EXPERIMENTAL STUDY

Dose–response of estrogen on bone versus the uterus in ovariectomized mice

Ulrike I L Mödder¹, B Lawrence Riggs¹, Thomas C Spelsberg², Daniel G Fraser¹, Elizabeth J Atkinson³, Rudolf Arnold⁴ and Sundeep Khosla¹

¹Endocrine Research Unit, ²Biochemistry and Molecular Biology and ³Biostatistics, Mayo Clinic College of Medicine, Rochester, Minnesota 55905, USA and ⁴Department of Internal Medicine, Division of Gastroenterology and Endocrinology, Philipps University, Marburg, Germany

(Correspondence should be addressed to S Khosla, Mayo Clinic, 200 First Street SW, 5-194 Joseph, Rochester, MN 55905, USA; Email: khosla.sundeep@mayo.edu.)

Abstract

Background: Estrogen is known to have important effects on both reproductive and non-reproductive tissues. Moreover, there is increasing interest in developing compounds that may have selective effects on bone versus reproductive tissues.

Methods: Since mouse models are often used in these studies, we administrated increasing doses of estradiol (E₂) (0 to 500 µg/kg/day) by slow release pellets to ovariectomized 6-month-old C57BL/6 mice and assessed skeletal and uterine responses following 2 months of treatment.

Results: The mice lost bone at multiple sites following ovariectomy (OVX); however, while the lowest E₂ dose of 5 µg/kg/day completely prevented loss of cancellous bone (at the lumbar spine and tibial metaphysis), it had no stimulatory effects on the uterus. Higher doses of E₂ resulted in further increases in bone mineral density, with eventual stimulation of the uterus at a dose of 40 µg/kg/day. By contrast, when 3-month-old C57BL/6 mice were administered the same doses of E₂ and studied after 1 month, the 5 µg/kg/day dose resulted in uterine hypertrophy, but was not able to prevent loss of cancellous bone.

Conclusions: Thus these results (i) provide data on the dose–response for the effects of E₂ on mouse bone and (ii) indicate that the relative effects of E₂ on bone versus the uterus are highly dependent on the particular experimental conditions used. This issue needs to be considered in evaluating agents with potential ‘selective’ effects on bone versus reproductive tissues.

European Journal of Endocrinology 151 503–510

Introduction

Estrogen plays an important role in both reproductive and non-reproductive tissues. In addition to its major role in regulating the uterus and other reproductive tissues, estrogen also influences pubertal growth, regulates bone maturation, and maintains bone mass in women and in men after puberty. The importance of estrogen for bone metabolism was perhaps best demonstrated by the description of the males with mutations in the estrogen receptor (1) or the P450 aromatase genes (2, 3). These patients all had osteoporosis, unfused epiphyses, and continuing linear growth into adulthood. Treatment of the patients with aromatase mutations with estrogen led to closure of the epiphyses and a dramatic increase in bone mass. It has also long been known that estrogen deficiency is the major cause of postmenopausal bone loss in women (4) and, based on more recent data, perhaps also in aging men (5, 6).

In recent years, the mouse has increasingly been used as a model to study mechanisms of bone loss as

well as estrogen action on bone. One reason is the availability of numerous inbred strains with differences in peak bone mass (7, 8) and susceptibility to bone loss following ovariectomy (OVX). In addition, a large number of genetically altered mice have been developed with important skeletal phenotypes, some involving defects in estrogen signaling pathways (9, 10). Of note, most of these mice have been bred into the C57BL/6 background strain.

Despite the importance of mice in general and the C57BL/6 strain in particular as tools to study estrogen action on bone, the precise dose–response of estrogen on bone and reproductive tissues in these mice has not been clearly defined. Moreover, these mice are often used to evaluate agents with potential tissue-specific actions (i.e. favorable effects on bone without effects on reproductive tissues), without clear data at present on the relative dose effects of estrogen on these tissues. In addition, some studies have used very high estrogen replacement doses following OVX, which often result in a sclerotic effect and a decrease

in endocortical circumference in mouse long bones (11, 12). Indeed, this has led some to question the relevance of the mouse model for studying postmenopausal bone loss (13).

The goal of the present study was to systematically to define the dose–response effect of estrogen on bone and the uterus in C57BL/6 mice. We also tested whether the estrogen dose–response in these tissues differed, depending on the particular experimental paradigm used.

