

# Genistein treatment increases bone mass in obese, hyperglycemic mice

Richard M Michelin<sup>1</sup>

Layla Al-Nakkash<sup>2</sup>

Tom L Broderick<sup>3</sup>

Jeffrey H Plochocki<sup>4</sup>

<sup>1</sup>Arizona College of Osteopathic Medicine, <sup>2</sup>Department of Physiology, <sup>3</sup>Laboratory of Diabetes and Exercise Metabolism, Department of Physiology, <sup>4</sup>Department of Anatomy, Arizona College of Osteopathic Medicine, Midwestern University, Glendale, AZ, USA

**Background:** Obesity and type 2 diabetes mellitus are associated with elevated risk of limb bone fracture. Incidences of these conditions are on the rise worldwide. Genistein, a phytoestrogen, has been shown by several studies to demonstrate bone-protective properties and may improve bone health in obese type 2 diabetics.

**Methods:** In this study, we test the effects of genistein treatment on limb bone and growth plate cartilage histomorphometry in obese, hyperglycemic ob/ob mice. Six-week-old ob/ob mice were divided into control and genistein-treated groups. Genistein-treated mice were fed a diet containing 600 mg genistein/kg for a period of 4 weeks. Cross-sectional geometric and histomorphometric analyses were conducted on tibias.

**Results:** Genistein-treated mice remained obese and hyperglycemic. However, histomorphometric comparisons show that genistein-treated mice have greater tibial midshaft diameters and ratios of cortical bone to total tissue area than the controls. Genistein-treated mice also exhibit decreased growth plate thickness of the proximal tibia.

**Conclusion:** Our results indicate that genistein treatment affects bone of the tibial midshaft in the ob/ob mouse, independent of improvements in the hyperglycemic state and body weight.

**Keywords:** obesity, hyperglycemia, genistein, ob/ob mice, bone

## Introduction

More than one-third of US adults are obese.<sup>1</sup> According to the Centers of Disease Control and Prevention, obesity-related conditions such as hypertension, heart disease, cancer, and type 2 diabetes mellitus (T2DM) are listed as the leading causes of preventable death. Obesity is also a significant risk factor for the development of diseases that affect the skeleton, such as T2DM. In a report by the World Health Organization, ~85% of patients with T2DM were found to be obese. Patients with T2DM also have elevated rates of limb bone fractures, even when controlling for weight and other risk factors.<sup>2-5</sup> T2DM-related loss of cortical limb bone without sufficient compensatory growth of cancellous bone reduces limb bone resistance to bending and elevates bone fracture prevalence in the T2DM population.<sup>6,7</sup>

The relationship between obesity-related T2DM and skeletal health is multifaceted. One important influence on this relationship is leptin, an adipokine released in response to insulin that helps regulate adipose and bone metabolism.<sup>8,9</sup> Leptin resistance due to hyperleptinemia has been implicated in the pathophysiology of insulin resistance and obesity-related T2DM.<sup>10-14</sup> Following onset, obese adolescents and obese females with T2DM display significant relative hypoleptinemia in comparison with obese individuals without T2DM.<sup>15,16</sup> Ob/ob mice that have a mutation in the gene encoding leptin display

Correspondence: Jeffrey H Plochocki  
Department of Anatomy, Arizona College of Osteopathic Medicine, Midwestern University, Glendale, AZ 85308, USA  
Tel +1 623 572 3715  
Fax +1 623 572 3679  
Email jploch@midwestern.edu

elevated insulin levels and T2DM-like hyperglycemia, as well as decreased cortical bone mass and size.<sup>17,18</sup> Ob/ob leptin-deficient mice also exhibit reduced metabolism of epiphyseal plates during growth.<sup>19</sup> Further confirming the role of leptin in the maintenance of bone health is the observation that leptin administration decreases the presence of adipocytes in rodent bone marrow, leading to a reduced production of inflammatory cytokines that promote osteoclastogenesis, thereby promoting bone formation and the maintenance of bone mass.<sup>20</sup> Thus, increased fracture risk associated with T2DM could, in part, be related to the role that leptin plays in skeletal tissue health and maintenance.

