Plum and Soy Aglycon Extracts Superior at Increasing Bone Calcium Retention in Ovariectomized Sprague Dawley Rats

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ABSTRACT: Plant-derived polyphenols have been shown to influence bone turnover and bone properties in the estrogen-depleted state. We used a crossover design in ovariectomized rats (n = 16 rats for each diet) to investigate the effect of supplementation of two doses each of blueberry, plum, grape, grape seed extract, and resveratrol on bone. We tested the aglycon and glucoside forms of genistein to quantify differences in efficacy on bone calcium retention. Rats were given an intravenous dose of 45Ca to prelabel bone, and bone calcium retention was assessed by urinary excretion of 45Ca:Ca ratio during an intervention period compared with nonintervention. Genistein aglycon increased bone calcium retention significantly (p < 0.05) more than the glucoside (22% vs 13%, respectively). Plum extract (0.45% w/w total dietary polyphenols) and resveratrol (0.2% w/w total dietary polyphenols) were also effective, increasing bone calcium retention by 20% (p = 0.0153) and 14% (p = 0.0012), respectively. Several polyphenol-rich diets improved bone calcium retention.

KEYWORDS: genistein aglycon, genistein glucoside, bone calcium retention, bone resorption, plant-derived polyphenols

INTRODUCTION

Various plant sources of polyphenolic compounds have been studied for their ability to prevent postmenopausal bone loss associated with estrogen deficiency. The link between soy consumption in the Asian diet and reduced fracture risk† has sparked many studies examining soy isoflavones and postmenopausal bone health. More recently, plum, blueberry, and grape products have also been implicated in preventing postmenopausal bone loss due to their polyphenolic components.7–6

In Western countries, soy is not consumed as often or in sufficient quantities to match the soy consumed in Asia, where beneficial effects to bone have been observed. In addition, soy in Western countries is typically consumed as an ingredient or in supplemental form that contains extracted isoflavones from soy rather than in whole soy foods.7 The ability of soy isoflavones to attenuate bone resorption in an estrogen deficient state remains controversial.8 Discrepancies have been attributed to a number of factors including an individual’s ability to convert daidzein to equol, the effect of food matrix on bioavailability of isoflavones compared with extracted isoflavones, and specific isoflavone composition. In foods, isoflavones exist as glycosides and require cleavage of the sugar moiety in the gut to be absorbed in the intestine.9 Although there have been contrary reports of bioavailability of glycoside compared with aglycone forms of genistein,10,11 there is interest in comparing glycoside to aglycon forms for bone health as the aglycon form was used in various studies that have demonstrated a strong bone preserving effect in postmenopausal women.12–15 The presence of the sugar moiety may have an influence on where the isoflavone is absorbed in the gut and how the isoflavone is subsequently conjugated inside the body.

Isoflavones and other phenolics have demonstrated estrogenic activity16,17 and are being investigated for their biological effect on the estrogen-depleted state. Depletion of estrogen results in a marked increase in osteoclastogenesis, causing bone resorption. During this postmenopausal period, bone formation lags behind resorption, and net bone loss ensues.18

There is emerging evidence to suggest that certain fruits, vegetables, and other dietary compounds found in the Western diet may be protective to bone, especially from prune, grape products, and blueberries.19–21 Grape seed extract has also shown promise in maintaining bone health as supplementation with grape seed extract was able to recover bone lost while on a calcium deficient diet in rats.22 Dried plum is one of the most extensively studied botanicals for its role in bone health because of a potentially anabolic effect in bone. Dried plum was very effective in protecting bone in ovariectomized (OVX) rats when compared with the effect of intermittent teriparatide (parathyroid hormone 1–34) administration, which is the most effective anabolic drug therapy currently available for osteoporosis treatment.3 Plum, as a daily supplement of 100 g/d dried plum for one year, was able to increase bone mineral density (BMD) in the spine and ulna of postmenopausal women compared with those who were supplemented with an equal amount of dried apple.23 While these extracts appear to be diverse, they are rich sources of specific polyphenol forms...
including quercetin, anthocyanins, proanthocyanidins, hydroxycinnamates, and resveratrol, which can be classified as possible bioactive agents responsible for observed bone health benefits associated with these foods. For example, resveratrol, found in grape products, extracts, and supplements, increased bone formation markers and decreased expression of receptor activator of nuclear factor kappa-B (RANK) in human primary monocytes, showing potential to alter osteoclastogenesis.

