terone levels affect physical, mental, and sexual activities, manifesting as a loss of muscle mass and bone strength, increased body fat, decreased energy, less interest in sex, erectile dysfunction, irritability and depression [2].

In contrast to women, men do not experience a sudden cessation of gonadal function comparable to the menopause, although there is a progressive reduction in hypothalamic–pituitary–gonadal (HPG) function in aging men, hence serum testosterone levels decline through both central (pituitary) and peripheral (testicular) mechanisms [3]. Serum total testosterone gradually declines with advancing age, particularly after the age of 50 [4]. Although serum testosterone levels are generally measured in the morning when they are at a peak, this circadian rhythm may be abolished or blunted in men with advancing age [3,4]. In healthy men, only 1–3% of biologically active steroids circulate freely, with the balance being tightly bound to sex hormone binding globulin (SHBG) or loosely bound to albumin. Free testosterone and the fraction bound loosely to albumin are readily available for entry into tissues. Unlike serum total testosterone, the concentration of SHBG significantly increases with age [4]. Consequently, serum free testosterone level decreases steeply, and this is considered to be more closely associated with the incidence of LOH than is the decline in total serum testosterone [5,6]. Moreover, physical and psycho-social stresses challenge homeostasis, increasing glucocorticoid secretion and decreasing testosterone levels [7].

Most patients who visit LOH clinics in Japan are in their 40s to 50s. They have more responsibilities than other generations, both at work and at home, placing them in a stressful environment. In Teikyo Hospital, many of the patients who came to LOH outpatient services were white-collar workers in their 40s to 50s, and half of them suffered from depression [8]. Japan is facing a rapidly increasing suicide rate in middle-aged men, mostly due to depression that may be associated with LOH. Previous studies of age-related testosterone decline have not focused on the testosterone levels in middle-aged men. We hypothesized that, aside from aging, environmental stressors such as overwork escalate the steep decline of free testosterone levels, leading to the onset of LOH. To investigate the complexity of declining testosterone levels in later life we examined the circadian rhythm of salivary testosterone levels in three age cohorts: 20s–30s, 40s–50s and 60s+. We used salivary testosterone as an alternative measure for evaluating serum free testosterone levels. Salivary testosterone level is a useful, non-invasive and repeatable method of assessing levels of free testosterone because testosterone is not bound to any protein in the saliva [9].

Subjects and methods

Subjects

This study was approved by our institutional review board. Sixty-four healthy, salaried men were included in this study upon written consent: 22 in a 20s–30s cohort (mean age = 30.32; range = 22–39), 32 in a 40s–50s cohort (mean age = 53.53; range = 40–59) and 10 in a 60s+ cohort. They were all white-collar workers in Tokyo. We also included 17 healthy retired men in their 60s–70s. The healthy 60s+ cohort thus consisted of 27 men in total (mean age = 66.25; range = 60–74). We compared the healthy 40s–50s cohort with 20 new LOH patients in their 40s–50s (mean age = 50.42; range = 41–57) whose calculated free testosterone, using the formula devised by the International Society for the Study of the Aging Male (ISSAM), was lower than 72 pg/ml, the generally acceptable lower limit considered to be normal for testosterone substitution [10]. We included information on both body mass index (BMI) and smoking habits, which could affect testosterone levels. The subjects were asked about their history of smoking. If a subject had quit smoking for more than 2 years, he was counted as a non-smoker, as being smoke-free for 2 years can eliminate many adverse effects on health [11].

Health-related quality-of-life

The 36-item Short-Form Health Survey (version 2: SF-36 v2) was used to evaluate each subject's health-related quality-of-life (QOL) [12]. The SF-36 produces scores for eight dimensions of health status, namely, Physical health (PH), Role-physical (RF), Body pain (BP), General health (GH), Vitality (VT), Social function (SF), Role-emotional (RE), and Mental health (MH). The scores for each dimension were assigned a mean (±SD) score of 50 (±10) on the basis of an

assessment of a general Japanese population without chronic conditions; individual scores could then be compared with the normalized scores for the general population.

Saliva collection

Subjects were provided with plastic sterile screw sputum processors to collect samples at 2-hourly intervals between 9 am and 9 pm. Subjects were asked to finish eating and brushing their teeth at least 1 hour before saliva sampling in order to avoid both food and blood contamination. Subjects rinsed their mouths with water three times and waited for a few minutes before expectorating at least 1 ml of saliva directly into a collection vial. Testosterone levels in saliva increase post-microinjury due to the brushing of teeth [1]. It is noteworthy that, in this study, the testosterone levels remained elevated over the baseline well after microinjury and even in samples that did not appear, visually, to be contaminated with blood. Importantly, the effect of microinjury was specific for testosterone. That is, after brushing, neither salivary cortisol nor dehydroepiandrosterone levels were different from the baseline levels [13]. Subjects were asked not to use sugar-free chewing gum, which can change salivary testosterone results [1].

Sample storage

Salivary free testosterone levels were to be determined using an enzyme-linked immunosorbent assay (ELISA; Demeditec Diagnostics, Germany). The instructions from Demeditec Diagnostics for the use of free testosterone in saliva ELISAs stated that saliva samples, in general, were stable at ambient temperature for several days. Therefore, mailing of such samples by ordinary mail without cooling should not create a problem.

Specifically, Demeditec Diagnosics had done stability studies of salivary testosterone with the following results:

- Stability at ambient temperature: up to 1 week
- Stability at 4 °C: up to 1 month
- Stability at −20 °C: no limitation was detected

Notwithstanding these results, Demeditec Diagnosics recommended deep freezing samples whenever a freezer was available to avoid any potential risk.

To be on the safe side, salivary samples were stored for up to 3 days at 4 °C in regular household refrigerators, to avoid bacterial growth that could interfere with antibody binding, and they were then shipped cooled and stored at -20 °C for up to 1 month in laboratory freezers until analyzed.

Hormone determinations

A previous study had shown that salivary concentration measured using a refined immunoassay was a reliable biomarker of serum free testosterone concentration [14,15]. Saliva testosterone levels were, therefore, measured using an ELISA (DE-SLV3013: Demeditec Diagnostics, Germany). The efficacy of using an ELISA was examined by the simultaneous measurement of free testosterone using liquid chromatography-mass spectrometry (LC-MS), which has high sensitivity and specificity.

Salivary testosterone, as measured by LC-MS and by ELISA, showed a strong correlation ($r = 0.84$), confirming the comparative validity with established kits of LC-MS for measuring salivary testosterone.

Statistics

SPSS (version 15.0) was used for statistical analysis. Since the data were normally distributed, One-Way Analysis of Variance tests (ANOVA; post-hoc t test) were used to compare the means of BMI, smoking rates and each dimension of QOL on the SF-36 between the three healthy cohorts.

We performed post-hoc t tests on the results from each of the 2-hour saliva samples to determine if there were any differences in hormone levels at a particular time of the day for each age group. For outliers whose testosterone levels were more than 1.5 interquartile ranges below Q1 or above Q3 on the Box-and-Whiskers plots, data were visually inspected for each subject. If there were explanatory events at unexpected peaks of testosterone levels, those values were excluded; if there were no explanatory events, data were included as likely to represent part of the normal fluctuation in the HPG axis in response to day-to-day events.

In order to examine circadian rhythm, we analyzed the data using repeated measures

Table 1 Baseline characteristic of the healthy subjects

	Age ± SD(years)	BMI ± SD	*P* value	Smoking rates (%)	*P* value
20s-30s (*n*=22)	30.32 ± 4.45	22.73 ± 2.46	0.47 (20s-30s vs 40s-50s); 0.83 (20s-30s vs 60+)	0.53 ± 0.51	0.99 (20s-30s vs 40s-50s); 0.19 (20s-30s vs 60+)
40s-50s (*n*=32)	53.53 ± 5.47	23.62 ± 2.37	0.83 (40s-50s vs 60+)	0.52 ± 0.51	0.16 (40s-50s vs 60+)
60+ (*n*=27)	66.25 ± 4.88	23.20 ± 1.98		0.21 ± 0.43	

Values given are Means ± SD.

ANOVA for each of the 2-hour samples, in accordance with previous work that used a similar design [16-18]. In addition, we used t-tests to compare the 40s–50s cohort with the LOH patients.

