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A diet containing high- versus low-daidzein does not affect bone density and osteogenic gene expression in the obese Zucker rat model

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Abstract

Phytoestrogens are nonsteroidal plant compounds with similar chemical structures to mammalian estrogen capable of mimicking the effect of estrogen in selective tissues. A diet rich in phytoestrogens is associated with a variety of health benefits including decreased risks for heart disease, breast cancer, and osteoporosis. Obesity has long thought to be associated with improved bone density due to increased mechanical loading, but recent literature suggests obesity may actually decrease bone health. Daidzein, a soy-derived phytoestrogen, has been shown to improve parameters of bone health in lean animal models of osteoporosis but has not been tested in obese animals. Following a one-week acclimation to a standard AIN-93G diet, 19 five-week-old female obese Zucker rats (OZR) were randomly assigned to a modified AIN-93G diet containing either high daidzein (HD, 0.121g/kg feed) or low daidzein (LD, 0.01g/kg feed). After 8 weeks, tibias and femurs were removed to assess true density (Archimedes principal), mechanical strength (three-point bending test), and femoral osteogenic gene expression. Serum was collected to assess osteocalcin and deoxypyridinoline. Our results indicated that there were no significant differences between the measures for tibial or femoral true density or mechanical strength for the rats in the HD and LD diet groups. Similarly, there were no significant differences in gene expressions related to osteogenic pathways, or serum biomarkers of bone formation and resorption. Overall, an increased dose of daidzein from soy protein supplementation does not elicit an improvement in markers of bone health in obese Zucker rats.

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Keywords

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INTRODUCTION

Obesity and osteoporosis are global health issues associated with significant morbidity and mortality. According to the most recent epidemiological data from the National Health and Nutrition Examination Survey (NHANES 2015–2016), greater than one-third (39.8%) of American adults are currently obese (body mass index, BMI ≥ 30 kg/m^2).[1] Additionally, obesity is now considered a disease, which independently increases risk for chronic diseases such as type 2 diabetes mellitus (T2DM), cardiovascular diseases, hypertension, stroke, and some types of cancer.[2]

Paradoxically, obesity has been historically associated with a protective effect against osteoporosis, presumably due to the link between obesity and increased bone mineral density (BMD), which is the primary predictor of osteoporosis.[3–5] The relationship between BMD and excess body weight has been attributed to possible mechanisms involving the mechanical loading effect on bone from increased body weight, as well as increased levels of adipose tissue-derived hormones, such as estrone and leptin.[6, 7] For example, estrone was shown to be positively associated with BMD in postmenopausal women.[6] Additionally, early in vitro studies have demonstrated a role for leptin in increasing osteoblast differentiation.[7] However, more recent analyses indicate that the detrimental effects of common obesity induced factors, such as increased inflammation and oxidative stress, may counteract the benefits of body weight on BMD.[8, 9] In support of this hypothesis, evidence from the Global Longitudinal Study of Osteoporosis in Women revealed similar prevalence and incidence of fragility fractures, particularly in the ankle, upper leg, and vertebrae, in postmenopausal women who were obese compared to non-obese. However, postmenopausal obese women were also significantly less likely to be receiving bone protective treatment, possibly due to the aforementioned misconception of intrinsic protection.[10]

Hormone replacement therapy (HRT) has proved to be the most effective treatment of osteoporosis, but presents several health risks including those for coronary artery disease, stroke, and certain forms of cancer.[11] The Women's Health Initiative study raised concerns about the safety of HRT, and, subsequently, women's usage of HRT was reported to significantly decrease.[12, 13] As a response to these reports, search for natural alternatives to prescription medications have increased.[14] Soy foods, in particular, have received considerable attention due to their rich source of health-promoting nutrients and phytochemicals, including isoflavones.[15] Isoflavones are phytoestrogens, or estrogen-like plant compounds found in soybeans and other leguminous plants may structurally and functionally mimic 17 β-estradiol (E_2), the most abundant form of human estrogen, by weakly binding to estrogen receptors (ERs) in estrogen responsive tissue.[16]

