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ABSTRACT: Serum androgens decline with age in normal men, despite normal or elevated bioactive serum gonadotropins, suggesting that primary testicular dysfunction occurs with aging. The authors further assessed the question of age-related testicular dysfunction by evaluating whether raising serum gonadotropins above the normal serum range for an extended time in healthy elderly men might result in bringing their gonadal function to a level similar to that found in young adult men. Five elderly (65 to 85 years old) and five young adult men (26 to 33 years old) were given 50 mg of clomiphene citrate (CC) twice a day for 8 weeks to stimulate gonadotropin production. During that time, testosterone (T), non-sex hormone-binding globulin bound T, and estradiol increased significantly in both age groups, while serum inhibin increased significantly only in the young adult men. The increases in serum androgens with CC administration were significantly greater in the young adult men than in the elderly men. These hormone changes occurred in the setting of serum gonadotropins that increased significantly in both age groups, although there was a tendency for the elderly men to have a smaller increase in luteinizing hormone. Despite 8 weeks of stimulation of the pituitary-gonadal axis by CC administration, the elderly men demonstrated significantly diminished testicular responses compared with the young adult men. Sertoli cell function, as determined by inhibin production, was more diminished in the elderly men than was Leydig cell function. These data strengthen the hypothesis that normal aging in men is accompanied by a decline in testicular function.

Key words: Testes, inhibin, gonadotropins, age.
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Normal aging in men is accompanied by several markers of testicular decline, including a decrease in total serum testosterone (T) and a decrease in that portion of serum T not bound to sex hormone-binding globulin (nSHBG-T; Bremner et al, 1983; Moroz and Verkhratsky, 1985; Nankin and Calkins, 1986; Tenover et al, 1987). With aging, there are also decreases in Leydig and Sertoli cell masses (Johnson et al, 1984; Neaves et al, 1984), a reduced relative or absolute serum T response to human chorionic gonadotropin (Harman and Tsitouras, 1980; Longcope, 1973), and a decline in daily sperm production (Neaves, et al, 1984).

The changes in testicular morphology and function with age occur in the presence of increased or similar levels of bioactive and immunoreactive gonadotropins, as compared with those found in young adult men (Moroz and Verkhratsky, 1985; Tenover et al, 1987; Harman and Tsitouras, 1980; Deslypere and Vermeulen, 1984; Winters and Troen, 1982; Baker et al, 1976). There seem to be no major alterations in luteinizing hormone (LH) pulse frequency or amplitude with aging in men (Tenover et al, 1987; Winters and Troen, 1982), although one study reported a small, but significant, slowing of LH pulse frequency with age (Deslypere et al, 1987). In addition, one study, using clomiphene citrate (CC) as a method of *in vivo* stimulation of gonadotropin secretion, demonstrated that a short course of CC (7 days) led to similar increases in LH pulse frequency and amplitude in young and elderly normal men, but that serum T levels increased less in the elderly men (Tenover et al, 1987). All these studies suggest that testicular dysfunction is a major component of the age-associated changes in the hypothalamic-pituitary-testicular axis. It is not known, however, if the decrease in testicular response, especially in response to increased gonadotropin stimulation produced by compounds such as CC, is due only to a decreased maximal response of the aging testes or is due, in part, to a delay in the time needed to maximally respond to increased gonadotropin stimulation.

To assess this question, healthy young adult and elderly men were given 100 mg of CC daily for 8 weeks. Clomi-

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phene citrate was used because it has been shown to lead to an increase in serum gonadotropins, largely by its effect on the hypothalamus (Winters et al, 1979; Adashi, 1984). The effects of chronic CC administration on serum levels of sex hormones, gonadotropins, and pulsatile LH release were measured and compared between the two age groups.

Materials and Methods

Subjects

Five healthy older men (65 to 85 years old; mean age ± SE, 73.0 ± 3.8 years) and five healthy young adult men (26 to 33 years old; mean age, 29.2 ± 1.6 years) were recruited from the community as volunteers. The study protocol was approved by the Human Subjects Committee of the University of Washington, and participants gave informed consent before entering the study. All men were white, nonsmokers, nonabusers of alcohol, within 10% of ideal body weight, and taking no medication. All were healthy as determined by medical history, physical examination, complete blood count, urinalysis, and blood chemistry, and had normal sized testes (at least 3.6 × 2.2 cm).

