Supplemental Dietary Racemic Equol Has Modest Benefits to Bone but Has Mild Uterotropic Activity in Ovariectomized Rats

Lee Cole L. Legette, 1 Berdine R. Martin, 1 Mohammad Shahnazari, 4 Wang-Hee Lee, 5 William G. Helferich, 6 Junqi Qian, 1 David J. Waters, 8 Alireza Arabshahi, 7 Stephen Barnes, 9 Jo Welch, 4 David G. Bostwick, 7 and Connie M. Weaver 4*

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4 Department of Foods and Nutrition and 1 Department of Agricultural and Biological Engineering, Purdue University, West Lafayette, IN 47907; 3 Department of Veterinary Clinical Sciences, Purdue University, West Lafayette, IN 47907; and 7 Department of Pharmacology-Toxicology, University of Alabama, Birmingham, AL 35294

Abstract
Soy isoflavones and their metabolites, with estrogenic activity, have been considered candidates for reducing postmenopausal bone loss. In this study, we examined the effect of dietary equol, a bioactive metabolite of the soy isoflavone daidzein, on equol tissue distribution, bone parameters, and reproductive tissue activity using an adult ovariectomized (OVX) rat model. An 8-wk feeding study was conducted to compare 4 dietary treatments of equol (0, 50, 100, 200 mg/kg diet) in 6-mo-old OVX female Sprague-Dawley rats. A dose response increase in tissue equol concentrations was observed for serum, liver, kidney, and heart, and a plateau occurred at 100 mg equol/kg diet for intestine. In OVX rats receiving 200 mg equol/kg diet, femoral calcium concentration was greater than those receiving lower doses but was still less than SHAM (P < 0.05), and other bone measures were not improved. Tibia calcium concentrations were lower in OVX rats receiving 100 and 200 mg equol/kg diet compared with the OVX control rats. Trabecular bone mineral density of tibia was also lower in equol-fed OVX rats. At this dietary equol intake, uterine weight was higher (P < 0.05) than in other OVX groups but lower than the SHAM-operated intact rats. The 200 mg/kg diet dose of dietary equol significantly increased proliferative index in the uterine epithelium. Dietary equol had no stimulatory effect on mammary gland epithelium. We conclude that in OVX rats, a dietary equol dose that had modest effect on bone also exerts mild uterotrophic effects. J. Nutr. 139: 1908–1913, 2009.

Introduction

In 2002, results of the Women’s Health Initiative Study revealed adverse effects of estrogen therapy, including increased risk for cardiovascular disease and breast cancer, despite positive benefits of reduced bone loss (1). This created interest in alternative choices with bone protective effects such as soy isoflavones and their metabolites (2). Soy isoflavones are commonly referred to as phytoestrogens due to their chemical structure, which is similar to estrogen. It has been postulated that soy isoflavones exert their actions through binding with estrogen receptor (ER)α-β (2) in osteoblast cells. This leads to suppression of osteoclast differentiation via osteoprotegerin (OPG) and subsequent inhibition of bone resorption (3). Specifically, OPG competes with receptor-activated nuclear factor κ receptor (RANK) for binding to RANK ligand, which is essential for osteoclast differentiation (3). A recent study showed that administering soy isoflavone genistein to osteopenic postmenopausal women for 2 y resulted in higher OPG: RANK ligand ratios and femoral neck bone mineral density (BMD) compared with a placebo group (4).

Some evidence suggests that soy isoflavone metabolites exert more potent effects on bone than their parent precursors (5). In

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9 Abbreviations used: BMC, bone mineral content; BMD, bone mineral density; ER, estrogen receptor; OPG, osteoprotegerin; OVX, ovariectomized; OVX-0, ovariectomized rats receiving 0 mg equol/kg diet in an AIN-93M diet; OVX-50, ovariectomized rats receiving 50 mg equol/kg diet in an AIN-93M diet; OVX-100, ovariectomized rats receiving 100 mg equol/kg diet in an AIN-93M diet; OVX-200, ovariectomized rats receiving 200 mg equol/kg diet in an AIN-93M diet; pQCT, peripheral quantitative computed tomography; RANK, receptor activated nuclear factor κ receptor; μCT, micro computed tomography.
Materials and Methods

