Original Article

Bone Formation Is Impaired in a Model of Type 1 Diabetes

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The effects of type 1 diabetes on de novo bone formation during tibial distraction osteogenesis (DO) and on intact trabecular and cortical bone were studied using nonobese diabetic (NOD) mice and comparably aged nondiabetic NOD mice. Diabetic mice received treatment with insulin, vehicle, or no treatment during a 14-day DO procedure. Distracted tibiae were analyzed radiographically, histologically, and by microcomputed tomography (μCT). Contralateral tibiae were analyzed using μCT. Serum levels of insulin, osteocalcin, and cross-linked C-telopeptide of type I collagen were measured. Total new bone in the DO gap was reduced histologically (P ≤ 0.001) and radiographically (P ≤ 0.05) in diabetic mice compared with nondiabetic mice but preserved by insulin treatment. Serum osteocalcin concentrations were also reduced in diabetic mice (P ≤ 0.001) and normalized with insulin treatment. Evaluation of the contralateral tibiae by μCT and mechanical testing demonstrated reductions in trabecular bone volume and thickness, cortical thickness, cortical strength, and an increase in endosteal perimeter in diabetic animals, which were prevented by insulin treatment. These studies demonstrate that bone formation during DO is impaired in a model of type 1 diabetes and preserved by systemic insulin administration. Diabetes 54:2875–2881, 2005

Type 1 diabetes is associated with several disorders of skeletal health, including decreased bone density, an increased risk for osteoporosis (1–6), and fragility fracture (7–9), as well as poor bone healing and regeneration characteristics (10), conditions which all rely, in part, upon an intramembranous component to bone formation. Increasing evidence suggests that skeletal abnormalities in type 1 diabetes may, in part, result from the detrimental effects of type 1 diabetes on bone formation. For example, decreased expression of transcription factors that regulate osteoblast differentiation have been demonstrated in animal models of type 1 diabetes (11). Numerous reports of bone histology in diabetic animals demonstrate decreased osteoblast number, osteoid volume, and mineral apposition rates (rev. in 12). In diabetic rats, plasma osteocalcin concentrations, a marker of osteoblast activity, acutely decline beginning on the 1st day of glucosuria (13). Similarly, serum concentrations of osteocalcin in children with newly diagnosed type 1 diabetes are significantly lower at the onset of disease (14). Serum markers correlated with bone formation (IGF-I, alkaline phosphatase, and osteocalcin) also are significantly lower in diabetic patients with osteopenia compared with those without osteopenia (2), and studies have demonstrated that lower bone mineral density (BMD) in type 1 diabetes is correlated with decreased markers of bone formation and more exaggerated dysregulation of the IGF system (15).

The present study was designed to test the hypothesis that type 1 diabetes specifically impedes intramembranous bone formation by using a model of tibial distraction osteogenesis uniquely modified for use in the nonobese diabetic (NOD) mouse, an animal model of autoimmune diabetes. The technique of distraction osteogenesis (DO) allows for the in vivo study of de novo intramembranous bone formation under varying physiologic and pathologic conditions (16). By pairing DO with the NOD model of autoimmune diabetes, the intent was to gain greater understanding of the role of metabolic derangements in the pathogenesis of altered bone metabolism in type 1 diabetes by comparing bone formation in nonconverting (nondiabetic) NOD mice with bone formation in diabetic NOD mice that received either insulin or vehicle treatment. In addition, bone volume, architecture, and strength in the contralateral tibia were examined to determine the short-term effects, if any, of type 1 diabetes on the intact skeleton.

RESEARCH DESIGN AND METHODS

Female 8-week-old nonobese diabetic mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were acclimated to a presterilized diet and water in autoclaved cages and maintained in a pathogen-free facility to maximize development of diabetes. Urine samples were monitored weekly for glucosuria using Keto-Diastix reagent strips (Bayer, Elkhart, IN). Diabetes was confirmed by the measurement of blood glucose at the time of surgery. In experiment 1, bone formation was compared in 7 untreated diabetic animals and 11 comparably aged nondiabetic mice (Fig. 1A and B and Fig. 2A and B). In experiment 2, bone formation was compared in 11 diabetic and 12 nondiabetic mice (Figs. 1C and D, 2D–F, and 3 and Tables 1 and 2). Diabetic animals in experiment 2 (n = 11) were alternately assigned to begin treatment with 18 U.S.C. Section 1734 solely to indicate this fact.

