Ascorbic acid insufficiency induces the severe defect on bone formation via the down-regulation of osteocalcin production

Won Kim1,2,*, Seyeon Bae1,*, Hyemin Kim1, Yejin Kim1, Jiwon Choi1, Sun Young Lim3, Hei Jin Lee1, Jihyuk Lee1, Jiyea Choi1, Mirim Jang1, Kyoung Eun Lee4, Sun G. Chung2, Young-il Hwang1, Jae Seung Kang1, Wang Jae Lee1

1Laboratory of Vitamin C and Immunology, Department of Anatomy, Seoul National University College of Medicine, 2Department of Rehabilitation Medicine, Seoul National University College of Medicine, Seoul, Korea, 3Department of Psychology, Boston College, Chestnut Hill, MA, USA, 4Division of Hematology-Oncology, Department of Internal Medicine, Ewha Womans University School of Medicine, Seoul, Korea

Abstract: The L-gulono-γ-lactone oxidase gene (Gulo) encodes an essential enzyme in the synthesis of ascorbic acid from glucose. On the basis of previous findings of bone abnormalities in Gulo−/− mice under conditions of ascorbic acid insufficiency, we investigated the effect of ascorbic acid insufficiency on factors related to bone metabolism in Gulo−/− mice. Four groups of mice were raised for 4 weeks under differing conditions of ascorbic acid insufficiency, namely, wild type; ascorbic acid-sufficient Gulo−/− mice, 3-week ascorbic acid-insufficient Gulo−/− mice, and 4-week ascorbic acid-insufficient Gulo−/− mice. Four weeks of ascorbic acid insufficiency resulted in significant weight loss in Gulo−/− mice. Interestingly, average plasma osteocalcin levels were significantly decreased in Gulo−/− mice after 3 weeks of ascorbic acid insufficiency. In addition, the tibia weight in ascorbic acid-sufficient Gulo−/− mice was significantly higher than that in the other three groups. Moreover, significant decreases in trabecular bone volume near to the growth plate, as well as in trabecular bone attachment to the growth plate, were evident in 3- or 4-week ascorbic acid-insufficient Gulo−/−. In summary, ascorbic acid insufficiency in Gulo−/− mice results in severe defects in normal bone formation, which are closely related to a decrease in plasma osteocalcin levels.

Key words: L-Gulonolactone oxidase, Ascorbic acid, Osteogenesis, Osteocalcin

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Introduction

Severe ascorbic acid-insufficiency (scurvy) results in defects in collagen synthesis [1], and is associated with connective tissue lesions such as vascular purpura, bleeding and gum abnormalities [2]. Given that organic bone matrix is composed mainly of type I collagen [3], ascorbic acid-insufficiency has been speculated to play a role in bone abnormalities. For example, inhibition of new bone formation after bone grafting, and a delay in bone regeneration after injury, have been observed in scurvy guinea pigs [4, 5]. Moreover, in humans, ascorbic acid-insufficiency causes bone abnormalities such as microfracture, osteolysis and...
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osteoporosis [6-8]. As a corollary, epidemiologic studies have shown that supplementation with ascorbic acid may enhance bone formation and overall bone health in humans [9, 10].

The synthesis of ascorbic acid from glucose in most animals negates the need for dietary intake of ascorbic acid by food. However, due to the lack of L-gulono-γ-lactone oxidase, an enzyme essential for the synthesis of ascorbic acid which catalyzes the conversion of L-gulono-γ-lactone to L-ascorbic acid [11-13], humans must obtain ascorbic acid in their diet. The ability of most experimental animals to synthesize ascorbic acid is a major impediment to the study of the in vivo effect of ascorbic acid in animals. Accordingly Gulo−/− mice, which contain a defect in the L-gulono-γ-lactone oxidase gene and are unable to synthesize ascorbic acid [14], are considered a useful model for the investigation of the in vivo effects of its supplementation. For example, we recently demonstrated the preventive effect of vitamin C on the development of acute hepatic inflammation in Gulo−/− mice [15].

During the course of a previous investigation of the general characteristics of Gulo−/− mice [16], skeletal changes, including chondrocostal junction thickening and multiple fractures, were clearly evident after 5 weeks withdrawal of ascorbic acid. To determine the specific mechanisms involved in these lesions, we investigated the effect of ascorbic acid insufficiency on bone metabolism in Gulo−/− mice.