Although the bioactive foods and ingredients described above have been shown to benefit bone by traditional measures of bone density and material properties, these approaches require chronic feeding and terminal measures. This has largely prevented comparisons among multiple diets and dose response effects. The aims of this study were to directly compare botanical sources and to study the effective dose of extracts to increase bone calcium retention in OVX rats using a novel technology that does not require sacrifice and evaluates responses quickly so that multiple comparisons can be efficiently tested. This involves prelabeling bones with $^{45}$Ca as we have done previously with $^{41}$Ca in human studies as a means of screening botanical extracts for their ability to improve bone calcium retention. By injecting rats with $^{45}$Ca, allowing the isotope to incorporate into bone, and quantifying the amount of $^{45}$Ca in urine, a direct measure of net calcium tracer lost from bone is obtained. The long half-life of $^{45}$Ca (163 days) allows multiple diets to be compared using a crossover design in the same rat, providing greater power to detect differences among diets. This method allows for screening of numerous diets on a relatively small number of rats during a short time period.

Figure 1. Study design. Forty-four rats were designated into one of three study arms. Rats in arm 1 ($n=16$) received blueberry and plum fruit powder and grape seed extract to compare the effect of polyphenols in the whole fruit. Rats in arm 2 ($n=16$) received whole fruit polyphenols from grape to compare against the isolated resveratrol polyphenols. Rats in arm 2 were also given genistein in the aglycon form and glycosylated soy to determine the effect of conjugation. Rats in arm 3 ($n=12$) were not given a dietary botanical to establish an unperturbed excretion of $^{45}$Ca excretion.

The method for determining bone calcium retention was derived from a well-established protocol using another bone seeking tracer, $^3$H-tetracycline, and adapted by us for calcium tracers in rats and humans. $^3$H-tetracycline was the tracer of choice initially because it was assumed that it assessed only bone resorption from prelabeled bones in rats because it attaches tightly to hydroxyapatite and was slow to release. However, we showed through kinetic modeling of both $^3$H-tetracycline and calcium tracers that both isotopes leave bone during bone resorption and can re-enter bone during bone formation. Our findings suggest that these two tracers give comparable values and that net bone calcium retention is a more accurate description of what is being measured with bone seeking tracers. Mühlbauer and Fleish evolved the method to use daily measures of urinary $^3$H-tetracycline from prelabeled bone of young rats given multiple injections of the tracer in order to continuously monitor bone resorption through 10-day diet periods and 10-day washout periods using a crossover design. We established that older OVX rats and a single isotope administration could be used in a study that evaluated effects of time of stabilization to OVX and time from dose on tracer behavior. We further validated urinary tracer excretion against bone disappearance of the tracer and adequacy of 10-day diet and washout periods. Quantifying the urinary tracer appearance of a bone label enables rapid screening and multiple comparisons of efficacy of diets designed to improve bone calcium retention.

We hypothesized that both source and dose effects would be observed through testing high and low doses of various extracts from grape seed, grape, and blueberry, as well as plum powder,
and resveratrol. We also used this screening method to determine whether genistein in an aglycon form is more effective at increasing bone calcium retention than its glucoside counterpart.

**MATERIALS AND METHODS**

**Chemicals.** A retrospective analysis of phenolic compounds from grape, blueberry, and plum extract was conducted after the conclusion of the study. Extracts were stored at −80 °C, reconstituted in 2% formic acid in ddH2O at ~1 mg/mL and analyzed for key phenolic components by LC−MS as described by Song et al.37

**Rats.** Forty-four OVX Sprague Dawley rats (3 month old) were shipped from Harlan (Indianapolis, IN) and given approximately 30 days to acclimate to their environment, and to stabilize hormone changes post-OVX, while being fed AIN93-M polyphenol-free diet (soybean oil was replaced with corn oil) and distilled water ad libitum. The rats were housed individually in a humidity- and temperature-controlled room with a 12 h light and 12 h dark cycle. Food intake was monitored twice weekly, and body weight was recorded weekly. All procedures performed on rats were approved by and in compliance with the standards set by Purdue University’s Animal Care and Use Committee.

