

LH, Non-SHBG Testosterone and Estradiol Levels during Testosterone Replacement of Hypogonadal Men: Further Evidence that Steroid Negative Feedback Increases as Men Grow Older

Stephen J. Winters¹ and Chenxi Wang² for the Fortigel Study Group

¹Division of Endocrinology, Metabolism and Diabetes, and ²Department of Epidemiology and Population Health

University of Louisville

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Corresponding author: Stephen J. Winters, M.D.

Division of Endocrinology, Metabolism and Diabetes
University of Louisville
ACB-3G11, 550 Jackson Street
Louisville, KY 40202, USA
Telephone 1-502 852-5237
FAX 1-502 852-4978
Email: sjwint01@louisville.edu

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Abstract

Previous evidence suggests that the testicular negative feedback control of GnRH-LH secretion may change as men age, and may thereby contribute to the hypogonadism that occurs as men grow older. To pursue this idea, we analyzed the results for 35 men with primary hypogonadism who were participants in an open-label multicenter study of testosterone replacement. LH, SHBG and total and free T and E concentrations were measured in blood samples at baseline and after 2% testosterone gel was applied daily for 2 wks. A 24h pharmacokinetic profile for T and E was obtained at the end of week 2. Age was a strong predictor of LH suppression during testosterone replacement ($r = -0.46$), and the effect could not be explained by obesity, SHBG or higher levels of total or non-SHBG testosterone or estradiol during treatment. In fact, both LH and non-SHBG testosterone levels were lower ($p < 0.05$) in older men receiving testosterone treatment. In addition, the strongest association overall was between the percent decline in LH and non-SHBG estradiol concentrations ($r = -0.39$). These data provide further evidence that suppression of LH secretion during testosterone treatment is greater as men age, and are consistent with the hypothesis that the hypogonadism of aging men is partly due to a change in gonadotropin negative feedback regulation. These results further suggest that estrogen receptor signaling might contribute to this effect.

Introduction

Testosterone deficiency is common as men grow older (Gray, et al., 1991, Harman, et al., 2001). Testosterone levels peak between ages 20 and 40 years, and then decline gradually by approximately 1% per year (Belanger, et al., 1994, Litman, et al., 2006). Sex hormone binding-globulin (SHBG) levels increase with aging so that the aging-related decline in non-SHBG testosterone concentrations, and by inference testosterone production, is more pronounced than is the change in total testosterone levels (Baker, et al., 1976, Mitchell, et al., 1995).

The mechanism for the decline in testosterone as men age is incompletely understood.

While there is evidence for a reduction in Leydig cell number and steroidogenic function (Mulligan, et al., 2001, Neaves, et al., 1984, Tenover, et al., 1987) and LH levels tend to increase with aging, LH levels generally remain within the normal range (Gray, Feldman, McKinlay and Longcope, 1991, Morley, et al., 1997) suggesting that GnRH-LH secretion is also altered (Deslypere, et al., 1987, Harman, et al., 1982, Veldhuis, et al., 1992, Winters and Troen, 1982). Results from several studies support the idea that GnRH secretion is reduced partly because of an aging-related increase in androgen negative feedback. Studies in which testosterone or dihydrotestosterone (DHT) were administered continuously iv (Winters, et al.,

1984), testosterone was administered as a patch (Winters and Atkinson, 1997), or men were treated with a DHT gel (Deslypere, Kaufman, Vermeulen, Vogelaers, Vandalem and Vermeulen, 1987) all found more effective suppression of LH in older men with no reduction in responsiveness to GnRH. Other studies, however, have concluded that testosterone negative feedback is either reduced (Muta, et al., 1981, Veldhuis, et al., 2004) or unaffected (Bhasin, et al., 2005, Liu, et al., 2006) by aging, whereas one study found greater LH suppression among older men during low-dose but less suppression during higher-dose testosterone enanthate treatment (Gentili, et al., 2002).

