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ORIGINAL PAPER

Using three-point bending to evaluate tibia bone strength in ovariectomized young mice

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Abstract It is well known that estrogen deficiency induces a deterioration of bone strength in aged females. The aim of this study is to determine the effect of estrogen depletion on tibia bone strength in sexually mature mice that are still undergoing skeletal maturation. At 8 weeks of age, C57BL/6 female mice underwent an ovariectomy (OVX) or sham (SHAM) surgery. Mice were killed at 2, 4, or 8 weeks post-surgery. Tibia length and cross-sectional area continued to increase in both treatment groups until 4 weeks post-surgery. Compared to SHAM mice, OVX mice demonstrated a significant reduction in uterine weight and plasma estrogen levels. Three-point bending was used to quantify the mechanical properties (breaking point, stress, stiffness, and elasticity) of the tibia. The tibias from the SHAM mice had a higher breaking point than all the age-matched OVX mice. At 8 weeks post-surgery, the tibias from the SHAM mice demonstrated higher elasticity, stress, and stiffness than the younger SHAM mice and the age-matched OVX mice. Compared to the SHAM mice, our study suggests that (1) there is a reduction in the mechanical strength of tibias from young OVX mice, and (2) the greatest decline in tibia strength of the OVX mice was once they reached skeletal maturity.

Keywords Elasticity · Stress · Stiffness · Bone · Three-point bending · Ovariectomy · Tibia

1 Introduction

Osteoporosis is characterized by reduced bone strength and is often seen in postmenopausal women and those who have age-related estrogen deficiencies. However, there are conditions when women experience depleted estrogen levels prior to menopause. For example, it is

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common for a woman to undergo a surgical ovariectomy (OVX) as a preventative measure against ovarian cancer or other ovarian pathologies [1]. Furthermore, individuals born with a deficiency of the cytochrome P450 gene for aromatase/estrogen synthase (CYP19 mutation), or estrogen insensitivity (estrogen receptor α gene mutation) lack the ability to produce adequate levels of estrogen or generate a normal in vivo response to estrogen, respectively [2, 3]. The objective of this study is to determine how the depletion of gonadal estrogen affects bone strength in young females. It is well known that estrogen stimulates and regulates pubertal bone growth, and thus is responsible for minimizing bone resorption and remodeling while promoting the thickening of the cortical long bone [4–6]. Therefore, females that have an estrogen deficiency or insensitivity (surgically induced or from a genetic disorder), demonstrate reduced bone mineral density, incomplete epiphyseal growth plate fusion, and an overall diminishment in skeletal integrity [4, 5].

In this study, we used the C57BL/6 J inbred mouse stain, which has been commonly used to evaluate the mechanical properties of the skeletal system [7–10]. These mice become sexually mature at approximately 6 weeks of age [11], and bone maturity is reached at 16 weeks of age [12–14]. Therefore, studies that evaluate adult bone strength and morphology utilize these mice at approximately 16 weeks of age [10, 15]. However, to our knowledge, no studies have evaluated the effect of circulating in vivo estrogen in sexually mature mice that are still undergoing skeletal maturation.

We used three-point bending to quantify the mechanical strength of the tibia [16]. The tibia is commonly used in three-point bending because of its relative cylindrical shape. With this technique, force can be applied to the bone until reaching its breaking point (i.e., maximal load), and the degree to which a bone will bend to resist facture (i.e., elastic modulus) can be calculated [16–18]. Additional properties used to measure the resistance to fracture of a compact bone are stiffness, stress, and strain [19]. This study improves upon previous studies [20], which typically quantify the mechanical strength of compact bone without regard to body size (i.e., body weight and tibial length). Body composition should be considered because the removal of gonadal estrogen production (i.e., ovariectomy) is typically associated with an increase in abdominal adipose deposition [21, 22]. An increase in adipose mass can induce strain on a bone, which will ultimately influence its mechanical strength [8].

