The Effect of Testosterone Replacement on Endogenous Inflammatory Cytokines and Lipid Profiles in Hypogonadal Men

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Testosterone has immune-modulating properties, and current in vitro evidence suggests that testosterone may suppress the expression of the proinflammatory cytokines TNFα, IL-1β, and IL-6 and potentiate the expression of the antiinflammatory cytokines IL-10. We report a randomized, single-blind, placebo-controlled, crossover study of testosterone replacement in which major improvements of clinical status and inflammatory disease have been attributed to the immunosuppressive effects of androgens compared with estrogens (1). Laboratory studies using animals with experimental induced inflammation have reported a beneficial response with androgens (2–4). Moreover, there are several case reports and small clinical trials of testosterone therapy in humans with rheumatoid arthritis and systemic lupus erythematosus in which major improvements of clinical status and inflammatory markers have been recorded (5–7). The mechanism by which testosterone effects these responses is uncertain, but laboratory evidence has suggested that testosterone suppresses proinflammatory cytokines and may up-regulate antiinflammatory cytokines. Despite the varied approach of these studies, the response to testosterone appears to be similar, so that testosterone incubated in cell culture attenuates the production of inflammatory cytokines such as TNFα, IL-1β, and IL-6 in human macrophages (8), human monocytes (9), human gingival fibroblasts (10), murine and human osteoblasts (11, 12), and human endothelial cells (13). Furthermore, antiinflammatory cytokines such as IL-10 are stimulated in the presence of testosterone (14, 15). There have been few in vivo studies with highly varied experimental approaches. Injection of bacterial endotoxin into castrated male mice increased the endogenous production of TNFα, which was abrogated by testosterone replacement (16). Macrophages taken from castrated mice subjected to trauma hemorrhage had increased production of IL-1β and IL-6 compared with sham-castrated animals subject to the same procedure (17). In the few human studies, elderly men with hypogonadism induced with gonadotropin hormone-releasing agonists developed significant increases in serum TNFα and IL-6 (18). Although testosterone replacement reduced the levels of proinflammatory serum cytokines the reduction did not achieve statistical significance. A cohort of 29 hypogonadal young men with delayed puberty was found to have increased levels of immune activation compared with age-matched healthy controls. The immune activation was normalized with androgen-stimulating therapy (19).

There have been two prospective studies of androgen therapy in men with partial androgen deficiency, but neither showed any convincing effects on inflammatory cytokines.
Ng et al. (20) used dihydrotestosterone, recombinant human chorionic gonadotropin, or a control in healthy men with total testosterone levels below 15 nmol/liter and found no changes in C-reactive protein or the soluble cellular adhesion molecules intracellular adhesion molecule-1 and vascular cell adhesion molecule-1. Lambert et al. (21) used megestrol acetate with testosterone injections, resistance training, or both in elderly eugonadal men, but no treatment effects on serum TNFα or leptin were found. To our knowledge there are no published data on the inflammatory/cytokine balance in postpubertal men with symptomatic androgen deficiency, nor is there any information on the effects of testosterone replacement on the cytokine balance in these hypogonadal men.

The aim/hypothesis of this study was to assess the effects of testosterone replacement on circulating inflammatory cytokines, antiinflammatory cytokines, and plasma lipids.

**Subjects and Methods**

This was a randomized, single-blind, placebo-controlled, crossover trial of testosterone replacement in hypogonadal men. The primary outcome was the serum level of TNFα. Eligible androgen-deficient men were recruited from a male andrology clinic before commencing testosterone replacement. Patients provided written informed consent and underwent a screening protocol before entry and randomization. Treatment began with either testosterone or placebo for 1 month, each comprising three injections given at 0, 14, and 28 d. Assessments were performed 2 d after the final injection in each phase. There was 1 month of no treatment (washout) between the two treatment phases.

Subjects were referred back to the andrology clinic after completion of the trial protocol for continuing management and testosterone replacement. There were no changes to concomitant medications during the study period.

**Setting**

This study was performed at the andrology outpatient department, Royal Hallamshire Hospital; Sheffield Cardiology Research Department, Royal Hallamshire Hospital Sheffield; and Center for Diabetes and Endocrinology, Barnsley District General Hospital, Barnsley.

**Subjects**

Patients were males referred to the andrology clinic who were androgen deficient and for whom androgen replacement was deemed clinically indicated by a consultant endocrinologist (T.H.J.; Table 1). Twelve of the patients had primary gonadal failure with elevation of gonadotropins above the normal range; one of these patients had Noonan’s syndrome, and two had Klinefelter’s syndrome. Four patients had hypogonadotropic hypogonadism with low gonadotropin levels; one of these had hemochromatosis, and the other three other had no underlying diagnosis, although pituitary magnetic resonance imaging was normal in each case. The remaining patients (n = 11) had a mixed picture of hypogonadism, with low serum testosterone levels and gonadotropin levels within the normal range. The local regional ethics committee approved the protocol, and patients provided written informed consent.