Materials and Methods

Mice

Gulo+/− breeding pairs were obtained from the Mutant Mouse Regional Resource Center, MMRRC (University of California, Davis, CA, USA). We determined the genotypes of the offspring by polymerase chain reaction as recommended in the literature [14]. Gulo−/− and C57BL/6 wild type (WT) mice were maintained in specific pathogen free conditions in the animal facility at the Seoul National University College of Medicine. Twelve-week-old male mice were used in this experiment, and the protocol was reviewed and approved by Ethics Committee of the Seoul National University.

Supplementation and withdrawal of ascorbic acid

Gulo−/− mice were supplemented with ascorbic acid (3.3 g/l, Sigma, St. Louis, MO, USA) in water to maintain the general health of the mice for 12 weeks. The mice were divided into three groups. One group received continuous ascorbic acid supplementation until the end of the experiment, referred to as “ascorbic acid-sufficient Gulo−/− mice.” The second group underwent complete withdrawal of ascorbic acid supplementation for the last 3 weeks of the experiment, referred to as “3-week ascorbic acid-insufficient Gulo−/− mice.” The third group underwent a withdrawal of ascorbic acid supplementation for the last 4 weeks of the experiment, referred to as “4-week ascorbic acid-insufficient Gulo−/− mice.” To determine the general physiological changes in Gulo−/− mice in response to ascorbic acid insufficiency, the body weight of all mice was measured at the beginning and end of the experiment.

Biochemical analysis

At the conclusion of the experiment, blood was collected from the intra-orbital plexus with a heparinized capillary tube. Plasma was obtained from each blood sample by centrifugation at 14,000 rpm for 30 minutes. The plasma levels of calcium, phosphorus, and alkaline phosphatase (ALP) were measured with a chemistry analyzer (Hitachi 7070, Hitachi Science Systems, Ltd., Hitachinaka-shi, Japan). The plasma level of osteocalcin was measured with a mouse-specific enzyme-linked immunosorbent assay kit following the manufacturer’s protocol (Biomedical Technologies, Inc., Stoughton, MA, USA).

Determination of tibia dry weight

Mice were sacrificed by cervical dislocation under ether anesthesia. The left leg was dissected and the surrounding soft tissues were removed. After de-fatting with a mixed solution of chloroform/ethanol (2:1) for 24 hours, the tibia was flushed out with saline to remove the remnant marrow element. The fat-free tibia was then dried in an oven at 80°C over 48 hours. The tibia dry weight was measured with a microbalance (AEX-200G, Shimadzu Co., Kyoto, Japan).

Histologic examination

The dissected right tibia was fixed in 4% paraformaldehyde for 24 hours after removal of the surrounding soft tissues. Decalcification was performed with a 10% ethylenediaminetetraacetic acid solution for 5 days [17]. The fixed and decalcified tibia was embedded in paraffin and sectioned at 4 μm. Paraffin bone sections were stained with hematoxylin and eosin (Sigma) according to the manufacturer’s instruction.

Statistical analysis

Results are presented as the mean±SD. Differences among
the groups were tested using the Kruskal-Wallis test, and subsequent comparisons of each group were performed using a Mann-Whitney test. Data were analyzed using PASW for Windows ver. 18.0 (SPSS Inc., Chicago, IL, USA). All statistical outcomes were based on a two-sided test, and a $P<0.05$ was regarded as statistically significant.

**Results**

*Decreased weight of Gulo$^{-/-}$ mice after 4 weeks of ascorbic acid withdrawal*

No significant differences in the body weight between

![Graph A](A) Baseline body weight (g)

*WT, Gulo$^{-/-}$ + AsA, 3w Gulo$^{-/-}$, 4w Gulo$^{-/-}$*

![Graph B](B) Final body weight (g)

*WT, Gulo$^{-/-}$ + AsA, 3w Gulo$^{-/-}$, 4w Gulo$^{-/-}$*

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Fig. 1. Mice weight at baseline and at the conclusion of the experiment. The weight of the wild-type (WT), ascorbic acid-sufficient Gulo$^{+}$ mice (Gulo$^{+}$ + AsA), and ascorbic acid-insufficient Gulo$^{-}$ mice (Gulo$^{-}$) was measured at baseline and after 3 or 4 weeks of ascorbic acid withdrawal. (A) No significant differences in weight were observed among the groups at baseline. (B) Weight was significantly decreased in Gulo$^{-}$ mice after 4 weeks of ascorbic acid insufficiency compared with ascorbic acid-sufficient (−) mice. Data are presented as the means±SD, and each group included 5 animals. **P<0.01.

