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ORIGINAL PAPER

Insulin does not rescue cortical and trabecular bone loss in type 2 diabetic Goto-Kakizaki rats

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Abstract In type 2 diabetes mellitus (T2DM), the decreased bone strength is often associated with hyperglycemia and bone cell insulin resistance. Since T2DM is increasingly reported in young adults, it is not known whether the effect of T2DM on bone would be different in young adolescents and aging adults. Here, we found shorter femoral and tibial lengths in 7-month, but not 13-month, Goto-Kakizaki (GK) T2DM rats as compared to wild-type rats. Bone μCT analysis showed long-lasting impairment of both cortical and trabecular bones in GK rats. Although insulin treatment effectively improved hyperglycemia, it was not able to rescue trabecular BMD and cortical thickness in young adult GK rats. In conclusion, insulin treatment and alleviation of hyperglycemia did not increase BMD of osteopenic GK rats. It is likely that early prevention of insulin resistance should prevail over treatment of full-blown T2DM-related osteopathy.

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Introduction

Diabetes mellitus (DM) is a globally common non-communicable disease which has deleterious effects on many organ systems, e.g., cardiovascular and nervous systems, kidney, and bone structure and strength [1]. Several investigations into type 1 (T1DM) and type 2 DM (T2DM) mostly showed similar outcomes, i.e. both types of DM cause impaired osteoblast and osteoclast functions, abnormal formation and alignment of collagen in bone matrix, resulting in weakening of bone mechanical properties and increased fracture risk [2–4]. Specifically, hyperglycemia and cellular insulin resistance as well as DM-associated cytokines [e.g., tumor necrosis factor-α (TNF-α) and interleukin (IL)-1 and IL-6] often suppress bone-forming activity of osteoblasts, but accelerate osteoclast activity to resorb mineralized bone [5–7]. Meanwhile, an increase in circulating glucose level induces the production of advanced glycation end products (AGE) in the bone matrix [3, 4, 8], thereby compromising bone elastic property and its ability to repair microcracks.

In general, T2DM is the most common form of DM that accounts for 90–95% of diabetic patients [1]. It results from insulin resistance and relative rather than absolute insulin deficiency. Most T2DM patients develop obesity or high body fat distribution with onset later in life (~55–57 years) [9–11]. Although there are numerous studies of bone change under T2DM condition in various diabetic animal models, most studies have been performed in adolescent or young adult animals with relatively short periods of hyperglycemia [12–14]. Thus, evidence that reports the final outcome of bone change in late adulthood or aging rats is scant.

Several therapeutic strategies are used to limit diabetic progress as well as improve quality of life, e.g., dietary control, exercises, antidiabetic drug therapy, and insulin replacement therapy. Because insulin can improve whole body glycemic control by reducing endogenous glucose production, fasting blood glucose and hemoglobin A1c (HbA1c) [15–19], insulin injection is the treatment of choice for T2DM patients with poor glycemic control or poor response to antidiabetic drugs [20]. There has been a report that insulin action in osteoblasts played a role in the maintenance of bone structure [18]. Normally, insulin is administered to patients with T1DM as well as to patients in the later stages of T2DM [20–22]. Since bone deterioration might have occurred earlier in T2DM, perhaps just after the onset of insulin-resistant pre-diabetic condition [23], it is not known whether early insulin therapy in the adolescent would be effective in restoring bone structure.

In the present study, we aimed to investigate (1) whether in T2DM bone structure was permanently impaired from adulthood to aging, and (2) whether insulin therapy during the adolescent period could restore bone loss in Goto-Kakizaki (GK) rats. GK rats were used in this study because they are a non-obese T2DM substrain of Wistar rats that manifest hyperglycemia with insulin resistance and stable fasting hyperglycemia of ~130–150 mg/dL [24–26]. Their non-obese characteristic also minimizes the positive effect of body weight on bone formation, which is often observed in obese T2DM models.

Materials and methods

Animals

The experiment was divided into two parts, i.e., experiment 1 (7- and 13-month experiments) and experiment 2 (insulin treatment experiment). For the aging experiment (experiment 1), 1-month-old female GK rats and age-matched wild-type (WT) Wistar rats were used, while in the insulin treatment experiment, 6-week-old female GK and WT rats were used. All rats were purchased from the Center for Laboratory Experimental Animals, Japan. They were housed in stainless steel cages under a 12:12-h light–dark cycle. Room temperature was ~22–24 °C with relative humidity of ~50–60%. They were fed standard chow (Perfect Companion, Thailand) and reverse osmosis water ad libitum. All animals were cared for in accordance with the Mahidol University policy for the care and use of animals for scientific purposes. This study has been approved by the ethics committee of the National Laboratory Animal Center and the Animal Care and Use Committee of the Faculty of Science, Mahidol University.

