ORIGINAL RESEARCH

CURRENT DEVELOPMENTS IN NUTRITION

Nutrition and Exercise Physiology

Bone Strength Is Improved with Genistein Treatment in Mice with Diet-Induced Obesity

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ABSTRACT

Background: High caloric intake of saturated fat and refined sugars accelerates the development of obesity and diabetes and increases bone fracture risk. Some evidence suggests that consumption of a diet rich in phytoestrogens like genistein has the potential to strengthen bone biomechanical properties. Its bone-strengthening properties may mitigate fracture risk associated with metabolic conditions like obesity and diabetes, especially when combined with exercise.

Objective: In this study, we test the effects of genistein, exercise training, and combination treatment on biomechanical properties of cortical bone in mice fed a high-fat, high-sugar (HFHS) diet.

Methods: Eighty C67BL6 mice (40 females, 40 males) aged 6 wk were treated for 12 wk with an HFHS diet containing 60% fat and drinking water with 4.2 g/L sugar (55% sucrose, 45% fructose). Subgroups of the mice were also treated with genistein and/or moderate exercise (treadmill running). Genistein was incorporated into the HFHS diet (600 mg genistein/kg HFHS) and exercise was performed daily for 30 min, 5 d/wk ($n = 10$ females, 10 males per group). Three-point bending mechanical testing and quantitative fluorescence microscopy were conducted on femurs to measure bone strength and matrix quality.

Results: Mechanical testing revealed HFHS-fed mice treated with genistein, either alone or combined with exercise, had femurs that exhibited increased postyield displacement and reduced stiffness during 3-point bending in comparison with mice only treated with the HFHS diet. Femurs of genistein-treated mice also exhibited greater ultimate force required to achieve fracture. Quantitative fluorescence showed genistein reduced advanced glycation end product accumulation in bone matrix. Exercise treatment alone had no effect.

Conclusions: Treatment with genistein, either alone or in combination with exercise, improves fracture resistance in mice fed an HFHS diet by improving bone matrix quality and increasing bone strength. *Curr Dev Nutr* 2019;3:nzz121.

Keywords: obesity, bone, exercise, genistein, fracture, advanced glycation end products

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Abbreviations used: AGE, advanced glycation end product; CTBF, corrected total bone fluorescence; HFHS, high-fat, high-sugar.

Introduction

The effects of obesity on bone fracture risk are multifactorial and complex. Obesity is known to have a positive impact on reducing hip and pelvic fracture risk in humans with elevated BMI (1, 2). However, this protective effect in obese individuals primarily benefits trabecular bone and may not extend to cortical bone in the limbs such as the proximal humerus, which has a fracture risk 30% greater than in normal and underweight individuals (2, 3). Further, reports have suggested the effects may be dependent on diet and treatment duration. A review of the effects of high-fat diet–induced obesity in humans and animal models found short-term increases in bone mass that were reversed over time (4). The addition of high amounts of sugar to the high-fat diet did not exacerbate the bone loss, at least in animal models (5, 6).

Obesity combined with hyperglycemia and hyperinsulinemia, as with metabolic syndrome or type 2 diabetes, is also associated with an

elevated bone fracture risk in human patients relative to the healthy population (7–9). Yet, studies of type 2 diabetics that do not specify obesity as a factor show cortical bone fractures in the limb are relatively uncommon, although limb bones like the tibia and radius demonstrate significantly reduced cortical bone strength (10, 11). These diverse findings highlight the need for further investigation into the relation between diet-induced obesity and fracture risk of cortical bone.