It is well documented that estrogen replacement therapy (ERT) has been effective in reducing or reversing postmenopausal bone loss and in reducing the risk of osteoarthritis (OA).<sup>21–24</sup> Although the exact mechanisms are still unclear, it has been suggested that estrogen may interact in an indirect fashion with leptin to affect fat utilization.<sup>25</sup> However, recent studies have shown that ERT is correlated with increased risk of breast, ovarian, and endometrial cancers, as well as cardiovascular disorders.<sup>26,27</sup> Genistein is a phytoestrogen found in soybean products currently being studied as a potential alternative treatment to ERT due to its reported positive effects on postmenopausal depression, bone metabolism, and tumor growth, as well as its use as a dietary supplement to attenuate symptoms of menopause.<sup>28–31</sup> Several studies have raised concerns about the effectiveness of genistein treatment on postmenopausal bone maintenance and the relationship of genistein treatment with cancer risk and other health problems, while others suggest that genistein increases bone mineral density (BMD), and any deleterious effects are related to age and dose dependent or drug interactions with cancer interventions.<sup>32–36</sup> Longitudinal studies lasting a year or more have shown that genistein treatment may exhibit tumor-suppressing qualities, does not alter the expression of the breast cancer susceptibility genes, and may decrease the prevalence of chromosomal aberrations, along with other health benefits.<sup>32,37–40</sup>

Structurally, genistein resembles estrogen and can bind to estrogen receptors with an affinity 100- to 1,000-fold less than that of estradiol.<sup>41</sup> While the mechanism of action of genistein on bone is not yet fully understood, its positive effects on bone are most likely a direct result of greater binding affinity for ER- $\alpha$  compared with ER- $\beta$ , leading to the mineralization phase of bone formation.<sup>42,43</sup> With previous animal studies demonstrating increased BMD and increased bone fracture strength with genistein treatment, as well as clinical studies demonstrating increased BMD

in postmenopausal women with phytoestrogen treatment, it can be presumed that genistein, when ingested, aids in the regulation of bone formation and resorption.<sup>44–50</sup> In addition to increased bone loss and fracture risk, diabetes has also been linked to OA. OA is a degenerative disease characterized by the loss of collagens and proteoglycans as the main structural molecules of articular cartilage.<sup>51</sup> There has yet to be conclusive evidence labeling diabetes as a risk factor for OA; however, obesity has shown to be a risk factor for both conditions, highlighting the need for interventions that can address both OA and diabetes. Epidemiological studies have shown that the prevalence, incidence, symptoms, and severity of OA increase after menopause and are more severe in women.<sup>52–55</sup> These findings indicate a common relationship between OA and estrogen and suggests that genistein treatment could be beneficial to the health of cartilage.

To the best of our knowledge, genistein as a dietary treatment to counteract the negative effects on bone and cartilage seen in obese individuals with T2DM has yet to be explored. The aim of this study is to investigate the effects of genistein treatment on tibial bone remodeling in female leptin-deficient ob/ob mice, which exhibit the type 2 diabetic phenotype.

## Methods

### Animals

The study utilized female ob/ob mice (B6.V-Lep/J) purchased from Jackson Laboratory (Bar Harbor, ME, USA) at 4–5 weeks of age. These mice display leptin deficiency, severe obesity, hyperinsulinemia, and hyperglycemia in a phenotype similar to human patients with T2DM. All mice consumed food and water ad libitum and were housed in an animal facility maintained at a room temperature of 22°C with a 12-hour light/dark cycle. Body weight was measured weekly during the study, and the general health was monitored biweekly. The Institutional Animal Care and Use Committee at Midwestern University approved the study. Animal care followed the guidelines set forth in the National Institutes of Health's *The Guide for the Care and Use of Laboratory Animals* published in 2011.

### Study design

We followed the methodology previously described by Al-Nakkash et al.<sup>56</sup> Briefly, mice were randomly assigned to a genistein-containing or standard rodent diet for a period of 4 weeks. The genistein-treated group consisted of ten mice, and the control group consisted of nine mice; one tibia was excluded due to fracture during the embedding process. The specially formulated genistein-containing diet was prepared

in powder form by Dyets Inc., (Bethlehem, PA, USA), which contains 600 mg genistein/kg. Diets had an energy content estimate of 16.28 kJ/g and contained 20.3 g protein, 66 g carbohydrate, and 5 g fat. Mice were allowed to eat ad libitum. Genistein at the concentration of 600 mg/kg was chosen due to its high bioavailability and maintenance of plasma concentrations comparable to those of soy-based human diets. This is based on earlier work demonstrating that genistein in humans is readily absorbed, with genistein concentrations in plasma of  $\sim 2 \mu\text{mol/L}$ .<sup>57–59</sup> Furthermore, it has previously been reported that the consumption of 750 mg/L genistein also generates plasma genistein concentrations of  $\sim 2 \mu\text{mol/L}$  in mice.<sup>60</sup> Mice were monitored during the experiment, and no mice exhibited distress indicative of treatment intolerance. After 4 weeks of dietary treatment, mice were sacrificed with  $\text{CO}_2$  asphyxiation followed by bilateral pneumothorax, and the hind limb bones were dissected. Blood was collected and centrifuged at 14,500 rpm for 5 minutes, stored at  $-80^\circ\text{C}$ , and later analyzed for glucose using a commercially available kit (Autokit Glucose; Wako Pure Chemical Industries, Osaka, Japan).