Exclusion criteria for trial patients were age greater than 18 yr with a clinical indication for testosterone replacement. Exclusion criteria were elevation of prostate-specific antigen (PSA) above the age-adjusted normal range, malignancy, and any acute or chronic inflammatory condition.

**Randomization, drug treatment, and blinding**

We used a standard regimen of testosterone therapy in hypogonadal men, which comprised fortnightly injections of depot testosterone. Testosterone (100 mg testosterone esters/ml; Sustanon 100, Organon, West Orange, NJ) or placebo (1 ml 0.9% normal saline) in an identical syringe was given by deep im injection every fortnight by a member of the research staff (C.J.M. or P.J.P.) in the cardiology research department, blinded to the identity of the injection, and the drug was drawn up away from the patient. The order of drug administration was allocated using blocks of computer-generated numbers.

**Table 1. Baseline characteristics**

<table>
<thead>
<tr>
<th>Clinical characteristic</th>
<th>Normal range</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>61.6 ± 9.3</td>
<td>36–78</td>
</tr>
<tr>
<td>BMI (kg/m²) (20–25)</td>
<td>29.0 ± 6.9</td>
<td>21.5–57</td>
</tr>
<tr>
<td>Total testosterone (nmol/liter) (&gt;12 nmol/liter)</td>
<td>4.39 ± 1.24</td>
<td>0.9–17</td>
</tr>
<tr>
<td>Bioavailable testosterone (nmol/liter) (&gt;2.5 nmol/liter)</td>
<td>2.42 ± 1.07</td>
<td>0.2–6.4</td>
</tr>
<tr>
<td>FSH (IU/liter)</td>
<td>12.4 ± 10.5</td>
<td>1.9–46.8</td>
</tr>
<tr>
<td>LH (IU/liter)</td>
<td>8.6 ± 12</td>
<td>1.2–59.5</td>
</tr>
<tr>
<td>SHBG (nmol/liter)</td>
<td>16.9 ± 7.1</td>
<td>8.7–32.7</td>
</tr>
<tr>
<td>Total cholesterol (mmol/liter)</td>
<td>4.86 ± 1.11</td>
<td>3.3–7.6</td>
</tr>
<tr>
<td>TNFα (pg/ml)</td>
<td>5.78 ± 8.4</td>
<td>1.05–37</td>
</tr>
<tr>
<td>IL-1β (pg/ml)</td>
<td>0.41 ± 0.49</td>
<td>0.03–1.679</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>4.7 ± 4.9</td>
<td>0.7–23.7</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>3.3 ± 4.3</td>
<td>0.63–1.7</td>
</tr>
<tr>
<td>Androgen Deficiency Questionnaire Score (out of 10)</td>
<td>6.7 ± 2.3</td>
<td>4–10</td>
</tr>
</tbody>
</table>

BMI, Body mass index; ACE, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-Coenzyme A.

**Subject assessment**

All assessments were performed in the cardiology research department in the morning before 1000 h. At the screening visit all patients and controls were required to complete a detailed questionnaire recording their medical history and current medications. Subjects were asked to complete an androgen deficiency screening questionnaire (ADAM). The androgen screening questionnaire is a 10-statement true/false answer sheet. A positive screen is defined as a score of 3 or more out of 10, or a single positive response to any one of two highly specific questions: do you have reduced libido (sex drive)? or do you have reduced ability to maintain erections?

Blood was drawn at each visit for total testosterone, SHBG, and full lipid profiles. Blood samples for gonadotropin and PSA determinations were taken only at the screening visit. Blood for serum cytokine measurements was taken in chilled serum gel tubes and allowed to clot standing in ice. Serum was separated using a centrifuge, and plasma was stored at −80 °C until analysis. Total testosterone and SHBG were measured by enzyme immunoassay (DRG Instruments, Marburg, Germany). Bioavailable testosterone was assayed using a modification of the method described by Tremblay and Dube (22). In this method testosterone is removed from the test serum by the addition of activated charcoal and is replaced by [3H]testosterone which binds to SHBG and other plasma proteins in equimolar concentrations as native testosterone.
one. SHBG-bound testosterone is then precipitated out of the sample by the addition of cold ammonium sulfate. The remaining \(^{3}H\)testosterone, comprising the unbound fraction and the fraction weakly bound to albumin, is then measured using a \(\beta\)-counter. The intra- and interassay coefficients of variation for total testosterone were less than 5% and less than 6%; SHBG, less than 9% and less than 12%; and bioavailable testosterone, less than 5% and less than 8%. PSA, gonadotropins, and lipid profiles were measured with a standard hospital assay; low-density lipoprotein (LDL) cholesterol was calculated with the Friedwald equation LDL = total cholesterol – [high-density lipoprotein (HDL) + triglycerides/5.2]. Serum TnF\(\alpha\), IL-1\(\beta\), and IL-6 were assayed with high sensitivity quantitative sandwich enzyme immunoassay (Quantikine, R&D Systems, Abingdon, UK); the minimum detectable level is 0.1 pg/ml, and the intra- and interassay coefficients of variation were both less than 10%. Serum levels of proinflammatory cytokines should be undetectably low in 25% of young normal subjects.

