

Original article

Effects of teriparatide on bone in autochthonous transgenic model mice for diabetes mellitus (Akita mice)

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ARTICLE INFO

Article history:

Received 24 June 2019

Received in revised form

10 November 2019

Accepted 23 November 2019

Available online 10 December 2019

Keywords:

Diabetes mellitus

Akita mouse

Teriparatide

Bone mineral density

Bone strength

Bone quality

ABSTRACT

Objectives: The purpose of this study is to evaluate the effects of teriparatide (TPTD) on bone mineral density (BMD), bone strength, and bone quality in Akita mouse models of diabetes mellitus.

Methods: Twelve-week-old female Akita mice and control mice (C57/BL/6NCRSlc) were divided into 4 groups: control mice treated with vehicle (n = 7) or TPTD (n = 6); and Akita mice treated with vehicle (n = 6) or TPTD (n = 7). TPTD or vehicle was administered subcutaneously 3 times a week for 8 weeks. Blood glucose, serum sclerostin, total tibial BMD, femoral shaft bone strength, and bone quality using Fourier-transform infrared spectroscopy imaging were evaluated.

Results: No significant differences in serum sclerostin levels were evident among these groups after 8 weeks of treatment. TPTD significantly increased BMD in control mice (+12.7%, $P = 0.02$) and Akita mice (+29.2%, $P = 0.001$) compared with vehicle. Maximum load and stiffness were significantly higher in Akita mice treated with TPTD than in Akita mice treated with vehicle (+56.6%, $P = 0.03$ and +90.5%, $P = 0.02$, respectively). On Fourier-transform infrared spectroscopy imaging, the mineral/matrix ratio was significantly lower in Akita mice treated with vehicle than in control mice (−12.2%, $P = 0.02$), and TPTD treatment significantly increased the mineral/matrix ratio ($P = 0.003$).

Conclusions: TPTD thus improved BMD and bone strength in both control mice and Akita mice, with improvements in the mineral/matrix ratio among Akita mice.

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1. Introduction

Type 1 diabetes mellitus (DM) is an important cause of secondary osteoporosis, and the number of DM patients is increasing as the overall population ages. Treating secondary osteoporosis due to DM is important to prevent fragility fractures, which commonly cause patients to become bedridden. A meta-analysis revealed that both type 1 and 2 DM are risk factors for fractures at proximal femur when compared with no DM [1]. Insulin stimulates the differentiation of osteoblasts and accelerates bone formation [2,3], so the loss of bone mineral density (BMD) in type 1 DM is considered to be caused by insulin deficiency. However, the risk of fracture in type 1 DM is reportedly much higher than that due to loss of BMD

alone [4]. These studies demonstrate that both type 1 and 2 DM worsen bone fragility, independent of the loss of BMD.

Improvements in not only bone volume, but also bone quality may thus be essential to treat DM-induced osteoporosis. Teriparatide (TPTD) has been reported to exert anabolic effects on bone in normal animals [5], osteoporotic animals [6], and humans [7]. TPTD increases BMD and bone metabolic markers and prevents fragility fractures among postmenopausal osteoporosis patients [8–10]. However, the effects of TPTD on BMD, bone strength, and bone quality in DM-induced osteoporosis have not been fully elucidated.

Various DM model animals, such as the WBN/Kob rat and Goto-Kakizaki rat, have been used to evaluate the effects of TPTD on DM osteoporosis [11,12]. Furthermore, one of the animal models for type 1 DM is the streptozotocin-induced diabetic rat model (STZ rat). We have reported that TPTD improved BMD and bone strength in STZ rats [13]. However, STZ rats sometimes develop DM to varying degrees of severity among individual rats [14]. In the present study, Akita mice were used to evaluate the effects of TPTD on bone metabolism and bone quality in DM. Akita mice are an

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Peer review under responsibility of The Korean Society of Osteoporosis.

autochthonous transgenic model for DM and show strong glucose intolerance [15]. Our previous study reported decreases in BMD (-12.5% at 14 weeks), bone strength (-32.3% at 18 weeks), and serum sclerostin levels (-32.0% at 18 weeks) in Akita mice when compared to non-DM mice [16]. However, the effects of treating osteoporosis on bone have not been evaluated in Akita mice. The purpose of this study is to evaluate the effects of TPTD on BMD, bone strength, and bone quality in DM-induced osteoporosis using Akita mice.