Experimental design

Experiment 1 (7- and 13-month experiments)

To determine whether T2DM permanently impaired bone structure from early adulthood until aging, 1-month-old GK and WT rats were used. After a 7-day acclimatization, they were nursed until reaching the age of 7 and 13 months ($n = 10$ per group). Blood was collected to determine plasma ionized calcium by using ion-selective electrodes (model Stat Profile CCX; Nova Biomedical, Waltham, MA, USA). Ten left femora and ten left tibiae were collected from all rats, and bone length was measured with a vernier caliper. Ex vivo micro-computed tomography (μCT) analysis of the tibiae was performed to obtain volumetric bone cortical and trabecular parameters. The timeline of this experiment is shown in Fig. 1a.

Experiment 2 (insulin treatment experiment)

To determine whether insulin therapy could restore bone mass in T2DM animal model, 6-week-old female rats were divided into 3 groups, i.e., WT rats, GK rats, and insulin-treated GK rats (GK + Ins; $n = 10$ per group). After a 7-day acclimatization, intraperitoneal glucose tolerance test (IPGTT) was performed. Blood was collected at 15, 30, 60, and 120 min from all animals to determine the blood glucose levels using Accu-Chek Active Test Strips (Roche Diagnostics, Germany). At week 16, rats in the GK + Ins groups were daily injected subcutaneously with 4.6 U/kg/dose insulin glargine (Gla-100, Lantus; Sanofi-Aventis, Germany) 3 doses/day, and blood glucose was monitored weekly. This insulin glargine administration regimen has been validated for successfully lowering blood glucose. Specifically, in our pilot study, blood glucose levels were monitored at days 1, 4, and 7 of treatment. Since it was found that this dose of insulin glargine effectively decreased blood glucose, this dose was used for the entire experiment.

All groups of animals were nursed until 28 weeks of age. In vivo μCT analysis of the tibiae was performed at various time points, i.e., 20, 24, and 28 weeks (4, 8, and 12 weeks after treatments, respectively), and week 8 was used as a baseline (0 week; baseline control). After euthanasia, blood was collected to determine plasma ionized calcium, and the duodenum was removed to determine transepithelial calcium flux by the Ussing chamber technique [27]. The timeline of this experiment is depicted in Fig. 1b.

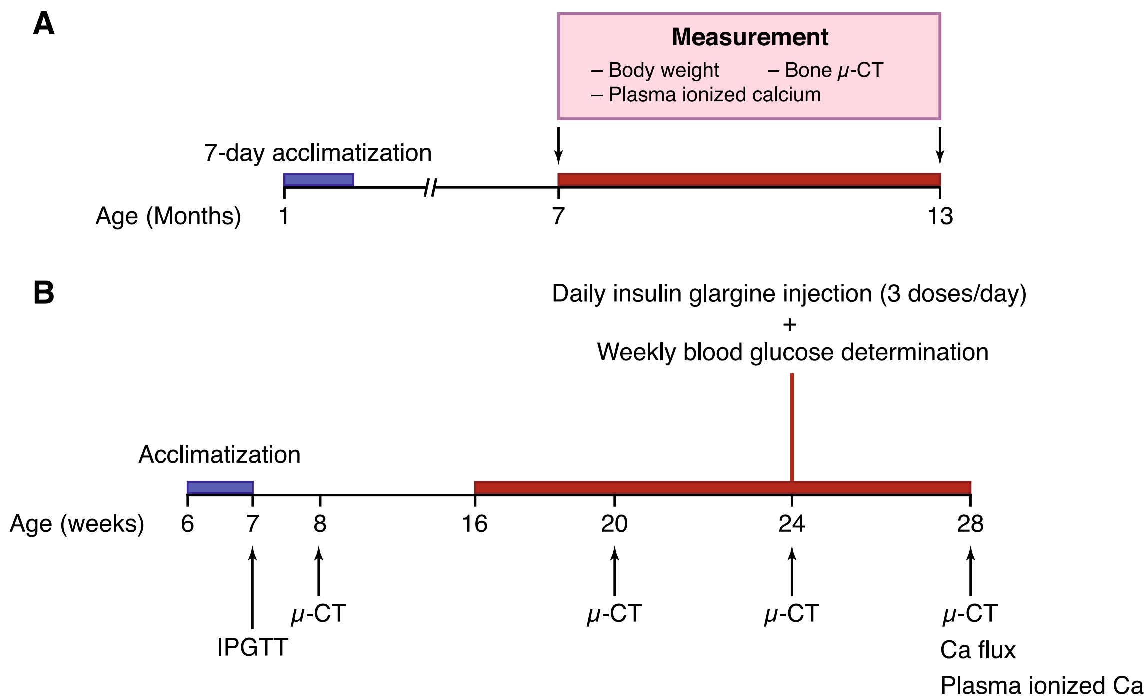

Fig. 1 Timelines of experiment 1 (**a**) and 2 (**b**). *Ca* calcium, *IPGTT* intraperitoneal glucose tolerance test, *μCT* micro-computed tomography

