.05	−0.00	−0.08
$CARauc_G$	−0.01	−0.03	0.00	−0.01	−0.01	−0.07	−0.01

Note: The bolded text indicates significant correlations by Spearman's correlation analysis.
a,b,c, and d represent $p<0.05$, $p<0.01$, $p<0.001$, and $p<0.0001$, respectively.
The post hoc power calculations indicated that the statistical power (1-β) of a sample size of CAR responders ($n=155$) varied ranging from 0.64 to 0.85 for the correlation between indices of testosterone secretion (AT-30, AT-60, and $Tauc_G$) and age and ranging from 0.69 to 0.99 for the correlation between indices of testosterone secretion (AT-30, AT-60, and $Tauc_G$) and AMS subscores.

showed typical CAR were divided into three groups based on their age (aged ≤ 39 years, $n=29$; aged 40–59 years, $n=99$, or aged ≥ 60 years, $n=27$) considering the age-related decline of testosterone and prevalence of symptoms suggestive of LOH [35,36]. Figure 3(A,B,C), and 3D show the differences in cortisol and testosterone secretion levels and patterns between classified three subgroups classified by age (aged ≤ 39, 40–59, and ≥ 60 years). Two-way ANOVA revealed a significant effect of group on testosterone ($F_{2,456}$ = 3.39, $p<0.05$) and cortisol levels ($F_{2,456}$ = 16.81, $p<0.001$) (Figure 3(A,B)). The patterns of testosterone secretion were similar ($F_{4,456}$ = 0.50, $p>0.05$), but those of cortisol secretion were significantly different among the subgroups ($F_{4,456}$ = 3.20, $p<0.05$) (Figure 3(A,B)). The Subgroup aged ≥ 60 years had significantly reduced levels of AT-0, AT-30, AT-60, $Tauc_I$, $Tauc_G$, AC-30, $CARauc_I$, and $CARauc_G$ compared with the Subgroup aged ≤ 39 years ($p<0.01$, one-way ANOVA followed by post-hoc test) (Figure 3(A–D)).

In order to examine AMS score-related differences in testosterone and cortisol levels, the 155 individuals who showed typical CAR were divided into three groups based on their total AMS score: no/little (total AMS score ≤ 26, $n=49$), mild (total AMS score 27–36, $n=49$), or moderate to severe (AMS total score ≥ 37, $n=57$) [22]. Figure 3(E–H) shows the differences in cortisol and testosterone secretion levels and patterns between subgroups classified by total AMS scores (scores ≤ 26, 27–36, and ≥ 37). Two-way ANOVA revealed a significant effect of total AMS scores on testosterone levels ($F_{2,456}$ = 4.47, $p<0.05$) but not on cortisol levels ($F_{2,456}$ = 2.17, $p>0.05$) (Figure 3(E,F)). The subgroup with a total AMS score of 27–36 and ≥ 37 had significantly reduced levels of AT-30, AT-60, and $Tauc_G$ compared to the subgroup with a total AMS score ≤ 26 ($p<0.05$, one-way ANOVA followed by *post hoc* test) (Figure 3(E,G)). Meanwhile, the patterns of testosterone and cortisol secretions were not different among the subgroups classified by the total AMS score ($F_{4,\ 456}$ < 0.35, $p>0.05$ by two-way ANOVA) (Figure 3(E,F)), and the levels of $CARauc_I$ and $CARauc_G$ were comparable among the subgroups ($F_{2,\ 15}$ < 1.55, $p>0.05$ by one-way ANOVA) (Figure 3(H)).

3.5. Correlation of the individual AMS item score with testosterone levels and age in participants exhibiting typical CAR

Table 3 shows the correlations of each AMS item's score with age or testosterone levels in participants exhibiting typical CAR. Age positively correlated with three sexual items (AMS items #15–17; $p<0.05$). Both AT-30 and $Tauc_G$ levels showed significant negative correlations with all five sexual items (AMS items #12

Table 3. Correlation between the score of individual AMS items and age and testosterone levels in CAR responders.

