Bone Mass Is Preserved and Cancellous Architecture Altered Due to Cyclic Loading of the Mouse Tibia After Orchidectomy*

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ABSTRACT:

Introduction: The study of adaptation to mechanical loading under osteopenic conditions is relevant to the development of osteoporotic fracture prevention strategies. We previously showed that loading increased cancellous bone volume fraction and trabecular thickness in normal male mice. In this study, we tested the hypothesis that cyclic mechanical loading of the mouse tibia inhibits orchidectomy (ORX)-associated cancellous bone loss.

Materials and Methods: Ten-week-old male C57BL/6 mice had in vivo cyclic axial compressive loads applied to one tibia every day, 5 d/wk, for 6 wk after ORX or sham operation. Adaptation of proximal cancellous and diaphyseal cortical bone was characterized by μ CT and dynamic histomorphometry. Comparisons were made between loaded and nonloaded contralateral limbs and between the limbs of ORX ($n = 10$), sham ($n = 11$), and basal ($n = 12$) groups and tested by two-factor ANOVA with interaction.

Results: Cyclic loading inhibited bone loss after ORX, maintaining absolute bone mass at age-matched sham levels. Relative to sham, ORX resulted in significant loss of cancellous bone volume fraction (-78%) and trabecular number (-35%), increased trabecular separation (67%), no change in trabecular thickness, and smaller loss of diaphyseal cortical properties, consistent with other studies. Proximal cancellous bone volume fraction was greater with loading (ORX: 290% , sham: 68%) than in contralateral nonloaded tibias. Furthermore, trabeculae thickened with loading (ORX: 108% , sham: 48%). Dynamic cancellous bone histomorphometry indicated that loading was associated with greater mineral apposition rates (ORX: 32% , sham: 12%) and smaller percent mineralizing surfaces (ORX: -47% , sham: -39%) in the final week. Loading resulted in greater BMC (ORX: 21% , sham: 15%) and maximum moment of inertia (ORX: 39% , sham: 24%) at the cortical midshaft.

Conclusions: This study shows that cancellous bone mass loss can be prevented by mechanical loading after hormonal compromise and supports further exploration of nonpharmacologic measures to prevent rapid-onset osteopenia and associated fractures.

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INTRODUCTION

OSTEOPOROSIS AS A result of hormonal loss or aging manifests itself after menopause in women and around the seventh to eighth decade in men.^(1–3) Although osteoporosis is more prevalent in women, the effects on men often result in greater morbidity and mortality, principally because related fractures occur later in life.⁽³⁾ The abrupt loss of sex steroids also predisposes men to osteoporosis if left untreated. Androgen deprivation therapy by

surgical or chemical castration to treat prostate cancer is associated with a 50% increase in osteoporotic fracture risk at corticocancellous sites in the hip, wrist, and spine within 5 yr of commencement.⁽⁴⁾

Osteoporotic fracture risk is greatest at cancellous skeletal sites,^(5,6) but small increases in bone mass at these sites can significantly reduce fracture incidence after treatment. For example, a 6% increase in BMD at the spine reduced vertebral fracture incidence by 50% after 3 yr of bisphosphonate treatment.⁽⁷⁾ Other pharmacologic agents also reduce fracture risk by inhibiting bone loss and stimulating new bone formation.^(1,7–11) However, the inhibition of cancellous bone loss without the side effects inherent to systemically administered drugs presents an attractive way to preserve mass, architecture, strength, and ultimately frac-

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ture resistance. Mechanical loading has been shown to enhance the quantity of cancellous bone mineral acquisition during prepubertal growth⁽¹²⁾ and might offer an alternative treatment to prevent bone loss in older individuals.^(13–17)

Our ability to noninvasively characterize cancellous bone changes is limited clinically, so preclinical experiments play an important role in osteoporosis research. Animal models allow bone mass and architecture changes to be distinguished after withdrawal of sex hormones by orchidectomy (ORX) and ovariectomy (OVX) and allow therapies to inhibit or reverse the resulting bone changes to be tested. In mice, both ORX and OVX result in rapid loss of cancellous bone mass and alteration of architecture.^(18–24) Osteoblast function seems to be maintained after gonadectomy,^(25,26) suggesting that concomitant stimulation of bone formation may be able to counteract the accompanying bone mass deficit.⁽²⁷⁾

Showing efficacy of a mechanical loading treatment to counteract osteoporosis at cancellous sites and in older or hormonally compromised animals is critical. Increased cortical bone formation caused by applied cyclic loading has been established in healthy growing and young adult mice.^(28–33) We previously developed an *in vivo* tibial mechanical loading model that induced a site-specific 15% increase in cancellous bone volume fraction and a 12% increase in trabecular thickness in the proximal tibia of the male C57BL/6 (B6) mouse.⁽³¹⁾ Using a similar loading approach, other investigators confirmed trabecular thickening at the same site in similarly aged young adult female B6 mice⁽³²⁾ and showed an increased cortical bone formation response after immobilization.^(33,34) Our controlled loading approach can now be applied to a preclinical hormonal compromise model of cancellous osteoporosis such as induced by ORX or OVX.

