Neonatal Exposure to Daidzein, Genistein, or the Combination Modulates Bone Development in Female CD-1 Mice

Jovana Kaludjerovic and Wendy E. Ward*

Department of Nutritional Sciences, Faculty of Medicine, University of Toronto, Toronto, Canada M5S 3E2

Abstract

Neonatal exposure to genistein (GEN), an isoflavone abundant in soy, favorably modulates bone mineral density (BMD) and bone strength in mice at adulthood. The study objective was to determine whether early exposure to a combination of the soy isoflavones daidzein (DAI) and GEN that naturally exists in soy protein-based infant formula results in greater benefits to bone at adulthood than either treatment alone. Male and female CD-1 mice (n = 8–16 pups per group per gender) were randomized to subcutaneous injections of DAI (2 mg/kg body weight · d⁻¹), GEN (5 mg/kg body weight · d⁻¹), DAI + GEN (7 mg/kg body weight · d⁻¹), diethylstilbestrol (DES; positive control) (2 mg/kg body weight · d⁻¹), or control (CON) from postnatal d 1–5 and were studied to 4 mo of age. BMD, biomechanical bone strength, and bone microarchitecture were assessed at the femur and lumbar vertebrae (LV). Females treated with DAI, GEN, DAI + GEN, or DES had greater (P < 0.05) BMD at the LV compared with CON and vertebra in the DAI and DES group were more resistant to compression fractures. Microstructural analyses demonstrated that treatment with DAI and GEN resulted in greater (P < 0.05) trabecular connectivity and trabecular thickness, respectively, than the CON. In conclusion, neonatal exposure to DAI and/or GEN had a positive effect on the skeleton of female mice at adulthood, but, compared with individual treatments, DAI + GEN did not have a greater benefit to bone in females or males. J. Nutr. 139: 467–473, 2009.

Introduction

Osteoporosis is a silent disease characterized by a low bone mineral density (BMD)¹ that predisposes an individual to fragility fractures that are associated with considerable morbidity and mortality. With the rapidly aging population, it is estimated that 1 in 2 adults will be at risk for osteoporosis-related fractures by the year 2020 (1). Thus, there is an urgent need to develop prevention strategies to reduce the risk of fragility fracture during aging. A prevention strategy may include optimizing peak bone mass (PBM), the maximum amount of bone mineral acquired at the end of skeletal maturation (2,3). Although it is estimated that 75% of PBM is attributable to genetic factors (4–6), there exists an opportunity for nutrition to modulate PBM (7–10).

Endogenous hormones are critical for the normal development of bone metabolism in males and females. The skeleton may be particularly sensitive to exogenous hormones or food components with potential hormonal activity (i.e. soy isoflavones) when endogenous levels of hormones are low (11,12), as occurs during postnatal life and during aging (11). Short-term exposure to estrogen or compounds with estrogen-like activity during early stages of development can have positive effects on bone health at adulthood. Administration of diethylstilbestrol (DES), a synthetic estrogen, to female mice for the first 5 d of postnatal life resulted in higher BMD and greater bone matrix formation at adulthood (13). A subsequent study reported that exposure to DES during development reduced the number and activity of osteoclasts that contribute favorably to bone mass (14). Recent findings from our laboratory demonstrated that exposure to genistein (GEN), an isoflavone abundant in soy, programs bone metabolism during early life in the CD-1 mouse model at young adulthood (15). Offspring treated with GEN had higher BMD at the lumbar spine, translating into stronger vertebrae that were more resistant to compression fractures. Studies in rodents have focused solely on individual isoflavones, particularly GEN, without examining the effects of isoflavone combinations that naturally exist in soy protein (15,16). For this reason, the combination of daidzein [DAI] and GEN at a ratio equivalent to that occurring in soy protein-based infant formula was used in this study.
Many infants in North America are fed formula within the first 6 mo of life, with a large portion of infants (up to 20%) fed soy protein-based infant formula (17). Recent data indicates that 90% of North American women initiate breast-feeding, but 25% of infants are formula fed by 4 wk postpartum (18). By 3–4 mo postpartum, 62–70% of infants are formula fed (19,20) and by 6 mo postpartum, as high as 89% of infants are exposed to infant formula (20). The levels of isoflavones in 5 different soy protein-based infant formulas ranged from 32 to 47 mg isoflavones/L of formula (21,22). Thus, infants fed soy protein-based formula were exposed to 5.7–11.9 mg isoflavones/kg body weight \(^{-1}d^{-1}\) during the first 4 mo of life. This level of isoflavones is at least 10 times the level of exposure in adults consuming a traditional Asian or vegetarian diet (50 mg/d isoflavones) (23,24). Because infancy is a critical stage of development and sensitive to hormonal stimuli, it is biologically plausible that isoflavones can result in long-term programming effects. Thus, early exposure to isoflavones may provide a preventive strategy that optimizes PBM and bone strength, thereby potentially decreasing the risk of developing osteoporosis and fragility fractures later in life.