Results

First, we examined the circadian rhythm of testosterone levels in three healthy cohorts. Then we compared age-matched LOH patients with the healthy 40s–50s cohort to see whether there were any similarities.

Healthy cohorts

Subjects' characteristics

For each age group, the average age, BMI, smoking rate, health-related QOL (as measured by SF-36 v2), and the *P* values for the post hoc t tests are shown in Tables 1 and 2.

There were no statistically significant differences in either BMI or smoking rates among the three groups.

On the SF-36 v2, there was a statistically worse QOL in relation to body pain in the 40s–50s age group than in the 20s–30s cohort.

Salivary testosterone levels

A comparison of values for each age group from each 2-hour sample is shown in Table 3. Post-hoc analysis showed that there were significantly lower testosterone levels in the 40s–50s cohort than in the 20s–30s cohort at all time points, except for 7:00 pm ($P = 0.03$ for 9:00 am; $P < 0.001$ for 11:00 am; $P = 0.004$ for 1:00 pm; $P < 0.001$ for 3:00 pm; $P = 0.044$ for 5:00 pm; $P = 0.070$ for 7:00 pm; $P = 0.046$ for 9:00 pm). There were only two time block samples in which the testosterone levels were lower in the 60s+ cohort than in the 20s–30s cohort ($P = 0.007$ for 9:00 am; $P < 0.001$ for 3:00 pm). There were no significant differences in mean testosterone levels at any time blocks between the two older cohorts (40s–50s and 60s+).

Analysis of the circadian rhythm

The repeated-measures ANOVA showed significant main effects of time in the 20s–30s cohort

Table 2 Health-related quality-of-life using SF-36 v2 in healthy subjects

	Physical function	Role-physical	Body-pain	*P* value	General health	Vitality	Social function	Role-emotional	Mental health
20s-30s	57.38 ± 2.37	55.32 ± 2.21	58.45 ± 4.31*	*0.025	52.48 ± 8.21	51.36 ± 9.05	54.11 ± 5.39	52.31 ± 6.01	52.0 ± 6.67
40s-50s	54.84 ± 2.79	55.11 ± 3.03	51.13 ± 7.39*		52.54 ± 10.07	55.88 ± 6.40	51.62 ± 8.33	54.97 ± 2.19	53.32 ± 7.56
60+	54.88 ± 2.92	55.03 ± 3.69	54.98 ± 6.75		52.21 ± 6.54	56.18 ± 7.18	52.46 ± 6.54	53.53 ± 4.23	53.10 ± 7.71

Values given are Means ± SD. * $P = 0.025$.

Table 3 Comparison of testosterone levels from each 2-hour sample for each age group

	9 am	*P* value	11 am	*P* value	1 pm	*P* value	3 pm	*P* value
20s-30s	66.82 ± 32.49	* 0.03	63.17 ± 23.59	*** <0.001	55.25 ± 10.60	** 0.004	61.47 ± 24.77	*** <0.001
40s-50s	47.33 ± 20.35	** 0.007	36.89 ± 7.30	0.135	37.60 ± 15.40	0.126	29.16 ± 13.22	*** <0.001
60+	41.17 ± 26.18	0.704	50.77 ± 28.11	0.075	44.54 ± 21.98	0.348	35.54 ± 13.47	0.414

	5 pm	*P* value	7 pm	*P* value	9 pm	*P* value
20s-30s	51.58 ± 25.89	* 0.044	45.51 ± 19.06	0.070	45.72 ± 18.87	* 0.046
40s-50s	36.64 ± 12.87	0.232	32.81 ± 13.38	0.490	32.50 ± 13.57	0.230
60+	41.04 ± 24.33	0.741	38.54 ± 20.21	0.524	36.72 ± 20.82	0.696

Values given are Means ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(Hotteling's Trace = 2.750, $F = 3.667$, $P = 0.047$) and the 40s–50s cohort (Hotteling's Trace = 2.539, $F = 4.231$, $p = 0.022$), confirming a circadian pattern. However, there was no main effect of time in the 60s+ cohort (Hotteling's Trace = 0.516, $F = 1.117$, $P = 0.404$), indicating the lack of circadian rhythm.

The repeated-measures ANOVA for the 20s–30s and 40s–50s cohorts showed significant group-by-time interactions (Hotteling's Trace = 1.068, $F = 4.096$, $P = 0.002$), suggesting that the two groups had a different circadian rhythm pattern.

Comparison of the healthy 40s–50s cohort with LOH patients

LOH patients had significantly lower testosterone levels than the healthy 40s–50s cohort at 11am ($P = 0.016$), 7 pm ($P = 0.024$) and 9 pm ($P = 0.029$). In LOH patients, there was no circadian rhythm (Hotteling's Trace = 6.131, $F = 3.061$, $P = 0.19$). Despite the similarity of testosterone levels, the scores for each domain of SF-36 v2 were significantly lower in LOH patients than in the 40s–50s cohort (Table 4).

In summary, the mean testosterone levels in the 40s–50s cohort were the lowest at almost every time-point among the healthy cohorts and similar to those in LOH patients. A circadian rhythm of salivary testosterone levels was maintained in the 20s–30s and 40s–50s cohorts. However, their circadian patterns differed. The circadian rhythms were lost in the 60s+ and LOH patients.

Discussion

Gender-focused health care has been gaining ground through the understanding of the biological, behavioral, and socio-cultural factors that account for differences in the health of

Table 4 Scores from the SF-36 v2 for the 40s-50s cohort and the LOH patients

	Physical function	Role-physical	Body-pain	General health	Vitality	Social function	Role-emotional	Mental health
40'–50'	54.84 ± 2.79	55.11 ± 3.03	51.13 ± 7.39	52.54 ± 10.07	55.88 ± 6.40	51.62 ± 8.33	54.97 ± 2.19	53.32 ± 7.56
LOH	45.25* ± 7.56	37.77** ± 10.63	40.55** ± 7.24	35.42** ± 4.38	35.12** ± 7.92	32.43** ± 11.94	32.83** ± 16.26	39.37** ± 6.83

Values given are Means ± SD. LOH, late-onset hypogonadism. * $P < 0.01$, ** $P < 0.001$.

men and women. The health care community has paid much less attention to men's health than to woman's so that the health care of men has tended to be piecemeal and somewhat uncoordinated [19].

Striking differences in life expectancy and susceptibility to certain diseases between men and women point to opportunities for understanding and improving the health of men. In Japan, the average life expectancy was 78.6 years for men and 85.6 years for women in 2005 [20]. Although the life expectancy gap between men and women has gradually narrowed over several decades, it remains approximately 7 years and merits better understanding.

Previous studies have shown that a steep decline in serum free testosterone contributes to the onset of LOH [21,22]. Our preliminary study revealed two findings: (1) testosterone levels in healthy middle-aged Japanese men were low, even though they maintained a circadian rhythm; (2) the circadian rhythm was lost in the 60s+ cohort and in LOH patients.

There is a progressive reduction in HPG function in aging men [3]. Testosterone secretion declines through both central (pituitary) and peripheral (testicular) mechanisms and its circadian rhythm becomes blunt or diminished [23]. Our study was consistent with previous studies. In addition, our study showed that a progressive reduction in HPG function may occur in LOH patients as well as in aging men, which induces the loss of circadian rythmicity in saliva testosterone levels.

The decline of serum free testosterone is associated with aging [22] as well as with stressors that challenge the homeostasis of the endocrine environment. Stress and other conditions that elevate circulating adrenocorticotropine hormone (ACTH) and cortisol levels lead to depressed testosterone levels in animals and in men [24,25]. Excessive exposure to cortisol initiates apoptosis in rat Leidig cells, potentially contributing to the suppression of testosterone levels [26].

From a cross-cultural perspective, Japanese workers have reported higher levels of job stress, anxiety, depression, and psychosomatic tendencies [27,28].