In this study, we desired to determine the mechanical strength of the tibia in ovariectomized mice over an 8-week period until skeletal maturity is reached at 16 weeks of age. Using three-point bending, we determined the maximal load, elasticity, stress, and stiffness of each tibia. Also, the cortical bone area and marrow cavity area were determined. We hypothesized that the in vivo depletion of estrogen in young mice would reduce bone strength, as has been similarly shown in skeletally mature mice.

2 Materials and methods

2.1 Animals

Six-week-old C57BL/6 female mice were acquired from Envigo (Indianapolis, IN, USA) and housed in the animal facility at the University of Central Arkansas (Conway, AR, USA). The mice were maintained on a 12-h light/dark cycle, kept at room temperature, and given food (Tekland 8640 rodent diet; Envigo) and water ad libitum. Mice were acclimated for 1–2 weeks before surgery. When the mice were 8 weeks old, a standard dorsal ovariectomy (OVX) or

sham (SHAM) surgery was conducted. For surgery, mice were anesthetized with inhaled isoflurane (1–4%) and weighed. The mice were killed at 2, 4, or 8 weeks post-surgery using CO_2 asphyxiation. All procedures were approved by the Institutional Animal Care and Use Committee.

After euthanization, the body weight was recorded and the uteri dissected from the mice. The tibias were dissected from the lower limbs of the mice, cleaned of any soft tissue, and stored at 20 °C until experimentation. Blood was collected via cardiac puncture (tuberculin syringe, 26G) and placed in a microtube coated with heparin (Sarstedt AG & Co, Germany). The blood samples were centrifuged at 2000 × *g* for 10 min. After centrifugation, the plasma was removed and stored at −20 °C. The plasma was shipped on dry ice to the Reproductive Ligand Assay and Analysis Core at the University of Virginia Medical School (Charlottesville, VA, USA) for the analysis of plasma estrogen (17β-estradiol; Calbiotech mouse estradiol assay, CA, USA).

2.2 Three-point bending

The modified, three-point bending apparatus used in this study has recently been described [16]. Before experimentation, the bones were soaked in a phosphate buffer solution for 24 h. The tibia was placed in a holder that fixed the ends of the bone in place while a measured amount of force was applied perpendicular to the midpoint of the anterior side of the tibial diaphysis. Force was applied (at a rate of 0.498 $mm \cdot s^{-1}$) using a flat-tipped wedge that was connected to a motorized force transducer (World Precision Instruments, FL, USA), which was controlled by a Hayden Kerk IDEA Drive Interface program. Force was amplified by a transbridge amplifier (World Precision Instruments) and recorded by WinDaq data acquisition software (DATAQ Instruments, OH, USA).

2.3 Structural properties

The tibial length was measured using a digimatic digital caliper (Mitutoyo, IL, USA). The midpoint of the tibia was sectioned using an IsoMet1000 Precision Saw (Buehler, IL, USA) equipped with an IsoMet 15HC diamond wafering blade. Each bone section was imaged using a Leica MZ6 microscope (IL, USA) equipped with an OptixCam digital camera. The image analysis program, ImageJ (NIH, USA) was used to measure the lateral and medial diameter, cortical area, and cavity area.

Using the bone dimensions and three-point bending data, the elasticity of the bone was calculated. Elasticity (*I*) is defined as the bone's ability to deform in response to an applied force where F_{avg} is the maximal load, L is the length between endpoints, V_{max} is the bone's horizontal displacement, and I is the moment of inertia (2) [16]. The mice tibias were treated as elliptical tubes when calculating moment of inertia.

$$\frac{F_{avg} \cdot L}{48 \cdot V_{max} \cdot I} \tag{1}$$

$$\frac{\pi}{4}\left(a_o {b_o}^3 - a_i {b_i}^3\right) \tag{2}$$

Stress (3) essentially represents the area of bone over which the force was applied, and strain (4) is the deformation of the bone due to stress.

$$\frac{F_{avg} \cdot L}{\pi(a_o b_o - a_i b_i)} \tag{3}$$

$$\frac{Stress}{Elastic\ Modulus} x\ 100\ \% \tag{4}$$

$$\frac{F_{avg}}{V_{max}} \tag{5}$$

The bone toughness, or resistance to bending, is described by stiffness (5). The V_{max} was important for calculating both elasticity and stiffness and was not able to be experimentally measured. We defined the V_{max} as the distance the wedge traveled after 25% of the experimental time had elapsed. Our V_{max} is comparable to those obtain by others [7, 17]. The maximal load, elasticity, stress, and stiffness data were normalized for body weight and tibia length.