Rats. Seventy-eight virgin female Sprague Dawley rats (6 mo old) were purchased from Harlan, of which 62 were OVX and 16 were SHAM operated 2 d before shipment. Rats were housed in individual cages in a temperature- and humidity-controlled room with lights on a 12:12-h on:off cycle. An AIN-93M diet (17) and distilled water were provided ad libitum for an acclimation period of 2 d. All procedures were approved by and performed in accordance with the rules of Purdue University’s Animal Care and Use Committee.

Diet and equol supplement. Equol powder (50% R-equol, 50% S-equol; Supplemental Fig. 1) was produced as previously described (6) and mixed evenly with AIN-93M base diet (17) ingredients (Dyets) at the following concentrations: 0, 50, 100, and 200 mg/kg diet. Doses were selected based on a pilot study in rats aimed to achieve serum human equol levels, 0–1, 3–39, and 5–49 μmol/L, observed in postmenopausal women in response to dietary equol supplement for those unable to produce equol naturally.

Our aim was to determine the effect of dietary equol on bone metabolism in ovariectomized (OVX) rats. Such a study became feasible only with the recent capability to synthesize equol (6). A dietary dose ranging study allowed insight into the potential benefits of dietary equol supplement for those unable to produce equol naturally.

pQCT measurements. pQCT analysis was performed with the Stratec peripheral quantitative computed tomograph (model Research SA+ of Norland Stratec XCT, Stratec Electronics). Three cross-sectional sites (1.0 mm thick) from distal, midshaft, and proximal sites of bones, determined at 12, 50, and 88% of the length of from distal, were scanned using a 0.46-mm collimation (4 × 105 counts/s) and a 0.08-mm voxel size. Thresholds for segmentation of trabecular and cortical bone were set at 300 mg/cm2 and 900 mg/cm2, respectively.

Bone architecture was assessed by micro computed tomography (μCT) analysis using a Scanco microCT tomograph (μCT 40, Scanco Medical). Eight femurs were randomly selected from each of the following groups: SHAM, OVX-0, and OVX-200. Cancellous and cortical bone of femurs was evaluated using methods and parameters described previously (20). Cancellous bone at the distal femur was scanned with an isotropic resolution of 16 μm obtained from 0.2 down the growth plate extended for 62 slices. In the femur, 62 contiguous slices were contoured and imaging proceeded proximally covering a distance of 1 mm.

Bone calcium content. After mechanical testing, right femurs and tibias were dissolved in concentrated nitric acid overnight and analyzed for total calcium content using atomic absorption spectrometry (PE S100, Perkin Elmer).

Reproductive tissue analysis. The extent of epithelial proliferation in mammary glands and uterine epithelium was determined by visually scoring formalin-fixed tissue sections. Each specimen was scored on scale from 0 to 3, with 0 and 2 equivalent to the extent of epithelial proliferation in OVX and SHAM controls, respectively. In addition to visual scoring, the proliferative index in these tissues was determined by proliferative cell nuclear antibody (PCNA) immunohistochemistry using a technique previously reported (12). The proliferative index was defined as the percentage of cells with unequivocal nuclear immunostaining.

Statistical analysis. Data were analyzed using SAS (version 9.1, SAS Institute). The effect of equol on bone parameters was determined by dietary treatment, rats were killed by excess CO2. Blood was drawn via the portal vein and serum was stored at −80°C for isoflavone analysis. Various tissues, including kidney, liver, whole intestine, and heart (flushed with 50 mL saline), were excised and stored at −80°C for isoflavone analysis. The uterus and caudal mammary glands were collected and stored in 10% buffered formalin for histopathologic examination and assessment of epithelial proliferation. Right femurs and tibias were wrapped in saline-soaked gauze and stored at −20°C for evaluation of bone mechanical properties. Left femurs and tibias were collected and placed in 70% ethanol and stored at 4°C for scanning by peripheral quantitative computed tomography (pQCT).