AMS Item #	Age	AT-30	$Tauc_G$	$Tauc_I$
1	−0.05	**−0.16**[a]	−0.14	−0.03
2	−0.08	−0.11	−0.11	−0.06
3	−0.15	−0.04	−0.01	0.04
4	0.08	0.02	0.01	−0.06
5	−0.09	−0.15	−0.15	0.02
6	−0.06	**−0.17**[a]	−0.16	−0.06
7	−0.04	−0.10	−0.10	−0.05
8	−0.13	**−0.21**[a]	−0.16	−0.13
9	−0.16	**−0.23**[b]	**−0.20**[a]	−0.06
10	0.08	**−0.36**[d]	**−0.31**[c]	−0.13
11	−0.07	**−0.18**[a]	**−0.16**[a]	−0.08
12	0.13	**−0.16**[a]	**−0.23**[b]	−0.12
13	−0.01	−0.09	−0.07	−0.04
14	0.08	**−0.20**[a]	**−0.16**[a]	−0.14
15	**0.51**[d]	**−0.28**[c]	**−0.23**[b]	**−0.20**[a]
16	**0.49**[d]	**−0.30**[c]	**−0.25**[b]	**−0.22**[b]
17	**0.36**[d]	**−0.26**[c]	**−0.21**[b]	**−0.23**[b]

Note: The bolded text indicates significant correlations by Spearman's correlation analysis.

a,b,c, and d represent $p<0.05$, $p<0.01$, $p<0.001$, and $p<0.0001$, respectively.

AMS question #1: Decline in your feeling of general well-being, #2: Joint pain and muscular ache, #3: Excessive sweating, #4: Sleep problems, #5: Increased need for sleep, often feeling tired, #6: Irritability, #7: Nervousness, #8: Anxiety, #9: Physical exhaustion/lacking vitality, #10: Decrease in muscular strength, #11: Depressive mood, #12: Feeling that you have passed your peak, #13: Feeling burnt out, having hit rock-bottom, #14: Decrease in beard growth, #15: Decrease in ability/frequency to perform sexually, #16: Decrease in the number of morning erections, #17: Decrease in sexual desire/libido.

and #14–17) ($p<0.05$), and $Tauc_I$ levels showed significant negative correlations with three sexual items (AMS items #15–17; $p<0.05$). Additionally, AT-30 and $Tauc_G$ levels had a significant negative correlation with somatic (AMS items #1, 9, and 10) and psychological (AMS items #6, 8, and 11) items ($p<0.05$) (Table 3).

The levels of AT-30, $Tauc_G$, and $Tauc_I$ in participants exhibiting no CAR were not correlated with sexual items; however, the levels of $Tauc_G$ showed a significant negative correlation with two somatic items (AMS items #1 and 9) and one psychological item (AMS item #11) ($p<0.05$) in participants exhibiting no CAR (data not shown).

4. Discussion

This study hypothesized that testosterone secretion responds to morning awakening in men, similar to increased cortisol levels after awakening. Consistent with this hypothesis, we observed a concomitant increase in cortisol and testosterone levels during the post-awakening period in the subgroup of participants exhibiting typical CAR. This result is in line with a previous study that showed sharp increases in 17β-estradiol and progesterone levels within the first hour after awakening in women with regular menstrual cycles and typical CAR [20]. Some studies have reported similar observations, showing a simultaneous increase in testosterone and cortisol levels after awakening [37] and a similarity in acrophase values and patterns of testosterone and cortisol circadian rhythms [38]. We additionally observed that there was no discernible increase in testosterone levels after the awakening period in the subgroup of participants exhibiting no typical CAR, which was similar to the findings of a previous study that showed that after the awakening period, 81.7% of cases had a decrease in testosterone levels and the other cases had a rise in testosterone levels in a study group consisting of 108 men with a group mean CARi of 1.7 nmol/L (range from −5.6 to 11.6 nmol/L and negative CARi in 37.5% of cases) [39]. The results of the present and previous studies suggest that morning awakening from nocturnal sleep may act as a stimulant for the HPA and HPG axes, and the timely collection of a set of saliva samples after the post-awakening period is needed to assess the HPG axis response to morning awakening in adult men.

