Combined treatment with minodronate and vitamin C increases bone mineral density and strength in vitamin C-deficient rats

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Abstract

Objectives: Reduced bone quality caused by vitamin C deficiency in older persons may lead to incidental fragility fractures during bisphosphonate treatment, although bisphosphonate increases bone mineral density (BMD). This study aimed to evaluate the effects of minodronate and ascorbic acid (Aa) on BMD, bone quality, and bone strength in Aa-deficient osteogenic disorder Shionogi (ODS) rats.

Methods: Six-month-old ODS rats were divided into four groups (n = 20 per group): (1) Aa supplementation (Aa+); (2) Aa-deficient (Aa−); (3) Aa supplementation and minodronate administration (Aa+ + Mino); and (4) Aa-deficient and minodronate administration (Aa− + Mino). BMD, bone strength, bone histomorphometry, and bone quality determined using Fourier transform infrared spectroscopy imaging (FTIRI) were evaluated after 4 and 8 weeks.

Results: BMD was significantly higher in the Aa+ + Mino group than in the Aa− group (p < 0.05). Bone strength was significantly higher in the Aa+ and Aa+ + Mino groups than in the Aa− group (p < 0.05). Furthermore, bone strength was significantly higher in the Aa+ + Mino group than in the Aa− + Mino group (p < 0.05). Minodronate treatment irrespective of Aa supplementation significantly decreased bone resorption compared with the Aa− and Aa− groups (p < 0.05). No significant differences in the parameters evaluated by FTIRI were observed between the groups.

Conclusions: Aa supplementation improved bone strength in ODS rats. Combined treatment with minodronate and Aa, but not minodronate alone, improved bone strength and increased BMD. Aa is required for bone health because it is essential for osteoblast differentiation.

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Keywords: Bone mineral density; Bone strength; Minodronate; Vitamin C

1. Introduction

Fragility fractures in older people are related to the impairment of their quality of life or reduced life expectancy and comprise an important clinical issue in aging societies. Prevention of fragility fractures is a very important clinical topic. Regarding the prevention of fragility fractures, oral administration of nitrogen-containing bisphosphonates reduced the relative risk of vertebral fractures from 36% to 49%, and that of nonvertebral fractures from 16% to 40% by increasing bone mineral density (BMD) in postmenopausal women [1−5]. Among nitrogen-containing bisphosphonates, minodronate was at least 10 times more potent than alendronate in inhibiting bone resorption by preventing the function of osteoclasts in vitro [6,7] and by increasing BMD in ovariectomized rats in vivo [8]. Minodronate clinically reduced the vertebral fracture risk by 59% in postmenopausal women over 2 years [9]. However, although BMD is increased with minodronate therapy, minodronate cannot completely prevent new vertebral or nonvertebral fractures, particularly in aged patients [9].

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One possible cause of fragility fractures associated with bisphosphonate treatment is the impairment of bone quality. Bone quality is a broad term encompassing the factors affecting the structural and material properties of bone. Material properties are explained by the mineral/matrix ratio, crystallite maturity and size, collagen cross-links, and micromdamage.

Ascorbic acid (Aa; vitamin C) has been gaining attention as a candidate for determining the material properties. Previous in vitro studies have shown that Aa is essential for osteoblast differentiation in animals and humans [10,11]. Aa is an indispensable coenzyme for the hydroxylation of lysine and proline residues that are necessary for collagen triple-helix formation, and the presence of collagen triple helices is essential for the promotion of osteoblast differentiation [12–15]. Aa also modulates the expression of transcription factors such as osterix, which are critical for osteoblast differentiation [16]. However, the in vivo effects of Aa on bone quality, such as the mineral/matrix ratio or crystallite maturity and size, remain unknown.

The blood level of Aa is lower in older people because of decreased absorption from the intestine and reabsorption from the kidney [17–20]. Thus, we speculated that a reason for incidental fragility fractures during treatment with bisphosphonates could be impairment of bone quality caused by Aa deficiency in older people [17]. Therefore, we hypothesized that treatment with minodronate and Aa may exert additive effects on bone strength via increased BMD and improved bone quality under Aa-deficient conditions in vivo.

To confirm this hypothesis, we used congenitally Aa-deficient osteogenic disorder Shionogi (ODS) rats that are unable to produce Aa. These rats cannot survive without Aa supplementation, and their Aa levels are controlled by Aa supplementation [21]. The purpose of this study was to evaluate the effects of minodronate and Aa on BMD, bone quality, and bone strength in Aa-deficient ODS rats. Bone quality was evaluated by Fourier transform infrared spectroscopy imaging (FTIRI), which provides a way to explore bone quality at multiple bone hierarchical levels [22].