![Graph A](A) Calcium (mg/dl)

*WT, Gulo$^{-/-}$ + AsA, 3w Gulo$^{-/-}$, 4w Gulo$^{-/-}$*

![Graph B](B) Phosphorus (mg/dl)

*WT, Gulo$^{-/-}$ + AsA, 3w Gulo$^{-/-}$, 4w Gulo$^{-/-}$*

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Fig. 2. Plasma levels of calcium and phosphorus after ascorbic acid withdrawal. Plasma levels of calcium (A) and phosphorus (B) were measured in 3-week (3w Gulo$^{-}$) and 4-week (4w Gulo$^{-}$) ascorbic acid-insufficient Gulo$^{-}$ mice. No significant differences were observed among the groups. The data are presented as the means±SD, and each group included 5 animals. WT, wild-type.
Decreased plasma osteocalcin levels in Gulo<sup>−/−</sup> mice in response to ascorbic acid withdrawal

We hypothesized that the loss in body weight of the Gulo<sup>−/−</sup> mice in response to ascorbic acid withdrawal might be related to defects in bone metabolism. To test this hypothesis, we measured the plasma levels of calcium, phosphorus, ALP and osteocalcin in each of the experimental groups at the termination of the experiment. Plasma levels of calcium (Fig. 2A) and phosphorus (Fig. 2B) did not exhibit any significant differences among the groups. The plasma level of ALP, a known non-specific marker of bone formation, was higher in ascorbic acid-sufficient Gulo<sup>−/−</sup> mice than in mice in the other groups, although this failed to reach statistical significance (Fig. 3A). However, plasma levels of osteocalcin, a specific marker of bone formation, were significantly lower in both 3- and 4-week ascorbic acid-insufficient Gulo<sup>−/−</sup> mice, compared to WT and ascorbic acid-sufficient Gulo<sup>−/−</sup> mice (Fig. 3B).

Effect of ascorbic acid insufficiency on tibia dry weight in Gulo<sup>−/−</sup> mice

To examine the net effect of ascorbic acid-insufficiency on bone metabolism, tibia dry weight was measured at...
the conclusion of the experiment (Fig. 4A). The average tibia dry weight in ascorbic acid-sufficient \textit{Gulo}\(^{-}\) mice was significantly higher than that in the other three groups. No significant differences were among the WT, 3-week and 4-week ascorbic acid-insufficient \textit{Gulo}\(^{-}\) mice with respect to dry tibia weight. To adjust for the effect of general growth difference, we compared the ratio of the tibia weight to final body weight between the WT and ascorbic acid-sufficient \textit{Gulo}\(^{-}\) mice. This ratio was significantly higher in the ascorbic acid-sufficient \textit{Gulo}\(^{-}\) mice compared to WT mice (Fig. 4B).

**Effect of ascorbic acid insufficiency on trabecular bone architecture in \textit{Gulo}\(^{-}\) mice**

To evaluate alterations in bone architecture in response to ascorbic acid withdrawal, we next carried out histological examination of mice in the four groups. We observed the growth plate of the tibia in all four groups, indicating that bony growth was not complete at that age (Fig. 5). In both WT and ascorbic acid-sufficient \textit{Gulo}\(^{-}\) mice, metaphyseal trabecular bone was well organized (Fig. 5A, B) and trabecular bone was attached to the growth plate (Fig. 5E, F). However, in both the 3- and 4-week ascorbic acid-insufficient \textit{Gulo}\(^{-}\) mice, trabecular bone volume was significantly decreased near to the growth plate (Fig. 5E, F), and there was a significant decrease in trabecular bone attachment to the growth plate (Fig. 5G, H). These findings indicate a derangement in new bone formation in response to ascorbic acid insufficiency.