2. Methods

2.1. Animals

Twelve-week-old female Akita mice (Japan SLC, Shizuoka, Japan) and C57/BL/6NCRSlc mice (Japan SLC) were used as controls (CON) in this study. Animals were housed in a controlled environment at 22°C with a 12-hour light/dark cycle. Mice were paired and allowed free access to water and standard food (CE-2; CLEA Japan, Tokyo, Japan) containing 1.14% calcium, 1.06% phosphorus, and 250 IU of vitamin D₃ per 100 g.

2.2. Experimental design

Mice were divided into 4 groups: (1) control group treated with vehicle (CON + Veh; $n = 7$); (2) control group treated with TPTD (CON + TPTD; $n = 6$); (3) Akita mouse group treated with vehicle (AKM + Veh; $n = 6$); and (4) Akita mice treated with TPTD (AKM + TPTD; $n = 7$). TPTD (h-PTH; Asahi Kasei Pharma Corp., Shizuoka, Japan) or vehicle (0.1% mouse albumin) was administered subcutaneously at a dose of $80\text{-}\mu\text{g/kg}$ body weight 3 times a week for 8 weeks. The dose of TPTD was determined based on previous studies [17,18]. Mice were euthanized under anesthesia with an intra-abdominal injection of ketamine (Sankyo, Tokyo, Japan) and xylazine (Zenoa, Fukushima, Japan). Before sacrifice, body weight was measured. After sacrifice, blood, right femur and bilateral tibiae were harvested. The left tibia was stored in 10% formalin and used for BMD measurement. The right tibia was fixed in alcohol and used for FTIR. The right femur and serum were stored at -20°C . The right femur was used for 3-point bending tests to measure bone strength, and serum was used for measurements of biochemical parameters. The animal experimentation protocols were approved by the animal committee at our institute (approval number: a-1-2417). All animal experiments conformed to the guidelines for animal experimentation of our institute.

2.3. Biochemical parameters

Blood was collected from the inferior vena cava. After measuring blood glucose levels (mg/dL) (Antsense III; Horiba, Kyoto, Japan), the remaining blood samples were centrifuged at 15,000 rpm for 20 minutes to separate the serum. Serum sclerostin (pg/mL) was measured using an enzyme-linked immunosorbent assay kit (Mouse Sclerostin ELISA; ALPCO Diagnostics, Salem, NH, USA).

2.4. Bone mineral density

Total BMD (mg/cm^2) of the left tibia was measured by dual-energy X-ray absorptiometry (QDR-4500; Hologic, Waltham, MA, USA).

2.5. Biomechanical analysis

Bone strength of the right femur was evaluated with a 3-point bending device using a mechanical testing machine (MZ-500S;

Maruto, Tokyo, Japan). The femur was placed horizontally on a 2-point holder (6-mm span) with the anterior aspect facing up, and load was applied to the midshaft with a crosshead speed of 10 mm/minutes until fracture occurred. Maximum load (N) and stiffness (N/mm) were calculated using the software supplied with the testing machine (CTR win; System Supply, Nagano, Japan).

