Genistein and a Soy Extract Differentially Affect Three-Dimensional Bone Parameters and Bone-Specific Gene Expression in Ovariectomized Mice 1-3

Yan Zhang, 4,5 Qi Li, 4 Hoi-Ying Wan, 6 William G. Helferich, 7 and Man-Sau Wong 4,6 *

4Shenzhen Research Institute of The Hong Kong Polytechnic University, State Key Laboratory of Chinese Medicine and Molecular Pharmacology, Shenzhen, Guangdong 518057, PRC; 5Department of Medicine, Division of Biological Sciences, University of Chicago, Chicago, IL 60637; 6Central Laboratory of the Institute of Molecular Technology for Drug Discovery and Synthesis, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong, PRC; and 7Department of Food Science and Human Nutrition, University of Illinois, Urbana-Champaign, IL 61801

Abstract

Soy isoflavone preparations, such as purified genistein and a soy extract (Novasoy), were reported previously to exert beneficial effects on bones. Our purpose in this study was to compare the effects of genistein and Novasoy on 3-dimensional trabecular bone parameters and the expression of bone-specific genes in ovariectomized (OVX) mice. The sham-operated mice were fed the control diet and OVX mice were fed diets containing genistein or Novasoy or the control diet, with or without 17β-estradiol treatment, for 5 wk. Trabecular bone parameters of tibias were measured by microcomputed tomography and gene expression was assayed by real-time PCR. Consumption of diets containing genistein or Novasoy partially prevented the ovariectomy-induced increase in body weight but did not alter the uterus weight of the OVX mice. Novasoy, but not purified genistein, significantly preserved trabecular bone mass, bone volume, and trabecular bone separation in the proximal tibial metaphysis. Purified genistein decreased mRNA expression of receptor activator of nuclear factor-κB ligand (RANKL), carbonic anhydrase II, and cathepsin K and enhanced the ratio of osteoprotegrin:RANKL mRNA expression in the tibial head of the OVX mice. In contrast, the diet containing Novasoy suppressed the OVX-induced increase in serum alkaline phosphatase but did not alter bone-specific gene expression of tibia. Our study demonstrated that a soy extract containing a similar level of genistein in the form of Novasoy is more effective than purified genistein in improving tibial trabecular bone quality in OVX mice, but the mechanism of action might be distinct from that of genistein. J. Nutr. 139: 2230–2236, 2009.

Introduction

Osteoporosis is a major worldwide public health problem that poses a great economic burden to society as well as to families of patients who suffer from related fractures and have reduced functional independence (1,2). The higher consumption of soy by the Asian population is thought to be one of the contributing factors for the lower incidence of osteoporosis-related fractures in the region (3–6). Data from both human and animal studies using soy isoflavones are mostly supportive of the hypothesis that a diet high in soy exerts beneficial effects on bone health (7–13). Recently, due to the reported risk of breast, endometrial, or ovarian cancer development associated with the use of estrogen replacement therapy, many postmenopausal women have turned to soy isoflavones and considered it as an alternative to estrogen replacement therapy for the prevention and treatment of osteoporosis (14,15).

Many studies indicated that soy isoflavone-rich extracts have bone-preserving functions in postmenopausal women (10,12) and estrogen-deficient animals (13). Novasoy, a commercial isoflavone-enriched product that contains 40% isoflavones and 60% other naturally occurring soy proteins, is a natural alternative for easing the transition through menopause. Novasoy contains soy saponins, protein, some carbohydrates, and other minor components besides isoflavones. To our knowledge, there is not yet any comparison of the efficacy between genistein and Novasoy on bone loss.
Genistein was found to stimulate the growth of subcutaneously implanted MCF-7 cells in ovariectomized (OVX) athymic mice, which had no T cells and did not reject tumor or other cells transplanted from mice (16). On the other hand, genistein exhibited tumor inhibition in intact nude mice fed with estrogen pellets and orthotopically implanted with MCF-7 cells (16). These contradictory results might be due to the differences in the estrogen environment used in the studies. In the former study, genistein was estrogenic and had a proliferative effect on breast tissue in a low estrogen environment, whereas in the latter study, it had an antiproliferative and possibly antiestrogenic effect in a high estrogen environment. The matrix or composition of soy-derived compounds in diet could influence genistein-stimulated tumor growth in OVX athymic mice (17) and mammary cancer in mouse mammary tumor virus-neu mice (18). A recent report indicated that the cancer-inducing ability of soy isoflavones could be greatly altered or blunted in the presence of soy and microarchitecture of a diet containing Novasoy and a diet will affect the osteoprotective effects of soy remains poorly understood.

