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Original article

Teriparatide and etelcalcetide improve bone, fibrosis, and fat parameters in chronic kidney disease model rats

Shun Igarashi [a], Yuji Kasukawa [b,*], Koji Nozaka [a], Hiroyuki Tsuchie [a], Kazunobu Abe [a], Hikaru Saito [a], Ryo Shoji [a], Fumihito Kasama [a], Shuntaro Harata [a], Kento Okamoto [a], Keita Oya [a], Naohisa Miyakoshi [a]

[a] Department of Orthopedic Surgery, Akita University Graduate School of Medicine, 1-1-1, Hondo, Akita, 010-8543, Japan
[b] Department of Rehabilitation Medicine, Akita University Hospital, 1-1-1, Hondo, Akita, 010-8543, Japan

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ABSTRACT

Objectives: Chronic kidney disease (CKD) complicated by secondary hyperparathyroidism (SHPT) is associated with an increased risk of fragility fractures. Etelcalcetide (EC) is a treatment for SHPT that reduces serum parathyroid hormone (PTH) levels. However, the effects of combined treatment with osteoporosis drugs such as teriparatide (TPTD) remain unclear. This study investigates the combined effects of EC and TPTD on bone in CKD model rats.

Methods: The CKD model was established in 8-week-old male Wistar rats by feeding them a 0.75% adenine diet for 4 weeks. At 20 weeks of age, the rats were divided into 4 groups (N = 9–10 in each group): CKD group (vehicle administration), TPTD group (30 μg/kg, 3 times/week), EC group (0.6 mg/kg, daily), and Comb group (TPTD and EC combined). EC was injected for 12 weeks starting at 20 weeks of age, and TPTD was injected for 8 weeks starting at 24 weeks of age. After treatment, the followings were evaluated: bone mineral density, bone strength, biochemical tests, bone and fat histomorphometry, and micro-computed tomography.

Results: In CKD model rats, the combination of EC and TPTD was more effective in increasing cortical bone thickness and bone strength and inhibiting porosity. In addition, the combined treatment decreased bone marrow adiposity and fibrosis, and it increased bone mass and improved bone microstructure in trabecular bone.

Conclusions: With the observed benefits such as improved bone mass, bone strength, structural properties, and bone marrow adiposity, combination therapy may be a potential way to improve bone fragility in CKD.

1. Introduction

The number of patients with osteoporosis and chronic kidney disease (CKD) is increasing as the population ages, and there is a high rate of the 2 occurring together [1,2]. The risk of fragility fractures in patients with CKD has been reported to be considerably higher than in the general population, [3]. Since CKD patients have a higher incidence of fractures than predicted by bone mineral density (BMD), the increased risk of fracture in CKD patients is attributed to abnormalities in bone quality in addition to bone loss [4,5]. It has been reported that the incidence of fracture is higher when the disease progresses to stage 4 or higher compared to CKD stage 3 or lower [6]. Effective treatments of bone fragility associated with CKD are therefore essential to maintain activities of daily living (ADL) and quality of life (QoL), particularly in advanced-stage CKD and dialysis patients.

CKD-mineral bone disorder (CKD-MBD), one of the factors associated with bone fragility in CKD, is a clinical manifestation of the triad of biochemical abnormalities, bone loss, and vascular calcification due to prolonged and sustained decline in renal function [7]. In early stages (stages 2–3) of CKD, phosphodiesis by fibroblast growth factor 23 (FGF23) and suppression of 1, 25-hydroxylated vitamin D lead to stimulation of secondary hyperparathyroidism (SHPT) [8,9], which compensates for the phosphorus overload caused by CKD. When the compensatory mechanism fails in stage 4–5 CKD, serum phosphorus levels increase, and serum calcium (Ca) levels decrease to maintain the balance between Ca and phosphorus, both of which act to further

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* Corresponding author.
E-mail address: kasukawa@doc.med.akita-u.ac.jp (Y. Kasukawa).

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promote SHPT. In addition, bone lesions in CKD include osteitis fibrosa due to SHPT, osteomalacia due to vitamin D deficiency, and a mixture of both, as well as aplastic osteopathy [7]. SHPT promotes bone resorption and bone formation by increasing the continuous secretion of PTH, with a higher degree of bone resorption, decreased bone mass, and increased fibrous components [10]. Particularly in cortical bone, increased bone resorption leads to deterioration of bone structure, such as porosity, leading to increased fracture risk in CKD [4,7].

Therefore, it is very important to improve SHPT in patients with CKD. Recently, peptide agonists of the calcium-sensing receptor (CaSR), known as calcimimetics, have been used to treat SHPT in dialysis patients [11,12]. The calcimimetics suppress PTH synthesis and secretion by acting on the CaSR, which becomes dysfunctional in SHPT. The calcimimetics have recently improved the outcome of CKD-MBD in patients treated with hemodialysis [13,14]. Etelcalcetide (EC) and cinacalcet are typical calcimimetics. EC suppresses PTH secretion more persistently than cinacalcet, and because it is administered intravenously, it is expected to improve compliance and gastrointestinal tolerability compared to cinacalcet [15]. However, because CKD patients often present with a variety of bone metabolic pathologies, there is no certainty regarding the effect of calcimimetics on fracture inhibition [16]. For these reasons, the need for combined treatment of osteoporosis in addition to CKD-MBD management has recently been identified for the appropriate management of bone lesions in advanced-stage CKD and dialysis patients [17].

However, there is insufficient evidence on the safety and efficacy of pharmacological treatment of osteoporosis in patients with advanced CKD. Although teriparatide (TPTD) treatment, involving intermittent administration of PTH, can increase trabecular bone mass and inhibit new vertebral fractures [18], further administration of PTH with TPTD is discouraged in the case of SHPT, in which endogenous PTH secretion is increased. Based on these background factors, there are few reports on the efficacy of TPTD in CKD and dialysis patients, and the detailed efficacy of TPTD for fracture suppression in advanced-stage CKD has not been clarified. In other words, the safety and efficacy of TPTD in SHPT patients, whose PTH secretion is controlled by EC, are not fully understood. In this study, the hypothesis is that TPTD for advanced-stage CKD, in which PTH secretion is suppressed by EC, has more positive effects on bone, such as increased bone mass and improved bone microstructure, in addition to improving bone metabolic abnormalities associated with CKD-MBD, without adversely affecting mineral metabolism or renal function. Therefore, the purpose of this study is to evaluate the safety and effects of EC in combination with TPTD on bone in a rat model of adenine-induced advanced-stage CKD.

2. Methods

2.1. Animal model and experimental design

Eight-week-old, male Wistar rats (N = 9–10) (Charles River Laboratories Inc., Tokyo, Japan) were housed in a controlled environment (temperature 23 ± 2°C, humidity 40 ± 20%) with a 12-hour light-dark cycle with free access to water and rat food. The details were described in previous studies and followed for the selection of rat species and sex [19]. Rats were treated with a 0.75% adenine diet (Oriental Yeast Co., Ltd., Tokyo Japan) for 4 weeks until 12 weeks of age, followed by a standard rodent chow (CE-7; Clea Japan, Tokyo, Japan) diet to generate CKD model rats (CKD group). The 4-week treatment with the adenine diet was decided based on previous studies [19,20], which reported that 4-week treatment with the adenine diet induced non-progressive, irreversible renal failure.