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with insulin implants (n = 6) (LinBit sustained-release insulin implant pellets; LinShin, Scarborough, Canada) or vehicle (n = 5) (blank palmitic acid micro-crystal implants; LinShin) at the time of surgery. All research protocols were approved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences.

The confirmed diabetic mice and age-matched nondiabetic NOD mice were anesthetized with sodium pentobarbital (71 mg/kg, day 1). In experiment 2 only, insulin or vehicle implants were inserted under the mid-dorsal skin using a 12-gauge trocar. A titanium ring fixator was placed on the left tibia, and a mid-diaphyseal osteotomy was created, as previously described (16). The fibula was fractured by direct lateral pressure. The periosteum and dermal tissues were closed, and buprenex (0.1 mg/kg) was given postoperatively by intramuscular injection for analgesia. Distraction was initiated on day 4 (3 days latency after surgery) at a rate of 0.075 mm twice a day (0.15 mm/day) and continued for 14 days. Mice were killed on postoperative day 17. The distracted tibiae were harvested for radiographic and histological studies, and the contralateral nondistracted tibiae were analyzed using microcomputed tomography (μCT) and biomechanical testing.

Biocenological analyses. In experiment 2, serum was obtained on the day of killing for measurement of glucose, insulin, osteocalcin, and cross-linked C-telopeptides of type I collagen. Insulin was measured using Luminex xMAP technology with a multiplex assay kit (Mouse Adipokine LINCplex Panel; Linco Research, St. Charles, MO). Osteocalcin was measured by enzyme immunosorbent assay (Mouse ELA kit; Biomedical Technologies, Stoughton, MA). Degradation products of the C-telopeptide of type I collagen were measured by enzyme immunoassay (RayLAPS; Osteometer Bio Tech, Herlev, Denmark).

Radiographic and histological analysis. After at least 48 h of fixation in 10% neutral buffered formalin, the left tibiae were removed from the fixators for high-resolution single-beam radiography: a Xerox Micro50 closed system radiography unit (Xerox, Pasadena, CA) was used at 40 kV (3 mA) for 20 s using Kodak X-OMAT film. For quantification, the radiographs were video recorded under low-power (1.25× objective) magnification, and the area and density of mineralized new bone in the distraction gaps were evaluated using National Institutes of Health Image Analysis 1.62 software. The distraction gap was outlined from the outside corners of the two proximal and the two distal cortices forming a quadrilateral region of interest (ROI). The mineralized new bone area in the gap was determined by outlining regions with radio density equivalent to or greater than the adjacent medullary bone. The percentage of new mineralized bone within the distraction gap was calculated by dividing mineralized bone area by total gap area. The distracted tibiae were then decalcified in 5% formic acid, dehydrated, and embedded in paraffin, as previously described (17,18). Longitudinal sections (5-7 microns) were stained with hematoxylin and eosin. Sections were selected for analysis to represent a central gap location. As detailed above, a quadrilateral ROI was outlined and recorded. New bone was defined as all organized ostoid/sinoidal columns. Both the proximal and distal endosteal new bone matrix was outlined and the area was recorded. The percentage of new bone area within the DO gap was calculated by dividing the new bone matrix area by the total distraction gap area. Adjacent sections were then used for immunohistochemistry.

Immunohistochemistry. A primary polyclonal antibody specific for insulin receptor α and normal rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The biotinylated secondary antibody was purchased from Vector Laboratories ( Burlingame, CA). Sections were deparaffinized and rehydrated. Slides were then rinsed in phosphate buffered saline (pH 7.4) with 0.02% Triton-X 100 for 3 min to permeabilize the tissue and incubated for 10 min in a 1% BSA and 2% nonfat dry milk solution to block nonspecific binding. After washing, the slides were incubated with Peroxidixtech (for 5 min and washed twice again. The primary antibody (insulin receptor α, diluted 1:200) or rabbit IgG (for negative control; diluted 1:100) was applied and incubated at 4°C for 16 h. After washing, a biotinylated goat anti-rabbit secondary antibody (diluted 1:100) was applied for 20 min, followed by the application of hors eradish peroxidase–conjugated streptavidin for 20 min. Color was developed using Nova Red. The sections were counterstained with hematoxylin, dehydrated, and mounted using permanent mounting media.