Animal studies showed that the SCN transmits information about the time of the day to neuroendocrine neurons *via* neurotransmitters, such as arginine vasopressin (AVP) and vasoactive intestinal polypeptide (VIP) [40]. The rhythmic release of AVP from SCN terminals controls the rhythmic secretion of corticotropin-releasing hormone and adrenocorticotropic hormone (ACTH) [40–42]. The circadian signal generated in the SCN is transmitted to the adrenal gland *via* sympathetic outflow, which increases adrenal responsivity to ACTH [43]. Based on the similar organization of SCN-related neural pathways between rodents and humans [40], it has been suggested that the SCN-mediated activation of the neuroendocrine pathway, which stimulates ACTH secretion and the sympathetic nervous system that modulates adrenal sensitivity for ACTH, is involved in the initiation of the CAR [18,44]. Meanwhile, the majority of gonadotropin-releasing hormone neurons contacted by SCN efferent neurons are located in the preoptic area in rodents [45–47], and the SCN coordinates the timing of the gonadotropin-releasing and luteinizing hormone secretion *via* a rhythmic release of VIP and AVP [48]. Although a direct neuronal connection between the SCN neurons and testis has not been reported, the existence of a multisynaptic neural pathway between the hypothalamic paraventricular nucleus and testis indicates that the autonomic nervous system is involved in the circadian activity of the HPG axis [49]. Based on these similarities between SCN-related neural and neuroendocrine pathways in the HPA and HPG axes, it is conceivable that the increase in testosterone levels after awakening is likely to be a phenomenon analogous to the CAR in the HPA axis.

Elevated hormone levels produced in the HPA axis are known to have suppressive effects on the HPG axis function [50]. However, the present study observed a concomitant increase in cortisol and testosterone levels and no significant association between cortisol and testosterone levels during the post-awakening period. Previous studies have shown that the HPG axis exhibits a differential response to short-term and long-term increased cortisol levels. For example, oral administration of dexamethasone (1 mg prior to sleep) abolishes the circadian rhythmicity of cortisol but not that of testosterone and gonadotropin in healthy young men [51]. Testosterone and gonadotrophin levels during the daytime and the levels of nocturnal testosterone rise are not affected by the short-term oral treatment of cortisol (60 mg initial dose, then 30 mg every two hours for six hours). However, the levels of nocturnal but not daytime testosterone are increased, and gonadotropin levels are reduced by long-term oral treatment of cortisol (60 mg initial dose, then 30 mg every two hours for 24 h) in normal adult men [52]. It has been reported that, due to severe temporal stress, baseline testosterone but not gonadotropin levels in adult male patients after major surgery were significantly reduced and recovered to baseline testosterone levels in healthy controls one week after surgery [53]. Therefore, it is conceivable that testosterone levels might be reduced by chronically elevated cortisol levels and severe temporal stress but not by a short-term increase in cortisol levels. Meanwhile, the relationship between the concentrations of steroids provides insight into their origin and interdependence [54]. Because testosterone is not a direct or indirect precursor of cortisol synthesis and *vice versa*, the lack of correlation between cortisol and testosterone indices in the subgroup of participants exhibiting typical CAR indicated that cortisol and testosterone were not synthesized by the same origin and did not share the same precursor.

We also hypothesized that the levels of testosterone secretion during the post-awakening period would decrease with aging in men. In agreement with this hypothesis, age showed a significant negative correlation with AT-0, AT-30, AT-60, and $Tauc_G$ levels in the subgroup of participants exhibiting typical CAR. These results corroborate an earlier study that showed a circadian testosterone acrophase closer to the wake-up time (around 07:30–08:00 h) and a less pronounced circadian testosterone rhythm in older men (55–64 years) than in younger men (23–33 years) due to the reduced testosterone levels around the circadian acrophase [13]. We additionally observed a significant negative correlation between age and cortisol levels after awakening in the present study. These results are similar to a previous study that demonstrated a significant negative correlation between age and CARi and CARauc levels [55]. However, our results are inconsistent with the findings of two other studies that showed no significant effect of age on CAR [16,30]. The age-related concomitant decrease in testosterone and cortisol levels during the post-awakening period suggests the potential effect of age-related functional changes in the SCN because of the decline in the number of VIP-producing neurons after 40 years of age [56]. VIP neurons are among the photic recipient neurons of the SCN [57], which coordinate the daily neuronal activity rhythm of gonadotropin-releasing hormone-producing neurons [58], corticotropin-releasing hormone-producing neurons [59], and pre-autonomic neurons in the hypothalamus [60].

