RESEARCH

Testosterone inhibits aneurysm formation and vascular inflammation in male mice

Bo-Kyung Son¹², Taro Kojima¹, Sumito Ogawa¹, Masahiro Akishita¹

¹Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
²Institute of Gerontology, The University of Tokyo, Tokyo, Japan

Correspondence should be addressed to BK Son: son-tky@umin.ac.jp

Abstract

Abdominal aortic aneurysm (AAA), one of the pathological phenotypes of vascular aging, is characterized by aortic dilation with impaired arterial wall integrity. Recent epidemiologic studies have shown that men with AAA have lower serum testosterone compared to men without. However, the underlying mechanisms remain unclear. In this study, we investigated the effects of testosterone on AAA formation using a murine AAA model under the conditions of depletion and administration of testosterone. In wild-type male mice (C57BL/6J), AAA was induced by CaCl₂ application and angiotensin II infusion at 5 weeks after castration. Exacerbated AAA formation was seen in castrated mice, compared with sham-operated mice. Histological analysis revealed marked infiltration of macrophages in the destroyed aorta and IL-6/pSTAT3 expression was significantly elevated, suggesting that AAA development by castration is attributable to pronounced inflammation. Conversely, both 4-week and 9-week administration of testosterone significantly prevented AAA formation, and improvement of histological findings was confirmed. Aortic F4/80, Il-1b and Il-6 expression were significantly inhibited both by testosterone administration. Indeed, mice with implanted flutamide exhibited exacerbated AAA formation and aortic F4/80, Il-1b and Il-6 expression were significantly increased. Taken together, these results demonstrate that testosterone depletion and AR blockade precede AAA formation, and conversely, testosterone administration could suppress AAA formation by regulating macrophage-mediated inflammatory responses. This anti-inflammatory action of testosterone/AR on AAA formation might provide a mechanistic insight into the vascular protective actions of testosterone and suggest that its proper administration or selective AR modulators might be novel therapeutic strategies for this aortic pathology.

Introduction

With advancing age, circulating testosterone declines gradually in men (Feldman et al. 2002, Liu et al. 2007). This decline has been suggested to be causally related to poor health outcomes, including increased risk of cardiovascular events and mortality (Lorenz et al. 2007, Soisson et al. 2012). The beneficial action of testosterone in atherosclerosis has been extensively investigated and is well accepted. For example, a low serum testosterone level is associated with an increase in carotid intima-media thickness, a marker of preclinical atherosclerosis (Tsujimura et al. 2012) and, further, advanced atherosclerosis in the form of aortic calcification (Hak et al. 2002).
These vascular protective actions of testosterone are further evidenced by experimental studies in which castration and androgen receptor (AR) knockout in mice increase atherosclerosis, and this increase is abolished by physiological testosterone replacement on an ApoE-deficient background (Bourghardt et al. 2010).

Abdominal aortic aneurysm (AAA), which is characterized by aortic dilation with impaired arterial wall integrity, is one of the significant features of vascular pathology with advancing age. In many cases, AAA co-exists with atherosclerosis within the same individual, commonly exhibiting severe immune and inflammatory cell infiltrates at lesion sites. Regarding the actions of androgens in this aortopathy, a recent epidemiological study showed that men with AAA had lower serum total and free testosterone compared with men without AAA. Further, in multivariate analysis adjusting for potential confounders, serum-free testosterone level was negatively associated with AAA (Yeap et al. 2010). However, in experimental studies, detrimental effects of androgen on the formation and progression of AAA have been suggested in castrated ApoE-deficient mice with AAA induction by angiotensin II (AngII) infusion (Henriques et al. 2004, Zhang et al. 2015). These controversies are presumed to be due, in part, to lack of a reliable animal model.

Because inflammation is a hallmark of AAA, we induced an inflammatory AAA murine model in wild-type mice by modification of our previous finding (Son et al. 2015). Using this AAA model, in the present study, we sought to unravel the effects of testosterone on AAA formation and found that castration promoted AAA formation through exacerbated infiltration of inflammatory macrophages and IL-6 and IL-1β upregulation. Conversely, testosterone administration inhibited AAA formation by suppression of macrophage-mediated inflammatory responses. Furthermore, AR blockade by flutamide implantation could also induce AAA formation through increased expression of IL-6 and IL-1β. Collectively, the anti-inflammatory actions of testosterone/AR on AAA formation might provide a novel therapeutic strategy for prevention or treatment of this aortic pathology.

