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Characteristics of bone strength and metabolism in type 2 diabetic model Tsumura, Suzuki, Obese Diabetes mice

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ABSTRACT

Objective: Type 2 diabetes mellitus (T2DM) is a metabolic disease characterized by hyperglycemia, hyperinsulinemia, and complications such as obesity and osteoporosis. The Tsumura, Suzuki, Obese Diabetes (TSOD) mouse is an animal model of spontaneous obese T2DM. However, bone metabolism in TSOD mice is yet to be investigated. The objective of the present study was to investigate the effects of T2DM on bone mass, metabolism, microstructure, and strength in TSOD mice.

Methods: We determined the following parameters in TSOD mice and Tsumura, Suzuki, Non-obesity (TSNO) mice (as controls): serum glucose levels; serum insulin levels; bone mass; bone microstructure; bone metabolic markers; and bone strength. We also performed the oral glucose tolerance test and examined histological sections of the femur. We compared these data between both groups at pre-diabetic (10 weeks) and established (20 weeks) diabetic conditions.

Results: Bone strength, such as extrinsic mechanical properties, increased with age in the TSOD mice and intrinsic material properties decreased at both 10 weeks and 20 weeks. Bone resorption marker levels in TSOD mice were significantly higher than those in the control mice at both ages, but there was no significant difference in bone formation markers between the groups. Bone mass in TSOD mice was lower than that in controls at both ages. The trabecular bone volume at the femoral greater trochanter increased with age in the TSOD mice. The femoral mid-diaphysis in TSOD mice was more slender and thicker than that in TSNO mice at both ages.

Conclusions: Bone mass of the femur was lower in TSOD mice than in TSNO mice because hyperinsulinemia during pre-diabetic and established diabetic conditions enhanced bone resorption due to high bone turnover. In addition, our data suggest that the bone mass of the femur was significantly reduced as a result of chronic hyperglycemia during established diabetic conditions in TSOD mice. We suggest that bone strength in the femur deteriorated due to the reduction of bone mass and because the femoral mid-diaphysis was more slender in TSOD mice.

1. Introduction

Diabetes is associated with an increased risk of fragility fractures (Janghorbani et al., 2007; Melton 3rd et al., 2008). Albright and Reifenstein (1948) were the first to report the presence of low bone mineral density (BMD) and a high incidence of fractures in diabetic patients. More recently, a meta-analysis showed that BMD is reduced in patients with type 1 diabetes mellitus (T1DM) but is increased in patients with type 2 diabetes mellitus (T2DM) (Vestergaard, 2007). T1DM has been shown to be associated with reduced BMD and insulin deficiency (Nyman et al., 2011; Silva et al., 2009). In contrast, studies were more inconsistent in patients with T2DM, who do not exhibit insulin deficiency, with some showing a similar BMD (Hampson et al., 1998; Tuominen et al., 1999), a higher BMD (Hanley et al., 2003; Strotmeyer et al., 2004), or even a lower BMD (Gregorio et al., 1994; Yaturu et al., 2009) compared to non-diabetic patients. There are

Abbreviations: T2DM, type 2 diabetes mellitus; TSOD, Tsumura, Suzuki, Obese Diabetes; OCN, osteocalcin; TRAcP5b, tartrate-resistant acid phosphatase 5b; TSNO, Tsumura, Suzuki, non-obesity; BMD, bone mineral density; T1DM, type 1 diabetes mellitus; CSMI, cross-sectional moment inertia; BMC, bone mineral content; PBS, phosphate-buffered saline; micro-CT, micro-computed tomography; OGTT, oral glucose tolerance test

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several factors which might explain such disparities, including differences in the severity and duration of the disease, along with the relative impairment of glucose metabolism (Vestergaard, 2007), or the fact that hyperinsulinemia can accelerate factors related to bone formation (Ashraf et al., 2013). These findings were reported in clinical investigations and studies involving animal models of T2DM (Fajardo et al., 2014). In T2DM, low bone mass may occur because of the impairment of glucose metabolism and high bone mass due to elevated insulin levels, as this can promote anabolic activity.

Several studies have used T2DM animal models to investigate the relationship between fragility fractures and diabetes, although a suitable animal model for T2DM is yet to be established. The Tsumura, Suzuki, Obese Diabetes (TSOD) mouse strain was first developed in 1992 through the selective inbreeding of obese ddY mice (Suzuki et al., 1999). Analysis showed that only male TSOD mice showed hyperglycemia, hyperinsulinemia, urinary glucose, and obesity (Suzuki et al., 1999). In contrast, the Tsumura, Suzuki, Non-obesity (TSNO) mouse strain was simultaneously established from ddY mice as a control, and exhibits neither obesity nor hyperglycemia (Hirayama et al., 1999). In TSOD mice, three quantitative trait loci were identified on the chromosome that determines genetic blood glucose levels (Nidd4 on chromosome 11), controls body weight (Nidd5 on chromosome 2 and Nidd6 on chromosome 1), and insulin levels (Nidd5 on chromosome 2) (Hirayama et al., 1999). Furthermore, it is evident that the existing literature does not address issues related to the skeletal phenotype in either TSOD or TSNO mice.

Therefore, in the present study, we used TSOD mice to investigate the effect of T2DM progression on various key aspects of skeletal integrity, including bone mass, bone metabolism, bone microstructure, and bone strength. These parameters were characterized during prediabetic conditions (age: 10 weeks) and during established diabetic conditions (age: 20 weeks).

2. Material and methods

2.1. Animals

We studied male TSOD and TSNO mice (The Institute for Animal Reproduction, Ibaraki, Japan) (n = 6/group) from 6 weeks of age to 10 or 20 weeks of age. The mice were housed and had free access to food (CE-2; Clea Japan Inc., Tokyo, Japan) and water. The animal room was maintained at 22 ± 2 °C with a 12-h light (8:00–20:00) and dark (20:00–8:00) cycle. Before the mice were sacrificed by cervical dislocation at 10 and 20 weeks of age, we collected serum samples that were refrigerated until each analysis was carried out. A blood sample from each mouse was taken from the cavernous sinus with a capillary. Both femurs were removed from each mouse and cleaned of muscles and tendons. The right femur was wrapped in gauze soaked in phosphate-buffered saline (PBS) and stored at −40 °C until three-point bending test and micro-computed tomography (micro-CT) scanning were performed. The left femur was fixed in 10% neutral buffered formalin. Experimental protocols were approved by the Guidelines for the Care and Use of Laboratory Animals (Prime Minister's Office Directive No. 228, 2015).

