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Short Term Effects of Vitamin C and Teriparatide on Bone Mineral Density and Strength in Vitamin C-Deficient Rats

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Abstract

Introduction: Vitamin C (Ascorbic acid; Aa) is considered to be essential for bone formation and its serum level is thought to decrease with age. Thus, Aa deficiency may be one cause of osteoporotic fragility fracture during teriparatide (TPTD) treatment of osteoporosis. This study aimed to evaluate the effects of Aa and TPTD administration on Bone Mineral Density (BMD) and bone strength in Aa-deficient Osteogenic Disorder Shionogi (ODS) rats.

Materials and Methods: Six-month-old ODS rats were divided into four groups (n = 10 per group): (1) Aa-deficient (Aa^-);(2) Aa-supplemented (Aa^+); (3) Aa-deficient and TPTD-administered (Aa^-+T); and (4) Aa-supplemented and TPTD-administered (Aa^++T). BMD and bone strength were evaluated after 8 weeks of treatment.

Results: BMD was significantly higher in the Aa^+, Aa^-+T, and Aa^++T groups than in the Aa^- group (P = 0.0001~0.002). Only the Aa^++T group showed significantly higher bone strength than the Aa^- group.

Conclusion: Thus, single administration of Aa or TPTD improved BMD, but did not improve bone strength in the short term.Combined treatment with Aa and TPTD improved both BMD and bone strength. Aa supplementation is important for maintenance of bone strength in osteoporosis of elderly people.

Keywords: Osteoporosis; Ascorbic acid; Teriparatide

Introduction: Osteoporosis and related bone fractures are a major global health issue. Several previous studies have reported that lower bone mineral density (BMD) or vertebral osteoporotic fractures are related to increased mortality [1-3]. Therefore, treatment of osteoporosis has been considered important in aged patients. Teriparatide (TPTD), which is one of the medicines used to treat osteoporosis, has shown significant effects on BMD by stimulating bone formation and reducing incidences of fragility fractures of the vertebral body [4-6]. However, treatment with TPTD cannot completely prevent osteoporotic fragility fractures in elderly osteoporotic patients.

Vitamin C (Ascorbic acid; Aa) deficiency may cause osteoporotic fragility fractures during TPTD treatment. Although Aa is essential for the differentiation of osteoblasts [7,8], it has been reported that the serum level of Aa decreases with age [9-12]. Thus, this age-related decrease in the serum level of Aa may have negative effects on TPTD treatment of osteoporosis. However, the effects of TPTD on BMD and bone strength under an Aa-deficient condition are completely unknown.

In this study, to evaluate the short-term effects of TPTD on BMD and bone strength under an Aa-deficient condition, we used an animal model of Aa deficiency, osteogenic disorder Shionogi (ODS) rats. The exact purpose of the present study was to evaluate the effects of TPTD on the BMD of the femur and vertebrae, as well as on the bone strength at the mid-diaphysis and the distal metaphysis of the femur in these Aa-deficient ODS rats.

Materials and Methods

Animals

ODS rats (Clea Japan, Tokyo, Japan) are genetically deficient for the synthesis of Aa because they lack L-gulono-γ-lactone oxidase,

and as a result develop body weight loss, scurvy, and osteoporosis [13,14]. When ODS rats are fed an Aa-free diet, their polypeptide hydroxyproline levels, which are known to be related to collagen synthesis [15] decrease below those in normal rats after one week and are about one-third of the normal level after two weeks [13,14]. When Aa is added to their drinking water, the scorbutic symptoms of these rats resolve in a few days. The rats were housed in a controlled environment at 22 ^{0}C with a 12-h light/dark cycle. They were pair-fed and allowed ad libitum access to water and standard ascorbic acid-free food (CE-2; Clea Japan) containing 1.14% calcium, 1.06% phosphorus, and 250 IU vitamin D3 per 100 g of food, as described previously [16,17]. Rats received 2 mg/mL Aa (Iwaki Pharma, Tokyo, Japan) in their drinking water for 16 weeks from birth. At 4 months of age, they received 0.5 mg/mL Aa in their drinking water [18] for 8 weeks to create an Aa-deficient condition.