At 20 weeks of age, the rats were divided into 4 groups: CKD group (vehicle administration), TPTD group (30 μg/kg subcutaneous injection of teriparatide (TPTD), 3 times/week), EC group (0.6 mg/kg subcutaneous injection of EC daily), and Comb group (TPTD and EC combined) (Fig. 1). Vehicle or EC was injected for 12 weeks from 20 weeks of age in the CKD group or the EC group, respectively, and TPTD was injected for 8 weeks from 24 weeks of age with prior administration of vehicle for 4 weeks from 20 weeks of age in the TPTD group. The Comb group was treated with EC for 4 weeks from 20 weeks of age and with EC and TPTD for 8 weeks from 24 weeks of age. The non-CKD control (Cont group) was also included without the adenine diet and treated with vehicle for 12 weeks from 20 weeks of age. The protocols for all animal experiments were approved in advance by the Animal Experimentation Committee of our institute (permit No. a-1-0271), and all subsequent animal experiments adhered to the Guidelines for Animal Experimentation of our institution.

2.2. Body weight measurement

Body weight (BW) was measured weekly from the beginning (20 weeks of age) until the end of the experiment.

2.3. Administration of TPTD and EC

TPTD (teriparatide acetate, LKT LABS, St. Paul, MN, USA) was dissolved in saline containing 0.1% rat serum albumin, and a dose of 30 μg/kg of BW was administered subcutaneously 3 times per week for 8 weeks according to a previously reported protocol [21,22]. EC (etelcalcetide hydrochloride) was provided by ONO Pharmaceutical, Co. Ltd. (Osaka, Japan). EC was dissolved in vehicle (1 mass/volume percent (m/v%) glycine, 1 m/v% d (+)-trehalose dihydrate, 0.27 m/v% disodium succinate hexahydrate, 2 m/v% d-mannitol, 0.9 m/v% benzyl alcohol, pH 4.5), and a dose of 0.6 mg/kg of BW was administered subcutaneously daily for 12 weeks based on a previously reported protocol [23].

2.4. Tissue preparation

After sacrifice, the right femur was fixed in 10% neutral-buffered formalin (Wako Chemical Industries, Osaka, Japan) until preparation for BMD measurement and micro-computed tomography (CT) measurements. The left femur was dissected free of soft tissue, wrapped in

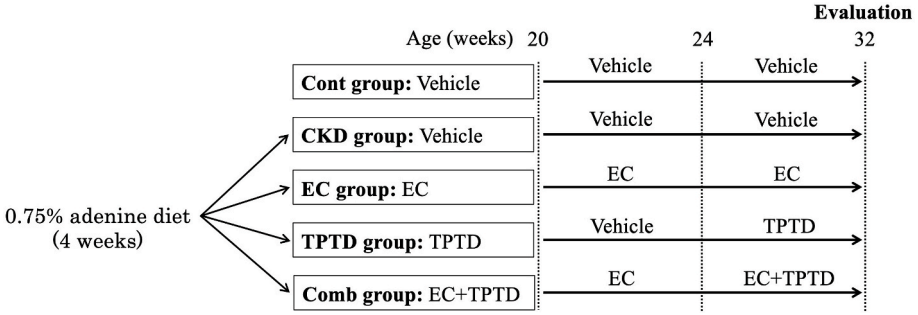

Fig. 1. Experimental protocol. Cont: non-CKD control rats, CKD: CKD rats administered vehicle, EC: CKD rats administered etelcalcetide, TPTD: CKD rats administered teriparatide, Comb: CKD rats administered EC and TPTD.

gauze moistened with saline, and frozen at −80°C until biomechanical testing. The bilateral proximal half of the tibia was decalcified with neutral 10% ethylene diamine tetraacetic acid for approximately 4 weeks and then embedded in paraffin. Subsequently, 3-μm-thick, mid-frontal slices were sectioned and stained with hematoxylin and eosin for bone histomorphometry.

2.5. Serum chemistry

At 32 weeks of age, blood samples were collected from the vena cava of rats 24–36 h after administration of EC. Blood samples were centrifuged at 3000 rpm for 30 minutes at 4°C to separate the serum, which was then stored in disposable aliquots at −80°C until analysis. Blood urea nitrogen (BUN), creatinine (CRE), calcium (Ca), and inorganic phosphorus (IP) levels were measured using a Hitachi Automatic Biochemical Analyzer 7180 (Hitachi Ltd., Tokyo, Japan). Intact-parathyroid hormone (I-PTH) levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Rat Intact PTH ELISA Kit, Immutopics, Inc. San Clemente, CA, USA).

2.6. Bone histomorphometry

Bone histomorphometric analysis at the proximal tibia was performed at 200 × magnification using a semi-automated graphic system (Histometry RT CAMERA; System Supply, Nagano, Japan). Measurements were obtained 400 μm caudally from the lowest point of the growth plate and 100 μm medially from the endosteal surface. Bone histomorphometric parameters were calculated and expressed according to published methods [24]. The standard parameters evaluated were bone volume (BV/TV, %), osteoid surface (OS/BS, %), eroded surface (ES/BS, %), and fibrosis volume (Fb.V/TV, %).

2.7. Fat histomorphometry

Adipocyte volume per total bone marrow volume (Ad.V/Ma.V, %), number of adipocytes per unit area of marrow (N.Ad/Ma.V, number of cells/mm^2), and volume of each adipocyte per number of adipocytes (Ad.V/N.Ad, μm^2) were evaluated as parameters of fat histomorphometry in the bone marrow of the proximal tibia, as described previously [21,25,26]. The measurement range of the fat histomorphometry was the same as that of the bone histomorphometry.

2.8. Micro-computed tomography analysis

The excised right femur was secured in a specimen holder. Micro-CT was performed using the Cosmo Scan GX II (Rigaku Corporation, Tokyo, Japan) according to the manufacturer's instructions with an isotropic voxel size of 36 μm, energy of 90 kVp, and current of 88 μA. The acquired images were rendered using TRI/3D BON software (Ratoc System Engineering Co., Ltd., Tokyo, Japan). Osteoporosis assessment was performed based on total area (Tt.Ar, mm^2), cortical area (Co.Ar, mm^2), cortical volume (Ct.V, mm^3), cortical area/total area (Ct.Ar/Tt.Ar, %), cortical thickness (Ct.Th, mm), and cortical porosity (Co.Po, %) at the mid femur and bone volume/tissue volume (BV/TV, %), trabecular thickness (Tb.Th, mm), trabecular number (Tb.N, 1/mm), trabecular separation (Tb.Sp, mm), structure model index (SMI), and degree of anisotropy (DA) at the distal femur. The measurement range of the mid femur was a 1000-μm-high region centered on the midpoint of the entire femur. The measurement range of the distal femur was 2000 μm cranially from 500 μm from the reference line connecting the two ends of the growth plate of the sagittal image.