We hypothesized that mechanical loading would inhibit the cancellous bone loss associated with ORX in growing mice. To test this hypothesis, we used the loading approach previously shown to be osteogenic in growing male mice.⁽³¹⁾ Cyclic compressive loads were applied to the mouse tibia every day, 5 d/wk, for 6 wk starting 3 days after ORX. On the completion of the loading protocol, bone mass and architecture were assessed in the cancellous metaphysis and cortical diaphysis of the loaded and nonloaded control tibias using μ CT.

MATERIALS AND METHODS

We studied the adaptive response to 6 wk of loading in the tibia of growing C57BL/6J male mice (B6; Jackson Laboratories) after ORX ($n = 13$) or a sham operation ($n = 13$). This mouse strain was chosen because the B6 responds to mechanical loading and provides the background for many strains currently used to study bone adaptation.^(30–35) Before surgery, mice were acclimated to our facility for 2 wk. The Institutional Animal Care and Use Committee at the Hospital for Special Surgery approved all surgical and mechanical loading protocols.

ORX or sham surgery was performed under an intraperitoneal-injected anesthetic/analgesic cocktail (0.7 ml/kg)

consisting of ketamine (100 mg/ml; Fort Dodge Animal Health), xylazine (20 mg/ml; Lloyd Laboratories), and acepromazine maleate (10 mg/ml; Boehringer Ingelheim) mixed in the proportion of 3:3:1. Fur around the scrotal sac was clipped, and the skin prepped with alcohol and betadine. A single midline skin incision was made on the sac through which each of the two testes was palpated and removed from the body cavity. Incision of the inguinal tunic was followed in the ORX procedure by bilateral scalpal excision of the testes and electric cautery of cut vessels. Tunic and skin incisions were sutured (“closed” castration). Body mass measurements made immediately pre- and post-surgery indicated consistent loss >0.5 g because of excision of the testes from ORX mice only. Body mass of sham-operated mice was unchanged. Subcutaneous injection of buprenorphine hydrochloride (0.05 mg/kg; Reckitt Benckiser) provided postsurgical analgesic. Mice completely recovered from anesthesia before return to a cage.

All mice were 10 wk old (± 1 day) at the beginning of the experiment when mechanical loading was started to match our previous study.⁽³¹⁾ A separate, nonoperated, and nonloaded basal control group ($n = 12$) was killed at the beginning of the experiment to account for growth. Mice were caged in groups of three or four and fed *ad libitum*. Body mass was measured daily. Five mice were lost over the 6-wk period to surgical- and anesthesia-related complications, leaving 33 mice for analysis: ORX ($n = 10$), sham ($n = 11$), and basal ($n = 12$).

The number of mice per group was based on a power analysis considered before the experiment based on the effect we measured in intact animals.⁽³¹⁾ Ten mice per group would provide >90% power to detect a change of 15% with mechanical loading using a two-factor repeated-measures design (gonadal status as a between-subjects factor and mechanical loading as a within-subjects factor, $\alpha = 0.05$, SD = 10% of mean). Power calculations were made using a repeated-measure ANOVA panel (PASS 6.0; NCSS).

In the ORX and sham animals, 6 wk of mechanical loading started after a 3-day postoperation recovery period. Cyclic compressive loading was applied noninvasively at the ends of the left tibia using a custom loading device.⁽³¹⁾ A 0.2-N preload was applied immediately before dynamic compression to maintain the initial limb position. The applied waveform was a load ramp of 0.075-s duration to a peak load of 4.6 N, followed by a symmetric unload ramp of 0.075-s duration. Peak-to-peak mechanical strain at the midshaft was calculated to be ~1200 microstrain based on *in vivo* calibration.⁽³¹⁾ The dwell between each load cycle lasted 0.100 s at the preload level of 0.2 N. Cyclic loads were applied at 4 cycles/s, representing the stride frequency of the mouse.⁽²⁸⁾ Mechanical loading continued for 1200 cycles, yielding a loading period of 5 min applied every day for 5 d/wk (Monday through Friday). The force magnitude and rate were ~50% greater than used in our previous study; the alteration was intended to elicit a greater response.⁽³¹⁾ Loads were applied with the mouse under isoflurane anesthetic (2.0% in 1 liter/min O₂; Baxter Pharmaceutical Products), and recovery from the anesthetic

FIG. 1. Illustration of analysis VOI and their spatial relationship. Each box encloses a 0.5-mm-thick section (43 μ CT slices). Diaphyseal volumes of interest were located at 25%, 50%, and 75% of the tibia length. The whole bone volume of interest included all slices (~ 1550) along the entire length.

required ~ 10 min. Mice had unrestricted cage activity between mechanical loading sessions.