According to the free hormone hypothesis (Mendel, 1989), steroids bound to SHBG are unable to enter cells to regulate receptor signaling. Because SHBG levels increase as men age, analysis of non-SHBG steroid levels may provide additional insight into the testicular steroid control of LH secretion that exceeds the information provided by total steroid hormone levels. In this study we examined LH concentrations at baseline and after two weeks of testosterone replacement with a 2% testosterone gel in men with primary hypogonadism, and related the change in LH levels with increasing subject age to circulating levels of total and non-SHBG testosterone and estradiol. The findings provide further support for the idea that the gonadotropin disturbance in aging men is partly due to an alteration in the responsiveness of hypothalamic-pituitary unit to regulation by sex steroids.

Methods

Subjects

Subjects were participants in a multicenter open-label study of testosterone replacement for hypogonadal men using a 2% testosterone gel (Tostrex, ProStrakan Pharmaceuticals, Galashiels, UK). Men were excluded if they had a history of malignancy, marked benign prostatic hypertrophy, sleep apnea, or uncontrolled diabetes, or if they were being treated with androgens, or with antiandrogens or alpha receptor antagonists for urinary dysfunction. The protocol was approved by the Institutional Review Committees of the participating institutions. Subjects provided written informed consent, and received a stipend for participating in the study. The investigators who enrolled subjects are listed in the Appendix.

The overall study enrolled 201 men who had a screening testosterone level below 250 ng/dl or two values less than 300 ng/dl. These values were selected by the industry sponsor. Of the 201 men, 35 men with LH levels that exceeded the laboratory reference range for LH (greater than 11 IU/L) are the subjects of this report. Men with elevated LH levels were selected for study to allow for an accurate assessment of the suppression of LH at week 2 whereas men with normal LH levels at baseline often had undetectable

LH levels after 2 wks of testosterone treatment. Participants were 22-73 years of age, and their body mass index ranged from 24-37.1 kg/m². One man with morbid obesity (BMI of 45.6 kg/m²) was excluded. Blood samples were drawn in the morning on day 0 prior to the first treatment dose. Subjects then applied 3 gm of 2% testosterone gel each morning to the inner thighs. On day 14, blood was drawn for the measurement of LH and SHBG at time 0, the gel was applied, and blood samples were drawn for testosterone determination at 0, 0.5, 1, 2, 4, 6, 8, 10, 12, 15 and 24 h, and estradiol was measured in the samples drawn at 0, 2, 4, 6 and 24h, according to the industry protocol.

Assays

Immunoassays were performed at Esoterix Center for Clinical Studies (Calabasas Hills, CA, USA). Testosterone was measured by immunoassay after extraction from serum using hexane: ethyl acetate (90:10). The intra-assay variation of assay pools was <9.4%, and the between assay variation was <8.8%. Samples with >5% deviation between replicates were re-assayed. SHBG was measured with an immunoradiometric assay using a pair of monoclonal antibodies. The within- and between-assay coefficients of variation were <12% and <14%, respectively. Estradiol levels were measured by RIA after extraction of the samples (1ml) with hexane:ethyl acetate followed by LH-20 column chromatography. Samples with less than 57% recovery were re-assayed. The estradiol level at baseline was ≤ 5 pg/mL in 11/35 men (31%). The intra- and interassay coefficients of variation were <15% and <16%. LH and FSH were assayed by immuno-chemiluminescence using paired monoclonal antibodies. The within assay coefficient of variation across the reference range were <10%, and the between assay precision was below 10% for LH and 22% for FSH. Non-SHBG-testosterone and estradiol levels were calculated from the levels of testosterone or estradiol and SHBG, as described by Sodergard et al (Sodergard, et al., 1982). The K_D for testosterone for SHBG was defined as 1 x 10⁹ L/M, and for albumin, 3 x 10⁴ L/M. The constants used to calculate the level of non-SHBG-estradiol were 6.8 x 10⁸ L/M for SHBG, and 6 x 10⁴ L/M for albumin. The albumin concentration was fixed at 4.0 gm/dl.

Data Analysis

Results are presented as the Mean ± SEM. Differences among groups were determined by ANOVA and post-hoc Fisher's test. The change in LH level from day 1 to day 14 was determined using the paired t-test. The association between the percentage change in LH and the AUC for non-SHBG-testosterone and non-SHBG-estradiol was evaluated further by linear regression analysis with adjustment for age and BMI. The area under the curve (AUC_{24h}) was calculated using Graph Pad Prism software (La Jolla, CA).

