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Genistein Delivered as a Once Daily Oral Supplement Had No Beneficial Effect on the Tibia in Rat Models for Postmenopausal Bone Loss

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Abstract

Objective—Estrogen deficiency following menopause results in rapid bone loss, predisposing women to osteoporotic fractures. Genistein, a phytoestrogen present in high concentrations in soy, is an ingredient in dietary supplements aggressively marketed for bone health. However, the efficacy of soy extracts in reducing bone loss in a recent, long-duration clinical trial in postmenopausal women was disappointing. To better understand the failure of soy extracts to consistently induce a robust skeletal response in women, we investigated the long-term (5 months) effects of genistein, administered as a daily oral supplement, on 1) its efficacy to prevent cancellous bone loss in skeletally mature virgin Long-Evans rats ovariectomized (ovx) at 7 months of age, and 2) its efficacy to improve cancellous bone mass and architecture in aged retired breeder rats ovx at 16 or 22 months of age.

Methods—Rats within each age group were randomly assigned into one of 3 treatment groups (n=7–12 rats/group); 1) vehicle control, 2) 485 µg/day genistein, or 3) 970 µg/day genistein, resulting in serum genistein levels of 0.18 ± 0.10 , 0.76 ± 0.15 , and 1.48 ± 0.31 µM, respectively. Total tibia bone mass and density were evaluated using dual energy absorptiometry whereas

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cancellous bone mass and architecture in the tibial metaphysis and cortical bone mass and architecture in the tibial diaphysis were evaluated by micro-computed tomography.

Results—Oral genistein administered as a dietary supplement did not influence the cumulative effects of ovx, aging and/or reproductive history on cancellous and cortical bone mass and architecture.

Conclusions—Serum levels of genistein similar to those in women consuming a high soy diet are ineffective in prevention or treatment of bone loss in rat models for postmenopausal osteoporosis.

Keywords

osteoporosis; soy isoflavone; phytoestrogen; rat bone; microcomputed tomography

Introduction

Genistein, an isoflavone present in high concentrations in soy, is classified as a phytoestrogen due to its ability to bind to and activate estrogen receptors (ERs). In the legume, genistein functions as a chemoattractant for N₂ fixing *rhizobium* bacteria and has additional functions as an endocrine disruptor to reduce predation. As a chemoattractant, genistein attracts *Bradyrhizobium japonicum* to the plant by activating the bacterial nodD gene, which in turn promotes expression of other nod genes [1]. The transcriptional products of these genes, nod factors, are bacteria-to-plant signaling molecules that are required for bacterial infection and plant root nodule organogenesis and subsequent rhizobia–legume symbioses and N₂ fixation [2]. The mechanism by which genistein induces nodD genes in bacteria has many similarities to the gene regulatory pathway in animals involving nuclear receptor ligand interactions. Since ligand binding regions of nodD in bacteria and ER in animals exhibit significant homology, it is likely that they originated from a common ancestor protein [3].

Genistein, in addition to binding to ERs in animals, has the capacity to interact with other nuclear receptors, including peroxisome proliferator-activated receptors in vertebrates and the ecdysone receptor in arthropods [4]. At high concentrations, genistein inhibits tyrosine kinase activity induced by binding of natural ligands to epidermal growth factor receptor, platelet-derived growth factor receptor, insulin receptor and kit receptor [5]. These findings suggest that genistein has the potential to influence numerous hormone-mediated pathways.

Hormonal regulation of physiological processes involves tight feedback control. The unregulated introduction of an exogenous ligand that can bind to a hormone receptor may disrupt physiological signaling through that receptor. By acting as an endocrine disruptor, genistein has been shown to impair reproduction in mice and molting in arthropods [6–8]. The ability to reduce predation by disrupting critical functions in vertebrate and arthropod herbivores would be of value to the evolutionary success of the legume.

Non-physiological activation of ERs in select tissues may also confer context-specific benefits to vertebrates. For example, although a normal physiological process in humans, menopause results in greatly decreased serum estrogen levels and, as a consequence, rapid

bone loss [9]. Hormone replacement is an effective pharmacological intervention to prevent the bone loss. Phytoestrogens like genistein, by virtue of their ability to bind to and activate ERs in bone cells, have the potential to have a similar beneficial effect [10]. However, whether this occurs with levels of dietary and supplemental intake of genistein is controversial. In the present study, we modeled the effects of oral genistein administered as a once daily dietary supplement on bone density, mass and architecture. Specifically, we determined the effect of long-term oral genistein on cancellous bone in the proximal tibial metaphysis and on cortical bone in the tibial diaphysis in skeletally mature ovariectomized (ovx) 7-month-old virgin rats, and in aged ovx 16- and 22-month-old retired breeder rats. The mature ovx rat has accurately predicted the effects of estrogen agonists, partial agonists, and antagonists on cancellous bone architecture and turnover in the human skeleton and is recommended by the FDA as a preclinical model to evaluate the safety and efficacy of drug interventions to prevent or treat postmenopausal osteoporosis [11].