2.4 Statistical analysis

All data are expressed as the mean ± S.E. for the number of mice used (Table 1). A log transformation was used on nonparametric data. Data were analyzed using a two-way analysis of variance to detect the effect of age and treatment, and $p < 0.05$ was considered significant. Post hoc analysis was conducted on the effect of age (Tukey HSD) and treatment (Student's t test with a Bonferroni adjusted alpha) with $p < 0.05$. Statistical analysis was conducted with JMP 12.1.0 (SAS Institute Inc., Cary, NC).

3 Results

The surgical removal of the ovaries (OVX) at 8 weeks of age resulted in a progressive increase ($p < 0.001$) in body weight over the next 8 weeks compared to SHAM mice (Fig. 1a). This

Table 1 Tibial bone structural dimensions

Age (weeks)	Weeks post-surgery	Treatment	No. of animals	Tibial length (mm)	Cortical area (mm^2)	Cavity area (mm^2)
10	2	SHAM	8	16.59 ± 0.11[a]	0.47 ± 0.01 [a,b]	0.30 ± 0.02
		OVX	4	16.52 ± 0.18 [a]	0.31 ± 0.06 [a,b]	0.31 ± 0.06
12	4	SHAM	16	16.98 ± 0.11	0.55 ± 0.02	0.29 ± 0.01
		OVX	12	17.24 ± 0.12	0.59 ± 0.02	0.33 ± 0.02
16	8	SHAM	10	17.06 ± 0.13	0.54 ± 0.01	0.30 ± 0.02
		OVX	7	17.23 ± 0.19	0.56 ± 0.01	0.32 ± 0.03

Two-way ANOVA followed by Bonferroni post hoc test, $\alpha = 0.05$

[a] Both treatments at 2 weeks post-surgery are different ($p = 0.001$) than 4 and 8 weeks post-surgery

[b] Treatments are different ($p = 0.001$) at 2 weeks post-surgery

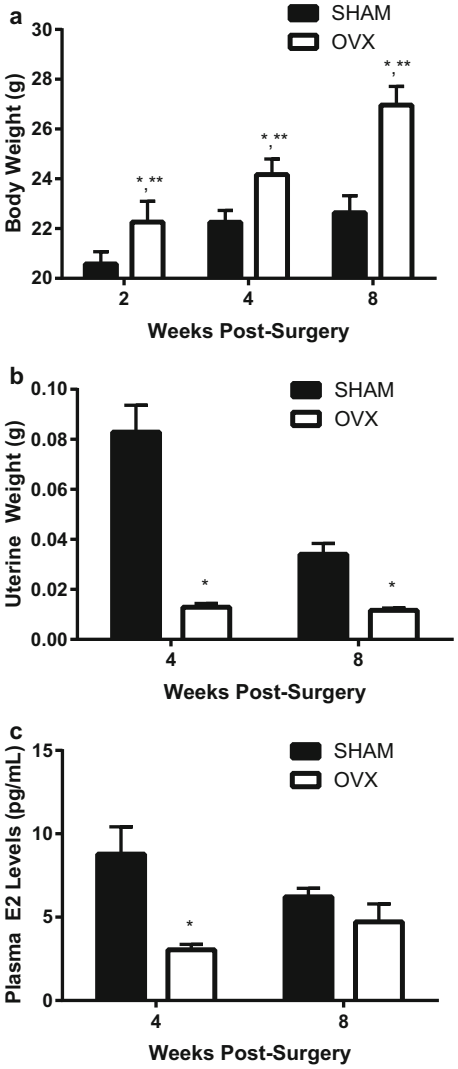

Fig. 1 Physiological measurements due to bilateral ovariectomy (OVX). Mice underwent OVX or SHAM surgery at 8 weeks of age. In all graphs (**a**–**c**), significance is defined as $p < 0.05$ using a two-way ANOVA followed by post hoc analysis. *Represents OVX is $p < 0.05$ from the SHAM treatment group at each age group. **Indicates $p < 0.05$ from both SHAM and OVX treatment groups across ages