2.9. BMD measurement

BMD of the femur was measured using dual-energy X-ray absorptiometry (Horizon® DXA System; Hologic, Bedford, MA, USA). Each region was scanned in "small animal" mode, with the "regional high resolution" scan option. Femoral BMD was measured at the proximal, middle, and distal thirds of the femur.

2.10. Biomechanical testing

Mechanical testing of the left femoral shaft was performed at room temperature using a material testing machine (MZ500S; Maruto, Tokyo, Japan). The mid-diaphysis of the femur was stabilized by placing it on 2 supports of the test apparatus placed 18 mm apart. The load of a three-point bending test was applied in the anteroposterior direction midway between the 2 supports. Load–displacement curves were recorded at a crosshead speed of 5 mm/minute. The maximum load (N), stiffness (N/mm), breaking energy (N mm), and breaking time (s) were calculated using software for measuring bone strength (CTR win. Version 1.05; System Supply, Nagano, Japan), as described previously [27]. In addition, the elastic modulus (GPa), as a tissue-level mechanical property, was calculated using the formula reported previously [28]. Following the 3-point bending test, the distal part of the femur was evaluated using a compression test, as previously described [29]. Load–displacement curves were recorded, and the maximum load (N), stiffness (N/mm), and breaking energy (N mm) were calculated using the same software.

2.11. Statistical analysis

All data are expressed as means ± standard deviation (SD). A Kolmogorov-Smirnov test showed that all data were normally distributed. The sample size for each group is N = 9–10. Student's *t* test was used to compare data between the Cont and CKD groups. The differences between CKD groups such as CKD, EC, TPTD, and Comb groups were evaluated by one-way analysis of variance (ANOVA) and analyzed by Tukey's multiple comparison test as a post hoc test. All statistical analyses were performed with EZR [30], which is a modified version of R commander designed to add statistical functions frequently used in biostatistics. Values of $P < 0.05$ values were considered significant.

3. Results

3.1. Body weight and biochemistry

To evaluate the health status of the animals, BW and its changes during the experiment were measured. The results for BW are shown in Table 1. At the beginning of treatment, the CKD group weighed significantly less than the Cont group ($P < 0.01$), but there were no significant differences among the CKD, TPTD, EC, and Comb groups. At the end of the experiment, BW and weight gain were significantly lower in the EC and Comb groups than in the CKD and TPTD groups ($P < 0.05$ to $P < 0.01$).

In addition, blood biochemical tests were performed to evaluate the effects of EC and TPTD at 32 weeks of age. The results for serum biochemical markers are shown in Table 1. Compared with Cont rats, serum BUN and CRE were significantly higher in the CKD group ($P < 0.01$), reflecting the deterioration of renal function, but EC and TPTD had no significant effect on serum BUN and CRE. EC decreased serum Ca compared to the CKD and TPTD groups ($P < 0.01$). Compared with Cont group rats, I-PTH levels were significantly higher in CKD group rats ($P < 0.01$). Serum I-PTH levels in EC group and Comb group rats were significantly lower compared with CKD group rats ($P < 0.05$).

3.2. Bone and fat histomorphometry

To evaluate the changes in trabecular bone and adipose tissue by the adenine-induced CKD model and treatment with EC and/or TPTD, bone and fat histomorphometry was performed on histologic sections of the proximal tibia. The histological sections are shown in Fig. 2. At lower magnification, trabecular bone volume was decreased in CKD rats

Table 1
Body weight and serum biochemical markers.

	Cont	CKD	EC	TPTD	Comb	ANOVA
	N = 10	N = 10	N = 9	N = 9	N = 10	P-value
Body weight (g)						
Baseline	609.3 ± 34.8	513.9 ± 29.4[a']	490.7 ± 19.8	514.1 ± 24.2	484.5 ± 33.4	< 0.001
Endpoint	704.1 ± 34.8	603.9 ± 33.5[a']	536.9 ± 33.2[b']	594 ± 37.5[c']	532.8 ± 34.8[b'd]	< 0.001
Body weight change (%)	15.6 ± 2.9	17.5 ± 2.6	9.4 ± 3.6[b']	16.1 ± 4.1[c']	11.2 ± 2.1[b'd']	< 0.001
BUN (mg/dL)	20.7 ± 2.5	36.7 ± 8.2[a']	30.6 ± 3.4	36.8 ± 9.5	35.7 ± 7.4	0.257
CRE (mg/dL)	0.33 ± 0.03	0.67 ± 0.16[a']	0.60 ± 0.08	0.68 ± 0.20	0.56 ± 0.08	0.203
Ca (mg/dL)	11.4 ± 0.4	11.2 ± 0.4	10.5 ± 0.3[b']	11.3 ± 0.4[c']	10.8 ± 0.5	0.002
IP (mg/dL)	7.6 ± 1.2	7.4 ± 0.7	6.5 ± 0.7	6.9 ± 0.9	7.4 ± 1.3	0.143
I-PTH (pg/mL)	655.0 ± 294.9	1845.3 ± 587.4[a']	1098.9 ± 254.9[b]	1420.3 ± 822.3	1129.6 ± 378.6[b]	0.002

Values are presented as means ± standard deviation. N = 9–10 in each group.
Cont: non-CKD control rats, CKD: CKD rats administered vehicle, EC: CKD rats administered etelcalcetide, TPTD: CKD rats administered teriparatide, Comb: CKD rats administered EC and TPTD, Bodyweight change: percentage change in body weight in each group from baseline, BUN: blood urea nitrogen, CRE: creatinine, Ca: calcium, IP: inorganic phosphorus, I-PTH: intact parathyroid hormone.
[a'] $P < 0.01$ vs Cont group (Student's *t*-test).
[b] $P < 0.05$, [b'] $P < 0.01$ vs CKD group, [c'] $P < 0.01$ vs EC group, [d] $P < 0.05$, [d'] $P < 0.01$ vs TPTD group (Tukey's multiple comparison test).

compared to control rats (Cont group), and trabecular bone volume was restored in TPTD and Comb rats, but not in EC rats (Fig. 2A). At higher magnification, adipose tissue volume was increased in CKD and EC compared with Cont and decreased in TPTD and Comb groups, and fibrosis volume was increased in CKD and TPTD compared with Cont and decreased in EC and Comb groups (Fig. 2B).

CKD rats showed increased bone formation (OS/BS) (Fig. 2D), bone resorption (ES/BS) (Fig. 2E), and fibrous changes (Fb.V/TV) (Fig. 2F), as well as bone marrow lipogenesis (Ad.V/Ma.V and N.Ad/Ma.V) (Fig. 2G, I), compared with control rats (Cont group) ($P < 0.05$ to $P < 0.01$). In addition to the changes in bone turnover, the increased bone marrow adiposity and fibrosis resulted in decreased trabecular bone volume (BV/TV) (Fig. 2C) in CKD rats.

