Bone mass and soy isoflavones in socially housed, premenopausal macaques¹–³

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ABSTRACT
Background: Soy consumption is associated with a lower incidence of hip fracture in Asian than in Western women, an effect often attributed to estrogen-like compounds (isoflavones) in soy. It is not known whether premenopausal soy exposure initiated in adulthood can increase bone mass and thereby reduce fracture risk.

Objective: We aimed to determine whether a high-isoflavone soy diet influences bone mass in soy-naïve, premenopausal cynomolgus monkeys (Macaca fascicularis).

Design: Ninety-four skeletally mature females were randomly assigned to consume diets whose protein content came from either high-isoflavone soy or casein and lactalbumin. Animals were socially housed. Bone mass and circulating isoflavone concentrations were measured at baseline and 19 and 31 mo after the start of treatment; bone biomarkers were measured at baseline and 31 mo.

Results: There were no significant differences at any timepoint in whole-body bone mineral content between casein-fed (112.5 ± 2.1, 119.2 ± 1.9, and 120.7 ± 2.1 g) and soy-fed (117.2 ± 2.1, 122.4 ± 2.0, and 125.4 ± 2.3 g; P = 0.12) monkeys. Similar results were seen for spinal bone mineral density (casein-fed: 0.46 ± 0.01, 0.50 ± 0.01, and 0.52 ± 0.01 g/cm²; soy-fed: 0.47 ± 0.01, 0.51 ± 0.01, and 0.52 ± 0.01 g/cm²; P = 0.30) and bone biomarker measurements—bone-specific alkaline phosphatase (soy-fed: 82.3 ± 4.1 and 63.2 ± 3.4 ng/mL; casein-fed: 94.1 ± 4.5 and 61.7 ± 4.3 ng/mL; P = 0.22) and C-terminal crosslink of type 1 collagen (soy-fed: 0.944 ± 0.06 and 0.89 ± 0.08 nmol/L; casein-fed: 0.97 ± 0.07 and 0.78 ± 0.06 nmol/L; P = 0.20).

Conclusion: A soy diet high in isoflavones does not significantly affect bone characteristics in initially soy-naïve premenopausal monkeys.

INTRODUCTION

Fully half of all postmenopausal women will experience an osteoporosis-related fracture and associated morbidity (1, 2). Current methods for preventing osteoporosis and associated fractures include hormone replacement therapy (HRT), bisphosphonates, and selective estrogen receptor modulators (SERMs). However, these postmenopausal treatment modalities all have limitations or potential adverse side effects. Premenopausal interventions that promote maximal peak bone mass could increase resistance to future fracture and thereby reduce the need for postmenopausal treatment (3, 4).

Estrogen plays a critical role in the premenopausal acquisition and maintenance of peak bone mass (5–8). This observation, in turn, has led to speculation that the estrogen-like compounds (isoflavones) contained in soy protein may reduce resorption and enhance peak bone mass acquisition (9, 10). A series of observational studies evaluating premenopausal Asian women suggested that habitual consumption of soy protein or isoflavone is associated with the preservation of bone mass (11–13). However, at least one observational study of premenopausal Chinese women did not find such an association (14), and a randomized trial showed that isoflavone exposure had no effect on bone density among relatively young (21–25 y) women, most of whom were white (15).

The geographic epidemiology relating soy consumption to health benefits is based in part on Asian populations with extensive lifetime exposure to soy that begins in utero, that is much lower (but not zero) during breastfeeding, and that is elevated again when solid foods are consumed (16, 17). In contrast, typical Western persons, if raised on soy formula, experience high isoflavone exposure only during infancy; if not raised on soy formula, they are generally soy-naïve until adulthood (18). Soy supplementation to adults with such different histories of soy exposure could potentially have divergent effects on estrogen-sensitive tissues such as bone (18, 19).