**Study Design.** The study design (Figure 1) was a randomized, crossover intervention trial to evaluate 12 different polyphenolic-containing diets on bone turnover. The rats were randomized into one of three groups: (1) 16 rats received six diets of plum, blueberry, and grape seed extract at two doses each, and Novasoy (glycosylated soy) and Fosteum (genistein aglycon) at a single dose, and (3) 12 rats received no treatment to establish unperturbed 45Ca excretion over 61 days to acclimate to their environment, and to stabilize hormone changes post-OVX, while being fed AIN93-M polyphenol-free diet38 (Table 1) was used as a basal diet approximately equal macronutrient, vitamin, and mineral content. The modified AIN93-M diet replaced with corn oil to prevent an excess of CO2.

**Diet.** All diets were formulated to be isocaloric and to have approximately equal macronutrient, vitamin, and mineral content. The AIN93-M polyphenol-free diet38 (Table 1) was used as a basal diet with the replacement of soybean oil with corn oil to prevent confounding from residual isoflavones. A higher amount of corn starch than in the original AIN93-M diet was used to facilitate the incorporation of up to 25% fruit powder, while maintaining the ability to pellet the diets. Research Diets, Inc. (New Brunswick, NJ), 16 rats with 10 days of 24 h urine samples for each rat. The intervention and washout collections were repeated for 6 total intervention/washout cycles, and the total duration of the study was 189 days. Throughout the baseline, intervention, and washout, urine was collected for 24 h for 10 days during the intervention and 6 days during the washout. Urine was analyzed as a 24 h sample and was not pooled over multiple days. Food intake and body weight were monitored every 5 days. Blood was taken at the end of each diet and washout via the saphenous vein, and serum was stored at −80 °C to assay for bone-specific alkaline phosphatase. The urine collected at the end of each intervention and washout period was stabilized with 1% ascorbic acid at a 4:1 ratio immediately after the 24 h collection period, stored at −80 °C, and used to assay for total polyphenols and cross-linked N-telopeptides of type I collagen (NTx). Rats were killed using an excess of CO2.

**Study timeline for arrival of ovariectomized (OVX) rats, dosing with 45Ca, and urine and blood collection pattern.**

![Figure 2](dx.doi.org/10.1021/jf403310q)
dry blended the extracts and fruit powders into the basal diet prior to pelleting the diets. Each extract or fruit powder was added to the diet to create two doses of varying polyphenolic content and stored at −20 °C. The diets were formulated based on the goal of delivering 0.2% and 1% w/w total polyphenols for each extract where possible (Table 2). The total polyphenol content in the extracts was determined using the Folin–Ciocalteu method, however, levels of resveratrol and soy isoflavones were added based on the manufacturers’ specified levels of resveratrol and total isoflavones, respectively.

We could achieve this goal with grape seed extract (Sensient, Indianapolis, IN) and grape skin extract (Sensient, Indianapolis, IN), which were both formulated to deliver 0.2% and 1% w/w total dietary polyphenols in the low and high doses, respectively. The blueberry and plum powder had lower phenolic content; therefore, these were added to the diet at levels that have been shown to be effective in bone. Additional 2960 IU of vitamin D/kg diet to match Fosteum (genistein aglycon).

| Botanical and Total Polyphenol Composition in Modified AIN93-M Diet | % w/w diet |
|---------------------------------------------------------------|------------|
| **Botanical (Total polyphenols in extract)**                  | **Product** | **Total polyphenols** |
| grape seed extract (82.7)                                     | high       | 1.2                     |
|                                                               | low        | 0.25                    |
| plum** (2.22)                                                 | high       | 0.45                    |
|                                                               | low        | 0.9                     |
| blueberry** (1.65)                                            | high       | 0.4                     |
|                                                               | low        | 0.15                    |
| grape (32.8)                                                   | high       | 0.9                     |
|                                                               | low        | 0.2                     |
| resveratrol (99.9)                                            | high       | 0.2                     |
|                                                               | low        | 0.1                     |
| genistein aglycon (6.67)                                      | aglycon    | 0.04                    |
| glycosylated soy** (4.00)                                     | glucoside  | 0.4                     |
|                                                               |            | 0.04                    |

Corn starch and cellulose in the AIN93-M diet were replaced by carbohydrates and fiber from plum powder. Corn starch in the modified AIN93-M diet was replaced with maltodextrin from blueberry powder. Additional 2960 IU of vitamin D/kg diet to match Fosteum (genistein aglycon).