2.2. Serum glucose level and oral glucose tolerance test

Serum glucose levels were measured using the Wako glucose CII-test (Wako Pure Chemical Industries, Osaka, Japan). An oral glucose tolerance test (OGTT) was performed on each mouse at 10 and 20 weeks of age. All mice were weighed and then deprived of food for the previous 20 h. A glucose (2 g/kg) solution was given by weight, and blood samples were taken from the cavernous sinus before and at 30, 60, and 120 min after administration of the glucose solution. In addition, the glucose area under the curve (AUC), an index of whole glucose excursion after glucose loading, was calculated in accordance with a previous study (Sakaguchi et al., 2016).

2.3. Biochemical analyses

Serum insulin levels were measured with the Mouse Insulin ELISA KIT (AKRIN-011T, Shibayagi, Gunma, Japan). Serum osteocalcin (OCN) levels were measured with Mouse Osteocalcin EIA Kit (BT-470, Biomedical Technologies Inc., Stoughton MA, USA). Serum tartrate-resistant acid phosphatase form 5b (TRAcP5b) levels were measured with Mouse TRAP™ Assay (Immunodiagnostic Systems Inc., Fountain Hills AZ, USA).

2.4. Determination of bone mineral content and bone mineral density of the femur

Bone mineral content (BMC) and BMD of the greater trochanter and the mid-diaphysis of the right femur in all mice were measured using dual X-ray absorptiometry with an apparatus for small animals (DICHROMA SCAN DCS-600; ALOKA, Tokyo, Japan) at both 10 and 20 weeks of age. The mice were anesthetized by intraperitoneal injection of chloral hydrate (400 mg/kg) and the measurements were performed with extended hip and knee joints, i.e. flexion of each.

2.5. Micro-computed tomography measurements

Bone microstructure in the greater trochanter and the mid-diaphysis of the femur were assessed with micro-CT (SMX-90CT, SHIMADZU, Kyoto, Japan) at 23 μm × 23 μm × 23 μm voxel size with an X-ray power source of 90 kV and 110 μA. All bones were thawed to room temperature and placed in PBS during scanning. The greater trochanter of the femur was scanned at constant intervals of 23 μm in a region 460 μm in length from the inferior border of the femoral head, and the mid-diaphysis was scanned at the same intervals and length in a region of the central part of the femur. Then, the trabecular bone fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), and trabecular spacing (Tb.Spac) were measured in the greater trochanter. The cortical bone volume (Ct.V), total bone volume (Tt.V), cortical volume fraction (Ct.V/Tt.V), cortical porosity (Ct.Po), cortical thickness (Ct.Th) and cross-sectional moment inertia (CSMI) were measured in the mid-diaphysis respectively. Three-dimensional measurements and structural analyses were performed with bone analysis software, TRI/3D-BON (RATOC System Engineering, Tokyo, Japan). The images were binarized with a threshold range between 1 and 255 (gray values), and then each parameter was measured automatically according to a software program. Grayscale images were segmented using a median filter to remove noise with a fixed threshold to extract bone components.

2.6. Biomechanical analyses

Extrinsic mechanical properties and intrinsic material properties of the femoral mid-diaphysis were determined using a three-point bending test. The site of testing was matched to micro-CT sampling sites for the femoral mid-diaphysis (centered at 50% of total bone length). Prior to the three-point bending test, anteroposterior surface diameters were measured at the femoral mid-diaphysis using calipers for the calculation of toughness. Bones were thawed at room temperature and placed in PBS until tested. The span between the lower supports was 10 mm for the femur, which was oriented posterior side down. Quasi-static, displacement-controlled loading (2 mm/min) was applied to the upper surface (anterior for femur) until a fracture was caused using a mechanical testing machine (EZtest; SHIMADZU, Kyoto, Japan). All bones were kept moist with PBS immediately prior to testing to maintain hydration. All data were analyzed with software (Factory SHiKiBU2000; SHIMADZU, Kyoto, Japan). Extrinsic mechanical properties included ultimate force (maximum load during the test), fracture

force (load at which fracture occurred), stiffness (slope of the linear portion of the load-displacement curve), and work to failure (area under the load-displacement curve to fracture). Intrinsic material properties included the ultimate stress (maximum stress during the test), fracture stress (stress at which fracture occurred), and elastic modulus (stress-strain curve to fracture). Toughness (area under the stress-strain curve to fracture) was calculated as previously described (Mashiba et al., 2000).

2.7. Histological sections and staining

The left femur of each mouse was fixed in 10% neutral buffered formalin and subsequently degreased in 99% alcohol for 5 h. After washing and decalcification for 24 h in K-CX decalcifying liquid (Falma, Tokyo, Japan), the samples were embedded in paraffin wax. Then, 2-μm vertical serial slices were prepared using a microtome (SM2010R, Leica Biosystems, Germany), and sections were stained with hematoxylin and eosin (Muto pure chemicals, Tokyo, Japan).

2.8. Statistical analyses

All statistical analyses were performed with SPSS statistics version 24 (IBM, Armonk, NY, USA). All data were expressed as mean ± standard error of the mean. Two-way analysis of variance was used to compare differences among TSOD mice and their respective age-matched controls, followed by post-hoc Bonferroni tests for pairwise comparisons of significant variables. Linear regression and Pearson's product-moment correlation coefficient tests were performed to identify linear correlations between glucose or insulin and bone metabolism markers, bone mass, and microstructure parameter. For all experiments, $P < 0.05$ was considered statistically significant.

3. Results

3.1. Body weight, serum glucose levels, serum insulin levels, and the oral glucose tolerance test

Body weight was significantly higher in TSOD mice than in control mice at both 10 and 20 weeks of age ($P < 0.0001$ and < 0.0001, respectively), and was significantly higher at 20 weeks of age than at 10 weeks of age in both groups (TSNO: $P = 0.001$; TSOD: $P < 0.0001$) (Table 1). Serum glucose levels were significantly higher in TSOD mice than in control mice at 20 weeks of age ($P < 0.0001$) and were significantly higher at 20 weeks of age than at 10 weeks of age in the TSOD mice ($P < 0.0001$). However, there was no significant difference between the two groups in terms of serum glucose levels at 10 weeks of age (Table 1). Serum insulin levels were 5.2 times higher in TSOD mice than in control mice at 10 weeks of age ($P = 0.073$) and 6 times higher at 20 weeks of age ($P = 0.01$). Serum insulin was 1.4 times higher at 20 weeks of age than at 10 weeks of age in the TSOD mice ($P = 0.052$; Table 1).

Table 1
Body weight, serum glucose and insulin levels in TSNO and TSOD mice at 10 and 20 weeks of age.