2. Materials and methods

2.1. Animals

Four-month-old female ODS rats (mutant Wistar rats; Clea Japan, Tokyo, Japan) were used [23]. Normal rats can synthesize Aa; however, ODS rats have a hereditary defect in ability to synthesize Aa because they lack L-gulono-γ-lactone oxidase [24,25]. When ODS rats are fed an Aa-free diet, their polypeptide hydroxyproline levels, which are known to be related to collagen synthesis [26], decrease below those in normal rats after 1 week and are about one-third of the normal level after 2 weeks [24,25]. When Aa is added to drinking water, the scorbutic symptoms of these rats resolve in a few days. The rats were housed in a controlled environment at 22 °C with a 12-hour/12-hour light/dark cycle. They were pair-fed and allowed ad libitum access to water as well as standard Aa-free food (CE-2; Clea Japan) containing 1.14% calcium, 1.06% phosphorus, and 250 IU vitamin D3 per 100 g of food as previously described [27,28]. The rats received 2 mg/ml Aa (Iwaki Pharma, Tokyo, Japan) in their drinking water for 4 months. At the age of 8 months, they received 0.5 mg/ml Aa in their drinking water [21] for 4 weeks to create Aa-deficient conditions.

2.2. Experimental design

After a 4-week experimental period of feeding with Aa-deficient (0.5 mg/ml) drinking water, the rats were divided into the following four groups (n = 20 per group): (1) Aa supplementation (Aa+) group, which received 2 mg/ml Aa-supplemented water; (2) Aa-deficient (Aa−) group, which received 0.5 mg/ml Aa-deficient water; (3) Aa supplementation and minodronate (Aa+ + Mino) group, which received Aa-supplemented water and minodronate; (4) Aa-deficient and minodronate (Aa− + Mino) group, which received Aa-deficient water and minodronate. In addition to these four groups, Wistar rats of the same age (n = 20) were included as a control group. Minodronate (Astellas Pharma, Tokyo, Japan) was dissolved in 0.1 M sodium acetate buffer (pH 7.2) containing 0.1% bovine serum albumin. This was administered subcutaneously once a week for 4 or 8 weeks (0.15 mg/kg body weight) [8].

The 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 tibias were harvested. Our animal experimental protocols were approved by the Animal Committee of our institution, and all animal experiments conformed to the “Guidelines for Animal Experimentation” of our institution.

2.3. BMD and sample preparation

The BMD of the right femur was measured in 2.5-mm proximal regions of the femur by dual energy X-ray absorptiometry (QDR-4500; Hologic Inc., Waltham, MA, USA). Thereafter, the right femur and bilateral tibias were fixed in neutral-buffered formalin until preparation for histological examination and FTIRI. The left femurs were dissected of soft tissue, wrapped in gauze moistened with saline, and frozen to −80 °C until biomechanical testing.

2.4. Bone histomorphometry

The left proximal halves of the tibias were decalcified in neutral-buffered 10% ethylene diamine tetra-acetic acid for 3 weeks and embedded in paraffin. Next, 3-μm-thick mid-frontal sections were prepared for hematoxylin and eosin (H & E) staining for cancellous bone histomorphometry.

Bone histomorphometric analyses of H & E-stained sections of the left tibia were performed using a semiautomatic graphic system (Histometry RT Camera; System Supply, Nagano, Japan). Measurements were obtained in regions that were 390 μm from the lowest point of the growth
2.5. Biomechanical testing procedure

Mechanical testing of the rat femoral shaft and femoral neck was performed at room temperature using a materials testing machine (MZ500S; Maruto, Tokyo, Japan).

For stabilization, 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. A load—displacement curve was recorded at a crosshead speed of 5 mm/min. The maximum load (N), breaking energy (N·mm), and breaking force (N) were calculated using software for the measurement of bone strength (CTR win. Ver. 1.05; System Supply). The maximum load was defined as the highest load, and the breaking force was defined as the force at which breakage occurred on the load—deformation curve. The breaking energy was defined as the area under the load—deformation curve up to the point at which breakage occurred.

After the three-point bending test, the proximal part of the femur was evaluated using a compression test of the femoral neck. The breaking force on the load—deformation curve was calculated as the phosphate sub-band 1,030/C0 and the maximum load, breaking energy, and breaking force on the load—displacement curve were calculated using the same software.