Serum and tissue equol measurement. Serum and tissue concentrations of equol were quantified using a highly sensitive and specific electrospray ionization liquid chromatography-multiple reaction ion monitoring MS method as described previously (19). There were modifications in conditions of chromatography as listed below. Chromatography was carried out on a 30–2.0-mm i.d. Phenomenex Phenyl-Hexyl column using mobile phase consisting of a gradient of 10–80% acetonitrile in 10 mmol/L ammonium acetate over 5 min with a flow rate of 0.4 mL/min. Equol was detected using the transition of its m/z 241 molecular ion and its m/z 119 fragment ion. Tissue samples were prepared by removing 0.2–0.250 g of tissue from sample and homogenization in 2 mL of an 80% methanol, 0.01% (wt/v) ascorbic acid solution. Extraction techniques were described previously (19).

Bone density, size, and mechanical properties. Right femurs and tibias were measured for length and width at the midshaft using an ABS digimatic solar caliper (Tri-State Instrument Service). Bone density was measured by underwater weighing using a density determination kit and Mettler Toledo analytical balance. Bone strength was assessed using a 3-point bending test on a TA-XT2 Texture analyzer (Texture Technologies).

Micro computed tomography measurements. Bone architecture was assessed by micro computed tomography (μCT) analysis using a Scanco microCT tomograph (μCT 40, Scanco Medical). Eight femurs were randomly selected from each of the following groups: SHAM, OVX-0, and OVX-200. Cancellous and cortical bone of femurs was evaluated using methods and parameters described previously (20). Cancellous bone at the distal femur was scanned with an isotropic resolution of 16 μm obtained from 0.2 down the growth plate extended for 62 slices. In the femur, 62 contiguous slices were contoured and imaging proceeded proximally covering a distance of 1 mm.

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Results

**Body and uterine weight.** All OVX groups except OVX-200 had significantly higher body weight than the SHAM despite pair feeding. However, there was no significant effect of equol supplementation on body weight of OVX rats (Fig. 1A). Dietary intake was similar among all OVX groups, which were pair-fed to the mean intake of SHAM rats. Feed efficiency ratios were similar among OVX-0 (0.036 ± 0.023), OVX-50 (0.046 ± 0.020), and OVX-100 (0.035 ± 0.023) but significantly higher than for SHAM (0.090 ± 0.019) and OVX-200 (0.210 ± 0.024) rats. Uterine weight in all OVX groups was significantly lower than in SHAM rats (Fig. 1B). In OVX rats, the uterine weight was greater only in the OVX-200 group (55% higher than OVX-0; \( P \leq 0.05 \)) but was still substantially less than in SHAM (72% lower; \( P \leq 0.05 \)).

**Equol analysis.** Equol levels of 50, 100, and 200 mg/kg diet of OVX rats resulted in total serum (\( S/R \)) equol concentrations increasing with increasing dose of dietary equol (Fig. 1C). Liver, kidney, and heart tissue concentrations also increased with increasing dose of dietary equol. Equol levels in the intestine increased in all equol groups compared with OVX-0, but the increase was not dose dependent (data not shown).

**Bone calcium concentration, size, and mechanical properties.** Femoral density measured by underwater weighing was higher (\( P < 0.05 \)) in the SHAM group than in the OVX groups but was not affected by equol in the diet (Table 1). There were no group differences in femoral length, width, or breaking force. SHAM rats had higher (\( P < 0.05 \)) femoral calcium concentration than OVX-0 (Fig. 1D). Among all OVX groups, only the OVX-200 group maintained a femoral calcium concentration similar to SHAM rats and higher (\( P < 0.05 \)) femoral calcium concentration than OVX-0 rats.

There was no significant decrease in tibial density in response to ovariectomy, but the OVX-50 group had a lower tibia density from SHAM. There were no group differences in tibia length, width, or breaking force. SHAM rats had a significantly higher tibia calcium concentration than all OVX rats. Among OVX rats, OVX-100 and OVX-200 had significantly lower tibia calcium concentrations than the OVX-0 and OVX-50 groups (Fig. 1D).