Experimental design

After an 8-week experimental period of feeding with Aa-deficient (0.5 mg/mL) drinking water, the 6-month-old ODS rats were divided into the following 4 groups (n= 10 per group): (1) Aa-deficient group (Aa^- group), which was given 0.5 mg/mL Aa-deficient water and administered vehicle of TPTD; (2) Aa-supplemented group (Aa^+ group), which was given 2 mg/mL Aa-supplemented water and administered vehicle of TPTD; (3) Aa-deficient and TPTD-administered group (Aa^- + T group), which was given Aa-deficient water and administered TPTD; and (4) Aa-supplemented and TPTD-administered group (Aa^+ + T group), which was given Aa-supplemented water and administered TPTD. TPTD (Asahi Kasei Pharma Corporation, Shizuoka, Japan) was dissolved in saline containing 0.1% rat serum albumin and was administered subcutaneously once a week for 8 weeks (30 µg/kg body weight). After 8 weeks, these animals were euthanized under anesthesia with an intra-abdominal injection of ketamine (Sankyo, Tokyo, Japan) and xylazine (Zenoaq, Fukushima, Japan), and the bilateral femurs and lumbar vertebrae were harvested. All animal experimental protocols were approved by the Animal Committee of our institute, and all animal experiments conformed to the "Guidelines for Animal Experimentation" of our institute.

Preparation of bone

The right femur and the lumbar vertebrae were fixed in 10% neutral-buffered formalin until preparation for BMD measurement. The left femur was dissected of soft tissue, wrapped in gauze moistened with saline, and frozen to -80 ^{0}C until biomechanical testing.

Bone mineral density

BMD of the right total femur and of the lumbar vertebrae (L4-5) was measured using dual energy X-ray absorptiometry (QDR-4500, Hologic Inc.; Waltham, MA, USA). The BMD of the total femur was measured and the region of interest for BMD measurement at (L4-5) was determined manually.

Biomechanical testing

Mechanical testing of the left femur was performed at room temperature using a materials testing machine (MZ500S; Maruto, Tokyo, Japan). For stabilization of the femur, the mid-diaphysis of the femur was placed on two supports of the test apparatus that were 20 mm apart. The load of a three-point bending test was applied in the anteroposterior direction midway between the two supports. Load-displacement curves were recorded at a crosshead speed of 5 mm/min (Figure 1). The breaking force (N), breaking energy (N.mm), maximum load (N), and stiffness (N/mm) were calculated using software for the measurement of cortical bone strength (CTR win. Ver. 1.05; System Supply, Nagano, Japan). After the three-point bending test, the distal part of the femur was evaluated using a compression test. Load-displacement curves were recorded and the breaking force (N), breaking energy (N.mm), maximum load (N), and stiffness (N/mm) were calculated as a cancellous bone strength using the same software.

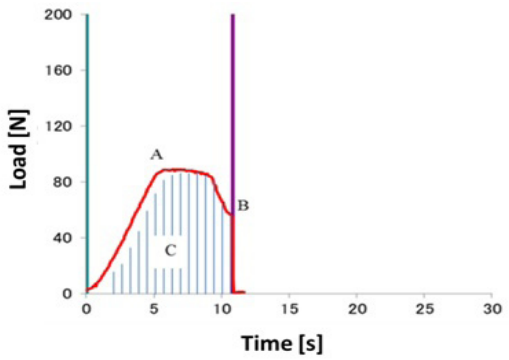

Figure 1: Typical load-displacement curve in the biomechanical test. The maximum load (N) is defined as the highest load (A), and the breaking force (N) is defined as the force at which breakage occurs (B) on the load-displacement curve. The breaking energy (N.mm) is defined as the area under the load-displacement curve up to the point at which breakage occurs (C). The stiffness was calculated as a secondary parameter by using software.

Statistical analyses

Results are expressed as means ± standard deviation. Intergroup differences were assessed by one-way analysis of variance (ANOVA). Following ANOVA, Scheffe's multiple comparison method was used as a post-hoc test. Differences with a P value < 0.05 were considered statistically significant. All statistical analyses were performed using the Statistical Package for the Biosciences software (SPBS v9.54; Murata& Yano 2002) [19].

