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Tissue selective effects of bazedoxifene on the musculoskeletal system in female mice

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Abstract

The actions of selective estrogen receptor modulators are tissue dependent. The primary objective of the current study was to determine the tissue selective effects of bazedoxifene (BZA) on the musculoskeletal system of ovariectomized (OVX) female mice, focusing on the strengths of muscle-bone pairs in the lower hindlimb. Treatment with BZA after ovariectomy (OVX+BZA) did not prevent body or fat mass gains ($P < 0.05$). *In vivo* plantarflexor muscle isometric torque was not affected by treatment with BZA ($P = 0.522$). Soleus muscle peak isometric, concentric and eccentric tetanic force production were greater in OVX+BZA mice compared to OVX+E₂ mice ($P \leq 0.048$) with no effect on maximal isometric specific force ($P = 0.228$). Tibia from OVX+BZA mice had greater cortical cross-sectional area and moment of inertia than OVX mice treated with placebo ($P < 0.001$), but there was no impact of BZA treatment on cortical bone mineral density, cortical thickness, tibial bone ultimate load or stiffness ($P \geq 0.086$). Overall, these results indicate that BZA may be an estrogen receptor agonist in skeletal muscle, as it has previously been shown in bone, providing minor benefits to the musculoskeletal system.

Key Words

- ▶ bone
- ▶ ovariectomy
- ▶ physical activity
- ▶ SERM
- ▶ skeletal muscle

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Introduction

As women age and progress through menopause, they experience a myriad of physiological and systemic changes including decreases in skeletal muscle function and bone health, increased incidence of hot flashes, and alterations in body composition, such as increased abdominal adiposity. There is evidence that menopausal hormone therapy is effective in mitigating these undesirable changes (Anderson *et al.* 2004, North American Menopause Society 2004, Greising *et al.* 2009, Ronkainen *et al.* 2009). However, since the Women's

Health Initiative raised concerns about the increased risk for thromboembolic events, stroke, and breast cancer for women using hormone therapy, the use of exogenous estrogens has declined substantially (Ettinger *et al.* 2012). This decline has prompted the development of a class of drugs known as selective estrogen receptor modulators (SERM). The ideal SERM will have an estrogenic effect on the target tissue(s), while at the same time have anti-estrogenic, or no effect, in non-targeted tissues. Bazedoxifene acetate (BZA) is a third-generation SERM.

It was approved in the European Union for the prevention of postmenopausal osteoporosis in 2009, and then by the United States Food and Drug Administration in 2014 for use in combination with conjugated estrogens for treatment of moderate to severe hot flashes, as well as prevention of postmenopausal osteoporosis.

Results from the Selective Estrogen, Menopause, and Response to Therapy (SMART) trials indicate treatment with BZA and conjugated estrogen increases bone mineral density, improves sexual functioning in women with vulvar/vaginal atrophy, reduces the number and severity of hot flashes, prevents endometrial hyperplasia, and does not affect mammographic breast density (Pickar *et al.* 2009, Pinkerton *et al.* 2009, Harvey *et al.* 2013, Pinkerton *et al.* 2014).

While such clinical investigations have shown several positive effects of BZA, additional pre-clinical studies are necessary to further determine effects on targeted and non-targeted tissues, as well as the mechanisms through which BZA elicits these effects independent of a simultaneous estrogen treatment. In terms of efficacy on the skeleton, both rodent and primate models show treatment with BZA following ovariectomy (OVX) increases total bone mineral density (BMD), reduces OVX-induced bone turnover, and increases bone volume and trabecular thickness, particularly of the vertebrae (Kharode *et al.* 2008, Komm *et al.* 2011, Saito *et al.* 2015, Smith *et al.* 2015). Work by Borjesson *et al.* implicates estrogen receptor alpha (ER α), specifically the ER α -activation function-1, as a mechanism through which BZA elicits its effects in bone (Borjesson *et al.* 2013, Börjesson *et al.* 2016). While it appears BZA functions through ER α to mediate positive effects on BMD and turnover, there has been a minimal investigation into the effect of BZA on the mechanical properties of bone such as ultimate load and stiffness (Borjesson *et al.* 2013, Börjesson *et al.* 2016).