	10 weeks		20 weeks	
	TSNO	TSOD	TSNO	TSOD
n	6	6	6	6
Body weight (g)	31.6 ± 0.6	45.7 ± 1.0[b]	38.5 ± 1.0[d]	59.1 ± 1.2[b, d]
Serum glucose (mg/dl)	177.6 ± 6.0	184.6 ± 6.3	144.2 ± 1.2	349.9 ± 35.1[b, d]
Serum insulin (ng/ml)	2.7 ± 0.4	14.1 ± 4.9	4.0 ± 0.4	23.8 ± 6.2[a]

Values are presented as the mean ± S.E.M; n = 6 per group. Statistical differences ([a]$P < 0.05$ and [b]$P < 0.01$ vs TSNO mice, [c]$P < 0.05$ and [d]$P < 0.01$ vs 10 weeks of age).

Serum glucose levels, measured 120 min after glucose administration at 10 weeks of age were significantly higher in TSOD mice than in control mice ($P = 0.02$; Fig. 1A). Furthermore, TSOD mice showed markedly impaired glucose tolerance at 20 weeks of age (0 min: $P = 0.07$; 30 min: $P = 0.054$; 60 min: $P = 0.023$, 120 min: $P = 0.034$; Fig. 1B). The AUC was significantly higher in TSOD mice than in control mice at 20 weeks of age ($P = 0.001$), although there was no significant difference between the two groups at 10 weeks of age (Fig. 1C).

3.2. Bone metabolism

The OCN level, which represents a marker of bone formation, did not differ significantly between the two groups at 10 or 20 weeks of age, but were significantly lower at 20 weeks of age than at 10 weeks of age in both groups (TSNO: $P < 0.0001$; TSOD: $P = 0.038$; Fig. 2A). TRAcP5b level, which represents a marker for bone resorption, was significantly higher (by a factor of 1.4–4.0) in TSOD mice than in control mice at both 10 and 20 weeks of age ($P < 0.0001$ and < 0.0001, respectively), but were significantly lower at 20 weeks of age than at 10 weeks of age in both groups (TSNO: $P < 0.0001$; TSOD: $P = 0.001$; Fig. 2B).

3.3. BMC and BMD

BMC at the femoral greater trochanter was significantly lower in TSOD mice than in control mice at 20 weeks of age ($P = 0.002$) and was also significantly lower at 20 weeks of age than at 10 weeks of age in TSOD mice ($P = 0.032$); however, there was no significant difference between the two groups at 10 weeks of age (Fig. 3A). BMC at the femoral mid-diaphysis was significantly lower in TSOD mice than in control mice at both 10 and 20 weeks of age ($P = 0.013$ and 0.004, respectively; Fig. 3B). BMD at the femoral greater trochanter was significantly lower in TSOD mice than in control mice at both 10 and 20 weeks of age ($P = 0.016$ and 0.002, respectively), but was significantly higher at 20 weeks of age than at 10 weeks of age in control mice ($P = 0.024$). There was no significant difference in TSOD mice when compared between the two ages (Fig. 3C). BMD at the femoral mid-diaphysis was also significantly lower in TSOD mice than in control mice at both 10 and 20 weeks of age ($P = 0.025$ and 0.001, respectively), but was higher at 20 weeks of age than at 10 weeks of age in control mice ($P = 0.084$). There was no significant difference in TSOD mice when compared between the two ages (Fig. 3D).

3.4. Bone microstructure

The trabecular bone volume fraction at the femoral greater trochanter was significantly higher in TSOD mice than in control mice at 20 weeks of age ($P < 0.0001$) and was also significantly higher at 20 weeks of age than at 10 weeks of age in TSOD mice ($P = 0.01$), although there was no significant difference between the two groups at 10 weeks of age (Table 2). The trabecular thickness at the femoral greater trochanter was significantly higher in TSOD mice than in control mice at 20 weeks of age ($P = 0.048$), although there was no significant difference between the two groups at 10 weeks of age (Table 2). The trabecular number at the femoral greater trochanter was significantly higher in TSOD mice than in control mice at 20 weeks of age ($P < 0.0001$) and was also significantly higher at 20 weeks of age than at 10 weeks of age in TSOD mice ($P = 0.003$), although there was no significant difference between the two groups at 10 weeks of age (Table 2). The trabecular separation at the femoral greater trochanter was significantly lower in TSOD mice than in control mice at 20 weeks of age ($P = 0.001$) and was also significantly lower at 20 weeks of age

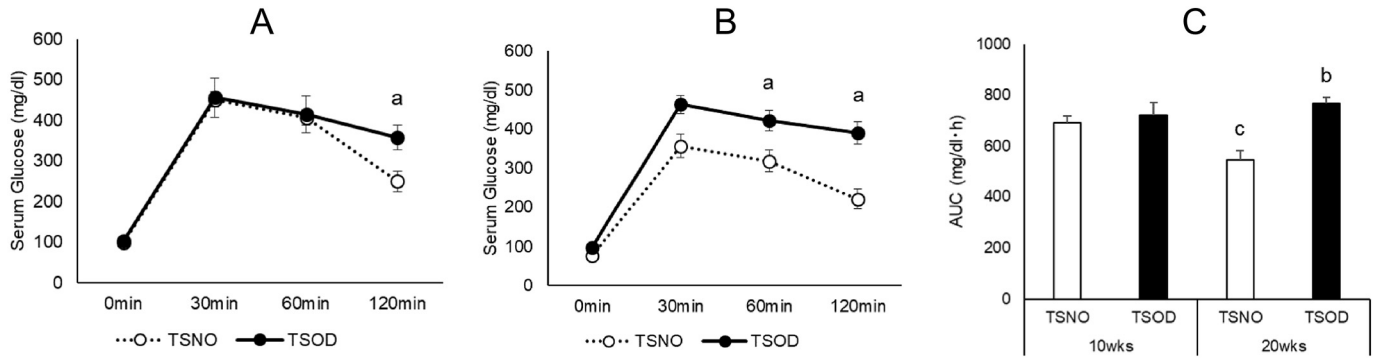

Fig. 1. OGTT in TSNO and TSOD mice; 10 weeks of age; A, 20 weeks of age; B, AUC; C. Data are presented as the mean ± S.E.M; n = 6 per group. $^{a}P < 0.05$ and $^{b}P < 0.01$; vs TSNO mice, $^{c}P < 0.05$ and $^{d}P < 0.01$; vs 10 weeks of age.

