Characteristics of Bone Strength and Metabolism in Type 2 Diabetic Model Nagoya Shibata Yasuda Mice

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Summary

We evaluated the suitability of Nagoya Shibata Yasuda (NSY) mice as an animal model for examining the influence of a glucose metabolism disorder on bone integrity, using Institute of Cancer Research (ICR) mice as controls. We selected six NSY and ICR mice each that were matched for weight, and measured serum glucose levels, serum insulin levels, and conducted an oral glucose tolerance test. Histological sections of the femurs of both mouse lines were prepared, and the bone strength, mass, and microstructure of the femur were compared, along with bone metabolism. Serum glucose levels were significantly higher in the NSY mice than in the control mice, but body weight and serum insulin levels did not differ between the groups. Bone mass, microstructure, and strength of the femur, and bone metabolism were lower in the NSY mice than in the control mice. In the cortical bone of the femur in the NSY mice, several parts were not stained with eosin, demonstrating a strong negative correlation between serum glucose levels and bone mineral density; however, there was a negative correlation between serum glucose levels and bone metabolic markers. The bone turnover rate in the NSY mice was decreased by hyperglycemia, resulting in a thinner and shorter femur, reduced cortical and trabecular areas, and lower bone mass compared to those of the control mice. Collectively, these results suggest deteriorated bone strength of the femur in NSY mice, serving as a useful model for studying the link between glucose metabolism and bone integrity.

Keywords: Nagoya Shibata Yasuda mouse, bone strength, bone metabolism, type 2 diabetes mellitus, bone mass, bone microstructure
1. Introduction

Diabetes mellitus (DM) is a metabolic disease characterized by chronic hyperglycemia as the main symptom, along with chronic complications such as neuropathy, nephropathy, and retinopathy. Osteoporosis is a disease characterized by low bone strength resulting in low bone mineral density (BMD) and bone quality, which increases the risk of fractures. Recently, a meta-analysis demonstrated that patients with both type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) have an increased risk of hip fracture risk as compared with non-DM patients.1, 2) therefore, understanding the link between DM and bone metabolism disorder is required to best treat secondary osteoporosis in DM patients. Insulin promotes osteoblast proliferation and differentiation;3) consequently, the deficiency of insulin in T1DM leads to impaired bone formation, abnormal mineralization, deteriorated bone microstructure, increased bone fragility, and decreased peak bone mass.4) However, the fracture risk in T1DM appears to be much higher than that associated with the loss of BMD alone.2) Moreover, the BMD is not necessarily lower in subjects with T2DM compared to that of individuals without DM despite the increased fracture risk.5) Such evident deterioration of bone fragility caused by diabetic osteoporosis is indicated by not only the decreased BMD but is also associated with decreased bone quality.

T2DM animal models are often used in basic research on diabetic osteoporosis. A review of bone fragility in T2DM rodent models published in 2014 generally classified the models into obese or lean types according to differences in body weight compared to a control group.6) However, the reduction of body mass in lean T2DM animal models results in a reduced skeletal system, which limits the possibility of examining the direct effect of glucose metabolism disorder on decreasing bone strength and BMD. Moreover, if the skeletal system is also reduced in the obese T2DM animal models than in the control groups, T2DM would be considered to have decreased bone strength and BMD; however, the influence of
dyslipidemia cannot be separated from that of glucose metabolism disorder.\textsuperscript{7,8} After a comprehensive search of the literature, we could not find a report that compared bone strength and bone metabolism using a T2DM animal model and control group matched for body weight. Therefore, to tease apart these effects and further elucidate the influence of deficiencies in glucose metabolism on bone integrity, we decided to focus on Nagoya Shibata Yasuda (NSY) mice.

The NSY mouse is a spontaneous T2DM animal model, which is an inbred strain generated from Institute of Cancer Research (ICR) mice, characterized by insulin resistance and deficiency of insulin secretion; however, its bone integrity characteristics remain unknown.\textsuperscript{9,10} However, unlike T2DM model mice, NSY mice do not show remarkable increases in body weight and serum insulin levels compared to those in ICR mice.\textsuperscript{10,11} Although several reports have used the inbred mouse strain C3H as a control for NSY mice,\textsuperscript{12–14} the body weight of the C3H mice is clearly different from that of age-matched NSY mice.\textsuperscript{9,12} Accordingly, we examined whether NSY mice would be a suitable animal model for studying the influence of glucose metabolism disorder on bone integrity, using the parental ICR mouse strain as a control.