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The blueberry powder (Sensient, Indianapolis, IN) was added at levels that have been shown to be effective in bone. The blueberry and plum powders were balanced by removing corn starch from the diet formula. Additionally, cellulose was removed to balance the added fiber in the plum powder. Resveratrol and isoflavones were added at lower levels than the fruit powders and extracts because they are isolated components rather than a complex botanical mixture. Resveratrol (Chromadex, Inc., Irvine, CA) was added at 0.1% and 0.2% w/w total dietary polyphenols. Novosoy (Archer Daniels Midland Company, Decatur, IL), which contains genistein glycoside plus other isoflavones, and Fosteum (Primus Pharmaceuticals, Inc., Scottsdale, AZ), which is genistein aglycon, were added to the diet to deliver an equivalent amount of total isoflavone content (0.04% w/w total dietary polyphenols) and to be consistent with the range of doses published in similar studies. The diets delivered 5000 mg/kg diet calcium and 5000 mg/kg diet total phosphorus. Vitamin D was added to the Novosoy (glycosylated soy) diet at 2960 IU/kg diet to match the vitamin D present in the Fosteum supplement.

The data for food intake, weight change, feeding efficiency, and calcium retention during the botanical diet period, and the mean of data from multiple periods (baseline and washout data points) creating a regression line as described by Medina-Romón et al. The Folin–Ciocalteu reagent (pH = 3.0). Strata-X polymeric reversed phase extraction tubes (Phenomenex Inc., Torrance, CA) were used for solid phase extraction. The tubes were initially washed with 3 mL of methanol, followed by 6 mL of phosphate buffer. The entire urine sample was applied to the column and washed with 6 mL of phosphate buffer, followed by 6 mL of 5% methanol. The samples were eluted with 6 mL of methanol, dried, and resolubilized with ethanol. Samples were then pipetted in triplicate onto a 96-well plate, along with gallic acid (Sigma-Aldrich, St. Louis, MO) in ethanol to create a standard curve, and an ethanol blank. The Folin–Ciocalteu reagent (2 N, Sigma-Aldrich, St. Louis, MO) was diluted to 0.2 N and added to the plate, and then samples were incubated for 7 min at room temperature. An equal amount of 7% sodium bicarbonate solution was added to the reaction, and the plate was incubated for 30 min prior to being analyzed by a spectrophotometer at 750 nm. Total polyphenols of the urine samples were calculated from the gallic acid standard curve and were reported as gallic acid equivalents.

**Statistical Analysis.** The 4Ca:Ca ratio was transformed using the natural logarithm to correct for skewness. For each rat, a simple linear regression model was fit through all of the nonintervention periods (baseline and washout data points) creating a regression line as illustrated in Figure 3A and Figure 3B. During the intervention period, a predicted value from the regression line for each observation of each rat was determined using the model described. The predicted value for each observation was subtracted from the experimental value measured during the botanical diet period, and the mean of differences was taken for that botanical diet of that rat. The means from each dietary intervention were averaged across all rats, and 95% confidence intervals were calculated. The values were exponentiated and reported in the original scale as percent improvement in calcium retention compared with baseline.

The data for food intake, weight change, feeding efficiency, ratio, body weight, and total polyphenols were normal and were analyzed without transformation. Means and standard deviations were calculated for these variables, a repeated measures analysis of variance...
RESULTS

Bone Calcium Retention. Percent change in bone calcium retention was determined by finding the difference between the $^{45}$Ca:Ca ratio excreted during the botanical diet compared with the residual line of all control diet periods. The urinary $^{45}$Ca:Ca ratio of the control rats in arm 3 (Figure 1) verified that the regression line was not altered by diets in similarly aging rats (Figure 3A). The ratio of percent dose of $^{45}$Ca to milligrams of Ca in the 24 h urine samples and residuals for two diets for one rat in arm 1 is shown in Figure 3B. Using this method, bone calcium retention was significantly improved due to dietary intervention with glycosylated soy (13%; $p = 0.0166$), genistein aglycon (22%; $p = 0.0003$), resveratrol-high (14%; $p = 0.0012$), and plum-high (20%; $p = 0.0153$) compared with baseline (Figures 4A and 4B). The genistein aglycon supplement, Fosteum, increased bone calcium retention 2-fold compared to the corresponding low dose of the same dietary botanical as determined by ANOVA was performed, and differences were determined by contrasts between high and low doses within each extract, and between genistein aglycon and glycosylated soy. $t$ tests were performed for food intake, body weight, weight change, and feeding efficiency ratio to determine differences between control and botanical diets with a Bonferroni correction to adjust for multiple comparisons of means.

