Supraphysiological testosterone enanthate administration prevents bone loss and augments bone strength in gonadectomized male and female rats

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Yarrow JF, Conover CF, Purandare AV, Bhakta AM, Zheng N, Conrad B, Altman MK, Franz SE, Wronski TJ, Borst SE. Supraphysiological testosterone enanthate administration prevents bone loss and augments bone strength in gonadectomized male and female rats. Am J Physiol Endocrinol Metab 295: E1213–E1222, 2008. First published September 9, 2008; doi:10.1152/ajpendo.90640.2008.—High-dose testosterone enanthate (TE) may prevent hypogonadism-induced osteopenia. For this study, 3-mo-old male and female Fisher SAS rats underwent sham surgery, gonadectomy (GX), or GX plus 28 days TE administration (7.0 mg/kg). GX reduced serum sex hormones (i.e., testosterone, dihydrotestosterone, and estradiol) (P < 0.05) in both sexes and bone concentrations of testosterone (males only), and estradiol (females only). GX also elevated serum osteocalcin (females only), findings that are consistent with high-turnover osteopenia. GX reduced cancellous bone volume (CBV) and increased osteoid surfaces in tibia of both sexes. GX males also experienced reduced trabecular number and width and increased trabecular separation, whereas GX females experienced increased osteoblast and osteoid surfaces. Bone biomechanical characteristics remained unaffected by GX, except that femoral stiffness was reduced in females. In contrast, TE administration to GX rats elevated serum and bone androgens to supraphysiological concentrations in both sexes but altered neither serum nor bone estradiol in males. Additionally, TE did not prevent GX-induced reductions in serum or bone estradiol in females. TE also reduced markers of high-turnover osteopenia in both sexes. In males, TE prevented GX-induced changes in trabecular number and separation, CBV, and osteoid surfaces while diminishing osteoblast and osteoclast surfaces; however, these changes were not fully prevented in females. In both sexes, TE increased femoral length and femoral maximal strength to above that of Sham and GX animals while preventing the loss of femoral stiffness in females. In conclusion, TE administration appears protective of cancellous bone in male rats and augments cortical bone strength in both sexes.

androgen; osteoblast; osteoclast; osteoporosis; osteopenia

ANDROGENIC [e.g., testosterone and dihydrotestosterone (DHT)] and estrogenic (e.g., estradiol) hormones influence skeletal development and maintenance in both sexes (11, 54). In males (16, 48) and females (31), androgen deficiency is associated with reduced bone mineral density (BMD), which reduces skeletal strength and increases fracture risk. Administration of testosterone at a physiological replacement dose appears only modestly effective in improving BMD in hypogonadal males (44) and perhaps hypopituitary (i.e., androgen-deficient) females (30). However, testosterone exerts dose-dependent anabolic effects on bone (55, 58) and muscle (5, 6), suggesting that administration of higher-than-replacement (supraphysiological) testosterone may be required to completely prevent hypogonadal bone loss. As evidence, in male rats, supraphysiological testosterone administration successfully prevents orchietomy-induced skeletal catabolism (7, 8, 47), while the human skeletal responses to supraphysiological testosterone administration remain largely unknown. At least one study has reported that slightly-higher-than-replacement testosterone administration effectively reverses BMD loss in hypogonadal elderly men (2). Moreover, several studies suggest that supraphysiological testosterone administration to transsexuals prevents the bone loss associated with combined ovarietomy/hysterectomy and thus the near-complete ablation of gonadally derived estrogen (25, 33, 45, 52). Overall, supraphysiological testosterone administration may be capable of preventing androgen deficiency-related bone loss; however, the sex-specific skeletal responses following testosterone administration require clarification (26, 59).

Testosterone appears capable of inducing skeletal growth and maintenance through a variety of pathways, including 1) direct androgen receptor (AR) activation, 2) indirect AR or estrogen receptor (ER) activation following the irreversible 5α-reduction or aromatization of testosterone to DHT or estradiol, respectively, and/or 3) modulation of nongenotropic signal transduction pathways that do not require direct sex steroid-to-DNA interactions (54). However, the 5α-reduction of testosterone to DHT does not appear to be required for the development or maintenance of BMD, as previous research from Borst and colleagues (7, 8) indicates that coadministration of high-dose testosterone and the 5α-reductase inhibitor MK-434 prevents orchietomy (ORX)-induced skeletal catabolism and maintains skeletal growth in rats in the near absence of circulating DHT. Similarly, in humans, systemic administration of the type II (i.e., finasteride) (28, 29, 42) or type I and II (i.e., dutasteride) (1, 3) 5α-reductase inhibitors nearly abolish circulating DHT concentrations while maintaining BMD. In contrast, the aromatization of testosterone to estradiol and the subsequent ER activation are necessary for skeletal growth and maintenance, given that aromatase-knockout (ArKO) mice of both sexes develop osteopenia resulting from the absence of circulating estradiol (34, 36) and that several ER knockout mouse models also experience adverse skeletal development (23, 57). Likewise, in humans, estradiol is required for skeletal...
development, considering that congenital aromatase deficiency in males causes adverse bone growth that is treatable with estradiol administration but not supraphysiological testosterone (10, 38). However, in these same subjects, coadministration of estradiol and testosterone augments BMD to a greater extent than does estradiol alone (38). Thus, both testosterone and estradiol appear necessary for optimal skeletal development in both sexes (11), whereas DHT may not be required for skeletal homeostasis.