Materials and methods

Care of mice

Ten-week-old C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed in our animal unit until they were 3 and 6 months old respectively. The animals were housed in a temperature-controlled room ($22 \pm 2^\circ\text{C}$) with a daily light/darkness schedule of 12 h. During the experiment, the animals had free access to water and were pair-fed standard laboratory chow (Laboratory Rodent Diet 5001; PMI Feeds, Richmond, VA, USA) containing 0.95% calcium. The Institutional Animal Care and Use Committee (IACUC) approved all animal procedures.

Experimental design

Experiment 1 The first study was conducted in 6-month-old female C57BL/6 mice. The mice were assigned to seven groups ($n = 6\text{--}12$ per group) as follows: sham operated, OVX and implanted with a vehicle pellet, or OVX and implanted with 60-day release pellets delivering 5, 10, 20, 40 or 500 $\mu\text{g/kg/day}$ estradiol (E_2) (Innovative Research of America, Sarasota, FL, USA). Following 56 days of treatment, bone mineral density (BMD) was measured at the spine and femur using dual energy X-ray absorptiometry (DXA) and at the proximal tibial metaphysis using peripheral quantitative computed tomography (pQCT) (see below). After appropriate anesthesia (see below), blood was drawn by cardiac puncture and the animals were killed by inhalation of CO_2 . The uteri were excised and weighed.

Experiment 2 In the second study, 3-month-old C57BL/6 mice were used and the duration of the experiment was reduced to 1 month. The rationale for this was that if similar results were observed in younger mice treated for a shorter period of time that would be preferable for future studies, given the cost of animal care and maintenance. These mice were assigned to six groups ($n = 4\text{--}7$ per group) and were either sham operated, OVX and implanted with a vehicle pellet, or OVX and implanted with 60-day pellets delivering 5, 10, 20, or 40 $\mu\text{g/kg/day}$ E_2 (Innovative Research of America). BMD was measured by DXA and pQCT (see below) after 30 days of treatment,

the animals were killed by inhalation of CO_2 , and the uteri were excised and weighed.

Bone densitometry

For both the DXA and pQCT measurements, the mice were anesthetized with Avertin (2,2,2 tribromoethanol, 400 mg/kg, i.p.). For the DXA measurements, they were placed on the animal tray in a prone position on the Lunar PIXImus densitometer (software version 1.44.005; Lunar Corp., Madison, WI, USA). In all analyses, the bones of the skull were excluded. Calibration of the machine was performed daily using the hydroxyl apatite phantom provided by the manufacturer. After scanning, regions of interest were identified for more specific analyses. In repeatedly scanned mice (with repositioning between scans), the coefficients of variation for total body, lumbar, and femoral BMD were 4.9%, 2.7% and 4.3% respectively.

For the pQCT measurements the mice were placed in a supine position on the gantry of the Stratec XCT Research SA Plus using software version 5.40 (Nordland Medical Systems, Inc., Fort Atkinson, WI, USA). As for the PIXImus, calibration of the machine was performed daily with the hydroxyl apatite phantom provided by the manufacturer. The mice were positioned so that the total length of the femur and tibia were visible on the scout view. The scout view speed was set at 15.0 mm/s with a slide distance of 0.3 mm. Once the scout view was completed, the reference line for the CT scans was set at the most proximal point of the tibia. Slice images were set at 1.9 mm (proximal metaphysis of the tibia) and at the synopsis of the tibia and the fibula. The CT speed was set at 2.5 mm/s, voxel size of 100 μm , and slice thickness of 0.5 mm. After scanning, the CT slices were analyzed using peelmode 2, cortmode 1 and contour mode 1 to evaluate trabecular and cortical parameters. To determine the cancellous bone the threshold was set at 400 mg/cm^3 and for cortical bone at 710 mg/cm^3 . Coefficients of variation were 4.4% and 1.2% for proximal and distal tibial BMD respectively.

Serum estradiol measurements

Blood was drawn by cardiac puncture at the end of the study. The blood was allowed to clot and serum was collected by centrifugation at 8000 g at 4°C for 6 min. Estradiol concentrations were assayed using a double antibody radioimmunoassay from Diagnostic Products Corporation (DPC, Los Angeles, CA, USA; interassay coefficient of variation $<16\%$).