For each rat, NTx values were transformed to correct for skewness using the natural logarithm. Subsequently, the difference in NTx between intervention and washout was determined for each botanical diet as change in NTx. The means and 95% confidence intervals for the difference in NTx between intervention and washout was determined for each diet. A repeated measures ANOVA was performed followed by contrasts to determine differences between high and low doses of each diet, and between genistein aglycon and glycosylated soy. The means for each diet were transformed back to the original scale and reported as a ratio with 95% confidence intervals for (A) arm 1 and (B) arm 2. A value of “0” represents no change from the regression line computed from nonintervention periods. A confidence interval that excludes “0” indicates a significant change from the regression line at $\alpha = 0.05$. The * indicates significant difference ($p < 0.05$) between high and corresponding low dose of the same dietary botanical as determined by a $t$ test. For soy, the * indicates significant difference ($p < 0.05$) between genistein aglycon and glycosylated soy.

Food Intake and Body Weight. There was no significant difference in body weight between high and low doses of any botanical diet intervention despite some differences in the contributing factors (Table 3). In arm 1, plum-low increased the food intake, feeding efficiency ratio, and weight change compared with the plum-high diet ($p < 0.0001$). Blueberry-low increased the feeding efficiency ratio and weight change compared with the blueberry-high diet ($p = 0.05$). In arm 2, grape-low and resveratrol-high decreased the feeding efficiency ratio compared with control ($p < 0.05$), and glycosylated soy, grape-low, and resveratrol-high diets decreased the weight change of rats compared to those on the control diet ($p < 0.05$). There was a weak, but significant, positive correlation between change in body weight and net bone calcium retention ($r = 0.21; p = 0.002$); however, change in body weight was not correlated with NTx ($p = 0.69$).

Analysis of Extracts. Analysis of key phenolic compounds in grape, blueberry, and plum extracts highlighted some compositional differences between the different extracts (Table 4). The most prominent constituents in the plum...
Standard Deviation ± respectively, between low and corresponding high groups of the same extract diet as determined by contrasts after ANOVA. The assay for key compounds.

Grape seed extract was unavailable for analysis using LC-MS. Other stilbene derivatives (9.16 mg/g extract) have other stilbene derivatives (9.16 mg/g extract) compared with plum (0.20% and 0.45% total polyphenols, respectively) and plum (0.02% and 0.45% total polyphenols, respectively) as determined by contrasts after ANOVA. The † denotes significant difference from control with p < 0.05 as determined by t tests performed using a Bonferroni correction to adjust for multiple comparisons.

The aglycon soy diet significantly increased serum bone alkaline phosphatase compared with baseline (p < 0.0001). Blueberry, plum, soy, grape seed extract, grape-low, and resveratrol-high diets did not produce a significant change in NTx.

The aglycon soy diet significantly increased serum bone alkaline phosphatase compared with baseline (p = 0.03, data not shown); however, none of the other diets impacted bone alkaline phosphatase.

Urine Total Polyphenols. There was a significant dose response of urinary total polyphenols in blueberry (p = 0.005) and grape seed extract (p < 0.0001) interventions of arm 1 and grape (p = 0.0002) dietary intervention of arm 2 rats (Figure 6A and Figure 6B). There was no difference in total polyphenols between glycosylated soy and genistein aglycon (p = 0.27).

Table 3. Food Intake, Body Weight, Weight Change, and Feeding Efficiency Ratioa for Ovariectomized Rats Expressed As Mean ± Standard Deviation

