Effects of Habitual Chitosan Intake on Bone Mass, Bone-Related Metabolic Markers and Duodenum CaBP D9K mRNA in Ovariectomized SHRSP Rats

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Summary We have demonstrated that the habitual intake of chitosan can decrease bone mass in ovariectomized (OVX) SHRSP rats fed a low-Ca diet (0.1%). In the present study, we examined both the etiology of bone loss induced by dietary chitosan and the preventive effect of vitamin C supplementation. Rats were OVX and maintained on one of the following diets for 6 wk: 10% cellulose (CE), 10% chitosan (CH) or 10% chitosan with sodium ascorbate (CHVC). CH caused a significant reduction in bone mineral density (BMD) and stiffness in femurs and the fourth lumbar vertebrae (L4). There was no significant difference in intestinal Ca absorption between CH and CE, whereas CH intake significantly reduced intestinal P absorption. The bone loss in CH rats was accompanied with an increase in urinary Ca excretion and a decrease in serum Ca as well as a significant increment in serum PTH and 1,25(OH)2D3. The vitamin D receptor and calcium binding protein D9K mRNAs were also significantly increased in the duodenum of CH rats. Vitamin C supplementation to CH caused an increase in the Ca and P contents of femurs as well as BMD of the L4, with a decrease in urinary Ca excretion. These results indicate that dietary chitosan with low Ca intake possibly induces the loss of bone mass by enhancing urinary Ca excretion rather than by inhibiting Ca absorption, and that vitamin C supplementation could prevent bone loss caused by chitosan through the increment of retained Ca followed by suppression of urinary Ca excretion.

Key Words dietary chitosan, OVX rats, bone loss, serum 1,25(OH)2D3, CaBP D9K mRNA

Chitosan, a polymer of glucosamine and a type of water insoluble dietary fiber, is prepared by the deacetylation of chitin, which constitutes the exoskeletons of arthropods such as crabs, shrimp, lobsters and insects (1). Chitosan has been shown to have a hypocholesterolemic effect in humans and animals (2–5), and also an inhibitory effect on intestinal fat digestion and absorption in rats (6). It has also been reported to be of low toxicity (7), and has come to be widely used as a food supplement to lower blood cholesterol and prevent weight gain. However, in spite of a number of nutritional advantages, chitosan may serve as a chelator of several minerals and hence impair their bioavailabilities. Deuchi et al. discovered that decreased intestinal absorption of calcium (Ca) and bone mineral contents of femurs were induced in male rats when fed diets containing 5% chitosan for 2 wk (8). Moreover, Wada et al., in an experiment using 47Ca, observed that there was no discriminatory difference in fecal Ca excretion in rats fed chitosan or cellulose, but a significant increase in urinary Ca excretion with chitosan intake (9). These studies suggested that dietary chitosan might influence calcium and bone metabolism through inhibiting Ca absorption and/or by accelerating its urinary excretion.

The decrease in net Ca retention as a consequence of habitual intake of chitosan with a low-Ca diet may enhance the risk of osteopenia or osteoporosis by increasing bone loss, especially in postmenopausal women. Estrogen deficiency during menopause may affect lipid metabolism and result in hyperlipemia and obesity (10). Estrogen deficiency may also accelerate bone resorption and affect intestinal Ca absorption (11). Therefore, it is very important to investigate the effect of chitosan ingestion on bone status and Ca metabolism in postmenopausal women who may use chitosan more frequently as a diet supplement to prevent hyperlipemia and obesity. Nevertheless, the effect of long-term massive intake of dietary chitosan on bone status in a postmenopausal model and Ca-related metabolic parameters have not yet been well demonstrated. According to our recent study, habitual intake of dietary chitosan can reduce femoral bone mineral density (BMD) and strength in ovariectomized (OVX) rats fed a low-Ca (0.1%) diet (12). The present study was performed to explore the etiology of bone loss induced by dietary chitosan through determining Ca and phosphorus (P) balance, hormonal responses and biochemical markers of bone turnover in OVX rats fed chitosan or cellulose for over 6 wk. The expression of intestinal vitamin D-dependent calcium binding protein 9K (CaBP D9K) as
well as vitamin D receptor (VDR) mRNAs were also examined by reverse transcription-polymerase chain reaction (RT-PCR). Moreover, considering the recent epidemiological finding that vitamin C supplement might help increase bone mass in postmenopausal women (13), the investigation was also performed to confirm whether vitamin C supplementation would reduce the aggravating action of chitosan on bone loss.