Because of the pleiotropic actions of testosterone [1], it is reasonable to postulate that a cluster of signs and symptoms might accompany low testosterone levels. The European Male Aging Study reported that nine out of 32 suggested candidate symptoms significantly correlated with either serum total or free testosterone in men aged 40–79 [61]. Morning salivary testosterone levels were reported to be correlated with five ADAM and six AMS items in men aged 20–89 years [62]. However, many previous studies have reported no significant correlation between serum testosterone levels and the ADAM and AMS questionnaire scores in aging men, even though they showed an age-related decline in testosterone levels [63–65]. We observed in the present study that AT-30 and $Tauc_G$ levels had a significant negative correlation with sexual subscores and total AMS scores in the subgroup of participants exhibiting typical CAR but not in the subgroup of participants exhibiting no typical CAR. Additionally, the levels of AT-30 were correlated with three somatic, three psychological, and all five sexual items in the subgroup of participants exhibiting typical CAR. The findings of the present study suggest that determining testosterone levels in the set of saliva samples with typical CAR would likely be another method to evaluate the HPG axis function in men suspected of LOH.

Among the 17 AMS items, the scores of three sexual items (ability/frequency to perform sexually, number of morning erections, and sexual desire/libido) were concomitantly correlated with age and testosterone levels (AT-30 and $Tauc_G$) in the subgroup of participants exhibiting typical CAR. Our results agree with previous findings, which showed that three sexual symptoms (poor morning erection, low sexual desire, and erectile dysfunction) have a syndromic relationship with decreased testosterone levels in men aged

40–79 years [61]. Moreover, this study found that each of the three sexual symptoms had a higher absolute correlation coefficient with age than the AT-30 or $Tauc_G$ levels. The results indicated that besides testosterone levels, other age-related factors might also be involved in the decline in sexual function with advancing age in men.

Our study has some limitations that should be addressed. First, serum testosterone levels during the post-awakening period were not simultaneously assessed. Second, the present study did not objectively confirm the awakening and sampling times with electric devices. Therefore, the present findings should be considered preliminary and need to be confirmed in the sleep laboratory setting. Third, besides age, circulating total and free testosterone levels in men are known to be reduced by health-related factors, such as diabetes, hypertension, and chronic medication use, and psychosocial factors, such as unemployment and the loss of a spouse [66]. However, using the study's data set, this study could not evaluate the confounding roles of the health-related and psychosocial factors in testosterone levels during the post-awakening period. Fourth, there is a general belief that testosterone measurements based on gas chromatography-mass spectrometry or liquid chromatography-tandem mass spectrometry are more accurate and precise than immunoassay.

This study's findings are considered a reference for future mass spectrometry-based analyses.

5. Conclusion

The CAR is thought to be a consequence of SCN-mediated activation of the HPA axis [18,19,46]. Our research found that the HPG axis, like the HPA axis, may respond to morning awakening from nocturnal sleep, manifesting in testosterone levels during the post-awakening period. The levels of testosterone secretion during the post-awakening period were negatively correlated with age, total AMS scores, sexual subscores, and 11 AMS item scores. Of these 11 items, three sexual items (ability to perform sexually, number of morning erections, and sexual desire/libido) were also correlated with age. Despite the aforementioned limitations, our findings suggest that determining testosterone levels from the set of saliva samples in individuals with a typical CAR may provide a new approach to determining the HPG axis function in men.

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Author contributions

Jai-Young Park: conceptualization, methodology, and writing-original draft. Jeong-Ho Seok: funding acquisition. Kang-Su Cho: conceptualization, methodology, formal analysis. Jin-Sun Kim: formal analysis, data curation. Dong-Hyun Kang: formal analysis, data curation. Sang-Hwan Do: funding acquisition. Hyo-Seok Na: investigation, project administration. Soo-Ah Jang: project administration, resources. Ryun S. Ahn: methodology, writing-original draft.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

All raw data have been deposited in Mendeley Data, DOI: 10.17632/x5sgmd3tzv.1. Data set and any detailed information can be obtained from the corresponding author.

