Altered bone mass, geometry and mechanical properties during the development and progression of type 2 diabetes in the Zucker diabetic fatty rat

Rhonda D Prisby^{1,4,5,6}, Joshua M Swift¹, Susan A Bloomfield^{1,2}, Harry A Hogan³ and Michael D Delp^{1,7}

Departments of ¹Health and Kinesiology, ²Intercollegiate Faculty of Nutrition and ³Mechanical Engineering, Texas A&M University, College Station, Texas 77843, USA

⁴INSERM U890, Saint-Etienne F4023, France

⁵IFR 143, Saint-Etienne F42023, France

⁶Université Jean-Monnet, Saint-Etienne F42023, France

⁷Department of Applied Physiology and Kinesiology and the Center for Exercise Science, University of Florida, Gainesville, Florida 32611, USA

(Correspondence should be addressed to M D Delp; Email: mdelp@ufl.edu)

Abstract

Osteopenia and an enhanced risk of fracture often accompany type 1 diabetes. However, the association between type 2 diabetes and bone mass has been ambiguous with reports of enhanced, reduced, or similar bone mineral densities (BMDs) when compared with healthy individuals. Recently, studies have also associated type 2 diabetes with increased fracture risk even in the presence of higher BMDs. To determine the temporal relationship between type 2 diabetes and bone remodeling structural and mechanical properties at various bone sites were analyzed during pre-diabetes (7 weeks), short-term (13 weeks), and long-term (20 weeks) type 2 diabetes. BMDs and bone strength were measured in the femora and tibiae of Zucker diabetic fatty rats, a model of human type 2 diabetes. Increased BMDs (9–10%) were observed in the distal femora, proximal tibiae, and tibial mid-shafts in the

pre-diabetic condition that corresponded with higher plasma insulin levels. During short- and long-term type 2 diabetes, various parameters of bone strength and BMDs were lower (9–26%) in the femoral neck, distal femora, proximal tibiae, and femoral and tibial mid-shafts. Correspondingly, blood glucose levels increased by 125% and 153% during short- and long-term diabetes respectively. These data indicate that alterations in BMDs and bone mechanical properties are closely associated with the onset of hyperinsulinemia and hyperglycemia, which may have direct adverse effects on skeletal tissue. Consequently, disparities in the human literature regarding the effects of type 2 diabetes on skeletal properties may be associated with the bone sites studied and the severity or duration of the disease in the patient population studied.

Journal of Endocrinology (2008) **199**, 379–388

Introduction

The occurrence of osteoporosis in diabetic individuals has been recognized since the beginning of the twentieth century (Morrison & Bogan 1927, Albright & Reifstein 1948, Berney 1952). However, the observed rates of osteoporosis reported in insulin-dependent type 1 diabetes mellitus differ from those reported in non-insulin-dependent type 2 diabetes. While the association between osteoporosis and type 1 diabetes is well established (Silberberg 1986, Forst *et al.* 1995), the reported association between osteoporosis and type 2 diabetes is less clear. The literature documents bone mineral densities (BMDs) of type 2 diabetic patients that are diminished (Isaia *et al.* 1987), enhanced (Van Daele *et al.* 1995), or similar (Wakasugi *et al.* 1993) to those of non-diabetic control subjects. However, the reports of enhanced BMDs in type 2 diabetic individuals are often confounded by increased rates of obesity in these patients, as obesity is

positively associated with increased bone mass (Dalen *et al.* 1975). For example, reduced BMD has been reported in type 2 diabetic men, while no bone loss occurred in obese type 2 diabetic women (Buysschaert *et al.* 1992). Investigators have also recently shown type 2 diabetes to be associated with an increased risk of fracture (e.g., hip, proximal humerus, foot) in older adults (Meyer *et al.* 1993, Forsen *et al.* 1999, Nicodemus & Folsom 2001, Schwartz *et al.* 2001, Keegan *et al.* 2002, Ottenbacher *et al.* 2002). In the study of osteoporotic fractures, the BMD of older diabetic women was higher than control subjects, yet their relative risk for non-spinal fractures increased (Schwartz *et al.* 2001). Given the ambiguity in the human literature of the effects of type 2 diabetes on BMD (Isaia *et al.* 1987, Wakasugi *et al.* 1993, Van Daele *et al.* 1995), and the possible dissociation between BMD and fracture risk (Schwartz *et al.* 2001), the purpose of this investigation was twofold: 1) to determine whether declines in bone structural and mechanical properties are

observed in the Zucker diabetic fatty (ZDF) rat, a model of type 2 diabetes and 2) to determine whether possible declines in BMD and bone mechanical properties coincide with the onset and progression of the disease.

Materials and Methods

The experimental procedures conducted in this investigation complied with the Texas A&M University laboratory animal care committee rules and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Animals and procedures

Male diabetic (ZDF:Gmi fa/fa) and age-matched control (ZDF:Gmi +/?) rats were obtained (Charles Rivers Laboratories, Redfield, AR, USA) for the evaluation of BMD and biomechanical properties. These rats are characterized by their development of type 2 diabetes (i.e., hyperglycemia, impaired wound healing, neuropathy, nephropathy, insulin resistance, hyperinsulinemia, mild hypertension, hypertriglyceridemia, and hypercholesterol; Clark *et al.* 1983, Peterson *et al.* 1990a,b, Sparks *et al.* 1998, Vrabec 1998) when maintained on a Purina 5008 diet (Purina Labdiet Formulab 5008, Richmond, IN, USA). Three age groups were chosen for study: 7 weeks of age (pre-diabetes), 13 weeks of age (short-term diabetes), and 20 weeks of age (long-term diabetes; Clark *et al.* 1983, Peterson *et al.* 1990b, Sparks *et al.* 1998). The ages were chosen to correspond with insulin resistance (pre-diabetic state, 6–10 weeks of age), impaired glucose tolerance and fully diabetic (12 weeks of age), and hyperglycemic and glucose intolerant (19 weeks of age; Clark *et al.* 1983, Peterson *et al.* 1990b, Sparks *et al.* 1998). The inbred line of ZDF rats are distinct from the obese Zucker rat that are a model of the metabolic syndrome that maintain normal fasting blood glucose levels and do not develop type 2 diabetes until much later in life and at less predictable ages (personal communication, Charles River Laboratories; Frisbee & Delp 2006). The rats were housed in a temperature-controlled ($23 \pm 2^\circ\text{C}$) room with a 12 h light:12 h darkness cycle. Rats had access to water and Purina 5008 *ad libitum*.