To further explore the effect of premenopausal soy exposure on bone mass, we evaluated the responses of skeletally mature cynomolgus monkeys (Macaca fascicularis) to diets deriving most of their protein from either high isoflavone, isolated soy protein, or a casein-lactalbumin mixture. This animal model, which closely resembles women in menstrual cyclicity and with an ovarian hormone profile that closely resembles that of women in reproductive physiology, has been used extensively to assess the effects of dietary, hormonal, and behavioral manipulations on skeletal indices and cardiovascular disease risk (20). The primary objectives of the current study were to determine whether

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soy consumption was associated with alterations in whole-body bone mineral content (WBBMC), spinal (lumbar vertebrae 2–4) bone mineral density (BMD), or serum biomarkers of bone turnover in premenopausal subjects.

MATERIALS AND METHODS

Animals
The subjects were 94 female cynomolgus monkeys imported from Indonesia (Institute Pertanian Bogor, Bogor, Indonesia). Before shipment, all animals were radiographed. Only individuals exhibiting evidence of complete epiphyseal closure at the distal radius and ulna and the proximal tibia were used in the study. This stage of development generally occurs by 9 or 10 y of age in cynomolgus monkeys, at which time individuals are approximately equivalent to 25-y-old women (21, 22). Peak bone mass attainment lags behind epiphyseal closure, although its trajectory is generally set during the period between puberty and epiphyseal closure (23). Additional radiographs of the spine were examined for any predisposing disease or injury that could potentially confound any spine density measurements. Finally, all animals had no known prior exposure to soy and were thus comparable to fully mature, premenopausal women that are developmentally naïve to isoflavones.

All animal manipulations were performed according to the guidelines of state and federal laws, the US Department of Health and Human Services, and the Animal Care and Use Committee, Wake Forest University School of Medicine. Wake Forest University is fully accredited by the American Association for the Accreditation of Laboratory Animal Care.

Housing, diets, and assessments
After their arrival at our facility, the animals were placed in single cages for a federally mandated 30-d quarantine period. On release from quarantine, the animals were placed in 16 social groups of 5 or 6 animals each; the groups were approximately equivalent in body weight gradient. For these new monkeys, an isoflavone-free diet was introduced to control for any differences in body composition and composition. This was designed to model the high-fat, high-cholesterol diet often consumed by Americans and thought to contribute to both obesity and cardiovascular disease (24). Three months after the initiation of this diet, baseline values of WBBMC, spinal BMD, bone biomarkers, lipids, lipoproteins, isoflavones, and body weight were measured, and baseline menstrual cycle characteristics and social status (ie, dominant or subordinate) were determined.

Study design
After the 8-mo baseline period, social groups of monkeys were assigned for 31 mo to 1 of 2 treatment conditions by using a stratified randomization procedure that matched the conditions for WBBMC and BMD and the responses of plasma total and HDL cholesterol to the baseline (isoflavone-free) diet. Statistical equivalence between cohorts assigned to the 2 conditions was verified for WBBMC (t = 112.5 ± 2.1 and 117.2 ± 2.1 g, respectively; NS) and spinal BMD (0.464 ± 0.006 and 0.472 ± 0.006 g/cm²; respectively; NS). Animals in the 2 treatment conditions were fed diets that differed only in the major protein source: 1) soy, derived from isolated soy protein (SUPRO; Solae Corporation, St Louis, MO) (the soy group) and 2) casein and lactalbumin (CL group)—the same isoflavone-free diet consumed by all animals during the baseline period. Approximately 20% of protein fed to each condition was derived from wheat flour.