The effects of SERMs on skeletal muscle are largely unknown. This is important because skeletal muscle fibers, such as bone cells, contain estrogen receptors (Lemoine *et al.* 2003, Wiik *et al.* 2003, 2009). Some pre-clinical studies on mice have shown that OVX results in decreased skeletal muscle contractility, while treatment with estradiol (E₂) mitigates decreases (Moran *et al.* 2006, Greising *et al.* 2011a,b, Lai *et al.* 2016). Similarly, postmenopausal women who take estrogen-based hormone therapy have greater skeletal muscle strength than those who remain estrogen-deficient (Phillips *et al.* 1993, Greising *et al.* 2009, Ronkainen *et al.* 2009). Studies on female mice have also demonstrated that deletion of ER α , specifically in skeletal muscle fibers,

results in decreased skeletal muscle function (Ribas *et al.* 2016, Cabelka *et al.* 2019, Collins *et al.* 2018). While the SERM tamoxifen has been shown to be an estrogen receptor agonist in skeletal muscle as measured by positive effects on muscle strength (Warren *et al.* 2007), the extent to which BZA is an ER agonist in skeletal muscle is not known.

There is an inherent link between the function of the musculoskeletal system and physical activity. That is, as physical activity decreases so can skeletal muscle function and bone strength, whereas increasing physical activity can improve muscle function and bone strength. In mice, OVX causes significant decreases in levels of spontaneous physical activity and voluntary wheel running, while treatment with E₂ can reverse or prevent the inactivity (Gorzek *et al.* 2007, Greising *et al.* 2011a, Bowen *et al.* 2012). Spontaneous physical activity has previously been defined as the home-cage activity or other activity that is different from motivated behaviors such as wheel running (Garland Jr *et al.* 2011). If BZA were to function such as E₂ in terms of preventing OVX-induced declines in physical activity, that would be considered a beneficial effect. However, the extent to which BZA affects spontaneous physical activity in mice has been studied only minimally (Kim *et al.* 2014). Also, the extent to which BZA affects musculoskeletal function independent of any physical activity has not been thoroughly investigated. Thus, to more fully elucidate the tissue-specific properties of BZA, this study was designed to test the hypotheses that in ovariectomized mice BZA will (1) improve spontaneous physical activity over that of placebo, (2) be an ER agonist and have beneficial effects on skeletal muscle contractility, and (3) enhance bone structure and strength compared to treatment with placebo.

Materials and methods

Animals and experimental design

Thirty, 8-week-old, female C57BL/6 mice from Jackson Laboratories were obtained and group housed on a 14 h light:10 h darkness cycle, with free access to water and phytoestrogen free chow (Harland Teklad, 2919, Madison, WI, USA). After 3 week of acclimation and prior to any intervention, body composition was measured on conscious, 11-week-old animals (Echo MRI 3-in-1, Echo Medical System; Houston, TX, USA). Body mass was measured weekly, from the time of the first body composition measurement, for the duration of the study.

Mice were randomly assigned to one of three treatment groups ($n = 10$ each): OVX+placebo, OVX+BZA, and OVX+E₂. At 12–14 week of age mice underwent surgery. Two hours prior to ovariectomy surgery (Moran *et al.* 2006) mice received subcutaneous injections of 2 mg/kg slow release buprenorphine (ZooPharm, Windsor, CO, USA). Immediately following surgery, treatment pellets (placebo, BZA, or E₂) were implanted at the base of the neck via a 10-gauge trochar (Innovative Research of America, Sarasota, FL, USA). One mouse each in the OVX+E₂ and OVX+BZA groups were euthanized secondary to non-healing incisions after surgery.