Results

BMD (Table 1)

	Aa^-	Aa+	Aa- + T	Aa+ + T	P value of ANOVA
Femur (total)	0.211 ±0.004	0.230 ±0.015[a]	0.247 ±0.006[a',b]	0.262 ±0.010 [a',b',c]	< 0.0001
Lumbar vertebrae	0.204 ±0.004	0.224 ±0.006[a']	0.247 ±0.008[a',b']	0.241 ±0.005[a',b']	< 0.0001

Values are the mean ± standard deviation (SD).

Aa^-, ascorbic acid-deficient group; Aa^+, ascorbic acid-supplemented group; Aa^- + T, ascorbic acid-deficient and teriparatide-administered group; Aa^+ + T, ascorbic acid-supplemented and teriparatide-administered group

[a]P = 0.002, [a']P< 0.0001 vs. Aa^- group, [b]P = 0.004, [b']P <0.0001 vs. Aa^+ group, [c]P= 0.01 vs. Aa^- + T group.

Table 1: Comparisons of the BMD of the rat groups using ANOVA with Scheffe's multiple comparison method.

The BMDs of the total femur and of lumbar vertebrae were significantly increased by treatment with Aa and/or TPTD (P value by ANOVA, P < 0.0001). The BMDs of both the femur and of lumbar vertebrae in the Aa^+, Aa^- + T, and Aa^+ + T groups were significantly higher than those of the Aa^- group (P = 0.0001 ~ P = 0.002). TPTD treatment either with or without Aa significantly increased (P = 0.004 ~ P < 0.0001) the BMDs of the total femur and of lumbar vertebrae compared with those of the Aa treatment (Aa^+) group. Combined treatment with Aa and TPTD significantly increased (P = 0.01) the total femoral BMD compared with TPTD monotherapy (Aa^- + T group). On the other hand, there was no significant difference in the BMD of lumbar vertebrae between the Aa^- + T and the Aa^+ + T group.

Bone strength (Table2)

	Aa^-	Aa+	Aa^- + T	Aa^+ + T	P value of ANOVA
Three-point bending test					
Breaking force (N)	100.53 ±17.2	112.1 ±20.64	119.7 ±18.58	124.64 ±14.3[a]	0.026
Breaking energy (N.mm)	51.07 ±12.82	64.85 ±22.12	66.19 ±29.17	75.84 ±16.59	0.09
Maximum load (N)	110.34 ±19.32	128.21 ±15.2	126.61 ±16.64	135.14 ±16.09[a']	0.017
Stiffness (N/mm)	253.09 ±106.66	278.0 ±38.26	276.89 ±69.9	289.98 ±60.35	0.721
Compression test					
Breaking force (N)	144.27 ±43.66	145.69 ±41.64	156.52 ±57.39	231.76 ±75.18[a'',b,c]	0.003
Breaking energy (N.mm)	293.73 ±78.99	281.03 ±55.04	332.17 ±55.36	337.93 ±33.09	0.089
Maximum load (N)	202.04 ±34.28	213.47 ±29.8	237.12 ±48.05	267.52 ±42.18[a'',b']	0.003
Stiffness (N/mm)	218.93 ±86.63	205.33 ±57.88	269.59 ±91.73	170.84 ±77.46	0.063

All results are expressed as the mean ± standard deviation (SD).

Aa^-, ascorbic acid-deficient group; Aa^+, ascorbic acid-supplemented group; Aa^- + T, ascorbic acid-deficient and teriparatide-administered group; Aa^+ + T, ascorbic acid-supplemented and teriparatide-administered group.

[a]P = 0.04, [a'']P = 0.02, [a''']P = 0.01, [a''']P = 0.007 vs. Aa- group, [b]P = 0.016, [b']P = 0.036 vs. Aa+ group,[c]P = 0.043 vs. Aa^-+ T group

Table 2: Comparisons of the bone strength of the rat groups using ANOVA with Scheffe's multiple comparison method.

In the three-point bending test at the mid-diaphysis of the femur, the breaking force (P = 0.026) and maximum load (P = 0.017), but not the breaking energy or the stiffness, were significantly increased by Aa and/or TPTD treatmentas determined by ANOVA analysis. The breaking force and maximum load at the cortical bone in the Aa^+ + T group were significantly higher than those in the Aa^- group (P = 0.04 and P = 0.02, respectively). There was no significant differencein breaking energy or stiffness between any of the groups.