**MATERIALS AND METHODS**

**Animals and diets.** Twenty female SHRSP (Stroke-prone Spontaneously Hypertensive) rats aged 4 wk were purchased from Funabashi Farm Co. Ltd., Chiba, Japan. The animals were individually housed in a room with a 12-h/12-h light-dark cycle, where the temperature and humidity were maintained at 24±2°C and 50±5%, respectively. They were fed a commercial diet (MF, Oriental Yeast Co., Ltd., Japan) until 12-wk-old, and then ovariotomies were performed according to the procedure described previously in detail (14). The OVX rats were randomly assigned to three groups on the basis of body weight and fed one of the following experimental diets ad libitum for 6 wk: 1) 10% cellulose diet (CE; n=6), 2) 10% chitosan diet (CH; n=7), or 3) 10% chitosan diet supplemented with sodium ascorbate (CHVC; n=7). They were allowed free access to distilled water. The Ca contents in the diets were adjusted to 0.1%, though the other mineral contents were maintained as in AIN-93M (15) (Table 1). The chitosan used in the present study was purchased from Seikagaku Co., Tokyo, Japan. According to the supplier’s statement, the viscosity of chitosan was about 635 cP and the deacetylation value was about 84%. The body weight was recorded once a week and food intake recorded every other day. All the rats were transferred individually to a metabolic cage in the middle of the 6th week, and feces and urine were collected for 4 d. At the end of the experimental period, rats were sacrificed after overnight fasting under anesthesia with sodium pentobarbitate, and blood was collected. The serum obtained by centrifugal separation was stored at -30°C until analysis. Both right and left femurs and the fourth lumbar vertebrae (L4) were removed for measuring the bone status, since L4 was relatively bigger among the vertebrae and could be excised more precisely. Uterus, livers and abdominal fat were weighed. The duodenum was also excised for examining the expression of VDR and CaBP D9K mRNA. The animals were maintained in accordance with the Waseda University Guidelines for the Care and Use of Laboratory Animals.

**Biochemical measurements.** Serum alkaline phosphatase (Alp) was assayed by the colorimetric method using p-vitro-phenylphosphate as the substrate (ALP-K test kit, Wako, Japan). Serum Ca, inorganic phosphorus (iP) and magnesium (Mg) concentrations were also determined using commercial kits (Ca-test, iP-test and Mg-test kit, Wako, Japan), respectively. Serum 1α,25-dihydroxyvitamin D3 (1α,25(OH)2D3) and parathyroid hormone (PTH) levels in the CE and CH groups were assayed by radioimmunoassay (TFB, 1.25(OH)2 DRIA Kit, Immuno Diagnostic Systems Ltd., Boldon, UK) and by ELISA (Biotrak parathyroid hormone, rat, ELISA system, Amersham Pharmacia Biotech K.K., USA), respectively. Urinary deoxypyridinoline (Dpd) was quantified by ELISA (Osteolinks-DPD, MetraBiosystems Ltd., Wheatley, Oxon., UK).

**Measurement of Ca and P contents in the diets, feces and urine.** The diets and feces were lyophilized and then micropulverized. These powders were analyzed for Ca using an atomic absorption spectrometer (HITACHI, Model 7130) after wet ashing with sodium perborabitate, and blood was collected. The serum obtained by centrifugal separation was stored at -30°C until analysis. Both right and left femurs and the fourth lumbar vertebrae (L4) were removed for measuring the bone status, since L4 was relatively bigger among the vertebrae and could be excised more precisely. Uterus, livers and abdominal fat were weighed. The duodenum was also excised for examining the expression of VDR and CaBP D9K mRNA. The animals were maintained in accordance with the Waseda University Guidelines for the Care and Use of Laboratory Animals.