Histology

After killing, the uteri were excised and fixed overnight at 4°C in 10% (v/v) paraformaldehyde, rinsed, and stored in 70% (v/v) ethanol before routine processing

into paraffin wax. The sections of the uteri were prepared and stained with hematoxylin-eosin.

Statistical analysis

All data are presented as means $\pm$ S.E.M. The overall dose effect was tested by ANOVA. Pairwise comparisons between the 0 dose versus the other doses were made using the Dunnett method, which adjusts for multiple comparisons. The sham and OVX groups were compared using *t*-tests. A *P*-value of <0.05 was considered significant.

Results

Effects of increasing doses of E_2 on bone and uterus in 6-month-old C57BL/6 female mice treated for 2 months

We initially compared the effects of increasing doses of E_2 on bone and the uterus using OVX mice treated with 0, 5, 10, 20, 40 and 500 $\mu\text{g/kg/day}$ E_2 for 2 months. Estrogen deficiency caused by OVX significantly decreased spine and femur BMD (Fig. 1). However, treatment with an E_2 dose of as little as 5 $\mu\text{g/kg/day}$ was effective in preventing bone loss at both the spine and the femur in these mice (Fig. 1). Moreover, there was a dose-dependent increase in spine and femur BMD with higher doses (20 and 40 $\mu\text{g/kg/day}$) and even more so at a pharmacological E_2 dose of 500 $\mu\text{g/kg/day}$.

Figure 1 BMD (mg/cm^2) of the spine and the femur (measured by DXA) in 6-month-old female C57BL/6 mice following 56 days of either sham surgery or OVX plus pellets delivering 0, 5, 10, 20, 40 or 500 $\mu\text{g/kg/day}$ E_2 . Data are means $\pm$ S.E.M. *P < 0.05 vs 0 $\mu\text{g/kg/day}$, adjusted for multiple comparisons by the Dunnett method; ^a*P* < 0.05 ; ^b*P* < 0.01 vs sham.

Table 1 shows the values for volumetric BMD at the proximal tibial metaphysis (total, cancellous, and cortical) and the cortical volumetric BMD at the tibia-fibula junction as measured by pQCT in the various groups. In contrast to the spine (which also contains predominantly cancellous bone), cancellous BMD at the tibial metaphysis was not significantly lower in the OVX mice treated with vehicle as compared with the sham-operated mice. However, similar to the DXA measurements at the spine and femur, the 5 $\mu\text{g/kg/day}$ dose was sufficient to increase (above the level of the sham-operated mice) total BMD at the tibial metaphysis. In fact, the overall dose-response relationship at the tibia using pQCT was similar to that seen at the femur using DXA. The high dose of E_2 (500 $\mu\text{g/kg/day}$) clearly resulted in a sclerotic response at the tibial metaphysis, with a marked increase in bone mass (approximately 200% increase in cancellous BMD). As expected, the high dose stimulated endosteal bone formation and increased cortical thickness (data not shown). Cortical volumetric BMD was maintained at the level of the sham-operated mice by an E_2 dose of 5 $\mu\text{g/kg/day}$ at the proximal site and at the tibia-fibula junction of the tibia, with a further increase at higher doses (Table 1).

Figure 2 shows the changes in uterine wet weight in these mice. As expected, uterine weight decreased significantly following OVX. In contrast to the protective effect of very low doses of E_2 on cancellous and cortical bone, however, an E_2 dose of 40 $\mu\text{g/kg/day}$ was necessary to fully restore uterine weight in the OVX mice to the level of the sham-operated mice (Fig. 2). Figure 3 shows changes in uterine histology with the various doses of E_2 in these mice. The uteri of sham-operated mice showed tortuous endometrium with tall columnar epithelium, large endometrial glands, and the stroma had a significant infiltration with polymorphonuclear cells (Fig. 3A). By contrast, the endometrium from the OVX mice had cuboidal epithelium, the endometrial glands were small, and the stroma contained closely packed hyperchromatic cells with no cytoplasm within the stroma (Fig. 3B). At a dose of 10 $\mu\text{g/kg/day}$ E_2 , there was a shift from cuboidal to columnar epithelium. Endometrial glands again increased in size and contained a large amount of eosinophilic cytoplasm (Fig. 3C). However, a dose of 40 $\mu\text{g/kg/day}$ E_2 was necessary to produce tall columnar endometrial epithelium and stromal cells with abundant eosinophilic cytoplasm (Fig. 3D).