2. Materials and Methods

2.1. Animals

Male NSY (n = 13) and ICR (n = 16) mice (Japan SLC, Shizuoka, Japan), aged 4–20 weeks, were used in this study. The mice from each group were housed together (n = 4–6 mice/cage) and had free access to food pellets (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. At 20 weeks of age, six NSY mice and ICR mice each were selected so as to ensure no substantial weight difference between the groups. Prior to the sacrificing of the
mice by cervical dislocation at 20 weeks of age, blood samples were collected from each mouse from the cavernous sinus with a capillary and refrigerated until serum analysis. Both femurs of all mice were removed 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 conducting the three-point bending test and micro-computed tomography (micro-CT) scanning. The left femur was fixed in 10% neutral buffered formalin for subsequent histological analysis. All experimental protocols were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals and approved by the ethics committee (Prime Minister’s Office Directive No. 228, 2015).

2.2. Serum glucose levels and oral glucose tolerance test (OGTT)

Serum glucose levels were measured using the Wako glucose CII-test (Wako Pure Chemical Industries, Osaka, Japan), and the OGTT was performed on each mouse at 20 weeks of age. All mice were weighed and then deprived of food for the previous 20 h prior to testing. A glucose (2 g/kg) solution was given based on weight, and blood samples were collected 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.15)

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 (BMC) and BMD of the femur

The BMC and BMD of the trochanter and 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). 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-CT measurements

Bone microstructure in the trochanter and mid-diaphysis of the femur were assessed with micro-CT (SMX-90CT, SHIMADZU, Kyoto, Japan) at a 23 μm/pixel 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. To determine the optimal region of interest in the trochanter and mid-diaphysis of the femurs, specimens from all mice were scanned to obtain 20 serial slices (23-μm thick each). Multiple transverse images of the femur were scanned at the trochanter (a 460-μm-long region at the inferior border of the femoral head) and mid-diaphysis (a 460-μm-long region at the central part of the femur). Quantitative analyses were performed using TRI/2D-BON-C software (RATOC System Engineering, Tokyo, Japan). At all bone sites, the values from multiple slices were averaged to yield one value for each variable.

2.6. Biomechanical analyses

Structural and material properties of the femoral mid-diaphysis were determined using a three-point bending test. The site of testing was matched to the micro-CT sampling sites for the femoral mid-diaphysis (centered at 50% of the 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 the femur) until fracture 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 Factory SHiKiBU2000 software (SHIMADZU, Kyoto, Japan). Structural properties determined 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). Material properties measured 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.¹⁶

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. Two-micrometer vertical serial slices were prepared using a microtome (SM2010R, Leica Biosystems, Germany), and the sections were stained with hematoxylin and eosin (Muto Pure Chemicals, Tokyo, Japan).

2.8. Statistical analyses

Statistical analyses were performed with SPSS Statistics version 24 (IBM, Armonk, NY,
USA). All data are expressed as mean ± standard error of the mean (SEM). Two-tailed comparisons were made between diabetic and control mice. Independent group t-tests were used to compare means of the same variable between two groups when data met the requirements of equal variance (Levene’s test) and normal distribution (Shapiro-Wilk test). Welch’s t-test was used when variances were not equal. The effect of strain on the OGTT was evaluated using two-way ANOVA for time and strain, 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 and BMD. A P-value less than 0.05 was considered to be statistically significant for all experiments.

3. Results

3.1. Body weight, serum glucose levels, serum insulin levels, and OGTT

Table 1 shows the mean body weight and the serum glucose and insulin levels for both types of mice. Serum glucose levels were significantly higher in the NSY mice than in the control mice, whereas there were no differences in body weight and serum insulin levels. As shown in Fig. 1A, serum glucose levels were significantly higher in the NSY mice than in the control mice after administration of glucose solution at all time points (30 min, P < 0.0001; 60 min: P = 0.0001; 120 min, P = 0.0099), but fasting glucose levels were similar (P = 0.5418). The AUC was also significantly higher in the NSY mice than in the control mice (P = 0.0004; Fig. 1B).

3.2. BMC and BMD

BMD at the trochanter and mid-diaphysis of the femur were significantly lower in the NSY mice than in the control mice (P = 0.0011 and P < 0.0001, respectively; Fig. 2A and 2B).
Similarly, BMC at the trochanter and mid-diaphysis of the femur was significantly lower in
the NSY mice than in the control mice (P = 0.0062 and P < 0.0001, respectively; Fig. 2C and
2D).