The soy diet contained 1.88 mg total isoflavones/g protein and was fed at a concentration designed to provide the equivalent of a woman’s daily consumption of ≈129 mg aglycone units of isoflavone, assuming a daily intake of ≈1800 kcal (ie, 0.072 mg isoflavone/kcal). Monkeys were fed 120 kcal diet/kg body wt and, therefore, consumed ≈8.6 mg isoflavone/kg body wt. This caloric adjustment of dose accounts for the differences in metabolic rates between the monkeys and humans. The soy and CL diets were formulated to be isocaloric for the macronutrients [protein (19% of kcal), carbohydrate (37%), and fat (44%)] and comparable for cholesterol (0.20 mg/kcal), calcium (1322 mg/1800 kcal), and phosphorus (1293.1 mg/1800 kcal). During the treatment period, all animals were assessed for plasma lipids (HDL and total plasma cholesterol), lipoproteins, serum isoflavone concentrations, serum bone biomarkers, WBBMC, spinal BMD, social status, menstrual cyclicity, and ovarian hormones. Previous analyses indicated that soy had no significant effects on menstrual cyclicity or the reproductive hormone profile (24). Nine animals died during the start of the experimental diet and the final bone density determinations. These animals died from a variety of unrelated causes that could not be attributed to the experimental manipulation, including arterial disease, congestive heart failure, meningitis, acute enteritis, and oligoendroglia.

Bone densitometry and body weight
WBBMC (g) and spinal BMD (g/cm²) were measured at baseline (=6 mo before starting the experimental diet) and 19 and 31 mo after the start of the diet by using a dual-energy X-ray absorptiometry machine (DXA) XR-46; Norland, Fort Atkinson, WI) with DXA software (version 3.9.6; Norland). The monkeys were sedated with ketamine (10 mg/kg) and then given isoflurane. Measurements were made of the whole body and the lumbar spine (vertebrae 2–4) by using techniques described previously (25, 26). The CVs were 1.7% for WBBMC and 2.1% for spinal BMD.

Animals were sedated every 4–6 wk to determine body weight (model #815 scale; Chatillon, Largo, FL). The amount of diet fed was adjusted to changes in body weight to ensure that the animals continued to receive 120 kcal/kg (+ 10% wastage).

Isoflavone concentrations
Serum samples were collected on 3 occasions from each monkey for the measurement of circulating isoflavone concentrations. These collections occurred at baseline and 19 and 31 mo after the initiation of dietary treatment. Animals were fed the morning and then sedated 4 h after eating for blood collection. Blood was immediately processed, frozen, and protected from light until analysis. Serum isoflavones were analyzed by using liquid chromatography photo-diode array mass spectrometry that was slightly modified from a previously established method (27) to include equol in the panel of isoflavonoids (genistein, dihydrogenistein, daidzein, dihydrodaidzin), glycitein, and O-desmethylangolensin) and isotopically labeled internal standards (28, 29). Detection limits were previously found to be 1–15 ng/mL.
A linear mixed-effects modeling (LMM) approach was used to account for these intraclass correlations in the assessment of the main study outcomes (WBBMC, spinal BMD, and bone biomarkers). Compared with traditional repeated-measures analysis of variance, the LMM approach allows monkeys with incomplete data (such as animals who died during the study period) to contribute to the analyses. It also allows more flexible modeling of within-subject associations. In all of our models, no significant dietary treatment × dominance status interaction effect was found. Therefore, only the main effect of dominance status was adjusted for in the subsequent analyses. The effects of diet on WBBMC and spinal BMD were compared longitudinally with the main effects of diet and time and the diet × time interaction after adjustment for the corresponding body weight at each time-point. For the bone biomarkers BALP and CTX, the 24-mo follow-up measurements were modeled with the main effect of diet treatment and covariates including baseline measurements. Type 3 F tests were used to test the significance of the variables in the model, and all analyses were performed by using SAS software (version 9.1; SAS Institute, Cary, NC).

### RESULTS

Data from all subjects that started the study (n = 94) contributed to the analyses. Forty-eight animals were assigned to the CL condition and the remaining 46 to the soy condition.