In our study, the middle-aged cohort showed the worst QOL in relation to body pain on the SF-36 v2. This merits profound consideration. Cultural factors have an impact on men's attitude to seek health care. In Japan, like many parts of the world, masculinity is given priority in traditional men's values. Men are forced to be 'macho' and to show stoicism, independence, self-reliance, and strength [29]. This gender perspective prevents them from admitting to having any problems, especially with regard to showing mental problems such as fear or sadness [30]. Job strain has been reported to be associated with bodily pain [31]. Thus, if middle-aged men are placed in a stressful environment, socio-environmental factors may affect their mental health and thus influence physical health problems.

In Western countries, LOH is frequently reported as the result of an age-related decline in free and bio-available testosterone. Cross-cultural studies have shown that the pattern of age-related decline in male free testosterone, as represented by salivary levels, is not a uniform characteristic of all populations [32]. The etiology of LOH is a complex matter involving cultural and socio-environmental issues as well as individual biological changes.

Low testosterone in middle-aged men might lead to their future frailty as well as to the onset of LOH. Our study suggests that middle-aged Japanese men are susceptible to LOH and that there may be many LOH patients who are under-diagnosed and not treated.

In an extension of this research, we will include a paper interview in addition to the SF-36 in order to evaluate stress levels.

So far, there have been few interventions to raise the public awareness of LOH in Japan. In addition to education for health professionals to help them understand the complexity of LOH symptoms, the distribution of information and screening tests in workplaces and public facilities will enable middle-aged men to become aware that they should be screened for LOH. It worth screening the testosterone levels in middle-aged men and providing appropriate awareness of the risk of having a low testosterone level. Holistic approaches, including proper prescriptions for exercise, consultations for dietary habits, alternative medicine such as acupuncture and Chinese herbal medicine, hormone replacement therapy, and counseling to ease excessive stress will be needed to prevent the symptoms of LOH.

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The prevalence of depressive symptoms and other variables among frail aging men in New York City's Personal Care Services program

Mitsuko Yasuda, Shigeo Horie, Steven M. Albert and Bridget Simone

Keywords

Depressive symptoms

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Mitsuko Yasuda, MD, PhD, MPH
Department of Urology, Teikyo University, Tokyo, Japan

Shigeo Horie, MD
Department of Urology, Teikyo University, Tokyo, Japan

Steven M. Albert, PhD, MSc
Department of Sociomedical Sciences, Columbia University and Department of Behavioral and Community Health Sciences, University of Pittsburgh, USA

Bridget Simone, PhD, DrPH
New York City Human Resources Administration, New York, USA

E-mail: myasuda@med.teikyo-u.ac.jp

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Abstract

Background: New York City's Personal Care Service Program provides service-rich assistance to the frail elderly who would not be able to live at home without such support. However, gender-specific health care delivery has not been introduced. Depressive symptoms are common among elderly people. We conducted a cross-sectional study in order to investigate the prevalence of depressive symptoms and other variables among frail elderly men receiving personal care services.
Methods: Data were collected from administrative data available in the Human Resources Administration's computer system. Two hundred men aged 65 or older were randomly selected. We defined depressive symptoms by tracking the recording of depressed mood in the data system. We examined statistical differences in a variety of indicators between elderly men with and without depressive symptoms. Multiple logistic regression analysis was performed to determine which independent variables were associated with depressive symptoms.
Results: Of all cases, 10.5% had depressive symptoms. In multiple logistic regression models, the duration of services provision and hearing impairment were independently associated with depressive symptoms in frail elderly men.
Conclusion: The results of this study indicate the low prevalence of depressive symptoms among frail elderly men compared with previous studies. The duration of services provision was a protective factor for depressive symptoms, i.e. personal care services provided high quality Activities of Daily Living (ADL) support, which keeps frail elderly men living at home for as long as possible. The significance of hearing impairment, which can induce social isolation, also needs to be stressed as an indicator of depressive symptoms, even though there was no discrete measurement of social isolation included in the data.
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Introduction

Personal care services provide help to individuals in need of assistance with Activities of Daily Living (ADLs: e.g. bathing, dressing, personal grooming, getting out of bed or getting up from a seated position, eating, using the toilet etc) as well as household support, which are prerequisites for health and safety in their own homes. While personal care services – the Medicaid Optional Program – have been adopted by 26 states, 15 states have set limits

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on the hours that services are made available and 10 states have set cost caps [1,2]. New York is the only state that has not placed limits on service benefits and provides generous and service-rich assistance to the frail elderly in the community. The program currently serves approximately 53,000 clients daily through 969 vendors. The high intensity of services, including 24-hour personal assistance care, allows individuals who are medically eligible for nursing home placement to live in their own homes. Elderly clients are more likely to be disabled than older community residents who do not participate in personal care services [3]. Depressive symptoms affect 8–20% of elderly individuals in the community [4]. Older adults with medical illness, somatic impairment and social isolation are more likely to be depressed [5]. We hypothesized that medical illness, physical impairment, and social isolation would be associated with administrative reports of depressive symptoms in elderly men. We sought to investigate the prevalence of depressive symptoms among those men aged 65 or older who had received personal care services from New York City for more than 1 year, and to examine the relationships between depressive symptoms and a variety of indicators, such as age, medical and functional indicators, use of personal care services, and caregiver support, in order to determine the variables associated with depressive symptoms. To do this, we used administrative data, such as Medicaid administrative claims, medical requests (M11Q) and social assessments (M11S).

Methods

Data collection

To receive personal care services, people need to submit a medical request form (M11Q) filled out by their physicians, a social assessment (M11S) performed by case workers, and a nurse's assessment (M27r) to New York City's Human Resources Administration (HRA). These data are collected at nine Community Alternative System Agency borough offices (CASA offices), which serve the clients in their geographic areas, and are stored in the centralized computer database.

Subjects

Subjects were randomly drawn from the centralized computer database. In addition, we limited the subjects to those who were men aged 65 or older who had been receiving services for at least 1 year. Finally, we excluded clients who received only 'level 1' service (housekeeper services), since they were not receiving personal assistance care. Information on 200 elderly men was included in the study.

Definition of depressive symptoms

According to DSM-IV [6], major depression is defined as the presence of five or more out of nine symptoms (depressed mood, loss of interest or pleasure, eating disturbance, sleep disturbance, psychomotor agitation, fatigue, a feeling of worthlessness or guilt, poor concentration and suicidal ideation) during the same 2 week period. At least one of the symptoms must be either depressed mood or loss of interest/pleasure. This level of either specificity or sensitivity was unavailable in the administrative data. In the absence of the full set of nine DSM-IV criteria, any mention of depressive mood in the medical report was used as an indicator of depressive symptoms, since depressive mood and anhedonia are two gateway symptoms, only one of which is needed to constitute a depressive disorder.

Sociodemographic characteristics

Sociodemographic variables included age, living situation (living alone, living with caregivers or with non-caregivers), and caregiving support.

Administrative claims

The number of service hours that each client was receiving in July 2005 was assessed as the number of billed service hours. For duration of home care services, we retrieved information on all periods of home care services from the initial authorization to July 2005.

Medical status

Overall medical co-morbidity was indicated by the total number of ICD-9 (International Classification of Diseases) diagnoses.

Cognitive impairment

Three mental status indicators in the M11Q, namely disorientation to place/time, short-term memory impairment, and impaired judgment, were used to identify cognitive impairment. If the clients had at least one of these three impairments, they were categorized as having 'dementia'. Clients with none of the three impairments were categorized as 'no impairment'.

ADL status

ADL status was based on the number of needs in six ADLs: bathing, dressing, personal grooming, getting out of bed or getting up from a seated position, eating, and using the toilet.

Functional status

Sensory impairment (speech, visual, or hearing impairments), muscular impairment (dominant hand, upper extremities, lower extremities), and bladder incontinence were assessed to examine whether these impairments were associated with depressive symptoms.

Caregiver support

Data related to caregiver support were obtained from a social assessment (M11S). We investigated whether the clients lived with or without informal caregivers.

Statistical analysis

The data were analyzed using an SPSS 13.0 statistics package. Statistical differences in each variable between those clients with and without depressed mood were examined using a t-test or a chi-square test. The level of significance was set at $P < 0.05$ (two-sided). In order to evaluate the role of depressive symptoms as indicators, multiple logistic regression analysis was performed considering depressive symptoms as a dependent variable.