The overall study objective was to determine whether early exposure in mice to the combination of DAI and GEN at a ratio equivalent to the amount of soy isoflavones consumed by human infants fed soy protein-based formula results in greater bone mineral, biomechanical bone strength, and improved bone microarchitecture at young adulthood than in mice exposed to DAI or GEN alone.

Methods

Animals and treatment. Six-week-old outbred CD-1 mice (Charles River Laboratories) were bred harem style. Once females were identified as being pregnant, they were housed in individual cages under standard environmental conditions (12-h-light, 12-h-dark cycle; 23°C) and were fed a control diet (AIN93G, Dyets) that was devoid of any estrogenic compounds (25). Fresh water was provided every 2–3 d for ad libitum consumption. Litters with 8–12 pups were randomized to 1 of 5 groups: control (CON; vehicle), DAI (2 mg/kg body weight \(^{-1}d^{-1}\)), GEN (5 mg/kg body weight \(^{-1}d^{-1}\)), DAI+GEN (2 mg DAI/kg body weight \(^{-1}d^{-1}\)+ 5 mg GEN/kg body weight \(^{-1}d^{-1}\)), or DES (2 mg DES/kg body weight \(^{-1}d^{-1}\), positive control) treatment for the first 5 d of life. CON pups received corn oil, because it is used as the vehicle for isoflavones and DES (15). DAI, GEN, DAI+GEN, and DES were solubilized in 1 mL of dimethyl sulfoxide and suspended in corn oil. Each morning from postnatal d (PND) 1 to 5, treatments were administered by gavage to the pups. At PND 5, 1.5 h after the last injection, a subset of pups (n = 4–6 per treatment group) were killed by decapitation to verify the level of isoflavones (GEN, DAI and equol, a metabolite of DAI) using reverse-phase HPLC (26). Pups remained with mothers until weaning at PND 21. At weaning, 4 pups were housed per cage according to gender and fed a control diet (AIN93G, Dyets) (25).

Body weight was measured once weekly. A previous study from our laboratory showed that the BMD and biomechanical strength properties of femurs and lumbar vertebrae (LV) do not significantly change between 3 and 4 mo of age in male and female CD-1 mice, identifying that 4 mo of age is an appropriate stage of the life cycle to represent young adulthood (27). Mice were killed using carbon dioxide followed by cervical dislocation at 4 mo of age. Femurs and LV1–LV3 were cleaned of soft tissue and stored at ~80°C until analyses were performed. All experimental procedures respected the policies set out by the Canadian Council on Animal Care (28) and were approved by the University of Toronto Animal Ethics Committee, University of Toronto.