**Discussion**

Ascorbic acid is a well-known co-factor for collagen synthesis, and a variety of abnormalities in connective tissues, such as scurvy and chondrocostal fracture, occur under conditions of ascorbic acid insufficiency [1, 18]. The extensive physiological changes that occur in humans in response
to ascorbic acid insufficiency, including decreased total cholesterol, high density lipoproteins and catecholamine levels, and increased low density lipoproteins and weight loss [14], are recapitulated in Gulo\(^{-/-}\) mice. Several reports point to the role of ascorbic acid in the generation and maintenance of bone in animals. For example, the osteogenic disorder Shionogi rat is unable to synthesize ascorbic acid due to the lack of L-gulono-\(\gamma\)-lactone oxidase, reflecting the situation in humans and Gulo\(^{-/-}\) mice. The accumulation of malformed collagen inside the osteoblast rough endoplasmic reticulum in these rats in response to conditions of dietary ascorbic acid insufficiency, has been shown to result in detachment of osteoblasts [19]. In addition, reductions in bone hydroxyproline content and osteocalcin mRNA expression [20, 21], as well as in bone and serum ALP activity [21], have been reported in ascorbic acid-insufficient guinea pigs. Moreover, spontaneous fractures and severe impairment of osteoblast differentiation are characteristic of sfx mice, which cannot synthesize ascorbic acid due to deletion of the L-gulono-\(\gamma\)-lactone oxidase gene [22]. Collectively, these reports indicate that ascorbic acid is an important factor for maintaining the structure and function of bone, although the factors and mechanisms involved in this process have to date been largely unknown. Accordingly, we set out in this study to characterize the consequences for bone metabolism of ascorbic acid insufficiency in Gulo\(^{-/-}\) mice.

Four weeks of ascorbic acid insufficiency resulted in a decrease in body weight in Gulo\(^{-/-}\) mice (Fig. 1). However, there was no evident weight change in Gulo\(^{-/-}\) mice after 3 weeks of ascorbic acid withdrawal. This result is in agreement with our previous report on time- and organ-specific changes in in vivo ascorbic acid concentration in Gulo\(^{-/-}\) mice [16]. Interestingly, despite the absence of weight loss, there was a remarkable loss of trabecular bone volume near to the growth plate in Gulo\(^{-/-}\) mice after 3 weeks of ascorbic acid withdrawal (Fig. 5). Given that similar results have been reported in ascorbic acid-deficient guinea pigs [21, 23], it appears that ascorbic acid-insufficiency exerts its primary effect on bone metabolism.

ALP and osteocalcin are representative markers of bone formation [24]. Because plasma levels of both ALP and osteocalcin are increased by activation of osteoblasts, they are taken to directly reflect changes in bone formation in vivo [24, 25]. We observed significant decreases in plasma levels of osteocalcin, but not ALP, in Gulo\(^{-/-}\) mice in both the 3- and 4-week ascorbic acid insufficiency groups (Fig. 3A, B). This is conceivably due to the presence in the serum of these animals of an isoform of ALP expressed in the liver, which is one of the major sources of blood ALP [26]. Because ascorbic acid plays an important role in the production of a variety of essential enzymes, especially in the liver, continuous supplementation with ascorbic acid may result in increased ALP levels in mice in the ascorbic acid-supplemented Gulo\(^{-/-}\) group. While we observed no differences in serum calcium or phosphorus levels between any of the mouse groups (Fig. 2), osteocalcin production was decreased and bone volume was reduced in ascorbic acid-insufficient Gulo\(^{-/-}\) mice. It appears, therefore, that bone formation by osteoblasts was impaired in ascorbic acid-insufficient Gulo\(^{-/-}\) mice. Taken together, these data indicate that the abnormalities in bone formation in ascorbic acid-insufficient Gulo\(^{-/-}\) mice are related to a decrease in osteocalcin levels, although the exact mechanism involved requires further investigation.

Both tibia weight, and the ratio of the gross weight of the tibia to total body weight, were higher in ascorbic acid-sufficient Gulo\(^{-/-}\) than in WT and ascorbic acid-insufficient Gulo\(^{-/-}\) mice (Fig. 4A, B). Moreover, although it was not statistically significant, there was a trend towards an increase in plasma osteocalcin level in the Gulo\(^{-/-}\) ascorbic acid-sufficient mice. While the concentration of ascorbic acid in the drinking water (3.3 g/l) is higher than that intrinsically produced by mice, these data indicate that sufficient- or mega-dose uptake of ascorbic acid could enhance bone formation and maintain bone health.