3.3. Bone microstructure
As shown in Table 2, the total area, cortical area, periosteal perimeter, endosteal perimeter,
and cross-sectional moment inertia at the femoral trochanter and mid-diaphysis were
significantly lower in the NSY mice than in the control mice. Trabecular area and cortical
thickness at the femoral trochanter were lower in the NSY mice than in the control mice.
However, the cortical thickness at the femoral mid-diaphysis was not different between the
groups. In addition, the mean length, anteroposterior diameter, and transverse diameter of the
femoral mid-diaphysis were significantly lower in the NSY mice than in the control mice
(Table 3).

3.4. Bone metabolism markers
The serum OCN levels, as a bone formation marker, and serum TRAcP5b levels, as a bone
resorption marker, were significantly lower in the NSY mice than in the control mice (P =
0.0413 and P = 0.0087, respectively; Fig 3).

3.5. Bone strength
As shown in Table 4, the structural properties and material properties of the bone strength
parameters at the femur were lower in the NSY mice than in the control mice. In addition,
ultimate force, fracture force, elastic modulus, and toughness were significantly lower in the
NSY mice than in the control mice by 30%, 36%, 60%, and 80%, respectively.
3.6. Bone histology
In contrast to the histological preparation of the femur of control mice, several parts of the cortical bone of the femur of NSY mice were not stained with hematoxylin and eosin (Fig. 4). Moreover, serum glucose levels, but not insulin levels, were significantly negatively correlated with bone metabolism markers and BMD in the trochanter and mid-diaphysis of the femur (Table 5).

4. Discussion
Previous studies examining the bone integrity of diabetic animal models have demonstrated a decrease in bone mass and bone strength. It is well known that streptozotocin-induced T1DM rodent models are lean, and bone mass decreases with reduction of insulin secretion. However, several factors might affect the bone mass and strength in T2DM animal models besides hyperglycemia or oxidative stress, including body weight or insulin. Because of the positive correlation between body weight or body mass index and BMD, the obesity-induced increase of body weight contributes to the increase in bone mass. Insulin is well known as a hypoglycemic and anabolic hormone, which promotes bone formation upon interaction with the insulin receptor of osteoblasts. However, body weight and serum insulin levels of T2DM rodent models can be either significantly higher or lower than those of the control group. To clarify the factors contributing to the effects of DM on bone integrity besides body weight and insulin, we compared bone structure, mass, and density between NSY mice and ICR mice as a control group at 18–20 weeks of age with no difference in body weight and insulin level between groups. Consequently, we suggest that NSY mice can be a suitable T2DM animal model for examining the effect of hyperglycemia on bone integrity, using ICR mice as a control. To our knowledge, this is the first investigation to examine bone mass, metabolism, microstructure, and strength in NSY mice. Importantly, there was a
decrease in insulin secretion in the NSY mice despite the lack of a difference between the serum insulin levels; serum glucose levels were higher compared to those in the control. Thus, the NSY mouse is a T2DM animal model similar to the clinical condition of Japanese T2DM, which is not necessarily associated with obesity.

The National Institutes of Health indicated that bone strength can be determined by BMD and bone quality, with a contribution of 70% and 30%, respectively. Bone quality is reflected by structural properties such as bone size, geometry, microstructure and damage accumulation (e.g., microfractures), and material properties such as bone metabolism, mineralization and collagen fiber production. Accordingly, we compared bone mass, size, microstructure, metabolism, and strength between NSY and ICR control mice. The bone mass at the trochanter and mid-diaphysis of the femur was significantly lower in the NSY mice than in the control mice, and there was a strong negative correlation between serum glucose levels and BMD. Fushimi et al. reported that the serum glucose levels in NSY mice were significantly higher than those in the control ICR mice at 13 – 15 weeks of age. Accordingly, we suggest that NSY mice, at 20 weeks of age, had had chronic hyperglycemia for more than 5 weeks. In addition, calcium uptake and deposition of bone nodules were found to be inhibited by glucose in vitro. Indeed, several previous reports with T2DM rodent models showed a decrease in bone mass despite differences in body weight and serum insulin levels. Therefore, it has been reported that the loss of BMC is aggravated by the negative calcium balance in poorly controlled diabetes. Accordingly, we suggest that the glucose metabolism disorder in NSY mice directly contributes to the decrease of bone mass. Takagi et al. 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, but no such correlation was detected for BMD at the femoral mid-diaphysis. However, Fu et al. reported a positive correlation between BMD of the whole femur and serum glucose levels in...
the KKAy mouse. Thus, the discrepancy in the correlation of BMD and serum glucose levels in obese T2DM animal models may be related to the combination effects of the decreased bone mass due to the glucose metabolism disorder with an increase in bone mass caused by hyperinsulinemia and obesity.