Results

The characteristics of groups designated as being with and without depressive symptoms are shown in Table 1. In all, 10.5% of the subjects were defined as having depressive symptoms, considered as the presence of depressed mood. Differences did not achieve a statistical significance with age, intensity of services (hours of services provided per week), number of ADL disabilities, number of comorbid conditions, and cognitive impairment. Regarding functional status, elderly men with depressive symptoms were significantly more likely to be visually impaired (66.7% vs. 44.9%, $P = 0.49$, $n = 200$, df = 1), to have a hearing impairment (76.2% vs. 43.3%, $P = 0.004$, $n = 200$, df = 1), an upper extremity impairment (70.0 vs. 43.8%, $P = 0.026$, $n = 200$, df = 1) and bladder incontinence (65.0% vs. 38.8%, $P = 0.024$, $n = 200$, df = 1). The two groups did not differ in either living situation or caregiver support. Although the difference did not achieve statistical significance, it should be noted that elderly men without depressive symptoms were more likely to live alone (59.3% vs. 44.4%, $P = 0.687$, $n = 200$, df = 2).

The associations between the independent variables and depressive symptoms, based on logistic regression analysis, are shown in Table 2. The probability of having depressive symptoms decreased with duration of services (odds ratio (OR) = 0.86, 95% confidence interval (95% CI) = 0.75–0.97), and increased with hearing impairment (OR = 3.67, 95% CI = 1.18–11.84).

Discussion

Late-life depression is common. Estimates of the presence of depressive symptoms in community-dwelling elderly range from 8% to 20% [4]. Older adults with medical illness, somatic impairment, and social isolation are more likely to be depressed [5]. About 30–50% of nursing home residents and about 26–44% of housebound elderly adults have depressive symptoms [7,8]. Sex differences in depression are of clinical and scientific importance. These differences in depression prevalence rates have been reported previously with respect to phenomenology, level of distress and suffering, functional impairment, coping style, and treatment response [9]. Previous studies have shown that sex differences in brain neuroanatomy may be important in the pathophysiology of late onset depression. Men may be more susceptible than women to atrophy in the frontal subregions, which are related to geriatric

Table 1 Features of Medicaid personal care services male clients aged 65+ by depressive symptoms

Full sample (M11Q) $n = 200$	No depressive symptoms $n = 179$ (89.5%)	Depressive symptoms $n = 21$ (10.5%)
Demographics		
Age ± SD (years)	78.92 ± 7.66	81.10 ± 7.38
Personal care services		
Hours/week (mean ± SD)	54.40 ± 44.45	61.76 ± 47.85
Duration, years (mean ± SD)	7.60 ± 5.75	4.95 ± 3.29*
Medical status		
Number of diseases (mean ± SD)	5.01 ± 1.35	5.58 ± 1.63
Cognitive impairment		
No impairment, %	36.5	14.3
Dementia, %	63.5	85.7
Functional status		
Number of ADL disabilities (mean ± SD)	3.92 ± 1.66	4.33 ± 1.77
Sensory impairment		
Speech impairment, %	29.2	42.9
Visual impairment, %	44.9	66.7*
Hearing impairment, %	43.3	76.2**
Muscular impairment		
Dominant hand/arm impairment, %	29.2	40.0
Upper extremities impairment, %	43.8	70.0*
Lower extremities impairment, %	71.8	80.0
Bladder incontinence, %	38.8	65.0*
Socio-environmental status		
Living situation		
Live alone, %	59.3	44.4
Living with caregiver, %	33.0	44.4
Living with non-caregiver, %	7.7	11.1
Care-giving support		
No caregiver, %	14.3	22.2
Only live-in caregiver, %	22.0	33.3
Only out-of-home caregiver, %	52.7	33.3
Both in- and out-of-home caregiver, %	11.0	11.1

*$P < 0.05$, **$P < 0.01$.

depression [10]. Gender differences in depression after widowhood have also been reported [11]. Since women adapt more successfully than men, men who remain alone after losing their partners have a higher risk of developing symptoms of chronic depression.

In our study, despite the level of their disabilities, the prevalence of depressive symptoms among elderly men was low (10.5%). A previous study showed that the prevalence of depression in nursing home residents was very high [7]. Instead of being accommodated in nursing homes, living at home for as long as possible with home care services would allow frail elderly men to maintain their self-esteem and internal locus of control, loss of which might be associated with depression [12].

Depression and cognitive impairment are two of the most frequently observed medical problems in elderly people and often manifest as co-morbid conditions [13]. Approximately 30–40% of those with dementia will experience significant depressive symptoms sometime during the course of the disease [14]. Our study showed that 14.0% of cognitively impaired men (28 out of 200) had depressive symptoms. This seems low when compared with other studies. However, defining depression in demented individuals is a challenge for most health care providers [13]. Patients with dementia are unlikely to be able to express their distress when they feel depressed, and often present with somatic symptoms that are less obviously related to depression [15].

Table 2 Association of independent variables with depressive symptoms from multiple logistic regression analysis

Dependent variable Frequency of dependent variable	Depressive symptoms (21/200) OR (95% CI), P
Personal care services	
Duration of services	0.86 (0.75, 0.97)*, $P = 0.018$
Functional status	
Sensory impairment	
Visual impairment	1.14 (0.38, 3.49), $P = 0.82$
Hearing impairment	3.67 (1.18, 11.84)*, $P = 0.030$
Muscular impairment	
Upper extremities impairment	2.61 (0.86, 7.65), $P = 0.082$
Bladder incontinence	1.79 (0.63, 5.11), $P = 0.28$

OR, odds ratio; 95% CI, 95% confidence interval.
* $P < 0.05$.

Untreated depression in dementia can lead to negative consequences, such as a high rate of persistence and increased cognitive impairment, physical disability, social isolation, substance abuse, and suicide [16]. It is important that proper mental health check-ups are provided for cognitively impaired clients.

The high prevalence of hearing impairment among elderly men in our study (46.7%) was consistent with previous studies, whose prevalence rates ranged from 21–72% [17–19]. Hearing impairment leads to withdrawal from social activities and isolation. Previous studies have suggested that hearing impairment in elderly people has a significant correlation with depression [20–24]. Our study was consistent with these findings. People with hearing impairment were 3.67 times more likely to have depressive symptoms than people without a hearing impairment ($P = 0.03$). Hearing impairment needs to be stressed as an indicator of social isolation and the presence of depressive symptoms. Regular audiological check-ups to detect occult hearing impairment, and to provide mental health screening for people with hearing loss, need to be considered.

Depression and ADL disability contributed to their mutual risk [25,26]. ADL disability is a chronic stress producer because, by its very nature, it involves ongoing challenges to the accomplishment of daily tasks [27]. In our study, the number of ADL disabilities was not associated with depressive symptoms. The high prevalence of ADL disability in those making use of personal care services might be too great to allow the detection of any differences in ADL disability between people with and without depressive symptoms.

Epidemiological studies have been consistent in finding an inverse relationship between age and the prevalence of late-life depression [28]. In our study, age did not reach statistical significance in the relationship with depressive symptoms.

Social support is a protective factor for depression among the elderly [29]. Our study did not find any significant associations in the relationship between depressive symptoms and caregiver support.

For every additional year of personal care service use past that of the mean duration, elderly male clients were 14% less likely to be depressed ($P = 0.018$, 95% CI = 0.75–0.97). This suggests that personal care services programs can mitigate the development of depression by keeping clients in their own homes, where they prefer to be the most, for as long as possible.

The main limitation of this study was the potential unreliability of the information on depressive symptoms reported in the M11Q. Primary care physicians complete the M11Q based on their observations, reports from the visiting nurse or from the clients themselves. Delegating responsibility for completing the M11Q to physicians who are not directly involved with the day-to day care of the clients may result in M11Q data that do not reflect the real status of the clients. However, since we employed four indicators of depressive symptoms, this limitation would not account for significant relationships between duration of

services, hearing impairment and depressive symptoms.

Untreated depression in elderly persons negatively influences somatic impairment, adaptation to medical illness, and quality of life, and is associated with increased morbidity and mortality, including suicide [5].