2.6. Fourier transform infrared spectroscopy imaging

To evaluate bone quality, the right tibias were used for FTIRI. Each right tibia was fixed in alcohol, embedded in polymethylmethacrylate (PMMA), cut into 3-μm-thick sections, and mounted on barium fluoride infrared windows (SpectraTech, Hopewell Junction, NY, USA). The undecalcified sections were 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 specific regions of the tibial cross-sections in cortical and cancellous bone. For the bone area, five different images were recorded. The mean and standard deviation (SD) for five images in the bone area were calculated and compared. The values were then averaged for each area. The cross-sections were processed for FTIRI in a blinded fashion, and the codes identifying the groups were not broken until the time of the statistical analysis. Spectra were baseline-corrected and the PMMA spectral contribution was subtracted using OMNIC8, TQ Analyst (Thermo Fisher Scientific, Kanagawa, Japan), as previously described [31]. The mineral/matrix ratio, which measures bone mineral content (correlated to ash weight), was calculated from the integrated areas of phosphate (907–1,183 cm⁻¹) to amide I (1,586–1,710 cm⁻¹). The collagen cross-linking network maturity (XLR) was estimated as the peak intensity ratio of amide I sub-bands at 1660 cm⁻¹. The carbonate/phosphate ratio, which reflects the level of carbonate substitution in the hydroxyapatite (HA) crystal, was calculated as the integrated area of the ν2 carbonate peak (840–892 cm⁻¹) to that of the phosphate peak. The HA crystallinity, related to mineral crystal size and perfection as determined by X-ray diffraction, was calculated as the phosphate sub-band 1,030/1,020 cm⁻¹ peak intensity ratio.

2.7. Statistical analyses

All values are expressed as mean ± SD. One-way analysis of variance was used to test for statistical significance among the groups. The significance levels of differences between two groups among the Aa+, Aa−, Aa+ + Mino, and Aa− + Mino groups were assessed by the Scheffe multiple comparison method at 4 and 8 weeks. Probability (p) values of <0.05 were considered statistically significant.

3. Results

3.1. Minodronate increased bone mineral density in the presence of ascorbic acid (Table 1)

BMD was significantly increased at 4 and 8 weeks (p = 0.044 and 0.041, respectively) in the group that

| Table 1 Bone mineral density |
|-----------------------------|
|                          | Aa⁺ | Aa⁻ | Aa⁺ + Mino | Aa⁻ + Mino | ANOVA | Control |
| 4 weeks                   |     |     |           |            |       |         |
| BMD                       | 0.253 ± 0.041 | 0.241 ± 0.032 | 0.272 ± 0.051<sup>a</sup> | 0.257 ± 0.044 | <0.001 | 0.254 ± 0.052 |
| 8 weeks                   | 0.257 ± 0.042 | 0.236 ± 0.049 | 0.281 ± 0.037<sup>a</sup> | 0.261 ± 0.041 | <0.001 | 0.256 ± 0.041 |

All values are expressed as mean ± standard deviation.

ANOVA, one-way analysis of variance; Aa⁺, ascorbic acid supplementation group; Aa⁻, ascorbic acid-deficient group; Aa⁺ + Mino, ascorbic acid supplementation and minodronate administration group; Aa⁻ + Mino, ascorbic acid-deficient and minodronate administration group.

<sup>a</sup> p < 0.05 between Aa⁻ and Aa⁺ + Mino groups by the Scheffe multiple comparison method.
underwent AA supplementation and minodronate administration (AA⁺ + Mino group) compared with that in AA-deficient rats (AA⁻ group). Although the BMD in the AA⁻ group tended to decrease, the decrease was not statistically significant compared with the control and AA⁺ groups. Minodronate treatment without AA supplementation (AA⁻ + Mino group) failed to increase BMD at 4 and 8 weeks of treatment compared with that in the AA⁺ group.

3.2. Ascorbic acid increased biomechanical strength irrespective of minodronate administration (Table 2)

The maximum load, breaking force, and breaking energy determined by the mid-shaft three-point bending test were significantly higher in the AA⁺ group \( (p = 0.033, 0.033, \text{and } 0.032, \text{respectively}) \) and AA⁺ + Mino group \( (p = 0.030, 0.029, \text{and } 0.031, \text{respectively}) \) than in the AA⁻ group at 4 and 8 weeks. Furthermore, the maximum load, breaking force, and breaking energy in the AA⁺ + Mino group were significantly higher than in the AA⁻ + Mino group \( (p = 0.046, 0.048, \text{and } 0.045, \text{respectively}) \). Minodronate administration without AA supplementation (AA⁻ + Mino group) failed to increase these parameters compared with the AA⁻ group at 4 and 8 weeks.

Similarly, the maximum load, breaking force, and breaking energy of the femoral neck compression test were significantly higher in the AA⁺ group \( (p = 0.033, 0.032, \text{and } 0.033, \text{respectively}) \) and AA⁺ + Mino group \( (p = 0.031, 0.027, \text{and } 0.029, \text{respectively}) \) than those in the AA⁻ group at 4 and 8 weeks. The breaking energy, but not the maximum load or breaking force, in the AA⁺ + Mino group was significantly higher than that in the AA⁻ + Mino group \( (p = 0.048) \).