| variable | feeding efficiency ratio | food intake, g/day | body wt, g | wt change, g/day |
|----------|--------------------------|--------------------|------------|-----------------|
| arm 1 rats |                          |                    |            |                 |
| blueberry-low | 0.07 ± 0.05*            | 15.7 ± 1.3        | 354.2 ± 26.1 | 1.06 ± 0.70*    |
| blueberry-high | 0.03 ± 0.03             | 14.9 ± 1.44       | 351.4 ± 29.2 | 0.45 ± 0.56     |
| plum-low | 0.06 ± 0.05**           | 15.3 ± 2.2**      | 352.9 ± 22.1 | 0.92 ± 0.83**   |
| plum-high | −0.02 ± 0.07            | 12.7 ± 2.3        | 346.1 ± 21.6 | −0.20 ± 0.80    |
| grape seed extract-low | 0.03 ± 0.04         | 14.5 ± 1.0        | 351.1 ± 24.2 | 0.48 ± 0.64     |
| grape seed extract-high | 0.05 ± 0.03         | 15.1 ± 1.3        | 352.7 ± 25.0 | 0.78 ± 0.57     |
| arm 2 rats |                          |                    |            |                 |
| glycosylated soy | 0.03 ± 0.03            | 14.9 ± 1.1        | 377.0 ± 24.7 | 0.50 ± 0.40**   |
| genistein aglycon | 0.05 ± 0.04           | 15.0 ± 1.5        | 376.1 ± 28.2 | 0.74 ± 0.57     |
| grape-low | 0.06 ± 0.02†           | 16.0 ± 1.5        | 362.4 ± 22.5 | 1.01 ± 0.43†    |
| grape-high | 0.07 ± 0.03             | 17.0 ± 1.5        | 363.6 ± 20.3 | 1.14 ± 0.63     |
| resveratrol-low | 0.05 ± 0.03†         | 15.2 ± 1.5        | 360.1 ± 22.2 | 0.83 ± 0.60†    |
| resveratrol-high | 0.07 ± 0.03†         | 15.2 ± 1.5        | 362.6 ± 22.0 | 1.05 ± 0.48     |
| arm 3 control rats | 0.16 ± 0.06            | 15.2 ± 1.2        | 370.2 ± 20.1 | 2.78 ± 1.24     |

Table 4. Key Phenolic Compounds Identified in Extractsa

| analytes | mg/g extractb | plum | grape | blueberry |
|----------|---------------|------|-------|-----------|
| gallic   | 11.85         | 0.15 | 11.85 | 0.45      |
| ferulic  | 3.16          | 2.82 | 0.37  | 0.45      |
| caffeic  | 2.22          |      |       |           |
| stilbenoids |            |      |       |           |
| resveratrol |             |      |       |           |
| other stilbene derivatives | 9.16 |      |       | 2.10      |
| chlorogenic acids |       |      |       |           |
| 3-0-cafeoylquinic | 4.19 | 11.62 | 11.62 | 4.19      |
| caffeoylquinic (other forms) | 1.85 | 21.75 | 3.28  |           |
| flavonoids |               |      |       |           |
| catechin | 1.25          | 2.84 |      |           |
| epicatechin | 0.81         |      |       |           |
| quercetin-glucosides | 0.23 | 0.09 | 0.14  | 0.23      |
| genistein |               |      |       |           |
| daidzein | 1.85          | 0.94 |      |           |
| cyanidin-glycosides | 0.94 | 0.27 | 0.71  | 0.19      |
| acetylated cyanidins | 1.21 | 3.21 |      | 1.21      |
| peonidin-glycosides | 0.19 | 1.17 |      |           |
| acetylated peptide | 9.77 | 5.51 |      |           |
| delphinidin-glycosides | 2.60 | 2.12 | 2.12  |           |
| acetylated delphinidins | 7.10 | 7.66 |      |           |
| rutin-glycosides | 21.69 | 22.44 |      |           |
| acetylated rutinidins | 16.5 | 22.2 |      |           |
| total polyphenols | 16.5 | 328  | 22.2  |           |

Phenolic compounds were tested in duplicate using LC–MS41 to assay for key compounds. Resveratrol contained 295.12 mg resveratrol/g sample and grape seed extract contained 827 mg/g total polyphenols as determined using the Folin–Ciocalteu method. Grape seed extract was unavailable for analysis using LC–MS. Total polyphenols were determined using the Folin–Ciocalteu method.38

extract were chlorogenic acids (11.62 mg/g extract 3-0-cafeoylquinic and 21.75 mg/g extract other forms of caffeoylquinic), which were also notably higher than the content found in grape (3.28 mg/g extract chlorogenic acids) and blueberry (5.04 mg/g extract total chlorogenic acids). Grape and blueberry were both characterized as being higher in anthocyanins ($4.7 mg/g extract and 44.41 mg/g extract total anthocyanins, respectively) compared with plum (0.5 mg/g extract total anthocyanins); however, grape had a greater amount of gallic acid compared with blueberry (11.85 mg/g extract with 0.45 mg/g extract, respectively), and blueberry contained a higher level of chlorogenic acids than grape (5.04 mg/g extract compared with 3.28 mg/g extract, respectively). Grape extract did not contain resveratrol, but did have other stilbene derivatives (9.16 mg/g extract).