In order to improve the quality of personal care services, it is vital to develop interventions for screening and assessing depressive symptoms among elderly clients who are at risk, and to provide access to specialist mental health services. Intervention focused on elderly men with hearing impairment may be cost-effective, since they are at a high risk for the development of depressive symptoms in later life. We would stress that among various indicators in the administrative data, hearing impairment is important as an indicator of social isolation and the presence of depressive symptoms.

It is also important to introduce personal care services to frail elderly men as soon as they need help, as men tend to be unwilling to get any support because of their masculinity and unfamiliarity with accepting support [30]. Personal care services can improve the circumstances in which they can compensate for their disabilities.

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ORIGINAL ARTICLE

Testosterone decreased urinary-frequency in nNOS-deficient mice

Satoru Muto, Mitsuko Yasuda, Yutaka Kamiyama, Hisamitsu Ide and Shigeo Horie

Department of Urology, Teikyo University, Itabashi-Ku, Tokyo, Japan

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Correspondence:
Shigeo Horie, MD, Department of Urology, Teikyo University, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605, Japan.
E-mail: shorie@med.teikyo-u.ac.jp

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Summary

To observe the effect of testosterone on the frequency of urination in mice lacking neuronal nitric oxide synthase ($nNOS^{-/-}$), we compared the urination patterns between unanaesthetized male wild-type ($n = 27$) and $nNOS^{-/-}$ mice ($n = 50$) with or without testosterone treatment. Compared with wild-type mice, $nNOS^{-/-}$ mice showed a greater frequency of urination during a 24-h observation period (3.0 vs. 5.4 times/day, $p < 0.0001$) without any significant difference in the total voided volume or the functional voiding capacity. While testosterone treatment did not affect the urination patterns in wild-type, it decreased the daytime frequency of urination (5.4 vs. 3.7 times, $p = 0.0198$) and the nighttime urination (4.4 vs. 2.9 times, $p = 0.039$) in $nNOS^{-/-}$ mice. The $nNOS^{-/-}$ mice can be a useful animal model for urinary frequency. Testosterone improved the functional abnormalities in the voiding of $nNOS^{-/-}$ mice.

Introduction

The relaxation of bladder outlet regions on voiding and the maintenance of low pressure during urinary storage are prerequisite conditions for normal micturition. An accumulating body of evidence indicates that nitric oxide (NO) is an important physiological inhibitory neurotransmitter that mediates relaxation in the smooth muscle component (Andersson *et al.*, 1992; Thornbury *et al.*, 1992; Persson & Andersson, 1994). Nitric oxide is produced as a result of the conversion of the substrate L-arginine to L-citrulline by three isoforms of the enzyme NO synthase (NOS). The mechanism underlying NO-induced smooth muscle relaxation was found to be calcium-dependent, and cyclic guanosine monophosphate (cGMP) was identified as the second messenger. The individual NOS isoforms (eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase and iNOS, inducible nitric oxide synthase), have a unique subcellular location, structure, kinetics, regulation and function. There is increasing evidence that alterations in the NO–cGMP pathway play an important role in the development of lower urinary tract syndrome (LUTS) (Andersson *et al.*, 2002; Mamas *et al.*, 2003; Chertin *et al.*, 2004; Hedlund, 2005). Neuronally released NO has been suggested to be involved in the relaxation of the urethral smooth muscle during micturition (Buga *et al.*, 1989; Andersson & Persson, 1995; Burnett, 1995). By inhibiting the production of NO, both bladder hyperactivity and a decreased bladder capacity have been demonstrated (Persson *et al.*, 1992).

Indeed a targeted disruption of the nNOS gene has been demonstrated to result in voiding abnormalities (Burnett *et al.*, 1997). In $nNOS^{-/-}$ mice, urinary bladders develop hypertrophy because of deficient outflow relaxation. The urinary bladder and urethra do not relax in response to electrical field stimulation or L-arginine, the amino-acid substrate for NO. The $nNOS^{-/-}$ mice have urinary frequency and a decreased threshold of afferent firing of the bladder detrusor, thus indicating that nNOS is responsible for the relaxation of the bladder outlet regions on voiding and the maintenance of low pressure in urinary storage. These features observed in the abnormal urination of $nNOS^{-/-}$ mice are thus considered to be a plausible model for human overactive bladder.

Recently, several studies have shown testosterone (T) to induce the relaxation of smooth muscle cells by modulating both the nNOS activity and cGMP level. Androgens play a pivotal role in the erectile function by

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regulating both cGMP formation by NOS and degradation by phosphodiesterase 5 (PDE5) (Morelli *et al.*, 2005; Vignozzi *et al.*, 2005). In addition, testosterone has direct vasoactive properties. Testosterone has been shown to cause a dose dependant effect on the relaxation of vascular smooth muscles (Webb *et al.*, 1990; English *et al.*, 2001). It is caused either by a direct effect on the vascular smooth muscle, or by an effect on potassium channel (Deenadayalu *et al.*, 2001; English *et al.*, 2002). These lines of evidence prompted us to investigate the role of testosterone in the relaxation of bladder smooth muscle. To evade the effect of nNOS in the relaxation of bladder, $nNOS^{-/-}$ mice was examined to see the role of T. The purpose of the current study was to examine the effect of testosterone on the abnormal voiding of $nNOS^{-/-}$ mice.

Materials and methods

Animals

Experiments were performed in nNOS null mutant mice ($nNOS^{-/-}$) generated by Huang *et al.* (1993) and their wild-type littermates. All wild-type littermates ($n = 27$) and homozygous $nNOS^{-/-}$ males ($n = 50$) of C57Bl/6 strains with targeted disruption of exon2 of the nNOS gene, used in this study were derived from a heterozygous breeding pair. Both groups were housed and studied under identical conditions. At weaning, the animals were ear-tagged. Genomic DNA was extracted from the tails after digestion with proteinase K and E, and purification of DNA with ethanol. The genotype of each DNA sample was then determined by testing for the presence of wild-type or modified nNOS sequences by use of PCR. Animals were maintained on standard rodent chow and tap water at 23 ± 1 °C with a 12/12-h light-dark cycle (lights on at 6:00 AM). The mice always had free access to food and tap water. All care and handling of the animals were carried out in accordance with institutional guidelines.

Voiding behaviour

Male $nNOS^{-/-}$ and wild-type littermates aged 8–13 months and weighing between 30 and 40 gm were used in this study. Voiding studies were carried out after a 3-day period of acclimatization to the laboratory. Individual mice were placed in a mouse micturition cage. The urine collection funnel and faecal separation screen were placed on the bottom of the cage. Each scale was monitored using a remote computer running an application that permits recording information sent by the scale to a resolution of 1 sec. The voiding volume is expressed as weight with a resolution of 10 mg. When assuming a urine specific gravity of approximately, 1.1 mg urine has a volume of 1 mL.

This assumption was reasonable because animals with polydipsia tend to produce extremely dilute urine. The total amount of urine voided in 1 day was accumulated into a container. Voiding was studied continuously for a 10-day period.

Testosterone administration

After stable baseline voiding patterns had been established, 3.6 mg/g body weight testosterone enanthate (Teikoku Hormone Mfg, Tokyo, Japan) diluted in sesame oil or other vehicles were singly administered intraperitoneally to male wild-type littermates and $nNOS^{-/-}$. This dose was selected based on previous studies that examined the effect of androgen on the gene expression in the prostate (Mirosevich *et al.*, 1999, 2001). The voiding pattern of the treated mice was examined 1-week later. We examined the urinary frequency, functional bladder capacity and the urinary volume.

Statistics

The results are generally reported as the mean ± SD. Student's unpaired two-tailed *t* test was used compared with the micturition parameters between the control and obstructed groups, as well as between the wild-type littermates and $nNOS^{-/-}$. One-way ANOVA was used to analyse to compare the differences of the parameters among four groups (wild-type with or without testosterone and $nNOS^{-/-}$ with or without T). A probability level of <0.05 was considered to be significant.