Bone mineral content and BMD of femurs and LV1–LV3. Bone mineral content (BMC) and BMD of the left femur and LV1–LV3 were determined by dual energy X-ray absorptiometry (pSabre, Orthometrix) using a specialized software program (Host Software version 3.9.4; Scanner Software version 1.2.0). Femurs or LV1–LV3 were placed flat on the machine in a fixed position and were scanned in the air with the following parameters: speed, 2 mm/min; resolution, 0.01 mm \(\times\) 0.01 mm. The CV for femur and LV1–LV3 BMC were 1.4 and 4.0%, respectively and for femur and LV1–LV3 BMD were 1.8 and 4.4%, respectively.

Biomechanical strength properties of femurs and LV2. We measured the biomechanical strength properties of the right femur and LV2 using a materials testing system (Model 4442, Intron) and software (Series IX Automated Materials Tester, version 8.1.5.001) as previously described (15,29). Prior to testing, the right femurs and LV2 were hydrated in 0.9% saline solution for 2 h at room temperature to mimic physiological conditions.

Microarchitecture of the femur and LV4. Micro-computed tomography (GE Healthcare System, model no. MS0900325-0010) was used to analyze the microarchitecture of trabecular and cortical bone in females, but not males, because significant differences in BMD and biomechanical bone strength properties were observed only among females. Trabecular bone was evaluated at the femur head, femur midpoint, and the LV4 and cortical bone was assessed at the femur midpoint and femur neck. For each scan, a femur or LV was embedded inside a wet sponge and placed inside a specimen holder with water. For all scans, the X-ray source was set at 80 kV (tube voltage) and 80 mA (tube current), with an isotropic pixel size of 15 \(\mu\)m for femur and 8 \(\mu\)m for LV2. A 1-mm-thick aluminum filter was employed to minimize beam hardening effects. For femurs, 500 radiographic projections were acquired over an angular range of 180° with a fixed exposure time of 3 s/frame. For LV, which are smaller in size, 900 radiographic projections were obtained over 360° with a fixed exposure time of 3 s/frame.

To analyze a specific bone volume, a contoured region of interest (ROI) was created using the advanced ROI tool (MicroView version ABA 2.2). For femur neck analysis, the ROI was defined from the top of the growth plate to the narrowest part of the femur shaft. For femur midpoint and LV analyses, the total length of the femur or LV4 was measured and an ROI was chosen to represent a 1-mm-thick region around the midpoint of the bone. Measurements of cortical integrity included inner and outer cortical parameters, cortical thickness, cortical area, and bone marrow area. Measurements of trabecular integrity included bone volume/total volume (BV/TV; %), bone surface area/bone volume (BS/BV; mm\(^2\)/mm\(^3\)), trabecular thickness (Th;Th; mm), trabecular number (Tb.N; mm\(^{-1}\)), and trabecular separation (Tb.Sp; mm).

Statistical analyses. Statistical analyses were performed using SigmaStat (version 3.5). Results are expressed as means \(\pm\) SEM. One-way ANOVA was performed to determine differences in microstructural parameters of femur and LV4 in females. Two-way ANOVA was performed for all other outcomes with gender and treatment as the main effects and interaction effects (gender \(\times\) treatment) were assessed. Student-Newman Keuls test was used for comparison of multiple means when statistical differences were observed. Significance was defined as P \(\leq\) 0.05.

Results

Body weight and serum isoflavones. Body weight at weaning and PND 120 did not differ due to isoflavone intervention in either gender (data not shown). By design, serum isoflavone concentrations of mice treated with GEN and DAI were greater (P < 0.05) than all other groups (Table 1). Serum equol concentrations were negligible.