Several epidemiological studies support the observation that postmenopausal women with greater consumption of phytoestrogens have higher correlating values for BMD.[17–19] Other studies have shown similar findings in postmenopausal women, but show no effect in

premenopausal women.[20] Clinical interventions with isoflavones by Huang, et al.[21] and Chi, et al.[22] at 100 mg/day and 90 mg/day, respectively, showed that supplementation for 6 months or 1 year could significantly increase BMD in postmenopausal women. However, in the former study, a treatment of 200 mg/day did not produce the same results, perhaps indicating a dose dependence to treatment effect.[21] To date, there have not been any reported epidemiological or intervention studies observing the effects of isoflavones specifically on obese populations.

Early research hypothesized that isoflavones were likely responsible for the bone sparing effects of soy in ovariectomized (OVX) rat models of osteoporosis.[23] However, a study by Picheret et, al. indicated that mixed isoflavone consumption was not effective at increasing bone density in obese Zucker (*fa/fa*) rats (OZR).[24] Other research focused on identifying whether a single isoflavone could be responsible for the bone protective effects of soy. Daidzein, when compared directly to other major isoflavones or in combination, has been shown to be the most effective in preserving bone health in animal models of postmenopausal bone loss.[25, 26] Daidzein, and its metabolite equol, have been shown to suppress bone loss in young[27] and old rats[25], positively affect the biological markers of bone formation, osteocalcin, and bone resorption, deoxypyridinoline (DPD)[25], as well as preserve parameters of bone histomorphometry[26, 28] in OVX rats. While studies have elucidated daidzein's positive effects on bone health stratified by age, sex, and menopausal status, it remains unclear whether daidzein alone will exhibit a similar effect on an obese population. Therefore, the purpose of this study was to investigate whether a high or low daidzein diet will affect the bone density or osteogenic gene expression in female OZR. We hypothesized that a diet high in daidzein would results in greater bone density and osteogenic gene expression, relative to a low daidzein diet, in female OZR.

MATERIALS AND METHODS

Animal Procedures

All experiments were approved by the Institutional Animal Care and Use Committee (Protocol # 3642) at the University of Arkansas for Medical Sciences. Nineteen ($n = 19$) five-week-old female OZR were purchased from Harlan Industries (Indianapolis, IN), for use in this study. Rats acclimated to the housing facility for one week prior to beginning the 8-week diet intervention. Rats were individually housed in cages receiving 12-hour light and dark cycles and provided with *ad libitum* access to both feed and standard drinking water. Upon completion of the study, rats were anesthetized by exposure to carbon dioxide (CO_2) and subsequently euthanized via decapitation. Right femurs and tibias were dissected, cleaned of muscle and connective tissue, and snap frozen in liquid nitrogen and stored at −80°C. Left femurs and tibias were dissected, wrapped in saline (0.9% NaCl) soaked gauze, and stored at −20°C.

Diets

OZR were randomly assigned to either a high daidzein (0.121 g/kg; $n = 10$) modified AIN-93G diet , or a low daidzein (0.01 g/kg; $n = 10$) modified AIN-93G diet, and continued with this diet for 8 weeks thereafter as previously described[30]. High daidzein (HD) and low

daidzein (LD) modified AIN-93G diets were matched to levels of daidzein found in a high isoflavone soy protein isolate (HISPI, 0.121g daidzein/kg) diet or low isoflavone soy protein isolate (LISPI, 0.01g daidzein/kg) diet, accordingly. With the exception of the level of daidzein, the formulas for the diets were identical; thus, the low-daidzein diet group served as control. Each diet contained 3.8 kcal/g of feed. The percentage of kilocalories from macronutrients were as follows: 18.8% protein, 64% carbohydrates, and 17.2% fat. The compositions of both LD and HD diets are listed in Table 1.