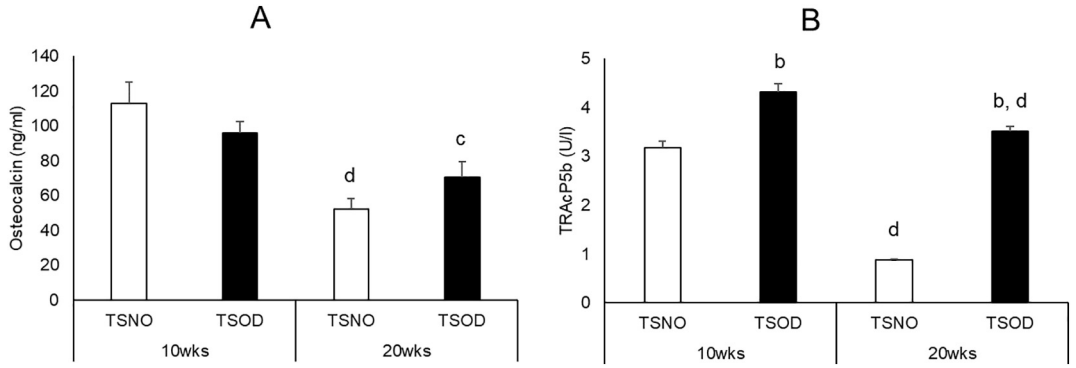

Fig. 2. Bone metabolism markers in TSNO and TSOD mice at 10 and 20 weeks of age; osteocalcin levels; A, TRAcP5b levels; B. Data are presented as the mean ± S.E.M; n = 6 per group. $^{a}P < 0.05$ and $^{b}P < 0.01$; vs TSNO mice, $^{c}P < 0.05$ and $^{d}P < 0.01$; vs 10 weeks of age.

than at 10 weeks of age in TSOD mice (P = 0.029), although was significantly higher at 20 weeks of age than at 10 weeks of age in control mice (P = 0.016) (Table 2). The trabecular spacing at the femoral greater trochanter was significantly lower in TSOD mice than in control mice at 20 weeks of age (P = 0.001) and was also significantly lower at 20 weeks of age than at 10 weeks of age in TSOD mice (P = 0.032), although was also significantly higher at 20 weeks of age than at 10 weeks of age in control mice (P = 0.02; Table 2). The cortical bone volume and cortical porosity at the femoral mid-diaphysis were not significantly different between the two groups at both 10 and 20 weeks of age (Table 2). The total bone volume at the femoral mid-diaphysis was significantly lower in TSOD mice than in control mice at both 10 and 20 weeks of age (P = 0.002 and 0.04, respectively), and was also significantly lower at 20 weeks of age than at 10 weeks of age in the controls (P = 0.003; Table 2). The cortical volume fraction at the femoral mid-diaphysis was significantly higher in TSOD mice than in control mice at both 10 and 20 weeks of age (P < 0.0001 and P = 0.001, respectively), and was also significantly higher at 20 weeks of age than at 10 weeks of age in TSOD mice (P = 0.041) and was also significantly higher at 20 weeks of age than at 10 weeks of age in control mice (P = 0.044). The cortical thickness at the femoral mid-diaphysis was significantly higher in TSOD mice than in control mice at both 10 and 20 weeks of age (P = 0.001 and 0.002, respectively). The CSMI at the femoral mid-diaphysis was significantly lower in TSOD mice than in control mice at 10 and 20 weeks of age (P = 0.009 and P = 0.023, respectively), although there was no significant difference between the two age points for TSOD mice (Table 2, Fig. 4).

3.5. Bone strength

There was no significant difference between the two groups in terms of extrinsic mechanical properties at 10 and 20 weeks of age, however ultimate force and fracture force were significantly higher at 20 weeks of age than at 10 weeks of age in TSOD mice (P = 0.028 and = 0.01, respectively; Table 3). Ultimate stress was significantly lower in TSOD mice than in control mice at 20 weeks of age (P = 0.003) and was significantly lower at 20 weeks of age than at 10 weeks of age in both groups (TSNO: P = 0.005; TSOD: P = 0.001; Table 3). Fracture stress was significantly lower in TSOD mice than in control mice at both 10 and 20 weeks of age (P = 0.027 and = 0.016, respectively; Table 3).

3.6. Bone histology

Fig. 5 shows typical histological sections of the femur from both groups of mice stained with hematoxylin and eosin. Compared to controls (Fig. 5A and B), the cortical bone of the femur in TSOD mice was thicker on general observation. In the cortical bone of the femur from TSOD mice, we identified many parts that were not stained with hematoxylin-eosin at both 10 and 20 weeks of age (Fig. 5C–F).

4. Discussion

A series of studies of bone metabolism using T2DM rodent models has shown that the degradation of bone strength associated with T2DM involves a reduction in BMD, reduced structural properties in the bone, and reduced levels of bone formation markers. However, when compared across existing studies, these results are inconsistent (Fajardo et al., 2014). Therefore, in the present study, we measured bone metabolism, mass, and microstructure of the femur in pre- and established-diabetic conditions in TSOD mice and investigated whether the strength of the femur in TSOD mice was influenced by these factors.

In the present study, we revealed that even by 10 weeks of age, TSOD mice had developed insulin resistance in typically pre-diabetic conditions, while at 20 weeks of age, TSOD mice were suffering from