Table 2

| Biomechanical test results | AA⁺ | AA⁻ | AA⁺ + Mino | AA⁻ + Mino | ANOVA | Control |
|----------------------------|-----|-----|------------|------------|-------|---------|
| 4 Weeks                    |     |     |            |            |       |         |
| Diaphysis three-point bending test |     |     |            |            |       |         |
| Maximum load (N)           | 118 ± 7\(^a\) | 95 ± 8 | 125 ± 9\(^b\) | 98 ± 6 | <0.001 | 116 ± 5 |
| Breaking energy (N-mm)      | 147 ± 5\(^a\) | 113 ± 6 | 161 ± 7\(^b\) | 129 ± 4 | <0.001 | 150 ± 5 |
| Breaking force (N)          | 69 ± 3\(^a\) | 45 ± 4 | 78 ± 6\(^b\) | 57 ± 4 | <0.001 | 73 ± 5 |
| Femoral neck compression test |     |     |            |            |       |         |
| Maximum load (N)           | 90 ± 6\(^a\) | 72 ± 5 | 95 ± 8\(^b\) | 78 ± 7 | <0.001 | 95 ± 5 |
| Breaking energy (N-mm)      | 84 ± 5\(^a\) | 67 ± 6 | 90 ± 8\(^b\) | 72 ± 7 | <0.001 | 86 ± 5 |
| Breaking force (N)          | 37 ± 5\(^a\) | 26 ± 4 | 42 ± 6\(^b\) | 29 ± 5 | <0.001 | 40 ± 4 |
| 8 Weeks                    |     |     |            |            |       |         |
| Diaphysis three-point bending test |     |     |            |            |       |         |
| Maximum load (N)           | 120 ± 6\(^a\) | 92 ± 7 | 128 ± 6\(^b\) | 101 ± 5 | <0.001 | 117 ± 7 |
| Breaking energy(N-mm)       | 150 ± 8\(^a\) | 111 ± 7 | 163 ± 6\(^b\) | 128 ± 7 | <0.001 | 152 ± 5 |
| Breaking force (N)          | 72 ± 3\(^a\) | 41 ± 4 | 79 ± 6\(^b\) | 58 ± 4 | <0.001 | 71 ± 5 |
| Femoral neck compression test |     |     |            |            |       |         |
| Maximum load (N)           | 93 ± 6\(^a\) | 69 ± 4 | 97 ± 8\(^b\) | 81 ± 7 | <0.001 | 95 ± 4 |
| Breaking energy(N-mm)       | 88 ± 4\(^a\) | 65 ± 5 | 94 ± 6\(^b\) | 71 ± 5 | <0.001 | 85 ± 7 |
| Breaking force (N)          | 39 ± 5\(^a\) | 25 ± 4 | 44 ± 6\(^b\) | 30 ± 4 | <0.001 | 41 ± 6 |

All values are expressed as mean ± standard deviation.
ANOVA, one-way analysis of variance; AA⁺, ascorbic acid supplementation group; AA⁻, ascorbic acid-deficient group; AA⁺ + Mino, ascorbic acid-supplementation and minodronate administration group; AA⁻ + Mino, ascorbic acid-deficient and minodronate administration group.
\(^a\) \( p < 0.05 \) vs. AA⁺ group.
\(^b\) \( p < 0.05 \) vs. AA⁻ + Mino group by the Scheffe multiple comparison method.

Table 3

| Bone histomorphometry | AA⁺ | AA⁻ | AA⁺ + Mino | AA⁻ + Mino | ANOVA | Control |
|-----------------------|-----|-----|------------|------------|-------|---------|
| 4 weeks               |     |     |            |            |       |         |
| BV/TV (%)             | 36.5 ± 3.2 | 31.6 ± 2.8 | 42.2 ± 3.1\(^a\) | 35.7 ± 3.7 | <0.001 | 38.1 ± 2.7 |
| OS/BS (%)             | 10.8 ± 1.1 | 8.9 ± 0.6 | 11.8 ± 0.8\(^a\) | 10.1 ± 0.5 | <0.001 | 10.9 ± 0.8 |
| ES/BS (%)             | 11.2 ± 0.4 | 11.5 ± 0.5 | 7.3 ± 0.6\(^b\) | 7.1 ± 0.4\(^b\) | <0.001 | 8.8 ± 0.6 |
| 8 weeks               |     |     |            |            |       |         |
| BV/TV (%)             | 37.1 ± 2.9 | 30.6 ± 2.5 | 44.3 ± 3.2\(^a\) | 35.8 ± 2.7 | <0.001 | 38.3 ± 2.9 |
| OS/BS (%)             | 10.3 ± 0.9 | 8.5 ± 0.5 | 12.1 ± 0.7\(^a\) | 10.3 ± 0.8 | <0.001 | 10.5 ± 0.9 |
| ES/BS (%)             | 11.5 ± 0.5 | 11.8 ± 0.4 | 7.1 ± 0.6\(^b\) | 7.5 ± 0.5\(^b\) | <0.001 | 8.9 ± 0.4 |

All values are expressed as mean ± standard deviation.
ANOVA, one-way analysis of variance; AA⁺, ascorbic acid supplementation group; AA⁻, ascorbic acid-deficient group; AA⁺ + Mino, ascorbic acid-supplementation and minodronate administration group; AA⁻ + Mino, ascorbic acid-deficient and minodronate administration group.
\(^a\) \( p < 0.05 \) vs. AA⁺ group.
\(^b\) \( p < 0.05 \) vs. AA⁻ group by the Scheffe multiple comparison method.
Minodronate without ascorbic acid supplementation (Aa− + Mino) treatment did not significantly increase these parameters compared with those in the Aa− group at 4 and 8 weeks.