Serum estradiol levels decreased significantly after OVX (Table 2). E_2 treatment at doses of 5 to 20 $\mu\text{g/kg/day}$ resulted in serum E_2 concentrations similar to the levels in the sham-operated mice (with differences between these doses being hard to see due to the variability of even this sensitive assay at these low levels). However, the 40 $\mu\text{g/kg/day}$ dose did result in serum E_2 levels that were approximately 4 times

Table 1 Volumetric (v) BMD at the proximal tibial metaphysis (total vBMD, cancellous vBMD, and cortical vBMD) and at the tibia-fibula (T/F) junction (cortical vBMD T/F) in 6-month-old female C57BL/6 mice following 56 days of either sham surgery or OVX plus pellets delivering 0, 5, 10, 20, 40, or 500 $\mu\text{g/kg/day}$ of E_2 , measured by pQCT. Data are means $\pm$ S.E.M.

	Total vBMD (mg/cm^3)	Cancellous vBMD (mg/cm^3)	Cortical vBMD (mg/cm^3)	Cortical vBMD T/F (mg/cm^3)
Sham	434.67 $\pm$ 6.26	172.8 $\pm$ 3.52	887.71 $\pm$ 7.65	1139.46 $\pm$ 7.94
OVX + 0 $\mu\text{g/kg/day}$	386.39 $\pm$ 5.64 ^c	173.18 $\pm$ 5.76	851.48 $\pm$ 4.86 ^c	1091.62 $\pm$ 6.94 ^c
OVX + 5 $\mu\text{g/kg/day}$	518.63 $\pm$ 32.21	280.11 $\pm$ 29.62	881.87 $\pm$ 14.32	1173.81 $\pm$ 17.90*
OVX + 10 $\mu\text{g/kg/day}$	492.70 $\pm$ 28.88	246.01 $\pm$ 16.34	899.11 $\pm$ 10.13*	1152.57 $\pm$ 15.36*
OVX + 20 $\mu\text{g/kg/day}$	517.85 $\pm$ 42.45*	266.82 $\pm$ 34.35	888.71 $\pm$ 12.74*	1159.06 $\pm$ 20.84*
OVX + 40 $\mu\text{g/kg/day}$	607.76 $\pm$ 44.92*	352.76 $\pm$ 48.38*	902.79 $\pm$ 9.03*	1177.86 $\pm$ 10.94*
OVX + 500 $\mu\text{g/kg/day}$	744.13 $\pm$ 53.68*	586.25 $\pm$ 59.46*	881.72 $\pm$ 8.61	1212.68 $\pm$ 185.89*

* $P < 0.05$ vs 0 $\mu\text{g/kg/day}$, adjusted for multiple comparisons by the Dunnett method; ^c $P < 0.001$ vs sham.

above the physiological level and levels were even higher at the pharmacological E_2 dose of 500 $\mu\text{g/kg/day}$ (Table 2).

Effects of increasing doses of E_2 on bone and uterus of 3-month-old mice treated for one month

In the second study, a different experimental paradigm was used in which 3-month-old mice were treated for only one month. As in experiment 1, the 5 $\mu\text{g/kg/day}$ dose was effective at preventing bone loss at the femur (which contains predominantly cortical bone) in the younger mice in experiment 2 (Fig. 4). Although the changes in cortical volumetric BMD at the tibia (proximal site and at the tibia-fibula junction) did not achieve statistical significance, the overall pattern was similar to that noted for the femur by DXA (Table 3). In contrast to cortical bone, however, a higher dose

(10 $\mu\text{g/kg/day}$) was necessary to prevent bone loss in these mice at the spine, which contains predominantly cancellous bone (Fig. 4). Similar to the findings at the spine, the 10 $\mu\text{g/kg/day}$ dose was also necessary in the 3-month-old mice in order to prevent loss of cancellous bone at the tibial metaphysis, although again, the individual changes in cancellous bone by pQCT were not significant (Table 3). However, in contrast to the 6-month-old mice treated for 2 months, uterine weight was restored to the level of the sham-operated mice even by the 5 $\mu\text{g/kg/day}$ dose in the 3-month-old mice treated for 1 month (Fig. 5).