Methods

The female Long-Evans rats used in this study to investigate the effects of genistein on bone metabolism comprised a subset of animals from a study evaluating the effect of oral genistein on cognitive function [12]. Long-Evans rats, although frequently used in cognitive research, are less commonly used in skeletal research. Therefore, validation studies were conducted to determine the effects of age, ovx, and reproduction on cancellous and cortical bone in this strain of rat. In all our studies, the animals were housed in temperature- and humidity-controlled rooms on a 12-hour light-dark cycle and all procedures were approved by the Institutional Animal Care and Use Committee. In all studies, animals were randomly assigned into their respective treatment groups.

Experimental Design

Effect of age and ovx on bone in Long-Evans virgin rats—Seventy two virgin female Long-Evans rats were used in the validation experiments. The rats were randomized by weight to their respective groups. Untreated rats (8 – 12/group) were sacrificed at 2, 7 or 24 months of age. Additional rats (5 – 9/group) underwent ovx or sham surgery at 2 months of age and were sacrificed 1 or 2 months later at 3 or 4 months of age, respectively, or underwent ovx or sham surgery at 4 months of age and were sacrificed 8 months later at 12 months of age.

Effect of reproduction on bone in Long-Evans rats—Retired breeder female rats can be osteopenic compared to age-matched virgin rats as a consequence of multiple pregnancies and lactations [11]. To determine bone status prior to ovx in retired breeder Long-Evans rats, we compared 6-month-old virgin rats, 12-month-old virgin rats, 12-month-old breeder rats retired from breeding at 8 months of age, and 12-month-old virgin rats ovx at 7 months of age (n = 7 – 10/group, total n = 35).

Effect of oral genistein on bone in virgin and retired breeder Long-Evans rats—Eighty nine female Long-Evans rats were used to investigate the effects of oral genistein on bone mass and architecture. The experimental protocol is described in detail elsewhere

[12]. In brief (Figure 1), the effects of oral genistein were evaluated in 7-month-old ovx virgin rats ($n = 7 - 10/\text{group}$), and in 16-month-old ($n = 8 - 10/\text{group}$) and 22-month-old ($n = 11 - 12/\text{group}$) ovx retired breeder rats. The rats were purchased and housed for 4 months (7 month-old virgins and 16 month-old retired breeders) or 8 months (22-month-old retired breeders) prior to ovx. During this time, the animals were fed standard rat chow. On the day of ovx, the diet was switched to an AIN-93G soy-free diet (Harlan-Teklad, Madison, WI) to avoid uncontrolled exposure to dietary phytoestrogens via feed. Treatment was administered using 97 mg fruit flavored sucrose tablets (TestDiet, Richmond, IN, #1811494) which contained either 0.5% genistein or sucrose only. For treatment administration, the rats within each age group (7, 16, or 22 months of age at start of treatment) were assigned randomly by using the same allocation probability for all groups into one of 3 groups; 1) vehicle control (two sucrose tablets), 2) 485 $\mu\text{g}/\text{day}$ genistein (one sucrose tablet and 1 genistein tablet delivering approximately 1.6 mg/kg/day of genistein), or 3) 970 $\mu\text{g}/\text{day}$ genistein (2 genistein tablets delivering approximately 3.2 mg/kg/day of genistein) and treated daily for 5 months. We are aware that randomization is never an infallible mechanism to achieve balanced groups. However, animals within each age group shared similar life courses (e.g., environment and diet). This alone diminishes concern over the prospect that unbalanced groups, with respect to measured and unmeasured covariates, were present in our study populations, and randomization adds to our confidence of a high probability of having balanced groups. For dosing, each rat was removed from its cage and given its dose individually. The tablets were consumed within a few seconds of presentation. For all studies, animals were sacrificed using CO_2 and tibiae were excised and preserved in 70% ethanol solution for analysis.

Dual-energy X-ray Absorptiometry (DXA)

Tibial bone mineral content (BMC, mg) and area (cm^2) were measured using DXA (Piximus, Lunar Corp., Madison, WI, USA). Bone mineral density (BMD) was calculated as BMC/area (mg/cm^2). One individual performed all DXA analyses. The coefficient of variation for repeat scans ($n=5$) of a single tibia is 1.9%, 1.1%, and 2.6% for area, BMC, and BMD, respectively.