Body Weight and Energy Intake

Following the acclimation period, rats were randomized to diet groups facilitating an equalization of baseline mean body weights between the groups, which has been reported elsewhere[29]. Rats were weighed twice weekly for the duration of the study. Feed intake was measured once weekly and occurred over two days. Body weight and energy intake were collected as previously described[30].

Determination of Bone Mineral Density

Left femurs and tibias were first hydrated in distilled water at reduced atmospheric pressure (15 in/Hg) for one hour. After hydration, the weights of the bones were measured both submerged in and out of water using the Mettler Toledo Density Kit ME-DNY-4. Bone densities (g/cm^3) were determined using Archimedes Principle and calculated by the following formula:

$$\text{Density}\left(\text{g/cm}^3\right) = \left(\frac{A}{A-B}\right) \times P$$

where A=weight of hydrated bone out of water, B=weight of hydrated bone submerged in water, and P=density of distilled water at 24°C.

RNA Isolation and Purification

A Spex 6700 freezer mill (SPEX Sample Prep, Metuchen, NJ) was used to pulverize the heads of right femurs, and extraction of RNA was executed by use of TRI Reagent (Sigma-Aldrich, Bellefonte, PA) solution per manufacturers protocols. After RNA extraction, purity and concentration were assessed by reading the absorbance at A260 nm and the ratio of A260/280 nm on a Nano-Drop 1000A Spectrophotometer (Fisher Scientific LLC, Hanover Park, IL).

Analysis of Osteogenic Gene Expression

Reverse transcription of femoral RNA to cDNA was performed using an iScript cDNA Synthesis Kit, according to the manufacturer's protocol. Genes of interest included TNF superfamily member 11 (*Tnfsf11*), TNF receptor superfamily member 11A (*Tnfrsf11a*), TNF receptor superfamily member 11B (*Tnfrsf11b*), NADPH oxidase 4 (*Nox4*), bone gamma-carboxyglutamate protein (*Bglap*), sclerostin (*Sost*), dickkopf WNT signaling pathway inhibitor 1 (*Dkk1*), 5' nucleotidase, ecto (*Nt5e*), Wnt family member 3A (*Wnt3a*), axin 1 (*Axin1*), runt-related transcription factor 2 (*Runx2*), and LDL receptor related protein 5 (*Lrp5*). Genes were normalized to the housekeeping gene, Glyceraldehyde-3-phosphate-

dehydrogenase (*Gapdh*). For each gene being investigated, a master-mix consisting of nuclease free water, the corresponding forward and reverse primer, and SsoAdvanced Universal SYBR Green Supermix was prepared. cDNA samples were then diluted to a concentration of 230 ng/μl based on calculations enabling two technical replicates. In a 384 well plate, 1μl of diluted cDNA and 9 μl of corresponding master-mix were added per well. After covering with MicroAMP Optical Adhesive Film and centrifuging at 1000 rpm for 1 minute, the samples were loaded into a ViiA Real-Time quantitative polymerase chain reaction (qPCR) Detection System set for 40 cycles. Each cycle was performed under the following conditions: denaturation of cDNA strands at 95ºC for 15 seconds, and annealing of the primer and extension of the complementary DNA at 60°C for 1 minute. Calculation of the Ct values and analysis of gene expression amplification plots was performed with the ViiA 7 Software Version 1.1. Differences in gene expression between diet groups were calculated using the delta-delta-CT method.

Analysis of Blood Biomarkers

Blood was collected and serum was separated by centrifuging tubes at 3000 × g at 4ºC and stored at −20ºC until time of analysis. Serum concentration values of deoxypyridinoline (DPD) and osteocalcin were measured with a MicroVue Bone tDPD Assay (Quidel, San Diego, CA; Catalog # 8032) and Rat Osteocalcin enzyme-linked immunosorbent assay (ELISA) Kit (Immunotopics, Inc., San Clemente, CA; Catalog # 60–1505), respectively. All assay procedures were administered according to manufacturers' protocols.