Dynamic histomorphometry was used to assess *in vivo* cancellous and cortical bone formation from fluorochrome labels. Intraperitoneal injections of calcein (20 mg/kg; Molecular Probes) and xylenol orange (90 mg/kg; Sigma-Aldrich) were administered at 10 and 3 days before death, respectively. Histomorphometric measures were made in a subset of mice from each of the two loaded groups ($n = 5/\text{group}$). Mice were killed by carbon dioxide inhalation 3 days after the last mechanical loading session.

BMC and architecture were compared between the loaded and contralateral control tibias of each mouse by quantitative μ CT scans as described previously (MS-8; GE Healthcare, 4 h/specimen).⁽³¹⁾ Scans were run at 90 kVp and 90 μ A (mean energy of ~ 40 keV). Voxel resolution was isotropic at 11.6 μm . Each scan included a mineral standard material (SB3; Gammex RMI) that allowed direct conversion of X-ray attenuation to BMC for each voxel.⁽³¹⁾ All μ CT data analyses were performed using custom code written in MATLAB (MathWorks, v. 6.5) with the exception of the cancellous morphometry, which was completed in Microview (GE Healthcare, v. 1.23). A global density threshold of 0.45 g/cm^3 (~ 1100 Hounsfield units) was used to separate mineralized tissue from the marrow and soft tissues.

μ CT volumes of interest (VOI) were chosen to compare local sites responding to mechanical loading. Based on previous findings⁽³¹⁾ and our hypothesis, five VOIs were defined: a cylinder of proximal metaphyseal cancellous bone; three mainly cortical diaphyseal volumes centered at 25%, 50%, and 75% of length (25%L, 50%L, and 75%L); and the whole bone (Fig. 1). All VOIs excluded the fibula. All but the whole bone VOI were 0.5 mm thick along the length of the tibia. The cylindrical VOI was defined to include only cancellous bone within the proximal metaphyseal endosteum, starting 35 μm distal to the most distal portion of the growth plate and extending 0.5 mm distally with a diameter of 1.24 mm.

Within the cylindrical cancellous VOI, bone volume fraction (BV/TV) and trabecular thickness (Tb.Th) and separation (Tb.Sp) were the primary μ CT variables of interest. BV/TV was the mineralized bone volume (BV) contained within the cylindrical volume (TV = 0.60 mm^3). 3D trabecular morphometry (Tb.Th and Tb.Sp) was directly computed by the distance transformation method.⁽³⁶⁾ Trabecular number was calculated according to the standard

relationship from 2D histomorphometry as the reciprocal of the sum of Tb.Th and Tb.Sp.⁽³⁷⁾ For Tb.Th and Tb.Sp, each voxel is associated with the center of a maximal sphere that will fit into bone or marrow space, respectively, and a volume-weighted average is calculated. Extreme cancellous bone loss resulted in the inability to make meaningful trabecular separation comparisons between limbs in seven ORX mice. The separation between some trabecular elements in these cases was larger than the thickness of the analysis volume of interest (0.5 mm). Therefore, the trabecular separation and trabecular number data reported under- and overestimate the true ORX mean, respectively.

For the diaphyseal VOIs, BMC and cross-sectional moments of inertia were the primary μ CT variables of interest. Tabulated results for total BMC and mean cross-sectional moments of inertia were calculated from the 43 consecutive slices ($43 \times 11.6\text{-}\mu\text{m}$ slice thickness = 0.5 mm) constituting each of the three cortical VOIs (25%L, 50%L, and 75%L). Cross-sectional moments of inertia were used to assess the adaptation of diaphyseal resistance to bending. The principal area moments of inertia (I_{max} and I_{min}) were directly computed by integrating the product of each bone pixel area and the square of pixel distance about orthogonal axes aligned with the long axis of the tibia.⁽³⁸⁾ Axis orientation with respect to a standard reference frame was tracked because angular orientation of principal axes may also change with mechanical loading.

For the whole bone VOI, BMC and tibial length were the primary outcome measures. Tibial length was determined as the distance between the most proximal and distal cross-sectional slices that contained bone.

Bone formation during the last week of mechanical loading was assessed with dynamic histomorphometric measures using the fluorochrome labels. After the successful μ CT scan of each hind limb, tibias were stripped of soft tissue, fixed, dehydrated in 100% ethyl alcohol, and embedded in plastic blocks (Poly/Bed 812 resin; Polysciences). Two perpendicular faces of each block were ground and polished (Ecomet 3; Buehler) to examine a cortical cross section cut near the mid-diaphysis (corresponding to a plane at 50%L; Fig. 1) and a cancellous sagittal section cut through the middle of the medial condyle (corresponding to the plane through the cancellous VOI; Fig. 1). Imaging was accomplished with a confocal microscope with digital detectors (Zeiss LSM 510, 1.8- μm isotropic pixel size with 512×512 pixel field of view [FOV]) that provided simultaneous mapping of the two fluorochrome labels. The z-depth was set for the thickness of each section at 4.5 μm . Multiple (~ 10) sections parallel to each block face and spaced at 4.5- μm intervals were imaged, and a single mid-stack section chosen for analysis. At least three FOVs were analyzed per cross-section, and multiple measurement of overlapping areas was avoided by digitally stitching adjacent FOVs together before analysis. The total area measured for each cancellous VOI was at least 0.62 mm^2 . Digital images were analyzed (BIOQUANT Image Analysis) by a blinded examiner. Mineralizing surface (MS) and mineral apposition rate (MAR) were measured, and bone formation rate (BFR) was calculated in sections corresponding to the cancellous and mid-diaphyseal (50%L) μ CT VOIs.