Bone Histomorphometry

For histomorphometric evaluation of cancellous bone in the validation studies, proximal tibiae were dehydrated in a graded series of ethanol and xylene, and embedded undecalcified in modified methyl methacrylate. Longitudinal sections (5 μm thick) were cut with a vertical bed microtome (Leica 2065) and affixed to slides. One section/animal was stained with toluidine blue. Bone volume/tissue volume (BV/TV, volume of tissue occupied by cancellous bone, %) was measured within the proximal tibial metaphysis as described [13]. The region of interest (2.9 mm^2) included secondary spongiosa only.

For histomorphometric evaluation of cortical bone, cross-sections (150 μm thick) were cut proximal to the tibiofibular junction with a low speed IsoMet saw (Buehler, Lake Bluff, IL, USA). Two sections per animal were ground to an approximate thickness of 20 μm and mounted unstained for microscopic assessment of cortical bone. Endpoints evaluated included cross-sectional area (area of cortical bone and bone marrow, mm^2), marrow area

(mm²), and cortical area (calculated as the difference between cross-sectional and medullary area, mm²).

All cancellous and cortical histomorphometric measurements were performed using the OsteoMeasure Analysis System (OsteoMetrics, Atlanta GA) and are reported using standard nomenclature [14]. One individual performed all histomorphometric analyses. The coefficient of variation for repeat measurements (n = 5) in a single tibia is 1.0% for bone volume/tissue volume.

Microcomputed Tomography

Microcomputed tomography (μCT) was used in the genistein dose response study for nondestructive three-dimensional evaluation of bone architecture. Tibiae were scanned at a voxel size of 16 × 16 × 16 μm using a Scanco μCT40 scanner (Scanco Medical AG, Basserdorf, Switzerland). The threshold for analysis was determined empirically and set at 245 (0–1,000 range) for both cancellous and cortical bone. One hundred and fifty slices (2.4 mm) were evaluated in the proximal tibial metaphysis. The volume of interest included secondary spongiosa only. Direct cancellous bone measurements included cancellous bone volume/tissue volume (BV/TV, volume of tissue occupied by cancellous bone, %), connectivity density (number of redundant connections per unit volume, mm⁻³), trabecular number (number of trabeculae intercepts, mm⁻¹), trabecular thickness (mean thickness of individual trabeculae, μm) and trabecular separation (the mean distance between trabeculae, μm) [15]. Twenty slices (0.32 mm) were evaluated in the tibial shaft 1.6 mm proximal to the tibiofibular junction. Direct cortical measurements included cross-sectional area (area of cortical bone and bone marrow, mm²), cortical area (mm²), marrow area (mm²), and cortical thickness (μm). One individual performed all μCT analyses. The coefficient of variation for repeat measurements (n = 5) in a single tibia is 1.1% for bone volume/tissue volume and 0.6%, 0.9%, and 1.1% for cross-sectional area, cortical area, and marrow area, respectively.

Statistical Analysis

Response variables BV/TV, cross-sectional area, marrow area, and cortical area, were modeled using separate one-way ANOVA analysis and comparisons were made using F tests and Tukey's honest significant difference method that maintained the joint level of confidence intervals (CIs) at 95%. Linear model assumptions were assessed using normal quantile plots of residuals, plots of residuals versus fitted values, Levene's test for homogeneity of variance, and the Anderson-Darling test of normality. In the genistein studies, one-way ANOVA was used to evaluate differences among treatment groups. If ANOVA distributional assumptions were not met, a Kruskal-Wallis test was applied. Statistical analysis was performed using SPSS 19.0 (SPSS Inc., Chicago, IL) and R version 2.12 [16]. Differences were considered significant at P<0.05. All data are expressed as mean ± SE.

Results

Effect of age and ovx on bone in virgin Long-Evans rats

The effect of age and ovx on BV/TV in proximal tibia metaphysis of virgin Long-Evans rats is shown in Figure 2. BV/TV increased between 2 and 3 months of age by an absolute value of 13.6% (95% CI: 6.3 – 20.8%; $p < 0.0001$), remained stable between 3 and 12 months of age (F test $p = 0.62$), and declined dramatically between 12 and 24 months of age by 19.7% (95% CI: 10.5 – 29.0%; $p < 0.0001$). Compared to age-matched sham controls, rats ovx at 2 months of age were severely osteopenic 1 and 2 months later when they were 3 and 4 months of age, respectively, with an age-adjusted estimated decrease in absolute BV/TV of 14.9% (95% CI: 11.9 – 17.9%; $p < 0.0001$). However, BV/TV at these time points was similar to 2-month-old ovary-intact animals (F test $p = 0.44$), indicating that ovx prevented the normal increase in BV/TV in the growing rats. In contrast, rats ovx at 4 months of age and sacrificed 8 months later (12 months of age) were osteopenic compared to baseline (ovary-intact 4-month-old) rats with decreased BV/TV of 20.7% (95% CI: 11.8 – 29.7%; $p = 0.0002$) as well as 12-month-old rats with decreased BV/TV of 19.3% (95% CI: 9.8 – 28.8%; $p = 0.0005$), indicating the ovx resulted in cancellous bone loss.