Overall, testosterone may prevent hypogonadal bone loss through direct interactions with skeletal ARs and/or indirectly through ER activation following aromatization in both sexes. However, direct measurements of the sex hormone content of bone have not been previously reported. Thus, the primary purposes of this study were 1) to determine the first-ever measurements of the bone sex steroid (i.e., testosterone, DHT, and estradiol) concentrations in intact, gonadectomized (GX), and GX plus testosterone enanthate (TE) (GX+TE) male and female rats, 2) to determine the sex-specific structural and histological cancellous bone responses resulting from supraphysiological TE administration in GX animals of both sexes, and 3) to evaluate the effects TE administration on the femoral biomechanical characteristics in GX rats. We hypothesized that TE administration would increase the bone testosterone concentrations and that these changes would prevent the cancellous bone loss associated with GX and ultimately augment the biomechanical characteristics of bone in both sexes.

METHODS

Animal care. Barrier-raised and viral pathogen-free Fischer SAS male and female rats aged 3 mo were obtained from Charles River Laboratories (Wilmington, MA). Animals were individually housed in a temperature- and light-controlled room on a 12:12-h light-dark cycle. Rats were fed an ad libitum diet of Purina rodent chow containing 3.3 kcal/g, distributed as 58.9% carbohydrate, 12.4% fat, and 28.7% protein (no. 5001; Purina Mills, St. Louis, MO) and tap water. All experimental procedures conformed to the ILAR Guide to the Care and Use of Experimental Animals and were approved by the Institutional Animal Care and Use Committee at the Gainesville VA Medical Center.

Surgery. Animals received bilateral closed orchietomy (ORX), ovariectomy (OVX), or sham surgery. ORX involved removal of testes, epididymis, and epididymal fat. OVX was performed through a dorsal midline incision and involved removal of the ovaries and the ends of the uterine horns. All surgeries were performed using aseptic procedures under isoflurane anesthesia. After surgery, rats received a nutritional supplement (Jello-O plus protein and fat) daily for 2 days to promote weight maintenance.

Hormone delivery. TE (Savient Pharmaceutical, East Brunswick, NJ), a slowly released testosterone ester, was dissolved in sesame oil containing 3.3 kcal/g, distributed as 58.9% carbohydrate, 12.4% fat, and 28.7% protein (no. 5001; Purina Mills, St. Louis, MO) and tap water. All experimental procedures conformed to the ILAR Guide to the Care and Use of Experimental Animals and were approved by the Institutional Animal Care and Use Committee at the Gainesville VA Medical Center.

Experimental design: study on skeletal responses to testosterone administration in GX male and female rats. Male and female Fisher SAS rats, aged 3 mo, were divided into six groups (n = 10/group). Males and females received either sham surgery plus vehicle (Sham), GX plus vehicle (GX), or GX plus TE (GX+TE). TE (7.0 mg per animal) and vehicle were administered intramuscularly on a weekly basis. On the 10th and 3rd days prior to being euthanized, rats were injected subcutaneously with 15 mg/kg declomycin and 15 mg/kg calcine, respectively, to label sites of bone formation (17). One day prior to euthanasia, rats were housed overnight in metabolic cages for urine collection. Rats were euthanized at day 28 via intraperitoneal pentobarbital sodium injection (120 mg/kg), and trunk blood was sampled for hormone analyses. Additionally, the femur, tibia, prostate, kidney, and plantaris muscle were excised, stripped of surrounding soft tissues, and stored for further evaluation.

Serum hormone analyses. Serum was stored at −80°C prior to hormone analyses. All hormones were assayed in duplicate using commercial kits. Testosterone was assayed using an EIA kit (Diagnostic Systems Laboratories, Webster, TX) with a sensitivity of 0.04 ng/ml and an intra-assay covariance of 5.7%. DHT was assayed using an EIA kit (Alpco Diagnostics, Salem, NH) with a sensitivity of 6 pg/ml and an intra-assay covariance of 5.4%. Estradiol was assayed using an ultrasensitive RIA kit (Diagnostic Systems Laboratories) with a sensitivity of 2.2 pg/ml and an intra-assay covariance of 7.4%. Osteocalcin was assayed using an ELISA kit (Biomedical Technologies, Stoughton, MA) with a sensitivity of 0.5 ng/ml and an intra-assay covariance of 4%.