μCT. Representative specimens of distracted tibiae were determined from the 2-dimensional (2-D) radiographs and imaged by μCT using a μCT-40 (Scanco, Bassersdorf, Switzerland) and the manufacturer’s software. A total of ~600 contiguous axial (cross-sectional) slices including the entire distraction gap and at least 0.5 mm of both proximal and distal host bone were obtained at 55 kV and 70 μA with a voxel size of 12.4 μm in all dimensions. To illustrate endosteal new bone, the endosteal surface of the host cortex at the proximal end of the distraction gap was outlined in the last cross-sectional image in which the cortex appeared intact. This outline was deleted and copied onto the slice in which ~50% of the host cortex was missing, defining the ROI for that slice and marking the proximal end of the distraction gap. This procedure was repeated for the distal end of the distraction gap. The ROI for each of the intervening slices was defined using an automated algorithm that interpolates gradual changes in the size and position of the manually defined ROIs, commonly known as morphing. The 2-D gray scale images within each ROI were stacked before applying a gray scale threshold and a 3-dimensional (3-D) noise filter to create a binary 3-D reconstruction. To illustrate the gap dimensions, a 3-D reconstruction of the proximal and distal ends of the cortex was created and overlaid on the endosteal new bone reconstruction. An analogous procedure was used to illustrate combined endosteal and intracortical new bone in which the ROI was defined using the periosteal perimeter of the host cortex instead of the endosteal perimeter.

Trabecular and cortical bone volume and architectural properties in the contralateral tibia from six nondiabetic, six insulin-treated diabetic, and four vehicle-treated diabetic mice were also evaluated by μCT. For trabecular analyses, the entire endosteal volume of the proximal tibia extending 1.24 mm
distal to the primary spongiosa was analyzed. Cortical analyses were performed at the tibial midshaft defined as the midpoint between the proximal and distal epiphyses. The gray-scale thresholds and noise filters were optimized specifically for murine trabecular or cortical analyses. Total volume, bone volume, and architectural indexes were calculated directly from the 3-D reconstructions.

**Biomechanical testing.** Cortical strength of the tibial midshaft was measured using 3-point bending analyses on a MTS Bionex 868 load frame operated via TestWorks version 4 software on a personal computer as described previously (19). The lower platens supported the bones with a span of 80% of the total bone length, and load was applied from the upper platen to the posterior aspect of the tibial midshaft. The peak load (N) and stiffness (N/mm) were recorded for each bone.

**Statistical analysis.** For statistical analysis, the unpaired Student’s t test was used to compare differences between groups for all serum analyses. Skeletal parameter comparisons (radiographic and μCT) were performed using one-way ANOVA and Student Newman-Keuls post hoc tests. All data are reported as means ± SE, and differences are considered statistically significant when \( P < 0.05 \).

**RESULTS**

A total of 41 mice (7 diabetic and 11 comparably aged nondiabetic animals in experiment 1 and 11 diabetic and 12 nondiabetic animals in experiment 2) underwent DO and were analyzed. At the time of surgery, the mean age of mice in experiment 1 was 15.9 ± 1.0 weeks for control animals and 16.9 ± 1.9 weeks for diabetic animals; the mean age of mice in experiment 2 was 22.2 ± 1.1 weeks for control animals, 19.3 ± 1.8 weeks in untreated diabetic animals, and 23.1 ± 1.1 weeks in insulin-treated diabetic animals (nonsignificant differences). At the time of killing, mean blood glucose measurements in the three groups were as follows: nondiabetic 187.0 ± 13.4 mg/dl, \( n = 23 \); untreated diabetic: 482.9 ± 51.6 mg/dl, \( n = 12 \); and diabetic + insulin: 261.0 ± 45.9 mg/dl, \( n = 6 \). Serum measurements for insulin, osteocalcin, and type I collagen C-telopeptide are shown in Table 1. Insulin levels confirmed relative insulinopenia in the vehicle-treated diabetic animals, with modestly higher insulin levels, compared with nondiabetic mice, noted in the insulin-treated group (−30% above controls). Serum osteocalcin concentrations were reduced in vehicle-treated diabetic animals compared with nondiabetic mice (\( P \leq 0.001 \)) and normalized with insulin treatment, while type 1 collagen C-telopeptide levels remained unchanged.