## Cross-sectional geometry of the tibia

Following sacrifice, tibias were collected, cleaned, and measured using a digital caliper. Tibias were fixed in formalin, dehydrated in 70% and 85% alcohol, and cleared using Histoclear (National Diagnostics, Atlanta, GA, USA), with each step involving two changes 24 hours apart. Infiltration step 1 was performed using Osteo-Bed Resin A, two changes 48 hours apart.

Infiltration step 2 was performed using Catalyzed Osteo-Bed Resin (100 mL Osteo-Bed Resin A, 1.40 g benzoyl peroxide), two changes 48 hours apart. Embedding solution (100 mL Osteo-Bed Resin A, 3.5 g benzoyl peroxide) was prepared ahead of time. Small amounts were poured into 19 vials that were then placed in a bead bath maintained at  $32^\circ\text{C}$  for 48 hours. After polymerization, tibias were removed from infiltration, marked at the midshaft, placed in each vial, and covered with embedding solution. The samples were again placed in a bead bath maintained at  $32^\circ\text{C}$  for 48 hours to polymerize. A single transverse section was taken at the midshaft of the tibias with an Isomet low-speed saw (Buehler, Lake Bluff, IL, USA). The sections were manually ground to a thickness of  $75 \mu\text{m}$  and digitally captured under microscopy at  $4\times$  magnification using a Nikon Eclipse 55i (Nikon Instruments, Melville, NY, USA). Histomorphometric properties of bone were calculated using the ImageJ v1.44 plugin MomentMacroJ (authored by M

Warfel and revised by S Serafin). These properties included the minimum and maximum second areas of moment ( $I_{\min}$  and  $I_{\max}$ , respectively), polar moment of area ( $J$ ), and area of cortical bone (CtAr). These cross-sectional geometric data are indicative of a bone's ability to resist deforming under mechanical loading. CtAr measures resistance to compressive loading,  $I_{\min}$  and  $I_{\max}$  measure resistance to bending loads in the minor and major axes, respectively, and  $J$  approximates torsional rigidity. Body mass, maximum radius (MaxRad), periosteal perimeter (PsPm), endosteal perimeter (EsPm), the location of the geometric mean on the X-axis ( $X_{\text{bar}}$ ) and Y-axis ( $Y_{\text{bar}}$ ), medullary area (MAR), total area of the cross-section (TtAr), ratios of cortical area to total area of the cross-section (CtAr/TtAr), and marrow area relative to the total area (MAR/TtAr) were recorded as well.

## Histomorphometry of the tibia

After the mice were sacrificed, tibia bones were dissected free of soft tissue and tibial length was measured with a digital caliper. The proximal half of the tibias were decalcified and fixed (Surgipath Decalcifier I; Surgipath Medical Industries, Grayslake, IL, USA) for 4 days. Once decalcified, liquid nitrogen was used to snap-freeze the tibias, and  $12 \mu\text{m}$ -thick coronal sections were made using a cryostat. After staining with aqueous fast green and toluidine blue (Sigma-Aldrich, Co., St Louis, MO, USA), the sections were digitally captured at  $40\times$  magnification (Nikon Instruments). Thickness of the growth plate and the calcified growth plate layer were measured in the middling of the joint using ImageJ.