**pQCT measurements.** In the midshaft femur and tibia, SHAM rats had significantly higher total BMC than all OVX groups. There were no differences among treatment groups for total BMD, cortical BMD, and cortical thickness at the midshaft femur and tibia.

For the distal femur, the SHAM group had significantly higher total BMC, total BMD, trabecular BMD, and cortical thickness than OVX-0 rats. There were no differences among OVX rats at the distal femur.

At the proximal femur, the SHAM group had significantly higher trabecular BMD than all OVX groups (Table 1).

![Figure 1](https://academic.oup.com/jn/article-abstract/139/10/1908/4670316)
TABLE 1 Bone characteristics of SHAM-operated and O VX rats fed diets containing various levels of equol

<table>
<thead>
<tr>
<th>Treatments</th>
<th>SHAM</th>
<th>O VX-0</th>
<th>O VX-50</th>
<th>O VX-100</th>
<th>O VX-200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Femur</td>
<td></td>
<td></td>
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<tr>
<td>Bone density, $g/cm^3$</td>
<td>1.56 ± 0.030a</td>
<td>1.51 ± 0.030b</td>
<td>1.50 ± 0.040b</td>
<td>1.50 ± 0.030b</td>
<td>1.52 ± 0.030b</td>
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<tr>
<td>Tibia</td>
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<tr>
<td>Bone density, $g/cm^3$</td>
<td>1.50 ± 0.040a</td>
<td>1.48 ± 0.030ab</td>
<td>1.46 ± 0.030b</td>
<td>1.46 ± 0.050ab</td>
<td>1.48 ± 0.030ab</td>
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<tr>
<td>Distal femur, $pQCT$</td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Total BMC, mmol Ca/g body wt</td>
<td>0.414 ± 0.082a</td>
<td>0.337 ± 0.051b</td>
<td>0.307 ± 0.025b</td>
<td>0.319 ± 0.042b</td>
<td>0.321 ± 0.041b</td>
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<tr>
<td>Trabecular BMC, mg/cm$^3$</td>
<td>558 ± 51.0a</td>
<td>448 ± 60.0b</td>
<td>410 ± 43.0b</td>
<td>414 ± 36.0b</td>
<td>425 ± 55.0b</td>
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<tr>
<td>Distal femur, $\mu CT$</td>
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<tr>
<td>Bone volume/tissue volume</td>
<td>0.250 ± 1.00b</td>
<td>0.090 ± 0.030b</td>
<td>0.090 ± 0.060b</td>
<td>24.0 ± 16.0b</td>
<td>2.61 ± 0.520b</td>
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<tr>
<td>Trabecular connectivity density, $1/mm^3$</td>
<td>78.0 ± 19.0b</td>
<td>19.0 ± 11.0b</td>
<td>19.0 ± 11.0b</td>
<td>24.0 ± 16.0b</td>
<td>2.61 ± 0.520b</td>
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<tr>
<td>Trabecular number, $1/mm$</td>
<td>4.28 ± 0.550b</td>
<td>2.39 ± 0.390b</td>
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<td>2.39 ± 0.390b</td>
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<tr>
<td>Proximal femur, $pQCT$</td>
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<tr>
<td>Trabecular BMC, mg/cm$^3$</td>
<td>543 ± 65.0a</td>
<td>481 ± 36.0b</td>
<td>454 ± 46.0b</td>
<td>466 ± 54.0b</td>
<td>486 ± 45.0b</td>
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<tr>
<td>Proximal tibia, $\mu CT$</td>
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<tr>
<td>Total BMC, mmol Ca/g body wt</td>
<td>0.270 ± 0.023a</td>
<td>0.255 ± 0.035b</td>
<td>0.255 ± 0.035b</td>
<td>0.263 ± 0.085b</td>
<td>0.230 ± 0.025b</td>
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<tr>
<td>Trabecular BMC, mg/cm$^3$</td>
<td>652 ± 48.0b</td>
<td>609 ± 43.0b</td>
<td>554 ± 30.0b</td>
<td>558 ± 31.0b</td>
<td>578 ± 40.0b</td>
</tr>
</tbody>
</table>

1 Values are means ± SD, n = 15-16. Means without a common letter differ, P < 0.05.
2 Bone density was calculated by underwater weighing.
3 $\mu CT$ measurements were only performed on SHAM, O VX-0 ppm, and O VX-200 ppm equol groups, n = 8.