Bone homogenization and hormone analyses. Following excision, the right tibia was homogenized, and sex hormones (i.e., testosterone, DHT, and estradiol) were extracted. Specifically, tibiae were cut into small pieces, pulverized with a liquid nitrogen-cooled Spex Certiprep freezer mill (Edison, NJ) for 2 min, and stored at −80°C until homogenization. Bone powder was homogenized in a 20-ml (i.e., 200 mg bone powder/4 ml solution) of 4°C titrated monobasic/dibasic Krebs-Ringer phosphate buffer that consisted of 116 mM NaCl, 10 mM phosphate, 4.5 mM KCl, 2.5 mM MgCl2, 1.3 mM CaCl2, 5% (w/v) glucose (pH 6.9), quantity sufficient to 1 liter with ddH2O plus 2 mM EDTA, and 4 mM DTT (Sigma-Aldrich, St. Louis, MO) via high-speed polytron (15 s) and probe sonication (30 s). The homogenate was diluted 1:2 in chloroform-methanol (2:1 vol/vol), vortexed for 45 s, and centrifuged at 1,500 rpm for 10 min to separate organic and inorganic aqueous layers. The layers were then separated, and the upper inorganic aqueous layer was re-diluted in 2 ml of chloroform, vortexed for 45 s, and recentrifuged at 1,500 rpm for 10 min. Following centrifugation, the bottom organic layers from both extractions were combined, dried under a gentle stream of nitrogen, and stored at −20°C. Prior to hormone analyses, bone extracts were reconstituted in testosterone-free standard assay buffer (Diagnostic Systems Laboratories, no. 10-4001), DHT-free standard assay buffer (Alpco Diagnostics, Calibrator A no. 11-DHT-280), or estradiol-free standard assay buffer (Diagnostic Systems Laboratories, no. 10-4801). Reconstituted samples were assayed in duplicate and on a single plate using the aforementioned sex-hormone analysis kits. In addition, the sensitivity of the estradiol RIA (Diagnostic Systems Laboratories, no. 10-4801) was increased by serial dilution of the lowest non-zero standard, which produced additional standard curve values below that contained in the commercial assay. Spiking homogenates with 3H-labeled testosterone, DHT, or estradiol demonstrated an extraction recovery of greater than 90% for all hormones. Spiking homogenates with unlabeled testosterone, DHT, or estradiol demonstrated that reconstituted bone extracts do not contain compounds that interfere with hormone assays.
**Analysis of deoxyxypyrindoline.** Deoxypyrindoline (Dpd), a degradation product of type I collagen and a specific marker for bone resorption, was measured using a Pyrlinks-D EIA kit (Quidel, Santa Clara, CA) with a sensitivity of 1.1 nmol/l and an interassay CV of 4%. Because the concentration of urine solutes is altered by water excretion, Dpd was normalized to urine creatinine and reported as nanomoles Dpd per millimole creatinine. Creatinine was measured using a colorimetric assay kit with an interassay CV of 2% (Sigma Chemical).

**Bone histomorphometry.** Following excision, the left tibiae were cut in half cross-sectionally with a Dremel Moto Tool (Racine, WI). The proximal tibiae were then placed in 10% phosphate-buffered formalin (pH 7.4) for 24 h before transfer to 70% ethanol and dehydration in increasing concentrations of ethanol. Bones were then embedded undecalciﬁed in modiﬁed methyl methacrylate (4) and sectioned longitudinally at 4 μm and 8 μm thicknesses with Leica/Jung 2050 or 2165 microtomes. The 4-μm bone sections were stained by the von Kossa method with a tetrachrome counterstain (Poly- sciences, Warrington, PA). Cancellous bone structural variables were measured in these sections with an OsteoMeasure (OsteoMetrics, Atlanta, GA) and the Bioquant Bone Morphometry Systems (Nashville, TN). The sample area, within the proximal tibial metaphysis, began 1 mm distal to the growth plate and excluded the primary spongiosa. The following bone variables were measured or calculated (17): cancellous bone volume (%), trabecular width (μm), trabecular number (#/mm), and trabecular separation (μm). The following fluorochrome-based indexes of bone formation (17) were measured in the unstained 8-μm bone sections of the proximal tibial metaphysis, using the Osteometrics system: mineralizing surface (%), mineral apposition rate (μm/day), and bone formation rate (surface referent, μm3/μm2·day−1). Osteoblast, osteoclast, and osteoid surfaces were measured in the stained sections and expressed as percentages of total cancellous bone surface.

**Bone mechanical strength.** After excision, the femora were weighed, immediately wrapped in saline-soaked gauze to prevent dehydration, and stored at −20°C to maintain the mechanical properties of the bone (37). Prior to mechanical testing, femora were thawed to room temperature and kept wrapped in saline-soaked gauze except during measurements. Also prior to mechanical testing, the femoral length was measured using a digital caliper (Mitutoyo, Aurora, IL). The midshafts of the left femora were then subjected to a medial/lateral three-point bending test using an MTS material testing machine (MTS Systems, Erden Prairie, MN) as described by Leppanen et al. (21). Before mechanical testing, a preload (10 N·0.1 mm−1·s−1) was applied on the medial surface of the femur using a steel cross-bar fixture. The bending load was applied at 1.0 mm/s until failure. From the load deformation curve, the following parameters were determined for the femoral shaft: breaking load, yield load, stiffness, and displacement. Bone mechanical strength is expressed both as force (measured in Newtons) and as stress (measured in N/mm2).

**Statistical analysis.** One-way ANOVAs (for normally distributed data) were used to separately analyze male and female tissue-speciﬁc data and Tukey’s post hoc test was performed when appropriate. The Kruskal-Wallis and Mann-Whitney tests were performed when data were not normally distributed. Independent-samples t-tests were used to evaluate differences in sex steroid concentrations between intact animals of both sexes, and paired-samples t-tests were used to evaluate intra-animal differences between serum and bone sex-hormone concentrations. Data that were more than two standard deviations from the mean were considered statistical outliers and were removed prior to analyses. Results are reported as means ± SE, and P < 0.05 was defined as the threshold of signiﬁcance. All data were analyzed with the SPSS v. 15.0.0 statistical software package.

