Oral Estradiol Impact on Mitigating Unloading-Induced Bone Loss in Ovary-Intact Rats

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BACKGROUND: The impact of the spaceflight environment on endogenous estrogen production in female crewmembers and the resulting impact on other adaptations, like bone loss, is an under-investigated topic. Hence, we investigated the interaction of exogenous 17-β estradiol (E2) treatment and disuse to test the hypothesis that E2 treatment would mitigate disuse-induced bone loss.

METHODS: There were 40 virgin female Sprague-Dawley rats (5 mo old) randomized to placebo (PL; 0 ppm E2) or estrogen (E2; 10 ppm E2) treatments, delivered via custom-made rodent diets; half of each group was randomized to either weightbearing (WB) or hindlimb unloading (HU) for ~39 d.

RESULTS: We observed expected lower values after HU (6–15%) in volumetric BMD and cross-sectional areas at the proximal tibia metaphysis (PTM, by pQCT), 20% lower %BV/TV (nonsignificant) at the PTM, and 11% lower femoral neck maximal load; none of these HU-induced impacts were modified by E2. Impaired PTM periosteal expansion was observed in all E2-treated rats, with smaller (−13 to −18%) cross-sectional areas. Midshaft tibial geometry was unaffected by E2 treatment, but large reductions (−73 to −81%) in periosteal bone formation indices were observed in E2-treated rats.

DISCUSSION: In summary, modest supplementation of exogenous E2 did not mitigate decrements in volumetric BMD, PTM cross-sectional geometry, or femoral neck strength observed with HU. However, numerous independent impacts of E2 treatment were observed, with significant suppression of periosteal bone formation indices. If maintained over time, this might impact negatively on cortical bone integrity during prolonged nonweightbearing.

KEYWORDS: disuse, histomorphometry, mechanical properties.

Mantri AV, Allaway HCM, Brezicha JE, Hogan HA, Bloomfield SA. *Oral estradiol impact on mitigating unloading-induced bone loss in ovary-intact rats*. Aerosp Med Hum Perform. 2021; 92(2):65–74.

The recent launch of NASA's Artemis program has brought new priorities to the forefront of America's space exploration and research efforts. The Artemis program aims to land the first woman and next man on the Moon by 2024 and establish sustainable missions by 2028. A critical physiological effect of spaceflight is a striking and rapid loss of bone mass and mineral content.[8,9,24] Current exercise protocols and improvements in dietary intake have successfully mitigated much of the spaceflight-associated bone loss in International Space Station crew. However, the design of the spacecraft for the return of humans to the Moon and Mars may reduce the footprint available for exercise equipment, increasing the need for pharmacological protocols that enhance exercise to preserve musculoskeletal health. Since optimal alternative countermeasures for spaceflight-associated bone loss are likely to vary between male and female astronauts, models for pre- and postmenopausal female and male astronauts must be used in on-going investigations.

Estrogen is an important hormone in the regulation of bone turnover and bone cell activity. It inhibits apoptosis in osteoblasts and osteocytes, induces apoptosis in osteoclasts, and inhibits the proliferation and differentiation of osteoclast precursors into mature osteoclasts (reviewed in Almeida et al.[3]).

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This manuscript was received for review in May 2020. It was accepted for publication in November 2020.

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DOI: https://doi.org/10.3357/AMHP.5668.2021

When provided in the form of hormone therapy, exogenous estrogen is well-known to have protective effects on bone in postmenopausal women, in whom endogenous estradiol is low.[3] In premenopausal women, low endogenous estradiol can occur in women with irregular and absent menstrual cycles. The use of transdermal 17-β estradiol in low-weight girls with anorexia nervosa, which can induce clinically significant bone loss, increases areal bone mineral density (BMD).[11] In oligoamenorrheic athletes, transdermal 17-β estradiol (with cyclic micronized progesterone) use results in a small reduction in P1NP (bone formation marker) and no change in NTx (bone resorption marker) over 12 mo.[1,18] However, transdermal 17-β estradiol is not a form of contraception; ovulation still occurs and young, sexually active women must be advised of the risks as they undergo treatment to recover absent menstrual cycles.

On Earth the requirements for maintaining bone health differ between men and women and with age.[3] Most animal research on disuse (during spaceflight and with ground-based models) has been conducted on either male or ovariectomized (OVX) female rats. Investigators have noted increased bone turnover and bone mineral loss in the hindlimbs of male and OVX female rats exposed to hindlimb unloading (HU);[10,13,22] however, the impact of disuse on bone in ovary-intact female rats has not been clearly determined.[2,19,21] Examinations of the effects of disuse on ovary-intact female rats have demonstrated an age-dependent impact on bone,[4,19,21] probably due to differences in baseline bone turnover. In young, skeletally immature animals (<4 mo old at HU initiation) total BMD decreases in the femur and tibia, with greater loss in cancellous vs. cortical compartments.[4,10,19] In skeletally mature animals (>4 mo old at HU initiation) there are reports of no impact of HU on total BMD; reduced cortical bone area in the proximal tibia;[2] reduced or no change in maximum load for midshaft femur or tibia;[2,13,22] reduced or no change in bone volume/tissue volume (%BV/TV), trabecular thickness (Tb.Th), and trabecular number in the distal femur metaphysis;[7,13] and reduced mineralizing surface/bone surface (%MS/BS)[2] and bone formation rate (BFR/BS)[7] of femoral and tibial periosteal surfaces. Interestingly, some investigations have determined that female rats experience less bone loss with HU than do males,[4] while others find no sex differences.[7]

Previous investigations examining the positive impact of exogenous estrogen on bone have used OVX rats to mimic the estrogen-deficient state of menopause, which does not effectively model the physiological effects of exogenous estrogen provided alongside endogenous ovarian estrogen production in premenopausal women. We are unaware of published data documenting alterations in plasma estradiol during spaceflight in female crewmembers. Given the propensity of many female crew to use hormonal contraceptives during missions to suppress menstrual bleeding,[14] it remains an open question as to whether endogenous estradiol production is altered by the spaceflight environment. Ground-based studies with ovary-intact adult female rats exposed to HU have demonstrated declines in plasma estradiol and reduced time spent in estrus after 38 d of HU in 3-mo-old virgin female rats.[23] We previously observed a (nonsignificant) 49% decline in circulating estradiol in 6-mo-old retired breeder females.[2] The causal impact of these declines in endogenous estrogen exposure on HU-induced bone loss has not been established. In young, virgin female rats randomized to a phytoestrogen or purified, phytoestrogen-free diet, there were no differences in the degree of bone loss induced by 35 d of HU.[21] However, those animals maintained on the phytoestrogen diet did exhibit reduced humeral and femoral longitudinal bone growth, indicating dietary sources of phytoestrogens are an important consideration when measuring bone endpoints.[21]

No previous investigations have tested the interaction of exogenous estrogen administration and disuse, nor the underlying biology behind changes in bone outcomes, in ovary-intact female rodents. Given that estrogen supports osteoblast activity and inhibits osteoclastic activity,[3] we hypothesized that oral 17-β estradiol treatment initiated prior to and continued during HU would mitigate disuse-induced bone loss in ovary-intact female rats.