2.6. Fourier-transform infrared spectroscopy imaging

To evaluate bone quality, right tibiae were used for Fourier-transform infrared spectroscopy imaging (FTIRI) [19]. Each right tibia ($n = 4$ per group) was embedded in polymethyl methacrylate (PMMA). The undecalcified sections were microtomed at a thickness of $3\text{ }\mu\text{m}$, placed on barium fluoride infrared windows (Spectral Systems, Hopewell Junction, NY, USA), and examined using a PerkinElmer Spotlight 400 Infrared Imaging System (PerkinElmer Instruments, Waltham, MA, USA) at a spectral resolution of 4 cm^{-1} . Mineral and matrix properties were assessed using FTIRI in a specific region of the tibial cross-sections of cancellous bone. The region included the area from the lowest point of the growth plate to 0.5 mm distally. For this region, 5 different points were extracted. Mean and standard deviation (SD) were calculated for the 5 points. Spectra were baseline-corrected, and the PMMA spectral contribution was subtracted using OMNIC8, TQ Analyst (Thermo Fisher Scientific, Kanagawa, Japan). The infrared spectrum was analyzed to determine 4 FTIRI parameters [20]: (1) mineral/matrix ratio, as the integrated region ratio of phosphate ($907\text{--}1,183\text{ cm}^{-1}$)/amide 1 ($1,587\text{--}1,711\text{ cm}^{-1}$), which reflects tissue mineral contents; (2) carbonate/phosphate ratio, as the region ratio of carbonate ($855\text{--}895\text{ cm}^{-1}$)/phosphate, which characterizes the extent of carbonate substitution into hydroxyapatite (HA) crystals; (3) HA crystallinity, as the intensity ratio of bands ($1,030/1,020\text{ cm}^{-1}$), characterizing crystal size and perfection as assessed by X-ray diffraction [21]; and (4) collagen maturity, as the intensity ratio of bands ($1,660/1,690\text{ cm}^{-1}$), reflecting the maturity of reducible collagen cross-links [22].

2.7. Statistical analyses

All values are presented as mean $\pm$ SD. Statistical analysis was performed using 1-way analysis of variance (ANOVA). Statistical differences among all groups were compared using Scheffe method for multiple comparisons. All statistical analyses were performed using Statistical Package for the Biosciences software (SPSS ver. 9.6; developed by Murata and Yano at our institution) [23]. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Body weight

At euthanasia, vehicle-treated Akita mice showed lower body weight (-11.6% , $P = 0.002$) compared to nondiabetic control mice. On the other hand, the body weights of TPTD-treated control and Akita mice were not significantly different (Table 1).

3.2. Blood glucose and serum sclerostin levels

Blood glucose levels in Akita mice treated with vehicle or TPTD were significantly higher than in the vehicle- or TPTD-treated control groups ($+133.9\%$, $P = 0.001$ and $+81.6\%$, $P = 0.03$, respectively). No significant differences in serum sclerostin level were seen among the 4 groups. In the TPTD-treated groups, serum sclerostin levels tended to be lower in control and Akita mice than in each of the vehicle-treated groups, but the differences were not

Table 1

Body weight, blood glucose levels, and serum sclerostin levels.

Variable	CON + Veh (n = 7)	CON + TPTD (n = 6)	AKM + Veh (n = 6)	AKM + TPTD (n = 7)	ANOVA
Body weight, g	21.5 ± 1.0	21.5 ± 0.8	19.0 ± 1.0 ^a	21.1 ± 1.0 ^b	P < 0.001
Blood glucose, mg/dL	170.3 ± 25.2	162.8 ± 16.1	398.3 ± 129.2 ^c	295.7 ± 63.6 ^d	P < 0.001
Serum sclerostin, pg/mL	303.6 ± 60.9	267.6 ± 23.3	282.3 ± 85.4	274.3 ± 63.2	NS

Values are presented as mean ± standard deviation.

NS, not significant; CON + Veh, control mice treated with vehicle; CON + TPTD, control mice treated with teriparatide (TPTD); AKM + Veh, Akita mice treated with vehicle; AKM + TPTD, Akita mice treated with TPTD; ANOVA, 1-way analysis of variance.

^a P=0.002 vs. CON + Veh.; ^b P=0.008 vs. AKM + Veh.; ^c P=0.001 vs. CON + Veh.; ^d P=0.03 vs. CON + TPTD by Scheffe multiple comparison methods.

significant (Table 1).

