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Genistein, a phytoestrogen, improves total cholesterol and Synergy®, a prebiotic, improved calcium utilization but there were no synergistic effects

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Abstract

Objective—Prebiotics and phytoestrogens have sparked great interest because evidence indicates that consumption of these dietary constituents leads to lower cholesterol levels and inhibition of postmenopausal bone loss. The aim of this study was to determine the effect of both a prebiotic (Synergy®) and phytoestrogen (genistein) on bone and blood lipid levels in an animal model of postmenopausal women.

Methods—A four week feeding study was conducted in 5 mo old ovariectomized (OVX) Sprague Dawley rats to examine the effect of genistein, Synergy® (prebiotic) and genistein + Synergy® on bone density and strength, calcium metabolism and lipid biomarkers. There were six treatment groups: SHAM Control, OVX Control, OVX rats receiving daily estradiol injections, and OVX rats receiving an AIN 93M diet supplement with either 200 ppm genistein, 5% Synergy®, or 200 ppm genistein + 5% Synergy®.

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Conflicts of Interest:

LeeCole Legette – No Conflict of Interest

Berdine Martin – No Conflict of Interest

WangHee Lee – No Conflict of Interest

Jon Story – No Conflict of Interest

Ali Arabshahi – No Conflict of Interest

Stephen Barnes – US patent on use of conjugated isoflavones and prevention of osteoporosis

Connie Weaver - Advisory board member for Pharmavite

Results—Rats receiving genistein had significantly lower total serum cholesterol concentration than OVX Control (17%), OVX rats receiving daily estradiol injections (14%), and OVX rats on the 5% Synergy® diet (19%). Synergy® improved calcium absorption efficiency (41%) compared to OVX Control. SHAM Control rats had significantly higher femoral bone density, as determined by underwater weighing, than all OVX groups. Genistein consumption restored total and trabecular BMD at the distal femur similar to levels of SHAM rats.

Conclusions—Genistein supplementation imparts modest heart health benefits and improves bone geometry at the distal femur, and prebiotic consumption (Synergy®) results in improved calcium utilization strength in ovariectomized rats but the combination produced no synergistic effects.

Keywords

genistein; prebiotics; cholesterol; bone rodents

Introduction

Postmenopausal women experience declines in bone and cardiovascular health primarily due to age related changes in metabolism and estrogen deficiency. Prebiotics and phytoestrogens have sparked great interest because evidence indicates that consumption of these dietary constituents leads to lower cholesterol levels and inhibition of postmenopausal bone loss. Prebiotics, including inulin, oligosaccharides, and short chain fructooligosaccharides (sc-FOS), have been shown to enhance mineral absorption and increase bone mineral density (1,2). Mixtures of inulin and oligosaccharides have been reported to impart more bone health benefits than other prebiotics (3). Our previous research demonstrated that chronic feeding of Synergy® (50:50 mix of inulin & sc-FOS) resulted in increased calcium absorption and bone mineral density as well as suppression of bone resorption in ovariectomized rats (4). Prebiotics exert their effects by augmenting anaerobic fermentation in the large intestine resulting in formation of short-chain fatty acids (5). These fermentation products (and other changes caused by polysaccharides) may alter the conditions of the large intestine by changing transit time, decreasing pH, and lowering the conversion of primary to secondary bile acids (5,6). It is postulated that prebiotics create optimal conditions for calcium absorption by lowering pH, inhibiting binding of calcium to bile acids, and increasing absorptive surface area in the large intestine (6). Actions of prebiotics in the intestine have also been associated with other health benefits including positive effects on lipid metabolism (7) and lower cardiovascular disease risk (8).