μCT analysis

For ex vivo scanning, the tibiae were wrapped with moist gauze and scanned at 65 kV, 615 μA (Skyscan 1178 high-speed in vivo/ex vivo μCT; Bruker MicroCT, Kontich, Belgium). For in vivo scanning, after being anesthetized by 50 mg/kg sodium pentobarbitone, the rats had their legs fixed with polystyrene foam before scanning. The region of interest (ROI) for trabecular and cortical regions were 1.360–5.610 and 14.110–18.360 mm distal to the proximal growth plate, respectively. The rotation angle was 0.54° at each step and voxel size was 85 μm^3 isotropically. Morphometric indices of cortical (tibial mid-shaft) and trabecular regions (tibial spongiosa) were cortical bone mineral density (BMD; g/cm^3), trabecular BMD (g/cm^3), cortical thickness (mm), cortical endosteal perimeter (mm) and medullary area (mm^2). Three-dimensional (3D) figures were reconstructed by NRecon Software (SkyScan, v.1.6.4.8) with ring artifact correction of 10 and a beam hardening correction of 30%. Serial 8-bit images were analyzed by CTAn software (v.1.14.4).

Measurement of transepithelial calcium flux

For ex vivo intestinal calcium transport [27], the duodenum was removed after a median laparotomy. The duodenum was cut longitudinally to expose the mucosa, which was later well rinsed by isotonic bathing solution. Then, the intestinal tissue was mounted in an Ussing chamber and bathed on both sides of the hemichambers with an isotonic bathing solution containing (in mmol/L) 118 NaCl, 4.7 KCl, 1.1 $MgCl_2$, 1.25 $CaCl_2$, 23 $NaHCO_3$, 12 D-glucose, and 2 mannitol (all purchased from Sigma) for 10 min. The solution in the mucosal hemichamber was then changed to the bathing solution containing ^{45}Ca (initial amount of 0.45 μCi/mL, final specific activity of 90 mCi/mol; catalog no. NEZ013; PerkinElmer, Boston, MA, USA). Unidirectional calcium flux ($J_{H\rightarrow C}$, nmol/h/cm^2) from the hot side (H; mucosal side) to the cold side (C; serosal side) was calculated by Eqs. 1 and 2:

$$J_{H\rightarrow C} = R_{H\rightarrow C}/(S_H \times A), \quad (1)$$

$$S_H = C_H/C_{To}, \quad (2)$$

where $R_{H\rightarrow C}$ is the rate of ^{45}Ca appearance in the cold side (cpm/h); S_H is the specific activity of the hot side (cpm/nmol); A is the surface area of the tissue (cm^2); C_H is the mean radioactivity of the hot side (cpm); and C_{To} is the total calcium content in the hot side (nmol). ^{45}Ca radioactivity was analyzed by a iquid scintillation spectrophotometer (model Tri-Carb 3100; Packard, Meriden, CT, USA). In the absence of a transepithelial calcium gradient (the same calcium concentration of 1.25 mmol/L in both hemichambers), the calcium flux represented active calcium transport in the mucosal-to-serosal direction.

Statistical analysis

Results are expressed as mean ± SE. Two sets of independent data were compared by unpaired Student's *t* test.

One-way analysis of variance (ANOVA) with Newman–Keuls multiple comparisons test was used for multiple sets of independent data. The level of significance for statistical tests was $P < 0.05$. All data were analyzed by GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

Results

In the adolescent period, plasma free ionized calcium of 7-month-old GK rats was markedly lower when compared to WT and the reduction lasted until 13 months. However, unlike WT rats, the plasma ionized calcium levels in GK rats did not decrease from 7 months to 13 months (Fig. 2). The body weight of WT rats increased with increasing age, whereas the lower body weight of GK rats did not change with age (Fig. 3a). Low body weight of GK rats could be partly due to shorter femoral and tibial lengths in 7-month-old GK rats (Fig. 3b, c). However, there was no difference in bone length between GK and WT at 13 months. Shorter long bones of 7-month-old GK rats suggested a possible deleterious effect of T2DM on body growth, particularly bone elongation in adulthood (7-month group).

Bone microarchitectural analyses by μCT revealed impairment of bone structure in GK rats, i.e., lower cortical BMD, trabecular BMD and cortical thickness when compared to WT (Fig. 4a–c). Moreover, the trabecular BMD in GK rats was markedly lower, by ~33 and ~49% in 7- and 13-month-old GK rats, respectively, possibly resulting from expansion of the medullary area (enlarged marrow cavities) and endosteal perimeter (Fig. 4d–f). Medullary areas were enlarged by ~40 and ~76% in 7- and 13-month-old GK rats, respectively (Fig. 4e, f).