3.3. BMD and biomechanical data

Total tibial BMD was significantly lower in vehicle-treated Akita mice than in vehicle-treated control mice (−17.2%, P = 0.02). TPTD treatment significantly increased total tibial BMD compared with that in vehicle-treated control mice (CON + Veh group) (+12.7%, P = 0.02) and Akita mice (AKM + Veh group) (+29.2%, P = 0.001). No significant differences in maximum load from 3-point bending tests were evident between vehicle-treated control mice and Akita mice. TPTD significantly increased maximum load in Akita mice (+56.6%, P = 0.03), but not in control mice, compared with that with vehicle treatment in each group. Stiffness was significantly higher in TPTD-treated groups than in vehicle-treated groups, in both control (+63.0%, P = 0.04) and Akita mice (+90.5%, P = 0.02) (Table 2).

3.4. Fourier-transform infrared spectroscopy imaging

The figures show typical FTIR findings for mineral/matrix ratio (Fig. 1) and collagen maturity (Fig. 2) in cortical bone. The yellow shaded part shows normal calcification or maturity, the green shaded part shows lower calcification or maturity, and the red shaded part shows higher calcification or maturity. With regard to the mineral/matrix ratio, fewer red areas were seen in vehicle-treated Akita mice (AKM + Veh) than in vehicle-treated control mice (CON + Veh) (Fig. 1). On the other hand, no significant difference in collagen maturity was evident between vehicle-treated control mice (CON + Veh) and Akita mice (AKM + Veh) (Fig. 2). In TPTD-treated groups, many red dots were seen in the mineral/matrix ratio when compared to vehicle-treated groups (Fig. 1). On the other hand, TPTD increased green dots, reflecting collagen maturity, in the area from the growth plate to proximally in both control and Akita mice groups (Fig. 2).

The mineral/matrix ratio was significantly lower in vehicle-treated Akita mice than in vehicle-treated control mice (−12.2%, P = 0.02). TPTD treatment significantly increased the mineral/matrix ratio (ANOVA, P = 0.003) (Table 3). TPTD treatment did not significantly increase the mineral/matrix ratio when compared with vehicle-treated groups in both control and Akita mice groups.

No other parameters (carbonate phosphate ratio, HA crystallinity, and collagen maturity) showed significant differences among groups.

4. Discussion

In the present study, BMD, bone strength, and mineral/matrix ratio were lower without significant changes in serum sclerostin levels in Akita mice, which have been used as a model for type 1 DM [24]. These results were consistent with previous studies showing that BMD, bone strength, and bone quality are impaired in type 1 DM [4]. However, serum sclerostin levels in Akita mice did not show significant changes. Several reports have described serum sclerostin levels in type 2 DM patients [25]. Gennari et al. [26] reported that serum sclerostin levels were significantly higher in the type 2 DM group than in the type 1 DM or normal groups. However, no reports have compared serum sclerostin levels in type 1 DM and non-DM, and consensus is therefore lacking regarding serum sclerostin levels in type 1 DM. Sclerostin is known to be secreted from more mature osteocytes, which are deeply embedded osteocytes [27]. In addition, hyperglycemia or deficiency of insulin induces cell apoptosis and decreases bone formation [28]. In view of these mechanisms, the present results suggest that serum sclerostin levels are not increased in Akita mice as a result of accelerated dysfunction or apoptosis of osteoblasts or osteocytes.

The present study evaluated the effects of TPTD on BMD, bone strength, bone quality as measured by FTIR, and serum sclerostin levels in Akita mice as a model of type 1 DM. TPTD therapy increased total tibial BMD in Akita mice. Previous studies have shown that TPTD improved BMD in DM model animals [13,29] and Torii-Leprfa rats [30], and bone metabolism turnover and secretion of parathyroid hormone (PTH) are known to be decreased in DM [31]. In theory, TPTD, which facilitates bone metabolism turnover, is useful for DM-induced osteoporosis.