On the day of tissue collection, animals were anesthetized with isoflurane (2%/oxygen balance), 2 ml blood was withdrawn via a cardiac puncture and the animal subsequently euthanized by the removal of the myocardium. Right femora, tibiae, and the spinal column were dissected free, wrapped in gauze soaked in PBS and stored at -80°C until computed tomography (CT) scans. Before ashing, the lumbar spines were simmered at 80°C in PBS to remove all soft tissue.

Peripheral quantitative CT (pQCT)

Tomographic scans were performed *ex vivo* on femoral necks, femoral mid-shafts, distal femora, and tibiae using a Stratec XCT Research-M device (Norland Corp., Fort Atkinson,

WI, USA). The calibration of this machine was performed prior to daily scans using a hydroxyapatite standard cone phantom to ensure measurement precision. All bones were thawed to room temperature and placed in PBS during scanning. To determine the optimal region of interest in the femoral neck, proximal femurs from two experimental animals chosen at random prior to the experiment were scanned to obtain 10–12 slices (each 0.5 mm thick) perpendicular to the neck's long axis. Those four serial slices near the center of the femoral neck, which when averaged provided the most representative values for total BMD for the entire region scanned were those collected on all experimental animals.

Similar studies have previously been performed in our laboratory to optimize the region of interest for the proximal tibia, distal femur, and mid-diaphysis sites. Multiple transverse images of the tibiae were scanned at the proximal metaphysis (4, 5, 6, and 7 mm from the proximal plateau) and mid-diaphysis (three slices, 2 mm apart, centered at 50% total bone length). In addition to the femoral neck site described earlier, femora were scanned at the distal metaphysis (4, 5, and 6 mm from the proximal and distal plateau) and mid-diaphysis (three slices, 1 mm apart, centered on 8 mm from distal end of the lateral epicondyles). Three slices of the third lumbar vertebral body centered at $\sim 50\%$ of its vertical height were scanned. Only L3 vertebrae from pre- and long-term-diabetic animals were available for these *ex vivo* scans. At all bone sites, the values from multiple slices were averaged to yield one value for each variable.

Scans were performed at 5 mm/s with voxel resolution of $0.07 \times 0.07 \times 0.05$ mm; analyses were performed using cut and peel modes of 3 and 2. Based on the manufacturer data, machine precision is ± 3.0 mg/cm³ for cancellous bone and ± 9.0 mg/cm³ for cortical bone. Reproducibility was determined from three repeat scans of six excised bones using multiple-slice scanning method. Each bone was repositioned after each scan. The coefficients of variation (CV) were $\pm 6\%$, $\pm 2\%$, and $\pm 4\%$ for cancellous BMD at distal femur, proximal tibia, and femoral neck sites respectively. The corresponding CVs for cortical BMD from mid-diaphyseal sites were $\pm 0.8\%$ and $\pm 0.4\%$ in the femur and tibia respectively.

Ashing of L4 vertebral body

The fourth lumbar vertebra from each animal, including all spines, was dried at 100°C and dry weight recorded. After 16 h at 600°C in an ashing oven that burned off all organic materials, an ash weight was recorded and expressed as a percent of dry weight.

Mechanical testing

Structural and material properties of mid-shaft femora and tibiae were determined using three-point bending tests. Mechanical testing was also conducted at the femoral neck.

Sites of testing were matched to pQCT sampling sites for femoral and tibial mid-diaphyses (50% of total bone length) and femoral neck (50% of neck length). Prior to three-point bend testing, anteroposterior (AP) and mediolateral (ML) surface diameters were measured at mid-diaphysis. Bones were thawed at room temperature and placed on metal pin supports with the upper loading pin centered and contacting the mid-diaphyseal testing site. The span between the lower supports was 15 mm for femora which were oriented anterior side down. For tibiae, the span was 18 mm and tibiae were oriented lateral side down. Quasi-static, displacement-controlled loading (2.5 mm/min) was applied to the upper surface (posterior for femur and medial for tibia) until fracture using a servo-controlled test machine (Instron 1125, 1000 lb load cell at 100 lb maximum). All bones were sprayed with PBS immediately prior to testing to maintain hydration. Displacements were monitored by a linear variable differential transformer interfaced with a personal computer (Gardener Systems software, Santa Ana, CA, USA). Raw data, collected at 10 Hz as load versus displacement curves, were analyzed with Table-Curve 2.0 (Jandel Scientific; San Rafael, CA, USA). Structural variables were obtained directly from load-displacement curves. The ultimate load (UL, in N) was defined as the maximum load sustained by the specimen, the stiffness (K, in N/mm) was defined as the slope of the linear portion of the curve, the energy absorbed (in N-mm) was determined as the area under the curve to UL, and the post-yield displacement (in mm) was the displacement from yield to fracture. Material properties were estimated by normalizing structural properties to bone geometry at the site of testing using the following equations from classical beam theory: elastic modulus (in GPa) = $K \times S^3 / (48 \times \text{CSMI} \times 1000)$; ultimate stress (in MPa) = $UL \times S \times (D/2) / (4 \times \text{CSMI})$, where CSMI is the mid-diaphysis cross-sectional moment of inertia (in mm⁴), D the mid-shaft bone diameter (in mm), and S the support span distance (in mm). CSMI values were averaged from three pQCT slices and were taken about the anatomic axis of bending, which was the ML axis for femora and the AP axis for tibiae. D was measured by calipers in the direction of loading (i.e., AP for femora and ML for tibiae).