BMC and BMD of intact LV1–LV3 and biomechanical strength of LV2. There was an effect of gender and treatment...
Human infants on BMC and BMD of LV1–LV3. Females had higher BMC (P < 0.001) and BMD (P < 0.001) than males (Table 2). All female groups receiving isoflavones or DES had a higher BMC (P < 0.005) and BMD (P < 0.001) than the female CON group. Males treated with DAI or GEN had a higher (P = 0.005) BMC of LV1–LV3 than males treated with DES. BMD of LV1–LV3 was lower (P < 0.001) in DES-treated males compared with all other groups (Table 2). There was an interaction of gender × treatment for LV1–LV3 BMC (P = 0.005) and BMD (P < 0.001). Peak load of LV2 was higher (P < 0.001) among females compared with males (Table 3). There was a gender × treatment effect with DAI and DES females having greater (P = 0.008) peak load compared with all other groups.

### BMC, BMD, and biomechanical strength properties of the femur.
Femur BMC was higher (P = 0.015) among males compared with females and there was an overall treatment effect due to DAI, as BMC was higher (P = 0.002) with DAI compared with the CON and DES groups (Table 2). Similarly, DAI resulted in greater (P < 0.001) BMD compared with all other groups (Table 2). There was also an interaction (P = 0.023) with DAI-treated females having higher femur BMD than all other groups, and males treated with DAI had higher BMD than DES-treated males (Table 2). Gender did not affect femur midpoint yield load and peak load (P > 0.05) (Table 3). There was an overall treatment effect (P = 0.034) of DES on femur midpoint yield load compared with CON but no gender × treatment interaction (P > 0.05) (Table 3). DAI treatment had an overall positive effect (P = 0.004) on femur midpoint peak load. Females treated with DAI or DES and males treated with DAI or DAI+GEN had greater (P = 0.039) femur midpoint peak load than the female CON and male DES groups, respectively. There was an overall treatment effect (P = 0.007), with all isoflavone groups (DAI, GEN, and DAI+GEN) and DES having greater ultimate stiffness at femur midpoint than the CON group (Table 3).

Gender affected yield load (P < 0.001), peak load (P < 0.001), and stiffness (P < 0.001) at the femur neck, with males having greater values than females (Table 3). For yield load, treatment with DAI, GEN, or DAI+GEN was higher (P = 0.030) than the DES group (Table 3). Males treated with DAI had higher (P = 0.006) femur neck yield load than DES, GEN, or CON males and DAI+GEN-treated males had higher femur neck yield load than DES-treated males (Table 3). DAI treatment had an overall effect (P = 0.002) on femur neck peak load compared with all other treatment groups (Table 3), with DAI males having higher (P = 0.002) femur neck peak load than all other groups (Table 3). The treatments did not affect femur neck stiffness (P > 0.05).

### Microarchitecture of the LV4.
Females treated with DAI, GEN, and DES had higher (P < 0.001) BV/TV at the LV4 compared with CON, and DAI+GEN had intermediate effects.
Treatment with DAI also resulted in higher ($P = 0.010$) BS/BV at the LV4 compared with all other treatment groups (Table 4). Tb.Th was higher ($P = 0.031$) among females treated with GEN and DAI+GEN compared with all other treatment groups (Table 4). Treatment with DAI and/or DES resulted in a higher ($P < 0.001$) Tb.N at the LV4 compared with CON, and DAI+GEN had intermediate effects (Table 4). All treatment groups had lower ($P < 0.001$) Tb.Sp at the LV4 compared with the CON, and female mice treated with DAI or DES had lower ($P < 0.001$) Tb.Sp than GEN- or DAI+GEN-treated mice (Table 4).

**Microarchitecture of femur neck.** At the femur neck, Tb.N was higher ($P = 0.040$) among DES-treated females compared with all other treatment groups (Table 4). Females treated with DAI and GEN had a higher ($P = 0.008$) Tb.Sp compared with the DES group, and DAI+GEN had intermediate effects (Table 4). The outer cortical parameter was higher ($P = 0.022$) among females treated with DAI, GEN, and DES compared with CON, and DAI+GEN had intermediate effects (Table 5). Treatment with DAI and GEN resulted in higher ($P = 0.002$) cortical area at the femur neck compared with CON and DES, and DAI+GEN had intermediate effects. Bone marrow area was higher ($P = 0.004$) among females treated with CON or GEN compared with DES, and DAI alone or with GEN had intermediate effects.