METHODS

Animals and Study Design

All procedures involving animals were reviewed and approved by the Texas A&M University Institutional Animal Care and Use Committee. All animal care was conducted in facilities accredited by the Association for the Assessment and Accreditation of Laboratory Care (AALAC). Skeletally mature, virgin female Sprague Dawley rats ($N = 40$) were obtained from Harlan Labs (Houston, TX) at 4 mo of age and allowed to acclimate to a purified, phytoestrogen-free pelleted diet (AIN-93M; Research Diets) for 4 wk prior to initiation of the study. Animals had ad libitum access to food and water throughout the study. All animals were housed in a temperature-controlled (23 ± 2°C) room with a 12-h light-dark cycle in an AAALAC-accredited animal care facility.

After 4 wk of purified diet acclimation, animals were randomized to one of two diets based on the AIN-93M rodent diet and maintained on this for the duration of the study: placebo (PL; 0 ppm 17-β estradiol) or estrogen (E2; 10 ppm 17-β estradiol) hormone treatments. At this time point, all rats were moved to single housing to allow for food intake measurements and to match the single-housing condition of rats subjected to HU. A week later, animals within hormone treatment groups were randomized to weight bearing (WB) or HU groups. The randomized loading treatment was initiated and maintained for, on average, 39 d (range: 35–42 d). This resulted in four combined study groups: placebo weight bearing (PL-WB), placebo hindlimb unloading (PL-HU), estradiol-treated weight bearing (E2-WB), and estradiol-treated hindlimb unloading (E2-HU). The fluorochrome calcein (Sigma Aldrich, St. Louis, MO, USA) was given as intraperitoneal injections 9 and 2 d prior to euthanasia to label mineralizing bone surfaces.

At the end of the HU treatment, animals were anesthetized with a ketamine-dexdomitor 2:1 cocktail (0.5 mL · kg^{-1}) before

being euthanized; all suspended animals were anesthetized before removal from the suspension apparatus to prevent any weight bearing by the unloaded hindlimbs. Following anesthetization, whole blood was collected via cardiac puncture and animals were then euthanized by decapitation, in accordance with the recommendations and guidelines of the American Veterinary Medical Association. Femora and tibiae were harvested from both hindlimbs, stripped of soft tissue, and stored for later analysis. The soleus muscle of the right hindlimb was collected and weighed. Uteri (with both horns) were dissected free of the ovaries and cervix and wet weight obtained; any unusually high levels of fluid within each uterus were noted. Uterine weights were recorded as a bioassay for estrogen activity.

Procedures

Hindlimb unloading treatment. On study day 15 (week 2) animals were randomized to loading treatment. Animals randomized to the WB group continued with normal cage activity in standard rat-sized shoebox cages. The unloading of microgravity was simulated by the well-accepted model of tail suspension,[12,20] in which each animal's hindquarters were suspended off the cage floor with a custom-made harness attached to the tail to disallow weight-bearing by the hindlimbs of the animal. Briefly, while the rat was under anesthesia (ketamine:dextomitor 2:1 cocktail), the tail was cleaned and dried thoroughly. A thin layer of adhesive (Amazing Goop, Eclectic Products, Los Angeles, CA, USA) was applied to the tail along the medial and lateral sides of the tail. A standard porous tape (Kendall, Mansfield, MA, USA) harness was pressed firmly to the glue and allowed to dry (~30 min). A paper clip attached the tail harness to a swivel apparatus on the rod spanning the top of a 46 × 46 × 46 cm cage; the rod was equipped with rubber stoppers located ~4 cm from each edge to disallow contact of the rats' hindlimbs with the cage walls. To prevent any contact of the hindlimbs with the cage floor, the height of the animals' hindquarters were adjusted, resulting in an approximately 30° head-down tilt.[6] The forelimbs of the animal maintained contact with the solid plastic cage floor, allowing the animal full access to the entire cage. All animals were monitored twice daily for health, including assessment of tail integrity.

Peripheral quantitative computed tomography for bone mineral density and geometry. Excised right tibiae and femurs were stored in phosphate buffered saline (PBS)-soaked cotton gauze and stored at −20°C until analysis. Once thawed, ex vivo peripheral quantitative computed tomography (pQCT) scans of the proximal tibia metaphysis, tibia diaphysis (midshaft), and femoral neck were completed on a Stratec XCT Research-M device (Norland Corp., Fort Atkinson, WI, USA). A voxel size of 70 μm, scanning beam thickness of 500 μm, and scanning speed of 2.5 mm · s^{-1} were used to obtain cross-sectional geometry and volumetric bone mineral density (vBMD). Before each use, the machine was calibrated with hydroxyl-apatite standard cone and cortical phantoms per manufacturer instructions.

Thawed whole tibiae were placed in a vial filled with PBS to maintain proper hydration during the scan, after which they were rewrapped in PBS-soaked gauze and returned to the −20°C freezer. At the metaphysis of the tibia, four adjacent transverse slices 2 mm distal to the growth plate were scanned; at the tibial diaphysis (50% of total tibial length) one transverse slice was scanned. The femoral neck was placed in a mold designed to hold the specimen in alignment with the scanning axis of the CT scanner and three adjacent transverse slices medial to the greater and lesser trochanter were scanned.

Scan slices were analyzed using Stratec software (version 6.00, Norland Corp., Fort Atkinson, WI, USA). Standardized analysis schemes were used for the metaphyses (contour mode 3, peel mode 4, contour threshold of 0.450 g · cm^{-3}, peel threshold of 0.800 g · cm^{-3}, and Cortmode 4, outer threshold of 0.450 g · cm^{-3} and inner threshold of −0.100 g · cm^{-3}), diaphyses (contour mode 1, peel mode 2, outer and inner threshold of 0.650 g · cm^{-3}, and Cortmode 2, outer threshold of 0.650 g · cm^{-3}), and femoral neck (contour mode 3, peel mode 2, outer threshold of 0.480 g · cm^{-3}, inner threshold of 0.710 g · cm^{-3}, and Cortmode 2, outer threshold of 0.300 g · cm^{-3}). Machine precision (based on manufacturer data) is ± 3.0 mg · cm^{-3} for cancellous bone and ± 9.0 mg · cm^{-3} for cortical bone.