Discussion

We demonstrate that, in 6-month-old C57BL/6 mice treated for 2 months, as little as 5 $\mu\text{g/kg/day}$ E_2 delivered via constant release pellets completely prevented loss of cortical and cancellous bone without a stimulatory effect on the uterus. Higher doses of E_2 further increased cortical and cancellous bone mass at various skeletal sites. In contrast to these findings, we also found that in 3-month-old mice treated for 1 month the 5 $\mu\text{g/kg/day}$ dose resulted in uterine hypertrophy but was ineffective in preventing loss of cancellous bone, and higher doses of E_2 were required to prevent bone loss. Collectively, these results (i) provide data on the dose-response of estrogen, using E_2 pellets, on bone and the uterus and (ii) demonstrate that the relative effects of estrogen on these tissues depends on the particular experimental paradigm used. Our findings in the younger mice are similar to the recent results of Andersson *et al.* (14), who also found that, in contrast to our data with the 6-month-old mice, there was a dramatic uterine response to very low doses of estradiol in 2-month-old mice, whereas both cancellous and cortical bone were less sensitive to the effects of estrogen in these mice.

We also found that whereas the 6-month-old mice had significant decreases in bone mass at the spine (which contains predominantly cancellous bone) following OVX, they did not lose cancellous bone (but did lose cortical bone) at the tibial metaphysis following OVX. Again, these findings were in contrast to

Figure 2 Uterine wet weights (mg) in 6-month-old female C57BL/6 mice following 56 days of either sham surgery or OVX plus pellets delivering 0, 5, 10, 20, 40 or 500 $\mu\text{g/kg/day}$ E_2 . Data are means $\pm$ S.E.M. * $P < 0.05$ vs 0 $\mu\text{g/kg/day}$, adjusted for multiple comparisons by the Dunnett method; ^c $P < 0.001$ vs sham.

Figure 3 Histological sections of uteri collected from sham mice (A), OVX mice (B), and OVX mice treated with 10 (C) or 40 µg/kg/day (D) E₂ for 56 days. At the end of the study the uteri from the mice were isolated and fixed in paraformaldehyde. (A) Uterus from a sham-operated mouse showing tortuous endometrium with tall columnar epithelium (arrows). (B) Uterus of an OVX mouse showing densely packed hyperchromatic cells within the stroma. (C) Uterus of a mouse treated with 10 µg/kg/day E₂ showing columnar endometrial epithelium (arrows) and hyperchromatic cells within the stroma. (D) Uterus of a mouse treated with 40 µg/kg/day E₂ showing tall columnar epithelium (arrows) and stromal cells with abundant eosinophilic cytoplasm. The sections were stained with hematoxylin-eosin. Magnification × 100.

the 3-month-old mice, who lost cancellous bone at both the spine and tibial metaphysis following OVX (although the findings at the tibial metaphysis did not achieve statistical significance). Of note, whereas spine BMD was similar in the 6- and 3-month-old mice ($57.3 \pm 1.0 \text{ mg/cm}^2$ vs $55.6 \pm 2.0 \text{ mg/cm}^2$, $P = 0.39$),

Table 2 Serum estradiol concentration in 6-month-old female C57BL/6 mice treated for 56 days with slow release pellets delivering 0, 5, 10, 20, 40, or 500 µg/kg/day of E₂. Data are means ± S.E.M.

	Serum E ₂ (pmol/l)
Sham	19.86 ± 1.98
OVX + 0 µg/kg/day	11.71 ± 0.48^c
OVX + 5 µg/kg/day	18.13 ± 2.64
OVX + 10 µg/kg/day	18.65 ± 3.45
OVX + 20 µg/kg/day	14.61 ± 1.28
OVX + 40 µg/kg/day	71.25 ± 28.49
OVX + 500 µg/kg/day	$184.39 \pm 88.43^*$

* $P < 0.05$ vs 0 µg/kg/day, adjusted for multiple comparisons by the Dunnett method; ^c $P < 0.001$ vs sham.

cancellous BMD at the tibial metaphysis was significantly lower in the 6-month-old as compared with the 3-month-old mice (Tables 1 and 3, $172.8 \pm 3.52 \text{ mg/cm}^3$ vs $216.26 \pm 8.14 \text{ mg/cm}^3$, $P < 0.001$). This suggests that cancellous bone at the tibia may be progressively lost with aging in these mice, so that by 6 months of age, OVX does not result in further loss of cancellous bone at this site, whereas cortical bone mass is still reduced following OVX (15, 16).