EC treatment significantly decreased bone formation (OS/BS) (Fig. 2D), bone resorption (ES/BS) (Fig. 2E), and fibrous change (Fb.V/TV) (Fig. 2F) parameters compared with those in CKD rats ($P < 0.01$). TPTD significantly decreased bone resorption parameters (ES/BS) (Fig. 2E) resulting in increased trabecular bone volume (BV/TV) (Fig. 2C) compared with CKD rats ($P < 0.05$ to $P < 0.01$). Although TPTD did not change the fibrous parameter (Fb.V/TV) (Fig. 2F), TPTD significantly decreased the adipose parameters (Ad.V/Ma.V and Ad.V/N.Ad) (Fig. 2G and H) compared with those in the CKD group ($P < 0.01$). Combination therapy with EC and TPTD significantly reduced bone marrow fibrosis (Fb.V/TV) (Fig. 2F) compared with CKD rats and TPTD alone ($P < 0.05$ to $P < 0.01$), significantly increased bone formation (OS/BS) (Fig. 2D) compared with EC alone ($P < 0.01$), and significantly increased bone volume (BV/TV) (Fig. 2C) compared with CKD rats and EC and TPTD alone ($P < 0.05$ to $P < 0.01$). In addition, the combination of EC and TPTD significantly suppressed bone marrow lipogenesis (Ad.V/Ma.V and Ad.V/N.Ad) (Fig. 2G and H) compared to CKD rats and EC alone, indicating a combined effect of these 2 treatments. Bone resorption parameters and fibrosis were significantly lower in the Comb group than in the CKD group, and bone formation parameters were preserved, suggesting that the metabolic abnormalities caused by fibrotic osteitis were improved.

3.3. Effects of combination therapy on micro-CT cortical bone parameters

Horizontal micro-CT images of the femoral diaphysis showed that rats in the CKD group had an enlarged medullary cavity and increased cortical porosity compared with normal rats in the Cont group. In addition, treatment with EC alone and the combination of EC and TPTD resulted in a decrease in cortical porosity, an increase in cortical bone thickness and an improvement in the shape of the medullary cavity in the CKD rats (Fig. 3A).

Analysis of the cortical bone of the femoral diaphysis by micro-CT showed a significant decrease in Ct.Ar/Tt.Ar (Fig. 3E) and a significant increase in Ct.Po (Fig. 3G) in rats in the CKD group compared with normal rats (Cont group). Ct.Ar/Tt.Ar was significantly increased ($P < 0.05$) (Fig. 3E), and Ct.Po was significantly decreased ($P < 0.01$) (Fig. 3G) in rats in the EC group compared with rats in the CKD group, indicating that increased bone resorption resulted in relative cortical bone thickness and porosity. Ct.Th in rats in the Comb group was significantly increased ($P < 0.01$) compared to rats in the CKD group (Fig. 3F), reflecting the finding that EC corrected PTH hypersecretion, thereby reducing the increase in bone resorption. Ct.Ar/Tt.Ar was significantly increased ($P < 0.05$ to $P < 0.01$), and Ct.Po was significantly decreased ($P < 0.05$ to $P < 0.01$) in Comb group rats compared to CKD and TPTD group rats, respectively (Fig. 3E, G), confirming an enhanced effect of the combination.

3.4. Effects of combination therapy on micro-CT trabecular bone parameters

Micro-CT horizontal section images of the distal femoral metaphysis showed that the trabecular bone microstructure was degraded in the CKD group compared with the Cont group, whereas the TPTD and Comb groups showed improved microstructure compared with the CKD group (Fig. 4A). Micro-CT analysis of the trabecular bone of the distal femur showed significantly reduced BV/TV ($P < 0.05$) (Fig. 4B) and Tb./N ($P < 0.001$) (Fig. 4D) and significantly increased Tb.Sp ($P < 0.01$) (Fig. 4E) and DA ($P < 0.05$) (Fig. 4G) in CKD group rats compared to normal rats (Cont group). Reduction in bone mass, rarefaction, and deterioration in structural properties were observed in the trabecular bone of CKD rats. BV/TV (Fig. 4B) and Tb.N (Fig. 4D) were significantly increased, and SMI (Fig. 4F) was significantly decreased in the TPTD and Comb groups compared to the CKD and EC groups ($P < 0.05$ to $P < 0.01$). Tb.Sp was significantly increased in the TPTD ($P < 0.01$) and Comb ($P < 0.01$) groups compared with the CKD group (Fig. 4E). Tb.Th was significantly increased in the Comb group compared with the CKD and EC groups ($P < 0.01$) (Fig. 4C). DA was significantly lower only in the Comb group than in the CKD group ($P < 0.01$) (Fig. 4G), confirming the enhanced effect of combined treatment on structural characteristics.

3.5. Effects of combination therapy on bone mineral density and bone strength

The results for femoral BMD are shown in Table 2. Compared with normal rats (Cont group), the BMDs of the whole femur and each region were significantly reduced in rats of the CKD group ($P < 0.05$ to $P < 0.01$). Compared with the CKD group, the TPTD and Cont groups showed significantly increased BMD of the whole femur, proximal and