Results

The micturitional characteristics of wild-type and $nNOS^{-/-}$ mice

The amount of urine output per day (0.84 ± 0.40 mL vs. 1.09 ± 0.55 mL, $p = 0.282$) and the functional bladder capacity (defined as the voided volume per void) (0.28 ± 0.17 mL vs. 0.21 ± 0.12 mL, $p = 0.061$) did not differ substantially between the wild-type and $nNOS^{-/-}$ mice. Within comparison to the wild-type mice, the $nNOS^{-/-}$ mice showed a greater frequency of urination during the 24-h observation period (3.00 ± 1.00 times vs. 5.40 ± 0.97 times, $p < 0.0001$) and also during the nocturnal period (2.50 ± 0.85 times vs. 4.40 ± 1.27 times, $p = 0.001$) (Table 1).

However, no significant differences were seen in the frequency of daytime voiding between the wild type and $nNOS^{-/-}$ mice (0.50 ± 0.71 times vs. 0.60 ± 0.70 times, $p = 0.750$).

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Table 1 The number of voids in wild-type and $nNOS^{-/-}$ mice

	WT (n = 27)	KO (n = 50)	p-value
Capacity			
Total micturition volume (mL/24 h)	0.84 ± 0.40	1.09 ± 0.55	0.282
Functional capacity (mL/void)	0.28 ± 0.17	0.21 ± 0.12	0.061
	WT (n = 10)	KO (n = 10)	
frequency			
No. of voids/24 h	3.00 ± 1.00	5.40 ± 0.97	<0.0001
No. of daytime voids	0.50 ± 0.71	0.60 ± 0.70	0.750
No. of nightime voids	2.50 ± 0.85	4.40 ± 1.27	0.001

WT, wild-type; KO, neuronal nitric oxide synthase ($nNOS^{-/-}$).

Effects of testosterone on the urinary frequency of $nNOS^{-/-}$ and the wild-type mice

Testosterone treatment did not affect either the amount of urine output per day (wild-type; 0.84 ± 0.40 mL vs. 0.64 ± 0.57 mL, $p = 0.420$, $nNOS^{-/-}$; 1.09 ± 0.55 mL vs. 1.01 ± 0.59 mL, $p = 0.782$) or the functional bladder capacity (wild-type; 0.28 ± 0.17 mL vs. 0.24 ± 0.13 mL, $p = 0.331$, $nNOS^{-/-}$; 0.21 ± 0.12 mL vs. 0.27 ± 0.10 mL, $p = 0.057$) in the wild-type and $nNOS^{-/-}$ mice. Regarding the frequency of daytime voiding, there were no significant differences before and after testosterone administration in the wild-type and $nNOS^{-/-}$ mice (wild-type; 0.50 ± 0.71 times vs. 1.33 ± 1.37 times, $p = 0.127$, $nNOS^{-/-}$; 0.60 ± 0.70 times vs. 0.86 ± 0.69 times, $p = 0.465$). However, in the $nNOS^{-/-}$ mice, testosterone administration significantly decreased the frequency of urination during both the 24-h observation period (5.40 ± 0.97 times vs. 3.71 ± 1.70 times, $p = 0.020$) and the nocturnal period (4.40 ± 1.27 times vs. 2.86 ± 1.86 times, $p = 0.039$). By one-way ANOVA, there were statistical differences in both the 24-h observation period (p =0.018) and the nocturnal period ($p < 0.001$) among four groups (wild-type with or without testosterone $nNOS^{-/-}$ mice with or without T) (Table 2).

Discussion with conclusions

In the present study, we confirmed the previous study that $nNOS^{-/-}$ mice had urinary frequency with a decreased functional urinary bladder capacity. Thus $nNOS^{-/-}$ mice can be a useful animal model for the exploration of the treatment of LUTS. Several lines of evidence may allow the speculation for the underlying mechanisms of the effect of the testosterone treatment on the voiding abnormalities of $nNOS^{-/-}$ mice.

First, testosterone has been shown to cause a dose-dependant effect on the relaxation of vascular smooth muscles (Webb *et al.*, 1990; English *et al.*, 2001). However, it remained unknown whether testosterone relaxes smooth muscle cell in a tonic manner. Secondly, there is evidence that androgens can regulate the expression of NOS enzymes in the corporeal tissue (Chamness *et al.*, 1995; Park *et al.*, 1999). As a result, in $nNOS^{-/-}$ mice, testosterone might possibly augment the expression of eNOS to compensate for the loss of nNOS. A previous study demonstrated that androgens maintain the erectile response by the pathways that are independent of NO but involve the synthesis of cGMP (Reilly *et al.*, 1997). Those hypotheses warrant further studies. The association of the late-onset hypogonadism and urinary frequency has been reported in literature. The findings of this study may indicate that testosterone potentially has a therapeutic effect on an overactive bladder by decreasing the nNOS expression in ageing males. The $nNOS^{-/-}$ mice showed an increased urinary frequency. Testosterone treatment significantly improved the urinary frequency for $nNOS^{-/-}$ mice. These findings may indicate that testosterone can improve bladder overactivity because of the loss of nNOS. Androgen replacement may therefore be a potentially useful and novel pharmacological target for patients with a decreased level of NO thus leading to urinary frequency.

Table 2 The voiding function in wild-type and $nNOS^{-/-}$ mice before and after testosterone administration

	WT (n = 27)	WT + T (n = 19)	p-value	KO (n = 50)	KO + T (n = 26)	p-value
Capacity						
Total micturition volume (mL/24 h)	0.84 ± 0.40	0.64 ± 0.57	0.420	1.09 ± 0.55	1.01 ± 0.59	0.782
Functional capacity (mL/void)	0.28 ± 0.17	0.24 ± 0.13	0.331	0.21 ± 0.12	0.27 ± 0.10	0.057
	WT (n = 10)	WT + T (n = 7)		KO (n = 10)	KO + T (n = 7)	
Frequency						
No. of voids/24 h	3.00 ± 1.00	2.71 ± 2.22	0.734	5.40 ± 0.97	3.71 ± 1.70	0.020
No. of daytime voids	0.50 ± 0.71	1.33 ± 1.37	0.127	0.60 ± 0.70	0.86 ± 0.69	0.465
No. of nightime voids	2.50 ± 0.85	1.33 ± 1.21	0.059	4.40 ± 1.27	2.86 ± 1.86	0.039

WT, wild-type; WT + T, wild-type after testosterone administration; KO, neuronal nitric oxide synthase ($nNOS^{-/-}$); KO + T, $nNOS^{-/-}$ after testosterone administration.

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Daniel C. Brennan, M.D.
Washington University School of Medicine
St. Louis, MO 63110
dbrennan@wustl.edu

Paula Buchanan, M.P.H.
Mark A. Schnitzler, Ph.D.
Saint Louis University Center for Outcomes Research
St. Louis, MO 63104

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DHEA and Testosterone in the Elderly

TO THE EDITOR: In their report on the effects of dehydroepiandrosterone (DHEA) and testosterone when used as antiaging supplements, Nair et al. (Oct. 19 issue)[1] conclude that low-dose testosterone replacement in elderly men has no "physiologically relevant beneficial effects on body composition, physical performance, [or] insulin sensitivity." However, this conclusion is premature, since the testosterone replacement administered failed to achieve physiologic testosterone levels throughout the study period (Fig. 2 of the article). Moreover, despite the marginal increase in testosterone levels achieved, improvements in fat-free mass, fasting insulin levels, and bone mineral density were observed.

Other studies of testosterone replacement, including those cited to support the authors' conclusions,[2] have shown a decrease in fat mass (12.5%) and an increase in lean mass (4%) when physiologic testosterone levels are achieved in elderly men. Studies of standard doses of testosterone in the treatment of testicular failure[3] have shown additional positive effects on muscle strength, physical performance,[4] and bone mineral density.[5] Large, long-term trials are clearly needed to assess the risks and benefits of testosterone replacement in elderly men, and caution should be exercised regarding the treatment of andropause in men. However, the serum testosterone level achieved should be within the normal range to assess the effect on outcome measures adequately.

Stephanie T. Page, M.D., Ph.D.
University of Washington
Seattle, WA 98195
page@u.washington.edu

Alvin M. Matsumoto, M.D.
Veterans Affairs Puget Sound Health Care System
Seattle, WA 98108

William J. Bremner, M.D., Ph.D.
University of Washington
Seattle, WA 98195

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TO THE EDITOR: The findings of Nair et al. cannot be generalized, because the study included relatively healthy subjects. To investigate the benefits and risks of androgen-replacement therapy, it is essential to make judicious choices regarding the subjects to be included in the research. In this study, the average baseline scores for the quality of life (on the Health Status Questionnaire [HSQ] and the Medical Outcomes Study 36-item Short-Form General Health Survey [SF-36]) of all the subjects were above 50 for both the physical and mental components. The average score on both instruments in the general U.S. population is 50.[1] The high scores of these subjects suggest that the

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study included healthier elderly persons than those who would be representative of the general elderly population.