The principle objective of the present study is to determine whether the protective effects on bone mineral density (BMD) and microarchitecture of a diet containing Novasoy and a diet containing genistein in OVX mice are comparable and if similar mechanisms are involved in mediating their protective actions in bone by studying their effects on bone specific gene expression.

Materials and Methods

Animals study design. Twelve-week-old female C57BL/6j mice (Guangzhou University of Traditional Chinese Medicine, Guangzhou, China) were housed in a specific pathogen-free room with alternating 12-h periods of light and darkness, a constant temperature of 23 ± 1°C, and humidity of 55 ± 5% upon arrival. The mice were acclimated for 2 wk during which time they were allowed free access to tap water and a phytoestrogen-free diet (D00031602). The latter was used as the control diet in this study and was prepared according to the AIN-93M formulation (D10012M) where corn oil was used instead of soybean oil (20) as a fat source to prevent any additional components of soy from being added to the diets. The mice were either dorsal OVX or sham-operated (Sham) under light ether anesthesia. Briefly, the mice were first placed into an induction chamber with ether. Once they had been adequately anesthetized, they were removed from the induction chamber and attached to a nose-cone mask with light ether for the duration of the surgery. Starting from 2 wk postsurgery, the mice were divided into 5 groups: Sham mice with control diet (Sham; n = 10), OVX mice with control diet (OVX; n = 10), OVX mice with control diet and orally administration of 17β-estradiol (E2; 2 mg/kg, n = 12), OVX mice with genistein (D06092701; 500 mg/kg diet, n = 12), and Novasoy (D09092702; 2500 mg/kg diet, n = 12) (Archer Daniels Midland). Novasoy contained 40% of isoflavones in the ratio of 1.3:1:0.3 of genistein:daidzein:glycitein, 7–12% protein, 4% ash, and 6% moisture. The evaluated products were added to a phytoestrogen-free diet to provide equal concentrations of genistein (aglycone equivalents). In addition, diets were isocaloric and nutritionally balanced for fat, protein, and carbohydrate contents. Genistein (500 mg/kg) will generate 2–3 μmol/L, total serum concentration (aglycone + conjugated form of genistein) in mice (21) and these levels are relevant to human soy product consumption (22). The diets were pelleted by dry extrusion and color-coded to ensure quality control during the experiment. The nutritional composition of different diets is shown (Table 1). All mice were individually caged and pair-fed with 3 g/d of the respective diet, the minimum mean food intake in mice during the recovery period. After 5 wk of treatment, blood was taken by cardiac puncture exsanguination. Serum was collected by centrifuging at 1800 × g for 15 min and stored at −80°C for further biochemical analyses. The uterus and bilateral tibias were aseptically removed. We weighed the uterins to confirm the success of the ovariecotomy and the right tibia was cleaned of any adherent soft tissues and stored at −80°C for the detection of gene expression. The left tibia was deposited in a tube with 10% formalin. All procedures were reviewed and approved for consideration of animal welfare by the Animal Ethics Committee of The Hong Kong Polytechnic University.

Table 1 Formulations and estimated nutrient composition of experimental diets

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<th>Nutrient</th>
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*Abbreviations used: ALP, alkaline phosphatase; BMD, bone mineral density; BS, bone surface; BV, bone volume; BV/TV, bone volume:total volume; CAII, carbonic anhydrase II; DA, degree of anisotropy; E2, 17β-estradiol; DPG, osteoprotegerin; OVX, ovariectomized; RANKL, receptor activator of nuclear factor-κB ligand; Sham, Sham-operated; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness.