Analysis of Mechanical Strength

Left femurs and tibias were thawed at room temperature, fully hydrated, and measured by caliper to ascertain dimensions for length, width, and height. A TA.XT Plus texture analyzer (Manufacture, City, State) with a 50-kg load cell and a three-point bending rig was used to analyze texture and mechanical properties of bone as an estimation for diaphysis strength and fracturability. Supported horizontally at both ends with a gap distance of 20 mm, femurs were placed with the posterior side facing down and the tibiae were placed on their lateral side. A 3mm width blade travelling 15mm downwards at 1mm/s compressed and fractured the bones on the anterior side at the midpoint between the supports. Force and displacement data, including ultimate load (N), displacement-to-ultimate (mm), stiffness (N/mm), and energy-to-ultimate (mJ) were recorded at 200 Hz by the Exponent software (Version 6, 1, 10, 0; Stable Micro System; Godalming, United Kingdom). In order to account for size variation in samples, Young's modulus of bend (GPa) was calculated as follows:

$$\text{Young's modulus of bend} = \frac{(L^3 \times F)}{(4 \times w \times h^3 \times d)}$$

where L = bone length, F = breaking force, w = bone width, h = bone thickness, d = breaking distance.

Statistical Analysis

All statistical analyses were conducted with IBM SPSS Statistics Version 22 (IBM, Armonk, NY). Statistical significance was determined at a p-value of $p \leq 0.05$. Student's *t*-tests were conducted to compare LD and HD mean body weights, bone mineral density, and osteogenic gene expression of femurs. Results are expressed in terms of mean ± standard deviation (SD).

RESULTS

Body Weight

Mean body weights between groups followed similar trends but began to differentiate over the course of the experiment[29]. At the end of the experiment (age 98 days), body weights (means ± SD) per group were 486 ± 30 g and 476 ± 24 g for LD and HD, respectively. Parametric testing on end-point mean body weights revealed that the difference between the LD and HD diet group means was not statistically significant ($p = 0.408$).

Energy Intake

The weakly mean ± SD kilocalorie intakes were 267 ± 97 kcal kg−1 for the LD group and 265.2 ± 93 kcal kg−1 for the HD group, which were not statistically significantly different according to parametric testing ($p = 0.9$). However, similar to the slight differentiation observed with average feed intake, the end-point mean kcal kg−1 for LD versus HD was 190 ± 13 kcal kg−1 and 176 ± 13.9 kcal kg−1 , respectively. Because the data in the LD group were not normally distributed, a Mann–Whitney U test was performed to compare the difference between the end-point kcal kg−1 values of the diet groups. Distributions of kcal kg−1 intake for LD and HD groups were not similar, as assessed by visual inspection. Energy intake for the LD group (mean rank = 12.67) trended towards being significantly higher than for the HD group (mean rank = 7.6): $U = 21.0$, $z = -1.96$, $p = 0.053$. Overall, kcal kg−1 intake between the diet groups did not significantly differ throughout the experiment.

Bone Mineral Density

True density measurements (g/cm^3) of tibias were 1.33±0.09 and 1.33 ±0.06 for the LD and HD groups, respectively ($p = 0.0835$) (Figure 1, A). Mean true density measurements of femurs were 1.36 ±0.04 for the LD group and 1.38±0.02 for the HD group ($p = 0.30$) (Figure 1, B).

Gene Expression

Gene expression results were expressed in terms of fold change in gene expression from a low to high daidzein treatment normalized to *Gapdh*, as well as by statistical differences in delta-delta CT values between the groups. No differences in gene expression were found for either measure (see Table 2).

Mechanical Strength

Biomechanical properties of strength were measured by the three-point bending test and expressed in terms of ultimate force, displacement, and energy-to-ultimate. Mean measurements for mechanical properties, including ultimate force in tibias ($p = 0.64$) or femurs ($p = 0.49$), displacement for tibias ($p = 0.19$) or femurs ($p = 0.16$), and energy-to-ultimate in tibias ($p = 0.36$) and femurs ($p = 0.09$), were not statistically significant between the LD and HD groups (see Table 3).