Epidemiological reports of a beneficial effect of mega-doses of ascorbic acid on bone health include a positive association between ascorbic acid intake and bone mineral density (BMD) of the spine and hip in postmenopausal estrogen/progestin trials [9]. Moreover, it has been reported that elevated total ascorbic acid intake is associated with a reduction of femoral neck and trochanter BMD loss in men with low calcium or low vitamin E intake [10]. In addition, femoral neck BMD in male nonsmokers has been shown to be positively correlated with total ascorbic acid intake [10]. As a caveat, however, other studies have failed to find a positive correlation between ascorbic acid and bone health [27, 28], suggesting that further studies on this association are warranted.

Well-organized trabecular bone was observed in WT mice and ascorbic acid-sufficient Gulo\(^{-/-}\) mice (Fig. 5A, B). In addition, the attachment of trabecular bone to the growth plate in these animals was intact (Fig. 5E, F). In contrast, in ascorbic acid-insufficient Gulo\(^{-/-}\) mice, we observed striking
reductions of both trabecular bone volume in the proximal metaphysis near the growth plate, and trabecular bone attachment to the growth plate (Fig. 5C, D, G, H). Because the area just distal of the growth plate is the most active site for new bone formation, we speculate that this is the reason that the reduction in the trabecular bone was more noticeable in the proximal metaphysis near the growth plate than at any other site. On the basis of the previous reports of trabecular bone reduction in ascorbic acid insufficient animal models [18, 23, 29], we anticipated that other bony abnormalities such as cortical bone thinning, growth plate reduction, and malalignment would occur after a more prolonged period of ascorbic acid withdrawal than was evaluated in this experiment.