1 Prepared by Research Diets, Inc., New Brunswick, NJ.
2 The mineral mix composition (AIN-93M) was as follows (amount in 35 g): 5 g Ca, 2 g P, 0.5 g Mg, 3.6 g K, 0.3 g S, 1.0 g Na, 1.6 g Cl, 4.0 g Mn, 0.2 mg Se, and 30.0 mg Zn.
3 The vitamin mixture composition (AIN-93) was as follows (amount in 10 g): 2.2 mg vitamin A (all-trans-retinyl palmitate), 25 μg cholecalciferol, 33.75 mg vitamin E (all-α-tocopherol acetate), 0.75 mg phylloquinone, 0.2 mg biotin, 25 μg cyanocobalamin, 2 mg folic acid, 30 mg nicotinic acid, 16 mg Ca pantothenate, 7 mg pyridoxine-HCl, 6 mg riboflavin, and 6 mg thiamin HCl.
considered significant.

mean expression in bone in this study. measured by micro-CT to avoid the possible underestimation of the gene relative mRNA expression in trabecular bones was corrected by BS as whole bone extract was derived from proximal tibial metaphysis, the smaller in area due to the ovariectomy-induced bone loss. Because the in which the active metaphyseal trabecular BS was likely to be much whole bone extract might underestimate the amount of activity in bones Supplemental Table (concentration-threshold cycle) was generated by the dilution of cDNA of copies of the targeted DNA in the samples, a relative standard curve curve was obtained at the end of each analysis. To determine the number genes. To discriminate specific from nonspecific PCR products, a melting 8 \[8 \text{C for } 15 \text{s}, \text{annealing at } 56 \text{C for } 20 \text{s, and extension } 72 \text{C for } 20 \text{s. The amplification cycle number was 45 for all target genes. To discriminate specific from nonspecific PCR products, a melting curve was obtained at the end of each analysis. To determine the number of copies of the targeted DNA in the samples, a relative standard curve (concentration-threshold cycle) was generated by the dilution of cDNA from the calibrator (Sham group). Data were normalized with glycer- aldehyde 3-phosphate dehydrogenase levels in the samples. The primer sequences used in the present study are available in Supplemental Table 1. A previous study by Westerlind et al. (25) suggested that the use of whole bone extract might underestimate the amount of activity in bones in which the active metaphyseal trabecular BS was likely to be much smaller in area due to the ovariectomy-induced bone loss. Because the whole bone extract was derived from proximal tibial metaphysis, the relative mRNA expression in trabecular bones was corrected by BS as measured by micro-CT to avoid the possible underestimation of the gene expression in bone in this study.

Statistical analysis. The data from these experiments were reported as mean ± SEM for each group. All statistical analyses were performed using PRISM version 4.0 (GraphPad). If variances associated with each experimental mean were unequal (Barlett's test for homogeneity of variances), the data were log-transformed before analysis. Inter-group differences were analyzed by 1-way ANOVA and only followed by Tukey’s multiple comparison test as a post test to compare the group means if the overall P-value was <0.05. Differences of P < 0.05 were considered significant.

Results

Body weight and uterus index. The body weight of the OVX mice at 2 wk after surgery was greater than that in the Sham group (P < 0.01). Administration of 17β-estradiol to the OVX mice prevented the weight gain induced by ovariectomy (Fig. 1). Neither genistein nor Novasoy altered the body weight of the OVX mice within the first 4 wk of treatment. However, by the end of the diet treatment (5 wk), the ovariectomy-induced increase in body weight in mice was reduced by the consumption of diets containing genistein or Novasoy (P < 0.05).