Consistent with our findings in C57BL/6 mice, Gaumet *et al.* (17) found that the skeletal response to estrogen was similar in young (6 month) versus old (12 month) rats, but the uterus of the old rats was much less responsive to estrogen as compared with the young rats. It is also of interest that recent data indicate that very low doses of estrogen can prevent bone loss in elderly postmenopausal women without significant uterine stimulation (18, 19). Collectively, our findings and these other findings in rats and in humans indicate that whereas the skeleton retains

Figure 4 BMD (mg/cm²) of the spine and the femur (measured by DXA) in 3-month-old female C57BL/6 mice following 30 days of either sham surgery or OVX plus pellets delivering 0, 5, 10, 20 or 40 µg/kg/day E₂. Data are means ± S.E.M. *P < 0.05 vs 0 µg/kg/day, adjusted for multiple comparisons by the Dunnett method; ^a*P* < 0.05 vs sham. Note, the scale of the y-axis is the same as in Fig. 1.

sensitivity to estrogen throughout life, the uterus may become progressively less sensitive to estrogen with aging. In light of the recent findings from the Women's Health Initiative demonstrating adverse effects of estrogen plus progesterone on cardiovascular events and the risk of breast cancer (20), these data provide a further impetus to examine the effects of low doses of estrogen in elderly women, which may result in skeletal protection without adverse sequelae in other tissues.

We have found that the subcutaneous implantation of slow release pellets is the most convenient and efficacious way to deliver estrogen to mice. This avoids the handling and trauma associated with daily subcutaneous injections, as well as the possible peaks and troughs in serum E₂ levels associated with injections (21, 22). However, it is possible that bone versus the uterus may respond differently to a constant, low dose of estrogen as provided by subcutaneous pellets versus the peaks and troughs associated with daily injections.

Figure 5 Uterine wet weights (mg) in 3-month-old female C57BL/6 mice following 30 days of either sham surgery or OVX plus pellets delivering 0, 5, 10, 20 or 40 µg/kg/day E₂. Data are means ± S.E.M. *P < 0.05 vs 0 µg/kg/day, adjusted for multiple comparisons by the Dunnett method; ^b*P* < 0.01 vs sham.

Indeed, previous studies have found that in mature rats subcutaneous injections of E₂ (10 µg/kg/48 h) resulted in a distinct increase in uterine weight (21%) compared with sham-operated rats at doses that were less effective on bone, whereas similar doses of E₂ (18 µg/kg/day) given by subcutaneous pellets led to an increase in cancellous bone area with no increase of the uterine weight above the weight of the sham-operated rats (17, 23). Also of interest is the study by Erben *et al.* (24) in rats, where a dose of estrogen given by subcutaneous pellets (5.2 µg/kg/day) resulted in an increase in cancellous bone area with a concomitant decrease in uterine wet weight over the course of the treatment.

As previously demonstrated (12, 25, 26), we also found that a very high dose of E₂ (500 µg/kg/day) resulted in a dramatic increase in cancellous and cortical bone mass at the proximal tibia in the older mice. By contrast, the spine did not demonstrate this sclerotic

Table 3 Volumetric (v) BMD at the proximal tibial metaphysis (total vBMD, cancellous vBMD, and cortical vBMD) and at the tibia-fibula (T/F) junction (cortical vBMD T/F) in 3-month-old female C57BL/6 mice following 30 days of either sham surgery or OVX plus pellets delivering 0, 5, 10, 20, or 40 µg/kg/day of E₂, measured by pQCT.

	Total vBMD (mg/cm ³)	Cancellous vBMD (mg/cm ³)	Cortical vBMD (mg/cm ³)	Cortical vBMD T/F (mg/cm ³)
Sham	430.23 ± 4.85	216.26 ± 8.14	863.21 ± 8.15	1069.19 ± 8.10
OVX + 0 µg/kg/day	375.08 ± 13.09 ^c	203.85 ± 5.06	836.65 ± 10.05	1050.10 ± 13.47
OVX + 5 µg/kg/day	420.36 ± 23.35	201.20 ± 7.30	867.60 ± 15.02	1074.40 ± 13.10
OVX + 10 µg/kg/day	464.16 ± 21.94	217.58 ± 9.17	868.54 ± 14.83	1077.60 ± 8.37
OVX + 20 µg/kg/day	479.80 ± 34.46	233.88 ± 14.93	889.62 ± 18.01	1104.00 ± 14.60*
OVX + 40 µg/kg/day	529.43 ± 80.68	312.20 ± 61.35*	882.03 ± 19.03	1096.78 ± 19.62