Materials and methods

Mice

Male 8-week-old C57BL/6J mice were obtained from Sankyo Labo (Tokyo, Japan) and maintained on a 12:12-h light-darkness cycle and received food and water ad libitum. All experimental protocols were approved by the Ethics Committee for Animal Experimentation of the Graduate School of Medicine, the University of Tokyo and conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Department of Medicine, the University of Tokyo.

Castration and pharmacologic blockade of AR

Male wild-type mice were anesthetized with intraperitoneal ketamine (100 mg/kg) and xylazine (10 mg/kg). Both testes were moved into the scrotal sacs by gently applying pressure to the abdomen. An 8–10-mm incision through the skin was made along the midline of the scrotal sac. Another incision was made into the midline wall between the testes sacs under the covering membranes. The testis, vas deferens and epididymal fat pad were pulled carefully out through the incision. The blood vessels supplying the testis were clamped with a hemostat, and the testis was dissected away. The vas deferens and fat pad were cauterized and placed back into the scrotal sac. This procedure was repeated for the other testis. The incision through the skin was closed using wound clips. Mice in the sham-operated control group were anesthetized, incisions were made and sex organs were localized.

To block the AR, time-release flutamide pellets (25 mg/pellet, 60-day release, Innovative Research of America, Sarasota, FL, USA) were given to wild-type mice. In the control group, placebo pellets supplied by the same company were used.

AAA model

We induced AAA in wild-type mice through synergistic augmentation and prolonged hemodynamic stress by peri-aortic CaCl2 application and continuous infusion of AngII for up to 4 weeks (Kimura et al. 2014, Son et al. 2015). In detail, mice were anesthetized and underwent laparotomy at 10–13 weeks of age. The abdominal aorta between the renal arteries and bifurcation of the iliac arteries was isolated from the surrounding retroperitoneal structures, and 0.5 M CaCl2 was applied to the external surface of the infrarenal aorta and AngII (2000 ng/kg/min, osmotic minipump, Alzet) was infused. NaCl (0.9%) was substituted for CaCl2 and AngII was not infused in sham control mice. The aorta was rinsed with 0.9% sterile saline after 15 min and the incision was closed.

Administration of testosterone

Testosterone was administered as testosterone propionate (Sigma-Aldrich), because it is more lipophilic than

https://joe.bioscientifica.com
https://doi.org/10.1530/JOE-18-0646
© 2019 Society for Endocrinology
Published by Bioscientifica Ltd.
Printed in Great Britain

Downloaded from Bioscientifica.com at 11/18/2022 08:54:50PM via free access
testosterone and is absorbed much more slowly than testosterone after injection (Meikle et al. 1999). Long-term and short-term administration of TP was performed: 9-week administration of testosterone after castration and 4-week administration after AAA induction. To examine the dose dependence of testosterone's effects, two doses (long-term: 5 and 10 mg/kg, short-term: 5 and 50 mg/kg) were administered twice a week by subcutaneous injection. Control mice were administered the same volume of safflower seed oil (Sigma-Aldrich) at the same time. The doses of testosterone administration in this study were based on Zhao et al. (2014), Ren et al. (2017), and Zou et al. (2017) and the results of our preliminary experiment in the aorta.

Histological and immunohistochemical staining

Aortas from mice were embedded in paraffin, then 5-μm-thick serial sections were prepared for Elastic Van Giesson (EVG) staining. Digital images of aortas with a reference scale were used for absolute measurement of diameter. Paraffin-embedded sections were taken from the aorta for EVG and immunohistochemical staining. For immunohistochemical staining, after deparaffinization and blocking, serial sections were incubated with the following antibodies: F4/80 (1:100; rat; Serotec, Kidlington, UK) for macrophages or p-STAT3 (1:200; rabbit; Cell Signaling Technology), followed by biotinylated secondary antibodies (1:200; DAKO). For detection, anti-streptavidin-conjugated AlexFluor 488 or AlexFluor 594 (1:200; Invitrogen) was used. The nuclei were stained with 4′,6-diamidino-2-phenylindole (1:5000; Sigma-Aldrich) after the final series of washes.