Experimental Protocol

Before the inception of CC administration and at the end of 8 weeks of administration, the men were admitted to the Clinical Research Center of the University of Washington for frequent blood sampling. Blood was drawn every 10 minutes for 8 hours (8:00 or 9:00 AM to 4:00 or 5:00 PM) through an indwelling arm vein cannula as previously described (Tenover et al, 1987). The men were given 50 mg of CC twice a day for 8 weeks. Single blood samples were taken (between 8:00 and 10:00 AM) at the end of weeks 1, 2, 4, and 6 of CC administration. Blood was allowed to clot at room temperature, serum was separated, and the sample was frozen and stored at −20°C until assayed. Compliance with CC administration was monitored by pill counts at each visit.

Serum LH was measured in duplicate by radioimmunoassay (RIA) in all blood samples. Hourly blood samples from the 8-hour studies and blood from the single samples were measured in duplicate for T by RIA. Pools of the 8-hour studies were made by combining equal aliquots from each hour of the study, and these pools, plus the single samples, were assayed for estradiol, sex hormone-binding globulin (SHBG), inhibin, follicle-stimulating hormone (FSH) by RIA, and bioactive LH. All samples from one man were analyzed in the same assay for each hormone.

Hormone Assays

LH and FSH RIA—The RIAs for serum LH and FSH were described previously (Bremner et al, 1981) and use LER-907 as the reference standard. The limit of detectability of the LH assay was 3.2 μg/L and for the FSH assay was 12.5 μg/L. Intra-assay and interassay coefficients of variation for LH were 5.5% and 8.4%, respectively, and for FSH were 7.3% and 9.7%, respectively.

Testosterone and Estradiol—The RIAs for serum testosterone and estradiol also were described previously (Matsumoto et al, 1983a). Assay sensitivity for testosterone was 0.35 nmol/L and 29.4 pmol/L for estradiol. Intra-assay and interassay coefficients of variation were 5.1% and 9.8%, respectively, for testosterone, and 8.2% and 8.8%, respectively, for estradiol.

Sex Hormone-Binding Globulin (SHBG)—Serum SHBG was measured in duplicate by RIA using reagents provided by Farmos Diagnostica (Oulunsalo, Finland). Intra-assay and interassay coefficients of variation were 3.5% and 5.6%, respectively.

Non-SHBG-Bound Testosterone (nSHBG-T)—Serum nSHBG-T was calculated from the total molar concentration of testosterone and SHBG (using the RIA method) according to a modification of the mass action equation of Pearlman, as previously described (Plymate et al, 1981).

Inhibin—Serum inhibin was measured as previously described (Tenover et al, 1988a) in a heterologous double-antibody RIA using purified 31 kd bovine follicular fluid inhibin as tracer and antigen to generate antiserum. Recently, it was reported that both an α-subunit-derived dimeric protein (pro αC) and the inhibin α subunit cross-react in the inhibin RIA but show no inhibin bioactivity (Robertson et al 1989; Schneyer et al, 1990). How these or other cross-reacting substances might affect the ability of the RIA to discriminate true dimeric bioactive inhibin in human serum remains unclear. However, studies of the ratio of inhibin bioactivity to immunoreactivity in humans suggest that the interference of nonbioactive substances in the present inhibin RIA probably is slight (Robertson, et al 1988). Sensitivity of the assay was 100 U/L, with an interassay coefficient of variation of 10.2% and an intra-assay coefficient of variation (middle range of male quality control serum) of 3.3%.

LH Bioactivity—Bioactive LH (LH BIO) was measured in duplicate by the mouse Leydig cell *in vitro* bioassay (Matsumoto et al, 1983b) using LER-907 as the reference standard. Intra-assay and interassay coefficients of variation for the assay were 9.4% and 15.6%, respectively, and the assay sensitivity was 0.3 μg/L.