**RESULTS**

**Pilot experiment: verification of hormone delivery.** In male rats, ORX reduced serum testosterone by 97%, decreasing from 5.7 ± 1.4 ng/ml prior to surgery to 0.2 ± 0.1 ng/ml 2 days after surgery and remaining low throughout the course of 10 days (Fig. 1). Intramuscular TE administration to ORX animals elevated serum testosterone concentrations approximately four-fold above presurgical concentrations (4.4 ± 0.8 vs. 18.3 ± 14 ng/ml) within 2 days of injection. Seven days following ORX, a second TE injection was administered, which resulted in peak testosterone concentrations of 21.5 ± 1.1 ng/ml on day 8. Thus, once-weekly intramuscular TE injections resulted in sustained supraphysiological elevations in serum testosterone, with peak concentrations reaching approximately four- to five-fold higher than that observed in intact animals.

**Main experiment: serum sex steroid concentrations.** Serum testosterone was 3.3 ± 1.0 (Sham males), 0.2 ± 0.06 (GX males), 41.5 ± 3.1 (GX+TE males), 0.4 ± 0.1 (Sham females), 0.1 ± 0.03 (GX females), and 46.7 ± 4.1 ng/ml (GX+TE females) (Fig. 2). Serum testosterone was approximately eightfold higher in Sham males compared with females (P < 0.05). GX lowered serum testosterone by 99% in males (P < 0.05) and by 75% in females (P < 0.05). TE administration increased serum testosterone 12.5-fold over Sham in males (P < 0.05) and 116-fold over Sham in females (P < 0.05).

Serum DHT was 464 ± 131 (Sham males), 63 ± 6.7 (GX males), 9,824 ± 850 (GX+TE males), 37 ± 9 (Sham females), 76 ± 6 (GX females), and 11,915 ± 565 pg/ml (GX+TE females) (Fig. 2). Serum DHT was ∼3.3-fold higher in males compared with females (P < 0.05). GX reduced serum DHT concentrations by 86% in males (P < 0.05) and by 45% in females (P < 0.05). TE administration increased serum DHT 21-fold in males and 87-fold in females compared with respective Shams (P < 0.05).

Serum estradiol was 4.6 ± 0.8 (Sham males), 3.0 ± 0.4 (GX males), 6.4 ± 0.6 (GX+TE males), 8.8 ± 0.7 (Sham females), 3.6 ± 0.4 (GX females), and 5.3 ± 0.6 pg/ml (GX+TE females) (Fig. 2). Serum estradiol was 48% lower in males compared with females (P < 0.05). GX did not alter serum estradiol in males but reduced estradiol by 59% in females (P < 0.05). TE administration resulted in a trend toward increased serum estradiol (+39%, P = 0.08) in males. Con-

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**Fig. 1.** Weekly injection of testosterone enanthate (TE) elevates serum testosterone in a sustained manner. Orchectomized (ORX) male rats were injected with 7.0 mg TE im or vehicle under isoflurane anesthesia at the time of surgery and 7 days later (indicated by arrow). Blood samples were obtained from the tail tip prior to surgery and every 2 days thereafter for 10 days. Values are means ± SE, n = 5. Some error bars are within the width of the symbol.
versely, serum estradiol remained 40% below Sham concentrations in GX+TE females \( (P < 0.05) \).

**Bone sex steroid concentrations.** Tibial testosterone concentrations were 2.5 ± 0.6 (Sham males), 1.2 ± 0.2 (GX males), 24.1 ± 2.0 (GX+TE males), 0.8 ± 0.1 (Sham females), 0.8 ± 0.1 (GX females), and 30.5 ± 2.4 ng/g (GX+TE females) \( (P < 0.05) \). In Sham animals, tibial testosterone was threefold higher in males than in females \( (P < 0.05) \). Additionally, in Sham males, tibial testosterone concentrations were not different from accompanying serum concentrations; however, tibial testosterone concentrations were 12.5-fold greater than serum testosterone in GX males and 42% lower than accompanying serum testosterone concentrations in GX+TE males \( (P < 0.05) \). In females, tibial testosterone concentrations were higher than accompanying serum concentrations by 200% in Sham and sevenfold in GX animals \( (P < 0.05) \); whereas tibial testosterone concentrations of GX+TE females were 35% lower than accompanying serum testosterone concentrations \( (P < 0.05) \). Overall, GX reduced tibial testosterone in males \( (P < 0.05) \) but not in females. In males, TE administration increased tibial testosterone 20-fold above GX and 9.5-fold above Sham \( (P < 0.05) \). Similarly, in females TE increased tibial testosterone ~38-fold above both GX and Sham \( (P < 0.05) \).

Tibial DHT concentrations were 8,768 ± 1,399 (Sham males), 8,418 ± 1,595 (GX males), 16,755 ± 1,574 (GX+TE males), 5,392 ± 555 (Sham females), 5,857 ± 473 (GX females), and 22,433 ± 2,145 pg/g (GX+TE females) \( (P < 0.05) \). Tibal DHT was 63% higher in males than in females \( (P < 0.05) \). Additionally, tibial DHT concentrations were higher than accompanying serum concentrations in all groups of males (Sham: +39-fold, GX: +133-fold, GX+TE: +1.7-fold, \( P < 0.05) \) and females (Sham: +39-fold, GX: +77-fold, and GX+TE: +1.9-fold, \( P < 0.05) \). Tibial DHT was unaltered by GX in either sex, whereas TE administration increased tibial DHT by 91% in males and 416% in females \( (P < 0.05) \).