<table>
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<tr>
<th>Group</th>
<th>Uterus weight</th>
<th>Serum Ca</th>
<th>Serum P</th>
<th>Serum ALP</th>
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<td>Sham</td>
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<td>2.51 ± 0.04</td>
<td>2.09 ± 0.15ab</td>
<td>66 ± 6ab</td>
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<td>OVX</td>
<td>0.51 ± 0.02b</td>
<td>2.52 ± 0.04</td>
<td>2.46 ± 0.11a</td>
<td>75 ± 7a</td>
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<tr>
<td>OVX+E2</td>
<td>4.65 ± 0.42ab</td>
<td>2.56 ± 0.05</td>
<td>1.92 ± 0.10a</td>
<td>52 ± 2b</td>
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<tr>
<td>OVX+Genistin</td>
<td>0.62 ± 0.03b</td>
<td>2.58 ± 0.03</td>
<td>2.01 ± 0.11b</td>
<td>71 ± 5b</td>
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<tr>
<td>OVX+Novasoy</td>
<td>0.64 ± 0.03b</td>
<td>2.47 ± 0.04</td>
<td>2.08 ± 0.07b</td>
<td>48 ± 2b</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 9–10. Means in a column with superscripts without a common letter differ, P < 0.05.

The relative uterine weight of the OVX mice was significantly lower than that of the Sham mice (P < 0.001; Table 2), suggesting that the bilateral oophorectomy operations in these mice were successful. 17β-Estradiol, but not genistein or Novasoy, stimulated a dramatic increase in uterus weight in the OVX mice as anticipated (P < 0.001, vs. OVX).

Serum chemistries. Serum Ca concentrations did not differ among the 5 groups (Table 2). Serum P concentrations in the OVX group tended to be higher than those in the Sham group (P = 0.06). Administration of either 17β-estradiol (P < 0.01) or genistein (P < 0.05) reversed the OVX-induced elevation of serum P level in mice. Serum ALP is a biochemical marker of bone formation and is elevated during a high bone turnover state such as estrogen deficiency. Serum ALP levels in the OVX mice were dramatically reduced by treatment with 17β-estradiol or diets containing Novasoy (P < 0.01) but not by diet containing genistein.

Trabecular BMD and bone microarchitecture. BMD in the proximal tibial metaphysis in the OVX group was 18% lower than in the Sham group (P < 0.05; Table 3). This reduction was completely prevented in the OVX mice by the administration of 17β-estradiol (P < 0.01) or diet containing Novasoy (P < 0.05). However, proximal tibial BMD was not significantly induced in...
mice in response to treatment with a diet containing genistein, suggesting that the treatment with genistein only did not effectively prevent bone loss.

The connecting rods were well maintained in the Sham mice (Fig. 2). In the OVX group, however, the structure of trabecular bone network was markedly destroyed and many of the connecting rods were missing. Administration of either 17β-estradiol (a positive bone anabolic agent) or diet containing Novasoy to the OVX mice largely prevented trabecular bone loss and the 3D trabecular bone microarchitecture of the proximal tibia in these mice was maintained at a level similar to that in the Sham mice. Diets containing genistein could only partially improve the ovariectomy-induced bone structural damages in the OVX mice as revealed by the 3D pictures (Fig. 2).

Trabecular 3D parameters were used to evaluate the effects of different treatments on the microstructure of proximal tibial metaphysis in the OVX mice. Tb.Th was significantly lower in the OVX mice than in the Sham group (P < 0.05). 17β-Estradiol increased tibial Tb.Th (P < 0.01) and led to a 36% reduction in Tb.Sp (P < 0.01) of proximal tibia in the OVX mice. Similarly, a diet containing Novasoy could significantly reduce the Tb.Sp (P < 0.05) of proximal tibia in the OVX mice. In contrast, the diet containing genistein did not restore the trabecular microstructural parameters of proximal tibial metaphysis, such as Tb.Th, Tb.Sp, or Tb.Th, in the OVX mice. The architectural anisotropy (DA) of proximal tibial metaphysis in mice was induced by ovariectomy (P < 0.01) and was suppressed in the OVX mice in response to treatments with diets containing genistein or Novasoy (P < 0.05).

Ovariectomy reduced trabecular BV of proximal tibia as revealed by a reduction of BV:TV in the OVX mice compared with the Sham group (P < 0.05; Table 3). The OVX-induced loss of BV and BV:TV was completely inhibited by the administration of 17β-estradiol or diets containing Novasoy in mice (P < 0.05). However, a diet containing genistein did not increase the trabecular BV or BV:TV of proximal tibia in the OVX mice.