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References

1. Peterkofsky B. Ascorbate requirement for hydroxylation and secretion of procollagen: relationship to inhibition of collagen synthesis in scurvy. Am J Clin Nutr 1991;54(6 Suppl):1135S-40S.
2. Fain O. Musculoskeletal manifestations of scurvy: Joint Bone Spine 2005;72:124-8.
3. Niyibizi C, Eyre DR. Structural characteristics of cross-linking sites in type V collagen of bone: chain specificities and heterotypic links to type I collagen. Eur J Biochem 1994;224:943-50.
4. Cammine J, Armstrong L, Nade S. Osteogenesis after bone and bone marrow transplantation: studies of cellular behaviour using combined myelo-osseous grafts in the subscorbutic guinea pig. Acta Orthop Scand 1983;54:235-41.
5. Bourne G. The effect of graded doses of vitamin C upon the regeneration of bone in guinea-pigs on a scorbutic diet. J Physiol 1942;101:327-36.
6. Léone J, Delhinger V, Eyre DR. Structural manifestations of scurvy: a report of two cases. Rev Rhum Engl Ed 1997;64:428-31.
7. Shetty AK, Buckingham RB, Killian PJ, Girdany D, Meyerowitz R. Hemarthrosis and femoral head destruction in an adult diet faddist with scurvy. J Rheumatol 1988;15:1878-80.
8. Wapnick AA, Lynch SR, Settel HC, Charlton RW, Bothwell TH, Jowsey J. The effect of siderosis and ascorbic acid depletion on bone metabolism, with special reference to osteoporosis in the Bantu. Br J Nutr 1971;25:367-76.
9. Hall SL, Greendale GA. The relation of dietary vitamin C intake to bone mineral density: results from the PEPI study. Calcif Tissue Int 1998;63:183-9.
10. Sahni S, Hannan MT, Gagnon D, Blumberg J, Cupples LA, Kiel DP, Tucker KL. High vitamin C intake is associated with lower 4-year bone loss in elderly men. J Nutr 2008;138:1931-8.
11. Ohta Y, Nishikimi M. Random nucleotide substitutions in primate nonfunctional gene for L-gulono-gamma-lactone oxidase, the missing enzyme in L-ascorbic acid biosynthesis. Biochim Biophys Acta 1999;1472:408-11.
12. Nishikimi M, Fukuyama R, Minoshima S, Shimizu N, Yagi K. Cloning and chromosomal mapping of the human nonfunctional gene for L-gulono-gamma-lactone oxidase, the enzyme for L-ascorbic acid biosynthesis missing in man. J Biol Chem 1994;269:13685-8.
13. Nishikimi M, Kawai T, Yagi K. Guinea pigs possess a highly mutated gene for L-gulono-gamma-lactone oxidase, the key enzyme for L-ascorbic acid biosynthesis missing in this species. J Biol Chem 1992;267:21967-72.
14. Maeda N, Hagihara H, Nakata Y, Hiller S, Wilder J, Reddick R. Aortic wall damage in mice unable to synthesize ascorbic acid. Proc Natl Acad Sci U S A 2000;97:841-6.
15. Bae S, Cho CH, Kim H, Kim Y, Kim HR, Hwang YI, Yoon JH, Kang JS, Lee WJ. In vivo consequence of vitamin C insufficiency in liver injury: vitamin C ameliorates T-cell-mediated acute liver injury in Gulo(-/-) mice. Antioxid Redox Signal 2013;19:2040-53.
16. Kim H, Bae S, Yu Y, Kim Y, Kim HR, Hwang YI, Kang JS, Lee WJ. The analysis of vitamin C concentration in organs of gulo(-/-) mice upon vitamin C withdrawal. Immune Netw 2012;12:18-26.
17. Alers JC, Krijtenburg PJ, Vissers KJ, van Dekken H. Effect of bone decalcification procedures on DNA in situ hybridization and comparative genomic hybridization: EDTA is highly preferable to a routinely used acid decalcifier. J Histochem Cytochem 1999;47:703-10.
18. Sakamoto Y, Tukano Y. Morphological influence of ascorbic acid deficiency on endochondral ossification in osteogenic disorder Shionogi rat. Anat Rec 2002;268:93-104.
19. Hasegawa T, Li M, Hara K, Sasaki M, Tabata C, de Freitas PH, Hongo H, Suzuki R, Kobayashi M, Inoue K, Yamamoto T, Oohata N, Oda K, Akiyama Y, Amizuka N. Morphological assessment of bone mineralization in tibial metaphyses of ascorbic acid-deficient ODS rats. Biomed Res 2011;32:259-69.
20. Tsuchiya H, Bates CJ. Ascorbic acid deficiency in guinea pigs: contrasting effects of tissue ascorbic acid depletion and of associated inanition on status indices related to collagen and vitamin D. Br J Nutr 1994;72:745-52.
21. Mahmoodian F, Gosiewska A, Peterkofsky B. Regulation and properties of bone alkaline phosphatase during vitamin C deficiency in guinea pigs. Arch Biochem Biophys 1996;336:86-96.
22. Mohan S, Kapoor A, Singgh A, Zhang Z, Taylor T, Yu H, Chadwick RB, Chung YS, Donahue LR, Rosen C, Crawford
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Anat Cell Biol 2013;46:254-261

GC, Wergedal J, Baylink DJ. Spontaneous fractures in the mouse mutant sfx are caused by deletion of the gulonolactone oxidase gene, causing vitamin C deficiency. J Bone Miner Res 2005;20:1597-610.

23. Kipp DE, McElvain M, Kimmel DB, Akhter MP, Robinson RG, Lukert BP. Scurvy results in decreased collagen synthesis and bone density in the guinea pig animal model. Bone 1996;18:281-8.

24. Civitelli R, Armamento-Villareal R, Napoli N. Bone turnover markers: understanding their value in clinical trials and clinical practice. Osteoporos Int 2009;20:843-51.

25. Kim DY. Biochemical markers of bone turnover. Korean J Nucl Med 1999;33:341-51.

26. Green S, Anstiss CL, Fishman WH. Automated differential isoenzyme analysis. II. The fractionation of serum alkaline phosphatases into “liver”, “intestinal” and “other” components. Enzymologia 1971;41:9-26.

27. Wolf RL, Cauley JA, Pettinger M, Jackson R, Lacroix A, Leboff MS, Lewis CE, Nevitt MC, Simon JA, Stone KL, Wactawski-Wende J. Lack of a relation between vitamin and mineral antioxidants and bone mineral density: results from the Women’s Health Initiative. Am J Clin Nutr 2005;82:581-8.

28. Leveille SG, LaCroix AZ, Koepsell TD, Beresford SA, Van Belle G, Buchner DM. Dietary vitamin C and bone mineral density in postmenopausal women in Washington State, USA. J Epidemiol Community Health 1997;51:479-85.

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