Tibial estradiol was measured in four to seven samples per group due to the limited amount of bone and to low estradiol concentrations. Tibial estradiol concentrations were 0.9 ± 0.5 (Sham males), 1.1 ± 0.5 (GX males), 1.3 ± 0.6 (GX+TE males), 4.4 ± 1.2 (Sham females), 1.4 ± 0.3 (GX females), and 2.2 ± 0.4 pg/g (GX+TE females) \( (P < 0.05) \). Tibial estradiol was 80% lower in Sham males than in females \( (P < 0.05) \). Additionally, tibial estradiol concentrations were lower than accompanying serum estradiol concentrations in Sham and GX+TE males [Sham: −80% \( (P = 0.059) \), GX: −66% \( (not significant) \), GX+TE: −80% \( (P < 0.05) \)] and in all groups of female animals (Sham: −50%, GX: −61%, and GX+TE: −58%, \( P < 0.05) \). Neither GX nor TE administration altered tibial estradiol concentrations in males, whereas GX reduced tibial estradiol by 68% in females \( (P < 0.05) \).

**Body mass.** In males, GX caused a 33% reduction in weight gain over the course of 28 days \( (P < 0.05) \). In GX+TE males, weight gain was restored \( (P < 0.05) \) and was not different from Sham \( (44.3 ± 6.8 g \text{ for Sham, } 29.4 ± 5.9 g \text{ for GX, and } 52.0 ± 4.0 g \text{ for GX+TE}) \). In females, GX caused a 94% increase in weight gain. In GX+TE females, weight gain was increased a further 67% over GX, and weight gain in GX+TE was approximately threefold greater than in Sham \( (12.8 ± 1.2 g \text{ for Sham, } 24.9 ± 1.8 g \text{ for GX, and } 41.7 ± 3.2 g \text{ for GX+TE}) \). GX did not alter kidney mass in either sex (Table 1). However, in both sexes, TE administration resulted in an approximately 35% increase in kidney mass \( (P < 0.05) \). In both sexes, the mass of the plantaris muscle was unaltered by GX. Compared with GX, TE increased plantaris mass by 13% in males \( (P < 0.05) \) and by 12% in females \( (P < 0.05) \). In males, GX reduced prostate mass by 82% \( (P < 0.05) \). TE increased prostate mass twofold over Sham \( (P < 0.05) \) and 11-fold over GX \( (P < 0.05) \).

**Bone mechanical characteristics.** In females, GX produced a 16% reduction in tibial mass \( (P < 0.05) \); Table 2) but did not alter femoral mass. In males, GX did not alter either tibial or femoral mass. In females, GX reduced femoral stiffness by 10% vs. Sham \( (P < 0.05) \); Table 2) but did not alter other femoral biomechanical characteristics or femoral length in either sex. In females, TE completely prevented the GX-induced loss of tibial mass \( (P < 0.05) \) and augmented femoral mass by 15% compared with both Sham and GX \( (P < 0.05) \). In males, TE increased tibial mass by 10% vs. GX \( (P < 0.05) \) and produced a 23% increase in femoral mass vs. both Sham and GX \( (P < 0.05) \). In both sexes, TE increased femoral breaking load by 12–19% vs. GX animals \( (P < 0.05; \text{ Fig. 5}) \). Additionally, in females, TE increased femoral breaking load by 14% compared with Shams \( (P < 0.05) \) and prevented the

![Fig. 2. Effects of TE administration on serum testosterone, dihydrotestosterone (DHT), and estradiol concentrations in GX male and female rats. Rats received GX (ORX for males and O VX for females) vs. sham surgery on day 0 and were treated with injection of 7.0 mg TE/wk im. Serum sex hormones were decreased with GX and increased with TE treatment in both sexes. Values are means ± SE; \( n = 7–10 \) per group. \#P < 0.05 vs. Sham, *P < 0.05 vs. GX.](http://ajpendo.physiology.org/)

![Fig. 3. Effects of TE administration on bone (tibial) testosterone, DHT, and estradiol concentrations in GX male and female rats. Rats received GX vs. sham surgery on day 0 and were treated with injection of 7.0 mg TE/wk im. Bone testosterone was reduced in GX males and bone estradiol was reduced in GX females, whereas bone androgens were increased with TE treatment in both sexes. Values are means ± SE; \( n = 9–10 \) per group (androgens) and 4–7 per group (estri odial). \#P < 0.05 vs. Sham, *P < 0.05 vs. GX.](http://ajpendo.physiology.org/)
GX-induced reduction in femoral stiffness ($P < 0.05$). In both sexes, TE also increased femoral length by 3–5% vs. both Sham and GX ($P < 0.05$). Neither GX nor TE administration altered maximal femoral displacement or displacement at the time of yield in either sex (data not shown).

Systemic markers of bone remodeling. Gonadectomy increased urinary Dpd/creatinine excretion in males (+28% vs. Sham, $P < 0.05$) and females (+220% vs. Sham, $P < 0.05$) (Fig. 6A). Testosterone administration completely prevented the GX-induced elevations in Dpd/creatinine in males ($P < 0.05$) and partially prevented the elevation in Dpd/creatinine excretion in females (+56% vs. Sham, $P < 0.05$). Similarly, GX increased serum osteocalcin concentrations in females (+44% vs. Sham, $P < 0.05$) and resulted in a slight increase in osteocalcin in males (+20% vs. Sham) that did not reach statistical significance (Fig. 6B). TE administration completely prevented the GX-induced increase in serum osteocalcin in females ($P < 0.05$) and reduced serum osteocalcin in males ($-28\%$ vs. GX, $P < 0.05$).