Mechanical tests were also conducted on the femoral neck, a site of mixed cortical and cancellous bone. The proximal half of the femur was placed in a rigid (aluminum) fixture containing machined holes of various sizes. Specimens were inserted such that they were tightly seated up to the lesser

trochanter and oriented with the main axis of the femoral shaft vertical. Quasi-static loading was applied to the femoral head in a direction parallel to the femoral shaft (vertical) at a displacement rate of 2.5 mm/min (0.1 in/min) until complete fracture. Load-displacement data were collected and analyzed similar to the procedures described earlier for three-point bending, except only structural properties (UL and stiffness) are applicable in this case.

Statistical analysis

Two-way ANOVA was used to compare differences among ZDF rats and their respective age-matched lean controls, with Student-Neuman-Keuls *post hoc* tests applied when a significant *F* was achieved. Alpha levels of $P \leq 0.05$ were considered statistically significant and group differences of $P < 0.10$ are acknowledged. Data are represented as means $\pm$ S.E.M.

Results

Body mass was higher in the ZDF rats versus their lean age-matched controls at 7 and 13 weeks of age (Table 1). However, body mass in the long-term diabetic rats (20 weeks) was similar to lean controls. Blood glucose levels were not different between groups in the pre-diabetic condition, but were higher with short- and long-term type 2 diabetes (Table 1). Hyperinsulinemia occurred during the pre- and short-term-diabetic states (i.e., 7 and 13 weeks) in the ZDF rats (Table 1). Lengths of femora and tibiae were smaller in ZDF rats at all age groups, suggesting a small deficit in longitudinal growth even when total body weight was greater in the pre- and short-term-diabetic conditions (Table 2).

BMD and geometry

Appendicular mid-shaft bone (femur and tibia)

CSMI were the same (both femora and tibiae) in pre-diabetic animals versus lean controls, but lower with the progression of the disease (short-term diabetes, for tibiae) or with long-term type 2 diabetes (both femora and tibiae; Fig. 1A and C). Similarly, despite a higher cortical BMD in tibia in pre-diabetic animals, this measure of bone mass was lower with short- and long-term type 2 diabetes in both the femur and tibia (Fig. 1B and D).

Table 1 Body mass and plasma blood glucose and insulin levels

	7 weeks lean	7 weeks fatty	13 weeks lean	13 weeks fatty	20 weeks lean	20 weeks fatty
Body mass (g)	138 $\pm$ 5	171 $\pm$ 6*	286 $\pm$ 6	334 $\pm$ 13*	373 $\pm$ 5	368 $\pm$ 6
Blood glucose (mg/dl)	80 $\pm$ 4	102 $\pm$ 9	83 $\pm$ 4	230 $\pm$ 23*	91 $\pm$ 7	258 $\pm$ 15*
Insulin (ng/ml)	1.1 $\pm$ 0.2	4.8 $\pm$ 1.2*	1.3 $\pm$ 0.2	5.3 $\pm$ 1.5*	1.7 $\pm$ 0.3	1.8 $\pm$ 0.2

Values are mean $\pm$ S.E.M.; $n = 9-10$ per group. *Significant differences from age-matched lean controls ($P < 0.05$).

Table 2 Mechanical properties of the mid-shaft femur and tibia determined by three-point bending to failure

	7 weeks lean	7 weeks fatty	13 weeks lean	13 weeks fatty	20 weeks lean	20 weeks fatty
Femur						
Length (mm)	27.5±0.2	26.1±0.3*	35.2±0.2	33.3±0.3*	38.0±0.1	34.9±0.2*
Stiffness (N/mm)	88.4±6.9	76.3±4.8	245.4±10.3	202.9±12.6*	331.7±11.0	254.7±10.8*
Ultimate load (N)	57.0±3.0	56.0±2.1	128.1±3.2	123.9±3.6	168.9±7.0	137.1±6.7*
Elastic modulus (GPa)	2.25±0.17	1.82±0.11*	3.61±0.14	2.72±0.13*	3.53±0.16	3.22±0.13
Ultimate stress (MPa)	126.2±5.3	121.1±6.5	192.4±5.4	172.6±8.5	192.0±10.6	180.4±9.6
Energy to max force (mJ)	23.7±1.5	28.1±1.0	56.4±3.6	51.5±3.6	69.2±6.9	52.9±5.1*
Post-yield displacement (mm)	0.40±0.11	0.38±0.09	0.48±0.07	0.47±0.09	0.33±0.06	0.35±0.03
Tibia						
Length (mm)	31.2±0.4	29.6±0.3*	38.7±0.4	36.8±0.2*	41.4±0.2	38.5±0.2*
Stiffness (N/mm)	56.1±3.3	50.7±1.8	179.0±6.3	152.6±9.2*	214.5±7.7	176.3±10.6*
Ultimate load (N)	35.1±1.3	32.6±1.8	88.2±3.5	78.4±3.1*	116.8±4.4	90.5±2.8*
Elastic modulus (GPa)	5.45±0.46	5.07±0.25	8.21±0.43	8.72±0.66	7.26±0.42	8.42±0.46
Ultimate stress (MPa)	152.0±10.2	130.5±9.3	234.3±12.8	250.5±11.2	263.4±11.7	284.8±16.5
Energy to max force (mJ)	15.1±1.0	14.5±1.3	28.3±2.4	23.4±1.4	37.3±2.2	27.4±1.4*
Post-yield displacement (mm)	0.93±0.29	1.16±0.39	0.90±0.15	0.62±0.16	0.79±0.14	0.39±0.12

Values are mean±s.e.m.; n=9–10 per group. *Significant differences from age-matched lean controls (*P*<0.05).

Mixed cortical and cancellous bone (distal femur metaphysis, proximal tibial metaphysis, and femoral neck) Total BMD (including cortical shell and cancellous core) and cancellous BMD were assessed at three sites having mixed cortical and cancellous bone (Fig. 2). Pre-diabetic rats had increased cancellous BMD at the distal femur (+9%) and total BMD at the proximal tibia (+10%) versus

lean controls. However, this enhancement disappeared or was reversed by 13 weeks in the short-term diabetic condition. With long-term diabetes at 20 weeks, deficits in total and cancellous BMD occurred relative to lean controls at all three bone sites, ranging from a minimum of 6% deficit in cancellous BMD at the femoral neck to a 29% reduction in cancellous BMD at the distal femur.