Results

The clinical characteristics and serum hormone levels for the study subjects at baseline are shown in Table 1. Eight of the 35 men were older than 65 yrs, and 8 men were obese (BMI >30 kg/m²). Based on the entry criteria, LH and FSH levels were elevated, and testosterone levels were low when compared to values in normal young men.

Linear regression analysis revealed that SHBG levels at baseline increased slightly with increasing age ($r = 0.28$; $p > 0.05$), and decreased slightly ($r = -0.26$; $p > 0.05$) with increasing BMI. LH levels at baseline were inversely related to both total ($r = -0.28$) and non-SHBG-testosterone ($r = -0.37$) as well as to total ($r = -0.19$) and non-SHBG-estradiol ($r = -0.25$). The significant relationship ($p = 0.032$) between non-SHBG-testosterone and LH became non-significant after controlling for age ($p = 0.537$) or BMI ($p = 0.597$). There was a positive association between SHBG and LH levels, but it was not significant ($r = 0.10$).

Testosterone replacement for two wks increased testosterone levels 5-fold from 144 ± 15 to 723 ± 54 ng/dL ($p < 0.01$) for the group as a whole, and non-SHBG-testosterone increased 4.2-fold from 76.1 ± 8.7 to 318 ± 47.9 ng/dL. Estradiol levels rose 2.9-fold from 12.5 ± 1.8 to 36.2 ± 2.6 pg/mL ($p < 0.01$) due to bioconversion of the administered testosterone, and non-SHBG-estradiol levels rose 2.7-fold from 7.4 ± 1.1 to 19.7 ± 1.6 pg/mL ($p < 0.01$). SHBG levels were slightly but not significantly lower during testosterone treatment (53.4 ± 5.9 vs 57.3 ± 6.7 nmol/L; $p = 0.41$).

Mean LH levels during testosterone replacement decreased 28 percent ($p < 0.05$) from 22.5 ± 1.7 to 16.3 ± 2.5 IU/L, and FSH levels fell 35 percent from 35.2 ± 3.9 to 23.0 ± 3.3 IU/L ($p < 0.05$). The linear regression shown in Figure 1 reveals that the percent change in LH from baseline during testosterone replacement was inversely related to age ($r = -0.46$; $p < 0.01$).

To further analyze the impact of age on LH secretion, the subjects were divided into three identically sized subgroups (tertiles) on the basis of age. Figure 2 reveals that LH levels were reduced significantly during testosterone treatment in age tertiles II and III but there was no significant decline in LH in men in age tertile I.

Variables that might have influenced responsiveness to testosterone are summarized in Table 2. BMI was similar among men in the three age tertiles. SHBG levels at baseline were higher in middle aged and older men than in younger men, and SHBG was unaffected by testosterone replacement in all groups. 24h mean total testosterone levels at baseline and during testosterone replacement were similar in the

three groups. While there was a trend suggesting higher estradiol levels with age at baseline, this was not statistically significant. Non-SHBG testosterone and estradiol levels at baseline were unrelated to age. During testosterone replacement, however, non-SHBG-testosterone levels were significantly lower in men in tertiles II and III than in tertile I whereas non-SHBG estradiol levels were similar in all age groups.

Because variation in steroid hormone absorption and clearance among subjects might influence circulating hormone levels and thereby suppression of LH, we also related the change in LH to the area under the curve for total and free testosterone and estradiol on day 14. As shown in Table 3, the lack of change in LH during testosterone treatment in younger men was not explained by lower circulating steroid levels since the area under the curve for total testosterone, as well as total and non-SHBG-estradiol at week 2 was similar among all three groups. In fact, the area under the curve for non-SHBG testosterone, like the 24h geometric mean value, was lower in older men ($p=0.03$).