TABLE 1. CANCELLOUS PARAMETERS FOR CYLINDRICAL VOI LOCATED IN THE PROXIMAL METAPHYSIS OF THE TIBIA

Variable	Group	Loaded (left)		Nonloaded (right)		Δ (left – right)		Difference (%)	
BV/TV	Basal	0.21	(0.17, 0.25)	0.20	(0.15, 0.24)	0.011	(–0.016, 0.037)	8.5	(–3.8, 21)
	Sham	0.27	(0.23, 0.30)	0.18	(0.13, 0.22)	0.089 [†]	(0.046, 0.13)	68	(27, 110)
	ORX	0.15	(0.13, 0.17)	0.04*	(0.03, 0.05)	0.11 [†]	(0.10, 0.13)	290	(250, 330)
BMC (mg)	Basal	0.17	(0.15, 0.18)	0.16	(0.14, 0.19)	0.002	(–0.011, 0.015)	3.1	(–5.1, 11)
	Sham	0.21	(0.20, 0.22)	0.16	(0.14, 0.18)	0.052 [†]	(0.031, 0.072)	38	(21, 54)
	ORX	0.15	(0.13, 0.16)	0.08*	(0.07, 0.09)	0.071 [†]	(0.051, 0.090)	106	(57, 154)
Tb.Th (μ m)	Basal	42	(40, 44)	42	(39, 46)	–0.20	(–1.7, 1.3)	0.09	(–3.3, 3.5)
	Sham	63	(57, 70)	44	(39, 48)	19 [†]	(12, 27)	48	(27, 69)
	ORX	80*	(69, 92)	39	(37, 41)	42 ^{†‡}	(31, 52)	108	(85, 132)
Tb.Sp (μ m)	Basal	223	(215, 232)	222	(216, 228)	1.3	(–6.7, 9.2)	0.65	(–2.7, 4.0)
	Sham	242	(234, 250)	249 [§]	(236, 263)	–7.6	(–16, 1.2)	–2.7	(–6.3, 0.8)
	ORX (<i>n</i> = 3)	349*	(290, 408)	416*	(363, 469)	–67 ^{†‡}	(–133, –2)	–16	(–30, –2)
Tb.N (#/mm)	Basal	3.8	(3.7, 3.9)	3.8	(3.7, 3.9)	–0.01	(–0.11, 0.09)	–0.3	(–3.0, 2.4)
	Sham	3.3	(3.2, 3.4)	3.4 [§]	(3.3, 3.6)	–0.14	(–0.28, –0.01)	–3.8	(–7.7, –0.01)
	ORX (<i>n</i> = 3)	2.4*	(2.1, 2.8)	2.2*	(1.9, 2.5)	0.20	(–0.06, 0.47)	9.4	(–3.7, 22.4)

Data are presented as means (95% CIs). Basal group includes 10-wk-old mice with no loading. Sham and ORX are 16-wk-old mice with loading applied to the left tibia for 6 wk. Sample sizes were basal (*n* = 12), sham (*n* = 11), and ORX (*n* = 10) unless otherwise indicated.

p < 0.05: * vs. Right_{Sham} (significant ORX effects); [†] vs. Δ _{Basal} (significant load effects vs. basal left-right differences); [‡] vs. Δ _{Sham} (significant load effects in ORX vs. sham); [§] vs. Right_{Basal} (significant growth effects).

MS estimated the extent of actively mineralizing surface at the time of label administration and was the total extent of double label plus one half the extent of single label. MS was expressed as a ratio with bone surface (BS) as referent. MAR was the distance between midpoints of the two consecutive labels, divided by the time between label injections. BFR was calculated as the product of MAR and MS/BS.⁽³⁸⁾

To examine the effects of surgical treatment and mechanical loading, data were analyzed by two-factor ANOVA with interaction. The within-subject factor was side (left versus right tibia), and the between-subjects factor was group (basal, sham-operated, and ORX). The type I error rate (α) was set at 0.05. If the ANOVA interaction term between group and side was not significant, the main effects were examined. If the interaction term was significant, the conclusion was drawn that the effect of side was dependent on group. A significant interaction term justified comparing the difference between left (loaded in sham and ORX groups) and right (control in sham and ORX) among groups using Bonferroni-adjusted comparisons.⁽³⁹⁾ The null hypotheses were as follows: (Left-Right)_{basal} = (Loaded-Control)_{sham}; (Left-Right)_{basal} = (Loaded-Control)_{ORX}; and (Loaded-Control)_{sham} = (Loaded-Control)_{ORX}. The strength of this approach was that the change with mechanical loading in sham and ORX groups considered any naturally occurring left/right differences measured in mice at baseline. To test the possible effects of growth, a Bonferroni-adjusted comparison was made. The null hypothesis was as follows: Right_{basal} = Control_{sham}. Analysis of the effectiveness of ORX (Control_{sham} = Control_{ORX}) and bone formation measured at the end of the 6-wk experimental period excluded the basal group. Departures from normality were assessed graphically and by the Kolmogorov-Smirnov test. Statistical analyses were run using SYSTAT (SPSS Science, v. 9.0). Data tabulations were summarized with mean and 95% CI values.