References

[1] Bagatell CJ, Bremner WJ. Androgens in men–uses and abuses. N Engl J Med. 1996;334(11):707–714. doi: 10.1056/NEJM199603143341107.

[2] Kaufman JM, Vermeulen A. The decline of androgen levels in elderly men and its clinical and therapeutic implications. Endocr Rev. 2005;26(6):833–876. doi: 10.1210/er.2004-0013.

[3] Araujo AB, Esche GR, Kupelian V, et al. Prevalence of symptomatic androgen deficiency in men. J Clin Endocrinol Metab. 2007;92(11):4241–4247. doi: 10.1210/jc.2007-1245.

[4] Harman SM, Metter EJ, Tobin JD, et al. Longitudinal effects of aging on serum total and free testosterone levels in healthy men. Baltimore longitudinal study of aging. J Clin Endocrinol Metab. 2001;86(2):724–731.

[5] Huhtaniemi I. Late-onset hypogonadism: current concepts and controversies of pathogenesis, diagnosis and treatment. Asian J Androl. 2014;16(2):192–202. doi: 10.4103/1008-682X.122336.

[6] Miwa Y, Kaneda T, Yokoyama O. Correlation between the aging males' symptoms scale and sex steroids, gonadotropins, dehydroepiandrosterone sulfate, and growth hormone levels in ambulatory men. J Sex Med. 2006;3(4):723–726. doi: 10.1111/j.1743-6109.2006.00277.x.

[7] T'Sjoen G, Goemaere S, De Meyere M, et al. Perception of males' aging symptoms, health and well-being in