Because of the importance of the bioconversion of testosterone to estradiol in the feedback regulation of LH in men, we next compared the change in LH with the AUC for total and non-SHBG testosterone and estradiol (Figure 3). Overall, the percent change in LH was more strongly correlated to the AUC for non-SHBG-estradiol ($r = -0.39$; $p < 0.025$) than to total estradiol ($r = -0.29$; $p > 0.05$), and did not correlate negatively with either total or non-SHBG-testosterone. We also tested the association between the (AUC) non-SHBG-estradiol levels on day 14 with the LH change by multiple linear regression. For each 100 pmol-hr/L increase of non-SHBG-estradiol, LH decreased 2.7% ($p = 0.03$). The strength of this association did not change after adjustment for both age and BMI.

Discussion

The results from this study of men with primary testicular insufficiency provide further evidence that LH secretion is more effectively suppressed during testosterone replacement in middle age and older men than in young men. There is substantial evidence that testosterone regulates GnRH secretion in men (Finkelstein, et al., 1991, Kawakami and Winters, 1999, Pitteloud, et al., 2008), and experimental evidence that androgens and estrogens regulate kisspeptin neurons upstream of GnRH (Smith, et al., 2006). This mechanism may be altered with aging.

LH suppression in older men was greater in spite of higher SHBG levels. Greater LH suppression is unlikely to be due to an aging-related decline in testosterone clearance (Baker, Burger, de Kretser,

Hudson, O'Connor, Wang, Mirovics, Court, Dunlop and Rennie, 1976, Morimoto, et al., 1980), however, since non-SHBG-testosterone levels during testosterone replacement were lower, rather than higher, in older men. On the other hand, older men who were treated with a GnRH analog to suppress LH and endogenous testosterone production, and were replaced with testosterone enanthate, had higher total testosterone levels than did younger men (Coviello, et al., 2006). The reduced non-SHBG testosterone but similar total testosterone levels in older men in our study underscores the usefulness of calculating non-SHBG testosterone levels when evaluating the results of testosterone replacement therapies. The circulating level of testosterone during testosterone treatment represents the sum of the testosterone administered and that which is produced by the testes. Thus the most parsimonious explanation for lower LH and non-SHBG-testosterone levels in older men during testosterone replacement was enhanced LH suppression resulting in less endogenous testosterone production. While an aging effect on the absorption into the circulation of drugs applied transdermally would influence our results, this has not been found (Kaestli, et al., 2008).

Non-SHBG testosterone levels during testosterone replacement were lower with increasing age whereas non-SHBG estradiol levels were unchanged. These findings suggest an increase in aromatase activity with aging, a finding which could be due to the increase in adipose tissue as men grow older (Baumgartner, et al., 1988). Most studies have reported no change in circulating estradiol levels as men age (Araujo, et al., 2008, Belanger, Candas, Dupont, Cusan, Diamond, Gomez and Labrie, 1994) although decreases have also been found (Denti, et al., 2000). The level of endogenous estradiol is partly dependent on the production of testosterone substrate, but in our study all men received the same dose of testosterone.

SHBG levels are elevated in patients with testicular failure (Wieland, et al., 1980), and increase as normal men grow older (Vermeulen, et al., 1996). Testosterone replacement appears to lower the level of SHBG partly by a post-transcriptional mechanism inasmuch as testosterone treatment of orchietomized monkeys decreased circulating SHBG levels while SHBG mRNA expression levels in the liver were increased (Kottler, et al., 1990). In the current study, SHBG levels were higher in older men with testicular insufficiency at baseline whereas non-SHBG testosterone levels were comparable in all age groups. These findings imply that the rise in SHBG with aging is not solely due to low testosterone levels.

Suppression of LH secretion during testosterone treatment in the study group as a whole was most strongly associated with the circulating level of non-SHBG-estradiol. The importance of estradiol in the negative feedback regulation of gonadotropin secretion in men is well established (Sherins and Loriaux,