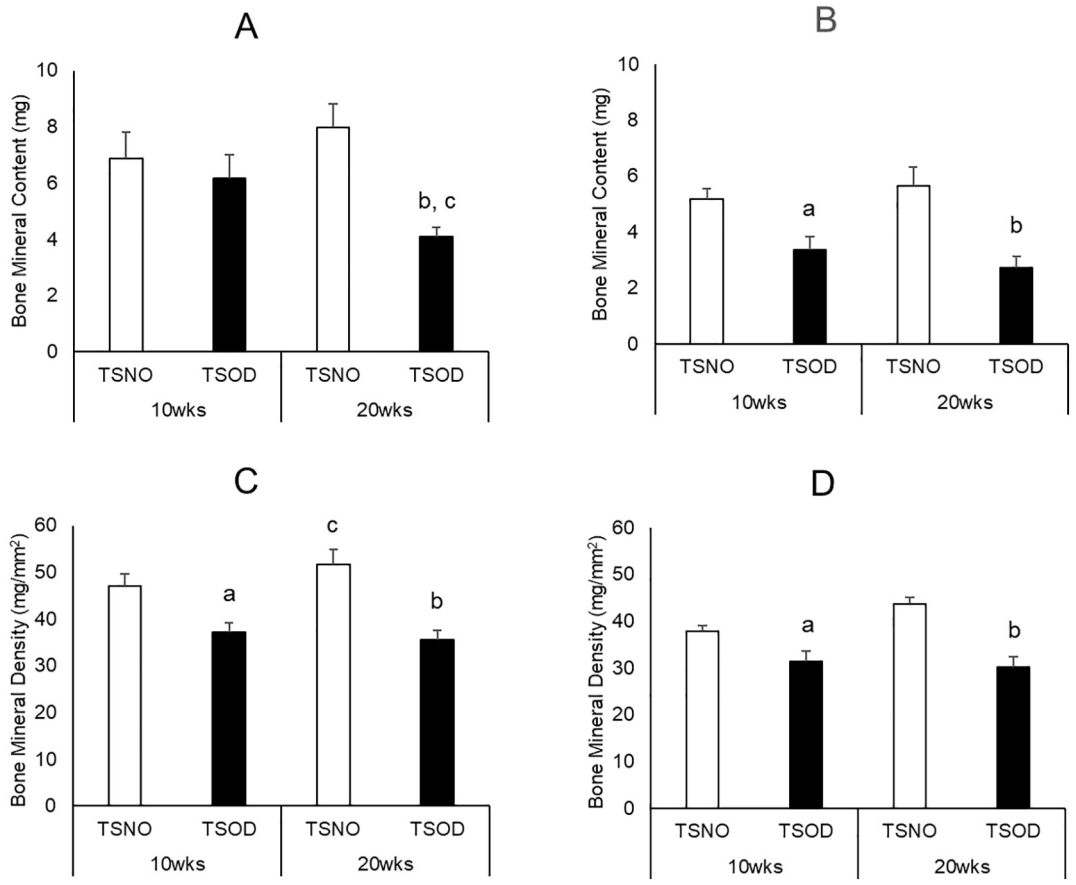

Fig. 3. Bone mineral content of the femur in TSNO and TSOD mice at 10 and 20 weeks of age; greater trochanter; A, mid-diaphysis; B. Bone mineral density of the femur in TSNO and TSOD mice at 10 and 20 weeks of age; greater trochanter; C, mid-diaphysis; D. Data are presented as the mean ± S.E.M; n = 6 per group. $^{a}P < 0.05$ and $^{b}P < 0.01$; vs TSNO mice, $^{c}P < 0.05$ and $^{d}P < 0.01$; vs 10 weeks of age.

chronic hyperglycemia in an established diabetic condition as determined by measurement of serum glucose and insulin levels, OGTT, and body weight. To the best of our knowledge, there have been no previous studies comparing bone metabolism at pre- and established diabetic conditions in spontaneous T2DM animal models. Accordingly, we measured serum levels of OCN and TRAcP5b to examine changes in bone metabolism in pre- and established diabetic conditions in TSOD mice. At both 10 and 20 weeks of age, the TRAcP5b levels of TSOD mice were significantly higher than age-matched control, although levels of TRAcP5b at 20 weeks of age were significantly lower than at 10 weeks of age in both groups of mice. In contrast, OCN levels at both 10 and 20 weeks of age were not significantly different when compared

Table 2
Trabecular and cortical bone microstructure assessed by micro-CT at the greater trochanter and the mid-diaphysis of the femur in TSNO and TSOD mice at 10 and 20 weeks of age.

	10 weeks		20 weeks	
	TSNO	TSOD	TSNO	TSOD
n	6	6	6	6
Great trochanter (trabecular)				
BV/TV (%)	34.69 ± 2.15	35.38 ± 4.54	26.21 ± 3.02	47.56 ± 2.92$^{b, c}$
Tb.Th (μm)	77.42 ± 3.93	70.84 ± 6.63	67.00 ± 2.80	78.98 ± 4.52^{a}
Tb.N (1/mm)	4.47 ± 0.12	4.94 ± 0.21	3.86 ± 0.29	6.03 ± 0.23$^{b, d}$
Tb.Sp (μm)	146.95 ± 8.06	133.65 ± 13.54	198.82 ± 20.97^{c}	88.13 ± 7.59$^{b, c}$
Tb.Spac (μm)	224.37 ± 6.17	204.48 ± 8.55	265.82 ± 18.63^{c}	167.11 ± 6.77$^{b, c}$
Mid-diaphysis (cortical)				
Ct.V (mm^3)	0.50 ± 0.02	0.50 ± 0.01	0.48 ± 0.01	0.51 ± 0.01
Tt.V (mm^3)	0.84 ± 0.02	0.73 ± 0.01^{b}	0.75 ± 0.01^{d}	0.71 ± 0.01^{a}
Ct.V/Tt.V (%)	59.86 ± 0.85	68.86 ± 1.00^{b}	63.36 ± 1.17^{c}	72.42 ± 1.58$^{b, c}$
Ct.Po (%)	0.23 ± 0.04	0.23 ± 0.04	0.26 ± 0.04	0.37 ± 0.10
Ct.Th (μm)	249.81 ± 5.88	291.04 ± 7.28^{b}	253.62 ± 8.10	305.60 ± 9.33^{b}
CSMI (mm^4)	0.33 ± 0.02	0.25 ± 0.01^{b}	0.29 ± 0.01	0.25 ± 0.01^{a}

Values are presented as the mean ± S.E.M. Statistical differences ($^{a}P < 0.05$ and $^{b}P < 0.01$ vs TSNO mice, $^{c}P < 0.05$ and $^{d}P < 0.01$ vs 10 weeks of age). BV/TV; Bone volume fraction, Tb.Th; Trabecular thickness, Tb.N; Trabecular number, Tb.Sp; Trabecular separation, Tb.Spac; Trabecular spacing, Ct.V; Cortical bone volume, Tt.V; Total bone volume, Ct.V/Tt.V; Cortical volume fraction, Ct.Po; Cortical porosity, Ct.Th; Cortical thickness, CSMI; Cross-sectional moment inertia.

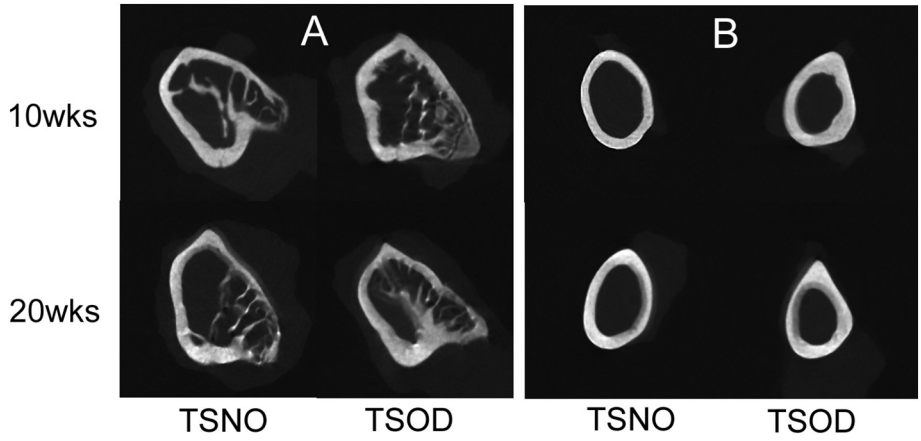

Fig. 4. Micro-CT images of the greater trochanter and the mid-diaphysis of the femur in TSNO and TSOD mice at 10 and 20 weeks of age; greater trochanter; A, mid-diaphysis; B.