elderly community-dwelling men is not related to circulating androgen levels. Psychoneuroendocrinology. 2004;29(2):201–214. doi: 10.1016/s0306-4530(03)00023-4.
[8] Spetz AC, Palmefors L, Skobe RS, et al. Testosterone correlated to symptoms of partial androgen deficiency in aging men (PADAM) in an elderly Swedish population. Menopause. 2007;14(6):999–1005. doi: 10.1097/gme.0b013e318057786b.
[9] Morley JE, Perry HM, 3rd, Kevorkian RT, et al. Comparison of screening questionnaires for the diagnosis of hypogonadism. Maturitas. 2006;53(4):424–429. doi: 10.1016/j.maturitas.2005.07.004.
[10] Basar MM, Aydin G, Mert HC, et al. Relationship between serum sex steroids and aging male symptoms score and international index of erectile function. Urology. 2005;66(3):597–601. doi: 10.1016/j.urology.2005.03.060.
[11] Chen W, Liu ZY, Wang LH, et al. Are the aging male's symptoms (AMS) scale and the androgen deficiency in the aging male (ADAM) questionnaire suitable for the screening of late-onset hypogonadism in aging Chinese men? Aging Male. 2013;16(3):92–96. doi: 10.3109/13685538.2013.805319.
[12] Cooke RR, McIntosh JE, McIntosh RP. Circadian variation in serum free and non-SHBG-bound testosterone in normal men: measurements, and simulation using a mass action model. Clin Endocrinol (Oxf). 1993;39(2):163–171. doi: 10.1111/j.1365-2265.1993.tb01769.x.
[13] Diver MJ, Imtiaz KE, Ahmad AM, et al. Diurnal rhythms of serum total, free and bioavailable testosterone and of SHBG in middle-aged men compared with those in young men. Clin Endocrinol (Oxf). 2003;58(6):710–717.
[14] Wang C, Nieschlag E, Swerdloff R, et al. Investigation, treatment and monitoring of late-onset hypogonadism in males: ISA, ISSAM, EAU, EAA and ASA recommendations. Eur J Endocrinol. 2008;159(5):507–514. doi: 10.1530/EJE-08-0601.
[15] Debono M, Ghobadi C, Rostami-Hodjegan A, et al. Modified-release hydrocortisone to provide circadian cortisol profiles. J Clin Endocrinol Metab. 2009;94(5):1548–1554. doi: 10.1210/jc.2008-2380.
[16] Pruessner JC, Wolf OT, Hellhammer DH, et al. Free cortisol levels after awakening: a reliable biological marker for the assessment of adrenocortical activity. Life Sci. 1997;61(26):2539–2549. doi: 10.1016/s0024-3205(97)01008-4.
[17] Wilhelm I, Born J, Kudielka BM, et al. Is the cortisol awakening rise a response to awakening? Psychoneuroendocrinology. 2007;32(4):358–366. doi: 10.1016/j.psyneuen.2007.01.008.
[18] Clow A, Hucklebridge F, Stalder T, et al. The cortisol awakening response: more than a measure of HPA axis function. Neurosci Biobehav Rev. 2010;35(1):97–103.
[19] Ishida A, Mutoh T, Ueyama T, et al. Light activates the adrenal gland: timing of gene expression and glucocorticoid release. Cell Metab. 2005;2(5):297–307. doi: 10.1016/j.cmet.2005.09.009.
[20] Ahn RS, Choi JH, Choi BC, et al. Cortisol, estradiol-17β, and progesterone secretion within the first hour after awakening in women with regular menstrual cycles. J Endocrinol. 2011;211(3):285–295. doi: 10.1530/JOE-11-0247.
[21] Kang JY, Park JY, Chun SI, et al. Puberty-related changes in cortisol, dehydroepiandrosterone, and estradiol-17β secretions within the first hour after waking in premenarcheal girls. Neuroendocrinology. 2014;99(3-4):168–177. doi: 10.1159/000363368.
[22] Heinemann LAJ, Zimmermann T, Vermeulen A, et al. A new 'aging males' symptoms' rating scale. Aging Male. 1999;2:105–114.
[23] Wood P. Salivary steroid assays - research or routine? Ann Clin Biochem. 2009;46(Pt 3):183–196. doi: 10.1258/acb.2008.008208.
[24] Jo KB, Lee YJ, Lee IG, et al. Association of pain intensity, pain-related disability, and depression with hypothalamus-pituitary-adrenal axis function in female patients with chronic temporomandibular disorders. Psychoneuroendocrinology. 2016;69:106–115. doi: 10.1016/j.psyneuen.2016.03.017.
[25] Stalder T, Kirschbaum C, Kudielka BM, et al. Assessment of the cortisol awakening response: expert consensus guidelines. Psychoneuroendocrinology. 2016;63:414–432. doi: 10.1016/j.psyneuen.2015.10.010.
[26] Gröschl M, Wagner R, Rauh M, et al. Stability of salivary steroids: the influences of storage, food and dental care. Steroids. 2001;66(10):737–741. doi: 10.1016/s0039-128x(01)00111-8.
[27] Corrie JE, Hunter WM, Macpherson JS. A strategy for radioimmunoassay of plasma progesterone with use of a homologous-site 125I-labeled radioligand. Clin Chem. 1981;27(4):594–599. doi: 10.1093/clinchem/27.4.594.
[28] Dettenborn L, Rosenloecher F, Kirschbaum C. No effects of repeated forced wakings during three consecutive nights on morning cortisol awakening responses (CAR): a preliminary study. Psychoneuroendocrinology. 2007;32(8-10):915–921. doi: 10.1016/j.psyneuen.2007.06.011.
[29] Federenko I, Wüst S, Hellhammer DH, et al. Free cortisol awakening responses are influenced by awakening time. Psychoneuroendocrinology. 2004;29(2):174–184. doi: 10.1016/s0306-4530(03)00021-0.
[30] Wust S, Wolf J, Hellhammer DH, et al. The cortisol awakening response - normal values and confounds. Noise Health. 2000;2(7):79–88.
[31] Okun ML, Krafty RT, Buysse DJ, et al. What constitutes too long of a delay? Determining the cortisol awakening response (CAR) using self-report and PSG-assessed wake time. Psychoneuroendocrinology. 2009;35(3):460–468. doi: 10.1016/j.psyneuen.2009.08.017.
[32] Dockray S, Bhattacharyya MR, Molloy GJ, et al. The cortisol awakening response in relation to objective and subjective measures of waking in the morning. Psychoneuroendocrinology. 2008;33(1):77–82. doi: 10.1016/j.psyneuen.2007.10.001.
[33] Stalder T, Lupien SJ, Kudielka BM, et al. Evaluation and update of the expert consensus guidelines for the assessment of the cortisol awakening response (CAR). Psychoneuroendocrinology. 2022;146:105946. doi: 10.1016/j.psyneuen.2022.105946.
[34] Stalder T, Bäumler D, Miller R, et al. The cortisol awakening response in infants: ontogeny and associations with development-related variables. Psychoneuroendocrinology. 2013;38(4):552–559. doi: 10.1016/j.psyneuen.2012.07.015.
[35] Handelsman DJ, Yeap B, Flicker L, et al. Age-specific population centiles for androgen status in men. Eur J Endocrinol. 2015;173(6):809–817. doi: 10.1530/EJE-15-0380.