**Bone status.** The BMD (g/cm²) of femurs and L4 were estimated by dual energy X-ray absorptiometry (DEXA, Lunar DPXL, USA, Software version 1.0C). The strength of both right and left femurs was measured using the compression test method (TK-252C, Muromachi Kikai Co., Ltd., Japan). In the three-point bending test, we first measured the length of femurs, put a mark in the middle of them, and then set the mark on the femur directly under the load shaft. The distance between bottom shafts was set at 1.2 cm and the loading was kept on the middle shaft of the femur until it began to fracture. The test was carried out with a cross-head speed of 5 mm/min and a load

| Table 1. Composition of the experimental diets (g/100 g). |
|-----------------|---------|---------|---------|
| Ingredients     | CE      | CH      | CHVC    |
| Cornstarch      | 41.3192 | 41.3192 | 39.8192 |
| Casein          | 14      | 14      | 14      |
| d-Cornstarch    | 15.5    | 15.5    | 15.5    |
| Sucrose         | 16      | 10      | 10      |
| Soybean oil     | 4       | 4       | 4       |
| Chitosan        | —       | 10      | 10      |
| Cellulose       | 10      | —       | —       |
| Mineral mixture (Ca free) | 3.5 | 3.5 | 3.5 |
| Vitamin mixture | 1       | 1       | 1       |
| L-Cystine       | 0.18    | 0.18    | 0.18    |
| Choline bitartrate | 0.25 | 0.25    | 0.25    |
| t-Butylhydroquinone | 0.0008 | 0.0008 | 0.0008 |
| CaCO3           | 0.25    | 0.25    | 0.25    |
| Vitamin C      | —       | 1.5     | —       |
| Total           | 100     | 100     | 100     |

1. AIN-93M mineral mixture without Ca. 2. AIN-93M vitamin mixture. 3. L(+)-Ascorbic acid sodium salt (Wako, Japan) was used. 4. Atomic absorption spectrometry.
range of 50 kg (Fig. 1). Ca and iP content (%) in the femur were determined as described previously (16).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Duodenum segments of the rats were removed immediately after sacrifice and rinsed with ice-cold phosphate-buffered saline (pH 7.4). The segments were cut open, minced and homogenized in tubes. Total ribonucleic acid (RNA) was extracted by the acid guanidium-phenol-chloroform (AGPC) method using RNAzol (4 M guadine thiocyanate, 0.1 M 2-mercaptoethanol and phenol, TEL-TEST, Inc., USA). A single-strand complementary deoxyribonucleic acid (cDNA) was prepared from 1 μg of heat-denatured total RNA with a First Strand cDNA Synthesis Kit (Pharmacia Biotech, USA) and used as a template for PCR. The specific oligonucleotide sequence of VDR, CaBP D9K and glyceraldehydephosphate dehydrogenase (GAPDH) primers and products size (bp) used in the PCR are shown in Table 2. cDNA was amplified for

| Sequence | Product size (bp) | Cycles | Tm (°C) |
|----------|------------------|--------|---------|
| CaBP-D9K (+) | 5'-ATGAGCGCTAAGAAATCTCCC-3' | 237 | 24 | 51 |
| CaBP-D9K (-) | 5'-TTGCTATACTTTTGAGAAA-3' | 280 | 24 | 53 |
| VDR (+) | 5'-TGACTTTGACCGGAACGTG-3' | 194 | 26 | 53 |
| VDR (-) | 5'-ATCATCTCCTCTTTACGGTG-3' | 194 | 26 | 53 |
| GAPDH (+) | 5'-OCATGAGAAGGCCGCGG-3' | 194 | 26 | 53 |
| GAPDH (-) | 5'-CAGAAGTTGTCAAGGATGACC-3' | 194 | 26 | 53 |

Statistical analysis. Data is expressed as means±SE. Statistical analyses were performed using the SPSS statistical program. The significance of the differences were determined by the LSD multiple-range test following one-way ANOVA, and the differences between the means were considered significant at p<0.05.

RESULTS

Body weight, organ weights and total food intake

Weight gain, total food intake and organ weights of rats fed CE, CH and CHVC diets for 6 wk are given in Table 3. There were no significant differences in body weight, organ weights nor food intake among the three groups. No visible abnormal appearances in organs were observed for any of the rats.