The estrogen pellet consisted of 0.18 mg 17 β -E₂, released over 60 days such that mice received approximately 3 μ g/day (Innovative Research of America). This E₂ dose was chosen to elicit physiological levels in mice (Nelson *et al.* 1981, Farr *et al.* 1995). The BZA pellet consisted of 1.44 mg BZA, released over 60 days such that the mice received approximately 24 μ g/day. This dosage of BZA has been reported to be effective in protecting against bone loss (Andersson *et al.* 2016a,b), and was based on body surface calculations used to determine human doses (Reagan-Shaw *et al.* 2008, Silverman *et al.* 2008). BZA, a generous gift from Pfizer Inc., was sent to Innovative Research of America to make BZA time-release pellets (Wardell *et al.* 2013).

Spontaneous physical activity was quantified using 24-h cage activity monitoring at 7 week of treatment, body composition was remeasured at 8 week of treatment, and then *in vivo* and *in vitro* skeletal muscle function was analyzed, immediately followed by sacrifice. At sacrifice, uteri, as well as subcutaneous and visceral fat pads, were dissected and weighed. Blood was then collected by cardiac bleed, and was allowed to clot at room temperature followed by centrifugation for 10 min at 10,000 g at room temperature. Serum, void of red cell hemolysis, was extracted, snap-frozen in liquid nitrogen, and then stored at -80°C until analyzed for E₂ or BZA. Lastly, tibial bones were removed, cleaned of all soft tissue, snap-frozen in tubes containing 1 mL PBS, and stored at -80°C until bone density and structure measurements and mechanical testing were completed.

The University of Minnesota Institutional Animal Care and Use Committee approved all protocols and animal care procedures. Measurements of serum BZA and E₂, physical activities, body mass and composition, *in vivo* and *in vitro* muscle physiology, and bone density and strength were conducted with investigators blinded to mouse treatment.

Serum E₂ concentration

Serum was pooled when necessary to reach >300 μL per sample to be analyzed, precipitated, eluted, and measured using liquid chromatography – tandem mass spectrometry (LC-MS/MS) as previously described (Le *et al.* 2018). Briefly, a micro LC column (2.7 μm , C18 90 Å, Halo Fused core, 100 $\times$ 0.3 mm, Eksigen, SCIEX; Framingham, MA, USA) was used in series with an AB SCIEX QTRAP 5500 mass spectrometer (Thermo Fisher Scientific). The E₂ reporter was identified by a m/z of 271.2 Da and quantified by the peak area of the chromatograph using MultiQuant™ (SCIEX). Mean serum concentrations of E₂ were: OVX+placebo = 4.7 pg/mL ($n = 4$, pooled samples), OVX+E₂ = 44.9 ± 27.9 pg/mL ($n = 5$, unpooled samples with SD) indicating successful ovariectomy and replacement of E₂ in the physiological range for female mice, respectively.

Serum BZA concentration

Serum samples were thawed on ice and an equal volume of acetonitrile was added to each. Samples were vortexed, place on ice for 10 min followed by centrifugation at 4°C and 20,817 g for 10 min, and the supernatant was used for analysis. Samples were spiked with 5 ng/mL BZA standard prior to analysis.

HPLC measurement of BZA was performed using a Shimadzu I-series 2030C with auto-sampler, RF-20A fluorescence detector and online degasser with 10 μL of each sample injected onto a Luna 5 μm C-18 100 Å, 250 $\times$ 4.6 mm column (Phenomenex, CA, USA). The mobile phase was generated by mixing (A) 10 mM KH₂PO₄ (pH 3) with (B) 10:90 water:acetonitrile (v:v) by pre-pump mixing to give 90% A and 10% B at 1 mL/min. BZA was eluted with a linear gradient from 10 to 90% B over 30 min and followed by 90% B for a further 10 min. The column was returned to the starting condition by 90–10% B gradient over 2 min and holding at 10% B for 3 min making a total run time of 45 min. BZA was detected at its natural fluorescence of 300 nm excitation and 380 nm emission (Chandrasekaran *et al.* 2010). The concentration and identity of BZA was compared to a standard, bazedoxifene acetate (Pfizer Inc.). Concentration was quantified by peak area using Shimadzu Class VP software. Serum BZA concentrations were (mean $\pm$ s.d.): OVX+placebo = 0.19 ± 0.47 ng/mL ($n = 4$), OVX+BZA = 3.62 ± 6.27 ng/mL ($n = 10$), and OVX+E₂ = -0.08 ± 0.37 ng/mL ($n = 4$). These BZA concentrations are consistent with previous reports (Chandrasekaran *et al.* 2010, Smitha *et al.* 2015).