Furthermore, we investigated whether early insulin treatment in adolescence could rescue bone microstructure in GK rats. Prior to μCT analyses, we determined glucose tolerance using IPGTT in 7-week-old GK rats and found

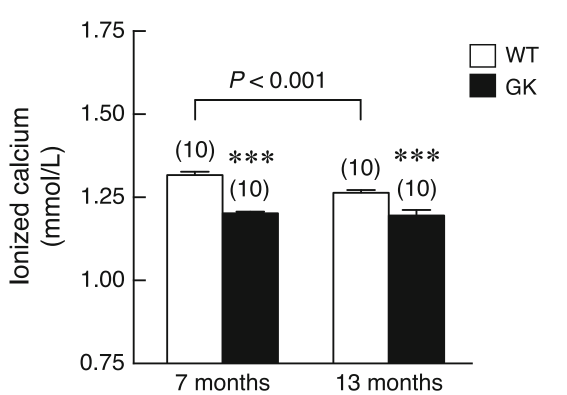

Fig. 2 Plasma ionized calcium of female WT and GK rats. *Numbers in parentheses* are numbers of animals. ***$P < 0.001$ vs. age-matched WT rats

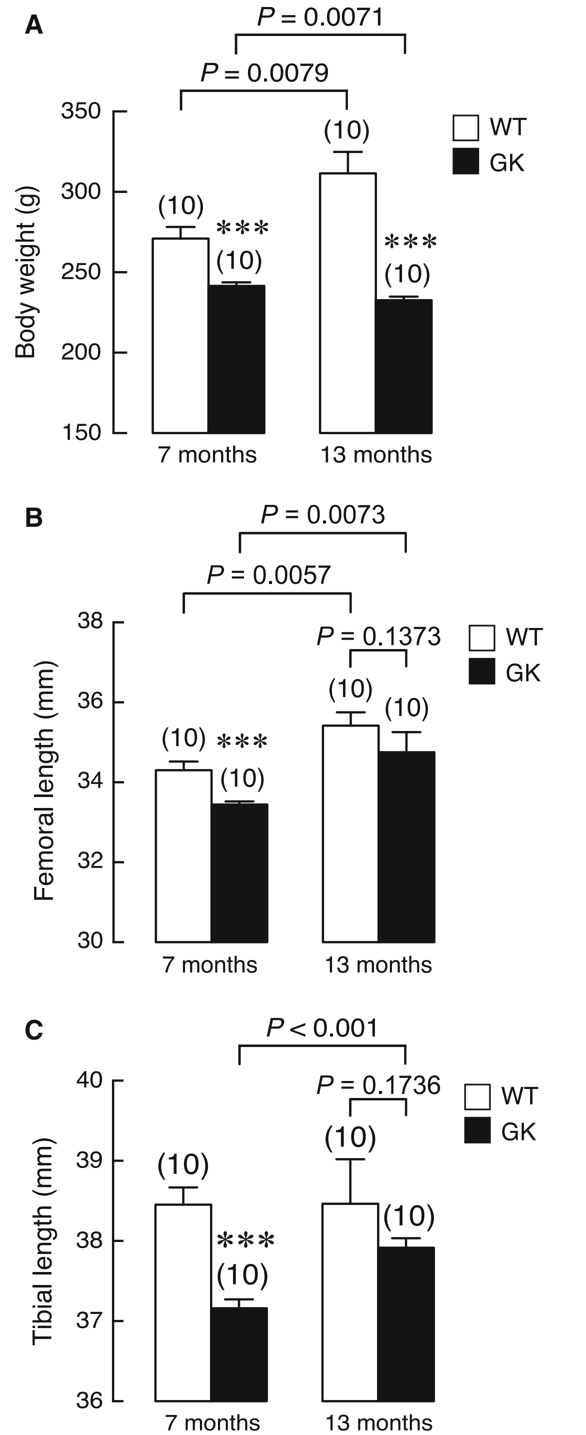

Fig. 3 Body weight (**a**), femoral (**b**) and tibial lengths (**c**) of 7- and 13-month-old female WT and GK rats. *Numbers in parentheses* are numbers of animals. ***$P < 0.001$ vs. age-matched WT rats

impaired glucose tolerance after 2 g/kg glucose loading (Fig. 5a, b), indicating the presence of insulin resistance in GK rats. After 12 weeks of daily insulin glargine injection, plasma glucose levels were restored to the normal range (Fig. 5c). Interestingly, plasma free ionized calcium