Table 3. Body weight, food intake and organ weights of OVX rats fed the experimental diets for 6 wk.

|            | CE                  | CH                  | CHVC                 |
|------------|---------------------|---------------------|----------------------|
| Initial body weight (g) | 188.3±3.0           | 192.5±4.0           | 192.4±3.7            |
| Final body weight (g)  | 230.0±3.7           | 232.1±4.9           | 230.2±2.6            |
| Body weight gain (g)   | 41.6±2.8            | 39.6±3.2            | 37.8±2.9             |
| Food intake (g/6 wk)   | 570±6               | 576±11              | 574±12               |
| Liver (g)             | 5.66±0.2            | 5.26±0.20           | 5.45±0.07            |
| Uterus (g)            | 0.12±0.02           | 0.10±0.01           | 0.11±0.02            |
| Abdominal fat (g)     | 7.99±0.48           | 7.53±1.01           | 6.53±0.48            |

Values are means±SE (CE: n=6, CH, CHVC: n=7). No significant difference was found among the three groups. CE: cellulose, CH: chitosan, CHVC: chitosan with vitamin C.
Table 4. Calcium and phosphorus balances of OVX rats fed the experimental diets for 6 wk.

|                  | CE          | CH          | CHVC         |
|------------------|-------------|-------------|--------------|
| Ca Intake (mg/4 d) | 46.4±1.3    | 52.4±2.1    | 51.5±1.0     |
| Fecal excretion (mg/4 d) | 28.7±1.6    | 29.7±1.9    | 27.7±1.4     |
| Urinary excretion (mg/4 d) | 5.2±0.3     | 12.6±0.6*** | 8.8±0.7***   |
| Total excretion (mg/4 d) | 33.9±1.6    | 42.4±2.0    | 36.5±1.5     |
| Absorption (mg/4 d) | 17.8±0.9    | 22.7±1.8    | 23.8±1.6     |
| Absorption rate (%) | 38.4±2.2    | 43.3±2.8    | 46.2±2.8*    |
| Retention (mg/4 d) | 12.6±0.8    | 10.1±1.8    | 15.0±1.6     |
| Retention rate (%) | 27.3±2.0    | 19.0±3.1*   | 29.1±3.0***  |

Values are means±SE (CE: n=6, CH, CHVC: n=7). ***p<0.001, **p<0.01, *p<0.05 vs. CE. ##p<0.01 vs. CH. Feces and urine were collected for 4 d. Absorption: Intake-fecal excretion. Absorption rate: [(Intake-fecal excretion)/intake]×100. Retention: Intake-total excretion. Retention rate: [(Intake-total excretion)/intake]×100. CE: cellulose, CH: chitosan, CHVC: chitosan with vitamin C.

Ca and P balance

The results of the Ca and P balance study through 4 d in the 6th week during the feeding period are shown in Table 4. There were no significant differences in Ca intake as well as fecal Ca excretion among the three groups. In the CH group, the Ca absorption rate was slightly greater than that in the CE group, but not significantly. The rats fed the CH diet with vitamin C supplementation showed a significant increase in Ca absorption rate, 46.2±2.8%, as compared to 38.4±2.2% in the CE counterparts (p<0.05). Ca retention rate was significantly lower in the CH group than in the CE group (19.0±3.1 vs. 27.3±2.0, p<0.05) because urinary Ca excretion was significantly increased to 12.6±0.6 mg/4 d by chitosan intake as compared to 5.2±0.3 mg/4 d in the CE group (p<0.001). The increased urinary Ca excretion caused by chitosan was significantly suppressed by vitamin C supplementation, from 12.6±0.6 to 8.8±0.7 mg/4 d (p<0.01), and the Ca retention rate also recovered to the level seen in the control group (CE).

The intake of P in the CE group was significantly higher than that in both chitosan groups (p<0.001) because the cellulose used in this study contained a remarkably large P content as compared to chitosan. In spite of a significantly lower intake of P in the CH and CHVC groups, fecal P excretion increased significantly to 42.3±2.1 mg/4 d in the CH group (p<0.05) and 44.3±2.6 mg/4 d in the CHVC group (p<0.05) as compared to 32.4±2.5 mg/4 d in the CE group. Consequently, the absorption and retention rates of P in both chitosan groups were significantly reduced by 10 (p<0.001) and 35% (p<0.001), respectively, as compared to those in the CE group.