increase in body weight was also due to the age of the mice in both OVX and SHAM mice ($p < 0.001$). In both surgical treatment groups, there was an increase in the length of the tibia ($p = 0.001$) until 12 weeks of age (i.e., 4 weeks post-surgery); after 12 weeks there was no additional increase in length (Table 1). Similarly, when each tibia was cross-sectioned at its mid-diaphysis, the area of the cortical/compact bone increased until 12 weeks of age in both treatment groups ($p = 0.001$, Table 1). In addition, mice 2 weeks post-OVX demonstrated less tibial cortical area than their age-matched SHAM mice. Even with the increase in compact bone structure, the area medullary cavity was similar between ages and surgical treatment groups. Figure 1b shows that ovariectomization (OVX) of the mice resulted in a decrease ($p < 0.001$) in uterine weight compared to SHAM mice. Similarly, as shown in Fig. 1c, the plasma estrogen (E2, 17β-estradiol) levels also declined with OVX at 12 weeks; however, at

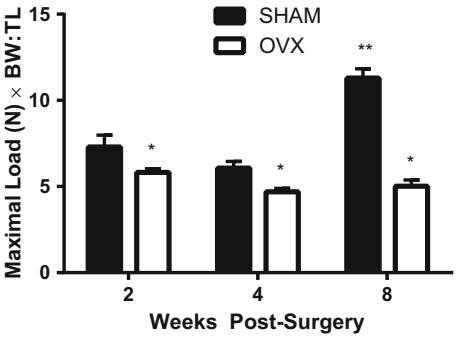

Fig. 2 Using three-point bending, less force was required to reach the fracture/breaking point of the tibia from ovariectomized (OVX) than age-matched SHAM mice. The mice underwent OVX or SHAM surgery at 8 weeks of age. A constant force was applied (rate of 0.498 mm · s^{-1}) to the mid-diaphysis of the tibia until fracture (max load) occurred. Data are normalized for the size of each individual mouse using the body weight-to-tibial length ratio (BW:TL). Significance is defined as $p < 0.05$ using a two-way ANOVA followed by post hoc analysis. *Represents OVX is $p < 0.05$ from the SHAM treatment groups at each age group. **Indicates $p < 0.05$ from both SHAM and OVX treatment groups across ages

16 weeks of age the SHAM mice estrogen levels declined to similar levels as the OVX mice. The correlation between uterine weight and plasma estrogen levels was significant ($p < 0.001$).

The three-point bending technique was used to assess the mechanical properties (fracture point, stiffness, and elasticity) of the tibia. A constant amount of force was applied to the midpoint of the tibia diaphysis to determine the maximum load that could be placed on the tibia until fracture/breakage occurred. Both the age ($p < 0.001$) and surgical treatment ($p < 0.001$) of the mice had a significant effect on the maximum load withstood by the tibias; thus, all OVX mice had a lower breaking point than the age-matched SHAM mice (Fig. 2). However, at 16 weeks of age (i.e., 8 weeks post-surgery) a greater force was required to reach the SHAM mice breaking point than the 10- and 12-week-old SHAM mice and the age-matched OVX mice.

At 16 weeks of age, the tibias from the SHAM mice also demonstrated a higher elasticity, stress, and stiffness than the younger SHAM mice and the age-matched OVX mice (Fig. 3). For all three parameters (i.e., elasticity, stress, and stiffness), the age of the mice and surgical treatment had a significant effect. The elasticity of the compact bone was only different between the SHAM and OVX mice at 16 weeks of age ($p < 0.001$); otherwise there was no difference between the two treatment groups at younger ages. The tibias from the SHAM mice were able to withstand more stress at every age than the OVX mice tibias ($p < 0.001$). Furthermore, SHAM mice at 16 weeks resisted more stress than any other treatment group ($p < 0.001$). The SHAM mice had tibias that demonstrated greater stiffness than OVX mice at 4 and 8 weeks post-surgery ($p < 0.001$).