RNA isolation and quantitative real-time PCR

Total RNA from aortic samples was extracted using a RNeasy fibrous tissue kit (Qiagen) according to the manufacturer's instructions. Then, 0.2–1 μg RNA was reverse-transcribed using Omniscript (Qiagen) according to the manufacturer’s instructions. Real-time PCR was performed using 2 μL of resulting cDNA per 25 μL reaction volume containing SYBR green I master (Thermo Fisher Scientific). Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control. PCR was performed on a 7300 Real-time PCR system (Applied Biosystems) in accordance with the manufacturer’s recommended procedure.

Western blot analysis

Mouse aortic specimens were homogenized with lysis buffer (T-PER, Thermo Fisher Scientific) containing protease inhibitors complex (Roche) and phosphatase inhibitors (Roche). Protein concentration was assayed using a BCA protein assay kit (Pierce), and 5 μg of protein were resolved with 7.5 or 12.5% SuperSept (WAKO, Osaka, Japan), and then transferred to a PVDF membrane. The blot was probed with primary antibodies: pERK1/2 (1:3,000), pSTAT3 (1:3,000), ERK1/2 (1:3,000), STAT3 (1:3,000) (all rabbit antibodies obtained from Cell Signaling Technology) and β-actin (1:3,000, mouse antibody, Sigma-Aldrich). Membranes were washed and incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology). Protein bands were detected using ECLplus (Thermo Fisher Scientific).

Blood pressure measurement

Systolic blood pressure was measured in conscious mice using a computerized tail-cuff method (BP-2000; Visitech Systems, Apex, NC, USA). Mice were acclimatized to the blood pressure machine for 1 week, and baseline measurements were obtained before implantation of an osmotic minipump. Blood pressure measurements were recorded at the same time of day, 5 days a week, throughout the study as described previously.

Measurement of plasma lipid profile

Cholesterol and triglyceride (TG) levels of lipoproteins in plasma were measured by gel-permeation high-performance liquid chromatography (GP-HPLC, LipoSEARCH) (Toshima et al. 2013). In detail, cholesterol and triglyceride (TG) levels were determined by their component peak analyses on the basis of lipoprotein particle sizes with the Gaussian curve fitting technique, and the particle sizes of each lipoprotein were calculated by their retention times on a chromatogram.

Enzyme-linked immunosorbent assay

Plasma level of IL-6 in mice was assayed using a commercially available quantikine ELISA kit (R&D systems) according to the manufacturer's instructions. All techniques and materials in this analysis were used in accordance with the manufacturer’s protocol. Optical density was quantified on a micro-plate-reader (Bio-Rad Laboratories). Plasma level of testosterone was measured using a kit with an EIA assay buffer (IBL, Gunma, Japan). All techniques and materials in this analysis were used in accordance with the manufacturer’s protocol. Optical density was quantified on a micro-plate-reader.
Testosterone/AR and AAA formation

BK Son et al.

Journal of Endocrinology

Statistical analyses

All data are presented as mean ± s.e.m. The statistical significance of differences between two groups was determined by Mann–Whitney test for non-parametric data after testing for normality by F-test analysis. For data containing multiple time points, two group comparisons at the same time point were performed. When comparing multiple groups, data were analyzed by Kruskal–Wallis non-parametric one-way ANOVA with Tukey’s post hoc test. All data were analyzed using Prism 6.0 (GraphPad Software, Inc.). A P value of less than 0.05 was considered significant.

Results

AAA formation is exacerbated in castrated mice

We initially found that castrated mice manifested a phenotype of exacerbated aortic aneurysm, compared with sham-operated mice, when subjected to AngII infusion with local application of calcium chloride (CaCl₂) (Fig. 1A). Aortic diameter was significantly enhanced in AAA-induced mice with castration (Fig. 1B). Histological findings also showed enlargement of the aortic lumen with fibrotic tissue deposition (Fig. 1C), implying that androgen deficiency by castration promotes the formation of AAA in wild-type mice.