24-h physical activity monitoring

Using open-field activity chambers (Med Associates Inc., St. Albans, VT, USA) for a 24-h period, spontaneous physical activities of individual mice were measured as previously described (Landisch *et al.* 2008, Greising *et al.* 2011a). Briefly, each mouse underwent a familiarization period by being housed in a mock chamber for a full day prior to initiating the 24-h cage activity monitoring on the second day. Activity data were acquired using Activity Monitor version 5 software (Med Associates), and a box size of three. Disruption of photo beam arrays in the chamber due to mouse movements register an activity count and duration. To note, an 'activity count' represents any single photo beam break and because multiple photo beams may break with one jump or vertical movement, those counts do not represent the actual number of times a mouse jumps or rears. One mouse from each of the OVX+placebo and OVX+E₂ groups had data excluded in the analysis secondary to software malfunction.

In vivo plantar flexor muscle function

Each mouse was anesthetized via isoflurane inhalation (1.5%) with an O₂ flow rate of 125 mL/min and *in vivo* contractility of the plantar flexor muscles (gastrocnemius, soleus, and plantaris) was assessed by stimulating the sciatic nerve. As previously described, the peroneal branch of the sciatic nerve was first severed to avoid recruitment of the dorsi flexor muscles (Baltgalvis *et al.* 2012). The ankle joint was positioned so that the foot was perpendicular to the tibia and the knee was stabilized with a clamp to inhibit lower limb movement. The footplate was attached to the shaft of a servomotor (300-LR; Aurora Scientific, Aurora, Ontario, Canada). Peak isometric torque was measured, followed by a series of concentric contractions at various angular velocities (1200–100°/s) of plantarflexion over a 40° excursion. Contraction of the plantar flexors was elicited via percutaneous stimulation of the sciatic nerve with platinum needle electrodes attached to a stimulator (E2–12 electrodes and S48 stimulator; Grass Telefactor, Warwick, RI, USA). While still anesthetized, mice received an intraperitoneal injection of sodium pentobarbital (100 mg/kg body mass) followed by *in vitro* contractility testing of the soleus muscle.

In vitro soleus muscle contractile function

Soleus muscle isolation and preparation have previously been described (Moran *et al.* 2005). Briefly,

the soleus muscle was placed in a 1.2 mL bath filled with oxygenated Krebs-Ringer buffer maintained at 25°C. The proximal muscle tendon was attached by 6–0 suture to a dual-mode muscle lever system (300B-LR; Aurora Scientific Inc.). Measures of peak isometric, concentric, and eccentric forces during tetanic stimulation were obtained as previously described (Greising *et al.* 2011b).

Cortical bone structure of the tibia

Cortical bone of tibia at the mid-diaphysis was assessed using a micro-CT system (XT H 225, Nikon Metrology Inc.). The scanner was set to a voltage of 120 kVp and a current of 61 µA, and bones were scanned using an isotropic 7.4 µm voxel size with a 708 ms integration time. A 0.5 mm region (approximately 68 slices) was scanned and the average relative bone density was determined at each slice using regression analyses derived from Image J (NIH) analysis based on standardized phantoms (Bruker micro-CT, Belgium). The average BMD across each of the 68 slices was calculated and reported for each tibia. CT Pro 3D (Nikon Metrology Inc.) was used to make 3D reconstruction volumes for each scan. VGStudio MAX 3.2 (Volume Graphics GmbH, Heidelberg, Germany) converted 3D reconstruction volumes to bitmap datasets for each scan. Morphometric analyses were completed with the SkyScan CT-Analyzer (Bruker micro-CT). At completion of scanning, bones were stored at –80°C until mechanical testing.