[36] Tan RS, Philip PS. Perceptions of and risk factors for andropause. Arch Androl. 1999;43(3):227–233. doi: 10.1080/014850199262544.
[37] Miyatake A, Morimoto Y, Oishi T, et al. Circadian rhythm of serum testosterone and its relation to sleep: comparison with the variation in serum luteinizing hormone, prolactin, and cortisol in normal men. J Clin Endocrinol Metab. 1980;51(6):1365–1371.
[38] Piro C, Fraioli F, Sciarra F, et al. Circadian rhythm of plasma testosterone, cortisol and gonadotropins in normal male subjects. J Steroid Biochem. 1973;4(3):321–329.
[39] Kuzawa CW, Georgiev AV, McDade TW, et al. Is there a testosterone awakening response in humans? Adapt Human Behav Physiol. 2016;2:166–183.
[40] Buijs RM, van Eden CG, Goncharuk VD, et al. The biological clock tunes the organs of the body: timing by hormones and the autonomic nervous system. J Endocrinol. 2003;177(1):17–26. doi: 10.1677/joe.0.1770017.
[41] Kalsbeek A, Perreau-Lenz S, Buijs RM. A network of (autonomic) clock outputs. Chronobiol Int. 2006;23(3):521–535. doi: 10.1080/07420520600651073.
[42] Perreau-Lenz S, Pévet P, Buijs RM, et al. The biological clock: the bodyguard of temporal homeostasis. Chronobiol Int. 2004;21(1):1–25.
[43] Ulrich-Lai YM, Arnhold MM, Engeland WC. Adrenal splanchnic innervation contributes to the diurnal rhythm of plasma corticosterone in rats by modulating adrenal sensitivity to ACTH. Am J Physiol Regul Integr Comp Physiol. 2006;290(4):R1128–1135.
[44] Fries E, Dettenborn L, Kirschbaum C. The cortisol awakening response (CAR): facts and future directions. Int J Psychophysiol. 2009;72(1):67–73. doi: 10.1016/j.ijpsycho.2008.03.014.
[45] de la Iglesia HO, Blaustein JD, Bittman EL. The suprachiasmatic area in the female hamster projects to neurons containing estrogen receptors and GnRH. Neuroreport. 1995;6(13):1715–1722. doi: 10.1097/00001756-199509000-00004.
[46] van der Beek EM, Horvath TL, Wiegant VM, et al. Evidence for a direct neuronal pathway from the suprachiasmatic nucleus to the gonadotropin-releasing hormone system: combined tracing and light and electron microscopic immunocytochemical studies. J Comp Neurol. 1997;384(4):569–579.
[47] Watson RE, Jr, Langub MC, Jr, Engle MG, et al. Estrogen-receptive neurons in the anteroventral periventricular nucleus are synaptic targets of the suprachiasmatic nucleus and peri-suprachiasmatic region. Brain Res. 1995;689(2):254–264. doi: 10.1016/0006-8993(95)00548-5.
[48] Kalsbeek A, Buijs RM. Output pathways of the mammalian suprachiasmatic nucleus: coding circadian time by transmitter selection and specific targeting. Cell Tissue Res. 2002;309(1):109–118. doi: 10.1007/s00441-002-0577-0.
[49] Gerendai I, Banczerowski P, Halász B. Functional significance of the innervation of the gonads. Endocrine. 2005; 20528(3):309–318. doi: 10.1385/ENDO:28:3:309.
[50] Chrousos GP, Gold PW. The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. JAMA. 1992;267(9):1244–1252. doi: 10.1001/jama.1992.03480090092034.
[51] Faiman C, Winter JS. Diurnal cycles in plasma FSH, testosterone and cortisol in men. J Clin Endocrinol Metab. 1971;33(2):186–192.