A significant decrease in bone formation was noted in the distraction gap of diabetic mice compared with nondiabetic mice, both radiographically and histologically (Fig. 1). In experiment 1, mineralization of the DO gap, as measured from the radiographs, showed a significant decrement in bone formation in diabetic mice compared with nondiabetic mice (Fig. 1A). Analysis of histological sections revealed a similar trend in the formation of

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**FIG. 2.** Graphic representation of bone formed during DO. Impairment of bone formation was confirmed using μCT on representative distraction gaps (five different gaps shown). These images show total endosteal and intracortical mineralized bone within the gap. Tips of the proximal (top) and distal (bottom) host cortices are shown for orientation. Diabetic NOD mice (B) displayed delayed bone formation during distraction osteogenesis, compared with nondiabetic mice (A). Inhibition of bone formation was prevented by concurrent insulin treatment during distraction. Representative control (D), vehicle-treated diabetic (E), and insulin-treated diabetic specimens (F) from experiment 2 are shown. A schematic representation of the bone forming unit is shown in panel C. Reproduced with permission of J. Aronson (20).
endosteal new bone columns (Fig. 1B). In experiment 2, bone formation was again impaired in diabetic mice (Fig. 1C and D). In contrast, the diabetes-induced inhibition of bone formation was prevented by treatment with insulin, as demonstrated both radiographically (Fig. 1C) and histologically (Fig. 1D). A more significant deficit in the formation of new bone columns was apparent by histological analysis than by radiographic analysis. This discrepancy is typical of DO analyses and reflects the fact that single-beam radiographic analysis will identify all sources of calcified material within the gap, hence overestimating mineralization in the gaps. (16,20)

As shown in Fig. 2, the delay in de novo bone formation in diabetic animals and the improvements in new bone volume with insulin replacement were confirmed when specimens were analyzed using μCT. Figure 2A and B depict representative μCT reconstructions of distraction gaps from nondiabetic NOD (A) or untreated diabetic (B) mice in experiment 1. Mineralized bone present within the nondiabetic gap was appreciably greater than that present within the gap of the untreated diabetic animal, consistent with the radiographic and histological data presented in Fig. 1. Figure 2C is a graphic demonstrating the microarchitecture that occurs during distraction. The bone-forming unit is described as the minimal structural element that comprises the basic biological unit of DO (20). As seen here, bone forming units can originate in parallel with the distraction force. These longitudinal bone columns can eventually bridge the gap, with new bone and mineralization emanating from the proximal and distal fracture sites. To better appreciate these phenomena, Fig. 2D–F illustrates only endosteal new bone in the context of the host cortices as described in RESEARCH DESIGN AND METHODS. In these figures, the bone shown lies within the future marrow cavity and represents intramembranous bone formation comprised of numerous bone forming units, as determined by previous histological analyses (20). In both Fig. 2D (nondiabetic NOD) and F (insulin-treated diabetic NOD), mineralization of these bone columns can be appreciated as they arise from both proximal and distal bone marrow. In contrast, the vehicle-treated NOD animal (Fig. 2E) shows minimal mineralized new bone in the distrac-

![Image of immunohistochemical demonstration of insulin receptors in a distraction gap.](image)

### TABLE 1

<table>
<thead>
<tr>
<th>Serum markers</th>
<th>Nondiabetic</th>
<th>Diabetic</th>
<th>Diabetic + insulin</th>
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<tr>
<td>Insulin (pmmol/l)</td>
<td>207.2 ± 87.3</td>
<td>118.6 ± 70.2</td>
<td>276.0 ± 51.6</td>
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<tr>
<td>Osteocalcin (ng/ml)</td>
<td>96.7 ± 8.3</td>
<td>41.9 ± 5.9</td>
<td>87.3 ± 7.8†</td>
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<tr>
<td>Type I collagen C-telopeptide (ng/ml)</td>
<td>22.3 ± 6.1</td>
<td>20.8 ± 2.8</td>
<td>22.9 ± 6.2</td>
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</tbody>
</table>

Data are means ± SE. *P < 0.05 vs. nondiabetic, †P < 0.05 vs. diabetic.