Serum Biomarkers

Serum markers of bone turnover, osteocalcin and DPD, were analyzed for comparison between the low and high daidzein diet groups. The mean concentration for serum osteocalcin was measured at 6.04±2.01 ng/ml in the LD and 5.50±2.29 ng/ml in the HD group. Mean concentration for serum DPD was measured at 11.73±1.93 nmol/L in the LD group and 13.19±1.04 nmol/L in the HD group. No statistically significant differences between the groups was observed for either osteocalcin or DPD. (Figures 2 A, B)

DISCUSSION

Although obesity is a disease and a chronic disease risk factor, historically obesity has been viewed as advantageous to bone health due to the protective effect of mechanical loading.[2, 3] Conversely, several epidemiological studies have shown similar risks for fragility fractures in obese populations, leading to the hypothesis that bone protection may be overridden by common obesity-induced factors such as oxidative stress and hormone dysregulation.[8, 10] The reported dangers of HRT have led many to seek natural alternatives to bone protective treatments, including isoflavones, of which daidzein (10 μg/[gram body weight] for 12 weeks and 50 μg/[gram body weight] for 4 weeks) has been shown to be the most effective in various animal models of bone loss.[25–27] However, it remained to be determined whether daidzein treatment could be beneficial to an obese population. Therefore, the objective of this study was to observe the effects of a low and high daidzein diet on the bone density and osteogenic gene expression in OZR. Importantly, the high daidzein dose (~ 10 μg/gram body weight/day) was chosen to mimic the lowest beneficial dose reported in previous studies[25].

Although previous studies have demonstrated a bone-protective effect of daidzein (10–50 μg/gram body weight/day) [25–27], and an increase in osteogenic gene expression in *in vitro* osteoblast cell cultures[30], the present study suggests there is no difference in bone density and osteogenic gene expression in groups treated with low (~ 1 μg/[gram body weight]) and high doses (~ 10 μg/[g body weight]) of daidzein. Unlike previous studies, in which daidzein increased bone density in OVX rat models, the results of our study indicate that the true bone densities of tibias and femurs were not significantly different between obese rats following LD and HD daidzein treatment. Similarly, we did not find any significant differences between the LD and HD groups regarding the expression of several genes involved in the maintenance of bone health. Lastly, daidzein supplementation did not significantly affect serum markers of bone formation, osteocalcin and bone resorption.

However, several results of this study support previous findings, which showed that a treatment of mixed isoflavones had no significant effect on measures of bone health in OZR, despite the relative low bone mass observed in the phenotype. Our results further support the hypothesis that the detrimental effects of obesity on bone health may be more attributable to oxidative stress and leptin resistance, both hallmarks of the OZR model, rather than a deficiency in estrogen like other models of osteoporosis. Therefore, it may be more appropriate in future research to explore treatments, which attenuate the effects of oxidative stress and inflammation when studying obesity-associated osteoporosis rather than traditional methods, which focus on hormone replacement.

Obese white adipose tissue contains an abundance of pro-inflammatory cytokines that can stimulate the differentiation and proliferation of osteoclasts through activation of RANKL and osteoprotogerin[31–33]. Furthermore, superoxide-driven oxidative stress, a common factor in obese adipose tissue, has been shown to stimulate osteoclastogenesis, bone matrix degradation and bone resorption[34–36]. Additionally, white adipose tissue-derived leptin, is increased with obesity and has been implicated in obesity associated osteoporosis, although results from in vivo studies are mixed.[37] A previous publication from this study revealed no difference in serum leptin between low and high daidzein treated OZR.[29] The lack of differences in body weight between groups in the present study may explain why we did not observe differences in measures of bone density and osteogenesis.