LH Pulse Analysis—Eight-hour LH pulse patterns were analyzed by a modification of the method of Santen and Bardin (1973). For each sampling series, measurement error was assessed on the basis of assay replicate variability, as determined by analysis of variance (ANOVA). An LH pulse was defined as an increase in the serum LH level from nadir to peak that was equal to or greater than four times the intra-assay coefficient of variation of assay replicates.

Statistical Analysis

Baseline value comparisons were made using Student's two-tailed unpaired t tests. Changes in hormone levels with time were analyzed using ANOVA with repeated measures.

Results

Table 1 shows the baseline pituitary and gonadal hormone values for the two age groups. Only serum inhibin was significantly different ($P < 0.05$) between the two groups at baseline, with lower levels seen in the elderly men. There was a tendency, however, for serum testosterone, nSHBG-T, and LH BIO to be lower and for the FSH RIA and estradiol to be higher in the elderly group.

With CC administration, serum FSH RIA, LH RIA, and

Table 1. *Baseline pituitary-gonadal hormone values in healthy young adult and elderly men*

	Young (n = 5)	Elderly (n = 5)
T (nmol/L)	18.0 ± 1.0	17.3 ± 1.0
nSHBG-T (nmol/L)	1.5 ± 0.2	1.1 ± 0.2
E_2 (pmol/L)	88.1 ± 14.7	102.8 ± 22.0
SHBG (μmol/L)	28.3 ± 1.1	35.0 ± 4.8
FSH RIA (μg/L)	150 ± 24	180 ± 30
LH RIA (μg/L)	32 ± 4	32 ± 3
LH BIO (μg/L)	359 ± 73	269 ± 70
Inhibin (U/L)	696 ± 63	465 ± 54*

Values are mean ± SE.
* $P < 0.05$ compared to young adults.

LH BIO increased significantly ($P < 0.001$) in both age groups (Fig 1). The stimulated levels of gonadotropins, especially LH, tended to be lower in the elderly group, but there was large individual variability and no statistical differences were found in either LH or FSH levels between the two age groups while on CC. In the elderly men, the mean maximal serum FSH RIA increased 49% above the baseline level to 268 ± 40 μg/L, which was 88% of the maximum serum FSH RIA level of 306 ± 71 μg/L obtained from the young adult men (Fig 1A). Mean serum LH RIA levels in the elderly men increased 169% above the baseline level to a maximum of 86 ± 20 μg/L, reaching 67% of the maximal response of 129 ± 32 μg/L seen in the young adult men (Fig 1B). Concomitantly, the maximum mean serum LH BIO level in the elderly men was 1,265 ± 81 μg/L, a 370% increase above the baseline level, but only 63% of the maximum LH BIO response of 2,001 ± 519 μg/L seen in the young adult men (Fig 1C).

Pulsatile LH characteristics were similar at the baseline level in the two age groups. The young adult men had an average of 4.7 ± 0.2 LH pulses/8 hours while the elderly men had 4.8 ± 0.2 LH pulses/8 hours. Mean baseline LH pulse amplitude was 15 ± 1 μg/L in the young and 16 ± 2 μg/L in the elderly men. After 8 weeks of CC administration, the mean LH pulse amplitude and number of LH pulses/8 hours increased significantly ($P < 0.05$) in both age groups. Although there was no statistically significant difference in these changes, the elderly men tended to have a lower average LH pulse amplitude (23 ± 5 μg/L) and mean pulse frequency (7.0 ± 0.5 pulses/8 hours) after CC administration than did the young adult men (mean LH pulse amplitude of 38 ± 9 μg/L and 8.4 ± 0.4 pulses/8 hours).