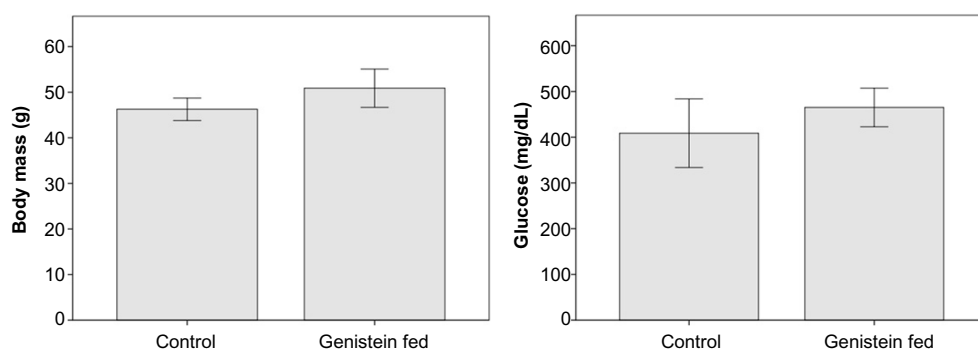
## Statistics

Data were shown as mean  $\pm$  standard error. The  $t$ -tests were used to identify significant differences between treatment groups. Kolmogorov–Simonov test for normality and Levene's tests of homogeneity of the variances were used to ensure assumptions of the  $t$ -tests were not violated. Cross-sectional geometric comparisons were adjusted for body mass. SPSS 19 was used for all statistical analyses (StataCorp LP, College Station, TX, USA). Values of  $P < 0.05$  were considered significant.

## Results

### Genistein does not affect body mass or serum glucose

While the genistein-treated mice had lower body mass overall in comparison with the control group, the difference was not significant ( $P=0.06$ ). Genistein-treated mice had serum glucose levels that were 12.9% lower than controls,



**Figure 1** The effects of genistein treatment on body mass and serum glucose in ob/ob mice.

**Notes:** There was no difference between control and genistein-fed mice in body mass ( $50.9 \pm 2.1$  g and  $46.3 \pm 1.2$  g, respectively,  $P=0.06$ ) or serum glucose in control ( $465.1 \pm 22.1$  mg/dL and  $408.7 \pm 37.5$  mg/dL, respectively,  $P=0.22$ ). Data are shown as mean  $\pm$  2 SE.

**Abbreviation:** SE, standard error.

but again this difference was not statistically significant ( $P=0.22$ , Figure 1).

## Genistein treatment increased bone mass of the tibia

Genistein treatment had no effect on tibial length (Table 1). Nor did genistein treatment affect CtAr,  $I_{\max}$ ,  $I_{\min}$ ,  $J$ , or the periosteal and endosteal perimeters. However, CtAr/TtAr and MaxRad were significantly elevated in genistein-treated mice in comparison with controls ( $P<0.05$ ). MAr was also not significantly elevated or decreased, but MAr/TtAr was significantly reduced in genistein-treated mice in comparison with controls ( $P<0.05$ ).  $X_{\text{bar}}$  was not significantly different

between treatment groups, but  $Y_{\text{bar}}$  was found to be significantly greater in genistein-treated mice ( $P<0.05$ ), indicating a change in location of the geometric mean of the sections.

## Genistein treatment decreases growth plate thickness in the proximal femur

Figure 2 shows micrographs of proximal tibial growth plates of control and genistein-fed mice. Growth plate thickness in genistein-treated mice was found to be significantly smaller in comparison with control mice ( $P<0.05$ ). Genistein treatment had no effect on calcified cartilage thickness (Figure 3).

## Discussion

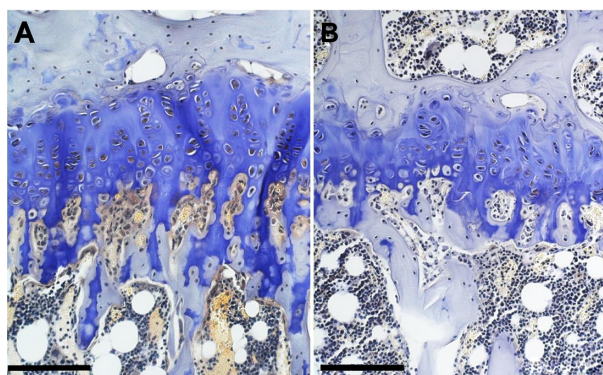
Earlier studies on the effect of leptin on skeletal tissue were inconsistent; however, a clearer picture is emerging that suggests leptin increases the expression of genes that promotes bone formation and decreases the expression of genes that promotes resorption of bone.<sup>10,61–65</sup> As a result, leptin-deficient ob/ob mice exhibit reductions in bone mass and bone formation

**Table 1** The effects of genistein treatment on cross-sectional geometric properties of the tibial midshaft in ob/ob mice