Table 3
Mechanical properties of the femoral mid-diaphysis determined by three-point bending test in TSNO and TSOD mice at 10 and 20 weeks of age.

	10 weeks		20 weeks	
	TSNO	TSOD	TSNO	TSOD
n	6	5	4–5	4–5
Extrinsic mechanical properties				
Ultimate force (N)	14.3 ± 0.7	12.8 ± 0.5	16.2 ± 1.0	15.6 ± 0.3[c]
Fracture force (N)	10.0 ± 1.6	7.4 ± 0.6	13.4 ± 2.1	12.3 ± 2.4[c]
Stiffness (N/mm)	26.4 ± 1.8	19.9 ± 2.7	24.9 ± 3.1	24.9 ± 1.4
Work to failure (Nmm)	9.8 ± 1.5	7.8 ± 0.6	7.2 ± 2.8	6.7 ± 2.9
Intrinsic material properties				
Ultimate stress (MPa)	273.9 ± 16.9	243.2 ± 11.1	222.7 ± 10.2[d]	173.0 ± 6.0[b, d]
Fracture stress (MPa)	182.6 ± 19.9	143.0 ± 16.2[a]	214.1 ± 13.5	158.9 ± 11.8[a]
Elastic modulus (Gpa)	9.7 ± 1.2	7.8 ± 1.5	5.4 ± 0.7	4.8 ± 0.8
Toughness (MJ/m^3)	0.32 ± 0.06	0.18 ± 0.02	0.29 ± 0.13	0.15 ± 0.02

Values are presented as the mean ± S.E.M. Statistical differences ([a]$P < 0.05$ and [b]$P < 0.01$ vs TSNO mice, [c]$P < 0.05$ and [d]$P < 0.01$ vs 10 weeks of age).

between the two groups. OCN levels at 20 weeks of age were significantly lower than at 10 weeks of age, although levels of OCN in TSOD mice at 20 weeks of age were higher than age-matched controls. Collectively, these data we indicate that bone turnover in both groups of mice decreased with advancing age, although bone turnover in the TSOD mice at 20 weeks of age occurred at a higher rate than in age-matched controls. We suggest that hyperinsulinemia is responsible for this effect because the levels of TRAcP5b and OCN levels were positively correlated with insulin levels at 20 weeks of age (Table 4). In a previous study, Ferron et al. (2010) showed that insulin signaling in osteoblasts promotes only the function of osteoclasts, although it is also established that insulin promotes osteoblast differentiation (Fulzele et al., 2010; Yang et al., 2010). Collectively, we observed that insulin regulates bone metabolic turnover. We believe that the preservation of TRAcP5b levels in TSOD mice at 20 weeks of age involved hyperglycemia because the TRAcP5b levels at 20 weeks of age showed a stronger positive correlation with glucose levels than with insulin levels (Table 4). A previous study on osteoclasts derived from db/db T2DM mice reported that the differentiation of osteoclasts was enhanced by hyperglycemia (Catalfamo et al., 2013). However, these results were not consistent with those of previous studies on bone metabolism in T2DM animal models although this may be due to the fact that these studies on bone metabolism were conducted in mice with low BMD (Devlin et al., 2014; Fu et al., 2015; Fujii et al., 2008; Hamann et al., 2011; Kawashima et al., 2009; Omi et al., 1998 Turner et al., 2013; Zhang et al., 2009;). We suggest that the increased functional ability of osteoclasts in pre-diabetic conditions in TSOD mice may involve hyperinsulinemia and that the function of osteoclasts is maintained by chronic hyperglycemia in established diabetic conditions. To our knowledge, this is the first investigation to examine bone metabolism during pre- and established diabetic conditions in the spontaneous obese T2DM animal model.

Our study also found that the BMD and BMC of the greater trochanter and mid-diaphysis of the femur decreased with age in TSOD mice but increased with age in control mice. At 20 weeks of age, serum glucose levels in TSOD mice were significantly higher than in control mice. Furthermore, both the BMD and BMC of the greater trochanter and mid-diaphysis of the femur at 20 weeks of age were negatively correlated with serum glucose levels (Table 4), and Takagi et al. (2012) also found a negative correlation between BMD in the proximal region of the femur and serum glucose levels in the obese T2DM KKAy mouse model. Accordingly, we suggest that chronic hyperglycemia caused a reduction in the BMD of TSOD mice because hyperglycemia interferes with the production of a mineralized matrix (Bai et al., 2004). Therefore, poorly controlled diabetes can induce a decrease in BMC (Gregorio et al., 1994). In addition, the BMD and BMC of the greater trochanter and the mid-diaphysis of the femur in TSOD mice at 10 weeks of age were already lower than that of age-matched controls. We suggest that obesity is not necessarily responsible for the reduction of BMD in TSOD mice at 10 weeks of age because body weight prevents bone loss; we observed a positive correlation between BMD and BMI (Akin et al., 2003; Bridges et al., 2005) and several studies have reported that the BMD decreased spontaneously in obese T2DM animal models (Omi et al., 1998; Takagi et al., 2012). In addition, hyperinsulinemia was not beneficial to the BMD of the femur in T2DM KKAy mice (Takagi et al., 2017). Accordingly, hyperinsulinemia may promote bone resorption via