**Microarchitecture of femur midpoint.** At the femur midpoint, Tb.N was lower ($P < 0.001$) among females treated with CON or GEN compared with DAI or DES, and DAI+GEN had intermediate effects (Table 4). BS/BV at the LV4 compared with CON, and DAI+GEN had intermediate effects (Table 5). Treatment with DAI and GEN resulted in higher ($P = 0.002$) cortical area at the femur midpoint compared with CON and DES, and DAI+GEN had intermediate effects. Bone marrow area was lower ($P < 0.001$) among females treated with CON or GEN compared with DES, and DAI alone or with GEN had intermediate effects.
area at the femur midpoint compared with CON or DES; GEN alone or in combination with DAI had intermediate effects (Table 5).

Visual representation of bone microarchitecture. Qualitative assessments (Fig. 1) revealed that females treated with DAI or DES had improved trabecular network at the LV, with GEN and DAI+GEN having intermediary effects. At the femur neck, females treated with DAI exhibited visibly greater cortical thickness than all other treatment groups and the female DES group had visibly improved cortical thickness compared with the CON group. At the femur midpoint, the thickness of the cortical wall was greater among females treated with DAI compared with the female CON group.

Discussion

Exposure to soy isoflavones during the first 5 d of life improved bone development in female CD-1 mice at young adulthood. Moreover, the circulating levels of isoflavones in our mouse model after 5 d of injections were similar to those in human infants fed soy protein-based infant formulas (21). The similar circulating levels of DAI and GEN and the negligible levels of equol may be because both neonatal mouse pups and human infants do not have fully developed bacteria in their intestine that can convert aglycones to secondary metabolites such as equol (22). Female CD-1 mice treated with DAI, GEN, or DAI+GEN had greater BMC and BMD at the LV-1-LV3 compared with CON, but not to each other, suggesting that isoflavones enhance accumulation of bone mineral at this site rich in trabecular bone. These effects are similar to those in female mice treated with DES, suggesting that soy isoflavones have a potential estrogen-like effect on bone development. Improvements in bone mineral among females treated with DAI and DES were translated into stronger individual LV2 that were more resistant to fracture. However, improvements in bone mineral induced by GEN or DAI+GEN did not result in significantly greater peak load at the

**TABLE 5** Cortical bone parameters in female CD-1 mice treated with isoflavones

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>DAI</th>
<th>GEN</th>
<th>DAI+GEN</th>
<th>DES</th>
<th>Treatment P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Femur neck</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Inner cortical parameters, mm</td>
<td>3.63 ± 0.107</td>
<td>3.675 ± 0.100</td>
<td>3.296 ± 0.422</td>
<td>3.618 ± 0.082</td>
<td>3.434 ± 0.299</td>
<td>NS²</td>
</tr>
<tr>
<td>Outer cortical parameters, mm</td>
<td>5.123 ± 0.142</td>
<td>5.739 ± 0.227</td>
<td>5.169 ± 0.718</td>
<td>5.506 ± 0.314</td>
<td>5.064 ± 0.455</td>
<td>NS²</td>
</tr>
<tr>
<td>Cortical area, mm²</td>
<td>0.811 ± 0.052</td>
<td>1.285 ± 0.126</td>
<td>0.994 ± 0.155</td>
<td>1.021 ± 0.087</td>
<td>0.801 ± 0.063</td>
<td>0.006</td>
</tr>
<tr>
<td>Marrow area, mm²</td>
<td>0.994 ± 0.053</td>
<td>1.018 ± 0.063</td>
<td>0.836 ± 0.131</td>
<td>0.961 ± 0.044</td>
<td>0.933 ± 0.092</td>
<td>NS²</td>
</tr>
<tr>
<td>Cortical thickness, mm</td>
<td>0.198 ± 0.007</td>
<td>0.288 ± 0.030</td>
<td>0.235 ± 0.034</td>
<td>0.243 ± 0.016</td>
<td>0.188 ± 0.013</td>
<td>0.007</td>
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</table>