4 Discussion

This study demonstrates that the loss of gonadal estrogen production (via an ovariectomy) induces a reduction of tibial strength in young female mice that are skeletally immature. Because most studies utilize skeletally mature mice (≥16 weeks old) to evaluate the decline in mechanical strength of compact bone, the objective of this study was to determine if estrogen

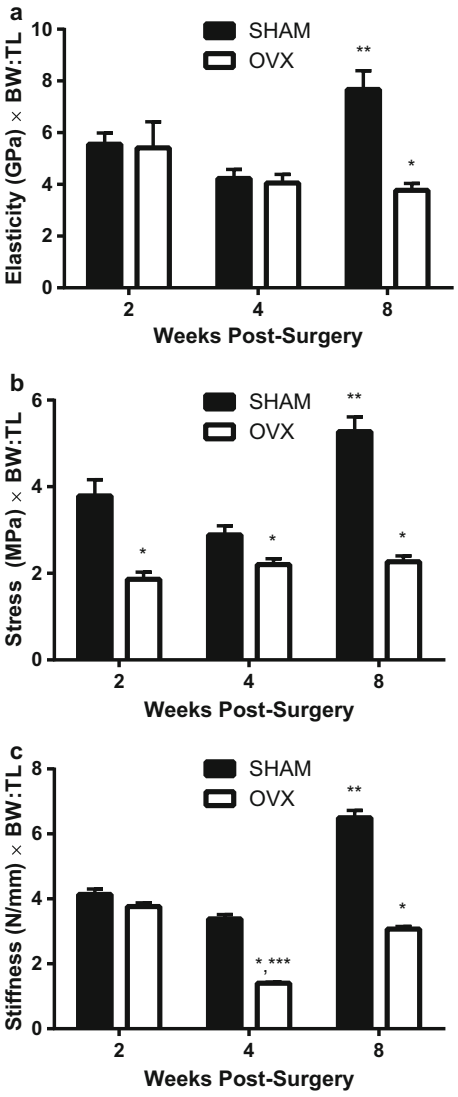

Fig. 3 Biomechanical properties (elasticity, stress, and stiffness) of the tibia between ovariectomized (OVX) and SHAM mice. In all graphs (**a**–**c**), significance is defined as $p < 0.05$ using a two-way ANOVA followed by post hoc analysis. Data are normalized for the size of each individual mouse using the body weight-to-tibial length ratio (BW:TL). Significance is defined as $p < 0.05$ using a two-way ANOVA followed by post hoc analysis. *Represents OVX is $p < 0.05$ from the SHAM treatment groups at each age group. **Indicates $p < 0.05$ from both SHAM and OVX treatment groups across ages. ***Indicates OVX is $p < 0.05$ from the other OVX treatment groups

depletion in skeletally immature mice could induce a similar decrease in cortical bone strength. In maturing females, the loss of estrogen is typically associated with increased bone turnover, whereby osteoclast activity will exceed osteoblast activity [10]. This is the first study to evaluate the effect of an ovariectomy on compact bone strength using skeletally immature mice.

Our results indicate that the tibia was still developing in 10-week-old mice (when compared to 12 and 16 weeks of age); this was suggested by the shorter tibial length and smaller cortical area. In addition, the body weight of our SHAM mice did not stabilize until 12 weeks of age. Similarly, Somerville et al. [13] used the tibia from C57BL/6 female mice and found that the tibial length, cortical area, and bone mineral density peaked and stabilized by 12 weeks of age. Both Somerville et al. [13] and Beamer et al. [14] suggest that skeletal maturity in these mice is