Moreover, physical exercise is expected to improve and maintain physical functioning in older people.[2,3] Not only androgen administration but also well-designed physical training is needed to improve the physical performance of elderly persons. The androgen level might be a mediator that could be elevated by exercise training, which would then increase physical performance. The administration of androgen in the absence of exercise may not be enough to improve physical performance among the elderly.

Mitsuko Yasuda, M.D., Ph.D.
Shigeo Horie, M.D.
Teikyo University
Tokyo 173-8605, Japan
shorie@med.teikyo-u.ac.jp

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TO THE EDITOR: DHEA was banned in 1985 by the Food and Drug Administration because clinical safety and efficacy data were lacking to support claims of cures for cardiovascular disease and aging. After the passage of the Dietary Supplement Health and Education Act in 1994, DHEA, which had not previously been labeled as a drug, again became available. It is amazing that a previously banned substance can now be sold directly to the public, and it speaks to the lack of oversight and protection afforded by the Dietary Supplement Health and Education Act.

Hormones have long been equated with youth by the public and are thus a favorite type of substance for marketing by the antiaging industry.[1] As one substance falls out of favor, another quickly replaces it: the miracle of melatonin[2] was replaced by the superhormone promise[3] of DHEA. The heir apparent now seems to be growth hormone, which, paradoxically, is illegal to distribute for antiaging uses but constitutes a market estimated at more than $600 million per year in the United States alone.[4]

Thomas T. Perls, M.D.
Boston University Medical Center
Boston, MA 02118
thperls@bu.edu

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TO THE EDITOR: The study by Nair et al. may be misleading. One problem arises from the age of the persons involved in the study. Women older than 60 years rarely have postmenopausal symptoms. In the absence of symptoms, how are the beneficial effects of treatment on the quality of life to be demonstrated? Similarly, one may question the use of testosterone in men older than 60 years.

The principal problem, however, is that Nair et al. treated laboratory values (low values of DHEA and testosterone), not — as is usual medical practice — symptoms. To return to the example of postmenopausal care for women older than 60 years, such an approach could be equated with indiscriminately treating unselected postmenopausal women, all of whom, of course, have low estradiol levels, with estrogen replacement, whether or not they are symptomatic. Whether such an unselected approach to treatment would ever reveal clinical benefits regarding the quality of life is questionable.

That DHEA can indeed positively affect certain physiological processes of aging has been suggested with regard to ovarian function.[1-3] Thus, nothing in the study by Nair et al. contradicts the value of further investigation of DHEA in specific conditions of aging.

Norbert Gleicher, M.D.
David Barad, M.D.
Center for Human Reproduction
New York, NY 10021
ngleicher@thechr.com

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Activation of Peroxisome Proliferator-Activated Receptor-γ and Retinoid X Receptor Inhibits Aromatase Transcription via Nuclear Factor-κB

WuQiang Fan, Toshihiko Yanase, Hidetaka Morinaga, Yi-Ming Mu, Masatoshi Nomura, Taijiro Okabe, Kiminobu Goto, Nobuhiro Harada, and Hajime Nawata

Department of Medicine and Bioregulatory Science (W.F., T.Y., H.M., M.N., T.O., K.G., H.N.), Graduate School of Medical Science, Kyushu University, Fukuoka 812-8582 Japan; Core Research for Evolutional Science and Technology (CREST) (T.Y., H.M., M.N., T.O., K.G., H.N.), Japan Science and Technology Corporation, Kawaguchi, Saitama 332-0012, Japan; Department of Endocrinology (Y.-M.M.), Chinese PLA General Hospital, Beijing 100853, China; and Department of Biochemistry (N.H.), School of Medicine, Fujita Health University, 470-1192 Aichi, Japan

Our previous studies demonstrated that a peroxisome proliferator-activated receptor (PPAR)-γ ligand, troglitazone (TGZ), and/or a retinoid X receptor (RXR) ligand, LG100268 (LG), decreased the aromatase activity in both cultured human ovarian granulosa cells and human granulosa-like tumor KGN cells. In the present study, we further found that a combined treatment of TGZ+LG decreased aromatase promoter II (ArPII) activity in both ovarian KGN cells and fibroblast NIH-3T3 cells in a PPARγ-dependent manner. Furthermore, the inhibition of both aromatase activity and the transcription of ArPII by TGZ+LG was completely eliminated when nuclear factor-κB (NF-κB) signaling was blocked by specific inhibitors, suggesting NF-κB, which is endogenously expressed in both fibroblast and granulosa cells, might be a mediator of this inhibition. Interestingly, activation of NF-κB by either forced expression of the p65 subunit or NF-κB-inducing kinase up-regulated ArPII activity. Positive regulation of aromatase by endogenous NF-κB was also suggested by the fact that NF-κB-specific inhibitors suppress basal activity of the aromatase gene. A concomitant formation of high-order complex between NF-κB p65 and ArPII was also observed by chromatin immunoprecipitation assay. Although activation of PPARγ and RXR affected endogenous expression levels of neither inhibitory κBα nor p65, it impaired the interaction between NF-κB and ArPII and the p65 based transcription as well. Altogether, these results indicate that activation of a nuclear receptor system, constituted by PPARγ and RXR, down-regulates aromatase expression through the suppression of NF-κB-dependent aromatase activation and thus provide a new insight in the mechanism of regulation of the aromatase gene. (*Endocrinology* 146: 85–92, 2005)

THE BIOSYNTHESIS OF estrogens is catalyzed by the enzyme complex referred to as aromatase cytochrome P-450, which aromatizes the A ring of C19 androgens to the phenolic A ring of C18 estrogens, resulting in loss of the C19 angular methyl group as formic acid (1). In humans, aromatase is present in many tissues, including ovary (2, 3), testis (4, 5), placenta (2), and brain (6, 7). The gene encoding the aromatase (*CYP19*) is extraordinarily long (more than 120 kb), with a coding region of approximately 30 kb, containing nine translated exons (II-X). One reason for this long gene is that the transcription of aromatase in different tissue is regulated by different promoters (8) (ovary: promoter II; placenta: promoter I.1; and adipose tissue: promoter I.4). The aromatase promoter II (ArPII) functions in the ovary under the control of FSH. In cooperation with Ad4BP/SF-1, FSH, via the cAMP-protein kinase A (PKA) pathway, stimulates aromatase gene expression in the ovary through promoter II.

It has been determined that estrogens contribute to the growth and development of some estrogen-dependent neoplasm, including breast, endometrial cancers, and some ovarian cancers (9, 10). Estrogens, especially those produced locally in the adipose stoma cells, exert a definite role in stimulating proliferation of breast tumor cells (11). In normal breast adipose tissue, the estrogen-producing aromatase gene is driven by a distal promoter I.4 (8), whereas in breast adipose tissue containing a tumor, there is a switch in the promoter, whereby the aromatase expression is regulated through the proximal promoter II. This shift results in elevated aromatase expression in the tumor or surrounding breast adipose tissue and subsequently elevated production of estrogen in local breast adipose tissue, thus leading to the development of breast cancer (12–16). These findings highlight the importance of promoter II, especially in breast cancer.

Peroxisome proliferator-activated receptor (PPAR)-γ is a nuclear receptor that has an essential role in adipogenesis and glucose homeostasis in response to its ligands, which are either naturally existing ligands like 15-deoxy-$\Delta^{12,14}$ prostaglandin J2 or synthetic thiazolidinediones. Besides relatively

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Abbreviations: APDC, Ammonium pyrrolidinedithiocarbamate; ArPII, aromatase promoter II; CAPE, caffeic acid phenethyl ester; ChIP, chromatin immunoprecipitation; CMV, cytomegalovirus; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; IκBα, inhibitory κBα; LG, LG100268; NF-κB, nuclear factor-κB; NIK, NF-κB-inducing kinase; NS, normal saline; PKA, protein kinase A; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; SDS, sodium dodecyl sulfate; TGZ, troglitazone.