Figure 1 Cross-sectional moment of inertia (CSMI) and cortical volumetric bone mineral density (vBMD) for femoral (A and B) and tibial (C and D) mid-shaft bone in type 2 diabetic fatty and lean control rats. White bars represent lean control rats (*n*=10/age group) and black bars represent type 2 diabetic fatty rats (*n*=9/age group). Data represent mean±s.e.m. *Statistical differences from age-matched lean controls (*P*≤0.05).

Axial bone (L3 and L4 vertebrae) Volumetric BMD of L3 vertebral body in the 7 weeks ZDF rats was 10% lower than that in lean controls; however, the difference in BMD narrowed by 20 weeks of age (Fig. 3A). When the bone mineral content of the entire L4 vertebra was assessed with ashing, no differences were observed in pre- and short-term-diabetic stages. At 20 weeks, the long-term diabetic ZDF rats exhibited a significantly lower ash weight in comparison to lean controls (Fig. 3B). The L4 vertebrae were visually smaller at 20 weeks in the ZDF rats (Fig. 3C), consistent with the reduced ash weights in this group.

Structural and material properties

Appendicular mid-shaft bone (tibia and femur)

Three-point bending to failure tests assessed mechanical properties of mid-shaft cortical bone (Table 2). Deficits in stiffness appeared in the femur and tibia in the short-term diabetic (13 weeks of old) ZDF rats (−17% and −14.5% respectively), when compared with lean controls at the same age. These deficits in stiffness were exacerbated in long-term diabetic rats (−23% and −18% respectively). UL, the maximal force absorbed by the bone, was 11% lower in the tibia in short-term diabetic rats but not in the femur. By 20 weeks of age, this deficit (versus lean controls) doubled for the tibia (−22%) and became apparent in the femur as well (−19%). These alterations are illustrated with idealized load–displacement curves utilizing yield, UL, and fracture data points in Fig. 4.

Figure 2 Total and cancellous volumetric bone mineral density (vBMD) in distal femur (A and B), proximal tibia (C and D), and femoral neck (E and F) metaphyseal sites. White bars represent lean control rats ($n=10$ /age group) and black bars represent type 2 diabetic fatty rats ($n=9$ /age group). Data represent mean $\pm$ S.E.M. *Statistical differences from age-matched lean controls ($P \leq 0.05$). †Denotes a tendency for statistical difference from age-matched lean controls ($P \leq 0.10$).

Toughness of mid-shaft bone, defined as energy absorbed to UL (estimated by area under the load–displacement curve up to displacement at UL) is reduced significantly in long-term diabetes by 23% and 26% in femora and tibiae respectively. Post-yield displacement, an indicator of bone ductility, was not different between ZDF and lean rats in the femur. Large variability in the tibia likely prevented post-yield displacement from achieving statistical significance in short-term diabetes (–31% versus lean controls) and long-term diabetes (–51% versus lean controls). Data for material properties, which describe mechanical properties of the bone tissue independent of its size or geometry, revealed that only elastic modulus of the femur was reduced by 19% and 25% in pre- and short-term-diabetic ZDF rats respectively.

Mixed cortical and cancellous bone (femoral neck)

Compressive loading of the femoral necks (which also involves bending and shear forces) was performed to assess mechanical properties at this site. UL was reduced 18% in the short-term ZDF rats versus lean controls and 44% in long-term diabetic animals (Fig. 5). Stiffness of the femoral neck was slightly higher in short-term diabetic rats at 13

Figure 3 Lumbar spine properties of type 2 diabetic fatty and lean control rats. (A) Cancellous volumetric bone mineral density (vBMD) of L3 vertebral body; (B) L4 vertebral ash weight (% dry weight). White bars represent lean control rats ($n=10$ /age group) and black bars represent type 2 diabetic fatty rats ($n=9$ /age group) (C) photograph of three representative sets of L4 vertebrae. *Statistical differences from age-matched lean controls ($P \leq 0.05$).

weeks (+16%), but with the progression of long-term diabetes stiffness was reduced (34%) versus that of the age-matched lean controls.

Discussion

Currently, there are conflicting reports in the literature regarding the effects of type 2 diabetes on BMD in humans (Isaia *et al.* 1987, Wakasugi *et al.* 1993, Van Daele *et al.* 1995), as well as an apparent dissociation between BMD and fracture risk in type 2 diabetic patients (Schwartz *et al.* 2001). These apparent

Figure 4 Idealized load-displacement curves utilizing actual load and displacement values at yield, maximal, and fracture forces from three-point bending to failure tests for mid-shaft femur (A, C, and E) and mid-shaft tibia (B, D, and F) in type 2 diabetic fatty rats and age-matched lean controls (refer to Table 2 for absolute values).

discrepancies could be the result of the type of bone studied (e.g., cancellous versus cortical) or the severity and duration of the disorder in the subject population studied. In addition, coexisting obesity in most type 2 diabetic humans confounds interpretation. Therefore, the purpose of the present study was to determine whether type 2 diabetes alters bone structural and mechanical properties in the ZDF rats, and to determine whether possible declines in BMD and bone mechanical properties coincide with the onset and progression of the disease. To our knowledge, this is the first investigation to examine the combined alterations in the structural and mechanical properties of the ZDF skeleton with the onset and progression of type 2 diabetes. The results demonstrate that in the pre-diabetic condition, which is associated with normoglycemia and hyperinsulinemia, there is an increase in tibial BMD (Figs 1 and 2). By contrast, the short- and long-term diabetic conditions that are associated with fasting hyperglycemia, predisposes femora and tibiae to diminished BMDs and mechanical integrity (Table 2, Figs 1, 2 and 5).