We further demonstrated that the bone turnover rate was significantly lower in NSY mice than in the control mice. Many previous studies of T2DM animal models have shown a decrease in the level of bone formation markers. In addition, Botolin et al. reported that hyperglycemia deteriorated the osteoblastic function in vitro. Terada et al. have shown that the proliferation and differentiation potency of osteoblasts was inhibited by high glucose culture media in vitro. Inaba et al. demonstrated that high glucose content in culture medium attenuates the stimulation of OCN gene expression by 1,25(OH)2D in an osteoblast-like cell line, MG-63, suggesting that hyperglycemia suppresses the production of OCN by osteoblasts. Based on this background, and the reduction of OCN levels that were negatively correlated serum glucose levels in NSY mice, we suggest that the glucose metabolism disorder deteriorated osteoblastic function. Alternatively, hyperglycemia might also play a role, since the levels of the bone resorption marker TRAcP5b were lower in the NSY mice and also negatively correlated with serum glucose levels. Tanaka et al. reported that the expression level of RANKL, which induces the differentiation of osteoclasts, decreased in a hyperglycemic culture medium. In addition, bone resorption markers and serum insulin levels in previous T2DM animal models were found to be significantly higher than those of the control groups. Therefore, the decrease in bone mass by acceleration of bone resorption might be related to the fact that insulin signaling in osteoblasts promotes only the function of osteoclasts, even though insulin is known to promote osteoblast differentiation. However, Fujii et al. reported that glycemic control by insulin ameliorated bone abnormalities in spontaneously diabetic Torii rats. Insulin has an
important role in bone turnover, but it has not been clarified whether the effects of insulin on bone metabolism with diabetes mellitus are similar to those in non-diabetics. Accordingly, we suggest that hyperglycemia decreases the differentiation of osteoclasts, which conflicts with the influence of insulin on bone metabolism in T2DM.

We further clarified that the femurs of the NSY mice were shorter and thinner than those of the control mice. The development of the epiphyseal plate in the long bone is largely responsible for longitudinal growth of the bone. Cartilage multiplies with growth and an epiphyseal plate is formed, while trabecular bone is supplied by endochondral ossification. An undifferentiated mesenchymal cell differentiates into a chondrocyte, and endochondral ossification begins to form the cartilage anlage. When a chondrocyte differentiates into a hypertrophic chondrocyte, the cells in the neighboring perichondria differentiate into osteoblasts and gradually replace calcified cartilaginous tissue with bone tissue. Accordingly, we assumed that the decrease in the longitudinal growth and trabecular bone of the femur in the NSY mice was caused by suppression of osteoblast activity, given the reduction of serum OCN levels. Moreover, the femurs in the NSY mice decreased not only with respect to longitudinal growth but also in the brachydiagonal growth direction, which has also been reported to be inhibited in many previous studies of bone fragility in T2DM animal models.\textsuperscript{17, 19, 36} We suggest that hyperglycemia inhibits ossification of the epiphyseal plate and periosteal ossification, which can explain the reduction in the length and thickness of the femur in the NSY mice despite the lack of a difference in body weight and serum insulin levels from the control.

In the bone, collagen fibers are linked by calcium phosphate. Following decalcification, bone collagen fibers appear red when stained with eosin. Given the substantial reduction of eosin staining in many parts of the femurs of NSY mice, our histological findings are further suggestive that the reduction in bone mass is due to a lack of
5. Conclusions

We suggest that the NSY mouse could serve as an appropriate T2DM animal model for examining the specific effect of hyperglycemia on bone integrity, using the ICR mouse as a control. Hyperglycemia contributed to a decrease in bone metabolism turnover in the NSY mice, resulting in a thinner and shorter femur, lower cortical and trabecular area, and lower bone mass. We suggest that these effects contribute to the deteriorated bone strength of the femur in NSY mice. This model can be particularly suitable for examining the effects of T2DM on bone fragility for the Japanese population, since NSY mice are not necessarily obese, thereby more accurately representing the characteristics of T2DM in Japan.