Consumption of the soy diet resulted in high concentrations of plasma daidzein, genistein, and equol (Table 1). Trace amounts of these compounds were detected in most monkeys at baseline and in the CL condition monkeys during the treatment period. The females included in this study had completed long bone growth. Nonetheless, all monkeys achieved an increase in bone mass across time (WBBMC from 114.86 ± 1.5 to 123.07 ± 1.6 g; P < 0.001; spinal BMD from 0.47 ± 0.00 to 0.52 ± 0.01 g/cm²; P < 0.001), a process accompanied by a significant, 20% increase in body weight that coincided with consumption of the experimental (high-fat) diets (from 2.92 ± 0.04 kg to 3.51 ± 0.07 kg, P < 0.001). The increases in bone mass and body weight occurred irrespective of dietary condition.

The effects of dietary treatment (soy or CL diet) on WBBMC and spinal BMD across time are shown in Figure 1 and Figure 2. Although the trend lines for both outcomes appeared to diverge over the course of the study, the treatment effects did not reach conventional significance as either a main effect (P = 0.12 for WBBMC and 0.29 for spinal BMD) or in interaction with time (P = 0.37 for WBBMC and 0.44 for spinal BMD). Nor did dietary treatment affect body weight, either as a main effect or in interaction with time (P > 0.20 for both; Figure 3).

### Serum biomarkers of bone metabolism

Baseline (6 mo before experimental diets) and 31-mo serum samples were collected and stored frozen at −70 °C until they could be assayed for serum bone biomarkers [bone-specific alkaline phosphatase (BALP) and C-terminal crosslink of Type 1 collagen (CTX)]. Serum BALP activity was measured by using an immunocapture assay (Metra BAP; Quidel Corp, San Diego, CA). Serum CTX was measured by using a commercially available enzyme-linked immunosorbent assay (Serum Crosslaps; Nordic Biosciences Diagnostics, Helev, Denmark) that was specific for the amino sequence EKAHD-β-GGR derived from the C-terminal telopeptide region of type I collagen. Intraassay and interassay CVs for serum BALP and CTX were <10%.

### Behavioral observations and dominance determinations

Because individual differences in social status were previously observed to influence bone density and reproductive hormones, status was evaluated in relation to soy treatment to determine whether there were any confounding effects (20). The social status of each animal in relation to others in her social group was based on data collected during weekly, 30-min observations beginning after social group formation and before the initiation of dietary intervention. Dominance and subordination were determined by the outcomes of fights, which are highly asymmetric, and they yield clear winners and losers as judged by specific facial expressions, postures, and vocalizations (30, 31). The female in each group that defeated all others was designated the first-ranking monkey. The female that defeated all but the first-ranking monkey was designated the second-ranking monkey, and so forth. Ranks tend to be highly stable under the experimental conditions described (32). For purposes of analysis, animals ranking first and second in groups of 5 were categorized as dominant, as were animals ranking 1, 2, or 3 in groups of 6; the remainder of the monkeys were considered subordinate.

### Statistical analyses

This study is a group randomized trial. Group size was determined by using power calculations with the NQUERY ADVISOR software (version 6.0; Statistical Solutions, Ltd, Saugus, MA). To detect a 15% difference between groups, with a 90% power, 45–47 animals are needed for each group. Data from monkeys within the same social group are expected to be correlated (either positively or negatively) with each other. Repeated measures taken over time for each monkey are correlated as well.

### Table 1

Plasma concentrations of genistein, daidzein, and equol in monkeys fed casein lactalbumin or high-isoflavone soy protein

<table>
<thead>
<tr>
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<th>Casein lactalbumin (n = 48)</th>
<th>High-isoflavone soy protein (n = 46)</th>
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<tr>
<td></td>
<td>Baseline 19 mo 31 mo</td>
<td>Baseline 19 mo 31 mo</td>
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<tr>
<td></td>
<td>nmol/L</td>
<td>nmol/L</td>
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<tr>
<td>Genistein</td>
<td>0.8 ± 0.5</td>
<td>0 ± 0</td>
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<td></td>
<td>4.6 ± 2.1</td>
<td>210.8 ± 25.0</td>
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<tr>
<td>Daidzein</td>
<td>2.4 ± 1.2</td>
<td>0.6 ± 0.4</td>
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<td></td>
<td>9.8 ± 2.6</td>
<td>293.6 ± 33.6</td>
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<tr>
<td>Equol</td>
<td>6.5 ± 0.5</td>
<td>0 ± 0</td>
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<td></td>
<td>7.6 ± 3.1</td>
<td>686.3 ± 63.0</td>
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All values are x ± SE.