Phytoestrogens also provide bone and heart benefits. Phytoestrogens, particularly soy isoflavones, have received much attention as an alternative therapy to estrogen for preventing age-related bone loss. Estrogen deficiency, which leads to reduced calcium retention, is the underlying etiology of osteoporosis in postmenopausal women. Earlier work in our lab showed that soy supplements containing genistein-like soy isoflavones were more effective than other commercial soy supplements in suppressing bone resorption in postmenopausal women (9). Other clinical studies have also shown that genistein supplementation helps mediate postmenopausal bone loss (10,11). The primary mode of action for genistein is thought to be through binding to estrogen receptor beta (ER- β), which could repair the imbalance in bone remodeling (bone resorption > bone formation), that occurs with advancing age and hormone deficiency, by suppressing osteoclastogenesis. Genistein raises osteoprotegerin (OPG) levels (12,13). OPG plays a crucial role in blocking osteoclastogenesis by serving as a decoy receptor for receptor activator of nuclear factor κ (RANK) ligand (14). However, other studies have shown that genistein can also influence bone remodeling by promoting bone formation as well as by exerting actions through

estrogen receptor alpha (ER- α) (15–17) suggesting further research is needed to clearly distinguish the effect of genistein on bone remodeling. Dietary phytoestrogens have also been shown reduce cardiovascular disease risk by lowering cholesterol levels (18). It is speculated that phytoestrogens exert heart health benefits by altering lipid metabolism. An *in vitro* study found that genistein lowers oxidative susceptibility of low density lipoprotein (LDL) (19).

Supplementing with a combination of phytoestrogens and prebiotics, sc-FOS and soy isoflavones, resulted in greater bone mineral density (20–22) in several rodent studies. Here we report a study with both a prebiotic (Synergy®) and phytoestrogen (genistein), which have independently shown bone sparing effects with OVX, in an animal model of postmenopausal women, to study mechanisms involved in bone and blood lipid levels in response to ovariectomy. The aims of this study were to determine 1) adaptation of calcium metabolism with prebiotic and genistein consumption and 2) the chronic effects of Synergy® and genistein on bone and heart health. We hypothesized that 1) Synergy® and genistein consumption would improved blood lipid levels and inhibit ovariectomy-induced bone loss by impacting calcium absorption and bone remodeling, respectively and 2) chronic consumption of both would have a greater effect on bone than consumption of Synergy® or genistein alone.

Materials and Methods

Animals

Ninety-six virgin female Sprague Dawley rats were purchased from Harlan (Indianapolis IN, USA) of which 80 were ovariectomized (OVX) and 16 were sham operated at 3 mo old. The animals remained on a maintenance rat chow diet for 2 mo to stabilize after OVX before shipment at 5 mo of age. Rats were housed in individual cages in a temperature and humidity-controlled room with lights on a 12h:12h on-off cycle. An AIN-93M (23) diet and distilled water were provided ad libitum for an acclimation period of two days. All procedures were approved by and performed in compliance with the rules of Purdue University's Animal Care and Use Committee.

Diet

Genistein (Indofine Chemical Company, Hillsborough, NJ, USA) and Synergy® (Orafti, Tienen, Belgium) were mixed thoroughly with AIN-93M base diet (23) ingredients (Research Diets, New Brunswick, NJ, USA) for the following dietary treatments: 200 mg genistein/kg diet, 50 g Synergy®/kg diet, and 200 mg genistein/kg diet + 50 g Synergy®/kg diet.

Study Design

All OVX rats were weighed and assigned to one of the following treatments: OVX–CON, OVX–E2, OVX–GEN, OVX–SYN and OVX–GEN+SYN to ensure similar mean body weight across OVX groups (n=16/group), where CON, E2, GEN and SYN indicate control, estradiol, genistein and Synergy®, respectively. SHAM, OVX–CON, and OVX–E2 groups were placed on AIN- 93M diet and OVX–GEN, OVX–SYN and OVX–GEN+SYN groups were on AIN-93M diets with 200 mg genistein/kg diet, 50 g Synergy®/kg diet, and 200 mg genistein/kg diet + 50 g Synergy®/kg diet, respectively. OVX–E2 rats also received biweekly subcutaneous injections of 25 μ g/kg BW estradiol (Sigma-Aldrich, USA) via a carrier solution of 4% DMSO in saline. After four weeks of dietary treatment (equivalent to one bone remodeling cycle), rats were fasted for 8h before sacrifice. Animals were euthanized by excess CO₂. Blood was drawn via the abdominal aorta and plasma was stored at –80°C for biochemical analysis. Cecal contents were removed and rapidly frozen in liquid

nitrogen before being stored at -80°C for subsequent SCFA analysis. The cecum was flushed twice with 12ml ice-cold saline and cecal wall weight was recorded. Right femurs and tibiae were wrapped in saline soaked gauze and stored at -20°C for evaluation of bone mechanical properties. Left femurs and tibiae were collected and placed in 70% ethanol and stored at 4°C for scanning by peripheral quantitative computed tomography (pQCT).