The current study is not without several limitations: 1) The intervention was relatively short in length. Perhaps we would have observed differences with a longer term daidzein intervention; 2) We may have seen differences with a higher dose of daidzein over the 8-week intervention period. However, the high daidzein group received ~ 10 μg/g body weight/day of daidzein, which would equate to 700 mg/day for a 70kg human, which is far beyond the dose (100–200 mg/d)[21,22] of other isoflavones that have shown to be protective against bone loss. Thus, increasing the dose may reduce the translational potential of the intervention; 3) Although the rats consuming the diet with a low level of daidzein are considered a control since we added less than 0.01 g daidzein/kg (low level of daidzein was chosen to match the amount of daidzein found in isoflavone-free or low-isoflavone soy protein isolate) this design did allow us to isolate the independent affects of obesity on bone loss, which may have allowed a greater chance of detecting measurable differences; 4) Moreover, the specific phenolic metabolites of daidzein were not quantified in urine or other tissues, which could provide insight into the lack of response observed in the current study.

In summary, results of the present study indicate that there is not a dose-dependent response between low and high daidzein treatment on bone density and markers of osteogenesis in female obese Zucker rats. Importantly, the mechanisms by which bone health is negatively affected in obese populations, remains to be fully elucidated, and may dictate treatments distinct from those used in hormone deficient osteoporosis.

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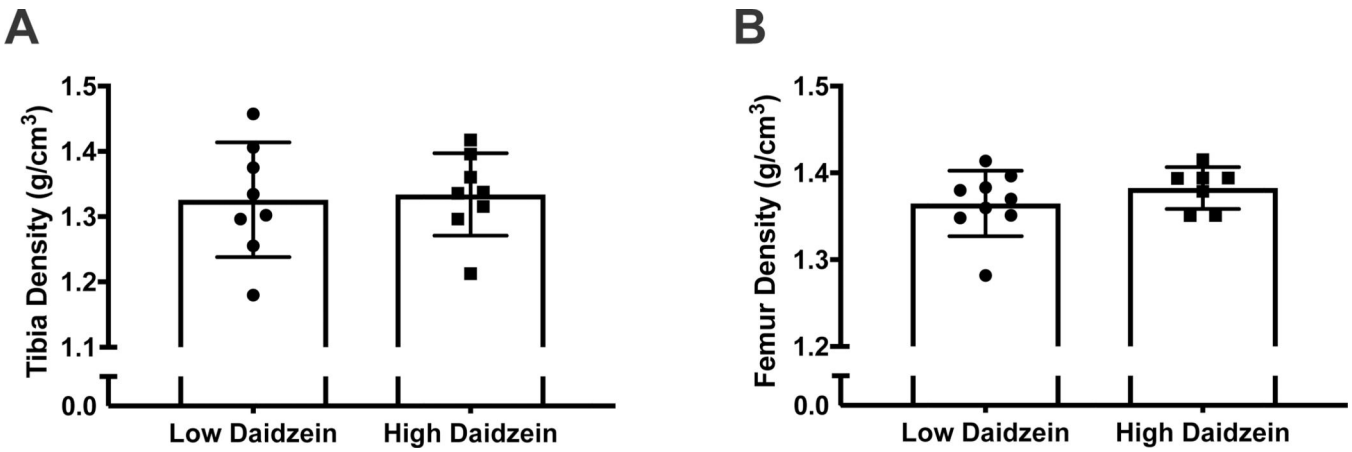

figure 1.

dietary daidzein concentration does not influence tibia and femur density. a) tibia and b) femur density in low (0.01 g/kg) and high daidzein (0.121 g/kg) fed ozr.