Bone parameters

The bone status in femurs and L4 is presented in Fig. 2. All of the measured properties of femurs decreased by 8% in BMD, 14% in strength, 8% in Ca content and 4% in P content as well as those of L4 being reduced by 13% in BMD and 26% in strength, respectively, in the CH group as compared to measurements of the CE group. Vitamin C addition to the CH diet induced a significant increase in femoral Ca contents as compared to the CH group (p<0.01). Vitamin C supplementation also significantly raised the femoral P content (p<0.05) and L4 BMD (p<0.05) in the CH group to the level of the CE group. The stiffness of L4 did not differ among the three groups, although measurements in the CH group tended to be lower than those of the other groups.

Serum parameters

Serum mineral levels and Ca-related metabolic parameters are shown in Table 5. Serum Ca levels in both chitosan groups (CH and CHVC), which caused a significant increase in urinary Ca excretion (Table 4), were significantly lower than that in the CE group (p<0.05). In contrast to serum Ca, serum P levels in the CH and CHVC groups were significantly higher than that in the CE group (p<0.05 and p<0.01, respectively). With respect to serum Mg, there was no significant difference among the three groups. Alp activity in the CH and CHVC groups was significantly lower compared to that in the CE group (p<0.001). The serum PTH level increased by 43% in the CH group as compared to that in the CE group, though no significant difference was observed. The serum 1α,25(OH)2D3 level was highest in
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Fig. 2. Effects of the experimental diets on bone properties of femurs and fourth lumbar vertebrae (L4) in OVX rats. Values are means±SE (CE: n=6, CH, CHVC: n=7). Femoral Ca contents: [Ca (mg)/dry femur weight (mg)]×100. Femoral P contents: [P (mg)/dry femur weight (mg)]×100. *** p<0.001, ** p<0.01, * p<0.05 vs. CE. ## p<0.01, # p<0.05 vs. CH. BMD: bone mineral density, L4: fourth lumbar vertebrae.

Table 5. Biochemical parameters in serum and urine of OVX rats fed the experimental diets for 6 wk.

|                | CE   | CH   | CHVC  |
|----------------|------|------|-------|
| Serum          |      |      |       |
| Calcium (mg/dL)| 10.4±0.1 | 9.6±0.3* | 9.4±0.2* |
| Phosphatase (mg/dL) | 4.0±0.8 | 6.7±1.1* | 8.0±1.0** |
| Magnesium (mg/dL) | 2.3±0.1 | 2.0±0.1 | 2.1±0.1 |
| Alkaline phosphatase (IU/L) | 142.7±20.1 | 67.8±2.9*** | 68.2±2.0*** |
| PTH (pg/mL)     | 26.2±9.9 | 46.2±6.3 | ---    |
| 1,25-Dihydroxvitamin D (pg/mL) | 102.4±12.7 | 359.7±18.6*** | 286.8±13.6***## |
| Urine           |      |      |       |
| Dpd/creatinine (nmol/mmol) | 418.5±19.0 | 447.0±26.8 | 474.7±13.3 |

Values are means±SE (CE: n=6, CH, CHVC: n=7). *** p<0.001, ** p<0.01, * p<0.05 vs. CE. ## p<0.01 vs. CH. CE: cellulose, CH: chitosan, CHVC: chitosan with vitamin C. PTH: parathyroid hormone. Dpd: deoxypyridinoline.

the CH group, being elevated approximately 3.5-fold compared to the control (CE) value. Urinary Dpy, one of the bone resorption markers, tended to be higher in the CH and CHVC groups as compared to that in the CE group, but the difference was not significant.

RT-PCR analysis of VDR and CaBP D9K mRNA expression in the duodenum

VDR and CaBP D9K mRNA expression in the duodenum were examined by RT-PCR analysis. The results were evaluated as the ratio of VDR to GAPDH mRNA and CaBP D9K to GAPDH mRNA (Fig. 3). Chitosan intake induced a significant increase in VDR and CaBP D9K mRNA expression in both the CH and CHVC groups as compared to the CE group (p<0.05). There was no significant difference between the CH and CHVC groups.