Inflammation contributes to advanced AAA formation in castrated mice

Because macrophage infiltration is one of the critical events involved in the underlying phenotype of castrated mice, we next investigated macrophage infiltration by histological analysis. Marked infiltration of F4/80-positive macrophages was seen in the destroyed aorta of castrated mice (Fig. 2A and C). Further, to characterize the infiltrated F4/80-positive macrophages, immunohistochemical analysis of pSTAT3 was performed and showed colocalization of nuclear pSTAT3 and F4/80 (Fig. 2B), implying that the enhanced AAA formation by castration

Figure 1

Enhanced aortic abdominal aneurysm (AAA) formation in castrated mice. (A) Representative whole aorta induced by 4 weeks of AngII infusion with CaCl₂ application in sham-operated (n = 7) and castrated (n = 8) mice. Arrowheads (white) indicate renal artery. (B) Quantification of infrarenal aortic diameter in sham and AAA-induced mice with or without castration (n = 3–5 per group). All values are presented as mean ± s.e.m. *P < 0.05, one-way ANOVA with Tukey’s post hoc test. (C) Histopathological analysis of infrarenal aorta (EVG). Scale bar, 100 µm. Arrowheads (black) indicate internal elastic lamina. A full colour version of this figure is available at https://doi.org/10.1530/JOE-18-0646.
was associated with inflammation involving expansion of inflammatory macrophages.

Elevated expression of *Il-6* and *Il-1b* were also seen in the aortic lesion (Fig. 2D) and circulating level of *IL-6* was elevated (Fig. 2E). The protein level of aortic pSTAT3, a downstream molecule of IL-6, was increased in AAA-induced mice with castration, while the level of STAT3 did not change (Fig. 2F and G). On the other hand, when we examined ERK1/2, a protein in the downstream signaling pathway of TGFβ that plays a central role in the pathogenesis of Marfan aortopathy, and phosphoERK1/2 (pERK1/2) was not affected by castration and AAA induction (Fig. 2F and G), thus suggesting that the TGFβ-mediated pathway is not critically involved in the underlying phenotype and that the IL-6/pSTAT3 pathway is.

**Long- and short-term administration of testosterone inhibits AAA formation**

We further examined whether administration of testosterone could inhibit exacerbated AAA formation by castration and whether dose-dependent inhibition of testosterone occurs. First, we tested two doses of testosterone (5 and 50 mg/kg) for 4 weeks after AAA induction (Fig. 3A). Both doses of testosterone administration significantly ameliorated the increased aortic diameter and pathological findings of aneurysm formation.
Aortic expression of F4/80, IL-6 and IL-1β were significantly inhibited by the high dose of testosterone (Fig. 3D). However, dose dependency was not seen at this time point. To further examine the dose dependency of testosterone administration, we next tested long-term testosterone administration (5 and 10 mg/kg) for 9 weeks after castration (Fig. 4A). Amelioration of both histological findings and aortic diameter was seen in a testosterone dose-dependent manner (Fig. 4B and C). Further, aortic F4/80 and IL-1β expression were also similarly inhibited in a dose-dependent manner (Fig. 4D), implying that the formation of AAA and inflammatory response could be inhibited dose dependently by long-term testosterone administration.

We also examined the effects of testosterone administration on lipid profile. With short-term administration, both cholesterol and triglyceride (TG) levels of each lipoprotein were slightly elevated, whereas those levels of each lipoprotein were slightly decreased with long-term administration (Supplementary Table 1, see section on supplementary data given at the end of this article). A significant decline in LDL-TG by long-term testosterone administration was seen, as in a previous report (Amano et al. 2017).

No difference in body weight was observed between AAA alone and AAA with castration mice with both short-term and long-term testosterone administration (Supplementary Fig. 1A and B), although a typical decline in body weight by AngII infusion was observed (Cassis et al. 1998). We also confirmed that blood pressure was not affected both by castration and testosterone administration, although a significant increase in blood pressure was observed by AngII infusion.
pressure was seen with AngII infusion for AAA induction (Supplementary Fig. 2A).

**Effects of AR blockade on AAA formation**

To further examine the involvement of AR in AAA formation, we used flutamide, a pharmacologic blocker of AR. Compared to placebo pellet-implanted mice with AAA induction, exacerbated formation of AAA was observed in flutamide-implanted mice. Intriguingly, aneurysm formation in the suprarenal aorta was also seen, which was not observed in AAA induced by castration (Fig. 5A and B). Both suprarenal and infrarenal aortic diameter were significantly increased by implantation of flutamide (Fig. 5C). Aortic F4/80, IL-6 and IL-1β expression were significantly increased in flutamide-implanted mice, compared with placebo-implanted mice (Fig. 5D).