In the compression test of the distal femoral metaphysis, breaking force (P = 0.003) and maximum load (P = 0.003) at the cancellous bone were significantly increased with Aa and/or TPTD treatmentas assessed by ANOVA analysis. The combined treatment with TPTD and Aa significantly increased the breaking force and the maximum load compared with the Aa^- group (P = 0.01 and P = 0.007, respectively) and the Aa^+ group (P = 0.016 and P = 0.036, respectively). Moreover, the combined treatment had an additive effecton breaking force compared to single administration of TPTD (Aa^- + T group) (P = 0.04). There was no significant difference in breaking energy or stiffnessbetween any ofthe groups.

Discussion

In the present study, we investigated the effects of Aa and TPTD on BMD and bone strength at cortical and cancellous lesions of the femur in an Aa-deficient rat model, ODS rats. Under an Aa-deficient condition, Aa or TPTD treatment recovered BMD, but not bone strength. Combined treatment with TPTD and Aa for 8 weeks improved femoral and lumbar vertebral BMD and also bone strength at the mid-diaphysis and distal metaphysis of the femur.

It is well known that Aa is a necessary factor for the differentiation of osteoblasts, and contributes to bone formation and increases in bone volume [20]. Aa supplementation in ODS rats significantly increased the BMD of the femur and of lumbar vertebrae in the present study. Another animal study showed that Aa supplementation increased the BMD of ovariectomized rats [21]. In addition, several clinical studies have demonstrated that vitamin C intake is positively associated with bone mass [22-24]. Based on

these results, Aa is considered animportant determinant of BMD.

TPTD significantly increased the BMD of the total femur and of lumbar vertebraeof ODS rats in the present study. This is the first study to report that TPTD recovers BMD under an Aa-deficient condition in an animal model. Compared to non-treated cells, Aa-treated osteoblasts display increased expression of transforming growth factor (TGF)-β, estrogen receptor (ER)-α, and osteopontin (OPN), which are regulators of bone formation [25]. Further more, Aa prevents the loss of osteoblast differentiation markers such as osteorix, osteocalcin, Runx2, and bone morphometric protein (BMP)-2 and stimulates bone formation [26]. On the other hand, TPTD has been considered to exert its anabolic effects on bone formation via downregulation of sclerostin expression in osteocytes and stimulation of the Wnt signaling pathway [27]. The mechanisms of the anabolic effects of Aa and TPTD on BMD might therefore be different, which would explain how TPTD could recover BMD under Aa-deficient conditions.

In the present study, combined treatment with Aa and TPTD showed an additive effect for increasing BMD at the cortical bone compared to the effect of TPTD single administration. Monotherapy of Aa or TPTD could not recover the cortical and cancellous bone strength in Aa-deficient rats. However, combined therapy with Aa and TPTD improved the cortical and cancellous bone strength parameters of breaking force and maximum load. These results indicated that Aa deficiency may be one of the causes of incidental insufficiency fractures during treatment of aged osteoporotic patients with TPTD.

Furthermore, the effect of combined TPTD and Aa supplementation on cancellous bone strength in the compression test at the distal femoral metaphysis was more obvious than that on cortical bone strength in the three-point bending test. Previous studies in humans indicated that TPTD causes cortical porosity for up to Twety four months after its administration [28,29], and this change may have influenced the observed difference between cortical and cancellous bone. Saito et al. investigated the relationship between the parameter of bone strength and bone structural and material properties after TPTD administration. Their results suggested that the total amount of enzymatic cross-links of collagen, which has an important influence on bone quality, was a determinant that was significantly associated with increased maximum load. The effects of TPTD or Aa on bone quality under Aa-deficient conditions will be investigated in future studies.

Conclusion

Treatment with Aa or TPTD for Eight weeks increased the BMD of the total femur and of lumbar vertebrae in Six-month-old ODS rats. Combined treatment with Aa and TPTD increased the BMD of the femur and of lumbar vertebrae as well as bone strength at the mid-diaphysis and distal metaphysis of the femur in ODS rats.

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Conflict of Interest

All authors have no conflict of interest to declare.

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