Biochemical Markers

Plasma genistein concentrations were quantified using a highly sensitive and specific electrospray ionization (17) liquid chromatography-multiple reaction ion monitoring (MRM) mass spectrometry method as described previously (24). Genistein was detected using the transition of its m/z 269 molecular ion to its m/z 133 fragment ion. Plasma total, high density lipoprotein (HDL), and LDL cholesterol levels were determined with use of commercial assays and the COBAS Integra 400 Analyzer (Roche Diagnostics Inc, Sommerville, NJ, USA).

Bone Density, Size and Mechanical Properties

Right femurs and tibiae were measured for length and width at the midshaft using an ABS digimatic solar caliper (Tri-State Instrument Service, Fort Wayne, TX). Bone density was measured by underwater weighing using a density determination kit and Mettler Toledo analytical balance (Mettler Toledo Inc., Columbus, OH). Bone strength was assessed using a 3- point bending test on a materials testing machine (Alliance RT/5, MTS Systems, Eden Prairie, MN) as described previously (25).

Bone Calcium Content

After mechanical testing, right femurs and tibias were dissolved in concentrated nitric acid overnight, diluted with ultrapure water and analyzed for total calcium content using atomic absorption spectrometry (PE S100, Perkin Elmer Inc., Boston, MA, USA).

Calcium Balance and Kinetics

An early metabolic balance (3d after start of treatment) was conducted to determine acute effects on calcium absorption and metabolism. Urine and feces were collected every 24h for 96h and analyzed for calcium. To assess chronic effects of dietary genistein and Synergy® on calcium absorption and metabolism, calcium kinetics and a late metabolic balance were performed during the last week of the feeding study as reported previously (24) with the following modifications in blood collection time points: 0, 5, 15, 30, 60, 80, 130, 180, 240, 480, 720, and 1440 min. Blood samples were centrifuged and serum was collected and stored at -80°C for ^{45}Ca analysis, as previously described by our group (25). For the late metabolic balance, urine and feces were collected every 24h for 96h following oral or IV dosing of ^{45}Ca . The radioactivity of ^{45}Ca was measured in urine and feces. The following parameters were calculated:

$$\text{Total Calcium Balance} = \text{Total Dietary Ca} - (\text{Fecal Ca} + \text{Urinary Ca})$$

$$\% \text{Net Calcium retention} = ((\text{Tracer in oral dose} - (\text{tracer in fecal} + \text{urine excretion})) / \text{Tracer in oral dose}) * 110\%$$

$$\% \text{Endogenous calcium secretion} = (\text{Tracer in fecal excretion of IV dosed rats} / \text{Tracer in IV dose}) * 100\%$$

Calcium absorption efficiency, bone Ca balance, bone formation rate and bone resorption rate was determined using kinetic modeling. For kinetic modeling analysis, blood, urine and fecal samples were fitted by the compartmental modeling approach, utilizing the WinSAAM (Simulation Analysis and Modeling) program (26) available through the National Institutes of Health with methods previously described (25).

pQCT measurements

pQCT analysis was performed with the Stratec peripheral quantitative computed tomograph (model Research SA+ of Norland Stratec XCT, Stratec Electronics, Pforzheim, Germany). 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 from the distal end, were scanned using a 0.46-mm collimation (4×10^5 counts/s) and a 0.08-mm voxel size. Thresholds for segmentation of trabecular and cortical bone were set at 500 mg/cm³ and 900 mg/cm³, respectively.

Short Chain Fatty Acid Analysis

Approximately 0.3 g of cecal contents was ground into a powder using a mortar and pestle. Samples were prepared and analyzed in duplicate. All samples were stored on dry ice during use and liquid nitrogen was added to mortar and pestle to keep samples frozen. Internal standard (500 μ l of 3 mM 2-ethylbutyric acid in 70% ethanol) was added to each sample and samples were stored at 4°C overnight. Samples were centrifuged for 20 min at 11,500 $\times$ g at 4°C. Supernatant (100 μ l) was removed from sample and placed in an injection vial along with 100 μ l of 70% ethanol. Immediately before injection, 10 μ l of 10% (v/v) H₃PO₄ was added to each injection vial. Samples were analyzed for acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate concentrations using a Hewlett-Packard model 6890 gas chromatograph with a flame ionization detector (Agilent Technologies, Palo Alto, CA) equipped with a 30 m, 0.53 mm ID capillary column coated with Hewlett-Packard Free Fatty Acids Phase under conditions described previously (27).