	Control (n=9)	Treated (n=10)	P-value
Tibial length (mm)	16.7 $\pm$ 0.11	16.53 $\pm$ 0.12	0.41
$I_{\max}$ (mm <sup>4</sup> $\times 10^{-2}$ )	4.78 $\pm$ 0.33	4.96 $\pm$ 0.25	0.67
$I_{\min}$ (mm <sup>4</sup> $\times 10^{-2}$ )	4.30 $\pm$ 0.33	4.47 $\pm$ 0.24	0.70
$J$ (mm <sup>4</sup> $\times 10^{-2}$ )	9.08 $\pm$ 0.67	9.47 $\pm$ 0.48	0.64
MaxRad (mm)	1.00 $\pm$ 0.20	1.22 $\pm$ 0.08	<b>0.05</b>
$X_{\text{bar}}$	0.71 $\pm$ 0.08	0.82 $\pm$ 0.20	0.21
$Y_{\text{bar}}$	0.70 $\pm$ 0.15	0.89 $\pm$ 0.19	<b>0.03</b>
PsPm (mm)	3.25 $\pm$ 0.06	3.29 $\pm$ 0.12	0.78
EsPm (mm)	2.27 $\pm$ 0.20	2.20 $\pm$ 0.21	0.52
CtAr (mm <sup>2</sup> )	0.73 $\pm$ 0.03	0.75 $\pm$ 0.02	0.53
MAr (mm <sup>2</sup> )	0.71 $\pm$ 0.02	0.70 $\pm$ 0.02	0.76
TtAr (mm <sup>2</sup> )	1.44 $\pm$ 0.05	1.46 $\pm$ 0.04	0.84
CtAr/TtAr (%)	50.5 $\pm$ 0.44	51.7 $\pm$ 0.34	<b>0.05</b>
MAr/TtAr (%)	49.4 $\pm$ 0.43	48.3 $\pm$ 0.33	<b>0.05</b>

**Notes:** Values are expressed as mean  $\pm$  SE. Bold values represent statistically significant P-values.

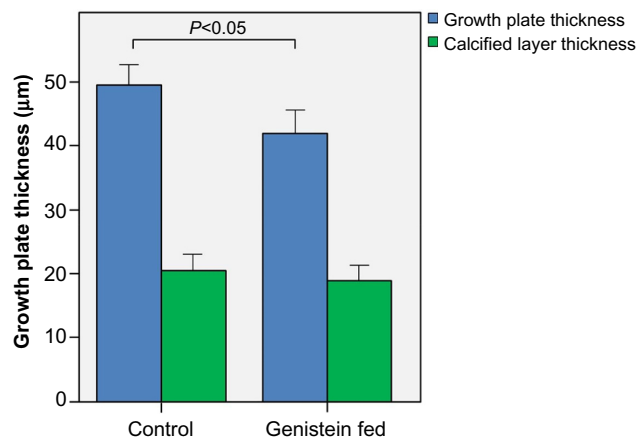
**Abbreviations:** MaxRad, maximum radius; PsPm, periosteal perimeter; EsPm, endosteal perimeter; CtAr, cortical area; TtAr, total area of the cross-section; MAr, medullary area; SE, standard error;  $I_{\max}$ , maximum second area of moment;  $I_{\min}$ , minimum second area of moment;  $J$ , polar moment of inertia;  $X_{\text{bar}}$ , X-axis geometric mean;  $Y_{\text{bar}}$ , Y-axis geometric mean.



**Figure 2** Micrographs of the proximal tibial growth plate of (A) control and (B) genistein-fed ob/ob mice.

**Notes:** Scale bar is 100  $\mu$ m. Toluidine blue and aqueous fast green stains were used, 40 $\times$  magnification.





**Figure 3** Growth plate thickness in control and genistein fed mice.

**Notes:** Growth plate thickness was significantly reduced in genistein-treated mice ( $42.7 \pm 1.98 \mu\text{m}$ ) in comparison with controls ( $49.3 \pm 1.96 \mu\text{m}$ ,  $P=0.03$ ). There was no difference in the thickness of the calcified cartilage layer between genistein treated ( $18.6 \pm 1.16 \mu\text{m}$ ) and control mice ( $21.0 \pm 1.14 \mu\text{m}$ ,  $P=0.20$ ). Data are shown as mean  $\pm$  2 SE.