1 Some values are missing due to incomplete data collection.
significant negative correlation between WBBMC and BALP bone mass had lower BALP concentrations, as indicated by the consumption of a diet high in soy protein and isoflavones alters DISCUSSION groups (Soy; CL) did not differ significantly between dietary treatment (Soy; CL; n = 46); the difference between animals in the 2 dietary conditions was not significant (P = 0.12). Linear mixed-effects modeling was used for statistical analyses.

At the end of the treatment period, the bone biomarkers BALP and CTX did not differ significantly between dietary treatment groups (P > 0.20; Table 2). However, individuals with greater bone mass had lower BALP concentrations, as indicated by the significant negative correlation between WBBMC and BALP (P < 0.05). Spinal BMD was also negatively associated with BALP, although the correlation coefficient was not significant (P < 0.10). Serum values of CTX were not significantly associated with indexes of bone mass (P > 0.20 for all).

The purpose of the present study was to determine whether consumption of a diet high in soy protein and isoflavones alters bone mass in skeletally mature, soy-naïve premenopausal monkeys. These data indicate that consumption of a soy protein-based diet containing isoflavones in an amount that approximates 129 mg/d for a woman resulted in substantially elevated plasma isoflavone concentrations. However, whereas WBBMC and spinal BMD increased in all individuals over time, there were no significant differences related to dietary treatment. Among other results, the soy diet had no significant effect on BALP, although, for all monkeys, that biomarker was significantly related to bone mass. All animals experienced a significant increase in body weight during the experiment, an effect that was independent of dietary treatment.

Asian women, whose diet typically contains soy, are postmenopausally at lower risk of hip fracture than are white women (33–35). This observation has led to the suggestion that lifelong consumption of soy isoflavones exerts a protective effect in postmenopausal women, either by increasing premenopausal peak bone mass or by decreasing the rate of postmenopausal bone loss. To date, studies done in premenopausal women or nonovariectomized animal models suggest that soy exposure has no consistent beneficial effect on bone in estrogen-normal individuals. For example, in one prospective trial, isoflavone supplementation for 1 y had no significant effect on bone mass among normally

TABLE 2
Plasma concentrations of bone-specific alkaline phosphatase (BALP) and C-terminal crosslink of type 1 collagen in monkeys fed casein lactalbumin or high-isoflavone soy protein

<table>
<thead>
<tr>
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<th>Casein lactalbumin (n = 48)</th>
<th>High-isoflavone soy protein (n = 46)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Baseline 31 mo</td>
<td>Baseline 31 mo</td>
</tr>
<tr>
<td>BALP (ng/mL)</td>
<td>94.1 ± 4.5</td>
<td>82.3 ± 4.1</td>
</tr>
<tr>
<td>CTX (nmol/L)</td>
<td>0.97 ± 0.07</td>
<td>0.89 ± 0.08</td>
</tr>
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1 All values are x ± SEM. Linear mixed-effects modeling was used for statistical analyses. There were no significant differences between dietary conditions at either time (P > 0.2 for both measurements).
cycling women in their 20’s (15). This study specifically evaluated the effect of isoflavones, because both the control (n = 13) and treatment (n = 15) groups received the same amount of alcohol-extracted (isoflavone-deficient) soy protein, whereas the treatment group received a supplement containing 90 mg isoflavones/d. In turn, this outcome is consistent with a premenopausal cohort study indicating that differences in isoflavone consumption are not associated with variation in bone mass (14). Similarly, studies using reproductively intact rats or mice usually do not find significant increases in bone mass after soy or isoflavone supplementation (36, 37).