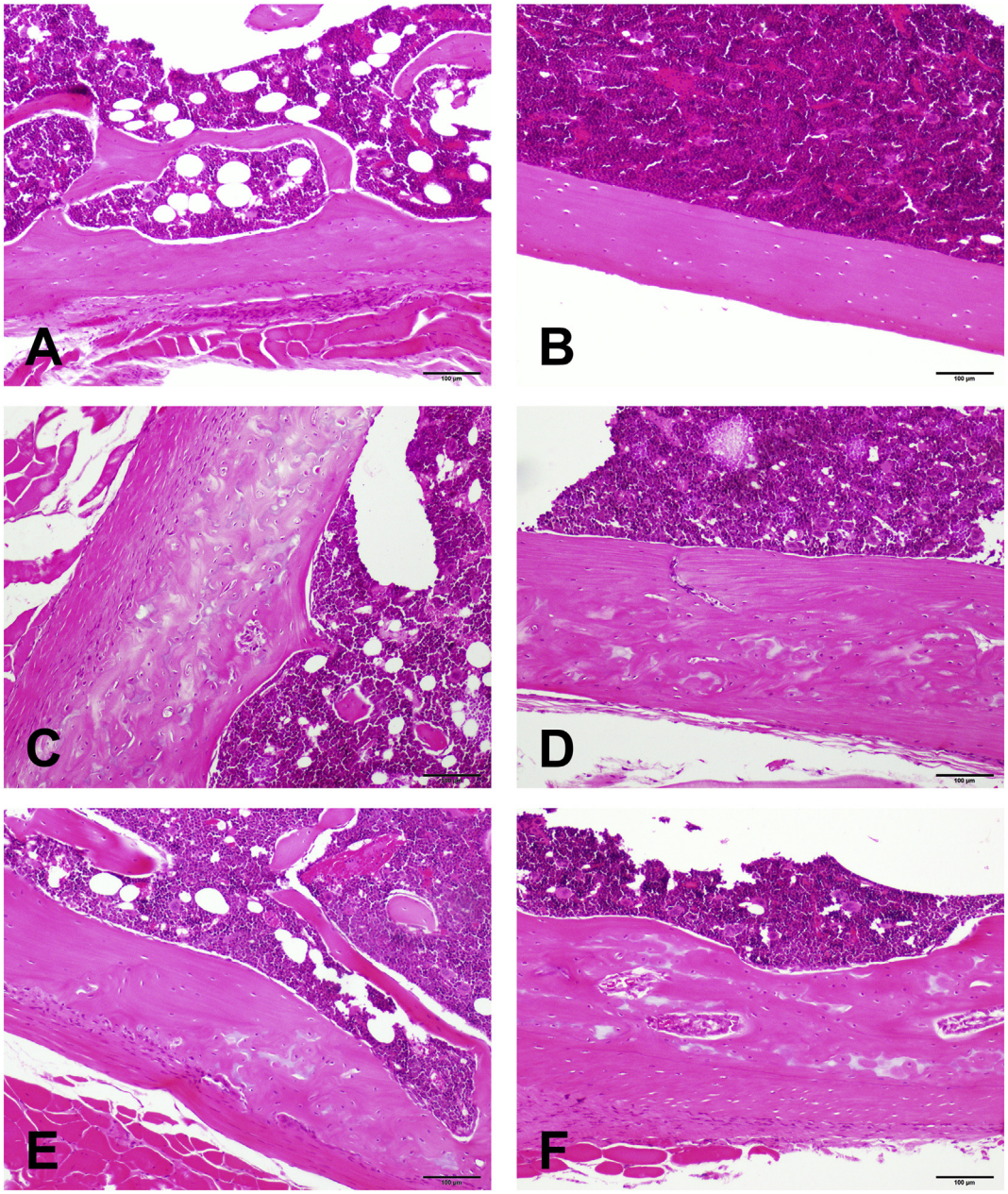

Fig. 5. Histology photomicrographs showing HE staining of the greater trochanter and the mid-diaphysis of the femur in demineralized paraffin sections. Representative photographs of histological sections from the greater trochanter; A and the mid-diaphysis; B of the femur in TSNO mice at 20 weeks of age. The greater trochanter; C and the mid-diaphysis; D of the femur in TSOD mice at 10 weeks of age. The greater trochanter; E and the mid-diaphysis; F of the femur in TSOD mice at 20 weeks of age. Scale bar; 100 μm.

the osteoclasts because TRAcP5b levels in TSOD mice during pre-diabetic conditions were already higher than in age-matched controls.

Total bone volume at the femoral mid-diaphysis in TSOD mice were significantly lower than in control mice at both 10 and 20 weeks of age, and cortical thickness in TSOD mice were significantly higher than in control mice at both 10 and 20 weeks of age, but cortical volume in TSOD mice were not difference between the two groups at both 10 and 20 weeks of age. Accordingly, we suggest that periosteal ossification was suppressed and endocortical ossification was enhanced at the cortical bone in TSOD mice. The cortical thicknesses of the femur in TSOD mice were higher than in control mice at both 10 and 20 weeks of age. Previous studies have shown that cortical thickness in the spontaneous T2DM animal model, KKAy (Fu et al., 2015), and in TallyHo mice (Devlin et al., 2014), were higher than in control mice, and that these mice developed hyperinsulinemia. We suggest that hyperinsulinemia results in an increase in cortical thickness because cortical thickness in KKAy mice showed a strong positive correlation with insulin levels (Fu et al., 2015). Furthermore, in the present study, cortical thickness was positively correlated with insulin levels at both 10 and 20 weeks of age (Table 4).

Enhancement of endocortical ossification in TSOD mice may be due to increased low mineralized bone tissue by proliferation of immature osteoblasts. BMD and BMC in TSOD mice were significantly lower than in control mice at both 10 and 20 weeks of age, and cortical bone volume and cortical porosity in TSOD mice were not significantly different between both groups at 10 and 20 weeks of age, although cortical thickness in TSOD mice was significantly higher than in control mice at both 10 and 20 weeks of age. In addition, we assumed that cortical thickness in TSOD mice increased due to immature osteoblasts. Since OCN is secreted by mature osteoblasts, if cortical thickness in TSOD

Table 4
Correlation analysis between serum glucose, insulin levels and bone metabolic markers, bone mass and microstructure parameter at 10 and 20 weeks.

Parameter	Glucose		Insulin	
	10 weeks	20 weeks	10 weeks	20 weeks
n	12	12	12	11
OCN				0.741[a]
TRAcP5b		0.906[b]	0.672[a]	0.687[b]
Greater trochanter BMD	−0.744[b]	−0.714[b]		
Mid-diaphysis BMD		−0.579[a]		
Greater trochanter BMC	−0.664[b]	−0.695[a]		
Mid-diaphysis BMC		−0.603[a]		
Ct.Th			0.736[b]	0.813[b]

[a]Correlation is significant at the 0.05 level. [b] Correlation is significant at the 0.01 level. The blank represents no significant correlation. OCN; Osteocalcin, TRAcP5b; Tartrate-resistant acid phosphatase form 5b, BMD; Bone mineral density, BMC; Bone mineral content, Ct.Th; Cortical thickness.

mice was increased by immature osteoblasts, the secretion of OCN did not significantly increase in TSOD mice. Furthermore, we investigated the histology of the femur in both groups of mice and found that many parts of the femur in TSOD mice at both 10 and 20 weeks of age were not stained with hematoxylin and eosin. In bone, collagen fibers are linked by calcium phosphate. Following decalcification, bone collagen fibers appear red when stained with the eosin stain. Consequently, our histological findings are suggestive of a reduction in bone mass because a lack of eosin staining indicated a lack of collagen fibers.

On the other hand, we suggest that the periosteal ossification of the femur in TSOD mice were decreased with increase osteoclasts because TRAcP5b levels in TSOD mice were significantly higher than in control mice at both 10 and 20 weeks of age.