Serum sclerostin levels were evaluated as an indicator of bone formation. Sclerostin is a glycoprotein secreted from osteocytes, and inhibits bone formation by inhibiting the canonical Wnt/β-catenin signaling pathway [32]. TPTD treatment showed lower serum sclerostin levels in control and Akita mice than in the vehicle-treated group, although the differences were not significant. In general, PTH secretion is known to decrease the expression

Table 2

Bone mineral density (BMD) and biomechanical data.

Variable	CON + Veh (n = 7)	CON + TPTD (n = 6)	AKM + Veh (n = 6)	AKM + TPTD (n = 7)	ANOVA
Total BMD, g/cm ²	0.055 ± 0.003	0.062 ± 0.002 ^a	0.048 ± 0.003 ^a	0.062 ± 0.005 ^b	P < 0.001
Biomechanical data					
Maximum load, N	15.5 ± 3.2	21.1 ± 5.6	12.2 ± 1.8	19.1 ± 3.6 ^c	P = 0.002
Stiffness, N/mm	58.3 ± 15.8	95.0 ± 28.9 ^d	46.1 ± 14.0	87.8 ± 23.2 ^e	P = 0.001

Values are presented as mean ± standard deviation.

CON + Veh, control mice treated with vehicle; CON + TPTD, control mice treated with teriparatide (TPTD); AKM + Veh, Akita mice treated with vehicle; AKM + TPTD, Akita mice treated with TPTD; ANOVA, 1-way analysis of variance.

^a P=0.02 vs. CON + Veh.; ^b P=0.001 vs. AKM + Veh.; ^c P = 0.03 vs. AKM + Veh.; ^d P = 0.04 vs. CON + Veh.; ^e P = 0.02 vs. AKM + Veh by Scheffe multiple comparison method.