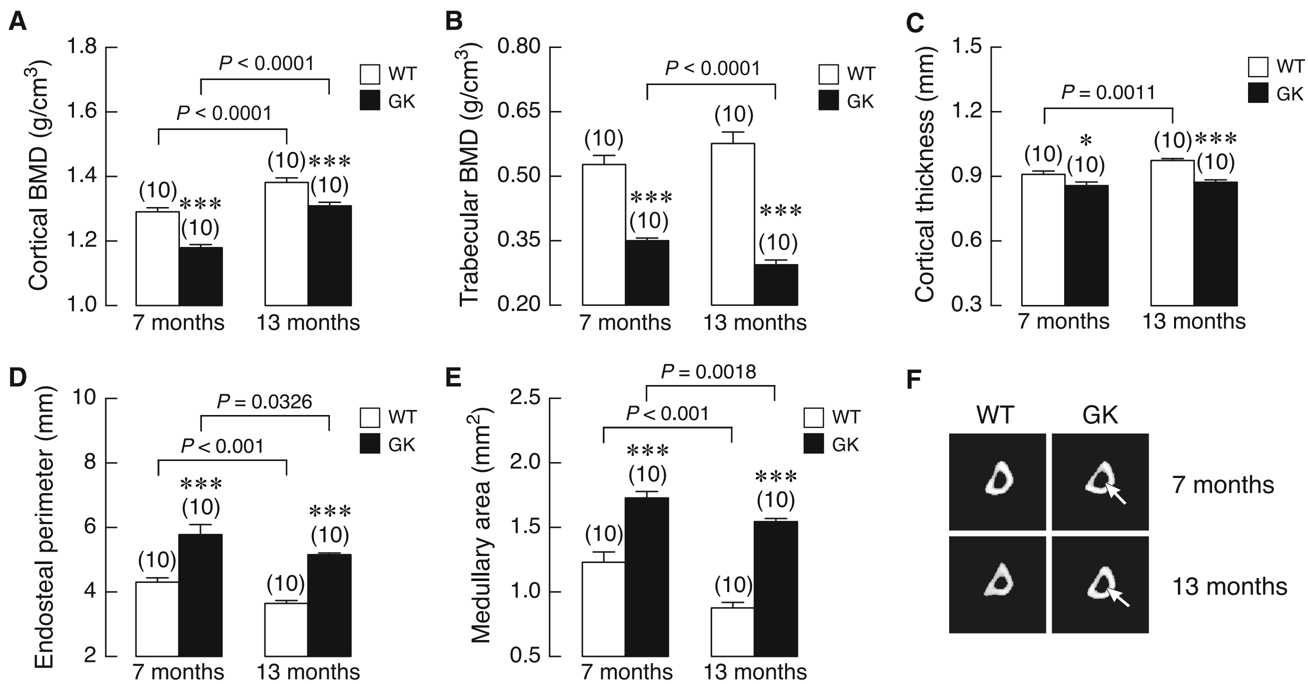

Fig. 4 Ex vivo μCT analyses in the tibiae of 7- and 13-month-old female WT and GK rats. **a** Cortical and **b** trabecular BMD, **c** cortical thickness, **d** endosteal perimeter, **e** medullary area, and **f** representative μCT images of the tibial cortical envelopes (midshaft). *Numbers in parentheses* are numbers of animals. *Arrows* indicate the enlarged marrow cavities in GK rats. *$P < 0.05$, ***$P < 0.001$ vs. age-matched WT rats

partially increased in insulin-treated GK rats with no changes in the transepithelial calcium transport across the intestine (Fig. 5d, e). However, longitudinal in vivo μCT analysis demonstrated that 12 weeks of daily insulin treatment in GK rats did not rescue trabecular BMD, cortical thickness, or medullary area (Fig. 6). In 12-week GK + Ins rats, trabecular BMD was further decreased when compared with GK rats, suggesting that T2DM permanently destroyed the cortical and trabecular bone, which could not be restored by early insulin treatment.

Discussion

It is evident that hyperglycemia and insulin resistance in T1DM and T2DM are able to impair bone structure and function by causing abnormal bone cell activities (cellular failure) and aberrant extracellular matrix structure and composition (matrix failure) (see [2] for review). In the present study, we demonstrated the effects of T2DM on longitudinal bone growth and BMD in adolescent (7-month-old) and late adult (13-month-old) GK rats. We found that GK rats had much lower body weight in both periods. Interestingly, although body weight of GK rats was much lower than WT rats, the final outcome of bone length in aging GK rats was not different from WT rats, indicating that T2DM might interfere with bone elongation only in the growing period. In other words, the shorter bone length in GK rats was observed in the young adult period, but later bone length reached the same length as in WT rats in the late adult period. Indeed, the reason for this evidence was unclear. There have been reports of both normal and impaired growth in DM individuals [28–30], which might link to DM-associated growth retardation [2, 30].