**Abbreviation:** SE, standard error.

rates, mimicking the phenotype seen in human diabetic patients.<sup>66</sup> Although leptin-deficient mice that undergo leptin treatment recover osteogenic capabilities, human diabetic patients are often found to have hyperleptinemia and are nonresponsive to leptin treatments due to leptin resistance, rendering leptin an ineffective antidiabetic treatment.<sup>67,68</sup>

ERT is another mode of treatment that has been considered due to its effectiveness at ameliorating hyperandrogenicity and improving glucose homeostasis and lipoprotein profile in postmenopausal women with T2DM.<sup>69</sup> ERT has also been documented as reducing or reversing postmenopausal bone loss and reducing the risk of OA.<sup>21–24</sup> However, due to the many risk factors that have been linked to chronic ERT, there has been increased interest and research on the effects of phytoestrogens as a potential antidiabetic treatment.<sup>29,30</sup> Previous nutritional intervention studies performed on animals and humans suggest that ingestion of phytoestrogens found in soy products can improve glucose control and insulin resistance.<sup>70</sup> Genistein is a phytoestrogen that has been suggested to be a potential natural antidiabetic agent that directly modulates pancreatic  $\beta$ -cell function via activation of the cAMP/PKA-dependent ERK1/2 signaling pathway as well as positively affect bone formation.<sup>41,42,71</sup> Increased BMD has been seen with genistein treatment in previous animal studies along with increased BMD in postmenopausal women with phytoestrogen treatment.<sup>44,45,47–50</sup> Genistein has also been noted to have a unique role in not only dampening osteoclastic markers but also stimulating osteoblastic markers in comparison with other available osteoporosis therapies that simply inhibit osteoclastic markers.<sup>46</sup> The aim of our study

was to examine the effect of dietary genistein treatment on attenuating bone loss in ob/ob mice. This is the first known study to examine the effects of genistein treatment on bone in the diabetic phenotype.

Genistein treatment did improve some indicators of bone strength in the ob/ob-treated mice of our study, but had little effect on others. Bone geometry did not adapt in a manner that improved cross-sectional geometric indicators of resistance to bending or torsional loads as indicated by a lack of significant differences in  $I_{\text{max}}$ ,  $I_{\text{min}}$ , and  $J$ . However, mice fed a genistein-rich diet demonstrated significantly greater maximum radius of the tibia from the geometric mean, MaxRad, suggesting increased periosteal growth at the tibial midshaft. Additionally, MAR/TtAr was significantly decreased in genistein-treated groups, suggesting endosteal growth reduced the size of the medullary cavity. It is unclear how these findings may translate to humans. Several clinical trials of genistein treatment in postmenopausal women have reported no effect on bone growth or maintenance.<sup>34,35</sup> However, we did not directly measure genistein consumption by the ob/ob mice, so it is unknown how genistein intake in our study compares to that of the postmenopausal women in these studies. Additionally, female subjects in these studies were not obese diabetics, potentially confounding direct comparisons with our findings. Finally, ob/ob mice are hyogonadal, and it is unclear if the estrogen-like effects of genistein treatment on bone are directly related to the diabetic state. More research is needed to determine how our findings translate to human T2DM patients.

Cartilage has been documented as an estrogen-targeted tissue, suggesting that phytoestrogens could pose as a potential treatment for OA.<sup>72</sup> The effect of genistein on cartilage has minimally been explored in the literature. To our knowledge, this is the first known study to examine the effect of genistein on cartilage in ob/ob mice. Our study found that genistein treatment significantly reduced growth plate thickness, but did not reduce tibial length. This is consistent with the findings of the recent study that the expression of the main components of the extracellular matrix cartilage (collagen type II and aggrecan) and chondrocyte proliferation decreased significantly in genistein-treated mice.<sup>73</sup> This is most likely due to genistein competing with local estrogen, leading to a decreased need for autocrine estrogen in cartilage metabolism.<sup>24</sup> Since genistein is a phytoestrogen and has a weaker effect, this leads to suppression of extracellular matrix synthesis and chondrocyte proliferation. Although the growth plate differences we identified between treatment groups were not associated with decreases in tibial length, any treat-

ment that impairs growth in diabetic children would warrant caution. If further investigation links genistein treatment to impaired growth due to disruption of the growth plate, its clinical value as a treatment for T2DM would diminish.

The results of our investigation support the hypothesis that genistein may slightly attenuate bone fracture risks seen in T2DM. Genistein treatment at 600 mg/kg increased periosteal and endosteal growth and led to greater cortical bone mass relative to total area of the midshaft cross-section. However, genistein treatment did not promote growth of growth plate cartilage.

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## Disclosure

The authors report no conflicts of interest in this work.

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