Histomorphometry analyses. Undemineralized left proximal and distal tibia were fixed in 4% phosphate-buffered formalin for 24 h then subjected to serial dehydration and embedded in methyl methacrylate (Aldrich M5, 590-9; Sigma-Aldrich, St. Louis, MO, USA). Embedded proximal tibia underwent serial frontal sectioning at 8 μm and 4 μm thick. The 8 μm sections were left unstained for flurochrome calcein label measurements. The 4 μm sections were treated with von Kossa stain and tetrachrome counterstain to measure static cancellous microarchitecture. Cross-sections of the embedded distal tibia closest to the midshaft (diaphysis) were made on an Isomet Low Speed Saw (Buehler, Lake Bluff, IL, USA) approximately 17 μm thick and left unstained for flurochrome calcein label measurements. All cancellous histomorphometric analyses were performed using OsteoMeasure Analysis System, version 3.3 (OsteoMetrics, Inc., Atlanta, GA, USA) within a defined region of interest starting 500 μm from the growth plate and extending distally within the endocortical edges, encompassing ~8 mm^2. All nomenclature for cancellous histomorphometry follows standard usage.[5]

Dynamic cancellous histomorphometric measures assessed at magnification ×200 included single-labeled surface, double-labeled surface, percent mineralized surface/total bone surface (%MS/BS), and interlabel distance for mineral apposition rate (MAR). Bone formation rate/total bone surface (BFR/BS) was calculated [MAR x MS/BS] within the OsteoMeasure Analysis System. When double label was absent in cancellous and cortical compartments, MAR and therefore BFR/BS was taken to equal zero. There were four animals missing double label for cancellous compartment measurements (one E2-WB, one PL-HU, two E2-HU).

Static cancellous histomorphometry measures were assessed at magnification ×400 and included bone volume as a percent

of total tissue volume (%BV/TV), Tb.Th., trabecular separation, and trabecular number. Relative osteoid (%OS/BS) and osteoclast (%Oc.S/BS) surfaces as a percent of total cancellous bone surface were measured as well. Cortical cross-sections were analyzed at magnification ×200 for %OS/BS and MAR, and BFR/BS calculated as above for the periosteal surface.

Plasma estradiol assay. Plasma was analyzed in duplicate by ELISA (Cayman Chemical; Ann Arbor, MI) for estradiol to verify the impact of the dietary estrogen treatment on endogenous estrogen levels. Manufacturer-reported intra- and interassay CVs are ± 6.5 and ± 12.3%, respectively.

Mechanical testing. Tibial 3-point bending and femoral neck axial loading tests were performed on those specimens used earlier for pQCT scanning that were brought to room temperature. All tests were performed on hydrated specimens using a material testing machine (Instron 3345, Norwood, MA, USA) with a 1000-N load cell. Load and displacement data were collected during tests (at 10 Hz) and analyzed using Bluehill software (version 2, Instron Bluehill) and a custom-written Matlab (version 9.3.0, The MathWorks, Inc., Natick, MA, USA) program.

Mechanical properties of the tibia diaphysis were evaluated using load-to-failure 3-point bending tests. The bone was placed lateral side down on metal pin (D = 3 mm) supports (L = 18 mm). The loading pin was centered above the lower supports and contacted the medial surface at the midpoint of the specimen (mid-diaphysis). Quasi-static loading was applied under displacement control (2.54 mm · min^{-1}) until complete fracture. Extrinsic or structural properties were determined from the load-displacement data directly as follows: stiffness (k; N · mm^{-1}) = the slope of the load-displacement curve in the linear region; maximum force (F_{max}; N) = the largest force achieved during the test before fracture; energy to fracture (U_f; mJ) = the area under the load-displacement curve for the whole test to final fracture. Cross-sectional moment of inertia (CSMI; mm^4) was determined from the ex vivo pQCT scans of each tibia at 50% of bone length (midshaft). The average rectangular CSMI (values reported in **Table I**) at the tibia midshaft was taken as one-half of the polar area moment of inertia from the pQCT scan. Intrinsic material properties were calculated according to classical beam theory. Elastic modulus (*EM*; MPa) and ultimate stress (*US*; MPa) were estimated using the following equations:

$$EM = \frac{kL^3}{48 * CSMI} \quad \text{Eq. 1}$$

$$US = \frac{F_{max}\, L\left(\frac{D_{ML}}{2}\right)}{4 * CSMI} \quad \text{Eq. 2}$$

where k is stiffness, L is the support span (18 mm), and D_{ML} is the measured medial-lateral periosteal diameter.

For femoral neck testing, each right proximal femur was placed upright with the diaphysis portion of the bone firmly inserted into a properly sized and fitted hole in a 1/2-inch thick aluminum plate fixture. A 10-mm cylindrical platen with a flat head was used to apply a load to the femoral head, parallel to the axis of the shaft of the femur. Quasi-static loading was applied under displacement control (2.54 mm · min^{-1}) until fracture occurred. The only outcome variable reported was the maximum force because the combination of compression, bending, and shear loading on the femoral neck makes intrinsic property estimation impractical.

Statistical Analyses

SPSS (IBM; Armonk, NY, USA) was used for all statistical analyses. Data were assessed for normality and outliers prior to analyses. All data are represented as mean ± SD. Differences between study groups were assessed with a 2-way ANOVA (hormone and loading treatments) for organ weights, pQCT, mechanical testing, and histology variables. Body weight and food consumption across the study was assessed with a repeated measures 2-way ANOVA (hormone and loading treatments) with a Greenhouse-Geisser correction.

RESULTS

Animals tolerated the tail suspension well, with no rats being removed from the study due to tail issues. Plasma estradiol (at experiment's end) was about twofold higher in those rats on the E2 treatment (1625 ± 961.6 pg · mL^{-1}) than those on the PL treatment [783.5 ± 427.7 pg · mL^{-1}; $F(1,24) = 5.470$, $P = 0.030$]. Based on the dietary content of the 17-β estradiol (0.01 gm%) and our food intake records, animals eating the E2 diet consumed an average of 98.3 ± 10.0 μg estradiol/d over the experimental period (range: 72.8–115.2 μg/d). No independent effect of HU on plasma estradiol was detected [$F(1,24) = 2.011$, $P = 0.172$]. All groups had equivalent body weights at study outset [average weight = 256 ± 19 g; $F(1,24) = 0.547$, $P = 0.467$] and consumed the same amount of food per day during facility acclimation [week 0; $F(1,24) = 0.281$, $P = 0.601$]. Starting the first week E2 diet treatment was initiated (week 1), E2-treated animals failed to gain weight over the remainder of the study (−12 ± 10 g), while PL-treated animals gained an average of 9 ± 20 g [$F(3.6,86.4) = 22.598$, $P < 0.001$, time*E2 effect; **Fig. 1A**]. Rats on E2 treatment ate less than PL-treated animals beginning the week of hormone treatment initiation [$F(4.9,110) = 11.315$, $P < 0.001$]; however, by the end of week 2 their food intake was very similar to that of PL-treated animals (**Fig. 1B**). There was no significant interaction (time*E2*loading) effect on bodyweight [$F(3.6,86.4) = 2.319$, $P = 0.070$] or food intake [$F(4.8,110) = 1.615$, $P = 0.164$].