The effect of age and ovx on cortical bone in tibia diaphysis is shown in Figure 3. Cortical bone area increased between 2 and 7 months of age by 1.3 mm² (95% CI: 0.9 – 1.8 mm²; $p < 0.0001$) (Figure 3A). The growth-related increase in cortical bone area was due to increased cross-sectional area of 1.2 mm² (95% CI: 0.7–1.8 mm²; $p < 0.0001$) (Figure 3B) with no change in marrow area ($p = 0.82$) (Figure 3C). There was an increase in marrow area between 12 and 24 months of age of 0.50 mm² (95% CI: 0.16–0.84mm²; $p = 0.001$), but this had no effect on cortical area. Marrow area in rats ovx at 4 months of age and sacrificed at 12 months of age was larger than marrow area in sham-operated rats sacrificed at 4 or 12 months of age by an estimated 0.3 mm² (95% CI: 0.03 – 0.6 mm²; $p = 0.03$) and 0.3 mm² (95% CI: 0.04 – 0.6 mm²; $p = 0.03$), respectively. Otherwise, no differences in cortical endpoints were detected with ovx.

Effect of reproduction on bone in Long-Evans rats

BV/TV in proximal tibial metaphysis of 12-month-old breeder rats retired at 8 months of age was lower than BV/TV in 6-month-old virgin rats (Figure 4). However, it was higher than BV/TV in 12-month-old virgin rats ovx at 7 months of age.

Effect of oral genistein on bone in virgin and retired breeder Long-Evans rats

Serum genistein was sampled from a subset of animals in each treatment and age group and results are reported in detail elsewhere [12]. In brief, age had no independent effect on serum genistein levels and values from the 3 different age groups/treatment were combined ($n = 25/\text{treatment}$). Serum genistein measured 0.18 ± 0.10 μM in the control group, 0.76 ± 0.15 μM in the 485 μg genistein/day dose group and 1.48 ± 0.31 μM in the 970 μg genistein/day dose group.

Seven-month-old ovx virgin rats treated for 5 months (Table 1)

Significant differences among the 3 treatment groups were not detected for tibial BMC, bone area, or BMD. All rats exhibited severe cancellous osteopenia as indicated by the low BV/TV. However, significant differences were not detected among the 3 treatment groups for any cancellous endpoints measured: BV/TV, connectivity density, trabecular number, trabecular thickness, or trabecular spacing. Similarly, significant differences among the 3 treatment groups were not detected for any cortical endpoints evaluated: cross-sectional area, cortical area, marrow area, or cortical thickness.

Sixteen-month-old ovx retired breeder rats treated for 5 months (Table 2)

Significant differences among treatment groups were not detected for tibial BMC, bone area, or BMD. All rats exhibited severe cancellous osteopenia as indicated by their low BV/TV. However, significant differences among treatment groups were not detected for any cancellous endpoint measured: BV/TV, connectivity density, trabecular number, trabecular thickness, or trabecular spacing. Similarly, significant differences were not detected among treatment groups for any cortical endpoint measured: cross-sectional area, cortical area, marrow area, or cortical thickness.

Twenty two-month-old ovx retired breeder rats treated for 5 months (Table 3)

Significant differences among the 3 treatment groups were not detected for BMC, bone area, or BMD. Likewise, significant differences among the 3 treatment groups were not detected for any cancellous or cortical endpoints measured.

Discussion

The goal of this study was to determine the effect of oral genistein on bone in a well validated rat model for postmenopausal osteopenia. The effect of long-duration (administered daily for 5 months) dietary genistein was evaluated on cancellous and cortical bone mass and architecture in skeletally mature virgin Long-Evans rats ovx at 7 month of age and in aging retired breeder Long-Evans rats ovx at 16 or 22 months of age. Dietary genistein increased serum levels of the isoflavone to μM concentrations, values achievable with a high soy diet in women [17, 18]. However, the treatment had no effect on bone mass, density or architecture in either skeletally mature ovx virgin rats (a model for rapid bone loss following attainment of peak bone mass) or in aged ovx retired breeder rats (a model in which ovx exaggerates bone loss associated with aging).