1973). This control mechanism is supported by the increase in circulating LH and FSH levels as well as plasma testosterone concentrations that occurs when estrogen receptor- α signaling is blocked (Winters and Troen, 1985) or estradiol production is suppressed by aromatase inhibitors (Marynick, et al., 1979) (Hayes, et al., 2000), and by the elevated gonadotropin and testosterone levels in a man with an inactivating mutation in the aromatase gene (Morishima, et al., 1995). Furthermore, estradiol replacement alone normalized serum gonadotropins in men treated with the aromatase inhibitor letrozole (Raven, et al., 2006). Because the portion of circulating estradiol that is calculated to be SHBG-bound is relatively small (Dunn, et al., 1981), most studies have related changes in LH to total circulating estradiol levels. The stronger relationship between the change in LH and non-SHBG-estradiol than with total estradiol levels in this study suggests, however, that SHBG binding of estradiol is important to the testicular feedback control of gonadotropin secretion. Similarly, many studies have shown that non-SHBG-estradiol is a better predictor of bone mineral density in older men than is total serum estradiol (Khosla, et al., 2002, van den Beld, et al., 2000). Our results suggest that non-SHBG estradiol levels provide useful information when assessing the impact of testosterone replacement.

One limitation of this study is the sample size. Therefore, we did not further adjust for other potential confounders such as the non-SHBG-testosterone and -estradiol levels at baseline.

Second, while a highly sensitive and specific immunoassay was used to measure estradiol, the accuracy of all estradiol immunoassays in men has been questioned, and the biological activity of the calculated non-SHBG-estradiol value is assumed, but not proven. Third, only single blood samples were available for LH before and during testosterone treatment, and frequent sampling for LH pulse analysis would have been substantially more informative. Finally, samples from individual subjects were not assayed in a single assay, and overall results may have been influenced slightly by between-assay variation.

In conclusion, we found that LH secretion in men with primary testicular insufficiency replaced with testosterone is more effectively reduced in older men, producing lower levels of non-SHBG testosterone in this group, and that the strongest predictor of the change in LH was the level of non-SHBG estradiol. These data provide further evidence for an age-related alteration in testosterone feedback control of GnRH-LH, and suggest that estrogen receptor signaling could contribute to this effect.

Appendix

U.S. Fortigel * Study Group (ProStrakan, Galashiels UK): John Agaiby, Gurnee, IL, Bruce D. Brazina, Tacoma, WA, Adrian S. Dobs, Baltimore, MD, Michael Godschalk, Richmond, VA, Elizabeth E. Houser, Austin, TX David W. Jones, Johnson City, TN Mark S. Kipnes, San Antonio, TX, Richard K. McDavid, Johnson City, TN, Harris H. McIlwain, Tampa FL, A. Wayne Meikle, Salt Lake City, UT, David D. Michie, Fort Myers, FL, A. Ola Odugbesan, Atlanta, GA, Horace M. Perry, St. Louis, MO, Jorge Pino, Birmingham, AL, Michael Reeves, Chattanooga, TN, Stephan C. Sharp, Nashville, TN, Ferril C. Smith, Austin, TX, Peter J. Snyder, Philadelphia, PA, Kevin Marion Tomera, Anchorage, AK, Richard L. Weinstein, Walnut Creek, CA, Norman Spencer Welch, Atlanta, GA, Stephen J. Winters, Louisville, KY.

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Table 1. Clinical characteristics and baseline hormone levels in men with primary testicular failure

	n=35	Normal Male Range
Age (yrs)	53.2 ± 2.4	
BMI (kg/m ²)	28.4 ± 0.77	
Testosterone (ng/dL)	144±15	290-1030
SHBG (nmol/L)	57.3±6.7	24-78
Estradiol (pg/mL)	12.5±1.8	< 36
Non-SHBG testosterone (ng/dL)	76.1±8.7	ND
Non-SHBG estradiol (pg/mL)	7.4±1.1	ND
LH (IU/L)	22.5±1.7	1.5-11
FSH (IU/L)	35.2 ± 3.9	2.0-9.2

Data are Mean ± SEM

Normal ranges were provided by the reference laboratory; ND, not determined.

Table 2. BMI, SHBG, total and free testosterone, and estradiol at baseline and after two weeks of treatment with 2% testosterone gel each day, by age tertiles.