The changes of BW by both placebo and flutamide implantation were not different (Supplementary Fig. 1C). Also, we confirmed that blood pressure was not affected by 5 weeks of implantation of flutamide and a further 4 weeks of AAA induction, compared with placebo implantation (Supplementary Fig. 2B).

**Discussion**

With respect to the effect of testosterone on AAA, there is a discrepancy between the beneficial effect in the clinical field (Yeap et al. 2010) and the deleterious effects in an experimental AAA model (Ailawadi et al. 2004). In the present study, we used an AAA model induced by prominent inflammation in wild-type mice and sought to address the underlying mechanisms of testosterone's...
action under conditions of depletion and administration. We report here that castration promotes AAA formation through marked macrophage infiltration and an increase in inflammatory cytokines such as IL-6, and conversely testosterone administration inhibits its formation through suppression of these inflammatory responses. Furthermore, in this AAA model, AR blockade by flutamide implantation could exacerbate AAA formation, which was caused by enhanced inflammation including IL-6 and IL-1β upregulation. Collectively, the anti-inflammatory actions of testosterone/AR inhibited AAA formation, which could provide a novel therapeutic strategy for prevention or treatment of this aortopathy.

Regarding reliable doses and duration of testosterone administration for AAA inhibition, in the present study, we found that long-term testosterone administration (5 and 10 mg/kg, 9 weeks), could dose-dependently suppress AAA formation. Furthermore, aortic F4/80 and inflammatory cytokines, especially IL-1β were similarly inhibited in a testosterone dose-dependent manner. When comparing short-term testosterone administration (5 and 50 mg/kg, 4 weeks), which lacks testosterone dose-dependent inhibition of AAA formation and inflammatory genes, it is feasible, at least based on this study, to administer 10 mg/kg testosterone for 9 weeks for AAA inhibition. We also performed preliminary tests of another method of testosterone administration using a silicon tube for 9 weeks, which exhibited a marked increase in serum testosterone level, but potent inhibition of inflammation was not seen (Supplementary Fig. 3 and 4), implying that further examination of testosterone administration, especially the dose, duration and method of supplementation, is needed.

For the mechanistical insight, suppressive effects of androgens on the immune system are well recognized (Kelly et al. 2013, Trigunaite et al. 2015). Observational evidence suggests that several proinflammatory cytokines including IL-1β, IL-6, TNFα and highly sensitive CRP are inversely associated with serum testosterone levels in patients with atherosclerosis, stable coronary arteries diseases, type 2 diabetes and hypogonadism (Yang et al. 2005, Maggio et al. 2006, Kapoor et al. 2007, Nettleship et al. 2007). Moreover, an association between circulating IL-1β levels and atherosclerotic burden has been demonstrated and found that patients with the highest IL-1β concentrations had lower endogenous testosterone
levels (Nettleship et al. 2007). Additionally, testosterone administration has been reported to significantly reduce TNFα and elevate the circulating anti-inflammatory IL-10 in hypogonadal men with CVD (Malkin et al. 2004a,b).

Especially regarding the immune cells, testosterone has been shown to inhibit TNFα, IL-1β and IL-6 released from cultured peripheral blood monocytes isolated from androgen-deficient men with type 2 diabetes (Corrales et al. 2006). Further, the physiological and supraphysiological concentrations of testosterone reduced the expression and secretion of TNFα and IL-1β in monocyte-derived macrophages obtained from a coronary heart disease age-relevant population (Corcoran et al. 2010). This notion is further strengthened by in vivo and in vitro experimental studies. For example, macrophages from castrated mice subjected to traumatic hemorrhage showed increased production of IL-1β and IL-6 compared with sham-operated mice (Wichmann et al. 1997). Conversely, testosterone attenuates the production of inflammatory cytokines such as TNFα, IL-1 and IL-6 in human monocytes (Li et al. 1993, Kuo et al. 2015) and macrophages (D’Agostino et al. 1999). In another study, IL-6 production was shown to be reduced in isolated human monocytes from a small healthy male population following in vitro testosterone treatment (Kanda et al. 1996). Of interest, more recently, it has been demonstrated that the atheroprotective effect of testosterone in male mice is T-cell dependent and that depletion of AR in epithelial cells results in increased thymus size and thymus-dependent atherosclerosis (Wilhelmson et al. 2018), suggesting immune cell-specific beneficial actions of testosterone.