3.3. Bone histomorphometry (Table 3)

BV/TV and OS/BS were significantly higher in the Aa+ + Mino group (p = 0.043 and 0.037, respectively) than in the Aa− group at 4 and 8 weeks. The ES/BS was significantly lower in the Aa+ + Mino and Aa− + Mino groups (p = 0.044 and 0.041, respectively) than in the Aa+ and Aa− groups.

3.4. Ascorbic acid caused a non-significant increase in the amount of mature collagen cross-linking in cancellous bone, but not in cortical bone

Fig. 1 shows typical FTIRI for the collagen cross-linking network maturity in cancellous and cortical bone. The yellow shaded part represents normal maturity, the green dots represent lower maturity, and the red dots represent higher maturity of the collagen cross-linking network. The collagen cross-linking network maturity tended to be higher in the Aa+ group than in the Aa− group.

Fig. 1. Typical Fourier transform infrared images for the collagen cross-linking network maturity in cancellous and cortical bone. The yellow shaded part represents normal maturity, the green dots represent lower maturity, and the red dots represent higher maturity of the collagen cross-linking network. The collagen cross-linking network maturity tended to be higher in the Aa+ group than in the Aa− group.
4 weeks

| Parameter                                               | Aa⁺ | Aa⁻ | Aa⁺ + Mino | Aa⁻ + Mino | ANOVA |
|---------------------------------------------------------|-----|-----|-----------|-----------|-------|
| Mineral/Matrix ratio                                     | 7.36 ± 1.19 | 7.01 ± 0.89 | 6.36 ± 0.80 | 8.02 ± 1.32 | NS    |
| Carbonate/phosphate ratio                                | 0.00724 ± 0.00060 | 0.00903 ± 0.00078 | 0.00835 ± 0.00059 | 0.00895 ± 0.00051 | NS    |
| HA crystallinity                                         | 1.078 ± 0.028 | 1.058 ± 0.026 | 1.042 ± 0.023 | 1.049 ± 0.028 | NS    |
| Collagen cross-linking network maturity                  | 4.702 ± 0.238* | 4.079 ± 0.231 | 4.364 ± 0.161 | 4.110 ± 0.148 | NS    |

5 weeks

| Parameter                                               | Aa⁺ | Aa⁻ | Aa⁺ + Mino | Aa⁻ + Mino | ANOVA |
|---------------------------------------------------------|-----|-----|-----------|-----------|-------|
| Mineral/Matrix ratio                                     | 8.11 ± 1.06 | 5.90 ± 1.01 | 6.92 ± 0.89 | 8.13 ± 1.22 | NS    |
| Carbonate/phosphate ratio                                | 0.00843 ± 0.00059 | 0.00875 ± 0.00063 | 0.00708 ± 0.00080 | 0.00894 ± 0.00073 | NS    |
| HA crystallinity                                         | 1.045 ± 0.032 | 1.066 ± 0.035 | 1.134 ± 0.031 | 1.041 ± 0.021 | NS    |
| Collagen cross-linking network maturity                  | 4.792 ± 0.231 | 4.137 ± 0.161 | 4.480 ± 0.172 | 4.398 ± 0.101 | NS    |

All values are expressed as mean ± standard deviation.
ANOVA, one-way analysis of variance; NS, not significant; Aa⁺, ascorbic acid supplementation group; Aa⁻, ascorbic acid-deficient group; Aa⁺ + Mino, ascorbic acid supplementation and minodronate administration group; Aa⁻ + Mino, ascorbic acid-deficient and minodronate administration group.

There were no significant differences in the parameters among the study groups. *p = 0.092 vs. Aa⁻ group by the Scheffe multiple comparison method.

The imaging in typical cases demonstrated the efficacy of Aa on bone quality (Fig. 1). However, there were no significant differences in any of the FTIRI parameters examined for cancellous bone among the study groups (Table 4). In cancellous bone at 4 weeks, the collagen cross-linking network maturity tended to be higher in the Aa⁺ group than in the Aa⁻ group (p = 0.092).

There were no significant differences among the study groups in any of the FTIRI parameters examined for cortical bone (Table 5).