Statistical Analysis

Data were analyzed using Statistical Analysis System (SAS Version 9.1, SAS Institute, Cary, NC). Plasma genistein concentrations were compared between OVX-GEN and OVX-GEN+SYN groups using a two-tailed t-test. Other treatment effects were determined by one way ANOVA and post-hoc comparisons using Dunnett's test (SHAM and OVX-CON served as control groups) after checking the normal distribution and constant variance assumptions of ANOVA. Due to group differences in body weight and calcium intake, ANCOVA analysis was used with body weight and/or calcium intake serving as a covariate when found to influence the model ($p \leq 0.10$). In the case of non-normal distribution, data underwent log transformation before analysis by one-way ANOVA and Dunnett's test. Significance was accepted at $p < 0.05$.

Results

Body and Cecal Wall Weight

Total food intake and calcium intake were significantly lower in rats consuming genistein compared to all other groups. The SHAM group had significantly lower body weight than any other OVX groups except OVX-E2 (Figure 1). Among OVX groups, OVX-E2, OVX-GEN, and OVX-GEN+SYN were similar in body weight and also had significantly lower body than OVXCON and OVX-SYN groups (Figure 1).

Rats consuming Synergy® (OVX-SYN, OVX – GEN + SYN) had a significantly higher cecal wall weight than OVX-GEN and SHAM rats and higher than OVX-CON, and OVX-E2 groups (Figure 2).

Biochemical Analysis

OVX-GEN rats had significantly lower plasma total cholesterol concentrations than OVX-CON (17%), OVX-E2 (14%), and OVX-SYN (19%) groups and similar concentrations were seen in SHAM and OVX-GEN+SYN groups (Figure 3). SHAM rats had significantly lower LDL cholesterol concentrations than OVX-CON, OVX-SYN and OVX-GEN+SYN groups and similar concentrations were seen in OVX-E2 and OVX-GEN rats. SHAM and OVX-E2 had similar HDL cholesterol concentrations but HDL concentrations were lower than those observed in OVX-CON, OVX-GEN, and OVX-GEN+SYN, and OVX-SYN groups.

Plasma genistein concentrations were significantly higher for rats consuming Synergy® with genistein compared to genistein alone (Figure 4).

Bone Calcium Content, Size, and Mechanical Properties

SHAM rats had higher femoral bone density than OVX-CON and OVX-E2 groups (Table 1). SHAM rats had significantly higher femoral breaking strength than OVX-CON, OVX-GEN, and OVX-GEN+SYN groups. Femoral breaking strength of OVX-E2 and OVX-SYN groups did not significantly differ from SHAM rats. SHAM, OVX-CON, and OVX-SYN rats had similar femoral length which was significantly higher than rats consuming genistein. There were no group differences in femoral width. Femoral calcium content was significantly higher in SHAM rats compared to all OVX groups except OVX-E2 rats. Among OVX groups, OVX-CON and OVX-E2 groups had significantly higher femoral calcium content than OVX-GEN, and OVX-GEN+SYN groups.

There were no differences in tibia bone density by underwater weighing, breaking strength, and width among groups. Similar to with the femur, tibia calcium content was significantly higher in SHAM rats compared to OVX groups. Among OVX groups, OVX-GEN+SYN rats had significantly higher tibia calcium content than OVX-GEN rats.

Calcium Balance

In the early metabolic balance, calcium absorption efficiency in the OVX-GEN+SYN group was significantly higher compared to OVX-CON rats (Table 2) but in the late metabolic balance, calcium absorption efficiency was similar among all OVX groups. Fecal calcium and calcium retention did not differ among all groups in either the early or late metabolic balances. However, OVX-SYN, OVX-GEN and OVX-GEN+SYN groups had higher urinary calcium than OVX-E2 rats in the early metabolic balance but not the late metabolic balance where levels were similar across all groups.