RESULTS

The cancellous bone of the proximal tibial metaphysis responded significantly to both ORX and applied loading. After ORX, BV/TV, BMC, and trabecular number were reduced by –78%, –52%, and –35%, respectively. Average trabecular separation was increased (67%), whereas trabecular thickness was unaffected (Table 1; Fig. 2). BV/TV and BMC in the loaded tibias were significantly greater versus the contralateral control limb and, for ORX mice, were not significantly different from the values of sham controls, showing that loading completely prevented bone mass loss in ORX mice. The magnitude of difference in bone volume fraction caused by loading was not significantly different in ORX and sham mice, indicating that gonadal status did not affect the absolute bone mass response to mechanical loading. In contrast, the magnitudes of differences in trabecular thickness and separation caused by loading in ORX mice were significantly different than in sham mice. Mechanical loading increased trabecular thickness to a greater extent in ORX (110%) compared with sham (48%) mice and attenuated the increased trabecular separation associated with ORX (–16%; Table 1). However, prevention of bone loss by loading was not accompanied by a preservation of structure; trabecular separation was increased and trabecular number was decreased in loaded ORX limbs compared with nonloaded sham limbs (Table 1; Fig. 2). The basal mice had no left-right differences in cancellous parameters. Growth-related modeling of the cancellous tissue was evident from significantly increased trabecular separation (12%) and decreased trabecular number (–10%), but no other cancellous parameters differed between the basal and sham right limbs (Table 1).

Although cancellous bone mass and architecture were clearly affected after ORX and 6 wk of loading, the results from dynamic histomorphometric measurements based on labels given in the final week of the experiment did not

FIG. 2. Anterior view of 3D reconstructions of 0.4-mm-thick frontal sections through the proximal metaphysis centered about the midtibial plateau of paired tibias from two 16-wk-old mice: one sham-operated and one ORX. Loading was applied to the lower left leg of each mouse for 6 wk. See Table 1 for measured cancellous parameters of all mice. Bar = 1 mm.

completely parallel these adaptations. Whereas cancellous labeling variables were not affected by ORX compared with sham, differences were present after loading (Table 2). In the loaded cancellous bone, the mineral apposition rates were greater (ORX: 32%, sham: 12%), whereas the percentage of mineralizing surfaces were smaller (ORX: -47%, sham: -39%), resulting in overall smaller bone formation rates (ORX: -45%, sham: -43%).

In contrast to the large effects in cancellous bone of the proximal metaphysis, the effects in the cortical diaphysis were demonstrably smaller due to both ORX and mechanical loading by μ CT measured mineral content and architecture (Fig. 3; Table 3). ORX reduced BMC similarly by 16–17% along the diaphysis at all three VOIs (25%L, 50%L, 75%L), and reduced the principal cross-sectional moments of inertia, $I_{\max}$ (50%L: -20%) and $I_{\min}$ (25%L: -24%). Comparison of loaded to nonloaded tibias in both ORX and sham mice showed that loading-related variables were significantly greater at the midshaft (50%L): BMC (ORX: 21%, sham: 15%), $I_{\min}$ (ORX: 19%), and $I_{\max}$ (ORX: 39%, sham: 24%). Loading results were similar at 25%L for BMC (ORX: 24%, sham: 19%), $I_{\min}$ (ORX: 45%, sham: 32%), and $I_{\max}$ (ORX: 21%, sham: 16%). At these two proximal cortical sites, no variables were significantly different between the mechanically loaded ORX and control sham tibias, showing that loading completely preserved both cortical mass and architecture in ORX mice. As in the cancellous VOI, greater cortical measures of BMC and architecture caused by mechanical loading were of equal magnitude regardless of gonadal status. In addition, the cortical parameters did not change with growth nor show left-right differences in the basal mice. No differences in the angle of orientation of the principal moments were found with gonadal status, loading, or growth.

Dynamic histomorphometry for diaphyseal surfaces at the cortical midshaft (50%L) showed results opposite those in cancellous bone. Despite greater cortical bone mass caused by 6 wk of mechanical loading, labeling in the final week was not affected (Table 2). However, the endocortical surface in ORX mice exhibited greater bone formation rates than in sham mice. Little label was detected on the periosteal surfaces, and double labels were only detected in the loaded tibias of sham mice (MAR: 0.35 ± 0.78 ; BFR: $0.02 \pm 0.06 \mu\text{m}/\text{d}$).