With CC administration, serum testosterone and nSHBG-T rose significantly ($P < .01$) in both age groups (Figs 2A and B). However, the changes in these serum androgens with time were significantly greater in the young adult men compared with the elderly men ($P < 0.0001$). By week 8 of CC administration, the maximal testosterone level (Fig 2A) achieved in the young adult group was 48.2

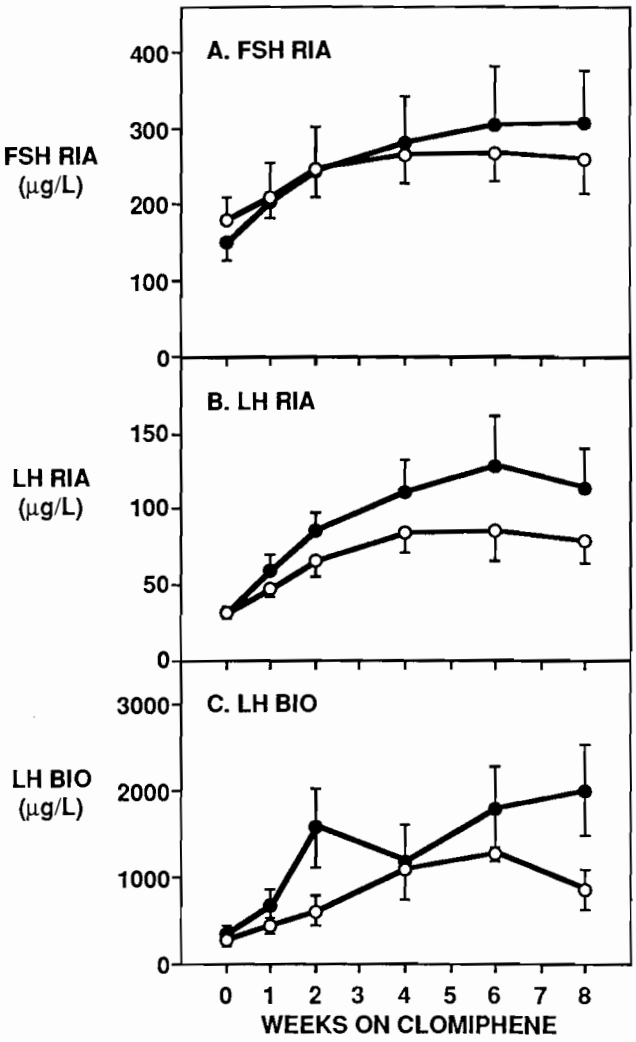

FIG. 1. Effect of clomiphene citrate administration on serum levels (mean ± SE) of FSH RIA (A), LH RIA (B), and LH BIO (C) in five young adult (filled circles) and five elderly (open circles) healthy men.

± 1.4 nmol/L (a 268% increase above baseline level), while the maximal testosterone level reached in the elderly group (week 6) was 34.3 ± 3.5 nmol/L, a 198% increase over baseline and 71% of the maximal value reached in the young adult group. The young adult men reached a maximal nSHBG-T level (Fig 2B) of 20.6 ± 3.2 nmol/L (1,410% increase) after 8 weeks of CC administration, while the elderly men reached a maximal level of 5.5 ± 2.4 nmol/L at week 4, representing a 512% increase over baseline but only 27% of the maximal response of the young adult group.

Serum estradiol levels increased significantly ($P < 0.001$) in both age groups with CC treatment (Fig 2C). Although the estradiol levels tended to increase more in the young adult group than in the elderly group, the changes in estradiol levels were not statistically different. In the young