Calcium Kinetics

Rats consuming both genistein and Synergy® had significantly higher bone formation and resorption rates than all other groups (Table 3). OVX-E2 and OVX-GEN rats had significantly lower bone resorption rates than OVX-CON and OVX-GEN+SYN groups. SHAM Control rats had a trend ($p=0.07$) for lower bone resorption rate compared to OVX-CON rats. Rats consuming only Synergy had significantly higher calcium absorption efficiency compared to OVX-CON (41%) and OVX-GEN rats. OVX-GEN+SYN rats had significantly higher bone balance than OVX-E2, OVX-GEN, and OVX-CON groups. There was also a trend ($p=0.08$) of higher bone balance for OVX-SYN rats compared to OVX-CON.

pQCT Measurements

The distal femur was more responsive to dietary treatment than either the midshaft or proximal femur. At the distal femur, SHAM rats had significantly higher total BMC and volumetric BMD (vBMD) and trabecular BMD than all OVX groups (Table 1). Genistein consumption with Synergy® resulted in a significant trend for higher trabecular BMD levels compared to OVX- CON.

At the midshaft femur, SHAM rats had significantly higher total BMC than OVX-CON and OVX-GEN+SYN groups as well as a trend ($p=0.09$) for OVX-GEN rats (Table 1). OVX-E2 and OVX-SYN rats had similar total BMC as SHAM rats. SHAM rats had significantly higher total BMD than OVX-CON and OVX-E2 rats. At the proximal femur, SHAM rats had significantly higher total and trabecular BMD than all OVX groups.

Short Chain Fatty Acid Analysis

OVX-SYN and OVX-GEN +SYN rats had significantly higher total SCFA concentration in cecal contents than SHAM rats (Table 4). Rats consuming Synergy® (OVX-SYN and OVX- GEN+SYN) also had significantly higher levels of propionate than all other groups. OVX-SYN rats also had a significantly higher acetate concentration than SHAM rats and higher acetate levels than other OVX groups. There were no significant differences in isobutyrate, butyrate, and isovalerate concentrations among groups.

Discussion

Research involving prebiotics and phytoestrogens has focused on bone and gut health but there has also been great interest in their heart health benefits (8,28). A meta-analysis of 40 randomized controlled trials showed that soy protein and isoflavone supplementation lowered serum lipids in a dose dependent manner (29). Inulin and FOS improved lipid profiles in animals at high doses, but clinical results have been mixed (30). In addition to cardiovascular disease, post menopausal women also have a higher risk of type 2 diabetes and insulin resistance (18). Our study findings indicate that genistein may play a role in ameliorating metabolic syndrome in postmenopausal women. In our study, genistein supplementation lowered total cholesterol while Synergy® consumption had no benefit to lipid levels. We also observed that genistein consumption as well as estradiol injections aided in preventing the increase in weight usually associated with estrogen deficiency. This corresponds with our previous findings that showed estradiol (25) as well as equol (24), a soy isoflavone metabolite, inhibited ovariectomy-induced weight gain. In OVX spontaneously hypertensive rats and Wistar control rats, genistein consumption for 4 weeks led to lower plasma lipids, increased HDL cholesterol and reduced insulin resistance (31).

In rats as well as humans, increases in BMD and BMC are related to risk of fracture. Modest benefits of genistein to BMD and BMC (32) as well as trabecular bone (33) have been reported but without benefit to bone strength or trabecular micro architecture (34,35). Clinical studies showed genistein supplementation improved the bone health of early-mid postmenopausal (1–5 y after menopause) women (average age = 50–55 y). Our study findings indicate that genistein aided in restoring total and trabecular vBMD at the distal femur to levels no different from the pre-menopausal state but few other benefits to bone were observed. Conflicting results in studies could be due to differences in treatment duration, length of stabilization after ovariectomy, method of administration and age at start of treatment. Most studies have been conducted in young animal models (1–3 mo) after receiving subcutaneous (s.c) injections of genistein directly or shortly after menopause (1–4 wks) for short treatment duration (3–4 wks) and showed improvement in femoral bone properties (36–38). Few studies in older animals have assessed the effect of genistein with