4. Discussion

In the present study, Aa deficiency caused lower bone strength without decreasing BMD and causing lower bone turnover as determined by bone histomorphometry in ODS rats. A possible cause of the weakened bone strength in Aa-deficient ODS rats is the impairment of bone quality, such as decreases in the collagen cross-linked network in the cancellous and cortical bone. However, no significant changes in these parameters were detected using FTIRI in the present study. To recover the decreased bone strength in ODS rats, minodronate and Aa were administered. Minodronate could not improve BMD or bone strength under Aa-deficient conditions, even with decreased bone resorption. Minodronate and Aa supplementation, but not minodronate without Aa supplementation, improved BMD and bone strength in ODS rats.

ODS rats, as a model of Aa deficiency, show reduced bone formation accompanied by deleterious effects in bone mechanical properties caused by decreased hydroxyproline in collagen synthesis [26,32–34]. Hasegawa et al. [35] reported that Aa deficiency causes several types of morphological deformities in bone mineralization and collagen, such as mis-assembly of the triple helices of collagenous α-chains. These observations indicate that Aa is essential for bone formation and collagen synthesis related to bone quality.

We evaluated bone quality by FTIRI in the present study. Aa supplementation did not increase the collagen cross-linking network maturity of cancellous bone or HA crystallinity of cortical bone in ODS rats. Collagen cross-linking network maturity is related to the ratio of non-reducible to reducible collagen cross-links, and HA crystallinity is a measure of crystal size assessed by X-ray diffraction [36]. It has been reported that the collagen cross-link ratio is partly responsible for the changes in the material properties of osteoporotic bone, contributing to clinically manifested fragility other than bone turnover alone [37].
Aa also exerts antioxidant effects in many tissues. It has been reported that Aa recovered bone volume, BMD, and bone strength under osteoporotic conditions caused by oxidizing stress in CuZn-SOD knockout mice [38]. In that study, Aa showed antioxidant effects associated with the recovery of bone strength in the fragile bone [38]. The findings of these previous studies indicate that Aa improved bone quality and increased bone strength. Although there were no significant differences in the collagen cross-linking network maturity of cancellous bone and HA crystallinity of cortical and cancellous bone in ODS rats after 4 and 8 weeks of treatment in the present study, the number of animals may not have been sufficient to detect the effects of Aa supplementation on bone quality evaluated by FTIRI.

Minodronate administration failed to increase BMD without concurrent Aa supplementation and did not affect the FTIRI results, although the bone resorption parameter ES/BS was significantly decreased in ODS rats. In addition, minodronate did not cause a significant increase in bone strength without Aa supplementation under Aa-deficient conditions. With regard to the effect of minodronate on bone quality, minodronate modifies bone mineral and matrix properties and their heterogeneity; however, the bone homogenization observed with minodronate use suggests that it also has negative effects on bone quality [39]. In contrast, minodronate prevented a change in the microarchitecture in ovariectomized monkeys, indicating that minodronate did not have negative effects on bone quality [40]. Although the effects of minodronate on bone quality remain unclear, minodronate may not exert a strong preventive effect on the incidence of fragility fractures caused by worsened bone quality, such as that under Aa-deficient conditions, which occur in some older patients with insufficient Aa. Aa deficiency could explain why some older patients have fragility fractures even while maintaining BMD during treatment with bisphosphonates.

There have been no clinical studies on the combined therapeutic effects of minodronate and Aa on the incidence of fragility fractures in older patients. Combined therapy with minodronate and Aa recovered the BMD and bone strength of ODS rats in the present study. It has occasionally been reported that fragility bone fractures increase with decreased blood levels of Aa [41] and that callus strength after fracture is increased by Aa supplementation [21]. The present findings indicate that combined minodronate and Aa therapy may decrease the incidence of fragility fractures in aged patients or those with worsened bone quality.

There are several limitations to this study. First, the blood level of Aa was not measured because blood was not extracted. Therefore, it is not possible to accurately judge the extent to which the blood level of Aa was decreased. Second, the number of samples was not sufficient to detect significant changes in parameters regarding bone quality evaluated by FTIRI among the treatment groups. Third, the model used in this study was not ovariectomized rats. Osteoporosis is common in older postmenopausal women, and thus ovariectomized ODS rats may be more suitable to evaluate the effects of Aa deficiency on BMD, bone quality, and bone strength.

In summary, Aa deficiency caused lower bone turnover and weakened bone strength without decreasing BMD in congenitally Aa-deficient ODS rats. Aa supplementation improved bone strength in ODS rats. Although minodronate increased BMD, but not bone quality, it did not recover the decreased bone strength in ODS rats. Combined treatment with minodronate and Aa supplementation improved bone strength with BMD increases in ODS rats.

Conflicts of interest
None.

Authors’ contributions
All authors contributed equally to the development of this article, providing intellectual input into the content and design, critical review, and final approval.