Biochemical Markers of Bone Turnover. Grape seed extract-high (p = 0.0056) and grape-high (p = 0.004) dietary interventions reduced NTx compared with each corresponding baseline period (Figure 5A and Figure 5B). Resveratrol-low significantly increased NTx compared with the corresponding baseline period (p = 0.0039). Blueberry, plum, soy, grape seed extract, grape-low, and resveratrol-high diets did not produce a significant change in NTx.

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**DISCUSSION**

In this crossover intervention trial in OVX rats, we identified that although both isoflavone-containing diets were effective at increasing bone calcium retention, the aglycon form of genistein was significantly more effective than the glucoside. In addition, we demonstrated that higher levels of resveratrol and plum (0.20% and 0.45% total polyphenols, respectively) increased bone calcium retention.
Dietary aglycon soy elicited an improvement to bone calcium retention that was approximately double the response from a diet with glycosylated soy (22% for aglycon soy compared with 13% for glycosylated soy), which supported our hypothesis that the aglycon form of genistein has the potential to lead to a higher presence of unconjugated genistein in the blood due to enhanced uptake in the gut and some absorption in the stomach. This is biologically important because unconjugated genistein has been shown to bind more strongly to estrogen receptor-β than its glucuronide metabolites, demonstrating the potential for a more estrogenic effect on bone. We were able to demonstrate the enhanced efficacy of the aglycon form of genistein on bone in an estrogen-depleted rodent model. Future research should directly compare the metabolites produced from consumption of aglycon and glycoside soy to more closely link this mechanism to the increase in bone calcium retention from the aglycon form of soy.

Estrogen in bone works primarily by decreasing the production of inflammatory cytokines (interleukin (IL)-1, IL-6, and RANKL and M-CSF (macrophage colony stimulating factor)) in osteoblasts and precursor cells to regulate osteoclast activity. A drop in estrogen leads to a prominent increase in osteoclast activity, which greatly increases bone resorption with a lag in subsequent bone formation. Although the estrogenic activity of phytoestrogens such as isoflavones has been shown to elicit an antiresorptive response on bone, we observed that the gut, it is conjugated to mostly glucuronic acid by both intestinal and hepatic enzymes. Consumption of the aglycon form of genistein has the potential to lead to a higher presence of unconjugated genistein in the blood due to enhanced uptake in the gut and some absorption in the stomach. This is biologically important because unconjugated genistein has been shown to bind more strongly to estrogen receptor-β than its glucuronide metabolites, demonstrating the potential for a more estrogenic effect on bone. We were able to demonstrate the enhanced efficacy of the aglycon form of genistein on bone in an estrogen-depleted rodent model. Future research should directly compare the metabolites produced from consumption of aglycon and glycoside soy to more closely link this mechanism to the increase in bone calcium retention from the aglycon form of soy.
genistein aglycon increased bone calcium retention, consistent with bone formation. This is evidenced by an increase in BAP, which is a marker of bone turnover and often an indicator of an anabolic effect to bone. Our finding suggests that the ability of isoflavones to reduce net bone loss works by stimulating the anabolic bone building effect to counter the increase in resorption caused by loss of estrogen, rather than by mitigating bone resorption only. Urinary calcium tracer excretion is specific to bone mineral balance and more precise than biochemical markers of bone turnover.

Our finding that short-term supplementation of dried plum at 20% w/w diet, delivering 0.45% w/w total dietary polyphenols (plum-high), to OVX rats resulted in a 20% increase in bone calcium retention was similar to the 15% increase in bone tracer retention observed by Mühlbauer et al. using a similar method in OVX rats with a lower percentage (8% w/w diet) of dried prune extract for 10 days. The effects we observed on bone calcium retention from plum is also consistent with doses reported in the literature to be protective to bone in OVX rats, i.e., 15% and 25% in postmenopausal women, plum supplemented at 100 g/d prevented bone loss and increased BMD; the control diet of 100 g/d dried apples also protected bone from loss, but to a lesser degree than dried plum supplementation. This level of dried plum intake would be equivalent to the 25% w/w diet given to rodents and similar to the 20% w/w diet given in this study, assuming that food intake in women totals approximately 400 g/d on a dry weight basis.