longer treatment duration with mixed results. Picherit *et al.* observed no effect of genistein in 12 mo old Wistar rats after administering a daily oral gavage (10µg genistein / g BW) directly after ovariectomy for 3 mo (39). Genistein supplementation provided only minimal benefits to bone micro architecture after daily s.c. injections in 7 mo old Sprague Dawley rats for 15 wks (33). Greater improvements in femoral BMC and BMD were observed in 9 mo Wistar rats after administering genistein (1 and 10 mg/ kg s.c. injections) 6 mo after ovariectomy for 12 weeks (32). Findings from our study as well as previous works suggest that to have bone health effects in stable postmenopausal women, genistein levels need to be higher than doses readily obtained through dietary supplementation. In contrast to genistein, Synergy® consumption improved calcium utilization as determined by the sensitive kinetic modeling approach. It has been proposed that prebiotics exert effects on bone health through improved mineral metabolism by increasing SCFA concentrations, which lowers intestine pH and causes higher mineral absorption. Suggestive of this hypothesis, Synergy® consumption led to higher SCFA concentrations in the cecum, increased calcium absorption efficiency, and higher bone calcium balance as determined by calcium kinetics which is consistent with previous findings (4). Calcium kinetics also showed that rats consuming genistein and Synergy® had higher bone turnover and calcium balance than other groups. However, rats consuming Synergy® only had higher femoral strength than rats consuming genistein and Synergy®, suggesting that the interaction between genistein and Synergy® needs to be further explored. Perhaps, examination of this interaction *in vitro* will expose modes of action on bone metabolism that accounts for effects seen *in vivo*. Increased net calcium absorption efficiency with Synergy®, when combined with genistein, was also observed early but not late in this study. Adaptation effects were also observed with sc-FOS (36).

Supplementation of Synergy® with genistein resulted in higher circulating genistein than genistein alone. Similarly, sc-FOS modified the absorption and enterohepatic recirculation of soy isoflavones in rats (37,38). Higher circulating phytoestrogen levels due to prebiotic consumption could be the main contributor to synergistic effects on femoral (21,22) and lumbar BMD (20) of sc-FOS and isoflavones. In our study, we did not observe synergistic effects of Synergy® (50:50 mix of inulin & sc-FOS) and genistein on bone or heart health which agrees with previous work examining inulin and isoflavones (23). This suggests that potential synergy between prebiotics and phytoestrogens may depend on chain length of oligosaccharides, i.e., sc-FOS vs. the long chains of inulin.

Limitations of our study include multiple outcome comparisons thereby decreasing our power to see individual effects. A longer duration of intervention though at least two remodeling cycles may have shown greater impact of treatment.

Conclusions

In summary, prebiotics, but not dietary genistein, influenced bone health except for distal femur vBMD, whereas genistein showed more impact on heart health. There were no extra health benefits with a combination of a prebiotic and genistein. Dietary genistein may play a role in cardiovascular events associated with menopause as a 200 mg genistein / kg diet dose improved cholesterol profiles in the postmenopausal rat model. This dose correlates to a diet high in soy (i.e., Asian/Vegetarian diet). It can also be obtained with commercially available dietary supplements. The minimal effect on bone observed with genistein supplementation, in stable ovariectomized rats, indicates that genistein may be not beneficial for post-menopausal women in the middle or latter stages of menopause for bone health. Synergy®, at 5% of the diet, increased calcium absorption but did not improve other bone health parameters. Further exploration into the effect of prebiotics on calcium metabolism and subsequent impact on bone properties is needed.

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FIG. 1.

Mean body weight of sham-operated and ovariectomized rats fed diets containing genistein and Synergy (n = 16 per group). Values are expressed as mean $\pm$ SE. Treatment groups were analyzed using ANOVA and Dunnett test, with the sham and OVX-CON groups serving as controls. ***Statistical significance at $P < 0.05$ from sham and OVX-CON controls.

ANOVA, analysis of variance, OVX-CON, ovariectomized rats control group; OVX-E₂, ovariectomized rats receiving estradiol treatment; OVX-SYN, ovariectomized rats receiving Synergy treatment; OVX-GEN, ovariectomized rats receiving genistein; OVX-GEN + SYN, ovariectomized rats receiving both genistein and Synergy.

FIG. 2.