Although in our experience male rats placed on HU reduce food intake over the first 7 d, we did not observe this in the females on HU; in fact, starting in week 3 (second week of HU), our HU animals ate significantly more than did WB animals [$F(4.8,110) = 9.092$, $P < 0.001$; Fig. 1B). This did not result,

Table I. Cross Sectional Geometry of the Tibia Assessed by Ex-Vivo Peripheral Quantitative Computed Tomography (pQCT).

	TREATMENT GROUPS				*P*-VALUES		
	PL-WB	PL-HU	E2-WB	E2-HU	INTERACTION	E2	LOADING
Proximal Tibia Metaphysis							
Area (mm^2)							
Total	14.4 ± 1.9	14.4 ± 1.3	13.4 ± 0.6	12.2 ± 1.3	0.265	**0.005**	0.250
Marrow	7.7 ± 1.3	8.4 ± 1.1	7.1 ± 0.5	6.6 ± 1.2	0.159	**0.008**	0.751
Cortical	6.7 ± 0.7	6.0 ± 0.5	6.3 ± 0.3	5.9 ± 0.4	0.924	**0.048**	0.001
Cortical Thickness (mm)	2.14 ± 0.14	2.14 ± 0.10	2.06 ± 0.04	1.96 ± 0.10	0.231	**0.005**	0.232
Periosteal Circumference (mm)	13.4 ± 0.9	13.4 ± 0.6	13.0 ± 0.3	12.3 ± 0.7	0.230	**0.005**	0.231
Tibia Diaphysis							
Cortical vBMD (mg/cm^3)	1420 ± 12	1408 ± 16	1414 ± 10	1406 ± 9	0.593	0.362	**0.035**
Area (mm^2)							
Total	5.7 ± 0.6	5.9 ± 0.3	5.8 ± 0.3	5.7 ± 0.4	0.352	0.827	0.816
Marrow	1.6 ± 0.3	1.7 ± 0.3	1.7 ± 0.2	1.6 ± 0.2	0.327	0.896	0.528
Cortical	4.2 ± 0.4	4.2 ± 0.2	4.2 ± 0.2	4.1 ± 0.2	0.532	0.606	0.808
Cortical Thickness (mm)	0.7 ± 0.02	0.6 ± 0.03	0.6 ± 0.03	0.6 ± 0.02	0.761	0.429	0.387
Periosteal Circumference (mm)	8.5 ± 0.5	8.6 ± 0.2	8.6 ± 0.2	8.5 ± 0.3	0.337	0.852	0.793
Endocortical Circumference (mm)	4.4 ± 0.4	4.6 ± 0.3	4.6 ± 0.2	4.5 ± 0.3	0.302	0.829	0.509
CSMI (mm^4)	3.2 ± 0.9	3.5 ± 0.4	3.1 ± 0.3	3.2 ± 0.6	0.523	0.303	0.379

Data presented as mean ± SD. Bolded *P*-values are $P < 0.05$.
CSMI = cross-sectional moment of inertia; Interaction = hormone*loading treatments; PL = 0 ppm 17-β estradiol in diet, E2 = 10 ppm 17-β estradiol in diet; Loading = loading treatment: WB = normal cage activity, HU = ~39 d of hindlimb unloading.

however, in increased body weight in HU rats as compared to WB groups; there was no independent impact of HU detected on animal bodyweight [$F(3.6,86.4) = 1.385$, $P = 0.249$; Fig. 1A].

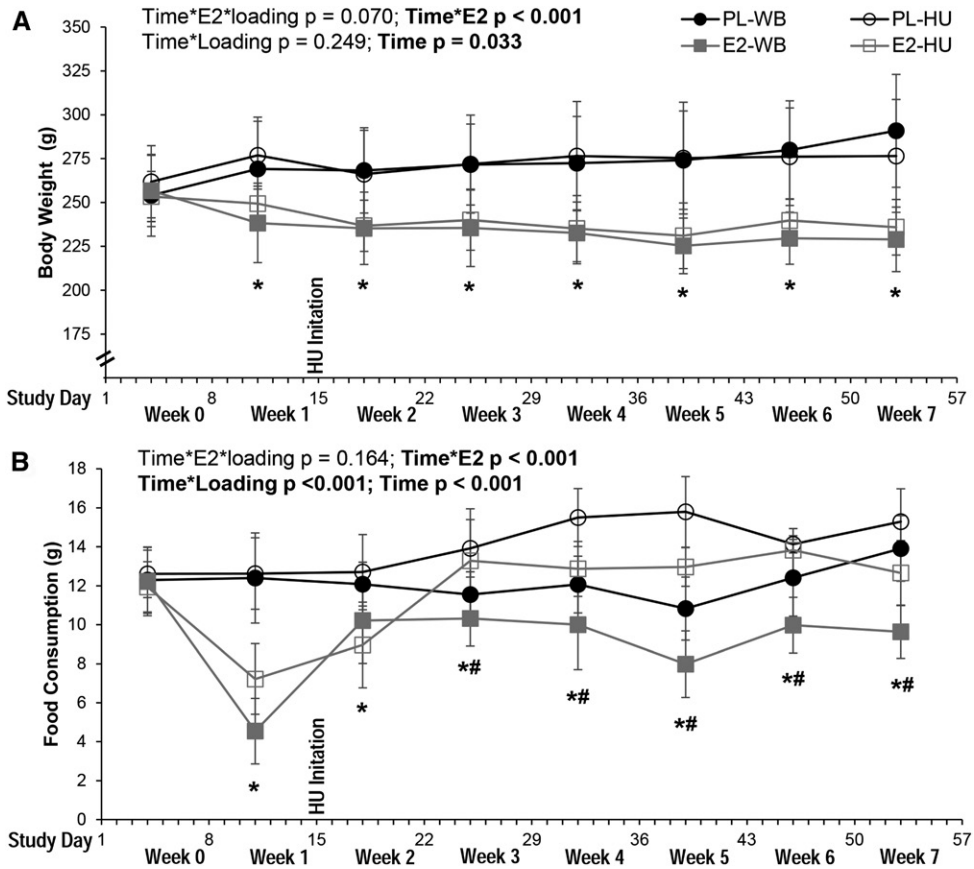

Fig. 1. A) Animal average weekly body weight and B) daily food consumption across the study. *E2 significantly different from PL within the week ($P < 0.050$); #HU significantly different from WB within the week ($P < 0.050$). PL = 0 ppm 17-β estradiol in diet, E2 = 10 ppm 17-β estradiol in diet; Loading = loading treatment: WB = normal cage activity, HU = ~39 d of hindlimb unloading.