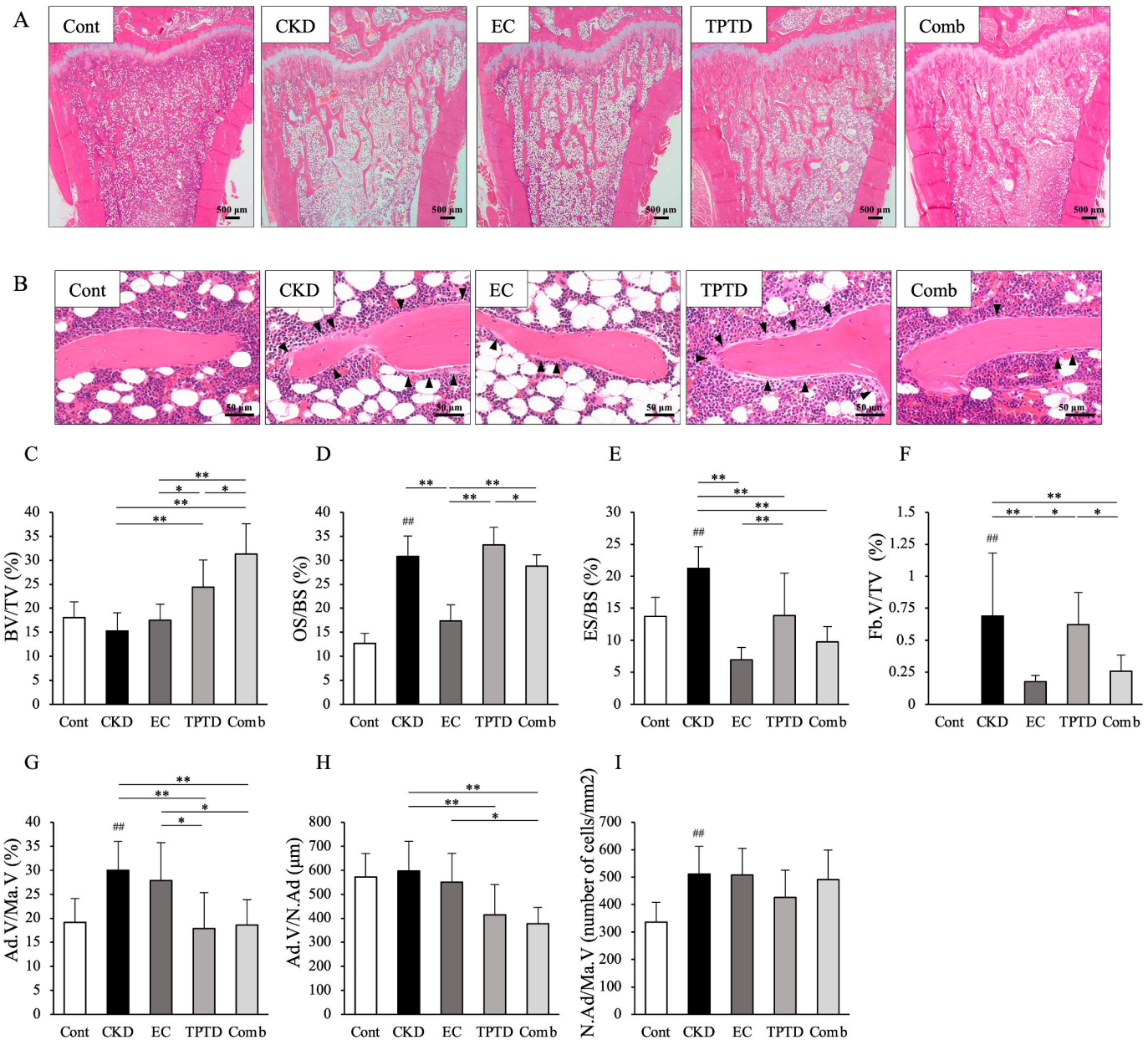

Fig. 2. Histological section of right proximal tibia stained with hematoxylin and eosin in the Cont, CKD, EC, TPTD, and Comb groups with 10 × magnification (A) and 200 × magnification (B). The black arrowheads in the images indicate peritrabecular fibrosis. (C) Bone volume (BV/TV), (D) osteoid surface (OS/BS), (E) eroded surface (ES/BS), (F) fibrosis volume (Fb.V/TV), (G) adipocyte volume per total bone marrow volume (Ad.V/Ma.V), (H) volume of each adipocyte per number of adipocytes (Ad.V/N.Ad), (I) number of adipocytes per unit area of marrow (N.Ad/Ma.V). Values represent the means ± SD (N = 9–10 in each group). ##: $P < 0.01$ vs Cont group (Student's *t*-test). *: $P < 0.05$, **: $P < 0.01$ (Tukey's multiple comparison test). Cont: non-CKD control rats, CKD: CKD rats administered vehicle, EC: CKD rats administered etelcalcetide, TPTD: CKD rats administered teriparatide, Comb: CKD rats administered EC and TPTD.

distal bone, but no significant changes in the cortical bone-dominated diaphysis.

Representative stress-strain curves from the three-point bending test are shown in Fig. 5A. In the 3-point bending test, the CKD group had significantly lower maximum load (Fig. 5B), breaking energy (Fig. 5D), and breaking time (Fig. 5E) compared to the Cont group ($P < 0.05$ to $P < 0.01$). The maximum load (Fig. 5B) was significantly higher in the TPTD and Comb groups than in the CKD group, and the breaking energy (Fig. 5D) and breaking time (Fig. 5E) were also significantly higher in the TPTD and Comb groups than in the CKD and EC groups, confirming an improved effect. Tissue-level osteomechanical properties assessed by the elastic modulus were significantly impaired in the CKD model rats (CKD group) compared to normal rats ($P < 0.05$) (Fig. 5F).

In the compression test at the distal metaphysis of the femur, the CKD group did not have decreased bone strength as assessed by maximum load (Fig. 5G), stiffness (Fig. 5H), and breaking energy (Fig. 5I) compared with those in the Cont group. TPTD and Comb treatment, but not EC treatment, significantly increased the maximum load (Fig. 5G) and breaking energy (Fig. 5I) compared with those in the CKD and EC groups ($P < 0.01$).

4. Discussion

The combined effects of EC and TPTD on osteoporosis and CKD-MBD due to CKD have not been adequately studied. To the best of our knowledge, the present study is the first to report them in a rat model of adenine-induced CKD. The combination of EC and TPTD more effectively increased cortical bone thickness and cortical bone strength,