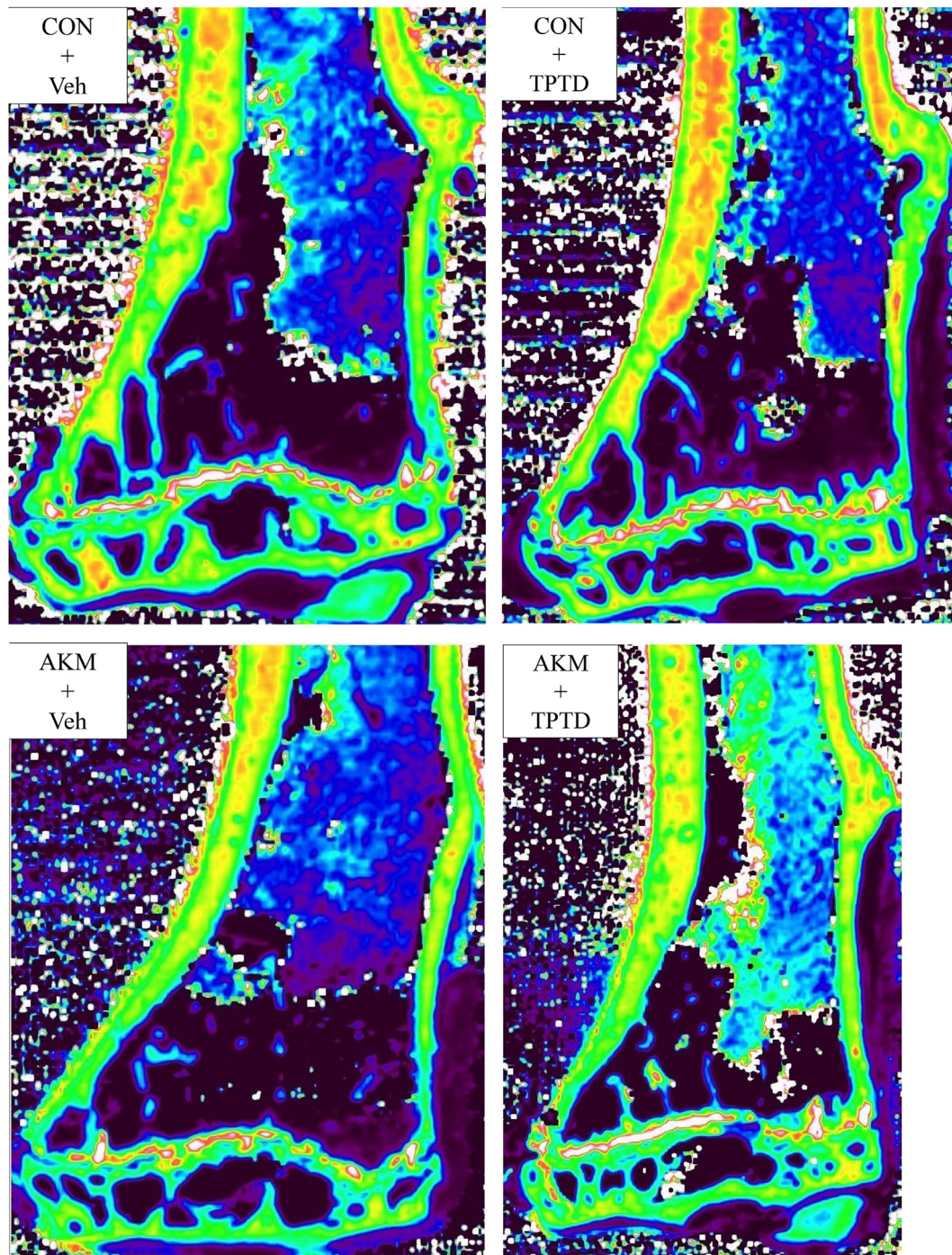


Fig. 1. Typical Fourier-transform infrared images for the mineral/matrix ratio in cortical bone. The yellow shaded part shows normal calcification, green dots show lower calcification, and red dots represent higher calcification of the mineral matrix. In TPTD-treated groups, red dots showed increased frequency compared to the vehicle-treated groups. CON + Veh, control mice treated with vehicle; CON + TPTD, control mice treated with TPTD; AKM + Veh, Akita mice treated with vehicle; AKM + TPTD, Akita mice treated with TPTD.

of sclerostin and increase bone volume [33]. Kousteni and Bilezikian [34] reported that intermittent administration of PTH activates the Wnt- β catenin signal pathway in osteoblasts and promotes the differentiation of osteoblasts. Hisa et al. [35] reported that suppression of sclerostin expression due to transmembrane protein 119 (Tmem 119), which increases β -catenin protein, influenced the acceleration of bone formation by TPTD. Based on the present results and the findings of previous studies, TPTD administration

stimulates bone formation in this animal model of type 1 DM.

In the 3-point bending test, TPTD significantly increased the maximum load in the Akita mice group, but not in the control group. On the other hand, stiffness was significantly higher in TPTD-treated groups than in vehicle-treated groups, in both control and Akita mice groups. Few reports have regarded the effects of TPTD on 3-point bending test results for long bones in DM model animals. Suzuki et al. [13] reported that TPTD significantly