	Tertile I n=12		Tertile II n=11		Tertile III n=12	
Age (yrs)	<46		47-62		>62	
BMI (kg/m ²)	27.6 ± 1.1		28.6±1.4		29.2±1.7	
	Baseline	Wk 2	Baseline	Wk 2	Baseline	Wk 2
SHBG (nmol/L)	37.5±4.2	40.2±5.4	69.8±10.6 ^b	58.0±9.6	65.5±15.5 ^b	65.0±14.0
Total Testosterone ng/dL	125±24	748±115 ^a	142±24	749±115 ^a	136±31	661±17 ^a
Non-SHBG testosterone ng/dL	77.0±16	480±111 ^a	74.7±14.5	258±51.1 ^{ac}	78.9±0.53	190±20 ^{ac}
Estradiol (pg/ml)	11±3	32±4 ^a	11±2	42±4 ^a	16±4	34±5 ^a
Non-SHBG Estradiol (pg/ml)	7±2	20±3 ^a	5±1	22±3 ^a	10±2	16±3 ^a

Data are mean ± SEM

a, p<0.05 vs baseline by paired t-test

b, p< 0.05 vs tertile 1, ANOVA and Fisher's test

c, p< 0.05 vs tertile 1, ANOVA and Fisher's test

Table 3. Area under the curve over 24h for total and free testosterone and estradiol levels on day 14 of treatment with 2% testosterone gel each day, by age tertiles.

	Tertile I	Tertile II	Tertile III
Age (yrs)	<46	47-62	>62
Total Testosterone (nmol-hr/L)	526±70	523±62	557±111
Non-SHBG testosterone (nmol-hr/L)	266 ± 44	194 ± 28	154 ± 17 ^a
Total Estradiol (pmol-hr/L)	2114±280	2503±210	2361±304
Non-SHBG Estradiol (pmol-hr/L)	1317±209	1361±165	1128±150

Data are mean ± SEM

^ap=0.03 vs tertile 1, ANOVA and Fisher's test.

Figure Legends

Figure 1. Relationship between the percent change in serum LH levels and age in men treated with a 2% testosterone gel for 14 days.

Figure 2. Mean serum LH levels before and after 2 wks of testosterone treatment in men by age tertile.

Figure 3. Relationship between the percent change in serum LH levels from baseline and the area under the curve for total and non-SHBG-testosterone or estradiol in men on day 14 of treatment with a 2% testosterone gel.

Figure 1

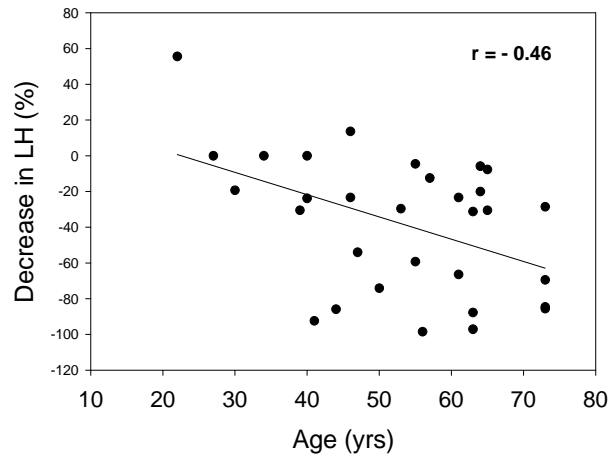


Figure 2

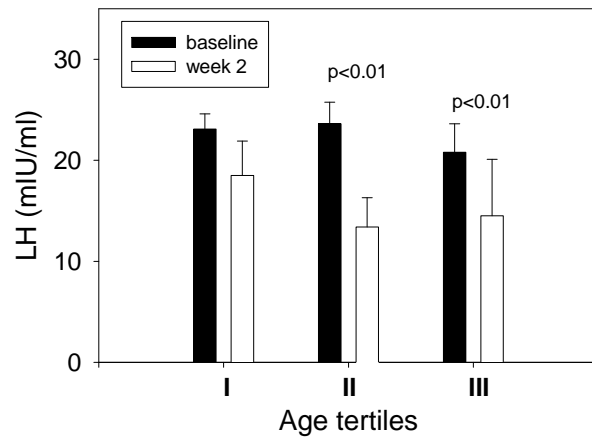


Figure 3