As in the diaphysis, the whole bone effects of ORX and loading were less than in the cancellous proximal metaphysis (Table 3). Tibial length was unaffected by ORX, but tibial BMC was reduced (-18%). This BMC loss was re-

stored to sham levels with loading. Tibial length increased slightly with mechanical loading in both ORX and sham-operated mice relative to their respective contralateral control limbs (1%). Significantly increased tibial length was also present as a result of growth over the experimental period; the sham control right limbs were longer (2%) than their basal counterparts.

Despite matching the groups by age (± 1 day) and body mass (24.2 ± 1.6 g) at the start of the experimental period, ORX was associated with a significantly lower body mass versus sham mice after 6 wk (-13%; 23.1 ± 1.3 g), consistent with previous reports and indicative of the adequacy of our surgical procedure.^(21,22) Over the same period, body mass of sham mice increased (10%; 26.7 ± 2.0 g). In addition, single, small (<0.5 mm) osteophytes were consistently observed on the distal, medial, and lateral surfaces of the calcaneus and often noted on the epiphyseal medial/lateral margins of the knee joint of loaded limbs. Seven mice from each loaded group were affected at the knee joint, and two ORX mice were affected on both femur and tibia.

DISCUSSION

Cyclic mechanical loading preserved cancellous and cortical bone mass in a preclinical model of rapid-onset male osteoporosis. After 6 wk of loading, the amount of bone in the proximal tibial metaphysis of ORX mice remained at sham control levels with an impressive thickening of trabeculae. However, cancellous architecture was not maintained, as indicated quantitatively by trabecular thickness, separation, and number and qualitatively by images of the proximal metaphysis (i.e., there were fewer trabeculae in ORX mice because trabecular separation remained elevated over sham levels despite a measured reduction with loading).

ORX of the B6 mouse produced bone mass and architecture deterioration consistent with previous studies and confirmed the adequacy of our surgical procedure. In the tibial proximal metaphysis, we found a 78% cancellous bone volume fraction loss 6 wk after ORX. Previous studies in similarly aged mice with B6 backgrounds established post-ORX cancellous bone area fraction loss in the proximal tibia to be 33% after 2 wk and 60–65% after 4 wk. In these studies, trabecular separation increased and trabecular thickness was not significantly affected, in agreement with our results.^(19,22–25) This loss of cancellous bone mass after ORX by complete elimination of individual trabeculae is also similar to findings from other rodent models and

TABLE 2. CANCELLOUS AND CORTICAL DYNAMIC HISTOMORPHOMETRY OF THE TIBIA

Variable	Group	Loaded (left)		Nonloaded (right)		Δ (left – right)		Difference (%)	
Cancellous proximal metaphysis									
MS/BS	Sham	0.07 [†]	(0.04, 0.10)	0.13	(0.06, 0.21)	–0.06	(–0.12, 0.00)	–39	(–71, –6.7)
	ORX	0.07 [†]	(0.03, 0.12)	0.15	(0.09, 0.22)	–0.07	(–0.13, –0.02)	–47	(–82, –13)
MAR	Sham	1.86 [†]	(1.50, 2.22)	1.67	(1.34, 1.99)	0.21	(–0.11, 0.52)	12	(–8.1, 32)
	ORX	1.81 [†]	(1.40, 2.22)	1.26	(0.54, 1.97)	0.55	(0.03, 1.07)	32	(–7.5, 72)
BFR	Sham	0.12 [†]	(0.08, 0.16)	0.21	(0.11, 0.31)	–0.09	(–0.18, –0.01)	–43	(–85, –2.4)
	ORX	0.12 [†]	(0.05, 0.20)	0.22	(0.08, 0.36)	–0.09	(–0.21, 0.03)	–45	(–95, 4.6)
Cortical diaphysis: endocortical surface (50%L)									
MS/BS	Sham	0.20	(0.09, 0.30)	0.26	(0.14, 0.38)	–0.06	(–0.19, 0.07)	–15	(–56, 26)
	ORX	0.34	(0.17, 0.50)	0.40	(0.23, 0.58)	–0.07	(–0.27, 0.14)	8.9	(–75, 93)
MAR	Sham	1.14	(0.75, 1.52)	1.21	(0.80, 1.63)	–0.08	(–0.42, 0.27)	–3.3	(–30, 23)
	ORX	1.10	(0.55, 1.64)	1.43	(1.04, 1.81)	–0.33	(–1.22, 0.56)	–10	(–59, 39)
BFR	Sham	0.20	(0.10, 0.30)	0.28	(0.15, 0.41)	–0.08	(–0.14, –0.02)	–27	(–43, –11)
	ORX	0.43*	(0.16, 0.71)	0.55*	(0.35, 0.75)	–0.11	(–0.49, 0.26)	17	(–90, 124)
Cortical diaphysis: periosteal surface (50%L)									
MS/BS	Sham	0.05	(0.01, 0.08)	0.05	(–0.01, 0.11)	–0.01	(–0.09, 0.08)	–11	(–74, 52)
	ORX	0.04	(0.01, 0.08)	ND		ND		ND	

Data are presented as means (95% CIs). Fluorochrome labels were administered 10 and 3 days before the completion of the 6-wk experiment. Sample sizes were $n = 5$ per group. Single labels were not detected (ND) on the periosteal surfaces of nonloaded tibias in ORX mice, and periosteal double labels were ND in ORX and the nonloaded tibias of sham mice.