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References
[1] Cranney A, Wells GA, Yetisir E, Adami S, Cooper C, Delmas PD, et al. Ibandronate for the prevention of nonvertebral fractures: a pooled analysis of individual patient data. Osteoporos Int 2009;20:291–7.
[2] Guyatt GH, Cranney A, Griffith L, Walter S, Krolicki N, Favus M, et al. Summary of meta-analyses of therapies for postmenopausal osteoporosis and the relationship between bone density and fractures. Endocrinol Metab Clin N Am 2002;31:659–79.
[3] Cranney A, Tugwell P, Zytaruk N, Robinson V, Weaver B, Shea B, et al. Meta-analyses of therapies for postmenopausal osteoporosis. VI. Meta-analysis of calcitonin for the treatment of postmenopausal osteoporosis. Endocr Rev 2002;23:540–51.
[4] Reginster J, Minne HW, Sorensen OH, Hooper M, Roux C, Blandi ML, et al. Randomized trial of the effects of risendronate on vertebral fractures in women with established postmenopausal osteoporosis. Vertebral efficacy with risendronate therapy (VERT) study group. Osteoporos Int 2000;11:83–91.
[5] Harris ST, Watts NB, Genant HK, McKeever CD, Hangartner T, Keller M, et al. Effects of risendronate treatment on vertebral and non-vertebral fractures in women with postmenopausal osteoporosis: a randomized controlled trial. Vertebral efficacy with risendronate therapy (VERT) study group. J Am Med Assoc 1999;14:1344–52.
[6] Dunford J, Thompson K, Coxon FP, Luckman SP, Hahn FM, Poulter CD, et al. Structure-activity relationships for inhibition of farnesyl diphosphate synthase in vitro and inhibition of bone resorption in vivo by nitrogen-containing bisphosphonates. J Pharmacol Exp Ther 2001;296:235–42.
[7] Lin JH. Bisphosphonates: a review of their pharmacokinetic properties. Bone 1996;18:75–85.
[8] Mori H, Tanaka M, Kayasuga R, Kishikawa K, Ito M. Efficacy of preventive and therapeutic treatments with minodronic acid on ovariectomized rat model of osteoporosis. J Pharmacol 2008;18:S33–48.
[9] Hagino H, Shiraki M, Fukunaga M, Nakano T, Takaoka K, Ohashi Y, et al. Number and severity of prevalent vertebral fractures and the risk of subsequent vertebral fractures in Japanese women with osteoporosis: results from the minodronate trial. J Bone Min Metab 2013;31:544–50.
Suzuki K, Kodama HA, Amagai Y, Yamamoto S, Kasai S. In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol 1983;96:191–8.

Segawa T, Nakada M, Fukuda M, Imai Y, Kinoshita Y, Fujita T. Effects of ascorbic acid on alkaline phosphatase activity and hormone responsiveness in the osteoblastic osteosarcoma cell line UMR-106. Calcif Tissue Int 1986;39:171–4.

Peterkovsky B. Ascorbate requirement for hydroxylation and secretion of procollagen: relationship to inhibition of collagen synthesis in scurvy. Am J Clin Nutr 1991;54:1135S–40S.

Harada S, Matsumoto T, Ogata E. Role of ascorbic acid in the regulation of proliferation in osteoblast like MC3T3-E1 cells. J Bone Miner Res 1991; 9:903–8.

Takeuchi Y, Nakayama K, Matsumoto T. Differentiation and cell surface expression of transforming growth factor-beta receptors are regulated by interaction with matrix collagen in murine osteoblastic cells. J Biol Chem 1996;271:3938–44.

Segawa M, Takeuchi Y, Fukumoto S, Kato S, Ueno N, Miyazono K, et al. Extracellular matrix associated bone morphogenetic proteins are essential for differentiation of murine osteoblastic cells in vitro. Endocrinology 1999;140:2125–33.

Xing W, Singgh A, Kapoor A, Alarcon CM, Baylink DJ, Mohan S. Nuclear factor-E2-related factor-1 mediates ascorbic acid induction of osterix expression via interaction with antioxidant-responsive element in bone cells. J Biol Chem 2007;282:22052–61.

Murata A, Kang KJ, Miyata S, Fujii J, Tamai H, Mino M, et al. Impaired vitamin C status of hospitalized elderly patients and its improvement by daily multivitamin supplementation. Vitamin 1999;7:903–52.

Richards T, Ball L, Rosenfeld T. Will an orange a day keep the doctor away? Postgrad Med J 2002;78:291–4.

Oeffinger KC. Scurvy: more than historical relevance. Am Fam Physician 1993;48:609–13.

Falch J, Mowé M, Behmer T. Low levels of serum ascorbic acid in elderly patients with hip fracture. Scand J Clin Lab Invest 1998;58: 225–8.

Alcantara-Martos T, Delgado-Martinez AD, Vega MV, Carrascal MT, Munuera-Martinez L. Effect of vitamin C on fracture healing in elderly Osteogenic Disorder Shionogi rats. J Bone Jt Surg Br 2007;89:402–7.

Paschalis P, Glass EV, Donley DW, Eriksen EF. Bone mineral and collagen quality in iliac crest biopsies of patients given teriparatide: new results from the fracture prevention trial. J Clin Endocrinol Metab 2005;90:4644–9.