In our AAA model, we found that F4/80-positive macrophages play critical roles in exacerbated inflammation, as suggested in our previous report (Son et al. 2015), and the IL-6/pSTAT3 signal pathway was stimulated by castration and, conversely, both short-term and long-term testosterone administration could inhibit this signal pathway. Mechanistically, given that IL-6 has been suggested to be an androgen-responsive gene by inhibiting the activation and binding of NFκB to its responsive element of IL-6 (Hofbauer et al. 1999), it is conceivable that transcriptional regulation of IL-6 by testosterone through AR contributes to the anti-inflammatory action of testosterone. However, a recent study using mice lacking AR in macrophages, smooth muscle cells (SMC) and endothelial cells with an ApoE−/− background showed that AR in macrophages and SMC mediates AAA formation through IL-1β and TGFβ1 transactivation (Huang et al. 2015). To clarify the molecular regulation of inflammatory responses by testosterone and AR, further investigation including identification of target cells and genes, is needed.

In contrast to our results, a previous study reported that castration prevented the occurrence of AAA in ApoE-deficient mice infused with AngII (Henriques et al. 2004), and further, DHT administration (0.16 mg/day) to castrated mice promoted AAA formation through AT1R mRNA abundance in the abdominal aorta (Henriques et al. 2008). This discrepancy might be caused by differences in mouse strains (ApoE−/− vs B6 wild-type mice), time point of castration surgery (simultaneous vs 5 weeks before AAA induction), AAA model (AngII infusion alone vs combined CaCl2 application and AngII infusion), quantification of AAA (quantification by three independent observers vs maximum external diameter of infrarenal aorta) and methods and duration of administration (5-week pellet implantation vs 4-week and 9-week subcutaneous injection). Of note, these previous studies demonstrated different effects of testosterone on atherosclerosis and AAA, implying the existence of different pathological mechanisms of these two aortic pathologies. However, in many cases, atherosclerosis co-exists with AAA pathology within the same person or mouse, and inflammation is the major common denominator playing crucial roles in both these pathologies, even though the molecular mechanisms of the initiation and maintenance of inflammation in these diseases are most likely different. In accordance, the anti-inflammatory actions of testosterone are relevant in vascular pathogenesis, and using an inflammatory AAA model, we could unravel these beneficial actions of testosterone in the vessel.

Regarding the effects of testosterone administration on lipid profile, in the present study, a significant drop of TG in LDL by testosterone administration was seen (Supplementary Table 1). Although the molecular role of testosterone is not clear, a similar reduction of LDL-TG by a high testosterone level has been demonstrated in male aromatase-knockout (ArKO) mice (Amano et al. 2017). Furthermore, high AR, SREBP1 and PPARα expression were observed in the liver of male ArKO mice. Thus, further investigation of the effect of testosterone on AAA through this action is warranted.

Taking our findings together, in the present study, using an inflammatory AAA model, we found that castration promotes AAA formation through inflammation involving expansion of inflammatory macrophages and IL-6 and IL-1β upregulation. Conversely, administration of testosterone inhibits AAA formation through suppression of macrophage-mediated inflammatory responses. Furthermore, AR blockade by flutamide implantation...
also could induce AAA formation through enhanced IL-6 and IL-1β expression. Collectively, anti-the inflammatory effects of testosterone/AR on AAA formation might provide a novel therapeutic strategy for prevention or treatment of this aortic pathology.

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/JOE-18-0646.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (177100000040, 187100000329, 167100000457, 160300000612).

Acknowledgements
The authors thank Hiroko Sasakawa and Naoko Yokoyama for technical assistance.

References


Hofbauer LC, Ten RM & Khosla S 1999 The anti-androgen, hydroxyflutamide and androgens inhibit interleukin-6 production by an androgen-responsive human osteoblastic cell line. **Journal of Bone and Mineral Research** 14 1330–1337. (https://doi.org/10.1038/srep04051)


Testosterone replacement in hypogonadal men. 