*P < 0.05 vs 0 µg/kg/day, adjusted for multiple comparisons by the Dunnett method; ^c*P* < 0.001 vs sham.

response to high doses of estrogen. Thus, there are important differences between the axial and appendicular skeleton in terms of the response to high doses of estrogen. The possible mechanism(s) for this are unclear, although it is of interest that studies with parathyroid hormone (PTH) have also shown different responses in axial versus appendicular sites. In rats treated with PTH, the relative BMD gain was highest at the distal femur and proximal tibia and lower at the lumbar vertebrae (27). In mice, however, PTH treatment augmented cancellous bone to a greater extent in the vertebral body than in the proximal tibia. In contrast, the same treatment added cortical bone in the tibia, a highly mechanically loaded site, but not in the cortex of the lumbar vertebrae, a less loaded site (16). Moreover, in light of recent evidence that the skeletal response to mechanical loading may, at least in part, be mediated by estrogen receptor- α (28), it is possible that the differential sensitivity of axial versus appendicular sites to the sclerotic effect of high doses of estrogen is related to weight bearing.

In summary, our study provides data on the dose-response of bone versus the uterus to estrogen. In addition, we found that the relative effects of estrogen on these tissues depends on the particular experimental conditions used. The latter finding is of particular relevance in interpreting results of studies on new compounds (e.g. selective estrogen receptor modulators) with putative selective effects on bone versus reproductive tissues.

Acknowledgements

We would like to thank Jesse Lamsam and Kelley Hoey for excellent technical assistance. This work was supported by grant AG004875 from the National Institute on Aging, National Institute of Health, Bethesda, MD, USA.