Long-Evans rats are frequently employed in cognitive research whereas Sprague-Dawley and Fisher rats are more commonly used as preclinical models in osteoporosis research. The similarity of the pattern of bone gain, maintenance, and age-related and ovx-induced bone loss in Long-Evans rats (current study) compared to Sprague-Dawley and Fisher rats [19–21] supports the use of Long-Evans rats in cross disciplinary research. The time course changes support our choice of 7-month-old Long-Evans rats to investigate the efficacy of long-duration genistein treatment in preventing ovx-induced cancellous and endocortical bone loss. Based on our findings, cancellous and cortical bone mass remain stable between 7 and 12 months of age. As a consequence, age-related bone changes during the age range

studied would not confound interpretation of our results. In younger rats, ovx-induced osteopenia was largely due to prevention of net accrual of cancellous bone, whereas 2-year-old animals exhibited pronounced age-related cancellous and endocortical bone loss. The mechanism for the age-related bone loss between 12 and 24 months of age is uncertain but may be related to the transition from regular estrus cycles to continuous anestrus, a condition similar to menopause in women [22].

Pregnancy and lactation is reported to result in reversible cancellous osteopenia in Sprague-Dawley rats [23, 24], a finding confirmed in the present study for Long-Evans rats. Specifically, BV/TV was lower in 12-month-old retired breeder rats than in 6-month-old virgins. However, BV/TV of retired breeder rats sacrificed at 21 and 27 months of age was even lower than BV/TV in 12-month-old retired breeder rats, indicating further bone loss. Although the skeleton of aged ovx rats becomes severely osteopenic, it remains responsive to bone anabolic agents [25]. As a consequence, the putative bone anabolic effect of genistein would be expected to result in an increase in bone mass compared to age-matched controls, which was not observed in the current study.

The effects of dietary soy, soy isoflavone extracts, and purified genistein have been evaluated on bone marrow stromal cells, osteoblast-like cells, and cultured osteoclasts *in vitro*, and *in vivo* in animal models and humans. Anti-resorptive and bone anabolic effects of genistein have been reported [26–29]. There is excellent *in vitro* evidence that genistein is a weak ER ligand and has higher affinity for ER β than ER α [30]. However, genistein had only a slight preference for transactivation of gene expression through ER β compared with ER α [31] and thus may show minimal discrimination between the two receptor subtypes. Genistein significantly increased uterine wet weight in wildtype mice but not in the ER α knockout mice, providing direct evidence that genistein can act through ER α [32]. This may be important to the rodent skeleton because ER α mRNA is present at higher levels in skeletal tissue than ER β and ER β signaling, by antagonizing ER α signaling, may lead to bone loss [33].

The precise actions of genistein on estrogen target tissues *in vivo* appear to be highly context-dependent; genistein can act as an estrogen agonist, an antagonist or have no effect [34–38]. Clinical studies reporting positive effects of soy and isoflavones extracted from soy on BMD in aging humans suggest that the isoflavones, particularly genistein and its metabolites, may mimic the bone sparing actions of estrogen. However, relatively small numbers of participants were studied for relatively short intervals and other similarly powered studies report either minimal or no effect of treatment in preventing age-related bone loss [39–41]. A meta-analysis concluded that the results of these studies do not support an important effect of isoflavones on bone health [42]. Moreover, a more recent long-duration randomized placebo-controlled, double-blind clinical trial reported no beneficial effects of soy isoflavones on bone health in postmenopausal women. In this clinical trial, no difference in BMD was detected between subjects receiving either soy isoflavone tablets (200 mg/day) or placebo after 2 years of treatment [43, 44]. Thus, whereas there is overwhelming evidence supporting the efficacy of hormone replacement therapy in preventing osteoporotic fractures, neither a reduction in bone loss nor a reduction in fracture rate has been convincingly demonstrated with ingestion of soy isoflavones in

postmenopausal women. Indeed, the latter would be extremely difficult to accomplish because of the large numbers of women required and cost associated with performing a fracture study.

Animal studies have played a critical role in preclinical evaluation of pharmaceuticals developed for the treatment of osteoporosis [11]. However, there is no consensus regarding the efficacy of genistein in preventing bone loss in animal models [45–50]. Depending upon experimental design, model system employed, mode of delivery, dose of genistein, and skeletal endpoint(s) measured, genistein has been reported to have a wide range of effects on the skeleton and reproductive tissues. The majority of studies investigating genistein's actions in animals were not designed to model the actions of either a once a day supplement or an isoflavone-rich diet on postmenopausal bone loss. Many animal studies reporting a positive skeletal effect of genistein used high doses of the compound, administered by injection (bypassing the low intestinal absorption efficiency of the phytoestrogen), used rapidly growing ovx animals (which does not mimic postmenopausal bone loss), focused on skeletal endpoints such as BMD (which do not detect cancellous bone loss), and/or used mice (a species that does not respond to exogenous estrogens in a manner similar to women). Thus, there is an important need to model the effects of soy, soy extracts and purified isoflavones using well validated preclinical animal models.