Normally, bone elongation depends on nutrient adequacy, e.g., calcium and zinc, as well as local and systemic factors, e.g., growth hormone (GH), insulin-like growth factor-1 (IGF-1), and insulin [31–34]. Specifically, GH cooperates with insulin to enhance growth plate chondrocyte proliferation and maturation through overexpression of endochondral bone formation-related genes, such as type 2 collagen and aggrecan [34, 35]. Therefore, relative insulin resistance in T2DM, which is caused by abnormal insulin signaling [19], could impair growth plate chondrocyte development or bone growth. Bone elongation is generally controlled by proliferation and differentiation of chondrocytes in the growth plate. The growth plate is divided into three zones, i.e., the resting zone with low mitotic activity chondroblasts that later migrate into the proliferative zone, where cells have high proliferative capacity. Proliferative chondrocytes become enlarged in the hypertrophic zone before undergoing apoptosis, and are replaced by osteoblasts that arrive with vascularization [31, 36, 37]. Therefore, it is possible that a decrease in bone

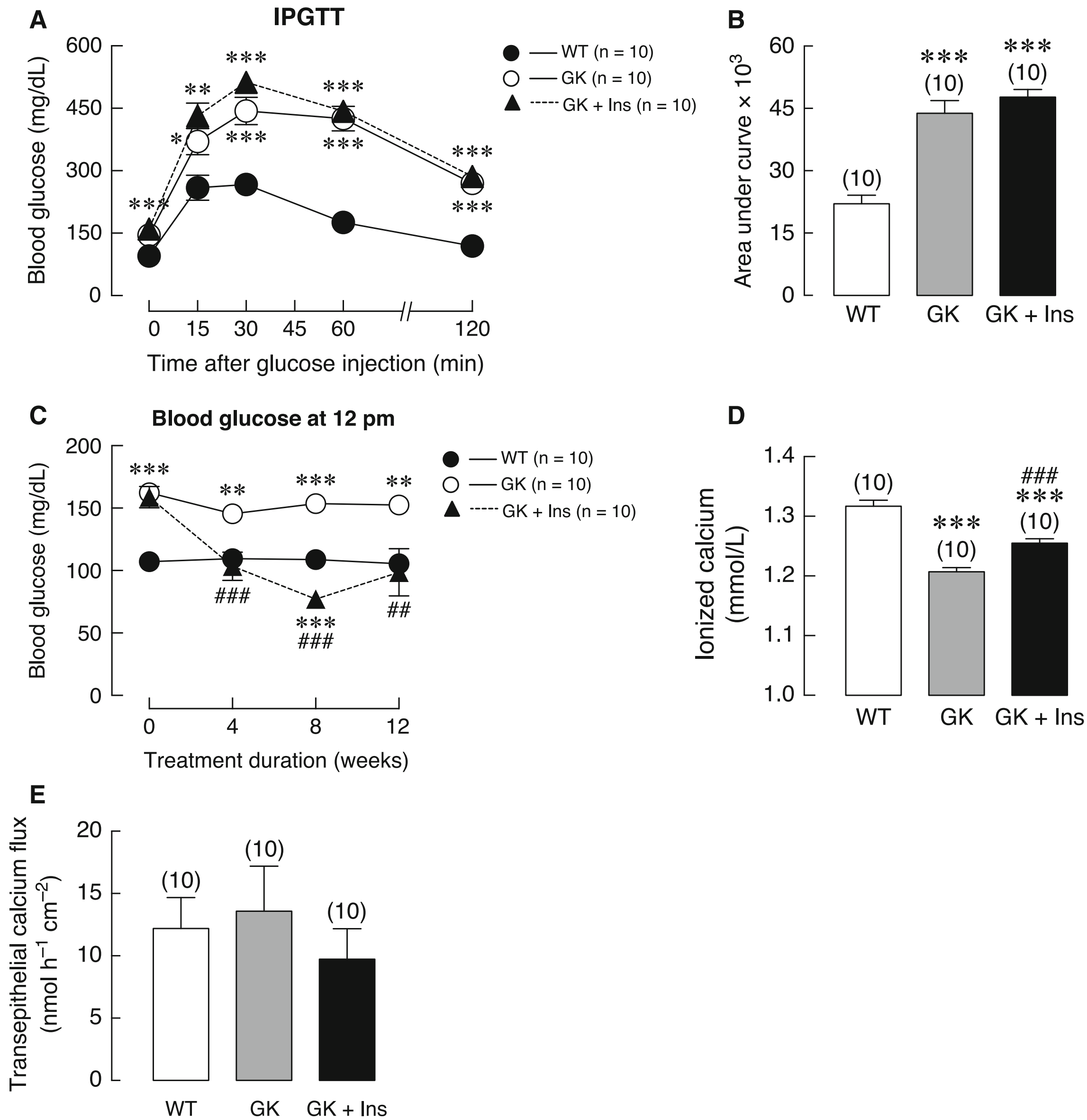

Fig. 5 Blood glucose profile of 7-week-old female WT, GK and GK + Ins rats. **a** Intraperitoneal glucose tolerance test (IPGTT) after administration of 2 g/kg glucose solution. Blood glucose was measured at various time points, i.e., 0, 15, 30, 60, and 120 min. **b** Area under the curve of IPGTT. **c** Weekly blood glucose at 0, 4, 8, and 12 weeks of insulin treatment. **d** Plasma ionized calcium. **e** Transepithelial calcium flux across the duodenum of 28-week WT, GK, and GK + Ins rats. *Numbers in parentheses* are numbers of animals. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. age-matched WT rats; ##$P < 0.01$, ###$P < 0.001$ vs. GK rats

growth in GK rats could result from delayed growth plate chondrocyte differentiation and/or premature chondrocyte apoptosis [14, 38].