**Bone-specific gene expressions.** Because active metaphyseal trabecular BS was likely to be much smaller in area in the OVX mice, the expression of bone-specific target genes in whole bone extract in the present study was corrected by the micro-CT-derived BS measurement of the tibial head. The mRNA expressions of osteoblast-specific genes, ALP and core binding factor 1, in proximal tibia of mice were downregulated by ovariectomy (P < 0.05; data not shown). In contrast, ovariectomy did not alter mRNA expression of the osteoclast-specific genes carbonic anhydrase II (CAII) or cathepsin K in mice (data not shown). Osteoblast-specific gene expressions in proximal tibia of mice did not differ among any treatment groups and the OVX group. Interestingly, the diet containing genistein downregulated the mRNA expression of CAII (P < 0.05) and cathepsin K (P < 0.01) in proximal tibia of the OVX mice. For the osteoprotegrin (OPG)/receptor activator of nuclear factor-κB ligand (RANKL) system (Fig. 3), ovariectomy downregulated the mRNA expression of OPG (P < 0.05; Fig. 3A) and reduced the ratio of OPG:RANKL (P < 0.05; Fig. 3C) in proximal tibia of mice. The expression of OPG (P < 0.01) and RANKL (P < 0.05; Fig. 3B) in the OVX mice was upregulated by 17β-estradiol, which finally led to an increase of the OPG:RANKL ratio (P < 0.05). Treatment with diet containing genistein decreased RANKL mRNA in proximal tibia of the OVX mice (P < 0.01), resulting in an increase in the ratio of

![Image](https://academic.oup.com/jn/article-abstract/139/12/2230/4670588/FIGURE_2)
Sham group. Means without a common letter differ, are the mean ± SEM, n = 9–10, and are expressed as fold of the Sham group. Means without a common letter differ, P < 0.05.

FIGURE 3 mRNA expression of OPG (A) and RANKL (B) and the ratio of OPG:RANKL (C) in the tibial head of Sham mice fed the control diet and OVX mice fed diets containing genistein or Novasoy or the control diet, with or without 17β-estradiol treatment, for 5 wk. Values are the mean ± SEM, n = 9–10, and are expressed as fold of the Sham group. Means without a common letter differ, P < 0.05.

OPG:RANKL mRNA expression (P < 0.05). In contrast, treatment with the diet containing Novasoy increased OPG mRNA expression (P < 0.05) but did not alter the ratio of OPG: RANKL mRNA expression in proximal tibia of the OVX mice.

Discussion

A study using 500 mg of genistein + daidzein/kg diet (genistein: daidzein 1:1) showed that femur BMD could be preserved in OVX CD1 mice (26). The results of the present study showed that the diet containing 500 mg/kg of genistein did not attenuate the decline of trabecular BMD or the destruction of microstructure in proximal tibia of the OVX mice, whereas the diet containing 500 mg/kg genistein in the form of Novasoy could preserve trabecular BMD and attenuate the deterioration of trabecular bone network in the OVX mice. Because Novasoy also contains other soy isoflavones such as daidzein and glycitein (27), these studies suggest that genistein alone might not be sufficient to protect people from ovariectomy-induced bone loss and that other isoflavones in soy such as daidzein are more important for achieving bone protective effects in vivo. This observation is in agreement with others (28,29) who reported that daidzein had greater modulatory effects on bone metabolism than genistein in OVX rats.

The positive effects of diets containing Novasoy or 17-β estradiol on BMD and microarchitecture of proximal tibia were associated with the suppression of serum ALP levels in the OVX mice. The results suggest that Novasoy, but not genistein alone, could mimic 17-β estradiol in suppressing the high bone turnover rate in the OVX mice. The superior effects of Novasoy on bone over genistein alone could be due to: 1) the higher total concentration of isoflavones (1000 mg/kg in the Novasoy-containing diet vs. 500 mg/kg in the genistein-containing diet), because 40% of Novasoy is isoflavones; 2) the presence of other isoflavonoids and nutritional ingredients in addition to genistein; and 3) higher bioavailability, as others reported that the bioavailability values for daidzein, genistein, and glycitein were significantly higher (up to 7-fold) in Novasoy compared with those of the aglycone forms (27). Taken together, the high amount of isoflavones, diversity, and bioavailability of the soy isoflavone components might account for the protective effects of Novasoy on bones.