Longitudinal growth was impaired in both femora and tibiae as early as 7 weeks of age in pre-diabetes, preceding the development of hyperglycemia. By 20 weeks of age, these long bones were 7–8% shorter than bones of age-matched lean controls. Interestingly, the increase in long bone diameters and CSMI observed in Goto-Kakizaki rats, another rodent model of type 2 diabetes (Ahmad *et al.* 2003), was not observed in the ZDF rats. In fact, data from the present study

Figure 5 Femoral neck structural mechanical properties in type 2 diabetic fatty and lean control rats. (A) Ultimate load and (B) stiffness. White bars represent lean control rats ($n=10$ /age group) and black bars represent type 2 diabetic fatty rats ($n=9$ /age group). Data represent mean $\pm$ S.E.M. *Statistical differences from age-matched lean controls ($P \leq 0.05$).

provide evidence for reduced radial growth as illustrated by smaller CSMI values in the long-term diabetic (20 weeks of old) rats. Lower ash weights of the L4 vertebral bodies of ZDF rats in the present investigation suggest that this effect on longitudinal and/or radial growth impacts on the axial skeleton as well.

The significant reduction in the cross-sectional geometry of the long bones is likely the major contributor to the reductions observed in the mechanical properties (UL and stiffness) of the short- and long-term diabetic rats. The estimated bending strength of the humeral and tibial diaphyses in Goto-Kakizaki rats has been reported to be higher relative to Wistar control animals (Ahmad *et al.* 2003). The present study offers a more complete determination of bone strength changes with type 2 diabetes, with direct measurement of mechanical properties during the three stages of development and progression of type 2 diabetes. It is unknown whether the different results of Ahmad *et al.* (2003) from 12-month-old Goto-Kakizaki rats are in part related to the severity and duration of the disease, or to key differences in this rodent model of type 2 diabetes with that of the ZDF rats.

Deficits in mid-shaft cortical BMD were also observed in long bones, but were relatively smaller than those in CSMI. For the significant differences in CSMI highlighted in Fig. 1A and C, percent reductions range from 15% to 30%, whereas the significant differences in BMD (Fig. 1B and D) are all less than 5%. One might expect alterations in BMD to impact more directly on material properties, since density is independent of bone size, but elastic modulus of the femur was the only material property affected by type 2 diabetes (and only at 7 and 13 weeks). No significant differences were observed for ultimate stress, i.e., the tissue-level strength. On the one hand, these results suggest a decoupling, or dissociation, between tissue mineralization and material properties. On the other hand, the percent differences in BMD are quite small, although statistically significant, yet material properties are generally not significantly different. To further explore this, correlations were determined between material properties (elastic modulus and ultimate stress) and BMD. When all age groups were included (pooling lean and

fatty animals), r^2 values ranged from 0.35 to 0.67 due mainly to the significantly lower values for the 7-week animals. Correlation r^2 values dropped to less than 0.15 when only the short- and long-term diabetic groups were included, further suggesting a dissociation.

Considering this dissociation, and even the different effects for the two long bones studied, suggest a major role for other determinants of bone functional properties (beyond cross-sectional geometry and BMD). The possibilities include the quality of the mineral, the bonding between mineral and collagen, and the properties of the organic matrix. One key candidate in this last category would be the accumulation of advanced glycation end-products (AGEs) as has been observed with chronic hyperglycemia in another rodent model for type 2 diabetes, the WBN/Kob rat (Saito *et al.* 2006). It is also interesting to note that energy absorbed to UL, an indicator of bone toughness, was decreased by 24–26% in the tibiae and femora of long-term diabetic rats. This property is determined not only by both the structural properties of stiffness and UL, but also by bone ductility, which may be reduced in the long-term diabetic rats, as indicated by numerical reductions in post-yield displacement.

Much of the published literature assessing BMD changes in diabetics utilizes dual energy X-ray absorptiometry, which cannot distinguish independent effects on cortical and cancellous bone. Using CT, the present study revealed more dramatic effects on cancellous bone compartments in appendicular bone with short-term (13 weeks) and long-term progression (20 weeks) of type 2 diabetes, particularly at the proximal tibia and distal femur. Axial bone may respond differently, since the deficit observed in L3 vertebral cancellous BMD in the pre-diabetic (7 weeks) ZDF rats disappeared by 20 weeks of age. In another investigation, reductions in BMD of the fourth and fifth lumbar vertebrae were observed in 20-week-old ZDF rats when compared with lean controls (Shibata *et al.* 2000). The results from Amir *et al.* (2002) also indicated that cancellous bone volume (determined histologically) in the distal femur and vertebral bodies was diminished when symptoms of diabetes were manifested for 1, 2, and 7–8 months. Cortical thickness of the femur and vertebra were not different at any time between the Cohen diabetic and control rats (Amir *et al.* 2002). CT data from ZDF rats of the present study suggest that the cortical shell is slightly diminished, since total BMD (assessing cortical shell plus cancellous core) at all three metaphyseal sites was affected to a similar degree as the cancellous BMD.

Collectively, data from the present study support the hypothesis that type 2 diabetes adversely affects bone structural and mechanical properties of the ZDF rats, and that the severity of these changes increases with the temporal progression of the disease. The ZDF strain is a diabetic rat model (Charles River Laboratories) and was chosen for study, because it allows for the examination of skeletal properties during the development of type 2 diabetes separate from those induced by obesity, which can have profound effects on bone structural and mechanical properties. Furthermore, the onset

and progression of type 2 diabetes have been well characterized in this strain and allows for the determination of bone properties with the corresponding changes in insulin resistance, impaired glucose tolerance and intolerance, and hyperglycemia, all of which can influence skeletal tissue (Clark *et al.* 1983, Peterson *et al.* 1990b, Sparks *et al.* 1998). By contrast, the Goto-Kakizaki rat is a strain that develops moderate type 2 diabetes (Janssen *et al.* 2004), and alterations in bone mechanical and structural properties in the most common mouse strain used for type 2 diabetes research, the db/db mouse (Wu & Huan 2007), have been previously characterized (Ealey *et al.* 2006).

Furthermore, the results of the current investigation suggest that disparities in the human literature regarding the effects of type 2 diabetes on skeletal properties may be associated with the bone sites studied and the severity or duration of the disease in the patient population studied. In addition to the greater fragility of bone associated with the progression of type 2 diabetes, the present study also demonstrates that some important effects (e.g., on longitudinal growth) may occur in the pre-diabetic hyperinsulinemic and euglycemic conditions.