References

- Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB & Korach KS. Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. *New England Journal of Medicine* 1994 **331** 1056–1061.
- Morishima A, Grumbach MM, Simpson ER, Fisher C & Qin K. Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. *Journal of Clinical Endocrinology and Metabolism* 1995 **80** 3689–3698.
- Carani C, Qin K, Simoni M, Faustini-Fustini M, Serpente S, Boyd J, Korach KS & Simpson ER. Effect of testosterone and estradiol in a man with aromatase deficiency. *New England Journal of Medicine* 1997 **337** 91–95.
- Albright F, Smith PH & Richardson AM. Postmenopausal osteoporosis. *Journal of the American Medical Association* 1941 **116** 2465–2474.
- Khosla S, Melton LJ III, Atkinson EJ, O'Fallon WM, Klee GG & Riggs BL. Relationship of serum sex steroid levels and bone turnover markers with bone mineral density in men and women: a key role for bioavailable estrogen. *Journal of Clinical Endocrinology and Metabolism* 1998 **83** 2266–2274.
- Khosla S, Melton LJ III & Riggs BL. Clinical review 144. Estrogen and the male skeleton. *Journal of Clinical Endocrinology and Metabolism* 2002 **87** 1443–1450.
- Beamer WG, Donahue LR, Rosen CJ & Baylink DJ. Genetic variability in adult bone density among inbred strains of mice. *Bone* 1996 **18** 397–403.
- Klein RF, Mitchell SR, Phillips TJ, Belknap JK & Orwoll ES. Quantitative trait loci affecting peak bone mineral density in mice. *Journal of Bone and Mineral Research* 1998 **13** 1648–1656.
- Sims NA, Clement-Lacroix P, Minet D, Fraslon-Vanhulle C, Gaillard-Kelly M, Resche-Rigon M & Baron R. A functional androgen receptor is not sufficient to allow estradiol to protect bone after gonadectomy in estradiol receptor-deficient mice. *Journal of Clinical Investigation* 2003 **111** 1319–1327.
- Modder UI, Sanyal A, Kearns AE, Sibonga JD, Nishihara E, Xu J, O'Malley BW, Ritman EL, Riggs BL, Spelsberg TC & Khosla S. Effects of loss of steroid receptor coactivator-1 on the skeletal response to estrogen in mice. *Endocrinology* 2004 **145** 913–921.
- Bain SD, Bailey MC & Edwards MW. The anabolic effect of estrogen on endosteal bone formation in the mouse is attenuated by ovariectomy: a role for the uterus in the skeletal response to estrogen? *Calcified Tissue International* 1992 **51** 223–228.
- Bain SD, Bailey MC, Celino DL, Lantry MM & Edwards MW. High-dose estrogen inhibits bone resorption and stimulates bone formation in the ovariectomized mouse. *Journal of Bone and Mineral Research* 1993 **8** 435–442.
- Turner RT. Mice, estrogen, and postmenopausal osteoporosis. *Journal of Bone and Mineral Research* 1999 **14** 187–191.
- Andersson N, Egecioglu E & Ohlsson C. Tissue-specific differences in response to estrogenic stimulation in ovariectomized mice. *Journal of Bone and Mineral Research* 2003 **18** S178.
- Halloran BP, Ferguson VL, Sims SJ, Burghardt A, Venton LL & Majumdar S. Changes in bone structure and mass with advancing age in the male C57BL/6J mouse. *Journal of Bone and Mineral Research* 2002 **17** 1044–1050.
- Zhou H, Iida-Klein A, Lu SS, Ducayen-Knowles M, Levine LR, Dempster DW & Lindsay R. Anabolic action of parathyroid hormone on cortical and cancellous bone differs between axial and appendicular skeletal sites in mice. *Bone* 2003 **32** 513–520.
- Gaumont N, Brailon P, Seibel MJ, Pointillart A, Coxam V & Davicco MJ. Influence of aging on cortical and trabecular bone response to estradiol treatment in ovariectomized rats. *Gerontology* 1998 **44** 132–139.
- Cummings SR, Yankov V, Ensrud KE, Ettinger B, Wallace R, Johnson K, Macer J, Vittinghoff E & Grady D. Ultralow estradiol increases BMD and decreases bone turnover in older women, particularly those with undetectable estradiol: the ULTRA trial. *Journal of Bone and Mineral Research* 2003 **18** S53 (Abstract).
- Utian WH, Burry KA, Archer DF, Gallagher JC, Boyett RL, Guy MP, Tachon GJ, Chadha-Boreham HK & Bouvet AA. Efficacy and safety of low, standard, and high dosages of an estradiol transdermal system (Eslim) compared with placebo on vasomotor symptoms in highly symptomatic menopausal patients. The Eslim Study Group. *American Journal of Obstetrics and Gynecology* 1999 **181** 71–79.
- Rossouw JE, Anderson GL, Lacroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM & Ockene J. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. *Journal of the American Medical Association* 2002 **288** 321–333.
- Edwards MW, Bain SD, Bailey MC, Lantry MM & Howard GA. 17 β -Estradiol stimulation of endosteal bone formation in the

- ovariectomized mouse: an animal model for the evaluation of bone-targeted estrogens. *Bone* 1992 **13** 29–34.
- 22 Sims NA, Morris HA, Moore RJ & Durbridge TC. Estradiol treatment transiently increases trabecular bone volume in ovariectomized rats. *Bone* 1996 **19** 455–461.
- 23 Sibonga JD, Bell NH & Turner RT. Evidence that ibuprofen antagonizes selective actions of estrogen and tamoxifen on rat bone. *Journal of Bone and Mineral Research* 1998 **13** 863–870.
- 24 Erben RG, Harti G & Graf H. Ovariectomy does not alter CD4 + / CD8 + ratio in peripheral blood T-lymphocytes in the rat. *Hormone and Metabolic Research* 1998 **30** 50–54.
- 25 Tobias JH, Chow J, Colston KW & Chambers TJ. High concentrations of 17 β -estradiol stimulate trabecular bone formation in adult female rats. *Endocrinology* 1991 **128** 408–412.
- 26 Samuels A, Perry MJ & Tobias JH. High-dose estrogen induces *de novo* medullary bone formation in female mice. *Journal of Bone and Mineral Research* 1999 **14** 178–186.
- 27 Kishi T, Hagino H, Kishimoto H & Nagashima H. Bone responses at various skeletal sites to human parathyroid hormone in ovariectomized rats: effects of long-term administration, withdrawal, and readministration. *Bone* 1998 **22** 515–522.
- 28 Lee K, Jessop H, Suswillo R, Zaman G & Lanyon L. Endocrinology: bone adaptation requires oestrogen receptor-alpha. *Nature* 2003 **424** 389.
-

Received 24 May 2004

Accepted 30 June 2004