Conflict of Interest

The authors declare no conflict of interest.
References


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26) Bouxsein ML, Devlin MJ, Glatt V, Dhillon H, Pierroz DD, Ferrari SL. Mice lacking beta-adrenergic receptors have increased bone mass but are not protected from deleterious skeletal effects of ovariectomy. *Endocrinology.*, **150**, 144-152 (2009).


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Table 1. Body weight, and serum glucose and insulin levels in ICR and NSY mice at 20 weeks of age.

<table>
<thead>
<tr>
<th></th>
<th>ICR</th>
<th>NSY</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>47.0 ± 0.9</td>
<td>46.3 ± 0.9</td>
<td>0.6059</td>
</tr>
<tr>
<td>Serum glucose (mg/dl)</td>
<td>125.1 ± 6.1</td>
<td>173.6 ± 7.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Serum insulin (ng/ml)</td>
<td>1.53 ± 0.39</td>
<td>1.29 ± 0.21</td>
<td>0.6133</td>
</tr>
</tbody>
</table>

Values are presented as the mean ± SEM; n = 6 per group.
Table 2. Cross-sectional structural properties assessed by micro-CT at the trochanter and the mid-diaphysis of the femur in ICR and NSY mice at 20 weeks of age.

<table>
<thead>
<tr>
<th></th>
<th>ICR</th>
<th>NSY</th>
<th>P value</th>
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<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
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</tr>
<tr>
<td><strong>Trochanter</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Tt. Ar (mm²)</td>
<td>4.14 ± 0.14</td>
<td>3.19 ± 0.03</td>
<td><strong>0.0013</strong></td>
</tr>
<tr>
<td>Ct. Ar (mm²)</td>
<td>2.55 ± 0.09</td>
<td>1.96 ± 0.06</td>
<td><strong>0.0003</strong></td>
</tr>
<tr>
<td>Tb. Ar (mm²)</td>
<td>0.58 ± 0.05</td>
<td>0.46 ± 0.03</td>
<td>0.0583</td>
</tr>
<tr>
<td>Ct. Th (μm)</td>
<td>376.50 ± 10.58</td>
<td>333.26 ± 13.09</td>
<td><strong>0.0279</strong></td>
</tr>
<tr>
<td>Ps. Pm (μm)</td>
<td>7884.69 ± 156.49</td>
<td>6982.53 ± 79.18</td>
<td><strong>0.0004</strong></td>
</tr>
<tr>
<td>Es. Pm (μm)</td>
<td>5380.14 ± 174.25</td>
<td>4542.80 ± 74.51</td>
<td><strong>0.0013</strong></td>
</tr>
<tr>
<td>CSMI (mm⁴)</td>
<td>1.61 ± 0.10</td>
<td>1.00 ± 0.02</td>
<td><strong>0.0021</strong></td>
</tr>
<tr>
<td><strong>Mid-diaphysis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tt. Ar (mm²)</td>
<td>2.57 ± 0.06</td>
<td>1.66 ± 0.04</td>
<td>&lt;<strong>0.0001</strong></td>
</tr>
<tr>
<td>Ct. Ar (mm²)</td>
<td>1.57 ± 0.05</td>
<td>1.21 ± 0.02</td>
<td>&lt;<strong>0.0001</strong></td>
</tr>
<tr>
<td>Ct. Th (μm)</td>
<td>312.59 ± 15.48</td>
<td>322.19 ± 3.03</td>
<td>0.5691</td>
</tr>
<tr>
<td>Ps. Pm (μm)</td>
<td>5808.97 ± 55.60</td>
<td>4719.46 ± 60.09</td>
<td>&lt;<strong>0.0001</strong></td>
</tr>
<tr>
<td>Es. Pm (μm)</td>
<td>3752.71 ± 150.19</td>
<td>2513.99 ± 46.41</td>
<td><strong>0.0002</strong></td>
</tr>
<tr>
<td>CSMI (mm⁴)</td>
<td>0.60 ± 0.02</td>
<td>0.30 ± 0.01</td>
<td>&lt;<strong>0.0001</strong></td>
</tr>
</tbody>
</table>

Values are presented as the mean ± SEM. Tt. Ar, total area; Ct. Ar, cortical area; Tr. Ar, trabecular area; Ct. Th, cortical thickness; Ps. Pm, periosteal perimeter; Es. Pm, endosteal perimeter; CSMI, cross-sectional moment inertia.
Table 3. Length of the femur, anteroposterior diameter, and transverse diameter of the femoral mid-diaphysis in ICR and NSY mice at 20 weeks of age.