FIG. 3. Immunohistochemical demonstration of insulin receptors in a distraction gap. A: H&E staining of a distraction gap from a nondiabetic NOD mouse. Bone formation within the DO gap is demarcated by the stippled outline. Adjacent sections from this specimen were immunostained with a primary polyclonal antibody to insulin receptor α (B) or normal rabbit IgG (C), as described in RESEARCH DESIGN AND METHODS. Red-brown staining represents immunoreactive insulin receptors, some of which are denoted by arrows (B).
Loss of cortical strength and degradation of structure at the tibial midshaft are prevented by insulin treatment.

### TABLE 3

<table>
<thead>
<tr>
<th></th>
<th>Nondiabetic</th>
<th>Diabetic + vehicle</th>
<th>Diabetic + insulin</th>
</tr>
</thead>
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<tr>
<td>Peak load (n)</td>
<td>10.09 ± 0.43*</td>
<td>8.92 ± 0.11</td>
<td>11.11 ± 0.22†</td>
</tr>
<tr>
<td>Stiffness (n/mm)</td>
<td>38.49 ± 2.52</td>
<td>32.33 ± 1.60</td>
<td>42.81 ± 1.52*</td>
</tr>
<tr>
<td>Periosteal perimeter (mm)</td>
<td>3.37 ± 0.06</td>
<td>3.50 ± 0.02</td>
<td>3.48 ± 0.05</td>
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<tr>
<td>Endosteal perimeter (mm)</td>
<td>1.57 ± 0.07*</td>
<td>1.81 ± 0.05</td>
<td>1.58 ± 0.04*</td>
</tr>
<tr>
<td>Periosteal diameter (mm)</td>
<td>0.522 ± 0.005</td>
<td>0.510 ± 0.003</td>
<td>0.520 ± 0.004</td>
</tr>
<tr>
<td>Endosteal diameter (mm)</td>
<td>0.247 ± 0.009</td>
<td>0.273 ± 0.004</td>
<td>0.250 ± 0.005</td>
</tr>
<tr>
<td>Cortical thickness (mm)</td>
<td>0.246 ± 0.005*</td>
<td>0.211 ± 0.007</td>
<td>0.248 ± 0.003*</td>
</tr>
</tbody>
</table>

Data are means ± SE. Peak load and stiffness were determined by 3-point bending, while the structural indices were measured using μCT as described in research design and methods. *Significant difference from diabetic + vehicle with \( P < 0.05 \); †significant difference from control and diabetic + vehicle with \( P < 0.05 \).
DIABETES AND NEW BONE FORMATION

We provide evidence of a significant reduction in bone formation, both histologically and radiographically, in diabetic NOD mice. In addition, we noted a reduction in serum markers of bone formation in diabetic animals compared with nondiabetic animals. Deficits in bone formation in the DO gap and reductions in osteocalcin were entirely prevented with 17 days of insulin treatment, despite persistent mild hyperglycemia. Furthermore, μCT analysis of distracted tibiae demonstrated that the mineralizing new bone emanating from within the future marrow cavity of the regenerating gap was almost undetectable in the diabetic animal, yet was significantly rescued with insulin treatment. Consistent with in vitro evidence of insulin receptor expression in osteoblast-lineage cultured cells (24–26), we have demonstrated insulin receptor expression on osteoblasts in all zones of the distraction gap in vivo. Together, these findings suggest that primary insulinopenia, as opposed to metabolic derangements such as hyperglycemia, may be more detrimental to the process of intramembranous bone formation.