Mean cecal weight of sham-operated and ovariectomized rats fed diets containing genistein and Synergy (n = 16 per group). Values are expressed as mean ± SE. Treatment groups were analyzed using ANOVA and Dunnett test, with the sham and OVX-CON groups serving as controls. *Statistical significance at $P < 0.05$ from sham rat group. ANOVA, analysis of variance, OVX-CON, ovariectomized rats control group; OVX-E₂, ovariectomized rats receiving estradiol treatment; OVX-SYN, ovariectomized rats receiving Synergy treatment; OVX-GEN, ovariectomized rats receiving genistein; OVX-GEN + SYN, ovariectomized rats receiving both genistein and Synergy.

FIG. 3.

Mean plasma total, HDL, and LDL cholesterol levels of sham-operated and ovariectomized rats fed diets containing genistein and Synergy (n = 16 per group). Values are expressed as mean $\pm$ SE. Treatment groups were analyzed using ANOVA and Dunnett test, with the sham and OVX-CON groups serving as controls. *Statistical significance at $P < 0.05$ from sham rat group. ANOVA, analysis of variance, OVX-CON, ovariectomized rats control group; OVX-E₂, ovariectomized rats receiving estradiol treatment; OVX-SYN, ovariectomized rats receiving Synergy treatment; OVX-GEN, ovariectomized rats receiving genistein; OVX-GEN + SYN, ovariectomized rats receiving both genistein and Synergy.

FIG. 4.

Mean plasma genistein levels of sham-operated and ovariectomized rats fed diets containing genistein and Synergy ($n = 16$ per group). Values are expressed as mean $\pm$ SE. Plasma genistein concentrations were compared between OVX-GEN and OVX-GEN + SYN groups using a two-tailed t test. Bars without common letters differ ($P < 0.05$). OVX-CON, ovariectomized rats control group; OVX-E₂, ovariectomized rats receiving estradiol treatment; OVX-SYN, ovariectomized rats receiving Synergy treatment; OVX-GEN, ovariectomized rats receiving genistein; OVX-GEN + SYN, ovariectomized rats receiving both genistein and Synergy.

Table 1
Bone characteristics of sham-operated and ovariectomized rats fed diets containing genistein and/or Synergy®.

	SHAM		OVX		
	CON	E2	SYN	GEN	GEN+SYN
Femoral bone density (g/cm ³) ¹	1.51±0.03	1.46±0.01 [*]	1.47±0.01 [*]	1.47±0.01 [*]	1.46±0.01 [*]
Femoral breaking strength (N)	176±3	162±3 [*]	170±3	160±3	157±3 [*]
Femoral Ca content (mg)	151±2	128±2 [*]	130±2 [*]	122±2 ^{*,**}	118±2 ^{*,**}
Tibia bone density (g/cm ³) ¹	1.53±0.02	1.52±0.02	1.48±0.02	1.49±0.02	1.48±0.02
Tibia breaking Strength (N)	111±4	114±3	118±4	116±4	117±4
Tibia Ca content (mg)	109±2	94±2 [*]	95±2 [*]	93±2 [*]	98±2 [*]
Distal Femur					
Total BMC (mg)	17.8±0.86	10.7±0.85 [*]	12.5±0.72 [*]	11.3±0.74 [*]	11.7±0.72 [*]
Total vBMD (mg/cm ³)	688±35	507±28 [*]	552±23 [*]	548±19 [*]	628±35
Trabecular vBMD (mg/cm ³)	552±21	338±24 [*]	359±20 [*]	374±17 [*]	449±29
Midshaft Femur					
Total BMC (mg)	10.1±0.16	9.31±0.16 [*]	9.75±0.13 [*]	9.64±0.14 [*]	9.49±0.13 [*]
Total vBMD (mg/cm ³)	1000±16	943±16 [*]	919±13 [*]	960±14 [*]	952±13 [*]
Proximal Femur					
Total vBMD (mg/cm ³)	1046±15	936±21 [*]	938±17 [*]	969±18 [*]	924±25 [*]
					934±15 [*]

Note: Values are adjusted mean ± SE.

*, ** denotes statistical significance at p<0.05 from SHAM and OVX Controls, respectively.