<table>
<thead>
<tr>
<th></th>
<th>ICR</th>
<th>NSY</th>
<th>P value</th>
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<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Length (mm)</td>
<td>17.42 ± 0.18</td>
<td>16.72 ± 0.07</td>
<td>0.0044</td>
</tr>
<tr>
<td>AP diameter (mm)</td>
<td>1.67 ± 0.03</td>
<td>1.22 ± 0.04</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TV diameter (mm)</td>
<td>2.18 ± 0.05</td>
<td>1.77 ± 0.05</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Values are presented as the mean ± SEM. AP, anteroposterior diameter; TV, transverse diameter.
Table 4. Mechanical properties of the femoral mid-diaphysis determined by a three-point bending test in ICR and NSY mice at 20 weeks of age.

<table>
<thead>
<tr>
<th></th>
<th>ICR</th>
<th>NSY</th>
<th>P value</th>
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<tr>
<td>n</td>
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<tr>
<td><strong>Structural properties</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Ultimate force (N)</td>
<td>25.31 ± 1.22</td>
<td>17.47 ± 0.66</td>
<td>0.0004</td>
</tr>
<tr>
<td>Fracture force (N)</td>
<td>24.09 ± 1.21</td>
<td>15.35 ± 0.53</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Stiffness (N/mm)</td>
<td>37.45 ± 3.03</td>
<td>29.21 ± 3.13</td>
<td>0.0876</td>
</tr>
<tr>
<td>Work to failure (Nmm)</td>
<td>8.99 ± 0.94</td>
<td>7.32 ± 0.57</td>
<td>0.1464</td>
</tr>
<tr>
<td><strong>Material properties</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ultimate stress (Mpa)</td>
<td>89.00 ± 4.39</td>
<td>88.92 ± 3.88</td>
<td>0.9896</td>
</tr>
<tr>
<td>Fracture stress (Mpa)</td>
<td>84.52 ± 3.57</td>
<td>78.13 ± 2.32</td>
<td>0.1647</td>
</tr>
<tr>
<td>Elastic Modulus (Gpa)</td>
<td>463.16 ± 39.71</td>
<td>184.94 ± 26.64</td>
<td>0.0002</td>
</tr>
<tr>
<td>Toughness (MJ/mm³)</td>
<td>58.01 ± 6.87</td>
<td>11.72 ± 1.99</td>
<td>0.0006</td>
</tr>
</tbody>
</table>

Values are presented as the mean ± SEM.
Table 5. Correlation of serum glucose and insulin levels with BMD and bone metabolism markers in mice at 20 weeks of age.

<table>
<thead>
<tr>
<th>Glucose</th>
<th>Insulin</th>
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<tbody>
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<tr>
<td>BMD, bone mineral density; OCN, osteocalcin; TRAcP5b, tartrate-resistant acid phosphatase form 5b.</td>
<td></td>
</tr>
</tbody>
</table>
Fig 1. Oral glucose tolerance test in ICR and NSY mice. (A) Serum glucose levels were measured at 30, 60, and 120 min after glucose loading. (B) Area under the curve (AUC); data are presented as the mean ± SEM (n = 6 per group). **P < 0.01 vs. age-matched controls.
Fig 2. (A, B) Bone mineral density of the femur in ICR and NSY mice at 20 weeks of age in the (A) trochanter and (B) mid-diaphysis. (C, D) Bone mineral content of the femur in ICR and NSY mice at 20 weeks of age in the (C) trochanter and (D) mid-diaphysis. Data are presented as the mean ± SEM (n = 6 per group). **P < 0.01 vs. age-matched controls.
Fig 3. Bone metabolism markers in ICR and NSY mice at 20 weeks of age: (A) serum osteocalcin levels; (B) serum TRAcP5b levels. Data are presented as the mean ± SEM (n = 6 per group). *P < 0.05, **P < 0.01 vs. age-matched controls.
Fig 4. Histology photomicrographs showing hematoxylin and eosin staining of the trochanter region and the mid-diaphysis of the femur in demineralized paraffin sections. Representative photographs of histological sections from (A) the trochanter region and (B) mid-diaphysis of the femur in ICR mice and of the (C) trochanter and (D) mid-diaphysis of the femur in NSY mice at 20 weeks of age. Scale bar, 200 μm.