Consistent with reports in T2DM, Muñoz et al. [30], who studied the heights of T1DM patients in different pubertal stages, reported that adult heights were eventually within normal range, but growth velocity was below average. The present finding of shorter bone length in 7-month-old GK rats was almost consistent with the aforementioned finding. Hence, catch-up growth was observed in 13-month-old GK rats in the present longitudinal study. This may be caused by both groups having completely passed the growth spurt period in which the growth hormone level is very high [39]. In addition, an optimal estrogen level during sexual maturation enhances skeletal growth [40–42]. Therefore, the accomplished growth spurt period might be a factor for catch-up bone growth of GK rats.

Furthermore, μCT analyses revealed that cortical and trabecular BMD and cortical thickness of GK rats were significantly lower than in WT rats from 7 until 13 months. The lower BMD in both cortical and trabecular portions led

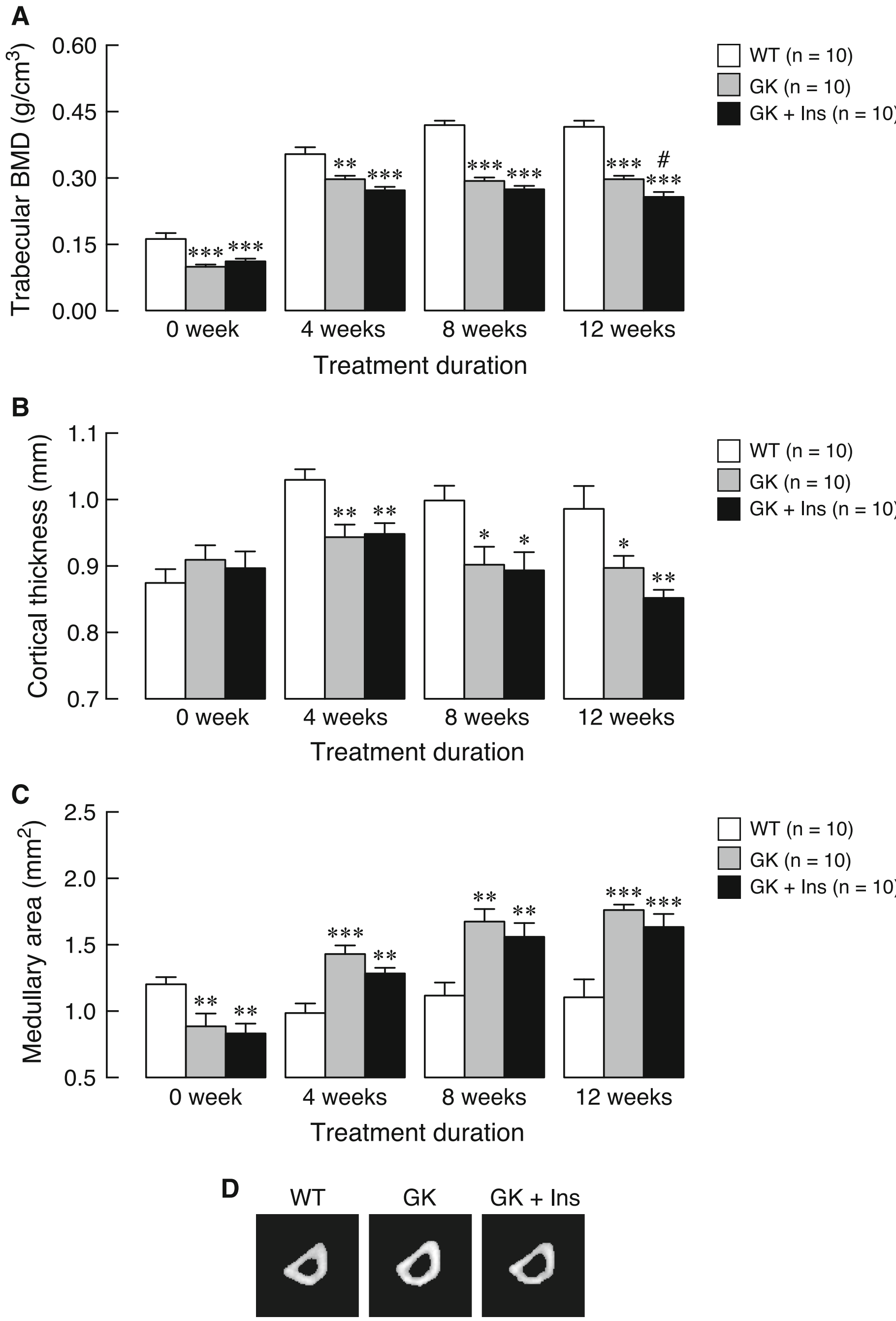

Fig. 6 Trabecular BMD (**a**), cortical thickness (**b**), medullary area (**c**), and representative µCT images of the tibial cortical envelopes (midshaft) (**d**) of female WT, GK, and GK + Ins rats as determined by in vivo µCT analyses. For the GK + Ins group, rats received daily subcutaneous injection of insulin glargine, while WT and GK rats were injected with normal saline (vehicle). In vivo µCT analyses were performed before insulin treatment (0 week; 8 weeks of age) and at 4, 8, 12 weeks after insulin treatment. *Numbers in parentheses* are numbers of animals. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. age-matched WT rats; #*P* < 0.05 vs. GK rats

to the expansion of the medullary area which persisted until 13 months, suggesting long-lasting negative effects of T2DM on bone. Therefore, we confirmed the T2DM-induced permanent bone loss by performing a longitudinal insulin rescue study by injections with insulin glargine 3 doses daily for 12 weeks (from 16 to 28 weeks of age). Strikingly, although insulin treatment could restore blood glucose towards the normal baseline, it was unable to recover bone density (both trabecular and cortical portions) in GK rats (Fig. 6), suggesting that insulin glargine could improve hyperglycemia but not insulin resistance in bone cells. The reason why insulin treatment failed to improve bone architecture may be due to several factors, e.g., different degrees of severity of insulin resistance in bone cells or bone-derived mesenchymal stem cells and other cell types (e.g., muscle cells). Recently, GK rats have been reported to exhibit insulin resistance with a decrease in insulin receptor expression in bone cells compared with WT rats [43]. Furthermore, prolonged accumulation of advanced glycation end products (AGEs) in the bone extracellular matrix and insulin resistance-related prolonged reactive oxygen species (ROS) production would continuously stimulate osteoclast survival and function, leading to the enhanced bone resorption [44, 45].