Several mechanisms may be involved in the observed alterations in bone structural and mechanical properties. These mechanisms include the effects of 1) hyperinsulinemia, 2) hyperglycemia and 3) leptin on skeletal tissue.

Hyperinsulinemia and skeletal tissue

Circulating insulin alters the metabolism and promotes the growth of many target tissues, including the skeletal system. In fact, insulin stimulates osteoblastic activity (Canalis *et al.* 1977, Raisz & Kream 1983), resulting in enhanced bone formation. Consistent with this effect, BMDs in ZDF rats of the present study were greater in the distal femora (Fig. 2B), proximal tibiae (Fig. 2C), and tibial mid-shafts (Fig. 1D) in the pre-diabetic state (7 weeks) when plasma insulin concentration was correspondingly higher. Regression analysis further indicated that there was a significant linear relationship between changing plasma insulin concentrations and femoral BMD in the ZDF rats (Fig. 6A). The relationship between BMD and insulin levels has also been reported in human type 2 diabetics, where BMD was positively correlated with fasting serum insulin concentrations (Rishaug *et al.* 1995). Furthermore, the increased BMDs at various skeletal sites have been reported in hyperinsulinemic individuals in the presence and absence of type 2 diabetes (Verhaeghe & Bouillon 1996). Taken together, these studies suggest that hyperinsulinemia contributes to increased BMD in both humans and rodents.

Hyperglycemia and skeletal tissue

The chronic hyperglycemia manifested in type 2 diabetes accelerates the nonenzymatic process of protein glycosylation, resulting in the formation and accumulation of AGEs. AGEs accumulate in bone with age (Miyata *et al.* 1996, Fratzl *et al.*

Figure 6 Scattergrams showing linear relationship between plasma insulin (A) or glucose (B) concentrations and cancellous bone mineral density (BMD) in the distal femur from 7-, 13-, and 20-week-old diabetic fatty rats. (A) $r=0.51$; $y=259+(13.51x)$; $P<0.002$ and (B) $r=0.674$; $y=434+(0.64x)$; $P<0.001$.

2004, Odetti *et al.* 2005) and have been suggested to contribute to skeletal fragility (Bailey *et al.* 1998, Schwartz 2003, Dominguez *et al.* 2005). Increased AGEs within the bone matrix make the tissue more brittle (less tough) by increasing the amount of collagen cross-linking (Wu *et al.* 2003, Boxberger & Vashishth 2004, Vashishth *et al.* 2004, Tang *et al.* 2005, Saito *et al.* 2006). In the WBN/Kob rodent model for type 2 diabetes, bone mechanical properties are significantly impaired despite maintained BMD, coincident with increases in glycation-induced pentosidine (Saito *et al.* 2006). The accumulation of AGEs is negatively correlated with ultimate strain (Hernandez *et al.* 2005), post-yield deformation (Wang *et al.* 2002, Boxberger & Vashishth 2004, Tang *et al.* 2005), and work to fracture (Viguet-Carrin *et al.* 2006). Although AGEs were not assessed in the present study, previous work has documented the increased skeletal AGEs and reduced bone strength in diabetic rats (Tomasek *et al.* 1994, Katayama *et al.* 1996). AGEs accumulation could also negatively influence bone through direct effects on osteoblasts (Katayama *et al.* 1996) and/or osteoclasts (Fong *et al.* 1993). For example, the accumulation of AGEs in bone matrix reduces bone formation rates and increases calcium efflux from calvariae, exacerbating bone resorption (Fong *et al.* 1993). In the present study, hyperglycemia was present at 13 and 20 weeks of age and corresponded with the declines in BMD observed during those time points (i.e., reduced BMDs in the femoral necks, distal femora, proximal tibiae, and femoral and tibial mid-shafts of the ZDF rats). Furthermore, regression analysis demonstrated that plasma glucose is negatively correlated with BMD (Fig. 6B). Interestingly, neither hyperglycemia nor bone loss was observed in the pre-diabetic (7 weeks) fatty rats, but rather, these animals often had greater BMDs versus lean controls, further supporting the temporal relationship between hyperglycemia, AGEs accumulation, and enhanced skeletal fragility with type 2 diabetes.

Leptin and skeletal tissue

Leptin resistance is a common characteristic of type 2 diabetes and ZDF rats are known to be hyperleptinemic (Liu *et al.* 2007) due to nonfunctional leptin receptors (Chua *et al.* 1996). There is

some evidence to indicate that leptin acts upon osteoblasts via a hypothalamic relay and neural mediators to down-regulate bone mass (Karsenty 2006). However, positive effects of leptin on bone mass have also been observed (Ducy *et al.* 2000). For example, impaired longitudinal bone growth and osteopenia have been observed with leptin deficiency or dysfunctional leptin receptors (Lorentzon *et al.* 1986, Steppan *et al.* 2000). These data are consistent with the decrements in femoral and tibial lengths and reduced BMD of ZDF rats observed in the present investigation. Furthermore, Liu *et al.* (2007) reported reduced bone formation following distraction osteogenesis in 9–11-week-old ZDF rats that was associated with hyperinsulinemia, hyperglycemia, attenuated serum osteocalcin levels, and leptin-signaling deficiency. Thus, the leptin-resistant status of ZDF rats indicates a potential role for this factor in modulating bone mass.

In conclusion, the present study demonstrates the diminished BMD and decreases in a number of bone mechanical properties in the femora and tibiae with the progression of type 2 diabetes in the ZDF rats. The alterations in BMD and bone mechanical properties were closely associated with the onset of hyperinsulinemia and hyperglycemia, which may have direct adverse effects on skeletal tissue. Therefore, disparities in the literature on human regarding the effects of type 2 diabetes on skeletal properties may be associated with the bone sites studied and the severity or duration of the disease in the patient population studied.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research was supported by National Aeronautics and Space Administration (NASA) Grants NAG2-1340 and NCC2-1166 and by NASA via the NASA Cooperative Agreement NCC9-58-H with the National Space Biomedical Research Institute.