Bone mechanical testing

Mechanical properties of the tibia at the mid-diaphysis were determined using three-point bending. A Mecmesin MultiTest 1-D test machine and a Mecmesin AFG-25 load cell (Mecmesin, West, Sussex, UK) was used as previously described (Novotny *et al.* 2011). Briefly, each tibia was thawed, length measured, and the mid-point was marked. Each bone was placed on its lateral side on two supports with 1 cm separation and a quasi-static load was applied to the mid-diaphysis at a displacement rate of 2 mm/min until failure. Load–displacement curves were analyzed for each bone using a custom-written TestPoint program (TestPoint version 7; Measurement Computing Corp., Norton, MA, USA) as previously described (Novotny *et al.* 2011, Mader 2014). Mechanical properties analyzed were ultimate load and stiffness.

Table 1 Twenty-four hour cage activities of ovariectomized mice treated with placebo, bazedoxifene, or 17 β -estradiol for 7 weeks.

	OVX+Placebo (n = 9)	OVX+BZA (n = 9)	OVX+E ₂ (n = 8)	One-way ANOVA P-value
Ambulation distance (m)	1211 (918)	549 (370) ^a	1155 (1129)	0.020
Ambulation time (min)	148 (37.4)	92.7 (43.8)	148 (70.3)	0.062
Jump count	1611 (484)	1423 (514)	2109 (757)	0.065
Vertical count	6206 (3315)	5400 (1558)	11,934 (13,109)	0.066
Active time (min)	284 (43.3)	221 (43.4)	290 (80.5)	0.042

Data are means (s.d.).

^aDifferent from OVX+Placebo.

Statistical analyses

To analyze the effects of treatment (OVX+placebo, OVX+BZA, OVX+E₂) and time (baseline/pre-surgery, OVX, weeks 1–8) on body mass and compositions, repeated measures two-way ANOVAs were performed. When main effects or interactions were significant ($P < 0.05$), Holm–Sidak *post hoc* tests were performed to determine which combination of conditions were different from one another. To assess differences in treatment on spontaneous physical activity, skeletal muscle contractility, fat pad masses, uterine masses, tibial bone structural and mechanical properties, one-way ANOVAs were performed. When an effect of treatment was significant, Holm–Sidak *post hoc* measurements were used to determine differences among treatments. When assumptions of equal variance or normality were violated, as assessed by Shapiro–Wilk testing, Kruskal–Wallis one-way ANOVA on Ranks was performed with Dunn's *post hoc* tests. For all *post hoc* testing, $P < 0.05$ is considered significant. Statistical analyses were carried out using SigmaStat version 3.5 (Systat Software, Inc., Point Richmond, CA, USA).

Results

Spontaneous physical activity

Following 7 week of treatment, 24-h spontaneous physical activities and time being active tended to be low in OVX+BZA mice with ambulation distance significantly less than OVX+placebo mice (Table 1). Although the ANOVA for total active time reached significance, *post hoc* tests did not identify any treatment group differing from another (Table 1).

Body mass and composition and uterine mass

All mice gained weight throughout the study with the amount of weight gained in each group dependent upon

time (interaction $P < 0.001$; Fig. 1A). OVX+placebo mice weighed more than OVX+E₂ mice at week 2 through 8. OVX+BZA mice weighed more than OVX+E₂ mice at week 3, and weeks 5–8, indicating that BZA treatment did not blunt weight gain as did treatment with E₂.