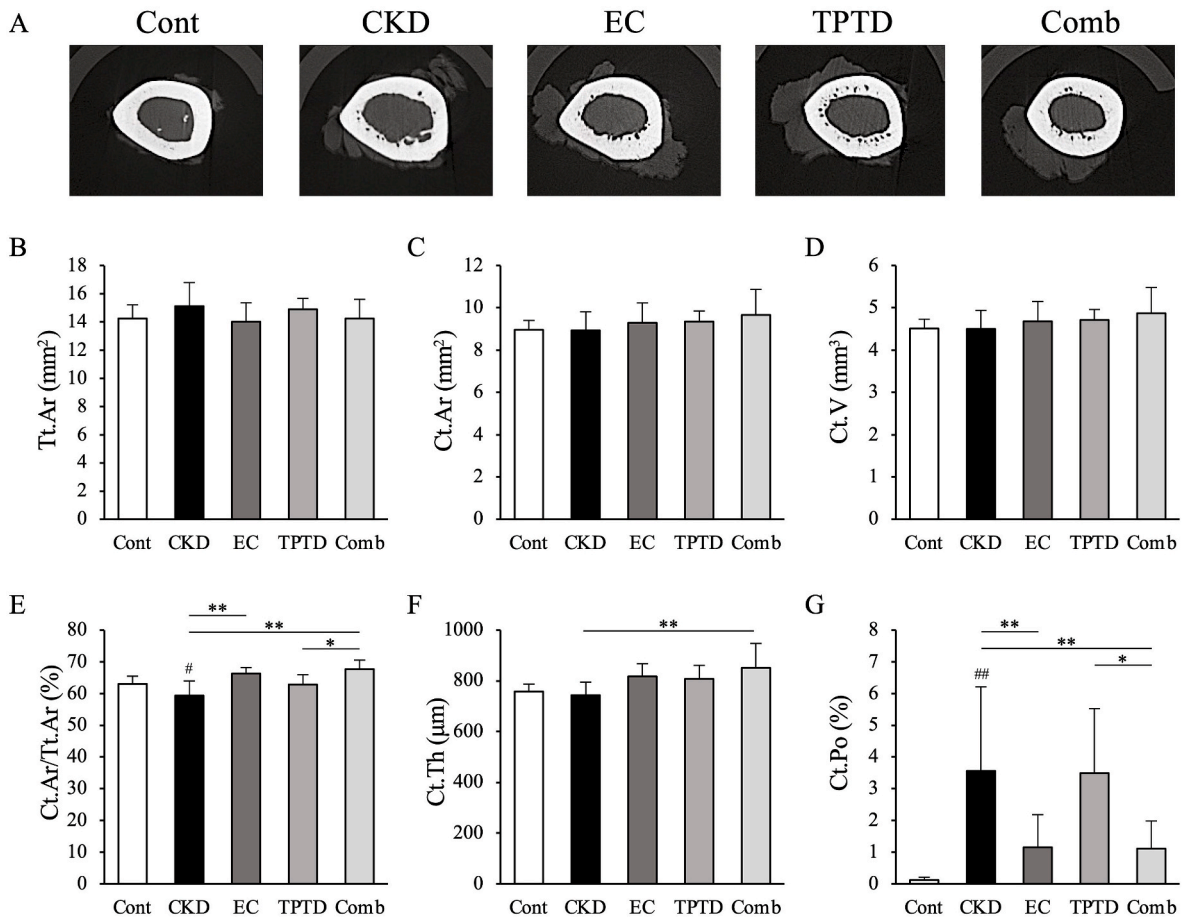

Fig. 3. (A) Representative micro-CT images of cortical bone transverse sections from each group. (B) Total area (Tt.Ar), (C) cortical area (Ct.Ar), (D) cortical volume (Ct.V), (E) cortical area/total area (Ct.Ar/Tt.Ar), (F) cortical thickness (Ct.Th), and (G) cortical porosity (Ct.Po). The measurement range of the mid femur was a 1000-μm-high region centered on the midpoint of the entire femur. Values represent the means ± SD (N = 9–10 in each group). #: $P < 0.05$, ##: $P < 0.01$ vs Cont group (Student's *t* test). *: $P < 0.05$, **: $P < 0.01$ (Tukey's multiple comparison test). Cont: non-CKD control rats, CKD: CKD rats administered vehicle, EC: CKD rats administered etelcalcetide, TPTD: CKD rats administered teriparatide, Comb: CKD rats administered EC and TPTD.

decreased cortical porosity, and reduced bone marrow adiposity and trabecular bone fibrosis and deterioration of trabecular bone structural properties without apparent adverse effects on renal function or mineral metabolism.

We have previously reported that this CKD model presents with increased serum levels of intact-PTH (I-PTH) and renal fibrosis, as stage IV of CKD and SHPT, and decreased systemic BMD, cortical bone microstructure, and cortical and trabecular bone strength [19]. Similar results were observed in the CKD rats in the present study. In addition, further micro-CT analysis showed increased cortical porosity in cortical bone and decreased bone mass, progressive osteoporosis, and increased anisotropy in trabecular bone. In the present study, the endpoint was evaluated at 32 weeks of age, and the progression of bone lesions due to CKD may have been more strongly observed. In the trabecular bone of the proximal tibia, increased bone marrow adiposity and fibrosis and high bone-turnover were observed.

In the present study, EC administration significantly reduced bone formation parameters, bone resorption parameters, and bone marrow fibrosis. In cortical bone, the inhibition of bone resorption resulted in increased cortical bone thickness and decreased cortical bone porosity on micro-CT analysis. However, the bone strength of both cortical and trabecular bone was not restored by EC treatment in the adenine-induced CKD rats. This may be due to the fact that porosity still remained to some extent with EC treatment, and its effect was considered to be mainly inhibition of the development and progression of new porosity in CKD. It has also been reported that EC was effective in inhibiting the development and progression of cortical bone porosity, but it was not effective in replenishing the existing porosity [13]. In SHPT, the risk of fracture was reported to be higher in cases of severely elevated serum I-PTH levels above 900 pg/mL [31]. A strong correlation has been observed between the severity of SHPT and the degree of cortical bone deterioration [23], and cortical porosity and thinning progress rapidly in CKD rats between 30 and 35 weeks of age [32]. Therefore, SHPT should be treated by calcimimetics. It would be possible to prevent cortical bone loss if EC could be administered from the early stages of CKD when PTH secretion begins to increase, but calcimimetics including EC have not been approved for the treatment of SHPT in the early stages of CKD [33]. Furthermore, due to the wide variety of pathologies associated with fracture risk factors in CKD and hemodialysis patients, a clear fracture-preventive effect of EC treatment alone cannot be expected. Therefore, Parfrey et al [13] reported that EC should be used in combination with osteoporosis medications such as bone resorption inhibitors or bone formation stimulators to maintain bone mass and replenish porosity. For these reasons, combination therapy with drugs for osteoporosis is needed to improve cortical bone porosity and improve bone strength to prevent fractures in addition to the EC treatment for SHPT.

TPTD significantly improved bone strength in cortical bone and increased BMD, reduced bone marrow adiposity, improved structural properties, and improved bone strength in trabecular bone. Although previous clinical studies have reported that TPTD was effective in postmenopausal osteoporosis in patients with mild or moderate renal