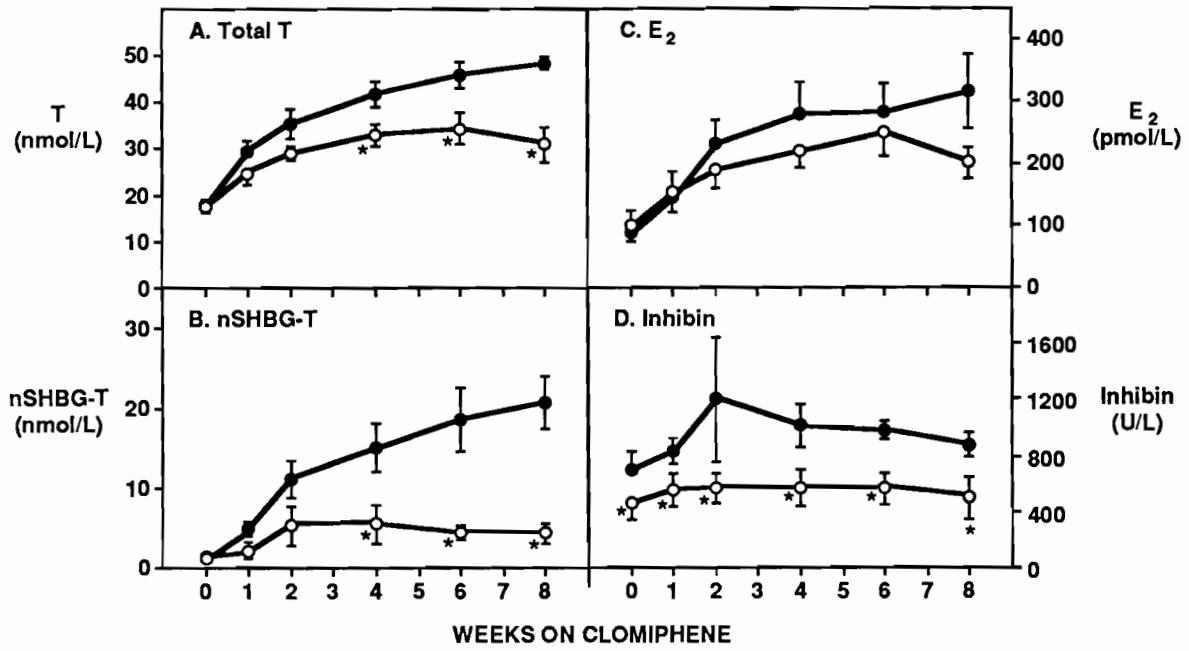

FIG. 2. Mean (± SE) serum levels of total testosterone (T; panel A), non-sex hormone-binding globulin bound-T (nSHBG-T; panel B), estradiol (E_2; panel C), and inhibin (panel D) in five young adult (filled circles) and five elderly (open circles) healthy men during 8 weeks of clomiphene citrate administration. *$P < 0.05$ compared to young adults.

adult men, serum estradiol levels increased to a maximum of 316 ± 59 pmol/L (360% of baseline) at week 8 of CC administration, while the maximum serum estradiol level achieved in the elderly men was 250 ± 37 pmol/L at week 6, representing a 196% increase over baseline levels.

Serum inhibin levels increased significantly ($P < 0.05$) with CC treatment only in the young adult men (Fig 2D). The maximum mean serum inhibin value reached in the young adult men was 1201 ± 200 U/L (week 2). Serum inhibin values in the elderly men also reached a maximum at week 2, but this level was 570 ± 45 U/L and not truly different from baseline levels. Serum SHBG levels did not increase over baseline values in either age group during the 8 weeks of CC administration.

Discussion

Using chronic (8-week) clomiphene citrate administration in both young adult and elderly healthy men to stimulate gonadotropins to reach serum levels well beyond those seen in the baseline state, we demonstrated that the elderly men tended to show a blunted LH response and significantly lower maximum serum levels of the predominant testicular hormones, testosterone and inhibin. In addition, even though LH and FSH serum levels increased with CC treatment in the elderly men, serum inhibin levels did not increase. These results strengthen the hypothesis that normal aging in men is accompanied by a decline in testicular function and suggest that Sertoli cell function, as determined by inhibin production, may be more affected by age than Leydig cell function.

Previous studies evaluating the response of young adult and elderly men to CC have used treatment periods of up to 1 week (Tenover et al, 1987; Winters and Troen, 1982; Tenover et al, 1988a). Other evidence suggests that more than 7 days may be required to achieve maximal testicular response to CC stimulation (Santen et al, 1971). The men in this study therefore were treated with CC for 8 weeks. For most hormones evaluated, nearly maximal serum levels appeared to be reached within the 8-week period, especially in the older group. With prolonged CC administration, the testicular hormone and gonadotropin serum levels obtained were much higher than in the 1-week stimulation studies, but the trend in changes and the relationship between the hormone levels in the elderly men were similar to the 1-week studies (Tenover et al, 1987, 1988a).