Acknowledgements

The authors gratefully acknowledge the technical assistance of Jan Stallone, Summer Bryant, and Alicia C B Allen for data collection and Mats I Nilsson for graphics preparation.

References

- Ahmad T, Ohlsson C, Saaf M, Ostenson C-G & Kreicbergs A 2003 Skeletal changes in type-2 diabetic Goto-Kakizaki rats. *Journal of Endocrinology* **178** 111–116.
- Albright R & Reifenshtein EC 1948 *The Parathyroid Glands and Metabolic Bone Disease; Selected Studies.*, Baltimore, MD: Williams & Wilkins.
- Amir G, Rosenmann E, Sherman Y, Greenfeld Z, Ne'eman Z & Cohen AM 2002 Osteoporosis in the Cohen diabetic rat: correlation between histomorphometric changes in bone and microangiopathy. *Laboratory Investigation* **82** 1399–1405.

- Bailey A, Paul RG & Knott L 1998 Mechanisms of maturation and ageing of collagen. *Mechanisms of Ageing and Development* **106** 1–56.
- Berney PW 1952 Osteoporosis and diabetes mellitus; report of a case. *Journal of the Iowa Medical Society* **42** 10–12.
- Boxberger J & Vashishth D 2004 Nonenzymatic glycation affects bone fracture by modifying creep and inelastic properties of collagen. *Transactions of the Orthopaedic Research Society* **29** 491.
- Buyschaert M, Cauwe F, Jamart J, Brichant C, De Coster P, Magnan A & Donckier J 1992 Proximal femur density in type I and type II diabetic patients. *Diabetes & Metabolism* **18** 32–37.
- Canalis E, Dietrich JW, Maina DM & Raisz LG 1977 Hormonal control of bone collagen synthesis *in vitro*: effects of insulin and glucagons. *Endocrinology* **100** 668–674.
- Chua SJ, Chung WK, Wu-Peng XS, Zhang Y, Liu SM, Tartaglia L & Leibel RL 1996 Phenotypes of mouse diabetes and rat fatty due to mutations in the OB (leptin) receptor. *Science* **271** 994–996.
- Clark J, Palmer CJ & Shaw WN 1983 The diabetic Zucker fatty rat. *Proceedings of the Society for Experimental Biology and Medicine* **173** 68–75.
- Van Daele PL, Stolk RP, Burger H, Algra D, Grobbee DE, Hofman A, Birkenhager JC & Pols HA 1995 Bone density in non-insulin-dependent diabetes mellitus. The Rotterdam study. *Annals of Internal Medicine* **122** 409–414.
- Dalen N, Hallberg D & Lamke B 1975 Bone mass in obese subjects. *Acta Medica Scandinavica* **197** 353–355.
- Dominguez L, Barbagallo M & Moro L 2005 Collagen overglycosylation: a biochemical feature that may contribute to bone quality. *Biochemical and Biophysical Research Communications* **330** 1–4.
- Ducy P, Amling M, Takeda S, Priemel M, Schilling AF, Beil FT, Shen J, Vinson C, Rueger JM & Karsenty G 2000 Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. *Cell* **100** 197–207.
- Ealey KN, Fonseca D, Archer MC & Ward WE 2006 Bone abnormalities in adolescent leptin-deficient mice. *Regulatory Peptides* **136** 9–13.
- Fong Y, Edelstein D, Wang EA & Brownlee M 1993 Inhibition of matrix-induced bone differentiation by advanced glycation end-products in rats. *Diabetologia* **36** 802–807.
- Forsen L, Meyer HE, Midtjell K & Edna TH 1999 Diabetes mellitus and the incidence of hip fracture: results from the Nord-Trøndelag Health Survey. *Diabetologia* **42** 920–925.
- Forst T, Pützner A, Kann P, Schehler B, Lobmann R, Schafer H, Andreas J, Bockisch A & Beyer J 1995 Peripheral osteopenia in adult patients with insulin-dependent diabetes mellitus. *Diabetic Medicine* **12** 874–879.
- Fratzl P, Gupta H, Paschalis EP & Roschger P 2004 Structure and mechanical quality of the collagen-mineral nano-composite in bone. *Journal of Materials Chemistry* **14** 2115–2123.
- Frisbee J & Delp MD 2006 Vascular function in the metabolic syndrome and the effects on skeletal muscle perfusion: lessons from the obese Zucker rat. In *Essays in Biochemistry: The Biochemical Basis of the Health Effects of Exercise*, pp 145–161. Ed. A Wagenmakers. London, UK: Portland Press.
- Hernandez C, Tang SY, Baumbach BM, Hwu PB, Sakkee AN, van der Ham F, DeGroot J, Bank RA & Keaveny TM 2005 Trabecular microfracture and the influence of pyridinium and non-enzymatic glycation-mediated collagen cross-links. *Bone* **37** 825–832.
- Isaia G, Bodrato L, Carlevatto V, Mussetta M, Salamano G & Molinatti GM 1987 Osteoporosis in type II diabetes. *Acta Diabetologica Latina* **24** 305–310.
- Janssen U, Vassiliadou A, Riley SG, Phillips AO & Floege J 2004 The quest for a model of type II diabetes with nephropathy: the Goto Kakizaki rat. *Journal of Nephrology* **17** 769–773.
- Karsenty G 2006 Convergence between bone and energy homeostases: leptin regulation of bone mass. *Cell Metabolism* **4** 341–348.
- Katayama Y, Akatsu T, Yamamoto M, Kugai N & Nagata N 1996 Role of nonenzymatic glycosylation of type I collagen in diabetic osteopenia. *Journal of Bone and Mineral Research* **11** 931–937.
- Keegan TH, Kelsey JL, Sidney S & Quesenberry CP Jr 2002 Foot problems as risk factors for fractures. *American Journal of Epidemiology* **155** 926–931.
- Liu Z, Aronson J, Wahl EC, Liu L, Perrien DS, Kern PA, Fowlkes JL, Thrall KM, Bunn RC, Cockrell GE *et al.* 2007 A novel rat model for the study of deficits in bone formation in type-2 diabetes. *Acta Orthopaedica* **78** 46–55.
- Meyer HE, Tverdal A & Falch JA 1993 Risk factors for hip fracture in middle-aged Norwegian women and men. *American Journal of Epidemiology* **137** 1203–1211.
- Miyata T, Kawai R, Taketomi S & Sprague SM 1996 Possible involvement of advanced glycation end-products in bone resorption. *Nephrology, Dialysis, Transplantation* **11** 54–57.
- Morrison LB & Bogan IK 1927 Bone development in diabetic children: a Roentgen study. *American Journal of the Medical Sciences* **174** 313–319.
- Nicodemus KK & Folsom AR 2001 Type 1 and type 2 diabetes and incident hip fractures in postmenopausal women. *Diabetes Care* **24** 1192–1197.
- Odetti P, Rossi S, Monacelli F, Poggi A, Cernigliaro M, Federici M & Federici A 2005 Advanced glycation end products and bone loss during aging. *Annals of the New York Academy of Sciences* **1043** 710–717.
- Ottenbacher KJ, Ostir GV, Peek MK, Goddwin JS & Markides KS 2002 Diabetes mellitus as a risk factor for hip fracture in Mexican American older adults. *Journals of Gerontology Series A: Biological Sciences and Medical Sciences* **57** M648–M653.
- Peterson RG, Neel MA, Little LA, Kincaid JC & Eichberg J 1990a Neuropathic complications in the Zucker diabetic fatty rat (ZDF/Drt-fa). In *Frontiers in Diabetes Research. Lessons from Animal Diabetes III*, pp 456–458. Ed. E Shafir. London: Smith-Gordon.
- Peterson RG, Shaw WN, Neel MA, Little LA & Eichberg J 1990b Zucker diabetic fatty rat as a model for non-insulin-dependent diabetes mellitus. *ILAR News* **32** 16–19.
- Raisz L & Kream BE 1983 Regulation of bone formation. *New England Journal of Medicine* **309** 29–35.
- Rishaug U, Birkeland KI, Falch JA & Vaaler S 1995 Bone mass in non-insulin-dependent diabetes mellitus. *Scandinavian Journal of Clinical and Laboratory Investigation* **55** 257–262.
- Saito M, Fujii K, Mori Y & Marumo K 2006 Role of collagen enzymatic and glycation induced cross-links as a determinant of bone quality in spontaneously diabetic WBN/Kob rats. *Osteoporosis International* **17** 1514–1523.
- Schwartz A 2003 Diabetes mellitus: does it affect bone? *Calcified Tissue International* **73** 515–519.
- Schwartz AV, Sellmeyer DE, Ensrud KE, Cauley JA, Tabor HK, Schreiner PJ, Jamal SA, Black DM & Cummings SR 2001 Older women with diabetes have an increased risk of fracture: a prospective study. *Journal of Clinical Endocrinology and Metabolism* **86** 32–38.
- Shibata T, Takeuchi S, Yokota S, Kakimoto K, Yonemori F & Wakitani K 2000 Effects of peroxisome proliferator-activated receptor- α and - γ agonist, JTT-501, on diabetic complications in Zucker diabetic fatty rats. *British Journal of Pharmacology* **130** 495–504.
- Silberberg R 1986 The skeleton in diabetes mellitus: a review of the literature. *Diabetes Research* **3** 329–338.
- Sparks JD, Phung TL, Bolognino M, Cianci J, Khurana R, Peterson RG, Sowden MP, Corsetti JP & Sparks CE 1998 Lipoprotein alterations in 10- and 20-week old Zucker diabetic fatty rats: hyperinsulinemic versus insulinopenic hyperglycemia. *Metabolism* **47** 1315–1324.
- Stephan C, Crawford DT, Chidsey-Frink KL, Ke H & Swick AG 2000 Leptin is a potent stimulator of bone growth in ob/ob mice. *Regulatory Peptides* **92** 73–78.
- Tang S, Sharan A, Novak E, Ford T & Vashishth D 2005 Nonenzymatic glycation causes loss of toughening mechanisms in human cancellous bone. *Transactions of the Orthopaedic Research Society* **30** 678.
- Tomasek J, Meyers SW, Basinger JB, Green DT & Shew RL 1994 Diabetic and age-related enhancement of collagen-linked fluorescence in cortical bones of rats. *Life Sciences* **55** 855–861.
- Vashishth D, Wu P & Gibson G 2004 Age-related loss in bone toughness is explained by non-enzymatic glycation of collagen. *Transactions of the Orthopaedic Research Society* **29** 497.