Calcium metabolism of sham-operated and ovariectomized rats fed diets containing genistein and/or Synergy® at the beginning and end of the feeding study

Table 2

Metabolic Balance		SHAM	OVX				
Parameters			CON	E2	SYN	GEN	GEN+SYN
Early Balance							
Net Ca absorption efficiency		0.16±0.001	0.12±0.002	0.16±0.01	0.20±0.001	0.15±0.001	0.25±0.00**
Dietary Ca Intake (mg)		352±10	394±11	326±10*	320±10*	295±7*	304±8*
Ca retention (mg)		54±6	38±8	53±12	69±10	49±7	72±12 +
Fecal Ca (mg)		270±8	320±9	273±14	244±17	257±7	238±8
Urinary Ca (mg)		3.28±0.02	3.84±0.03	2.31±0.02**	5.01±0.02	4.45±0.02	6.55±0.04*,**
Late Balance							
Net Ca absorption Efficiency		0.125±0.03	0.158±0.03	0.195±0.03	0.148±0.03	0.194±0.03	0.146±0.03
Dietary Ca Intake (mg)		295±9	292±8	275±8	252±8	235±7	260±7
Ca retention (mg)		19±8	36±7	37±7	19±8	34±8	32±7
Fecal Ca (mg)		245±8	231±7	227±9	246±7	231±8	233±7
Urinary Ca (mg)		4.32±0.52	3.31±0.55	3.39±0.46	3.20±0.02	3.03±0.03	2.62±0.02

Note: Values are adjusted mean ± SE.

*, ** denotes statistical significance at p<0.05 from SHAM and OVX Controls, respectively.

⁺ denotes a trend (0.05< p<0.1) from OVX Control.

Kinetic modeling parameters of calcium metabolism of sham-operated and ovariectomized rats fed diets containing genistein and/or Synergy®

Table 3

Kinetic Parameters	SHAM	OVX			
		CON	E2	SYN	GEN+SYN
Ca absorption efficiency	0.39±0.04	0.34±0.03	0.34±0.03	0.47±0.05**	0.31±0.03
Bone formation (mg Ca /d)	29±4	32±1 ^b	21±2**	33±4	22±3
Bone resorption (mg Ca/d)	12±2 ⁺	20±2	9±2**	15±2	12±2**
Ca balance (mg Ca /d)	14±2	14±1	12±1	23±3 ⁺	10±1
					21±1*,**

Note: Values are adjusted mean ± SE.

*, ** denotes statistical significance at p<0.05 from SHAM and OVX Controls, respectively.

⁺ denotes a trend (0.05< p<0.1) from OVX Control.

Short Chain Fatty Acid Concentrations ($\mu\text{mol/g}$ cecal contents) in cecal contents of sham-operated and ovariectomized rats fed diets containing genistein and/or Synergy®.

Table 4

SCFA	SHAM	OVX			
		CON	E2	SYN	GEN+SYN
Acetate	18 $\pm$ 7.9	25 $\pm$ 8.4	22 $\pm$ 7.5	27 $\pm$ 9.2*	22 $\pm$ 9.1
Propionate	4.8 $\pm$ 2.1	6.5 $\pm$ 1.8	6.0 $\pm$ 2.0	10 $\pm$ 3.1*,**	6.2 $\pm$ 3.1
Isobutyrate	0.4 $\pm$ 0.5	0.2 $\pm$ 0.3*	0.2 $\pm$ 0.3*	0.1 $\pm$ 0.2	0.2 $\pm$ 0.4*,**
Butyrate	3.9 $\pm$ 1.6	4.8 $\pm$ 1.6	4.6 $\pm$ 1.7	3.9 $\pm$ 1.4	4.6 $\pm$ 1.7
Isovalerate	0.9 $\pm$ 1	1 $\pm$ 1	1 $\pm$ 1	1 $\pm$ 1	0.7 $\pm$ 0.7
Valerate	1.2 $\pm$ 2.4	0.7 $\pm$ 0.6	0.4 $\pm$ 0.5	1.3 $\pm$ 1.1	0.4 $\pm$ 0.6
Total SCFA	29 $\pm$ 13	38 $\pm$ 12	34 $\pm$ 11	44 $\pm$ 14*	35 $\pm$ 14
					44 $\pm$ 19*

Note: Values are adjusted mean $\pm$ SE.

*, ** denotes statistical significance at $p < 0.05$ from SHAM and OVX Controls, respectively.