One factor that may help elucidate the effects of a high-fat, high-sugar (HFHS) diet on fracture risk is the composition of the bone extracellular protein matrix. An HFHS diet is associated with the accumulation of advanced glycation end products (AGEs) in the organic portion of bone matrix (12). AGEs are formed by nonenzymatic glycation of proteins, such as collagen in bone, and are known to increase fracture risk in humans and rodents when present in high concentrations in bone matrix (12, 13). Cross-linked collagen in bone tissue with a high concentration of AGEs stiffens the matrix, making it more brittle and susceptible to mechanical injury (12). Treatments centered on reducing AGE accumulation may ameliorate the risk of fracture. Although the mechanism of its action is not thoroughly understood, the phytoestrogen genistein has been previously shown to improve bone microarchitecture and attenuate fracture risk in rodent models (14–16). Whereas their effects are limited in healthy, young adult humans (17), phyto estrogens have been shown to improve fracture resistance and maintain, or even increase, bone mineral density in the aging female population (18–20). Genistein reduces AGE accumulation in multiple tissues including collagen-rich connective tissue of the kidney and lens capsule, and may similarly reduce AGE accumulation in bone matrix (21). Exercise is also believed to provide long-term benefits in relation to bone strength (22), which could be used to counteract the effects of obesity on long bone fracture risk. Although there is evidence to suggest exercise mitigates bone loss associated with high-fat diets in rats (23), it is unclear how it may affect the biomechanical properties of bone when combined with genistein treatment.

With an ever-growing global obese population (24), it is important to find therapeutic means that can combat the negative effects on bone health that are associated with an HFHS diet. In this study, we examined the effects of exercise, genistein treatment, or both in combination, on bone mechanical properties and matrix quality in mice fed an HFHS diet. We hypothesized that these treatments would improve bone strength and reduce AGE content in mice fed an HFHS diet.

Methods

Forty female and 40 male mice (C57BL6; Jax Labs) aged 6 wk were given an HFHS diet for a period of 12 wk. Mice of each sex were randomly divided into the following treatment groups: HFHS controls, HFHS + genistein, HFHS + exercise, and HFHS + genistein + exercise. Ten subjects were assigned to each group. These group sizes were chosen because they were sufficient to detect significant differences in bone morphology in our previous study using genistein treatment in a mouse model for obesity (15). The HFHS diet consisted of pellets with 60% fat, 20% protein, and 20% carbohydrate (Dyets Inc.), and 42 g/L sugar (55% fructose, 45% glucose) dissolved in drinking water administered ad libitum. Monitoring food and water consumption revealed a food intake of 2.97 ± 0.52 g/d and sugar uptake of 3.52 ± 0.21 g/d, which is sufficient to induce obesity, hyperglycemia, and hyperinsulinemia in C57BL6 mice in comparison with controls fed standard diet (25). Exercise training consisted of moderate-intensity running for 30 min/d for 5 d/wk for a total of 150 min/wk on an uninclined motorized treadmill with a speed of 12 m/min (Exer 3/6; Columbus Instruments). Genistein was incorporated in the HFHS diet at a dose of 600 mg genistein/kg HFHS diet (custom formulation, Dyets Inc.). This dose is sufficient to achieve a genistein plasma concentration similar to that observed in human subjects drinking a soy beverage with 2 mg/kg genistein (26, 27). The exercise protocol and genistein dose were chosen because they have been associated with significant improvements in cortical bone strength (15, 28, 29). The exercise training protocol was consistent with the recommendation for moderate-intensity exercise to control complications associated with obesity and diabetes (30). Approval for animal use was granted by the Institutional Animal Care and Use Committee at Midwestern University. Guidelines in the NIH's Guide for the Care and Use of Laboratory Animals were strictly followed. Animals were housed in a facility with a 12-h light/dark cycle and a temperature held constant at 22°C.