Hyperinsulinemia in TSOD mice may be associated with enhancement of bone resorption by an increase in osteoclasts because the TRAcP5b levels at both 10 and 20 weeks of age showed a positive correlation with insulin levels.

Due to the above reasons, cortical thickness may be significantly increased to compensate for suppression of periosteal ossification in TSOD mice.

In mice, age-related trabecular bone loss begins as early as 8 weeks of age (Glatt et al., 2007). In the present study, the trabecular bone volume fraction and number at the femoral greater trochanter was significantly higher in TSOD mice than in control mice at 20 weeks of age and was significantly higher at 20 weeks of age than at 10 weeks of age. In contrast, the trabecular separation and spacing at the femoral greater trochanter was significantly lower in TSOD mice than in control mice at 20 weeks of age and was significantly lower at 20 weeks of age than at 10 weeks of age, although these parameters in control mice at 20 weeks of age increased significantly with age. If the increase in trabecular bone volume in TSOD mice at 20 weeks of age was associated with obesity or hyperinsulinemia, the trabecular bone volume should have already increased at 10 weeks of age. In addition, many studies using T2DM animal models with hyperinsulinemia have reported a decrease in trabecular bone volume (Devlin et al., 2014; Fu et al., 2015; Kawashima et al., 2009; Reinwald et al., 2009; Williams et al., 2011). Accordingly, we could not identify a cause for increased trabecular bone volume in TSOD mice; we guessed that the effect of obesity and insulin takes longer to become detectable in the trabecular bone volume in TSOD mice.

TRAcP5b levels of TSOD mice at 10 weeks of age were significantly higher than those of control mice, while BMD, BMC, total bone volume and CSMI were all significantly lower. The skeletal fragility of TSOD mice during pre-diabetic conditions may be associated with hypercholesterolemia because hypercholesterolemia and obesity are evident in TSOD mice after just 5 weeks of age (Murotomi et al., 2014; Suzuki et al., 1999). In addition, several studies, using experimental animal models, reported that osteoclast activity (Prieto-Potín et al., 2013) or the number of tartrate-resistant acid phosphatase–positive osteoclasts (Sanbe et al., 2007) increased as a result of hypercholesterolemia. For example, Pelton et al. (2012) showed that mice with hypercholesterolemia exhibited reduced formation of cortical and trabecular bone in the femur via the promotion of osteoclastogenesis. In contrast, the cortical thickness of the femur in TSOD mice at 10 weeks of age was already significantly higher than in age-matched controls. These results may also be related to hypercholesterolemia in TSOD mice during pre-diabetic conditions because in a previous study, the administration of cholesterol to a mouse marrow mesenchymal system stem cell stimulated the process of differentiation and increased mRNA and protein levels of osteogenic lineage markers, increased alkaline phosphatase activity and more mineralized nodules (Li et al., 2013). Consequently, the increase in cortical thickness in TSOD mice may be a result of the increase in low mineralized bone tissue in the endosteal side adjacent to the bone marrow.

Ultimate and fracture force in TSOD mice at 20 weeks of age were significantly higher than at 10 weeks of age. These are extrinsic mechanical parameters that depend on intrinsic material properties and geometry (Mashiba et al., 2000). If bone materials are similar, when geometry is smaller, a tubular structure such as the femoral mid-diaphysis can easily fracture in response to even a minimum external force. The total bone volume of the femoral mid-diaphysis in TSOD mice was significantly lower than age-matched controls at both 10 and 20 weeks of age, although the cortical thickness of the femoral mid-diaphysis in TSOD mice were significantly higher than in age-matched controls at both 10 and 20 weeks of age. Accordingly, we were not able to clarify the cause because these results did not increase with age. However, cortical volume fraction may reflect extrinsic mechanical properties because cortical volume fraction in TSOD mice significantly increased with age. Ultimate and fracture stress of TSOD mice was already lower than in the control group at 10 weeks of age and remained significantly lower than controls at 20 weeks of age. These parameters are intrinsic material properties which are independent of cross-sectional size and shape (Mashiba et al., 2000). Intrinsic material parameters were calculated from load-displacement data using CSMI. When CSMI is smaller, a tubular structure such as the femoral mid-diaphysis can only cause lower stress (Turner and Burr, 1993). Accordingly, we suggest that a decrease in intrinsic material properties in TSOD mice are associated with low CSMI because CSMI of the femoral mid-diaphysis in TSOD mice were significantly lower than in control at both 10 and 20 weeks of age. In addition, the ultimate stress in TSOD mice decreased with age. Generally, in the case of decrease in intrinsic mechanical properties, extrinsic geometrical shape must be significantly increased to compensate for the poor bone tissue properties. However, extrinsic geometrical shape in TSOD mice did not change with age. According to the above results, we assumed that bone strength in TSOD mice were decreased compared to that in control mice.

The strength of bone is determined by a combination of quantitative, structural, and quality factors. In the current study of TSOD mice, low BMD and BMC were considered as quantitative factors, reduction of the total bone volume and the CSMI of the femoral mid-diaphysis were considered to be structural factors, and bone turnover with the promotion of bone resorption was considered to be a quality factor. Notably, BMD is frequently referred to as an important factor underlying bone strength because it accounts for approximately 70% of bone strength (National Institutes of Health Consensus Development Conference Statement, 2000); a low BMD was shown to have a significant effect upon the bone strength of TSOD mice.

However, the study has a few limitations. First, in the present study, serum glucose levels were not different between the two groups until 19 weeks of age, but serum glucose levels in the TSOD mice were significantly higher than those in the control mice after 20 weeks of age. Accordingly, long-term investigation seems necessary to clarify the

skeletal fragility by chronic hyperglycemia. Second, hip fracture is one of the fragility fractures in diabetes mellitus, but we could not perform biomechanical testing for the femoral neck or trochanter region using a mechanical testing machine in the present study. Since the femoral neck and the trochanter region of the mice were too small, we were not able to add pressure on the femoral neck. Third, we guessed that the alteration of the periosteal ossification was due to changes in age-related total bone volume. However, we think that additional investigations to obtain detailed data are necessary.

5. Conclusions

Our current data suggest that the BMD of the femur were significantly reduced in TSOD mice compared to in controls because hyperinsulinemia induced the enhancement of bone resorption by high bone turnover. In addition, our data suggest that the BMD and BMC of the femur were significantly reduced as a result of chronic hyperglycemia during established diabetic conditions in TSOD mice. Therefore, we suggest that the intrinsic material properties of bone strength in the femur in TSOD mice deteriorated as a result of reduced BMD and BMC and the slenderness of the mid-diaphysis of the femur.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Declaration of interests

None.

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