Makino S, Katagiri K. Osteogenic disorder rat. Exp Anim 2007;29:903–8.

Horio F, Ozaki K, Yoshida A, Makino S, Hayashi Y. Requirement for ascorbic acid in a rat mutant unable to synthesize ascorbic acid. J Nutr 1985;115:1630–40.

Konishi T, Makino S, Mizushima Y. What is the ODS rat? historical description of the characterization studies: vitamin C and the scurvy-prone ODS rat. Cancer Lett 1990;49:3–22.

Krae SM, Maizoz AJ, Harris JR ED. Urinary polypeptides related to collagen synthesis. J Clin Invest 1970;49:716–29.

Suzuki K, Miyakoshi N, Tsuchida T, Kasukawa Y, Sato K, Ito T. Effect of combined treatment of insulin and human parathyroid hormone (1-34) on cancellous bone mass and structure in streptozotocin-induced diabetic rats. Bone 2003;33:108–14.

Tamura Y, Miyakoshi N, Ito T, Abe T, Kudo T, Tsuchida T, et al. Long-term effects of withdrawal of bisphosphonate inodinate disodium (YM175) on bone mineral density, mass, structure, and turnover in the lumbar vertebrae of ovariectomized rats. J Bone Miner Res 2001;16:541–9.

Nakajima A, Nakajima F, Shimizu S, Ogasawara A, Wanaka A, Moriya H, et al. Spatial and temporal gene expression for fibroblast growth factor type I receptor (FGFR1) during fracture healing in the rat. Bone 2001;29:458–66.

Parfitt AM, Drezen MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, et al. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR histomorphometry nomenclature committee. J Bone Miner Res 1987;2:595–610.

Allison GG, Thain SC, Morris P, Morris C, Hawkins S, Hauck B, et al. Quantification of hydroxyxycinnamic acids and lignin in perennial forage and energy grasses by Fourier-transform infrared spectroscopy and partial least squares regression. Biosens Techol 2009;100:1252–61.

Hara K, Akiyama Y. Collagen-nerated abnormalities, reduction in bone quality, and effects of menatetrenone in rats with a congenital ascorbic acid deficiency. J Bone Miner Metab 2009;27:324–32.

Sakamoto Y, Takan Y. Morphological influence of ascorbic acid deficiency on endochondral ossification in osteosporidion shionogi rat. Anat Rec 2002;268:93–104.

Tsunenari T, Fukase M, Fujita T. Bone histomorphometric analysis for the cause of osteopenia in vitamin-deficient rat (ODS rat). Calcif Tissue Int 1991;48:18–27.

Hasegawa T, Li M, Hara K, Sasaki M, Tabata C, de Freitas PH, et al. Morphological assessment of bone mineralization in tibial metaphyses of ascorbic acid-deficient ODS rats. Biomed Res 2011;32:259–69.

Paschalis EP, Recker R, DiCarlo E, Doty SB, Atti E, Boskey AL. Distribution of collagen cross-links in normal human trabecular bone. J Bone Miner Res 2003;18:1942–6.

Paschalis EP, Tatakis DN, Robins S, Fratzl P, Manjubala I, Zoehrer R, et al. Lathyrisim-induced alterations in collagen cross-links influence the mechanical properties of bone material without affectig the mineral. Bone 2011;49:1232–41.

Nojiri H, Saita Y, Morikawa D, Kobayashi K, Tsuda C, Miyazaki T, et al. Cytoplasmic superoxide causes bone fragility owing to low-turnover osteoporosis and impaired collagen cross-linking. J Bone Miner Res 2011;26:2682–94.

Leoni Arbogast E, Allen MR, Burr DB, Vashishth D, Tang SY, González M. Vitamin deficiency and bone strength, and improves bone microarchitecture in osteoporosis and impaired collagen cross-linking. J Bone Miner Res 2011;26:2682–94.

González M. Vitamin deficiency and bone strength, and improves bone microarchitecture in osteoporosis and impaired collagen cross-linking. J Bone Miner Res 2011;26:2682–94.

Lathyrism-induced alterations in collagen cross-links influence the mechanical properties of bone material without affecting the mineral. Bone 2011;49:1232–41.

Mori H, Tanaka M, Sayasuga R, Matsuda T, Ochi Y, Yamada H, et al. Minodronic acid (YM175) on bone mineral density, mass, structure, and turnover in the lumbar vertebrae of ovariectomized rats. J Bone Miner Res 2001;16:541–9.

Martinez-González MA, Palma Pérez S, Delgado-Martinez AD, Martinez-González MA, De la Fuente Arrillaga C, Delgado-Rodriguez M. Vitamin C, vitamin B12, folate and the risk of osteoporotic fractures. A case control study. Int J Vitam Nutr Res 2007;277:359–68.