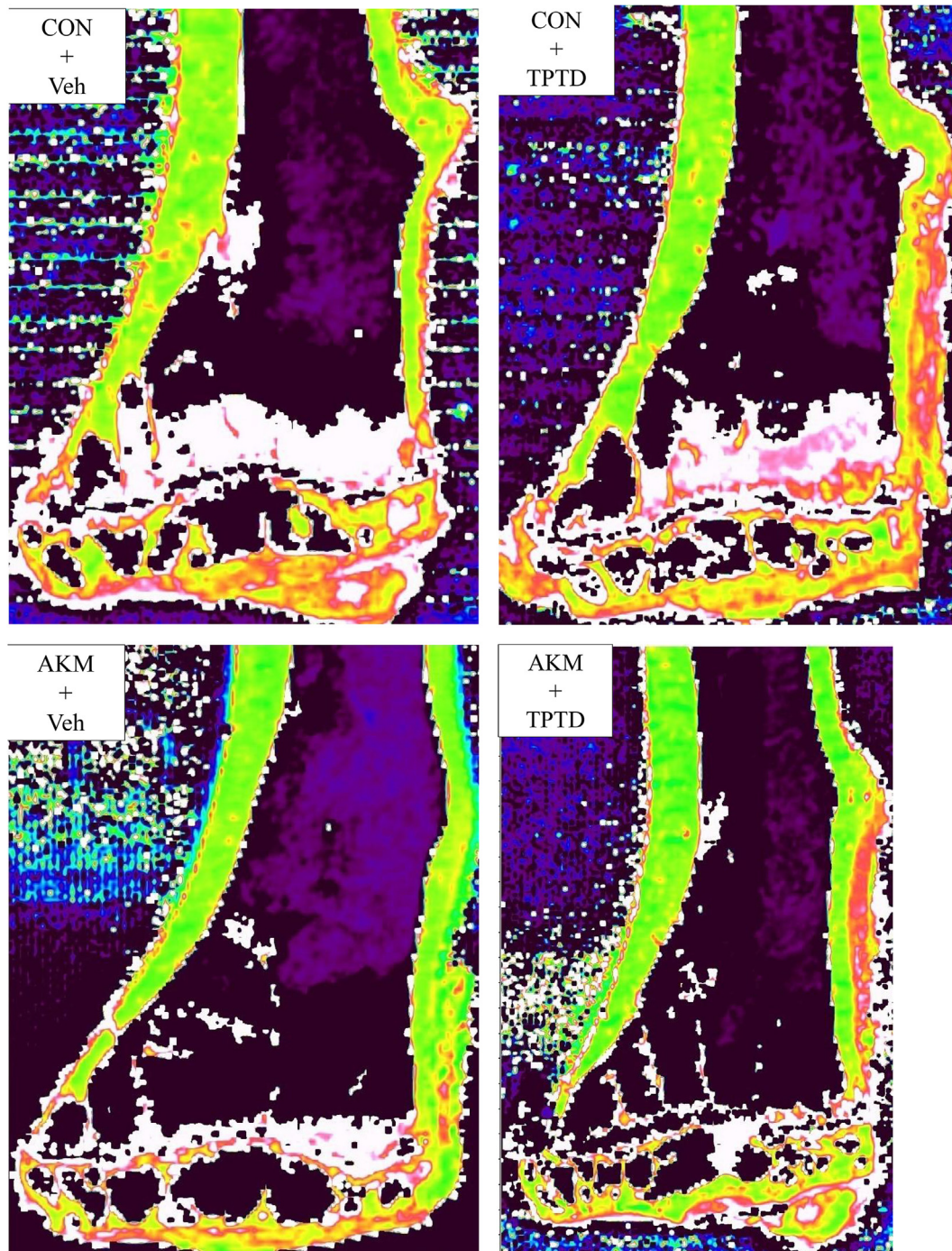


Fig. 2. Typical Fourier-transform infrared images for collagen maturity in cortical bone. The yellow shaded part shows normal maturity, green dots show lower maturity, and red dots represent higher maturity. TPTD increased green dots in the area from the growth plate to more proximally in both control and Akita mice groups. CON + Veh, control mice treated with vehicle; CON + TPTD, control mice treated with TPTD; AKM + Veh, Akita mice treated with vehicle; AKM + TPTD, Akita mice treated with TPTD.

increased the ultimate strength more in STZ rats than in control rats, but not stiffness. TPTD has been shown to improve bone microstructure in postmenopausal osteoporosis model animals and increases bone strength [36,37]. Regarding differences in maximum load and stiffness, Saito et al. [37] reported bone volume as the most important factor related to maximum load, while stiffness was mostly related to enzymatic mature or immature collagen-cross-links, which reflect bone quality. The present results suggest that TPTD improves bone quality much earlier than it increases bone volume.