At the end of the study, both E2- and PL-treated animals on HU had significantly smaller solei (on average, 69% lower weight) than did all WB animals [$F(1,24) = 92.97$, $P < 0.001$; **Fig. 2A**]. No independent effect of E2 treatment on solei weight was detected [$F(1,24) = 1.083$, $P = 0.308$]. The percent difference in soleus weight in E2-treated rats (−58%) was less than the percent difference observed in PL-treated animals [−83%; E2*loading interaction, $F(1,24) = 4.757$, $P = 0.039$]. Conversely, HU had no independent impact on uterine weight [$F(1,19) = 3.053$, $P = 0.138$], but E2-treated groups as expected exhibited significantly heavier uteri (~+29%) vs. PL-treated groups [$F(1,19) = 5.348$, $P = 0.032$; **Fig. 2B**], with no evidence of an interaction [$F(1,19) = 0.354$, $P = 0.559$].

Our skeletally mature, intact female rats demonstrated significant loss of bone mass with prolonged HU (35–40 d), as assessed by pQCT. We observed significant reductions in total [−9%, $F(1,24) = 10.589$, $P = 0.003$], cancellous [−15%, $F(1,24) = 6.137$, $P = 0.021$], and cortical shell [−9%, $F(1,24) = 10.614$, $P = 0.003$] vBMD (**Fig. 3A-C**) at the tibia metaphysis compared

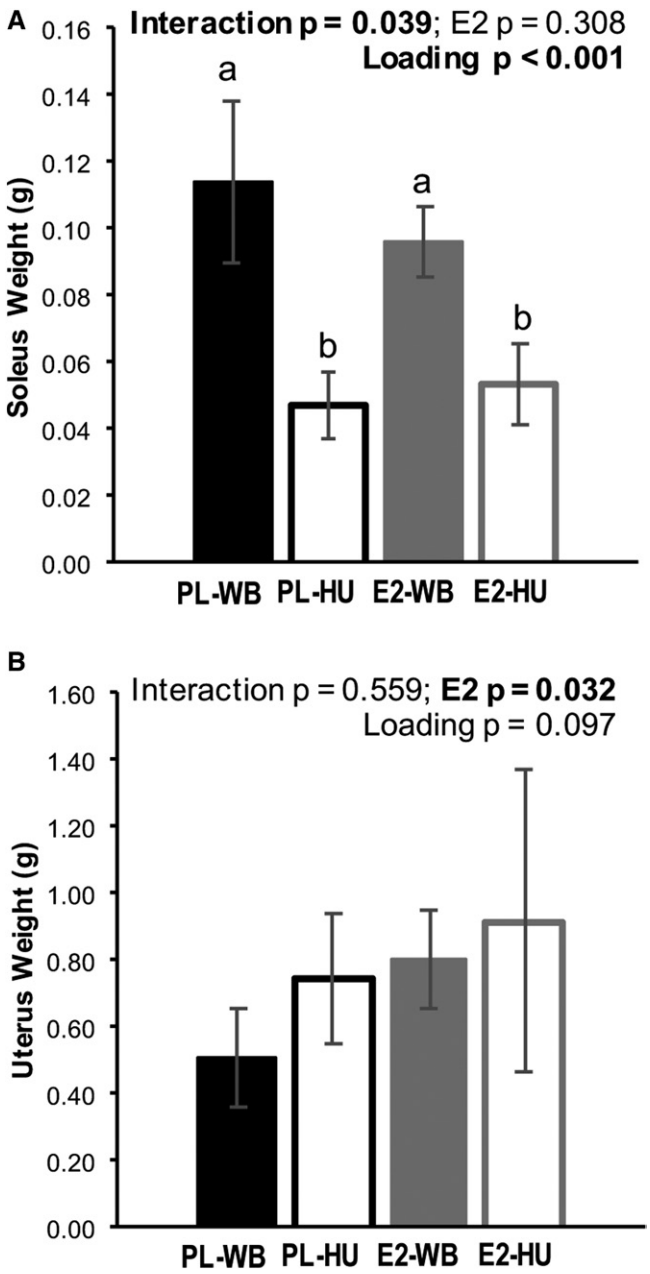

Fig. 2. A) Soleus weight and B) uterine weights in the animals. Bars with different superscript letters are significantly different ($P < 0.050$). Interaction = hormone*loading treatments; PL = 0 ppm 17-β estradiol in diet, E2 = 10 ppm 17-β estradiol in diet; Loading = loading treatment: WB = normal cage activity, HU = ~39 d of hindlimb unloading.

with WB animals. At the other mixed bone site, the femoral neck, only cancellous vBMD was significantly reduced (−6%) in HU animals [$F(1,24) = 1.106$, $P = 0.034$; **Fig. 3D-F**]. The thick cortical shell at this site appeared to be unaffected by unloading [$F(1,24) = 0.132$, $P = 0.720$], resulting in only small, nonsignificant reductions in femoral neck total vBMD [$F(1,24) = 0.547$, $P = 0.467$]. At the tibia mid-diaphysis, HU animals exhibited a slightly lower (−1%) cortical vBMD compared with WB animals [$F(1,24) = 4.970$, $P = 0.035$; Table I]. Other than an average 8% smaller cortical shell area [$F(1,24) = 16.31$, $P = 0.001$] at the proximal tibia metaphysis in HU animals (Table I), there were no significant changes in cross-sectional geometry measures at the proximal tibia metaphysis, midshaft diaphysis (Table I), or femoral neck (**Table II**) with this prolonged period of mechanical unloading.