### Statistical analysis and sample size determination

There are no pilot data on which to base a sample size calculation. However, in a study of lipid-lowering therapy in hyperlipidemic patients, active treatment resulted in a reduction of circulating TnF\(\alpha\) from 19.2 \(\pm\) 5.1 to 9.2 pg/ml. To detect half this reduction in a placebo-controlled, crossover study with 95% power and 5% significance, it was estimated that 22 patients would be required. It was anticipated that drop-out rates would be low, and therefore, we planned to recruit 30 patients. All data are presented as the mean \(\pm\) SD unless stated otherwise; analysis was performed with a statistical package (SPSS version 11.5, SPSS, Inc., Chicago, IL). Data were tested against a normal distribution using Kolmogorov-Smirnov tests. The serum levels of cytokines and most serum levels of cholesterol were nonparametric, so the Wilcoxon rank test was used for paired data analysis. A subgroup analysis of the response of TnF\(\alpha\) and IL-1\(\beta\) in the 20 patients with coronary disease was also performed. The effect remained highly significant; the difference by delta analysis for testosterone was \(-4.1 \pm 9.1\) vs. \(1.5 \pm 5.9\) pg/ml for placebo (\(P = 0.016\), corrected).

The testosterone phase of treatment was also associated with a reduction of total cholesterol and serum triglycerides (Table 2), although by analysis of the delta values, only the reduction in total cholesterol remained significant (Fig. 3). The reduction of LDL cholesterol with testosterone was not significant (\(-0.11 \pm 0.5\) vs. \(0.14 \pm 0.98\) mmol/liter; \(P = 0.3\)), but the reductions in total cholesterol and LDL cholesterol were positively correlated (\(r_{c} = 0.53\); \(P = 0.005\)), suggesting a relationship (Fig. 4).

There were no significant effects on HDL cholesterol (\(-0.02 \pm 0.12\) vs. \(-0.05 \pm 0.21\); \(P = 0.84\)).

### Discussion

This study found that 1 month of testosterone replacement given to men with symptomatic androgen deficiency resulted in statistically significant reductions of TnF\(\alpha\) and IL-1\(\beta\) compared with placebo, and there was a statistically significant increase in IL-10 by delta analysis. The baseline TnF\(\alpha\) was higher in the testosterone phase, but not significantly so. There were no treatment/period effect of significance. Two of the hypogonadal patients had very high basal TnF\(\alpha\) levels; both of these men had mild chronic congestive

### Table 2. Difference by treatment in 27 hypogonadal men (Wilcoxon matched pairs test)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Placebo (n = 27)</th>
<th>Testosterone (n = 27)</th>
<th>Baseline comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>4 wk</td>
<td>Baseline</td>
</tr>
<tr>
<td>TNF(\alpha)</td>
<td>2.68 (\pm) 1.76</td>
<td>4.18 (\pm) 5.0</td>
<td>0.4</td>
</tr>
<tr>
<td>IL-1(\beta)</td>
<td>0.33 (\pm) 0.37</td>
<td>0.5 (\pm) 0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>IL-6</td>
<td>4.9 (\pm) 5.89</td>
<td>4.8 (\pm) 3.5</td>
<td>0.23</td>
</tr>
<tr>
<td>IL-10</td>
<td>3.23 (\pm) 3.8</td>
<td>2.1 (\pm) 2.7</td>
<td>0.2</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>4.72 (\pm) 1.07</td>
<td>4.72 (\pm) 1.07</td>
<td>0.79</td>
</tr>
<tr>
<td>LDL</td>
<td>2.67 (\pm) 1.0</td>
<td>2.81 (\pm) 1.1</td>
<td>0.78</td>
</tr>
<tr>
<td>HDL</td>
<td>0.97 (\pm) 0.22</td>
<td>0.95 (\pm) 0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Cholesterol:LDL ratio</td>
<td>4.98 (\pm) 1.24</td>
<td>5.09 (\pm) 1.23</td>
<td>0.65</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>2.34 (\pm) 0.14</td>
<td>2.24 (\pm) 0.2</td>
<td>0.95</td>
</tr>
<tr>
<td>Total testosterone</td>
<td>6.7 (\pm) 4.5</td>
<td>8.7 (\pm) 9.5</td>
<td>0.18</td>
</tr>
<tr>
<td>Bioavailable testosterone</td>
<td>3.0 (\pm) 1.9</td>
<td>3.6 (\pm) 3.1</td>
<td>0.6</td>
</tr>
</tbody>
</table>
heart failure, a condition characterized by high basal TNFα values. A subgroup analysis was performed on the study group with these two patients removed, but the reduction in TNFα with testosterone by analysis of the delta remained significant. The TNFα response to testosterone of these patients was very sensitive, and both demonstrated large reductions compared with the effect of placebo. This is an intriguing finding, because the progression and prognosis of heart failure are linked to catabolic compounds such as TNFα, and there have been a number of attempts to reduce TNFα pharmacologically as a treatment for heart failure (23), although with limited clinical benefit (24). Preliminary data suggesting reductions of TNFα in heart failure patients with testosterone therapy should provoke interest in further studies in this group.