[52] Doerr P, Pirke KM. Cortisol-induced suppression of plasma testosterone in normal adult males. J Clin Endocrinol Metab. 1976;43(3):622–629.
[53] Carstensen H, Amér B, Amér I, et al. The postoperative decrease of plasma testosterone in man, after major surgery, in relation to plasma FSH and LH. J Steroid Biochem. 1976;4(1):45–55.
[54] Sanchez-Guijo A, Neunzig J, Gerber A, et al. Role of steroid sulfatase in steroid homeostasis and characterization of the sulfated steroid pathway: evidence from steroid sulfatase deficiency. Mol Cell Endocrinol. 2016;437:142–153.
[55] Kudielka BM, Kirschbaum C. Awakening cortisol responses are influenced by health status and awakening time but not by menstrual cycle phase. Psychoneuroendocrinology. 2003;28(1):35–47. doi: 10.1016/s0306-4530(02)00008-2.
[56] Hofman MA, Zhou JN, Swaab DF. Suprachiasmatic nucleus of the human brain: an immunocytochemical and morphometric analysis. Anat Rec. 1996;244(4):552–562.
[57] Lee HS, Billings HJ, Lehman MN. The suprachiasmatic nucleus: a clock of multiple components. J Biol Rhythms. 2003;18(6):435–449. doi: 10.1177/0748730403259106.
[58] Christian CA, Moenter SM. Vasoactive intestinal polypeptide can excite gonadotropin-releasing hormone neurons in a manner dependent on estradiol and gated by time of day. Endocrinology. 2008;149(6):3130–3136. doi: 10.1210/en.2007-1098.
[59] Jones JR, Chaturvedi S, Granados-Fuentes D, et al. Circadian neurons in the paraventricular nucleus entrain and sustain daily rhythms in glucocorticoids. Nat Commun. 2021;12(1):5763. doi: 10.1038/s41467-021-25959-9.
[60] Teclemariam-Mesbah R, Kalsbeek A, Pevet P, et al. Direct vasoactive intestinal polypeptide-containing projection from the suprachiasmatic nucleus to spinal projecting hypothalamic paraventricular neurons. Brain Res. 1997;748(1-2):71–76. doi: 10.1016/s0006-8993(96)01246-2.
[61] Wu FC, Tajar A, Beynon JM, et al. Identification of late-onset hypogonadism in Middle-aged and elderly men. N Engl J Med. 2010;363(2):123–135. doi: 10.1056/NEJMoa0911101.
[62] Morley JE, Hm3Rd P, Patrick P, et al. Validation of salivary testosterone as a screening test for male hypogonadism. Aging Male. 2006;9(3):165–169.
[63] Morales A, Spevack M, Emerson L, et al. Adding to the controversy: pitfalls in the diagnosis of testosterone deficiency syndromes with questionnaires and biochemistry. Aging Male. 2007;10(2):57–65.
[64] Tancredi A, Reginster JY, Luyckx F, et al. No major month to month variation in free testosterone levels in aging males. Minor impact on the biological diagnosis of 'andropause. Psychoneuroendocrinology. 2005;30(7):638–646. doi: 10.1016/j.psyneuen.2005.02.002.
[65] Tsujimura A, Matsumiya K, Miyagawa Y, et al. Comparative study on evaluation methods for serum testosterone level for PADAM diagnosis. Int J Impot Res. 2005;17(3):259–263. doi: 10.1038/sj.ijir.3901300.
[66] Travison TG, Araujo AB, Kupelian V, et al. The relative contributions of aging, health, and lifestyle factors to serum testosterone decline in men. J Clin Endocrinol Metab. 2007;92(2):549–555. doi: 10.1210/jc.2006-1859.