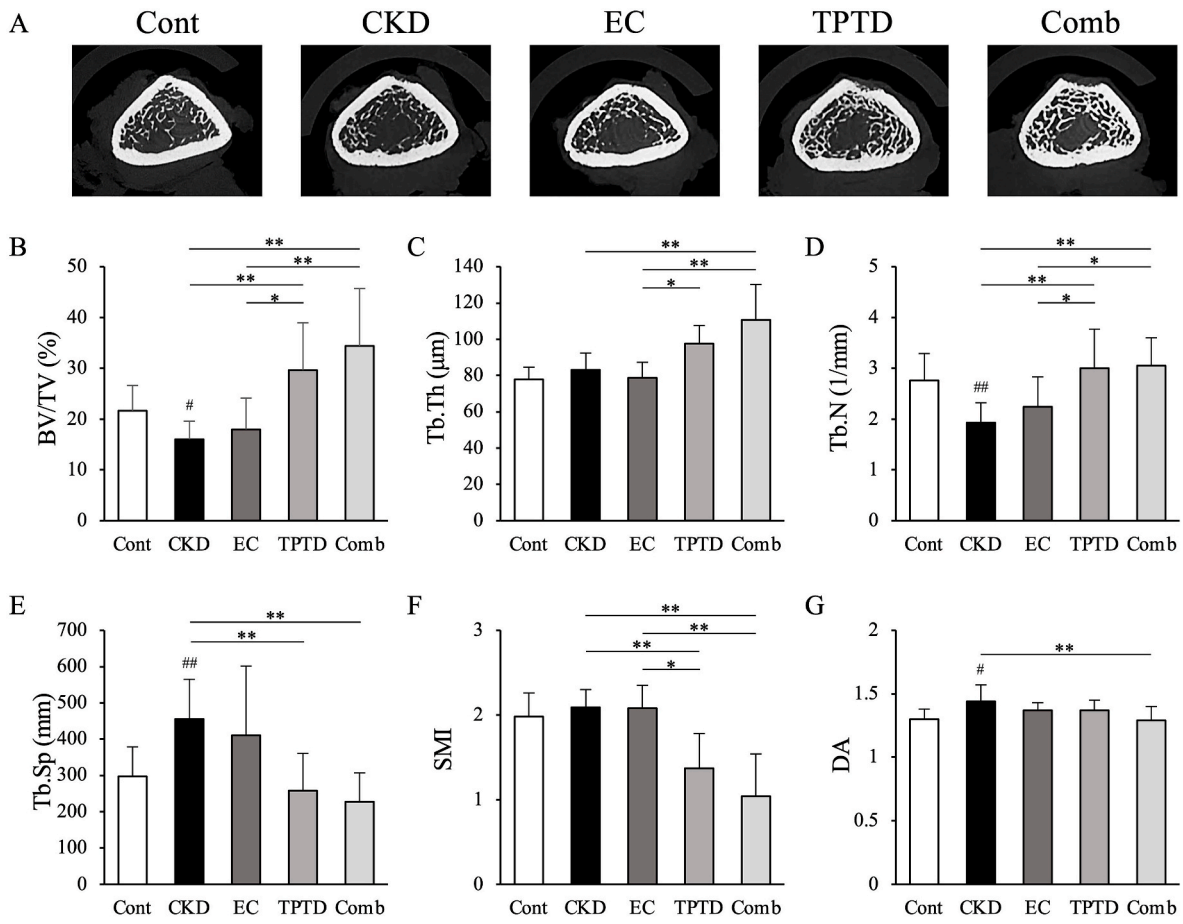

Fig. 4. (A) Representative micro-CT images of trabecular bone transverse sections from each group. (B) Bone volume (BV/TV), (C) trabecular thickness (Tb.Th), (D) trabecular number (Tb.N), (E) trabecular separation (Tb.Sp), (F) structure model index (SMI), and (G) degree of anisotropy (DA). The measurement range of the distal femur was 2000 μm cranially from 500 μm from the reference line connecting the 2 ends of the growth plate of the sagittal image. Values represent the means ± SD (N = 9–10 in each group). #: $P < 0.05$, ##: $P < 0.01$ vs Cont group (Student's *t*-test). *: $P < 0.05$, **: $P < 0.01$ (Tukey's multiple comparison test). Cont: non-CKD control rats, CKD: CKD rats administered vehicle, EC: CKD rats administered etelcalcetide, TPTD: CKD rats administered teriparatide, Comb: CKD rats administered EC and TPTD.

Table 2
Bone mineral density (mg/cm^2).

	Cont	CKD	EC	TPTD	Comb	ANOVA
	N = 10	N = 10	N = 9	N = 9	N = 10	P-value
Femur total	0.273 ± 0.009	0.259 ± 0.012$^{a'}$	0.260 ± 0.006	0.277 ± 0.009^{bc}	0.273 ± 0.013^{b}	< 0.001
Femur proximal	0.275 ± 0.008	0.258 ± 0.012$^{a'}$	0.257 ± 0.008	0.278 ± 0.011$^{b'c'}$	0.272 ± 0.011^{bc}	< 0.001
Femur middle	0.262 ± 0.010	0.250 ± 0.012^{a}	0.255 ± 0.013	0.264 ± 0.007	0.257 ± 0.016	0.237
Femur distal	0.279 ± 0.010	0.265 ± 0.012^{a}	0.266 ± 0.007	0.287 ± 0.011^{bc}	0.286 ± 0.015^{bc}	< 0.001

Values are presented as means ± standard deviation.N = 9–10 in each group.
Cont: non-CKD control rats, CKD: CKD rats administered vehicle, EC: CKD rats administered etelcalcetide, TPTD: CKD rats administered teriparatide.
Comb: CKD rats administered EC and TPTD.
[a] $P < 0.05$, [a'] $P < 0.01$ vs Cont group (Student's *t*-test).
[b] $P < 0.05$, [b'] $P < 0.01$ vs CKD group, [c] $P < 0.05$, [c'] $P < 0.01$ vs EC group (Tukey's multiple comparison test).

impairment (CKD stages 1–3) [34], the effect of TPTD therapy in CKD stage IV and SHPT was limited to animal studies. Ota et al [35] reported that TPTD has an anabolic effect on bone in CKD stage IV rats without adversely affecting mineral metabolism even under SHPT conditions. Similar results were generally obtained in the present study, except that OS/BS did not increase compared with the CKD group. On the other hand, a decrease in ES/BS was observed, resulting in an increase in trabecular bone volume. This may be due to differences in the type of teriparatide, the frequency, duration, and concentration of administration, and the timing of the evaluations. It seems that, in CKD model rats, even in the state of continuous hypersecretion of endogenous PTH, stimulation by intermittent administration of exogenous PTH has a predominant effect on bone formation.

Regarding the mechanisms of TPTD effects on bone in CKD with SHPT, basic and clinical studies have reported that PTH responsiveness to bone has been reduced due to decreased expression of the PTH-related peptide receptor (PTH1R) [36,37]. Furthermore, the relationship between increased serum PTH levels and decreased PTH1R expression in CKD model rats was not linear, and PTH1R expression remained decreased even when PTH was kept low by parathyroidectomy [38].