The current results in nonhuman primates are consistent with the foregoing data from humans and rats, and they support the conclusion that an isoflavone-rich soy diet does not improve bone mass indexes when fed chronically to fully mature, premenopausal individuals. Notably, there is no indication of a negative effect on bone, as might be expected had soy isoflavones interfered substantially with endogenous estrogenic activity. As reported elsewhere, consumption of the soy-based diet had no significant effects on menstrual cyclicity or the concentrations of reproductive hormones (24). Inasmuch as the present study focused on monkeys that had minimal prior exposure to soy and isoflavones, these observations are relevant to the likely effect of soy supplementation on typical premenopausal American women—a group of persons who generally consume little soy during the juvenile or adolescent years and who were not exposed in utero.

It has been suggested that the absence of effects in premenopausal subjects may relate to the inability of isoflavones to exert estrogenic effects when adequate endogenous estrogen is available (19). This hypothesis implies that soy isoflavones may exert an estrogen agonist–like effect in estrogen-deficient persons. In fact, ≥3 randomized trials in relatively young, postmenopausal women have indicated that soy isoflavones exert bone-sparing effects (38–40). In contrast, the Utrecht trial—a study in which women were an average of 18 y past menopause—did not find beneficial effects on bone after 1 y of treatment with a high-isoflavone soy supplement (41). However, a subgroup analysis in the same trial found that the soy supplement had a bone-sparing effect on the younger postmenopausal women. Studies with relatively young ovariectomized rats also indicate that isoflavone-rich soy protein prevents ovariectomy-induced bone loss (42, 43). The monkey data are equivocal on this point: the one published study indicated that soy isoflavones do not reduce bone loss after ovariectomy; however, that study did not have a nonsoy protein control (44). Taken together, the preceding data establish the possibility that high isoflavone soy protein may prevent postmenopausal bone loss if it were started early enough and continued for ≥18 mo (19).

With respect to the serum biomarkers BALP and CTX, we note that BALP was inversely associated with bone mass (for all animals), but there was no significant association between any bone mass measure and CTX. In the present study, then, elevations in bone mass possibly reflected reduced bone turnover (ie, lower BALP). As noted above, soy isoflavone consumption did not significantly alter serum bone biomarker concentrations. In view of the absence of soy effects on bone mass, this finding is not particularly surprising. As is consistent with the current observations, soy isoflavones have been found to have null or only modest effects on bone biomarkers in clinical studies involving men and women of reproductive age. Hence, soy protein and isoflavone supplementation did not alter BALP or deoxypyridinoline concentrations in young men (45, 46). Moreover, when Wangen et al (47) examined the effects of no, low, or high isoflavone intake on osteocalcin and deoxypyridinoline concentrations across the menstrual cycle, they found that isoflavone exposure affected only deoxypyridinoline concentrations (which it increased) and affected them only during the early follicular phase of the cycle (47).

Although radiographic evidence indicated that all females in the present experiment had completed long bone growth, all monkeys nonetheless experienced an increase in bone mass across the study. Similarly, women generally accumulate bone mass into their 30s, even though bone lengthening stops much earlier (23). In the current study, the outcome may also have been influenced by an increase in weight as the animals began consuming experimental diets that were calorically dense as well as high in fat. Another factor affecting bone mass may have been dietary calcium, because these monkeys received calcium at a dose equivalent to that received by a well-supplemented woman (ie, 1300 mg/d).

In summary, chronic consumption of a high-isoflavone soy protein diet did not significantly affect bone mass in skeletally mature, premenopausal monkeys. A similar null finding may be expected among women in industrialized countries, who are largely soy-naïve until adulthood and who are living in a calorically adequate environment.

The authors’ responsibilities were as follows—CJL (primary author): the collection and interpretation of the dependent measures; JRK (principal investigator): the design and overall conduct of the study; HC: the interpretation of results; CPJ: participation in the conception and design of the study; TCR: bone biomarker data; and AAF: the isoflavone analyses. None of the authors had a personal or financial conflict of interest.

REFERENCES