Three-point bending tests were conducted on the right femur after killing by inhalation of 100% CO_2 followed by cervical dislocation (29). Left femurs were retained for quantitative fluorescence analysis. Once cleaned of soft tissue, femurs were stored in 70% ethanol and refrigerated at 4°C until testing several days later. During mechanical testing, femurs were placed on supports positioned at each end of the bone. The distance between supports was adjusted for each specimen such that they were placed at the same relative distance from the ends of the bones. In this manner, load-to-fracture was standardized for variations in bone length and diameter in the breaking plane (28). A load was applied at a rate of 0.05 N/s in the posterior–anterior direction at midshaft using a round-edged tip (Model HP-5; Handpi Instruments Co. Ltd.). Femurs fractured consistently at, or near, midshaft where the load was applied. Data collected from the test included postyield displacement, stiffness, and ultimate force. Postyield displacement was the distance the bone was displaced as it yielded under loading until the time it fractured. It is a measure of whole-bone rigidity, with smaller values indicating greater brittleness. Stiffness was the slope of the load-versus-displacement curve and measured whole-bone resistance to displacement under loading. Increased stiffness is indicative of decreased ability to deform when stressed, or ductility. Ultimate force was the maximum load applied during the test, which was achieved at the time of fracture. It is a measure of whole-bone strength, because stronger bones are able to withstand greater applied forces.

AGE accumulation in bone matrix composition was assessed using quantitative fluorescence. Because AGEs are heterogeneous fluorescent derivatives of nonenzymatic glycation and modification of bone collagen, their concentrations in bone matrix can be quantified under fluorescence (31). Undecalcified femurs were sectioned at midshaft at a thickness of 100 μm using a slow speed saw (Isomet; Buehler) and imaged under fluorescence microscopy (Eclipse 55i, 370 nm excitation and 440 nm emission; Nikon Instruments). Corrected total bone fluorescence (CTBF) was calculated by multiplying the cross-sectional area of the bone by the mean gray value of the bone standardized to the mean background color using ImageJ version 1.8 (NIH). CTBF is positively correlated with AGE content in bone matrix, which in turn is corre-