To compare the effects of genistein and Novasoy on bone at the molecular level, we determined the mRNA expression of several important bone-specific genes that are involved in the process of osteoblast differentiation, osteoclastogenesis, and osteoclast actions in the proximal tibia of the OVX mice. Interestingly, the diet containing Novasoy could only induce OPG mRNA expression but not the expression of other bone-specific genes at the tibial head of the OVX mice despite the fact that it could significantly improve the BMD and bone microarchitecture at the same site of the mice. In contrast, the diet containing purified genistein alone could suppress the expression of RANKL as well as osteoclast-specific genes such as CAII and cathepsin K despite the fact that it could not preserve BMD or most of the microarchitectural parameters at the proximal tibia. These results showed that genistein may exert its effects at gene levels directly and rapidly in vivo within a short time. This was similar to results reported by others that changes of the gene expressions in response to genistein occurred rapidly within 3 d of treatment in vivo (30,31). However, it is unclear why the positive changes in bone parameters in the OVX mice fed the diet containing Novasoy were not associated with any significant changes in the expression of genes involved in osteoclast activities, because the levels of genistein in the diet were similar to those found in the diet containing purified genistein only. Further studies will be needed to delineate how the Novasoy-containing diet achieves its positive effects on bone at the molecular level as well as to determine the time-dependent effects and optimal duration for genistein to achieve bone protective effects in OVX animals.

The results of this study showed that the diet containing genistein could induce a significant reduction of mRNA expression of 2 important genes involved in osteoclastic bone resorption (32,33), namely CAII and cathepsin K in proximal tibia of the OVX mice. This result is consistent with the studies that...
demonstrated the inhibitory effects of pure phytoestrogen, such as coumestrol (34) and genistein (35), on the expression of osteoclast-specific genes in vitro. In particular, these observations were in agreement with a recent in vitro study in which genistein was reported to suppress RANKL signaling-related gene expression such as CAlI and cathepsin K in osteoclastic cells (35). Our study reported that genistein has suppressive effects on the expression of CAlI and cathepsin K in bone tissue of OVX mice, which may be caused at least partially by the attenuation of RANKL signaling in response to genistein treatment.

Our study demonstrated that feeding the diet containing purified genistein could lead to the downregulation of RANKL mRNA expression without altering OPG mRNA expression in the tibia of OVX mice. This result is in agreement with recently reported clinical studies in which lower levels of serum-soluble RANKL were found in postmenopausal women who received genistein treatment for 1 (36) or 3 y (37). The interaction of RANKL and RANK on the surface of osteoblasts and osteoclasts, respectively, is crucial for the induction of osteoclastogenesis (38). Either a decrease in the expression of RANKL or an increase in the expression of OPG, the soluble decoy receptor of RANKL, in osteoblasts could reduce the interaction between RANKL and RANK, thereby potentially suppressing the process of osteoclastogenesis. Thus, our study provides evidence to support that genistein might modulate osteoclastogenesis through its direct actions on regulating RANKL expression in osteoblastic cells in vivo. Furthermore, our study also shows that a Novasoy-containing diet could significantly induce OPG mRNA expression in the proximal tibia of OVX mice, suggesting that the actions of Novasoy on osteoclastogenesis were distinct from those of genistein.

In conclusion, the results of this study demonstrated that the diet containing soy extract in the form of Novasoy was more effective than the diet containing purified genistein in improving tibial trabecular BMD and bone microarchitecture in OVX mice. The potential difference between genistein and Novasoy on bone phenotype may be due to their differential effects on the regulation of bone metabolism and bone remodeling. However, it should be noted that there are some limitations in the application of the mice model for postmenopausal osteoporosis. These include the fact that the magnitude of the cancellous bone turnover is highly strain dependent and that only a minute amount of cancellous bone is present in mice (39). Moreover, clear differences between human and mouse physiology for the actions of estrogens and estrogen analogs on bone were previously demonstrated (40). Thus, further studies are needed to verify the bone protective effects of genistein and Novasoy in humans.

Acknowledgments

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Literature Cited