Bone structural histomorphometry. In males, GX reduced trabecular number by 52% and reduced trabecular width by 18% while increasing trabecular separation fivefold ($P < 0.05$; Table 3). In females, the effects of GX on trabecular structure were similar but less pronounced and did not reach statistical significance. GX reduced cancellous bone volume (CBV) by 72% in males and 54% in females ($P < 0.05$; Table 3 and Fig. 7, A–F). In GX males, TE increased trabecular number by 250% and reduced trabecular separation by 69%. TE prevented the GX-induced loss of cancellous bone volume in males (Table 3 and Fig. 7, $P < 0.05$) but not in females.

Fluorochrome-based bone histomorphometry. GX increased the rate of cancellous bone formation by 98% in females ($P < 0.05$; Table 3) and produced a strong trend toward increased cancellous bone formation rate in males (77% increase, $P = 0.053$). Similarly, GX increased cancellous mineralizing surface in females by 57% ($P < 0.05$) but not in males. GX did not alter mineral apposition rate, an index of osteoblastic activity, in either sex. Although some strong trends were observed, TE did not significantly reduce either bone formation or mineral apposition rate in GX males ($P = 0.06$ and 0.07, respectively) or in GX females. In females, TE prevented increases in mineralizing surface associated with GX and increased the mineral apposition rate by 25% vs. Sham ($P < 0.05$). TE did not alter other skeletal fluorochrome-based measurements in either sex.

**DISCUSSION**

Both testosterone and estradiol are known to induce skeletal effects in males and females (11, 54), while the sex-hormone dependent mechanism(s) underlying skeletal maintenance remains elusive, partially due to the interactions between testosterone and the 5α-reductase and aromatase enzymes. In hypogonadal males, testosterone replacement appears only modestly effective in improving bone quality (44), even in studies lasting up to 36 mo (40). However, higher-than-replacement testosterone administration has been shown to prevent hypogonadal bone loss in animal models (7, 8, 47) and to enhance BMD in hypogonadal elderly men (2). Likewise, testosterone administration prevents ovariectomy/hysterectomy-induced bone mineral deficits in humans (25, 33, 39, 45, 52) and animals (47) and elevates BMD in androgen-deficient females (30). In the present study, we report what appears to be the first-ever analyses of the sex hormone (i.e., testosterone, androstenedione, and estradiol) on bone remodeling in rodents.
DHT, and estradiol) content of bone. We also evaluated the effects of supraphysiological TE administration on the structural, histological, and biomechanical characteristics of bone in GX male and female rats. We found that TE administration completely prevented GX-induced cancellous bone loss in males but not in females. Additionally, TE administration increased bone length and enhanced bone strength and mass in roughly equal magnitudes between GX male and female rats. Overall, these results appear to be androgen mediated, as both the serum and bone androgens (i.e., testosterone and DHT) were elevated to supraphysiological concentrations, whereas neither serum nor bone estradiol concentrations were elevated with TE administration in either sex.

Our findings confirm previous reports from our laboratory (7, 8) and others’ (9) demonstrating a GX-induced body weight loss in male rats and GX-induced body weight gain in ad libitum-fed female rats (63), results that were both expected and that are a likely consequence of the near elimination of serum testosterone and/or estradiol. GX resulted in an expected loss of trabecular bone in males (15, 22, 60, 62), whereas the trabecular loss was less dramatic in females, perhaps due to the effects of increased body weight on bone (63). GX also produced expected prostate atrophy in males but did not alter muscle (plantaris) or kidney mass in either sex; all of these are consistent with previous findings (7, 8). Conversely, TE administration prevented trabecular bone loss in males, which appears consistent with previous research (58) and, at least partially, prevented high-turnover osteopenia in females, as indicated by reductions in urinary deoxypyridinoline/creatinine and serum osteocalcin compared with GX animals, although TE was unsuccessful in completely maintaining CBV in females. Previous research indicates that supraphysiological testosterone administration is capable of preventing cortical and cancellous bone loss in GX male rats (46, 47) and cortical bone loss in GX female rats (47). Overall, our results indicate that supraphysiological TE administration completely prevents cancellous bone loss in GX males, but not GX females, suggesting that physiological estradiol replacement may be necessary to completely prevent hypogonadism induced CBV loss in females.

The metabolic fate of circulating testosterone partially depends on its tissue-specific interactions with the 5α-reductase and aromatase enzymes (54). The results of our current study

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**Table 2. Bone mass, length, and mechanical properties**