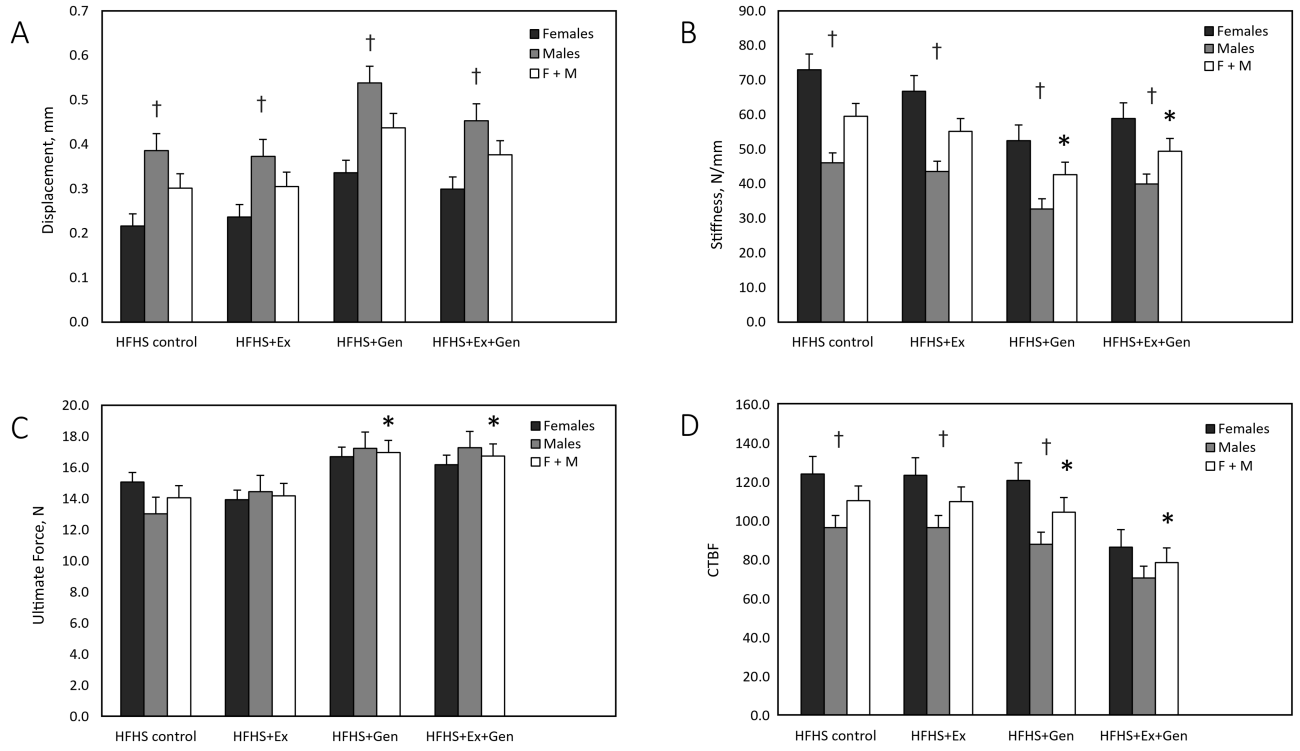

FIGURE 1 Three-point bending mechanical test of femurs for displacement (A), stiffness (B), and ultimate force (C). (D) CTBF was derived from quantitative fluorescence microscopy. Data shown are means ± SEs for females, males, and F + M. Bars are grouped by treatment group. *Significant ($P < 0.05$) difference with HFHS control group, sexes pooled. †Significant ($P < 0.05$) difference between males and females receiving the same treatment. CTBF, corrected total bone fluorescence; Ex, exercise; F + M, both sexes combined; Gen, genistein; HFHS, high-fat, high-sugar diet.

lated with cortical bone stiffness, and thus serves as an indicator of the quality of the bone matrix (28, 32, 33).

Two-factor ANOVA was used to test for significant treatment and sex effects for each of the variables measured, as well as a treatment × sex interaction. Dunnett's *t* test was used post hoc to identify significant differences between HFHS controls and the exercise and genistein treatment groups. Tests of normality and of equality of the variances were used to confirm that statistical assumptions of the analyses were not violated. Statistical significance was set at $P < 0.05$.

Results

Metabolic data from the mice in our study were previously published in our investigation into diet-induced splenomegaly (25). This study found mice fed an HFHS diet had greater body mass ($F = 6.86$, $P < 0.05$) and blood glucose concentrations ($F = 6.75$, $P < 0.05$) than lean controls fed standard unpurified diet. Mice fed an HFHS diet and treated with genistein, either alone or in combination with exercise, had reduced body mass ($F = 8.01$, $P < 0.05$) and plasma glucose concentrations ($F = 14.2$, $P < 0.01$) relative to HFHS controls. Exercise treatment alone did not significantly benefit body mass or plasma glucose concentrations (25).

The 2-factor ANOVA identified significant sex effects for displacement, stiffness, and CTBF ($P < 0.05$). Because the span distance of the supports was varied relative to bone length during 3-point testing, differences in biochemical properties were independent of size. Femurs of female mice exhibited reduced postyield displacement and greater stiffness and CTBF in comparison with those of males ($P < 0.05$). However, the lack of significant interaction between sex and treatment group for all variables ($P > 0.05$) indicated male and female mice responded similarly to treatment with exercise and genistein, both alone and in combination. Therefore, Dunnett's post hoc *t* tests were also conducted with the sexes pooled (**Figure 1**).

Three-point bending mechanical testing of cortical bone of the femur midshaft revealed a significant treatment effect for all variables in the analysis, including postyield displacement, stiffness, and ultimate force (Figure 1; $P < 0.05$). There was also a significant treatment effect for CTBF derived from quantitative fluorescence microscopy ($P < 0.05$). Post hoc analysis showed treatment with genistein, and genistein combined with exercise, significantly reduced femoral stiffness while increasing ultimate force under 3-point bending mechanical loading in comparison with HFHS controls (Figure 1; $P < 0.05$). The analysis also showed treatment with genistein, alone and combined with exercise, reduced the AGE content of bone matrix in mice fed an HFHS diet, as indicated by a significant decrease in CTBF relative to HFHS controls ($P < 0.05$). Together, these findings showed femurs of mice treated with genistein, both alone and combined with exercise, were less brittle and demonstrated greater whole-bone strength than those of HFHS controls. Exercise treatment alone had no effect on any of the measured