Uterine masses of OVX+placebo and OVX+BZA mice were low compared to OVX+E₂ mice ($P < 0.05$; Fig. 1B). This result indicates that BZA did not stimulate uterine hyperplasia as did E₂ in ovariectomized mice.

The percent of body mass that was lean mass changed over the course of the study depending on treatment (interaction $P < 0.001$; Fig. 2A). Within groups, OVX+placebo and OVX+BZA mice had less lean mass at week 8 compared to baseline, and at week 8 had less lean mass compared to OVX+E₂ mice (Fig. 2A). The opposite held for fat mass with mice treated with placebo or BZA, but not E₂, gaining fat over the 8-week study (Fig. 2B). The relative adiposity of OVX mice treated with placebo and BZA measured by MRI was corroborated by fat pad masses. Subcutaneous and visceral fat pads from OVX+placebo and OVX+BZA mice weighed 96–126% more than those from OVX+E₂ mice ($P < 0.05$; Fig. 2C).

Skeletal muscle contractile function

There was no difference in peak isometric torque produced *in vivo* by the plantar flexor muscles among the treatment groups ($P = 0.522$; Fig. 3A). Over a range of angular velocities, torque production differed at 800 and 1000°/s ($P \leq 0.020$; Fig. 3B) between OVX+placebo and OVX+E₂ mice ($P \geq 0.140$ for all other angular velocities; Fig. 3B).

Isolated soleus muscles from OVX+BZA mice weighed more and produced greater peak isometric, concentric and eccentric forces compared to OVX+E₂ mice (Table 2). Once normalized for size, isometric force did not differ significantly among treatment groups (specific P_o , Table 2).

Figure 1

Body masses of mice over the duration of the study and end point uterine masses. (A) Weekly body masses of ovariectomized mice differed among treatment groups depending on time (interaction $P < 0.001$). Significant results from *post hoc* testing between groups are denoted at week of treatment. (B) Uterine mass at sacrifice. Data are means $\pm$ s.d. *Different from OVX+placebo. #Different from OVX+BZA.

Cortical structural and mechanical properties of the tibia

Tibia were imaged at the mid-diaphysis to determine the impact of BZA on cortical bone structure. There was no difference among OVX mice treated with placebo, BZA, and E₂ in cortical BMD ($P = 0.528$; Fig. 4A). Cortical thickness of tibia also did not differ among groups ($P = 0.099$). Cortical cross-sectional area of tibia differed among groups ($P < 0.001$; Fig. 4B) with OVX+BZA and OVX+E₂ mice having greater areas than OVX+placebo, and tibia from OVX+BZA mice having less area than OVX+E₂ mice. Cortical cross-sectional moment of inertia of the tibia differed among groups as well ($P > 0.001$) being greater in OVX+BZA ($0.078 \pm 0.005 \text{ mm}^4$) than

Figure 2

Body composition of ovariectomized mice differed according to treatment group and time (interaction, $P < 0.001$) and mass of fat pads from ovariectomized mice was low in mice treated with estradiol. (A) Lean mass as a percent of total body mass differed between baseline (pre-surgery) and 8 week after ovariectomy within OVX+placebo and OVX+BZA groups as indicated by horizontal lines. At week 8, lean mass differed by treatment. (B) Fat mass as a percent of total body mass differed between baseline and 8 weeks after ovariectomy within OVX+placebo and OVX+BZA groups as indicated by horizontal lines. At week 8, fat mass differed by treatment. (C) Subcutaneous and visceral fat pad masses at sacrifice were affected by treatment. *Different from OVX+placebo within time (or within fat pad in C). #Different from OVX+BZA within time (or within fat pad in C). Horizontal bars indicate differences between baseline and week 8 within group.

Figure 3

In vivo muscle function of ovariectomized mice minimally differed among treatments. (A) Peak isometric torque of the plantarflexor muscles. (B) Torque production of the plantarflexor muscles at increasing velocities. Data are mean $\pm$ s.d. *OVX+E₂ different from OVX+placebo.