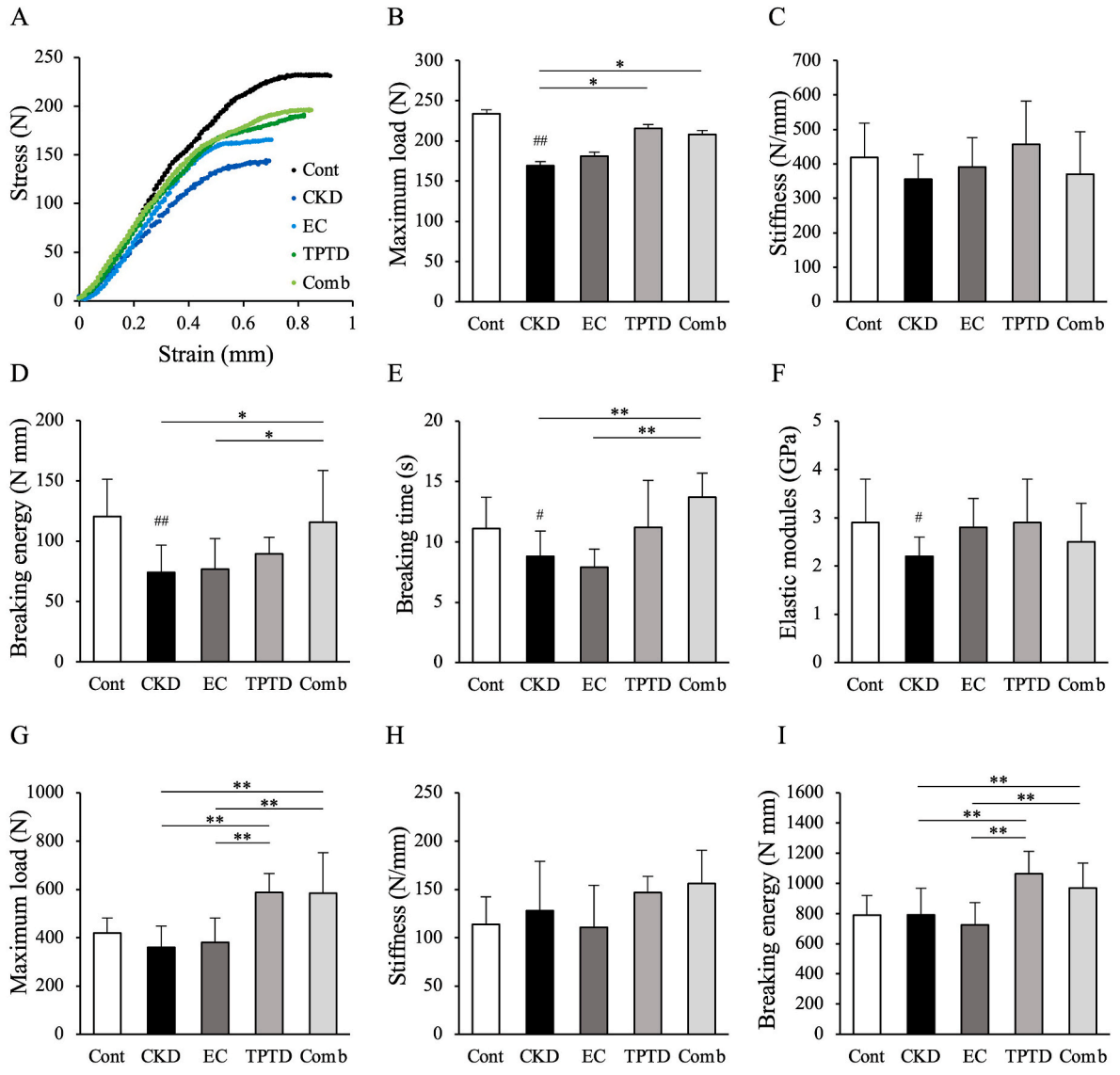

Fig. 5. (A) Representative stress–strain curves of the three-point bending test at the mid-shaft of the femur. (B) Maximum load, (C) stiffness, (D) breaking energy, (E) breaking time, and (F) elastic modulus of the three-point bending test at the mid-shaft of the femur. (G) Maximum load, (H) stiffness, and (I) breaking energy of the compression test at the distal metaphysis of the femur. Values represent the means ± SD (N = 9–10 in each group). #: $P < 0.05$, ##: $P < 0.01$ vs Cont group (Student's *t* test). *: $P < 0.05$, **: $P < 0.01$ (Tukey's multiple comparison test). Cont: non-CKD control rats, CKD: CKD rats administered vehicle, EC: CKD rats administered etelcalcetide, TPTD: CKD rats administered teriparatide, Comb: CKD rats administered EC and TPTD.

Since decreased PTH1R expression due to renal impairment was a contributing factor to abnormal bone metabolism in CKD, we hypothesized that PTH supplementation ameliorates bone dysplasia, which was one reason why TPTD was effective against SHPT in CKD rats. Based on these results, TPTD may be a useful treatment option. However, to date, the efficacy of TPTD in patients with advanced CKD, such as hemodialysis patients, has been limited to cases with reduced PTH secretion after parathyroidectomy and cases with a low metabolic turnover with reduced PTH secretion [39,40]. Furthermore, it is not practical to administer TPTD to untreated SHPT in clinical practice, and SHPT is a contraindication to the use of TPTD in Japan.

Therefore, the most important aspect of this study was to examine the effects of TPTD on bone microstructure and bone strength of cortical and trabecular bone in a CKD rat model in which SHPT was controlled by EC, which has not been investigated before. The results in the present study indicate that EC administration corrects the high metabolic turnover state caused by continuous PTH hypersecretion, and that TPTD can maintain the metabolic turnover from a state of bone resorption dominance to a state of bone formation dominance. Interestingly, in adipocyte metabolism, the effect of reducing adipocyte volume was also enhanced in the combined group. High concentrations of PTH have been found to promote adipocyte differentiation from mesenchymal stem cells [41]. On the other hand, intermittent administration of TPTD inhibits differentiation of mesenchymal stem cells into adipocytes and promotes differentiation into osteoblasts [42]. EC has been reported to enhance osteoblast activity in a non-PTH-dependent pathway [43]. In other words, the combination of EC and TPTD may be more effective in simultaneously inhibiting adipocyte differentiation and promoting bone formation. From the viewpoint of bone fracture prevention, it is important to note that the combination of EC and TPTD enhanced the improvement of bone strength of cortical bone. This may reflect the effect of improved porosity and thickness confirmed by micro-CT. However, parameters such as elastic modulus and stiffness, which are tissue-level mechanical properties, were not improved. In addition, the effect on BMD in the combination group was observed only in the trabecular bone area, suggesting that this effect may be mainly due to

TPTD. Although no apparent adverse effects were observed with combination therapy in the present study, further research is needed to address issues that are expected to occur in humans, such as increased gastrointestinal side effects and fluctuations in serum calcium levels and blood pressure after administration.

In recent years, basic experiments have suggested that uremic toxin is involved in the impairment of bone quality in renal failure. In a rat model of renal failure, femoral BMD was only slightly decreased, but bone dynamic viscoelasticity was significantly decreased with renal dysfunction [44,45]. Although TPTD has been reported to improve bone quality by restoring bone remodeling, significantly increasing collagen content and enzyme-dependent cross-links, and decreasing AGE cross-linked pentosidine [46], its effect on uremia-induced bone quality loss in CKD is unknown. Regarding the effects of EC on bone quality, EC improved material and mechanical properties at the tissue level in CKD rats (Cy/+) [47], and EC improved bone strength in a nephrectomized CKD rat model [23]. In the present study, there was no significant improvement effect of EC on material properties or mechanical properties. The decrease in material properties, mechanical properties, and bone strength in CKD cannot be explained by abnormal bone mineral metabolism alone, but it may be due to other related factors, such as uremic toxin, and may warrant further investigation.

This study has several limitations. First, it was not possible to measure or evaluate FGF23 or vitamin D, which are important in CKD-MBD; investigation of their blood kinetics, which have interactions with PTH, should be investigated in future studies. Second, bone metabolism markers in serum were not measured. The status of bone metabolism was determined by bone morphometry, but changes over time in bone formation and resorption markers due to administration of EC and TPTD need to be investigated in future studies.

5. Conclusions

In conclusion, this study shows that combination therapy provides a beneficial effect in improving bone deterioration in CKD that cannot be achieved with monotherapy; the cortical bone retention and bone metabolism turnover-improving effects of EC and the trabecular bone osteogenesis-promoting effects of TPTD go hand in hand, affecting both trabecular and cortical bone in CKD. Because they effectively improve health, they may represent a new option to combat skeletal fragility. To optimize the treatment and bone health of CKD patients, combination therapies should be considered as a future treatment modality.

CRediT author statement

Shun Igarashi: Investigation, Validation, Visualization, Writing - Original Draft. **Yuji Kasukawa**: Conceptualization, Methodology, Project administration, Writing - Review & Editing. **Koji Nozaka**: Conceptualization, Methodology, Writing - Review & Editing. **Hiroyuki Tsuchie**: Investigation, Formal analysis. **Kazunobu Abe**: Investigation. **Hikaru Saito**: Investigation. **Ryo Shoji**: Investigation. **Fumihito Kasama**: Investigation. **Shuntaro Harata**: Investigation. **Kento Okamoto**: Investigation. **Keita Oya**: Investigation. **Naohisa Miyakoshi**: Conceptualization, Funding acquisition, Supervision, Writing - Review & Editing.

Conflicts of interest

The authors declare no competing interests.

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