- Verhaege J & Bouillon R 1996 Effects of diabetes and insulin on bone metabolism. In *Principals of Bone Biology*, pp 549–561. New York: Academic Press.
- Viguet-Carrin S, Roux JP, Arlot ME, Merabet Z, Leeming DJ, Byrjalsen I, Delmas PD & Bouxsein ML 2006 Contribution of the advanced glycation end product pentosidine and of maturation of type I collagen to compressive biomechanical properties of human lumbar vertebrae. *Bone* **39** 1073–1079.
- Vrabec JT 1998 Tympanic membrane perforations in the diabetic rat: a model of impaired wound healing. *Otolaryngology – Head and Neck Surgery* **118** 304–308.
- Wakasugi M, Wakao R, Tawata M, Gan N, Koizumi K & Onaya T 1993 Bone mineral density measured by dual energy X-ray absorptiometry in patients with non-insulin-dependent diabetes mellitus. *Bone* **14** 29–33.
- Wang X, Shen X, Li X & Agrawal CM 2002 Age-related changes in the collagen network and toughness of bone. *Bone* **31** 1–7.
- Wu KK & Huan Y 2007 Diabetic atherosclerosis mouse models. *Atherosclerosis* **191** 241–249.
- Wu P, Koharski C, Nonnenmann H & Vashishth D 2003 Loading on non-enzymatically glycated and damaged bone results in an instantaneous fracture. *Transactions of the Orthopaedic Research Society* **28** 404.

Received in final form 18 August 2008

Accepted 22 August 2008

Made available online as an Accepted Preprint

28 August 2008