Non-obese and insulin resistance are important characteristics of GK rats [25]. Wei et al. [19] have provided evidence that insulin resistance caused perturbation of osteoblast function that notably affected whole-body glucose homeostasis. They demonstrated in mice lacking one allele of *Insr* in osteoblasts (*Col1a1-Insr*$^{+/-}$ mice) that bone-specific insulin resistance led to a decrease in circulating levels of bone-derived hormone osteocalcin, which is needed for optimal insulin sensitivity in muscle and white adipose tissue, thereby impairing glucose homeostasis [19, 46]. Importantly, osteocalcin as a non-collagenous extracellular matrix protein is largely responsible for hydroxyapatite binding in bone formation [47]. Thus, perturbation of insulin signaling could indirectly impair bone strength through a reduction in osteocalcin production [19, 48]. Furthermore, Wei et al. [19] noted that insulin resistance in high-fat diet-fed mice was developed from lipotoxicity-induced degradation of insulin receptors in osteoblasts. Therefore, a reduction in insulin receptor expression in osteoblasts probably causes ineffectiveness of insulin replacement therapy to recover BMD of GK rats.

Besides osteoblasts, osteoclasts are another target of insulin action. Thomas et al. [49] showed the expression of insulin receptor on mouse osteoclast-like cells. Consistent with our previous study in GK rats [14], bone histomorphometric analysis confirmed that DM reduced osteoblast function (e.g., osteoblast surface, mineralizing surface, and bone formation rate), while increasing osteoclast-mediated bone resorption (e.g., osteoclast surface and eroded surface). In addition, GK rats have been shown to increase mRNA expression of inflammatory cytokines, especially TNF-α, IL-1, and IL-6, all of which are known to be osteoclastogenic factors and might contribute to the enhanced bone resorption [50]. DM-induced bone resorption also caused the elevation of extracellular calcium in the bone microenvironment, which, in turn, enhanced differentiation of bone marrow stromal cells into adipocytes, and decreased osteoblast number and perhaps osteoblast-mediated bone formation [51].

Taken together, the present study showed the long-lasting negative effects of T2DM on cortical and trabecular bones during the stage of adulthood to the aging period. Early treatment with insulin in adolescent GK rats could not restore bone microstructure or BMD to normal, although it successfully abolished hyperglycemia. Therefore, early prevention of T2DM is exclusively the best way to control the T2DM-associated bone health deterioration. Limitations of the present study include the absence of data on the insulin tolerance test and bone cell insulin resistance. Moreover, in vitro and in vivo bone cell responses under diabetic condition and insulin treatment should be further investigated for a better understanding of the pathogenesis of diabetic osteopathy.

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Author contributions Conception and design of research—NC and KW. Performed experiments—RA, PS, WT, and KK. Analyzed data—RA, NC, KW, and NK. Drafted manuscript—RA, NC, KW, and NK.

Compliance with ethical standards

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Conflict of interest The authors declare that there is no conflict of interest.

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