In the present study, genistein was administered orally to model once daily dietary intake on postmenopausal bone mass and architecture. Oral delivery is important because the efficacy and precise actions of an estrogen receptor ligand depend upon bioavailability, concentration, affinity for the receptor and ability of the ligand receptor complex to attract transcriptional co-activators and co-inhibitors. Therefore, the delivery method of weak estrogens can drastically influence their actions on target tissues. For example, 16 α -hydroxyestrone, an important but weak endogenous estrogen in postmenopausal women, is a complete estrogen agonist on bone and reproductive tissues if administered once daily by subcutaneous injection. In contrast, infusion of 16 α -hydroxyestrone to model continuous endogenous production of the metabolite results in preferential actions of the estrogen on bone over reproductive tissues [51, 52].

Differential tissue effects of estrogen ligands are hypothesized to result from tissue-specific metabolism of the ligand. The significance of tissue level metabolism of steroid receptor ligands is beautifully illustrated by tibolone, a prodrug which, depending upon tissue, can be metabolized to products that bind to estrogen, androgen or progesterone receptors [53, 54]. Thus, the non-physiological methods of administering isoflavones commonly used in phytoestrogen research may not accurately model the effects of dietary consumption on estrogen target tissues. Therefore, in the present study we used the oral route of administration to deliver genistein at levels that could be achieved in the diet.

Prior studies often employed densitometry as the primary method for evaluation of the skeletal response to phytoestrogens. Furthermore, BMD was often the only densitometry endpoint reported. Densitometry unfortunately lacks sufficient sensitivity to dissociate changes in the highly responsive cancellous bone compartment from the much larger but less responsive cortical bone compartment. As clearly illustrated in the present study, bone

loss in ovx rats is largely confined to the cancellous compartment, and cancellous bone loss is especially important to the etiology of vertebral and hip fractures. Gonadal hormones, by acting differentially on the periosteal and endocortical bone envelopes, alter bone size as well as BMC [55], the two variables used to calculate BMD. Also, in growing animals, estrogens slow longitudinal bone growth by inhibiting chondrocyte proliferation and hypertrophy but they increase the cancellous bone volume fraction by suppressing resorption of primary spongiosa [56]. As a consequence, without supporting documentation (BMC and bone size), the physical significance of BMD is not easily interpretable. In the present study, DXA was used to evaluate bone mass (BMC) and areal density (BMD). However, we also used high resolution μ CT to evaluate cortical and cancellous bone architecture.

The mouse is being utilized with increasing frequency for investigating the actions of phytoestrogens, including genistein, on bone metabolism. The skeletal response of the mouse to estrogen is a sensitive bioassay for exogenous estrogens but, as reviewed previously [11], mice are a poor preclinical model for evaluating the differential actions of estrogenic compounds on bone and reproductive tissues. Two cases in point are that the mouse failed to predict the tissue-selective actions of the breast cancer drug tamoxifen and its metabolites on bone and reproductive tissues, or the inhibitory effects of estrogen on bone turnover universally observed in humans [57, 58]. Estrogens administered to mice neither replicate the skeletal actions of the hormone administered to humans nor the physiological actions of endogenous estrogen production in mice [59]. In mice, even weak exogenous estrogens are capable of inducing severe bone marrow failure and osteosclerosis [60]. Thus, the many studies demonstrating an increase in BMD in mice in response to phytoestrogens may in fact be detecting an exclusively mouse-specific skeletal pathology [61, 62].

We have reviewed the strengths and limitations of the ovx rat as a preclinical model for postmenopausal bone loss [11, 63]. Briefly, the rat has been valuable in accurately predicting the beneficial effects of the major classes of pharmaceuticals (e.g., estrogens, selective estrogen receptor modulators, bisphosphonates and parathyroid hormone) approved for prevention and/or treatment of osteoporosis. The rat has been particularly useful for modeling cancellous and endocortical bone loss. Because of the absence of Haversian remodeling in small rodents, the rat is less useful for evaluating the effects of these agents on intracortical bone remodeling.

The present studies focused on the efficacy of purified genistein in preventing ovx-induced cancellous and endocortical bone loss in tibia. It is possible, however, that other components of soy influence the bioactivity of genistein. Although the tibia is representative of the skeletal response to ovx and hormone replacement therapy in adult rats [64, 65], it is also possible that genistein exhibits greater effects at other skeletal sites. The present studies evaluated the efficacy of genistein in preventing bone loss following ovx; the studies provide no insight as to possible effects of genistein on bone mass and architecture if administered to ovary-intact rats. Finally, the present studies focused on blood levels of genistein that could be achieved via a once daily supplement similar to levels obtainable via a soy-rich diet. The studies do not address the possible skeletal effects of higher levels of genistein.