$p < 0.05$: * vs. Sham (significant ORX effects); [†] vs. Right (significant load effects).

FIG. 3. Principal cross-sectional area moments of inertia, $I_{\max}$ and $I_{\min}$, as determined by μ CT analysis for both sham and ORX mice. Plotted are mean $\pm$ SD diaphyseal values for loaded (left) and control (right) tibias from 25–75% of length (proximal to distal). $I_{\max}$ and $I_{\min}$ of loaded tibias were increased significantly ($p < 0.05$) proximally (in cross-sections left of the vertical dashed line), and this difference caused by mechanical loading decreased to near zero distally. The fibula was excluded from analysis but caused a discontinuity in $I_{\max}$ near 60% of length at anastomosis with the tibia.

mirrors the morphological traits associated with osteoporotic fracture in men.^(5,21,27,40,41)

Bone adaptation after both ORX and controlled loading support use of the methods we have developed for preclinical testing of mechanical loading interventions to combat rapid-onset osteopenia. The advantage of directly loading the tibia versus an exercise model is that mechanical-based adaptation to controlled loading is isolated from unintended systemic effects (i.e., neural, muscular, and cardiovascular).^(21,42,43) Additionally, controlled loading of a single limb, difficult to achieve in exercise studies, allows

stronger intraanimal comparisons against a contralateral nonloaded control limb and allows testing for the effects of different loading parameters. Loading the tibia offers a major advantage over the other standard mouse long bone loading model (ulna) because of the relatively large volume of cancellous bone in the proximal tibia.^(28–34)

Whereas examination of a cancellous compartment after single-limb, whole bone mechanical loading of ORX mice is novel, our study has some limitations. Modest cortical effects were shown with loading, and we concentrated our efforts on the more clinically relevant cancellous compart-

TABLE 3. TIBIAL LENGTH AND MINERAL CONTENT FOR THE WHOLE TIBIA AND CORTICAL DIAPHYSEAL VOIS

Variable	Group	Loaded (left)		Nonloaded (right)		Δ (left – right)		Difference (%)	
Whole tibia									
Length (mm)	Basal	17.61	(17.51, 17.72)	17.63	(17.52, 17.74)	–0.01	(–0.08, 0.05)	–0.075	(–0.42, 0.27)
	Sham	18.28	(18.12, 18.45)	18.05 [‡]	(17.86, 18.24)	0.23 [†]	(0.17, 0.30)	1.3	(0.97, 1.6)
	ORX	18.21	(18.07, 18.34)	18.00	(17.84, 18.17)	0.21 [†]	(0.12, 0.29)	1.1	(0.71, 1.6)
BMC (mg)	Basal	20.05	(18.53, 21.57)	19.98	(18.71, 21.25)	0.07	(–0.41, 0.56)	0.062	(–7.5, 7.6)
	Sham	24.59	(22.53, 26.65)	21.41	(19.06, 23.77)	3.17 [†]	(1.30, 5.04)	16	(7.2, 25)
	ORX	20.48	(19.31, 21.65)	17.60 [*]	(16.29, 18.91)	2.88 [†]	(1.99, 3.77)	17	(12, 22)
25% length from proximal end									
BMC (mg)	Basal	0.55	(0.51, 0.59)	0.55	(0.51, 0.58)	0.00	(–0.01, 0.02)	0.51	(–2.1, 3.1)
	Sham	0.65	(0.60, 0.71)	0.56	(0.49, 0.62)	0.10 [†]	(0.04, 0.15)	19	(8.5, 29)
	ORX	0.58	(0.55, 0.61)	0.47 [*]	(0.43, 0.51)	0.11 [†]	(0.09, 0.13)	24	(18, 30)
50% length from proximal end									
BMC (mg)	Basal	0.44	(0.40, 0.48)	0.45	(0.42, 0.48)	–0.01	(–0.03, 0.01)	–2.5	(–6.3, 1.4)
	Sham	0.55	(0.50, 0.60)	0.48	(0.43, 0.54)	0.06 [†]	(0.01, 0.12)	15	(3.4, 26)
	ORX	0.49	(0.46, 0.51)	0.40 [*]	(0.37, 0.44)	0.08 [†]	(0.06, 0.10)	21	(15, 27)
75% length from proximal end									
BMC (mg)	Basal	0.40	(0.38, 0.43)	0.41	(0.38, 0.43)	0.00	(–0.02, 0.01)	–0.89	(–3.5, 1.7)
	Sham	0.44	(0.40, 0.49)	0.42	(0.39, 0.46)	0.02	(0.01, 0.04)	4.9	(–1.0, 11)
	ORX	0.36 [*]	(0.34, 0.38)	0.35 [*]	(0.33, 0.38)	0.01	(–0.01, 0.02)	2.1	(–1.1, 5.3)

Data are presented as means (95% CIs). Basal group includes 10-wk-old animals with no loading. Sham and ORX are 16-wk-old animals with loading applied to the lower left leg for 6 wk. Sample sizes were basal ($n = 12$), sham ($n = 11$), and ORX ($n = 10$).