We observed multiple independent effects of oral E2 treatment on vBMD and cross-sectional geometry in the metaphyseal cortical bone compartment. Cortical shell vBMD at the proximal tibia was higher in all E2-treated animals [+6%; $F(1,24) = 4.305$, $P = 0.049$; Fig. 3C]; the main effect for E2 for total vBMD at this site almost reached statistical significance [$F(1,24) = 4.140$. $P = 0.053$]. No impact of E2 treatment on cancellous vBMD was detected [$F(1,24) = 0.482$, $P = 0.494$]. Notably, cross-sectional areas for total [−13%; $F(1,24) = 9.601$, $P = 0.005$], marrow [−18%; $F(1,24) = 8.461$, $P = 0.008$], and cortical [−13%; $F(1,24) = 10.027$, $P = 0.004$] compartments at this bone site were smaller in E2-treated rats compared with placebo-treated animals (Table I). Cortical shell thickness [$F(1,24) = 9.503$, $P = 0.005$] and periosteal circumference [$F(1,24) = 9.512$, $P = 0.005$] of the tibial metaphysis were also reduced by 6% in E2-treated animals. Interestingly, none of these bone geometry outcomes at the femoral neck were altered by E2 treatment (Table II). We could not detect any modification of the impact of mechanical unloading by E2 treatment (no significant interactions) on these vBMD or geometry outcomes.

Although cortical vBMD at the tibia mid-diaphysis was lower after HU, we could not detect any impact of HU on structural and material mechanical properties at this bone site (**Table III**). We did measure a consistent reduction (on average, 11%) in maximum force at the femoral neck [$F(1,24) = 7.407$, $P = 0.012$] after HU in pooled PL and E2 groups. We found no evidence for any impact of E2 treatment on this HU-induced decline in femoral neck strength.

HU animals exhibited on average a 20% lower cancellous %BV/TV after 39 d than did WB rats (**Table IV**), but this comparison was not statistically significant [$F(1,23) = 0.2971$, $P = 0.098$]. There was a reduced Tb.Th in all HU animals (~8%) compared with WB animals [$F(1,23) = 5.944$, $P = 0.023$]. There was no significant impact of this mechanical unloading on indices of bone formation activity (%OS/BS, %MS/BS, MAR, and BFR/BS) nor on bone resorption activity (%Oc.S/BS) in the cancellous compartment and on the periosteal surface of the midshaft tibia.

By contrast, there were distinct and significant differences observed after E2 treatment on %MS/BS [$F(1,19) = 5.080$, $P = 0.036$], MAR [$F(1,19) = 13.229$, $F = 0.002$], and BFR [$F(1,19) = 19.891$, $P < 0.001$] at the midtibia periosteal surface and on MAR in the proximal tibia cancellous bone [$F(1,22) = 5.749$, $P = 0.025$]. Mineral apposition rate appeared to be particularly impacted by E2 treatment, with reductions of 76% and 70% in MAR at the midtibial periosteal surface in WB and HU groups, respectively; %MS/BS was substantially reduced by E2 treatment in WB rats (−57%), but minimally in HU animals (−4%). These reductions in indicators of osteoblast vigor (MAR) and number (%MS/BS) on the midtibia periosteal surface resulted in much smaller BFR/BS values in

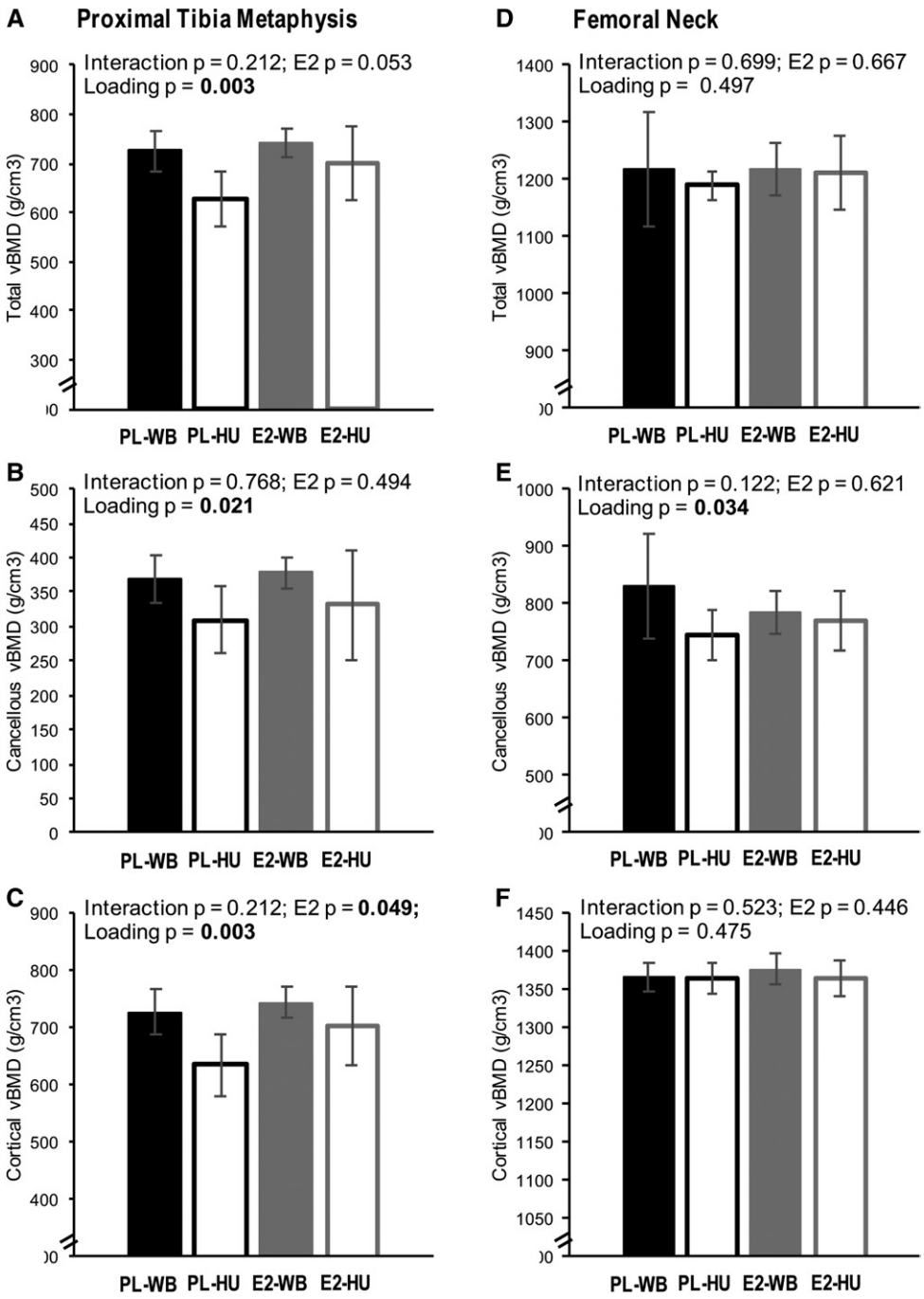

Fig. 3. Volumetric bone mineral density (vBMD) of the A–C) proximal tibia metaphysis and D–F) femoral neck assessed by peripheral quantitative computed tomography (pQCT). Total (A & D), cancellous (B & E), and cortical (C & F) vBMD. Interaction = hormone*loading treatments; PL = 0 ppm 17-β estradiol in diet, E2 = 10 ppm 17-β estradiol in diet; Loading = loading treatment: WB = normal cage activity, HU = ~39 d of hindlimb unloading.