reached by 16 weeks of age. In young mice, cortical bones will grow in length and diameter. An increase in tibial length will occur via endochondral ossification, in which, osteoclasts will facilitate the resorption of cartilage and osteoblasts will build the bone matrix. Osteoblasts will also deposit bone matrix along the periosteum [23]. After 2 weeks post-surgery (10 weeks of age), only the maximal load and stress withstood by the tibias was reduced in the ovariectomized mice. In contrast, once the mice reached skeletal maturity at 16 weeks of age, all four parameters (i.e., maximal load, elasticity, stress, and stiffness), which evaluate the mechanical strength of compact bone, were reduced in ovariectomized mice. As similarly reported by Somerville et al. [13], results from our SHAM mice show that age does increase the maximum load, elasticity, stress, and stiffness of the tibia. Ovariectomization of the mice appears to negate this age effect. Iwaniec and Turner [23] have recently described that with growth, weight gain, and the subsequent increase in bone mass, there is an adaptive skeletal response that is induced by the mechanical strain on the skeletal system. This adaptive response, which is sometimes referred to as the mechanostat theory and is not well understood, provides some insight into our results, which demonstrate that an increase in age increases the maximum load, elasticity, stress, and stiffness of bone.

In our study, the OVX mice did not demonstrate the increase in cortical bone strength due to skeletal maturity shown by the SHAM mice. There have been conflicting results that suggest that the elevation in follicle stimulating hormone (FSH) due to estrogen deficiency is associated with a decline in bone mass [24, 25]. However, Ozbek et al. [26] recently found that elevated levels of FSH due to hypogonadotropic and hypergonadotropic hypogonadism in adolescent girls did not have an impact on femur bone mineral density. Many of the estrogenic effects concerning the osteogenesis of bone mass have been shown to be mediated by estrogen receptor α (ERα) signaling mechanisms in osteoclasts. Therefore, we hypothesize that the blunted mechanical strength once the OVX mice reach skeletal maturity at 16 weeks of age is due to a downregulation of the intracellular events associated with ERα.

Nakamura et al. [27] demonstrated that the selective knockout of ERα ($ER\alpha^{-/-}$) in osteoclasts caused apoptosis and bone resorption via the Fas ligand (FASL) system in trabecular bone. Fas is a transmembrane protein that is associated with the tumor necrosis family (TNF) of receptors that induces apoptosis. Recently, using 12-week-old C57BL/6 mice, Wang et al. [28] found that OVX causes a downregulation of FASL in osteoblasts from the femur trabeculae via the proinflammatory cytokines, interferon-γ and TNF-α. They also demonstrated that FASL knockout osteoblasts caused an increase in osteoclast number, which led to a reduction in bone mass. It is unclear, but hypothesized, that these same mechanisms exist in cortical bone to promote osteoclast apoptosis with estrogen withdrawal [27]. ERα has also been implicated in the Wnt/Lrp/Fzd intracellular signaling mechanism that controls cortical bone mass; it has little effect on trabecular bone. Wnt16 is a glycoprotein that is derived from osteoblasts and binds to frizzled (Fzd) receptors and low-density lipoprotein 5/6 co-receptors [29, 30]. Todd et al. [30] found that OVX C57BL/6 mice demonstrated a 50% reduction in Wnt16 expression in the cortex of the femur. This reduction was prevented in OVX mice given 17β-estradiol supplementation.

As has been similarly described by others [10, 30], the ovariectomized mice demonstrated weight gain, a decline in uterus weight, and a decline in plasma estrogen levels. Most of our data are reported at 2, 4, and 8 weeks post-surgery; unfortunately, we were not able to report the plasma estrogen concentration at 2 weeks because the red blood cells were lysed in both experimental groups and an accurate reading could not be obtained. Even though there was an insignificant difference in plasma estrogen levels between the OVX and SHAM mice at

8 weeks post-surgery, our results indicated a significant correction between uterine weight and plasma estrogen levels at 4 and 8 weeks post-surgery. LeBlanc et al. [31] has found similar results using a SHAM and OVX rat model. Therefore, it appears that in intact mice and rats, there is a decline in plasma estrogen and uterine weight with an increase in age.

5 Conclusions

Overall, estrogen deficiency does reduce bone strength in skeletally immature mice. However, the effects are exacerbated once bones reach full maturity. This study provides foundational knowledge about how the inability to produce in vivo estrogen, or the bones' insensitivity to estrogen effects compact bone in younger females.

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