Treatment groups did not differ for total BMD, cortical BMD, and cortical thickness at the proximal femur.

At the proximal tibia, SHAM rats had significantly higher total BMD than all O VX groups (Table 1). Among O VX rats, all those fed dietary equol had significantly lower trabecular BMD compared with O VX-0 rats. Treatment groups did not differ for total BMD, cortical BMD, and cortical thickness at the proximal tibia.

$\mu CT$ measurements. At the distal femur, the SHAM group had significantly higher trabecular connectivity and significantly lower trabecular spacing than the O VX-0 and O VX-200 groups (Table 1). Treatment groups did not differ in cortical thickness, porosity, and cortical BMD at the midshaft of the femur.

Evaluation of mammotropic and uterotropic effects of equol. The SHAM group had significantly higher mammary gland epithelial mass on visual scoring than all O VX groups (Table 2). Among O VX groups, equol did not affect mammary epithelial proliferation as evident by PCNA immunostaining (Table 2). Treatment groups did not differ in cortical thickness, porosity, and cortical BMD at the midshaft of the femur.

Based on visual scoring of histologic sections of the uterus, the SHAM group had significantly greater uterine epithelial mass than all O VX groups. O VX-200 rats had significantly greater epithelial mass than O VX-0 and O VX-50 but not O VX-100 rats. PCNA immunostaining showed SHAM control, O VX-100, and O VX-200 rats had significantly higher uterine epithelial proliferative index compared with the O VX-0 and O VX-50 rats (Table 2). SHAM rats and O VX-200 rats had significantly higher uterine stroma cell proliferative index than O VX-0 and O VX-50 but not O VX-100 rats.

Discussion

Because soy is the most abundant source of isoflavones in the human diet, the 2 major soy isoflavones, daidzein and genistein, have been extensively evaluated for their impact on mitigating estrogen-related bone loss (11,21–24). Investigators have also started to examine soy isoflavone metabolites, such as equol, as potentially more biologically potent than their endogenous precursors (6,9,25). Here, we show in O VX rats that dietary intake of equol at a level of 200 mg/kg diet induces few changes in bone calcium concentration, specifically a beneficial increase in femoral calcium concentration but a negative decrease in tibia calcium concentration. Furthermore, this level of dietary equol intake exerted adverse effects in uterine, but not mammary, tissue in O VX animals.

Few studies have characterized humans by their ability to produce equol. Wu et al. (26) classified 68 study participants out of 122 as equol producers based on conversion rate of daidzein to equol present in fecal bacteria. They found that equol producers had a significantly lower percent change in total hip BMD after 24 wk of soy isoflavone treatment compared with nonproducers. Similarly, Sertell et al. (2) observed a 2.4% increase in lumbar spine BMD of equol producers (45% of participants) after a 2-y intervention with soy isoflavonoids compared with the 0.6% increase of nonproducers. Studies on the direct effect of dietary equol on bone health have been limited because of the high cost and limited availability of commercial sources. Consequently, the few studies that examined the effects of equol in vivo have administered it by subcutaneous injection or osmotic pump to conserve the compound rather than via the more physiological route of dietary intake (14,15). This may reflect the relationship between a metabolite produced in vivo and its health effects but does not address bioavailability of equol as a dietary supplement. Administration of 0.5 mg/μL equol, but not 0.1 mg in 1 μL, by osmotic pump inhibited O VX-induced bone loss of the whole body, femur, and lumbar spine in ddY mice (27). In our study, equol was fed to rats as a dietary supplement, so that serum concentrations corresponded to dietary intake (14,15). This may reflect the relationship between a metabolite produced in vivo and its health effects but does not address bioavailability of equol as a dietary supplement.
levels seen in previous studies with rodents receiving medium to high doses of dietary daidzein (28–30). Serum concentrations are also similar to levels in clinical participants (postmenopausal women) from our laboratory, classified as equol producers, who consumed between 120–150 mg isoflavones/d (20).