E2-treated WB (−91%) and E2-treated HU (−71%) animals vs. the paired PL groups. Bone formation rate in the cancellous bone compartment was 91% and 39% lower in E2- vs. PL-treated WB and HU animals, respectively; the main effect for estrogen in this case did not reach significance [$F(1,22) = 3.376$, $P = 0.079$]. The hormone-loading interaction terms were close to significance for both %MS/BS at the periosteal surface [$F(1,19) = 3.978$, $P = 0.061$] and for %OS/BS in the proximal tibia cancellous bone [$F(1,23) = 3.947$, $P = 0.059$]. Relative osteoid surface (reflecting new bone matrix formation) was halved in E2-WB animals, but increased by 85% in E2-HU rats vs. placebo-treated rats.

DISCUSSION

The purpose of this study was to evaluate the impact of oral 17-β estradiol, delivered via a customized rodent diet, on disuse-induced bone loss in skeletally mature, virgin female rats with intact ovaries. Our hypothesis that oral 17-β estradiol treatment would mitigate disuse-induced bone loss was not supported by our data, at least in terms of the most prominent densitometric and structure variables assessed. Interestingly, oral 17-β estradiol did significantly mitigate the typical loss of soleus weight in HU animals. We also observed significant decrements in metaphyseal and midshaft tibial cortical vBMD and trabecular thickness with ~39 d of unloading and an independent negative impact of oral 17-β estradiol on cortical bone formation indices that likely impacted on cortical bone geometry at the metaphysis.

Given estrogen's well-known impacts on inhibiting apoptosis in osteoblasts,[3] increasing circulating estradiol might be expected to diminish the bone loss incurred with mechanical unloading of HU, during which bone formation activity is severely depressed.[7,20] At the level of estrogen supplementation provided by our customized diet containing 10 ppm 17-β estradiol, we did not detect any impact on HU-induced losses in vBMD at the proximal tibia and femoral neck, sites rich in cancellous bone, nor on histomorphometric measures of cancellous bone volume (%BV/TV) and microarchitectural parameters, nor on the decline in maximal load observed at the femoral neck.

Table II. Cross-Sectional Geometry of the Femoral Neck Assessed by Ex Vivo Peripheral Quantitative Computed Tomography (pQCT).

	TREATMENT GROUPS				*P*-VALUES		
	PL-WB	PL-HU	E2-WB	E2-HU	INTERACTION	E2	LOADING
Area (mm^2)							
Total	3.8 ± 0.8	3.3 ± 0.5	3.6 ± 0.5	3.4 ± 0.4	0.557	0.673	0.122
Marrow	1.5 ± 0.6	1.3 ± 0.2	1.3 ± 0.3	1.2 ± 0.3	0.717	0.530	0.303
Cortical	2.8 ± 0.3	2.6 ± 0.3	2.8 ± 0.2	2.7 ± 0.2	0.629	0.117	0.095
Cortical Thickness (mm)	0.6 ± 0.09	0.6 ± 0.04	0.6 ± 0.05	0.6 ± 0.07	0.618	0.453	0.720
Periosteal Circumference (mm)	6.8 ± 0.7	6.4 ± 0.4	6.7 ± 0.4	6.5 ± 0.3	0.615	0.763	0.129
Endocortical Circumference (mm)	3.2 ± 1.2	2.9 ± 0.5	3.0 ± 0.7	2.8 ± 0.7	0.963	0.554	0.439

Data presented as mean ± SD.
Interaction = hormone*loading treatments; PL = 0 ppm 17-β estradiol in diet, E2 = 10 ppm 17-β estradiol in diet; Loading = loading treatment: WB = normal cage activity, HU = ~39 d of hindlimb unloading.

To our knowledge, we are the first to document multiple independent effects of E2 treatment on cortical bone geometry and bone formation indices on periosteal surfaces in ovary-intact HU rats, which effects have been well documented previously in E2-treated weightbearing animals.[15] Interestingly, the impact of E2 treatment on cortical periosteal bone in our hands was limited to metaphyseal cortical bone, with no impact observed at the tibia diaphysis (Table I). Highly significant main effects of E2 treatment were observed, with smaller cross-sectional areas (total, cortical, and marrow), cortical thickness, and periosteal circumference at the proximal tibia metaphysis. Even though midshaft tibial bone geometry was unaffected by E2 treatment, we detected large reductions at this site in MAR (~73%) and BFR/BS (~81%) in E2-treated rats, independent of loading status. This suggests that, were unloading continued for a longer period, this modest increase in circulating estradiol might ultimately have a negative impact on normal periosteal apposition in diaphyseal bone and result in reduced cortical and/or total cross-sectional area. We were not able to collect similar fluorochrome-based data on the periosteal surface of metaphyseal bone, but the significantly lower total and cortical areas are consistent with reductions in MAR and/or %MS/BS at this bone site. The smaller total and cortical areas at the proximal tibia in E2-treated animals likely explains the apparent positive impact of the oral 17-β estradiol treatment on vBMD of this bone compartment (Fig. 3).

Interestingly, estrogen's impact on cortical bone formation activity is highly surface-specific. Similar to the suppressive effect on periosteal bone formation rate that we observed in our skeletally mature virgin female rats, young male rats given low-dose 17-alpha ethinyl estradiol for 5 wk exhibited a 30% lower BFR/BS on ulnar periosteal surfaces, but a (nonsignificant) 17% increase in ulnar endocortical BFR/BS.[15] These very different impacts of ethinyl estradiol on murine osteoblasts on periosteal vs. endocortical surfaces mirror those seen during puberty in girls, when the rapid increase in circulating estrogen promotes bone formation on long bone endocortical surfaces (as mediated by estrogen receptor-alpha signaling), but suppresses periosteal expansion. Estrogen signaling impacts on cortical bone surfaces may also be sex-specific; in female mice, estrogen receptor-beta deletion results in greater periosteal expansion, whereas it has no effect on periosteal bone formation in male mice.[17] To our knowledge, there are no published data examining the impact of actual or simulated microgravity on estrogen receptor signaling; this is a key knowledge gap, given the multiple systems, including bone, impacted by estrogen signaling in both males and females.