The largest comorbid group was patients with coronary disease (n = 20); this included the two patients with congestive heart failure. Atherosclerosis is acknowledged to be a disease of chronic inflammation, and inflammatory cytokines such as TNFα and IL-1β are mediators of atheromatous plaque development and complications (25). Antiinflammatory cytokines such as IL-10 can regulate inflammation and may inhibit atherosclerosis (26, 27), and elevation of IL-10 after an acute coronary syndrome confers a favorable prognosis (28). Subgroup analyses in patients with coronary disease (n = 20) substantially weakened the results, with only TNFα retaining statistical significance. This analysis suggests that testosterone had an antiinflammatory effect in all patients, not just in a subgroup with established vascular disease.

The use of 3-hydroxy-3-methyl-glutaryl-coenzyme A enzyme inhibitors was high (n = 20). The reason for statin therapy was secondary prevention for vascular disease in most cases. Despite the presence of vascular disease, the dyslipidemia in these patients may reflect the underlying androgen status, because there are reports of dyslipidemia associated with hypogonadism (29, 30). However, the raised body mass index and high prevalence of type 2 diabetes (n = 9) are also likely to contribute. Testosterone replacement caused a significant reduction in total cholesterol and triglycerides, although by delta analysis only the reduction of total cholesterol remained significant. In general, our results agree with most studies of the effect of androgen replacement in cholesterol profiles, with modest reductions of total cholesterol and LDL cholesterol reported by a recent metaanalysis (31). We did not find a reduction in HDL cholesterol in our study that was documented in the meta-analysis performed by Whitsel et al. (31). This effect of testosterone on
HDL cholesterol is reported to be reduced or absent in hypogonadal or obese men (32, 33), and in our study may also be masked by the positive effects of testosterone on insulin resistance that have been reported in obese men (34) and in asymptomatic men with low serum testosterone levels (35).

We have shown that testosterone replacement induces an antiinflammatory shift in cytokine balance. This study is one of the very few in vivo studies and the only in postpubertal men with idiopathic disease to demonstrate a convincing antiinflammatory effect of testosterone. Moreover, the observed effect has a potentially beneficial action in men with coronary disease due to atherosclerosis. An anticytokine effect in this inflammatory condition may positively affect plaque biology and stability.

The observed reduction in total cholesterol may be related to the reductions in inflammatory cytokines, as cholesterol reduction with statins is itself associated with an anticytokine effect (36, 37). However the reduction of TNFα and IL-1β did not correlate with the reduction in total cholesterol or LDL cholesterol, suggesting the lack of a relationship. Furthermore, we cannot exclude an interaction between statin therapy and testosterone, because postmenopausal women treated with hormone replacement and statins have greater reductions in total cholesterol than those given statins alone (38, 39).

Conclusions

Testosterone replacement in men with symptomatic androgen deficiency is indicated to improve quality of life, maintain lean body mass, and preserve bone density. Although this trial enrolled a mixed population of hypogonadal men, an antiinflammatory effect of testosterone replacement may be of particular benefit to men with established vascular disease, because inflammation determines vascular risk in these individuals. Importantly, the prevalence of overt hypogonadism in men with coronary disease is high and may approach 25% (40), a figure far in excess of the background community prevalence (41). In addition, testosterone therapy has been found to improve symptoms and time to ischemic threshold in coronary disease (42–44) and congestive heart failure (45). The present study suggests that testosterone replacement in this potentially large group of men may beneficially alter cytokine balance and reduce total cholesterol. These effects may translate to prognostic benefit in addition to symptomatic improvements. Further prospective trials to determine safety and particularly cardiovascular outcomes are justified.

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