Even with 8 weeks of CC stimulation, the elderly men were never able to achieve serum testosterone levels that were as high as those of the young adult men. Since androgen clearance does not increase with normal aging (Baker et al, 1976; Vermeulen et al, 1972), the lower serum levels of androgens in the elderly men suggest decreased Leydig cell production. As previously reported with baseline and 1-week CC stimulation values, the differences between young and elderly men's androgens are more accentuated if nSHBG-T is evaluated rather than serum total testosterone (Nankin and Calkins, 1986; Tenover et al, 1987).

Although there was some increase in serum testosterone levels after CC treatment in the elderly group, there was no

increase in serum inhibin levels. A previous study reported a small increase in serum inhibin levels in elderly men given CC for 1 week (Tenover et al, 1988a). These data therefore imply that, in the elderly, serum inhibin level increases with CC treatment may not be sustained. Follicle-stimulating hormone is felt to be the major stimulator for inhibin secretion, although LH also may play a role (McLachlan et al, 1988). In both the elderly and the young adult men, serum levels of LH and FSH increased with CC treatment, yet inhibin levels failed to increase in the elderly men. Although there are no data on clearance rates of serum inhibin in elderly men unless CC administration results in an increase in inhibin clearance, the lack of a significant increase in serum inhibin with prolonged, increased gonadotropin stimulation suggests an age-related decline in Sertoli cell function or reserve that may be more pronounced than the decline in Leydig cell function.

As previously found with 1-week CC treatment studies (Tenover et al, 1987), serum estradiol levels in both young adult and elderly men increased with 8 weeks of CC treatment. Unlike previous studies (Tenover et al, 1987), serum SHBG levels did not significantly change in either age group after CC administration. The lack of change in SHBG serum levels occurred despite significant increases in serum estradiol levels, a major stimulator of SHBG production (Anderson, 1974), and changes in the testosterone:estradiol ratio that are similar to a previous report (Tenover et al, 1987). The exact reasons for the lack of change in overall SHBG levels with CC in this study are unclear.

In terms of gonadotropin production, both the young adult and elderly men were able to increase serum LH and FSH levels beyond the normal range with 8 weeks of CC stimulation. The increase in serum FSH levels in the elderly men was similar to that in the young adult men, but LH levels tended to be lower in the elderly men throughout the 8 weeks of treatment. Although the differences in LH levels between the two age groups did not reach statistical significance, the trend toward lower levels in the elderly men suggests that, in addition to an age-related decline in testicular function, there may be an age-related decline in hypothalamic-pituitary function as well.

The data on LH pulsatile characteristics obtained in this study are from 8-hour, rather than 24-hour, evaluations, and it is known that LH pulsatile secretion can vary over a 24-hour period. Nonetheless, LH pulsatile findings from this study are very consistent with previous 24-hour studies (Tenover et al, 1987, 1988b). At baseline levels, both LH pulse frequency and amplitude were similar in the two age groups and increased with CC administration. However, the elderly men tended not to have as much of an increase in the LH pulsatile parameters as did the young adult men.

Although the data presented here strongly suggest age-related primary testicular failure, they do not rule out some age-related alterations in hypothalamic or pituitary control of gonadal function. Other studies have suggested an age-related increase in hypothalamic sensitivity to steroid negative feedback (Moroz and Verkhratsky, 1985; Winters and Troen, 1982) and have reported that the pattern of LH pulsatile secretion is subtly changed with aging (Deslypere et al, 1987; Tenover et al, 1988b). Our findings that similar serum LH pulsatile patterns exist in young adult and elderly men, despite lower serum testosterone and nSHBG-T levels in the two age groups, and that elderly men tend to have lower CC-stimulated serum LH levels are consistent with the existence of a concurrent hypothalamic-pituitary defect in the aging male reproductive axis.

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