In general, we observed a moderate detrimental impact of unloading on bone mass and integrity in these skeletally mature, ovary-intact virgin female rats. After an average of 39 d unloading, vBMD of the total, cancellous, and cortical compartments at the proximal tibial metaphysis were lower by 9–15%, similar to the magnitude of declines we usually observe in skeletally mature, 6-mo-old male Sprague-Dawley rats (5–12%).[16,20] The cancellous compartment vBMD of the femoral neck in these female rats exhibited a smaller deficit of 6%, likely contributing to an 11% decline in maximum load at this site, similar to deficits in femoral neck strength observed by others in ovary-intact,

Table III. Mechanical Properties of the Tibia Diaphysis (3-Point Bend to Failure) and Femoral Neck (Axial Compression).

	TREATMENT GROUPS				*P*-VALUES		
	PL-WB	PL-HU	E2-WB	E2-HU	INTERACTION	E2	LOADING
Tibia Diaphysis							
Maximum Force (N)	91.8 ± 26.6	101.3 ± 7.2	100.6 ± 16.4	91.9 ± 6.9	0.149	0.958	0.945
Stiffness ($N \cdot mm^{-1}$)	235.4 ± 61.6	250.6 ± 27.4	260.7 ± 20.6	234.2 ± 30.5	0.168	0.761	0.703
Energy to Fracture (mJ)	48.6 ± 14.4	53.2 ± 19.6	52.2 ± 16.7	52.6 ± 21.4	0.782	0.842	0.735
Elastic Modulus (MPa)	11.2 ± 2.5	11.3 ± 1.3	12.1 ± 0.8	11.3 ± 0.8	0.386	0.443	0.591
Ultimate Stress (MPa)	240.1 ± 65.9	260.1 ± 20.9	256.0 ± 0.4	241.2 ± 26.1	0.260	0.922	0.868
Femoral Neck							
Maximum Force (N)	78.4 ± 7.7	68.7 ± 7.4	77.6 ± 8.6	70.6 ± 8.2	0.659	0.859	**0.012**

Data presented as mean ± SD. Bolded *P*-value is $P < 0.05$.
Interaction = hormone*loading treatments; PL = 0 ppm 17-β estradiol in diet, E2 = 10 ppm 17-β estradiol in diet; Loading = loading treatment: WB = normal cage activity, HU = ~39 d of hindlimb unloading.

Table IV. Histomorphometric Measures of Bone Formation Indices and Cancellous Microarchitecture of the Tibia.

	TREATMENT GROUPS				*P*-VALUES		
	PL-WB	PL-HU	E2-WB	E2-HU	INTERACTION	E2	LOADING
Proximal Tibia Metaphysis							
MS/BS (%)	2.06 ± 1.43	2.24 ± 0.88	1.13 ± 0.86	1.55 ± 1.31	0.802	0.086	0.521
MAR (um · d^{-1})	0.86 ± 0.66	0.94 ± 0.65	0.10 ± 0.06	0.60 ± 0.67	0.379	**0.025**	0.223
BFR/BS ($um^3/um^2/yr$)	5.80 ± 4.23	9.05 ± 6.50	0.51 ± 0.56	5.55 ± 8.82	0.711	0.079	0.097
BV/TV (%)	20.5 ± 2.6	16.4 ± 4.6	22.1 ± 4.4	19.4 ± 7.0	0.743	0.262	0.098
OS/BS (%)	0.30 ± 0.09	0.15 ± 0.14	0.13 ± 0.11	0.24 ± 0.24	0.059	0.551	0.721
OcS/BS (%)	2.4 ± 1.4	2.0 ± 0.9	2.3 ± 2.1	1.5 ± 0.7	0.676	0.594	0.243
Tb.Th (um)	53.3 ± 2.5	49.2 ± 4.1	51.8 ± 4.5	47.6 ± 5.4	0.959	0.376	**0.023**
Tb.Sp (um)	211.1 ± 40.8	265.6 ± 72.3	188.8 ± 40.7	221.4 ± 85.1	0.671	0.204	0.100
Tb.N (mm)	3.9 ± 0.5	3.3 ± 0.7	4.2 ± 0.6	4.0 ± 1.0	0.624	0.089	0.185
Tibia Diaphysis							
MS/BS (%)	30.78 ± 15.25	25.05 ± 2.85	13.09 ± 8.65	23.97 ± 7.81	0.061	**0.036**	0.544
MAR (um · d^{-1})	0.54 ± 0.31	0.43 ± 0.32	0.13 ± 0.06	0.13 ± 0.12	0.577	**0.002**	0.607
BFR/BS ($um^3/um^2/yr$)	59.44 ± 37.00	39.05 ± 29.40	5.28 ± 3.60	11.21 ± 10.97	0.206	**0.001**	0.477

Data presented as mean ± SD. Bolded ***P***-values are $P < 0.05$.

Interaction = hormone*loading treatments; PL = 0 ppm 17-β estradiol in diet, E2 = 10 ppm 17-β estradiol in diet; Loading = loading treatment: WB = normal cage activity, HU = ~39 d of hindlimb unloading.

MS/BS = mineralized surface/total bone surface; MAR = mineral apposition rate; BFR/BS = bone formation rate/total bone surface; BV/TV = bone volume/tissue volume; OS/BS = osteoid surface/bone surface; OcS/BS = osteoclast surface/bone surface; Tb.Th = trabecular thickness; Tb.Sp. = trabecular separation; Tb.N. = trabecular number.

skeletally mature female rats.[13] Interestingly, retired breeder female rats (maintained on normal rat chow containing phytoestrogens) exhibit no loss of total or cancellous vBMD at the proximal tibia metaphysis, but modest (5%) reductions in cortical shell area at this bone site, similar to that observed in the current study.[2] It should be noted that the highly variable, and sometimes quite low, baseline values for metaphyseal cancellous bone volumes may contribute to the lack of change observed in those retired breeder female rats.

In this study, we focused on the impact of oral 17-β estradiol treatment in combination with an extended duration HU protocol in skeletally mature, intact, female Sprague-Dawley rats. We found no evidence that this modest supplementation of exogenous estradiol mitigated the decrements seen in bone vBMD, cross-sectional geometry, or the decline in maximal load at the femoral neck observed with hindlimb unloading. Numerous independent impacts of our estrogen treatment were observed, with moderate suppression of bone formation indices at periosteal surfaces which, if maintained over time, might impact negatively on cortical bone integrity during the prolonged nonweightbearing of the spaceflight environment.

ACKNOWLEDGMENTS

The authors thank Alexandra Slavinsky Marich and Michael Junior, undergraduates of the Bone Biology Laboratory, for their assistance with the daily animal work involved in this publication. This work was a product of Dr. Anita V. Mantri's dissertation and was completed posthumously in her memory.

Financial Disclosure Statement: This study was supported by the National Biomedical Research Institute, the Huffines Institute for Sports Medicine and Human Performance, and the American College of Sports Medicine. The authors have no competing interests to declare.

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