In this dose response study on the effect of dietary equol on bone health, we found a modest effect on femoral calcium concentration. In contrast, dietary equol appeared to have a detrimental effect on tibia calcium concentration and trabecular BMD at the proximal tibia. Whole bone measures of the femur and tibia are largely cortical bone. Calcium absorption efficiency tended to be greater (P = 0.07) in the OVX-100 (0.338 ± 0.088) and OVX-200 (0.352 ± 0.124) groups than in the SHAM (0.300 ± 0.121), OVX-0 (0.280 ± 0.103), and OVX-50 (0.020 ± 0.037) groups. Skeletal site-specific results could be attributed to a differential response between cortical and trabecular bone. Alternatively, skeletal site-specific results could be due to differences in mechanical loading for the animal model used in this investigation. In this animal model, the tibia undergoes more mechanical stress than the femur (31). Saxon et al. (32) demonstrated that estrogen-like actions mediated through ERβ could inhibit bone formation under mechanical loading. Investigators applied daily loading to 16 wk-old mice with ERβ null mutations (ERβ−/−) and found that ERβ−/− mice had a 3.6-fold increase in bone formation after mechanical loading compared with wild type, ERβ+/+, mice. Additional research should examine the effect of estrogenic-like actions on bone mechanical signaling and the subsequent impact on bone health.

Our results also showed that despite pair feeding, only the highest dietary equol dose (200 mg/kg diet) helped maintain body weight at SHAM levels, probably by exerting estrogen-like effects on general metabolism. The highest dietary equol level was also associated with increased uterine weight. The weight differential between SHAM and OVX despite pair feeding (21) and uterotrophic effects of dietary equol (13) have been observed previously. A risk factor associated with estrogen therapy or compounds exerting estrogen-like effects is the growth stimulation of reproductive tissue, especially uterus. Increased uterine weight is a classical estrogen effect mediated by ERα (33–35). At equol levels of 0.1 or 0.5 mg/d, no effect on uterine weight was observed in OVX mice (27), whereas weight was increased in mice receiving equol injections at 12 and 20 mg/kg body weight per day (14). Similarly, Rachon et al. (15) observed increased uterine weight as well as higher proliferation in uterine epithelial cells after administering 2 doses of racemic equol (50 and 400 mg/kg unpurified diet) for 3 mo to OVX Sprague Dawley rats. In our study, only the highest dose of dietary equol, 200 ppm, caused a significant increase (55%) in uterine weight compared with the OVX control group as a reference as well as increased epithelial proliferation. Findings of uterotropic effects from dietary equol could be due to the fact that the only form of synthesized equol available is the racemic mixture and the R-equol isomer has an affinity for ERα, which is abundant in reproductive tissue. Moreover, S-equol has a greater affinity for ERβ than R-equol (6). Recently, Heemstra et al. (36) developed a method for total synthesis of S-equol. Future studies should determine whether greater efficacy without uterotrophic effects will occur with dietary administration of S-equol.

Our findings demonstrate that a 200-mg equol/kg diet dose in OVX rats has modest bone health benefits. Results also showed that a 200-mg equol/kg diet dose did not induce changes in the mammary epithelium but mildly stimulated the uterine epithelium. Our study suggests that the benefit:risk ratio of racemic equol has low promise as a commercial supplement. Given that S-equol is the natural bacterial product with a higher binding affinity for ERβ and not ERα compared with R-equol, future research should establish whether S-equol would have greater bone protective properties without reproductive tissue stimulation.

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### Literature Cited