Conclusion

In summary, the results of the 3 studies described here support neither a detrimental nor a protective effect of dietary genistein on bone mass, density and architecture in well validated preclinical models for postmenopausal bone loss. We cannot rule out the possibility that levels of genistein that exceed reasonable dietary intake may impact the skeleton. Herbivores and omnivores may have developed resistance to the endocrine disrupting effects of genistein, first by limiting its intestinal absorption and second by inducing its rapid excretion [66]. Although pharmacological administration of the isoflavone appears to be a strategy to bypass these defenses, based on the potential for serious detrimental side effects [67–69], it is not clear whether this is a desirable approach.

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Figure 1.

Experimental protocol: 3-month old virgin and 12- and 14-month old retired breeder rats were purchased and housed for 4 or 8 months prior to ovx at 7 months, 16 months, or 22 months of age, respectively. The rats within each age group were then randomly assigned to one of 3 groups by using the same allocation probability for all groups; 1) vehicle control, 2) 485 µg/day genistein, or 3) 970 µg/day genistein and treated daily for 5 months. Following necropsy, tibiae were evaluated for bone mass and architecture.

Figure 2.

Effect of age (dashed line) and ovariectomy (dotted lines) on cancellous bone volume/tissue volume (BV/TV) in the proximal tibia metaphysis determined by histomorphometry. Groups ($n = 8 - 12/\text{group}$) of untreated rats were sacrificed at 2, 7, or 24 months of age. Additional rats ($n = 5 - 9/\text{group}$) underwent ovx or sham surgery at 2 months of age and were sacrificed at 3 or 4 months of age or underwent ovx or sham surgery at 4 months of age and were sacrificed at 12 months of age. The lines approximate a best fit of the relevant data points. The lines between groups are meant to draw the eye to the different groups and to indicate some of the groups that were compared statistically. They are not meant to be used for statistical inference about the functional form between age and the response variables at unmeasured ages. Values are mean $\pm$ SE. ^aDifferent from aged-matched sham control, $P < 0.05$; ^bDifferent from baseline control, $P < 0.05$.

Figure 3.

Effect of age (dashed line) and ovariectomy (dotted lines) on cortical area (A), cross-sectional area (B), and marrow area (C) in the distal tibia diaphysis proximal to the tibia-fibular junction determined by histomorphometry. Groups ($n = 8 - 12$ /group) of untreated rats were sacrificed at 2, 7, or 24 month of age. Additional rats ($n = 5 - 9$ /group) underwent ovx or sham surgery at 2 months of age and were sacrificed at 3 or 4 months of age or underwent ovx and sham surgery at 4 months of age and were sacrificed at 12 months of age. As in Figure 1, the lines approximate a best fit of the relevant data points. The lines are

not meant to be used for statistical inference about the functional form between age and the response variables at unmeasured ages. Values are mean $\pm$ SE. ^aDifferent from aged-matched sham control, $P < 0.05$; ^bDifferent from baseline control, $P < 0.05$.

Figure 4.

Effects of reproductive status on bone volume/tissue volume (BV/TV) in the proximal tibia metaphysis determined by μ CT. Representative μ CT images are shown for 6-month-old virgin (A), 12-month-old retired breeder (B), 12-month-old virgin ovariectomized at 7 months of age (C) ($n = 7 - 10/\text{group}$). 12-month-old retired breeder rats were osteopenic compared to 6-month-old virgin rats, did not differ from 12-month-old ovary intact virgin rats, but had a higher BV/TV than 12-month-old virgin rats ovariectomized at 7 months of age (D). Values are mean $\pm$ SE. ^aDifferent from 6-month-old (mo) virgin, $P < 0.05$; ^bDifferent from 12-month-old (mo) virgin, $P < 0.05$; ^cDifferent from 12-month-old retired breeder, $p < 0.05$.

Table 1

Effects of genistein on BMC and BMD in total tibia and on cancellous bone in the proximal tibial metaphysis and cortical bone in the tibial diaphysis in ovariectomized virgin Long-Evans rats. The rats were ovariectomized at 7 months of age and treated with one of 2 doses of genistein for 5 months (until 12 months of age).

Endpoint	Ovariectomized Treatment Groups			ANOVA (P)
	Control (n = 7)	485 µg/d Genistein (n = 10)	970 µg/d Genistein (n = 10)	
Total tibia				
Area (cm ²)	1.80 ± 0.02	2.01 ± 0.08	1.83 ± 0.03	0.251
BMC (g)	0.259 ± 0.004	0.274 ± 0.008	0.259 ± 0.009	0.324
BMD (g/cm ²)	0.144 ± 0.003	0.138 ± 0.004	0.141 ± 0.004	0.483
Proximal tibial metaphysis (cancellous bone)				
Bone volume/Tissue volume (%)	2.9 ± 0.8	3.7 ± 0.7	5.0 ± 1.2	0.341
Connectivity density (1/mm ³)	3.2 ± 1.2	5.5 ± 1.8	8.3 ± 2.9	0.318
Trabecular number (1/mm)	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.2	0.964
Trabecular thickness (µm)	73 ± 4	77 ± 3	77 ± 2	0.600
Trabecular spacing (µm)	899 ± 90	973 ± 111	910 ± 89	0.852
Tibial diaphysis (cortical bone)				
Cross-sectional area (mm ²)	5.5 ± 0.1	5.5 ± 0.1	5.3 ± 0.2	0.651
Cortical area (mm ²)	4.2 ± 0.0	4.3 ± 0.1	4.2 ± 0.1	0.674
Marrow area (mm ²)	1.3 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	0.412
Cortical thickness (µm)	641 ± 11	667 ± 6	666 ± 12	0.181