$p < 0.05$: * vs. Right_{Sham} (significant ORX effects); [†] vs. Δ _{Basal} (significant load effects vs. basal left-right differences); [‡] vs. Right_{Basal} (significant growth effects).

ment. However, because no standardized testing methods of mouse cancellous bone strength exist, we were limited to assessing two surrogates of strength—mass and architecture. Cancellous bone strength also depends on the quality of the bone tissue itself, but the relative importance of these three factors is unknown. Therefore, the direct assessment of strength in the metaphysis, as well as the diaphysis, should be considered in future studies. Because of the complex loading environments involved in normal and osteopenic bone, the best assessment of strength may ultimately come from specimen-specific finite element modeling.^(44,45) Age at the time of intervention may influence adaptation, and the age of mice used in this study limits interpretation to the postpubescent and middle aged. We chose an age at which B6 mice are not rapidly growing (10 wk) to maintain consistency with our previous experiment.⁽³¹⁾ Additionally, we included a basal group to control for differences because of age. The growth spurt was nearly complete, as evidenced by the low rate of longitudinal growth over our 6-wk experimental period (~2%). However, body mass and trabecular separation were increased and trabecular number decreased without any intervention. The differences in trabecular parameters are indicative of modeling, which may have contributed to loading responses. The physiologic milieu (stem cells, growth factors, other cytokines, etc.) changes with age and could affect bone resorption, formation, and response to loading.

After ORX at any age, preserving cancellous architecture and fracture resistance from deterioration remains a challenge. However, several pieces of evidence from our study suggest that cancellous bone retains the capacity to respond. Accumulation of bone mass with mechanical loading was similar in both sham and ORX. However, in ORX mice, this accumulation represented a preservation of bone

mass that occurred despite a loss of cancellous surface area caused by decreased trabecular number. Therefore, the removal of whole trabeculae was offset by a loading-associated increase in mineral apposition rate, suggestive of greater osteoblast work, which resulted in trabeculae that were thickened beyond that even of sham-operated mice.

Our net histomorphometry results lend support to other data indicating the transient nature of cellular response after ORX in that bone formation rates at the end of the 6-wk experiment were similar regardless of gonadal status.^(20,26) These post-ORX bone formation rates in B6 mice are 8-fold lower than those measured by others after 4 wk, possibly indicating a dramatic decrease in cellular response as adaptation to new loading and cytokine environments is accomplished.⁽²²⁾ Mechanical loading may also suppress osteoclastic activity, as observed in exercise studies, which could partially account for both the preservation of cancellous bone mass and decrease in bone formation rates observed in the final week of our experiment.^(46,47)

The degree of mineral apposition resulting in thickened trabeculae may be driven by the magnitude of loading as previously shown for cortical bone.⁽³⁰⁾ The loading protocol used in this study was identical to our prior work except for the use of 50% higher loading strain magnitude (~1200 microstrain at midshaft).⁽³¹⁾ Because of consistency of our protocols, strain rate was also increased by the same 50%. As expected, this higher level of loading elicited a greater cancellous and cortical response in gonadally intact mice (48% versus 12% increased trabecular thickness). Based on this evidence and the similarity in the absolute bone mass responses to loading we measured in ORX and sham-operated mice, we speculate that trabecular bone mass after ORX may also depend on loading magnitude. Unexpectedly, the higher loading magnitude used also stimulated a

small increase in longitudinal growth of the tibia (~1%) and osteophyte formation in both ORX and sham mice. Higher magnitude (~3000 microstrain), long-term loading of the rat ulna similarly resulted in osteophyte formation, although longitudinal growth was reduced.⁽⁴⁸⁾ In these preclinical loading models, all parameters such as loading magnitude can be precisely controlled and require additional study.

In conclusion, this experiment showed that cyclic mechanical loading prevents cortical and cancellous bone loss that occurs because of ORX but also alters cancellous morphology. Our mechanical loading intervention after ORX partially prevented an increase in trabecular separation and substantially increased trabecular thickness to preserve cancellous bone mass. The mechanical consequences of this altered architecture require further study in both cancellous and cortical bone as does the determination of how modifications in the treatment regimen affect bone mass and morphology. Based on these data, loading offers an intriguing possible countermeasure to reduce osteoporotic fracture associated with periods of rapid bone turnover such as often occur after androgen-deprivation therapy is started for prostate cancer.

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