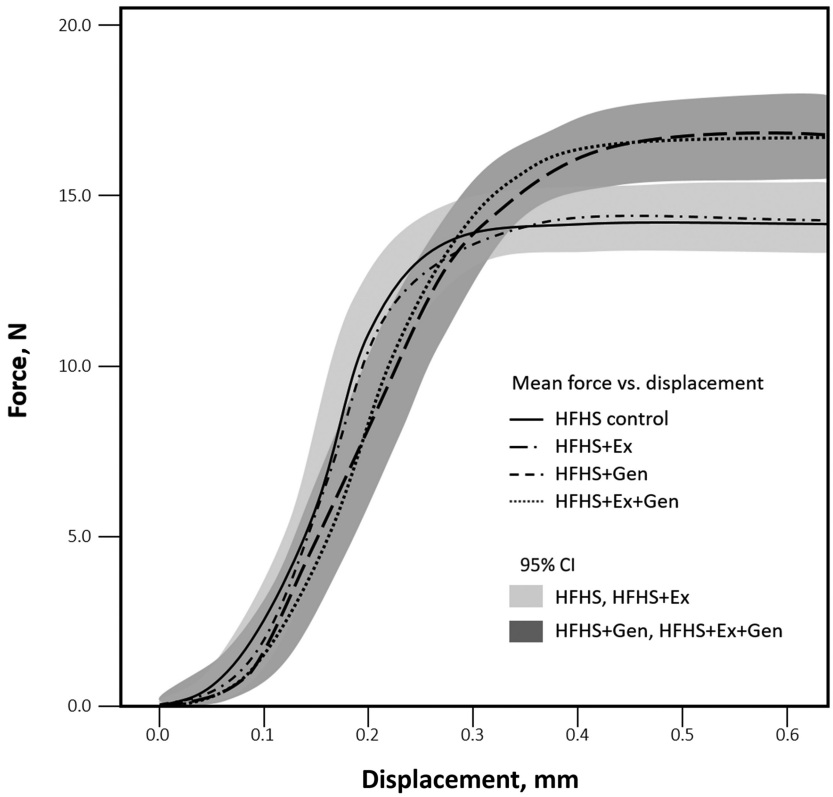

FIGURE 2 Mean force-versus-displacement curves for femurs by treatment group. The slope of the curve corresponds to the stiffness of the bone. The height of the curve represents the ultimate force. Curves derived for HFHS + Gen and HFHS + Ex + Gen mice are steeper and have greater heights than those for mice in the HFHS control and HFHS + Ex groups. Shaded areas represent 95% CIs for the means of the HFHS controls plus the HFHS + EX group, and the HFHS + Gen plus HFHS + Ex + Gen groups. Plots are derived from data points collected at 0.2-s intervals. Females and males pooled. Ex, exercise; Gen, genistein; HFHS, high-fat, high-sugar diet.

bone mechanical or matrix properties in comparison with HFHS controls ($P > 0.05$).

Plots of mean force-versus-displacement curves confirmed femoral response to loading was affected by treatment with genistein, both alone and when combined with exercise (**Figure 2**). The slopes of the force-versus-displacement curves, which correspond to whole-bone stiffness, were reduced in these mice relative to HFHS controls and mice treated with exercise alone, reflecting a greater capacity for deflection under loading. The heights of the force-versus-displacement curves, which correspond to ultimate force, were also steeper for genistein-treated mice than for HFHS controls and mice treated with exercise alone, indicating the ability to withstand greater force before fracturing. Consistent with these findings, stiffness was correlated with CTBF ($r = 0.84$), signifying AGE content in bone matrix had a positive relation with bone stiffness.