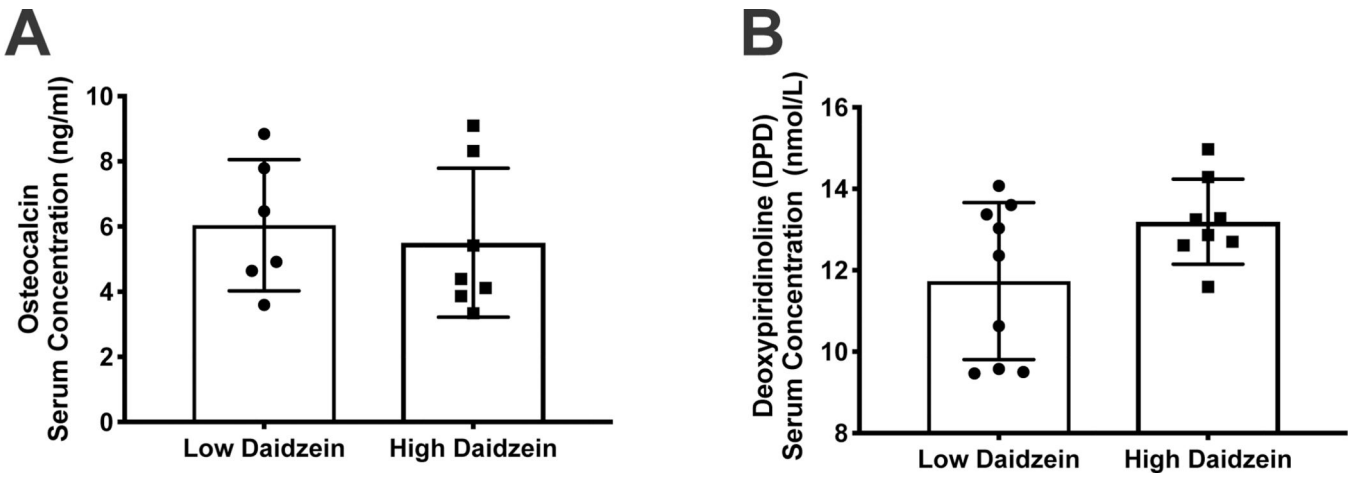

figure 2.

dietary daidzein concentration does not influence serum concentrations of osteocalcin and deoxypiridinoline. serum concentration of a) osteocalcin and b) deoxypiridinoline in low (0.01 g/kg) and daidzein (0.121 g/kg) fed ozr.

Table 1.

Diet Formula

Ingredient	g/kg[a]
Casein	200.0
L-cystine	3.0
Corn starch	397.5
Maltodextrin	132.0
Sucrose	100.0
Corn oil	70.0
Cellulose	50.0
Mineral mix, AIN-93G-MX	35.0
Vitamin mix, AIN-93G-VX	10.0
Choline bitartrate	2.5
TBHQ, antioxidant	0.014
Low daidzein	0.01
High daidzein	0.121

a: Grams of ingredient per kilogram of feed

Table 2.

Fold Change and Relative Significance of Osteogenic Gene Expression Between Obese Zucker Rats Fed Low and High Daidzein Diets

Gene	Fold Change	*P* value
RankL	1.038	0.439
Rank	0.946	0.645
OPG	1.038	0.363
Nox4	0.911	0.429
Bglap	1.115	0.173
Sost	0.992	0.909
Dkk1	0.989	0.502
Ctnnb1	1.001	0.931
Wnt3a	0.999	0.973
Axin1	0.870	0.485
Runx2	1.046	0.761
LRP5	0.996	0.99

Fold Change = fold increase or decrease between low and high daidzein gene expression as measured by rt-qPCR.

Table 3.

Measurements of Mechanical Strength of Femurs and Tibias Between Low and High Daidzein Groups

Measurement	LD Femurs	HD Femurs	LD Tibias	HD Tibias
Length (cm)	3.2 ± 0.13	3.1 ± 0.13	3.5 ± 0.13	3.6 ± 0.13
Ultimate Load (N)	91.8 ± 8.97	89.1 ± 6.62	57.2 ± 5.26	55.9 ± 5.27
Displacement to Ultimate (mm)	1.4 ± 0.37	1.7 ± 0.39	2.1 ± 0.71	1.6 ± 0.36
Energy to Ultimate (mJ)	68.8 ± 16.83	56.0 ± 11.80	30.8 ± 11.48	36.28 ± 10.94

All values expressed as mean ± standard deviation.