We did not find a significant effect on bone with diets of blueberry, grape seed extract, or grape extract in OVX rats. Evaluation of the phenolic profiles of these extracts reveals that chlorogenic acids are more prominent in plum compared to the other extracts. Both chlorogenic acid and dried plum powder have been found to have an inhibitory effect on the RANKL pathway involved in osteoclastogenesis. However, we did not observe a decrease in NTx, a marker of bone resorption, with dietary supplementation with dried plum.

Previous work demonstrated that blueberry at 5% w/w diet prevented the loss of whole-body BMD in OVX rats. Additionally, blueberry at 10% w/w diet fed to prepubertal rats prevented bone loss later in life; the suggested mechanism was that phenolic acids found in blueberry promoted osteoblast differentiation. Although sera phenolic acid derivatives were described, the phenolic profile of the blueberry extract was not reported. The lack of effect observed in our study could potentially be attributed to differences in blueberry phenolic profile and concentration of phenolic compounds in the extract. While abundant in anthocyanins, blueberry had lower levels of chlorogenic acids compared with the plum powder, which may have impacted the efficacy of the blueberry diet.

Grape seed extract when given with calcium was shown to have a protective effect on bone in adult and growing intact rats when given immediately following a low calcium diet. We tested grape seed extract in an estrogen-depleted rodent model and did not find an effect on bone. Catechins, the most abundant polyphenols present in grape seed extract, have been shown to have estrogenic activity and there is evidence to suggest that catechins from green tea have a role in preventing postmenopausal bone loss. However, the binding affinity of different catechins to estrogen receptor-β should be investigated further as this receptor more directly impacts bone.

We did not see an effect of grape extract (0.2−1% w/w total polyphenols in diet) on bone calcium retention; however, we did find that resveratrol-high, which delivered 0.2% dry matter as total polyphenols in the diet, increased bone calcium retention by 14%. Mühlbauer et al. fed rats 8% w/w diet red wine residue and observed a reduction in bone resorption; however, the measured total polyphenols and resveratrol content were not reported. Despite anthocyanins and gallic acid being more prominent in grape than the other extracts, our analysis of the phenolic profile of grape extract showed an absence of resveratrol, a compound which has been proven to have estrogenic activity and was effective as an isolated compound in our study. Further analysis comparing grape extracts with varying levels of resveratrol should be tested to determine if efficacy is due primarily to resveratrol.

Total polyphenols were significantly higher during the botanical interventions than during baseline and washout periods, suggesting that polyphenols were absorbed and cleared into urine from test diets. Total polyphenols were not significantly different during the washout compared with baseline, which suggests clearance of prior diet in the system and no carryover effect.

Biochemical markers of bone turnover were not always consistent with bone calcium retention response to diets. Grape seed extract-high and grape-high supplementation decreased NTx without a change in bone calcium retention. Resveratrol-low induced an increase in NTx, but had no significant effect on bone calcium retention. The increase in bone calcium retention for glycosylated soy, aglycon soy, plum-high, and resveratrol-high failed to lower NTx or increase bone alkaline phosphatase.

In our crossover study with OVX rats, dietary plum, resveratrol, genistein glucoside, and genistein aglycon increased bone calcium retention by 13−22%. To our knowledge we are the first to directly compare and report the enhanced effect of genistein aglycon on bone. Additionally, we confirmed efficacy of dried plum on bone and highlighted chlorogenic acids as the group of phenolic compounds contributing to increased bone calcium retention. Although we did not observe a bone protective effect with a resveratrol-devoid grape extract, we did observe a strong bone protective effect with resveratrol, which suggests that resveratrol may be the active component in other grape products that have been shown to have bone protective effects. Future studies could extend this work by evaluating the effect of specific classes of botanical polyphenolics on bone strength measures.

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ABBREVIATIONS USED

OVX, ovariectomized; BMD, bone mineral density; RANK, receptor activator of nuclear factor kappa-B; RANKL, receptor activator of nuclear factor kappa-B ligand; M-CSF, macrophage colony stimulating factor; IL, interleukin; NTx, cross-linked N-telopeptides of type I collagen; ANOVA, analysis of variance

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