Discussion

In aggregate, our findings suggest treatment with genistein, and genistein combined with exercise, but not exercise alone, decrease posttranslational modification of structural proteins in bone matrix, as indicated by decreased CTBF, despite daily intake of an HFHS diet. This improvement in bone matrix quality is strongly correlated with decreased bone stiffness, which in turn is correlated with increased capacity for postyield displacement under loading. These properties are manifested in an increased capacity to withstand loading, as indicated by greater ultimate force in the groups that received genistein as a treatment. In short, femurs of mice that received genistein in their HFHS diets were more ductile, providing them with greater resistance to fracture, than those of HFHS controls or HFHS-fed mice treated with exercise alone.

The relation between the AGE concentration in bone matrix and bone strength is well documented. Sugars like fructose react nonenzymatically with amino acids and other molecules, which subsequently undergo further rearrangement to become irreversibly cross-linked, forming AGEs. Diabetic patients with hyperglycemia accumulate AGEs in their bone tissue at an accelerated rate, which contributes to the poor bone quality and increased fracture risk observed in that population (34, 35). Bone matrix with high AGE content is more prone to fracture because cross-linked collagen in bone matrix alters its mechanical properties by stiffening the matrix, making it more brittle (36–38). Consistent with this observation, we found that HFHS-induced obesity led to a diabetic state characterized by hyperglycemia and hyperinsulinemia (25) that accelerated AGE formation in bone matrix, which increased bone stiffness and decreased the ability to yield when loaded. These factors

are associated with a reduced capacity to bear applied loads, making the bones more susceptible to injury by fracturing under less force than controls.

The beneficial effects of dietary genistein on bone health in the HFHS model are likely due to its role in inhibiting AGE formation, which is accelerated in the diabetic state (12). Phytoestrogens, such as genistein, act as chelating and trapping agents of intermediary molecules in glycation stages of AGE formation (21, 39, 40). Specifically, dicarbonyl compounds like glyoxal and methylglyoxal formed during reactions between sugars and the free amino groups of proteins are trapped by phytoestrogens like genistein (40). Such reactions in bone matrix would limit the formation and accumulation of AGEs that adversely affect the mechanical properties of bone. However, this action has yet to be tested in bone tissue directly and therefore warrants additional investigation.

Lastly, we found differences between female and male mice in their bone mechanical properties and matrix fluorescence representative of AGE accumulation. We found similar patterns of variation in their body mass and blood glucose concentrations (25). The mechanism behind these findings is unclear. Sex differences have been previously identified in the susceptibility to metabolic alterations associated with HFHS diet (25, 41). There is also evidence to suggest sex influences AGE formation and accumulation in tissues (42). Although the mechanisms behind these sex-related manifestations are not fully understood, one hypothesis is that endocrine disruption may be responsible, at least in part. Mice with increased exposure to AGEs have elevated serum testosterone, glucose, and insulin, and depressed estradiol concentrations (43). AGEs may interfere with ovarian steroidogenesis of testosterone and progesterone, an effect that is aggravated by insulin resistance (43, 44). These reports suggest metabolic and hormonal dysregulation may play a role in our findings, although more data are required to draw firm conclusions. Similarly, human and mouse bone cell responses to genistein should be characterized experimentally to identify differences that may exist that affect bone formation and resorption.

In summary, we found increased bone strength and reduced AGE content in mice fed an HFHS diet and treated with genistein in comparison with untreated mice fed the same diet. Similar effects of AGEs on bone strength in the obese, diabetic state have been documented in vivo in human patients (45, 46), although the extreme diet used in this study did not model the human diet. Although our findings show the potential for genistein as a therapeutic agent for skeletal complications associated with obesity and diabetes, more research is needed to elucidate the precise mechanism and benefits of genistein and exercise on human bone health. Their protective properties against glycation-associated changes to bone matrix and strength should be further explored.

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