¹ Values are means ± SEM. Means in a row with superscripts without a common letter differ, P < 0.05.
² NS, P ≥ 0.05.
LV2, suggesting that DAI and DES have a greater biological effect on programming of bone than GEN or DAI+GEN. Interestingly, the effects induced by DAI resembled those of DES, despite the fact that DES is 50,000 times more estrogenic than DAI (30). In contrast to females, effects of isoflavones in males were modest, with DAI being the only treatment to provide protection against fracture at the femur neck. No significant benefit or adverse effects were observed at the femur midpoint or LV among males treated with soy isoflavones.

Early exposure to DAI programs bone tissue such that trabecular connectivity, including increased Tb.N and decreased Tb.Sp of LV4, is improved in female mice. Improvements in trabecular connectivity are critical for the prevention of osteoporosis, because it reduces bone porosity and increases the structural support of bones. In situations where trabecular bone is disrupted, deposition of new bone matrix may merely thicken the remaining trabeculae rather than restore continuity, so the bone may never be able to return to normal strength (31). This is not the case with trabecular thinning, because it is a reversible phenomenon that can often be restored through dietary interventions (31). Neonatal exposure to GEN alone or in combination with DAI resulted in greater Tb.Th at the LV of adult female mice and also decreased Tb.Sp. The combination of DAI+GEN did not provide any added benefits to females at young adulthood than either treatment alone and its effects resembled those of GEN rather than DAI. This may be because GEN enters the circulation more quickly than DAI and has a 4- to 10-fold greater binding affinity for estrogen receptors than DAI (32), allowing it to more avidly bind to estrogen receptors and thereby interfere with some of the effects of DAI. Similar to the effects at the lumbar spine, females treated with DAI had higher femur BMC and BMD than all other treatment groups. Greater BMC among the DAI group was associated with higher peak load and stiffness at the femur midpoint compared with the CON group, with GEN and DAI+GEN having intermediary effects. Microstructural analyses of the femur revealed that all isoflavone groups resulted in greater cortical area at the femur midpoint compared with the CON group, with DAI providing the greatest benefit. In addition, treatment with DAI resulted in greater cortical and Tb.Th at the femur midpoint. Improvements in cortical and Tb.Th as well as cortical and trabecular area are associated with improved bone quality (33). Thus, it makes biological sense that mice treated with DAI withstand greater forces before fracture than all other treatment groups. Specific mechanisms of action need to be elucidated. Moreover, the mechanism by which the combination of isoflavones has differing effects from DAI alone needs to be determined.

Potential mechanisms include binding to estrogen receptors to induce estrogen-like effects and/or epigenetic changes in gene expression that may be mediated by alterations in DNA methylation patterns (34). DNA methylation can regulate expression of estrogen receptor-α (35) and proliferation of growth plate chondrocytes (36).

In summary, neonatal exposure to DAI, GEN, and DAI+GEN had a positive effect on bone development in female mice and neither a positive nor negative effect in males. Our findings indicate that neonatal exposure to DAI modulates trabecular connectivity at the LV in female mice at young adulthood and that GEN alone or in combination with DAI modulates Tb.Th at this site rich in trabecular bone. The combination of DAI+GEN does not provide added benefits to bone strength at the femur or LV beyond either treatment alone in both male and female mice and, surprisingly, its effects resembled those induced by GEN. Future studies should investigate the mechanisms by which DAI and GEN modulate bone metabolism and whether benefits to bone development at 4 mo of age can provide protection against the deterioration of bone tissue during aging that is associated with a decline in sex steroid production.

**Literature Cited**

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