OVX+placebo (0.068 ± 0.006 mm⁴) and OVX+E₂ mice (0.072 ± 0.006 mm⁴).

To determine if treatment with BZA improved bone strength, mechanical properties of the tibias were tested. Ultimate load differed among groups ($P = 0.033$) with OVX+E₂ being greater than OVX+placebo mice (Fig. 4C). There was no difference among groups for tibial bone stiffness ($P = 0.086$; Fig. 4D).

Discussion

This study addressed three hypotheses related to effects of BZA on the musculoskeletal system. First, we did not detect a difference in spontaneous physical activity among the groups and thus were unable to support our hypothesis that BZA treatment would improve spontaneous physical activity more than treatment with placebo. Despite the absence of treatment effect on spontaneous physical activity, we did find effects on body and fat masses. While treatment with placebo and BZA resulted in ovariectomy-induced gains in body and fat masses, treatment with E₂ maintained body mass after OVX. Second, BZA treatment preserved soleus muscle mass compared to treatment with E₂ contributing to slightly greater isometric, concentric and eccentric force production and providing some support for the hypothesis that BZA is an ER agonist in skeletal muscle. Third, although tibial cortical cross-sectional area and cross-sectional moment of inertia were improved with BZA treatment, there was no significant difference between mice treated with placebo and BZA for tibia cortical BMD, ultimate load, or stiffness, thus the hypothesis that BZA would positively affect cortical bone structure and strength was not supported.

Because the function of the musculoskeletal system and physical activity are inherently linked, our experiment first sought to determine if BZA treatment affected spontaneous physical activity. Ovariectomy in rodents typically results in decrements in both spontaneous physical activities as well as voluntary wheel running (Moran *et al.* 2006, Gorzek *et al.* 2007, Greising *et al.* 2011a, Bowen *et al.* 2012). Two main parameters, ambulation time and total active time, were not different by treatment group though ambulation distance was significantly lower in OVX mice treated with BZA compared to placebo. Previous studies also found no differences in locomotor activity in BZA-treated OVX mice compared to untreated OVX or ovary-intact mice (Barrera *et al.* 2014, Kim *et al.* 2014). Because wheel running shows robust responses to manipulation of estrogenic compounds in rodents (Moran *et al.* 2006, Gorzek *et al.* 2007, Greising *et al.* 2011a, Bowen *et al.* 2012), future studies should consider measuring this type of physical activity. Our results combined with previous studies indicate that any effect of BZA on body composition, skeletal muscle, or bone in mice are not likely related to changes in physical activity.

Inconsistent with previous studies, our results show that BZA did not prevent gains in body and fat masses after OVX. Prior studies reported that BZA prevented OVX-induced weight and fat mass gains in mice (Barrera *et al.*

Table 2 *In vitro* soleus muscle contractile properties of ovariectomized mice treated with placebo, bazedoxifene, or 17 β -estradiol for 8 weeks.

	OVX+Placebo (n = 10)	OVX+BZA (n = 9)	OVX+E ₂ (n = 9)	One-way ANOVA P-value
Muscle mass (mg)	8.48 (0.47)	8.69 (0.61)	7.59 (0.74) ^a	0.009
Peak isometric tetanic force (mN)	212 (26.3)	234 (38.0)	191 (20.7) ^a	0.048
Specific P ₀ (N/cm ²)	25.4 (2.88)	27.6 (4.80)	24.0 (2.08)	0.228
Peak concentric force (mN)	108 (18.4)	117 (24.0)	86.7 (15.1) ^a	0.009
Peak eccentric force (mN)	381 (49.2)	414 (66.2)	339 (39.8) ^a	0.020

Data are means (s.d.). Specific force (P₀) was calculated as peak isometric tetanic force normalized by muscle physiological cross-sectional area.

^aDifferent from OVX+BZA.