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<td></td>
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</tr>
<tr>
<td>Tibia mass, g</td>
<td>0.56±0.01</td>
<td>0.54±0.01</td>
<td>0.59±0.01*</td>
</tr>
<tr>
<td>Femur mass, g</td>
<td>0.69±0.01</td>
<td>0.70±0.02</td>
<td>0.86±0.01**</td>
</tr>
<tr>
<td>Femur length, mm</td>
<td>34.7±0.1</td>
<td>34.3±0.2</td>
<td>35.7±0.1**</td>
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<tr>
<td>Femur yield load, N</td>
<td>124±4</td>
<td>117±7</td>
<td>138±68*</td>
</tr>
<tr>
<td>Femur stiffness, N/mm</td>
<td>236±8</td>
<td>231±15</td>
<td>218±19</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Tibia mass, g</td>
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<td>0.41±0.00#</td>
<td>0.48±0.01*</td>
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<tr>
<td>Femur mass, g</td>
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<td>0.55±0.01</td>
<td>0.63±0.14#</td>
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<td>31.9±0.1</td>
<td>33.4±0.16#</td>
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<tr>
<td>Femur yield load, N</td>
<td>96±4</td>
<td>94±2</td>
<td>109±4*</td>
</tr>
<tr>
<td>Femur stiffness, N/mm</td>
<td>190±5</td>
<td>172±7#</td>
<td>209±11*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 9–10 samples/group. #P < 0.05 vs. Sham; *P < 0.05 vs. GX.
are unique in that we present what appear to be the first-ever direct measurements of bone sex hormone concentrations. We report that intact animals have similar testosterone concentrations in both serum and bone, whereas DHT is present in much higher concentrations in bone than in serum of both males and females. Conversely, bone estradiol concentrations of intact animals were significantly lower than accompanying serum concentrations regardless of sex. Additionally, we observed that surgical gonadectomy reduced bone testosterone in males and bone estradiol in females, an effect that mimics the serum hormone responses resulting from GX. In comparison, high-dose TE administration resulted in supraphysiological elevations in serum and bone androgens (i.e., testosterone and DHT) in males and females, indicating that administered testosterone undergoes a similar degree of systemic and skeletal-specific (49) 5α-reduction, regardless of sex. However, TE administration altered neither serum nor bone estradiol in males and did not prevent the reduction in bone estradiol in GX females. Our results suggest that circulating testosterone only slightly affects nongonadal skeletal production of estradiol in males, given that 1) GX did not reduce serum or tibial estradiol below Sham concentrations; 2) GX+TE did not elevate circulating estradiol above Sham concentrations; and 3) GX+TE did not elevate bone estradiol concentrations above that of Sham or GX animals, although serum estradiol was greater in TE-administered males than in respective GX animals. Similarly, in females, the reduction in serum and tibial estradiol following GX was not prevented by TE administration, indicating that a low systemic and skeletal-specific aromatization of testosterone also occurs in females. Thus, our results appear to indicate that the skeletal protection provided by high-dose TE administration is mediated primarily through direct androgen action in bone (49–51), as both serum and bone testosterone and DHT were elevated with TE administration without concomitant increases in either circulating or bone estradiol concentrations. However, our results should not be interpreted to reduce the obvious and important role of estradiol in mediating bone maintenance in both sexes, which others have previously reported (11, 36, 54, 61).

In the present study, GX did not alter bone length in either sex, while TE administration increased bone length to a similar magnitude in male and female rats. Our findings are consistent with previous reports indicating that testosterone augments bone length in both sexes (18, 19). van der Eerden et al. (51) have reported that skeletal growth plates express ARs, along with ERα and ERβ, whereas metaphyseal bone appears to express only ARs; thus, testosterone may have increased bone length through AR- and/or ER-mediated mechanisms, as others have reported (24, 32, 53, 56). We find it unlikely that the 5α reduction of testosterone to DHT was required for the androgen-mediated linear bone growth, given previous human (3, 28, 29, 42) and animal (7, 8) studies indicating that inhibition of the 5α-reductase enzyme does not alter bone turnover or BMD. However, we cannot completely eliminate the possibility that DHT stimulated linear bone growth, considering that 1) 5α reduction of testosterone resulted in considerably elevated serum and tibial DHT concentrations, and 2) DHT is capable of promoting longitudinal skeletal growth in GX rats in the absence of testosterone (12, 41). Conversely, it seems possible that the alterations in systemic and bone estradiol, at least partially, mediated the increased bone length in testosterone-treated animals, given the necessity for ER activation in the development and maintenance of male and female bone (54). The potential, estradiol-induced enhancements in bone length may have resulted from modulation of skeletal ERα or ERβ (20), which appear to exert divergent effects on skeletal growth (23, 24, 57). Specifically, transgenic mouse models demonstrate that ERα enhances linear bone growth, while ERβ restricts linear bone growth (23, 24, 57). Thus, the elevated bone length experienced in TE-treated animals may have resulted from 1) reduced skeletal ERβ activation following the incomplete restoration of serum and tibial estradiol associated with GX+TE administration and/or 2) ER activation following localized, skeletal-specific aromatization of testosterone to estradiol. Regardless, our results indicate that TE administration uniformly escalate the magnitude of linear bone growth, independently of sex.

Skeletal strength is primarily dependent on both cancellous and cortical bone quality and quantity (27), which are influenced by sex hormone-dependent and mechanical loading-induced regulation (43). We employed a three-point bending test on the femoral midshaft to evaluate the biomechanical characteristics of cortical bone (21). Consistent with previous literature (64), femoral strength deficits were not observed within 4 wk of GX, which may indicate that mechanical loading is sufficient to maintain cortical bone mass and femoral strength in the near absence of circulating sex hormones, at least during the relatively short duration of this study (43).