Data are mean ± SE

Table 2

Effects of genistein on BMC and BMD in total tibia and on cancellous bone in the proximal tibial metaphysis and cortical bone in the tibial diaphysis in ovariectomized retired-breeder Long-Evans rats. The rats were ovariectomized at 16 months of age and treated with one of 2 doses of genistein for 5 months (until 21 months of age).

	Ovariectomized Treatment Groups			
Endpoint	Control (n = 10)	485 µg/d Genistein (n = 8)	970 µg/d Genistein (n = 10)	ANOVA (P)
Total tibia				
Area (cm ²)	1.94 ± 0.02	1.95 ± 0.04	1.94 ± 0.03	0.970
BMC (g)	0.284 ± 0.007	0.286 ± 0.009	0.289 ± 0.005	0.868
BMD (g/cm ²)	0.146 ± 0.003	0.146 ± 0.002	0.149 ± 0.003	0.694
Proximal tibial metaphysis (cancellous bone)				
Bone volume/Tissue volume (%)	6.3 ± 1.5	5.9 ± 1.4	6.2 ± 1.1	0.982
Connectivity density (1/mm ³)	5.9 ± 1.3	7.1 ± 3.0	7.1 ± 2.1	0.900
Trabecular number (1/mm)	1.1 ± 0.1	1.3 ± 0.2	1.2 ± 0.2	0.750
Trabecular thickness (µm)	81 ± 3	82 ± 4	83 ± 3	0.934
Trabecular spacing (µm)	1007 ± 120	908 ± 136	983 ± 93	0.830
Tibial diaphysis (cortical bone)				
Cross-sectional area (mm ²)	6.1 ± 0.1	6.0 ± 0.2	5.8 ± 0.2	0.403
Cortical area (mm ²)	4.7 ± 0.1	4.4 ± 0.1	4.4 ± 0.1	0.133
Marrow area (mm ²)	1.5 ± 0.1	1.7 ± 0.2	1.4 ± 0.1	0.328
Cortical thickness (µm)	677 ± 16	638 ± 9	654 ± 17	0.180

Data are mean ± SE

Table 3

Effects of genistein on BMC and BMD in total tibia and on cancellous bone in the proximal tibial metaphysis and cortical bone in the tibial diaphysis in ovariectomized retired-breeder Long-Evans rats. The rats were ovariectomized at 22 months of age and treated with one of 2 doses of genistein for 5 months (until 27 months of age).

Endpoint	Ovariectomized Treatment Groups			ANOVA (P)
	Control (n = 11)	485 µg/d Genistein (n = 12)	970 µg/d Genistein (n = 11)	
Total tibia				
Area (cm ²)	2.08 ± 0.05	2.10 ± 0.07	2.21 ± 0.08	0.385
BMC (g)	0.281 ± 0.005	0.295 ± 0.009	0.295 ± 0.009	0.410
BMD (g/cm ²)	0.136 ± 0.004	0.141 ± 0.004	0.134 ± 0.003	0.435
Proximal tibial metaphysis (cancellous bone)				
Bone volume/Tissue volume (%)	4.8 ± 1	5.2 ± 0.9	3.6 ± 0.7	0.390
Connectivity density (1/mm ³)	4.1 ± 0.9	4.7 ± 1.1	3.6 ± 1.0	0.728
Trabecular number (1/mm)	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	0.360
Trabecular thickness (µm)	85 ± 4	83 ± 3	80 ± 3	0.589
Trabecular spacing (µm)	1135 ± 85	964 ± 65	1061 ± 75	0.279
Tibial diaphysis (cortical bone)				
Cross-sectional area (mm ²)	5.7 ± 0.2	5.7 ± 0.2	5.9 ± 0.1	0.687
Cortical area (mm ²)	4.3 ± 0.2	4.4 ± 0.1	4.3 ± 0.1	0.813
Marrow area (mm ²)	1.4 ± 0.1	1.3 ± 0.1	1.5 ± 0.1	0.085
Cortical thickness (µm)	607 ± 26	604 ± 17	571 ± 23	0.447

Data are mean ± SE