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well-known PPARγ-expressing tissues like adipose tissue, adrenal gland, and spleen (17–19), ovary (20) and granulosa cells (21, 22) also express an abundant amount of PPARγ, whose physiological role in these tissues is largely unknown. We previously reported that the PPARγ ligand, troglitazone (TGZ), especially together with the retinoid X receptor (RXR) ligand, LG100268 (LG), dose-dependently inhibits aromatase activity in granulosa cells (21, 23, 24).

In the present study, we extended our study to clarify the underlying mechanism whereby activation of a nuclear receptor system constituted by PPARγ and RXR down-regulates the aromatase gene. Herein we report an involvement of the transcriptional factor nuclear factor-κB (NF-κB) in the above mechanism as well as its importance in the regulation of aromatase expression through promoter II.

Materials and Methods

Materials

TGZ and LG were obtained from Sankyo Pharmaceuticals (Tokyo, Japan), and Ligand Pharmaceuticals Inc. (San Diego, CA), respectively. Caffeic acid phenethyl ester (CAPE), forskolin, and TNFα were all purchased from Sigma-Aldrich (St. Louis, MO). Ammonium pyrrolidinedithiocarbamate (APDC) was purchased from Wako (Osaka, Japan). All the above compounds (except CAPE and TNFα, which were dissolved in 50% ethanol and normal saline, respectively) were dissolved in dimethyl sulfoxide (DMSO), and the final concentration of solvents (DMSO, 50% ethanol or normal saline) in the cell growth medium was 0.1% (vol/vol). An equal volume of solvents was added to control cultures during cell treatment with chemicals.

Cell culture

We established a human ovarian granulosa-like tumor cell line, KGN, from a 63-yr-old female patient with invasive granulosa cell carcinoma (25). The cells grew as an adherent monolayer with stable proliferation. The cells possess properties similar to those of normal granulosa cells, including the expression of functional FSH receptor and a relatively high aromatase activity, which is PKA dependent (25). The cells were maintained in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) in an atmosphere of 5% CO_2 at 37 C. NIH-3T3 cells were purchased from the Japanese Cell Research Bank (Tokyo) and maintained in DMEM (high glucose) supplemented with 10% FBS at 37 C.

Aromatase assay

The aromatase activity was determined by measuring the [^{3}H]H_2O released on conversion of [1β-^{3}H]androstenedione to estrone, as described previously (21). The cells were precultured in 6-well plates in DMEM/F12 with 5% dextran-coated, charcoal-treated FBS for 48 h before treatment with chemicals. After the cells were treated with TGZ+LG, [1β-^{3}H]androstenedione was added, and the cells were then further incubated for 6 h. In the case of combined treatment with the NF-κB inhibitors, CAPE or APDC was added to cultures 2 h before an 8-h treatment with TGZ+LG. A second-round treatment consisting of 2 h of CAPE (or ADPC) followed by 8 h of TGZ+LG was carried out before addition of [1β-^{3}H]androstenedione. Extraction of medium (2.0 ml) and measurement of radioactivity in [^{3}H]H_2O for aromatase activity were done as described previously (21). The amount of radioactivity was then standardized by protein concentration, which was determined using a micro-BCA kit (Pierce Chemical Co., Rockford, IL) and expressed as picomoles per milligram protein per 6 h.

Plasmid constructions

The 4.0-kb ArPII was amplified by PCR from genomic DNA. After confirmation of the entire sequence by direct sequencing, the fragment was subcloned into PGL3-Basic vector (Promega, Madison, WI) to make the luciferase reporter plasmid PGL3-ArPII, in which the luc+ gene is driven by the 4.0-kb fragment of human ArPII. To construct the NF-κB luciferase reporter plasmid, pGL3-tk was first constructed by cloning the −109 to +37 region of the herpes virus thymidine kinase promoter into the *Bgl*II and *Hin*dIII sites of the pGL3-basic vector (Promega). A pair of oligonucleotides, 5′-TGGAAATTCCTGGAAATTCCTGGAAATTCC-3′ and 5′-TCGAGGAATTTCCAGGAATTTCCAGGAATTTCCA-3′, were annealed together, thus resulting in double-stranded oligonucleotides with both a blunt end and a *Xho*I compatible overhang, which were then ligated into the *Sma*I and *Xho*I sites of tk-Luc, thus giving rise to pGL3-NF-κB containing three copies of the NF-κB sites. The *Renilla* luciferase reporter plasmid phRL-cytomegalovirus (CMV), serving as an internal control in the dual-luciferase reporter assay, was purchased from Promega. Human p65 expression vector, pcDNA-p65, was provided by Dr. C. Scheidereit (Max Delbruck Center for Molecular Medicine, Berlin, Germany). pcDNA-NF-κB-inducing kinase (NIK) was provided by Dr. D. Wallach (Department of Biological Chemistry, The Weizmann Institute of Science, Rehovot, Israel). All plasmids were prepared from an overnight bacterial culture using the QIAfilter plasmid maxikit (Qiagen, Valencia, CA).

Relative luciferase reporter assay

For the relative luciferase reporter assay, 1.5 × 10^5 cells/well in 1 ml growth medium were seeded into 12-well plates, and 0.8 μg of PGL3-ArPII (or PGL3-NF-κB) and 2.0 ng of phRL-CMV were transiently cotransfected in each well using the Superfect transfection reagent (Qiagen) following the manufacturer's protocol. In the case of cotransfection, 0.15 μg of expression vector for p65 (pcDNA-p65) or NIK (pcDNA-NIK) was also added; the total amount of plasmid DNA added to each well was equalized using the empty vector: pcDNA-3.1. Twenty-four hours after transfection, the cells were treated with TGZ+LG for 24 h at the concentrations indicated in each figure. The cells were then lysed in 100 μl/well passive lysis buffer, and the luciferase assay was performed in accordance with the protocol of the dual-luciferase reporter assay system, using a Lumat LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). The firefly luciferase activity, produced by PGL3-ArPII in identically treated triplicate samples, was normalized for the *Renilla* luciferase activity produced by phRL-CMV. The data shown are representative of at least three independent experiments. In the case of cotreatment with the NF-κB inhibitors, cells were preincubated for 2 h with CAPE (working concentration 20 μg/ml) or APDC (working concentration 100 ng/ml) and then incubated for 10 h with TGZ+LG. Another round of 2 h of CAPE plus 10 h of TGZ+LG was carried out before the cells were lysed for luciferase assay.

Western blotting

NIH-3T3 and KGN cells treated with either TGZ+LG or DMSO were grown to subconfluent phase, washed with PBS, and actively lysed in 500 μl lysis buffer. Samples were subjected to electrophoresis on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were incubated with either a rabbit polyclonal antibody against the p65 subunit of NF-κB [NF-κB p65 (c-20): sc-372, Santa Cruz Biotechnology, Santa Cruz, CA] or a rabbit polyclonal antibody against inhibitory κBα (IκBα) (c-21: sc-371, Santa Cruz Biotechnology) and subsequently with a horseradish peroxidase-linked goat antirabbit IgG secondary antibody (Cell Signaling Technology, Beverly, MA). Detection was carried out using the ECL+Plus Western blotting detection system (Amersham Biosciences, Buckinghamshire, UK). Membranes were then visualized using a STORM 860 scanner (Molecular Dynamics, Sunnyvale, CA). Images were finally analyzed using ImageQuant software (Molecular Dynamics).

ChIP assays

These were performed by the chromatin immunoprecipitation (ChIP) assay kit (Upstate Biotechnology, Lake Placid, NY), according to the protocol provided by manufacturer with some modifications. Briefly, KGN cells were seeded in 10-cm^2 dishes and treated overnight with 10 μM TGZ + 1 μM LG or the solvent DMSO. After an additional treatment of 10 ng/ml TNFα or its solvent normal saline (NS) for 1 h, cells were cross-linked with 1% formaldehyde for 60 min, washed with chilled PBS, resuspended in 200 μl SDS lysis buffer, and sonicated six times for 10