To evaluate the effects of TPTD on bone quality, the present study used FTIRI analysis. TPTD treatment increased the mineral/matrix ratio compared with vehicle-treated groups, in both control and Akita mice groups, but other parameters (carbonate phosphate ratio, HA crystallinity, and collagen maturity) did not show significant differences among groups. Several reports have evaluated the effects of TPTD on bone quality using FTIRI. Paschalis et al. [38] reported that HA crystallinity and collagen maturity were lower in TPTD-treated groups than in placebo groups among postmenopausal osteoporosis patients. Excess maturity or aging of

Table 3
Fourier-transform infrared spectroscopy imaging values.

Variable	CON + Veh (n = 4)	CON + TPTD (n = 4)	AKM + Veh (n = 4)	AKM + TPTD (n = 4)	ANOVA
Mineral/matrix ratio	6.87 ± 0.23	7.01 ± 0.22	6.03 ± 0.19 ^a	6.66 ± 0.49	P = 0.003
Carbonate/phosphate ratio	0.00718 ± 0.0001	0.00730 ± 0.0001	0.00764 ± 0.0001	0.00769 ± 0.0001	NS
Hydroxyapatite crystallinity	1.11 ± 0.02	1.11 ± 0.01	1.10 ± 0.01	1.11 ± 0.04	NS
Collagen maturity	3.63 ± 0.15	3.54 ± 0.10	3.63 ± 0.13	3.59 ± 0.08	NS

Values are presented as mean ± standard deviation.

CON + Veh, control mice treated with vehicle; CON + TPTD, control mice treated with TPTD; AKM + Veh, Akita mice treated with vehicle; AKM + TPTD, Akita mice treated with TPTD; ANOVA, 1-way analysis of variance; NS, not significant.

^a P = 0.02 vs. CON + Veh by Scheffe multiple comparison method.

enzymatic cross-links has previously been shown as factors resulting in deterioration of bone strength [39], so the results of Paschalis et al. suggest that excess mature cross-links were broken by TPTD administration, and the production of normal mature cross-links increased. The present study found no significant difference in collagen maturity. However, in TPTD-treated groups, green dots, which show low maturity, were increased near the growth plate. This suggests that TPTD promoted the production of new, low-maturity cross-links. On the other hand, FTIR can evaluate only the maturity ratio of enzymatic cross-links. Bone strength has previously been shown to be decreased by increasing advanced glycation end-products (AGEs) due to oxidative stress [40], so quantitative evaluation of AGEs may be significant in DM osteoporosis.

Several limitations must be considered when interpreting the present study. First, histological examinations were not performed due to the limited number of samples. To evaluate the effects of TPTD on the bone microarchitecture in DM, bone morphometry or peripheral quantitative computed tomography might prove useful. Second, blood glucose and serum sclerostin levels were measured only at the endpoint of the protocol.

5. Conclusions

This study demonstrated for the first time the effects of TPTD on BMD, bone strength, and bone quality in Akita mice as a model of type 1 DM. The present results indicate that TPTD significantly improved BMD and bone strength, in both control and Akita mice groups, with improvements in the mineral/matrix ratio among Akita mice.

Author contributions

All authors have read the manuscript and have approved this submission. Study design: KO, NM and YK. Study conduct: KO, HK, CS and MF. Data collection: KO, TS and HK. Data analysis: KO, YK, TS and HK. Data interpretation: KO, NM, YK and HK. Drafting manuscript: KO, NM and YK. Revising manuscript content: NM, YK and YS. Approving final version of manuscript: KO, NM, YK, TS, HK, CS, MF and YS. NM takes responsibility for the integrity of the data analysis.

Conflicts of interest

Naohisa Miyakoshi has received consulting fees from Asahi Kasei Pharma Corporation. The other authors have no conflicts of interest to declare.

Acknowledgments

The authors would like to thank Asahi Kasei Pharma Corporation for providing TPTD and Ms. Matsuzawa for her support of our

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