### Table 3. Bone histomorphometry

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>GX</th>
<th>GX+TE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBV, %</td>
<td>7.2±1.2</td>
<td>2.0±0.4#</td>
<td>6.9±0.5*</td>
</tr>
<tr>
<td>Trabecular number, #/mm</td>
<td>2.1±0.2</td>
<td>1.0±0.3#</td>
<td>2.5±0.2*</td>
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<tr>
<td>Trabecular width, μm</td>
<td>38.9±2.6</td>
<td>30.7±2.5#</td>
<td>31.8±0.7#</td>
</tr>
<tr>
<td>Trabecular separation, μm</td>
<td>489±78</td>
<td>1.229±206#</td>
<td>385±30#</td>
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<tr>
<td>Osteoclast surface, %</td>
<td>6.9±0.2</td>
<td>6.9±0.4</td>
<td>5.2±0.4#</td>
</tr>
<tr>
<td>Osteoblast surface, %</td>
<td>23.9±3.0</td>
<td>30.7±1.5</td>
<td>14.7±1.6#</td>
</tr>
<tr>
<td>Osteoid surface, %</td>
<td>19.1±2.4</td>
<td>26.0±1.5#</td>
<td>12.2±1.4#</td>
</tr>
<tr>
<td>Mineralizing surface, %</td>
<td>19.3±4.4</td>
<td>25.1±3.9</td>
<td>18.8±1.8</td>
</tr>
<tr>
<td>MAR, μm/day</td>
<td>2.05±0.14</td>
<td>2.39±0.19</td>
<td>1.93±0.07</td>
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<tr>
<td>BFR/BS, μm3/μm2/day</td>
<td>33.6±8.3</td>
<td>59.4±9.1</td>
<td>36.2±3.6</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBV, %</td>
<td>17.0±3.3</td>
<td>7.9±2.0#</td>
<td>9.6±1.4</td>
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<td>Trabecular number, #/mm</td>
<td>3.6±0.5</td>
<td>2.2±0.6</td>
<td>3.4±0.5</td>
</tr>
<tr>
<td>Trabecular width, μm</td>
<td>46.4±4.5</td>
<td>37.1±1.7</td>
<td>33.8±1.7#</td>
</tr>
<tr>
<td>Trabecular separation, μm</td>
<td>282±48</td>
<td>583±181</td>
<td>327±52</td>
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<tr>
<td>Osteoclast surface, %</td>
<td>2.3±0.3</td>
<td>3.6±0.4</td>
<td>3.5±0.4</td>
</tr>
<tr>
<td>Osteoblast surface, %</td>
<td>12.4±1.5</td>
<td>24.3±2.4#</td>
<td>17.7±3.4</td>
</tr>
<tr>
<td>Osteoid surface, %</td>
<td>10.0±1.3</td>
<td>21.2±2.4#</td>
<td>14.7±3.1</td>
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<tr>
<td>Mineralizing surface, %</td>
<td>13.6±1.5</td>
<td>21.3±1.1#</td>
<td>15.0±1.5*</td>
</tr>
<tr>
<td>MAR, μm/day</td>
<td>1.21±0.07</td>
<td>1.41±0.08</td>
<td>1.51±0.09#</td>
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<tr>
<td>BFR/BS, μm3/μm2/day</td>
<td>16.2±2.3</td>
<td>32.1±3.1#</td>
<td>23.5±3.2</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 9–10 samples/group. #P < 0.05 vs. Sham; *P < 0.05 vs. GX. CBV, cancellous bone volume; MAR, mineral apposition rate; BFR/BS, bone formation rate (surface referent).
However, studies of longer duration have demonstrated that GX produces biomechanical deficits in bone, indicating that sex hormone-mediated skeletal regulation is necessary for bone maintenance (13, 14). In contrast, administration of high-dose TE increased maximal femoral strength, mass, and length in both male and female rats, suggesting that an elevation in total bone volume or perhaps periosteal bone formation occurred in these animals. As discussed above, we also observed that the GX-induced CBV loss was prevented by TE administration in male, but not in female, rats. It is possible that in females the effect of GX on cancellous bone was partially offset due to increased mechanical loading resulting from GX-induced body weight gain (63). TE administration also prevented the GX-induced reductions in femoral stiffness in females. Thus, it appears that TE administration produced rapid sex hormone-mediated bone accretion, which ultimately augments skeletal strength and/or stiffness.

In summary, TE administration increased both serum and bone androgen concentrations in GX male and female rats but did not alter either serum or bone estradiol concentrations. In addition, TE administration completely prevented GX-induced deficits in cancellous bone in male but not female rats. TE administration also appeared to rapidly augment cortical bone strength in both sexes, presumably through 1) direct androgen action and/or 2) ER-mediated actions associated with the aromatization of testosterone to estradiol. Our study demonstrates that high-dose TE administration induces substantial skeletal protection, including sex hormone-dependent augmentation of bone strength, mass, and length. However, we also observed a near doubling of prostate mass in male rats receiving TE, indicating at least one potential side effect of supraphysiological testosterone administration. Additionally, previous human trials have reported a variety of adverse side effects in older men following supraphysiological testosterone admin-

Fig. 7. Proximal tibial metaphyses from male Sham (A), GX (B), and GX+TE (C) rats and female Sham (D), GX (E), and GX+TE (F) rats. Note reduced mass of black-stained cancellous bone, indicative of cancellous osteopenia in GX rats of both sexes. TE treatment prevented loss of cancellous bone in males and tended to partially prevent cancellous bone loss in females in GX animals. (von Kossa-tetrachrome stain, ×40).
istration, including elevated hematocrit, leg edema, and increased incidence of prostatic events (6). Thus, future studies evaluating the safety and efficacy of supraphysiological TE administration appear necessary prior to recommending this pharmacological intervention as a viable therapy for hypogonadism-induced osteoporosis. Regardless, our study provides further rationale for the continued evaluation of selective androgen receptor modulators (35) and other anabolic therapies [e.g., combined testosterone + 5α-reductase inhibitor administration (2, 7, 8)] that may protect against hypogonadal osteopenia/osteoporosis while reducing the potential dose-dependent side effects associated with supraphysiological testosterone administration.

GRANTS
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REFERENCES