Examination of bone structure and strength in the contralateral tibiae demonstrated a surprisingly strong impact of short-term type 1 diabetes and insulin on the intact skeleton. In both trabecular and cortical compartments, bone volume and thickness were reduced by diabetes, and these changes were prevented by insulin treatment. However, other parameters of trabecular microarchitecture, which may affect strength independent of mass, volume, and density, were altered only in the insulin-treated diabetic mice. Without performing strength analyses directly on the isolated spongiosa, the mechanical (strength) impact of these structural changes remains unclear. However, results of the midshaft biomechanical tests demonstrate that insulin treatment prevents and even increases cortical strength. Recent studies using μCT and finite element modeling have demonstrated striking strain-dependent trabecular microarchitecture in mice (27).

However, the factors controlling architecture, independent of mass and volume, are completely unknown. Interestingly, the results presented here may have identified insulin and/or glycemic levels as the first agents known to modulate trabecular architecture.

Indirect evidence from clinical studies supports the concept that insulin deficiency, rather than chronic hyperglycemia, may contribute to the decrease in bone formation seen in diabetes. First, several studies have failed to demonstrate a consistent correlation between indicators of glycemic control and BMD in type 1 diabetes (1,2,4,5). Second, the effects of type 1 diabetes compared with type 2 diabetes on bone metabolism are discordant. While an increased incidence of osteopenia can be demonstrated among studies of type 1 diabetes (1–6), studies of type 2 diabetes, a state of hyperinsulinemia, more commonly demonstrate normal or increased bone density (3,28–30), despite the fact that years of untreated hyperglycemia could exist in individuals with type 2 diabetes. Interestingly, a direct correlation between BMD and endogenous insulin secretory capacity in type 2 diabetes has been observed (31). Similarly, Barrett-Conner et al. (32) found a positive association between bone density of the radius and spine and fasting insulin levels in nondiabetic postmenopausal women, again suggesting that hyperinsulinemia may preserve bone. Finally, several studies have demonstrated deficits in bone density, present either at the time of diagnosis (1,33) or fairly early in the course of disease, suggesting that prolonged exposure to systemic hyperglycemia alone is not a prerequisite. This is supported by the observation that diabetic osteopenia does not appear to progress faster over time than anticipated age-related decrements in bone density (34).

Several lines of evidence from in vitro bone cell cultures support the idea that insulin exerts direct anabolic effects on bone cells. For example, primary calvarial osteoblasts and multiple osteoblast-like cell lines express insulin receptors on the cell surface and have a high capacity for insulin binding (24–26). In response to physiological doses of insulin, cultured osteoblasts show increased rates of proliferation (35,36), collagen synthesis (24,37,38), alkaline phosphatase production (39,40), and glucose uptake (41,42). Insulin may also exert direct effects on osteoclasts; mature osteoclast-like cells in vitro express insulin receptors and exhibit reduced bone resorption in response to insulin stimulation (43). Taken together, these data support the idea that the actions of insulin in bone could be mediated directly via stimulation of osteoblasts in combination with inhibition of osteoclasts. In addition to the direct effects of insulin on bone cells, insulin may exert synergistic effects with other anabolic agents in bone, such as parathyroid hormone (44–46).

In summary, we have demonstrated that bone formation is clearly impaired in the NOD mouse model of diabetes and appears to be an insulin-dependent and reversible process. Specifically, impairment in bone formation can be ameliorated with insulin treatment, even in the face of persistent mild hyperglycemia. The observation that insulin receptor staining is evident throughout all cellular delineations of the distraction gap also suggests that the insulin signaling axis could be a physiological component of each phase of osteogenesis during DO. Evaluation of the contralateral tibiae, however, demonstrated some provocative findings. Specifically, while trabecular thickness and trabecular volume were enhanced in an insulin-treated diabetic model, we also noted decreases in connectivity density and trabecular number and increases in trabecular separation, findings that are indicative of osteoporosis. Together, this suggests that while insulin treatment appears to be capable of preventing the loss of cancellous bone volume and trabecular thickness, it may have other effects on trabecular microstructure. This observation is consistent with the fact that despite a discrepancy between the type 1 and type 2 diabetic populations and BMD, diabetic populations with both diseases uniformly appear to have higher risk for bone fracture (8,9,47). Consequently, the impact of insulin treatment (as well as hyperinsulinemia) on the biomechanical properties of the intact skeleton are still unclear, and further investigation is warranted.
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