2014, Kim *et al.* 2014). A difference between those studies and ours was delivery of BZA; we used subcutaneous time-release pellets whereas other studies used osmotic mini-pumps (Barrera *et al.* 2014) or oral gavage (Kim *et al.* 2014). It is possible that the delivery system or dose of the BZA plays a role in the effectiveness of minimizing weight gain, and that the resulting BZA serum concentration we report may have been below the therapeutic range. These results emphasize the importance of measuring and reporting concentrations of SERMs resulting from treatments. Consistent with previous studies (Barrera *et al.* 2014, Bernardi *et al.* 2014, Kim *et al.* 2014, Andersson *et al.*

2016a,b), our data clearly indicate that BZA treatment did not increase uterine mass.

A primary objective of our studies was to determine the effects of BZA on the musculoskeletal system. We are aware of only one other study that investigated the effects of a SERM on both skeletal muscle and bone functions. Previously, Warren *et al.* (2007) treated mice 30 days following OVX with tamoxifen for 60 days. No significant differences between tamoxifen- and E₂-treated groups were measured in rescuing maximal isometric tetanic force of the soleus muscle, micro-CT properties of the tibia, or mechanical properties of the tibia, from deleterious effects

Figure 4

Tibial bone properties from ovariectomized mice were marginally different among the treatment groups. (A) Cortical bone mineral density and (B) cortical cross-sectional area at the mid-diaphysis. (C) Mechanical properties, ultimate load and (D) stiffness. *Different from OVX+placebo. #Different from OVX+BZA.

of OVX. The conclusion was that tamoxifen is an estrogen receptor agonist in skeletal muscle as well as bone. The current study also evaluated soleus muscle contractility and tibial bone cortical and mechanical properties, with expanded analyses to include measures of *in vivo* plantar flexor muscle contractility. Our results did not show differences among ovariectomized mice treated with placebo, E₂, or BZA in plantar flexor torque production. Soleus muscles from OVX+BZA mice produced greater peak isometric, concentric and eccentric forces than did OVX+E₂ mice; however, once normalized for size, specific force did not differ among treatment groups.

Our imaging results of the mid diaphysis of the tibia showed that BZA increased cortical cross-sectional area and cross-sectional moment of inertia but did not increase cortical BMD. These results are consistent with the results from Andersson *et al.* (2016a) which showed that BZA increased trabecular but not cortical BMD. In contrast, Komm *et al.* (2011) demonstrated that BZA treatment was effective in preventing OVX-induced reduction in cortical BMD, however, this was after 52 week of treatment. Our study provided only 8 week of treatment. Additional studies have shown that BZA was effective in preventing OVX-induced decline in BMD, but these studies evaluated total BMD, that is, combining trabecular and cortical bone (Kharode *et al.* 2008, Bernardi *et al.* 2014).

There are only two studies that we are aware of which reported the effects of BZA on mechanical properties of bone. Borjesson *et al.* (2013), demonstrated that treatment with BZA increased maximal load to failure in femurs from orchidectomized male mice compared to femurs from control mice. In female mice, BZA treatment after OVX tended to increase cortical thickness and maximal load to failure but the increases did not reach statistical significance (Börjesson *et al.* 2016). Similarly, our analysis of bone mechanical properties measured no difference in ultimate load or stiffness between BZA and placebo treatment.

In summary, results of the current study show that BZA has minimal effects within the musculoskeletal system of female mice at the treatment dose used. The SERM caused modest improvements in soleus muscle mass and force production uniquely suggesting that BZA may be an estrogen receptor agonist in skeletal muscle. BZA had positive impacts on some but not all cortical bone structural properties, however, there was no effect of BZA on mechanical properties of tibia. BZA treatment to ovariectomized mice did not prevent body or fat mass gains as did E₂ treatment and thus overall did not demonstrate strong estrogenic properties in female mice. Further,

results from this study did not appear to be confounded by treatment-induced changes in physical activities by the mice. Future studies are needed to recognize the tissue-selective nature of SERMs, including investigation into effects on multiple tissues and physiological systems.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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