DISCUSSION

In the previous study, we obtained some evidence that the habitual intake of chitosan can decrease bone mass in ovariectomized (OVX) rats fed a low-Ca (0.1%) diet (12). The present investigation was undertaken to illuminate the influences of dietary chitosan on Ca and P balances as well as some biochemical markers related to bone loss, and to examine whether vitamin C supplementation could help to prevent bone loss caused by CH intake.

In the present study, ovariectomized SHRSP rats at the initial age of 12 wk were used as a postmenopausal model because bone mass begins to decline sponta-
neously after the age of 3 mo, and thereafter bone loss advances with aging. On the other hand, bone loss development with physiological aging is very small, and senile osteoporosis as seen in humans does not occur in normal rats (17). Therefore, OVX SHRSP rats are most likely to have similar responses to postmenopausal women who spontaneously lose bone mass with aging.

In the present study, chitosan feeding induced a significant reduction in BMD, strength, Ca and P contents in femurs as well as in the BMD and strength of L4 in OVX SHRSP rats subjected to low-Ca intake (Fig. 2). Chitosan has been reported to chelate Ca and other minerals, thus interfering with their intestinal absorption (8, 18). In the present experiment, rats were fed a diet containing 10% chitosan for 6 wk, and Ca and P balances were examined in the middle of the 6th week. The results obtained showed that urinary Ca excretion increased about 2.4-fold in the CH group as compared to that in the CE group even though there was no definite difference in the fecal Ca excretion, or Ca absorption rates between the CH and CE groups, as shown in Table 4. This evidence implies that chitosan operates to accelerate the excretion of endogenous Ca rather than to inhibit its intestinal absorption, and as a result, causes bone loss. Our results are consistent with those of Wada et al. (9), who found that whole body retention of $^{47}$Ca by rats on a 5% chitosan diet was significantly decreased due to enhanced urinary excretion of radiolabeled Ca. Chitosan induced hypercalcuria may reflect, in part, higher bone resorption. In fact, in the present investigation, urinary deoxypyridinoline (Dpd), a bone resorption marker, tended to be greater in the CH group than in the CE group (Table 5). The urinary Ca increment in the CH group was accompanied with a decrease in serum Ca and an increase in circulating PTH and 1,25-dihydroxyvitamin D (1,25(OH)$_2$D$_3$) levels (Table 5). These results suggest that increased urinary Ca excretion as the result of CH intake induced a decline in the serum Ca level, and as a consequence, PTH secretion and subsequent 1,25(OH)$_2$D$_3$ production were stimulated.

In the present study, the mechanism by which chitosan intake accelerates urinary Ca excretion could not be well explained, but one hypothesis could be proposed from the results of P balance: chitosan intake causes a significant reduction in intestinal P absorption (Table 4). That is, in response to a decrease in available iP in the body fluid caused by chitosan feeding, P and Ca are concurrently released from bone into the blood, and then relatively excessive Ca is excreted into urine. Indeed, we obtained experimental results that urinary Ca excretion was significantly increased in female Wistar rats fed a low-P (0.1%) diet for 4 wk as compared to that of a normal P (0.3%) diet (unpublished data). On the other hand, Wada et al. (9) suggested that absorbed fermentation products of chitosan by intestinal bacteria might stimulate Ca mobilization from the bone. Further study on the effect of dietary chitosan on Ca metabolism will be necessary in the future.

Although most of the published reports have described that dietary chitosan inhibits the intestinal absorption of Ca, and thus increases fecal Ca excretion (8, 18, 19), our present experiments show that there was no difference in intestinal Ca absorption between the CH and CE groups, at least after a prolonged period of feeding. The active transport of Ca requires the presence...
of CaBP D9K (20), and 1,25(OH)2D3 induces the synthesis of CaBP D9K via VDR (21, 22), thus contributing to intestinal Ca absorption. VDR and CaBP D9K mRNAs in the duodenum of rats were examined by RT-PCR analysis. In the present study, the rats fed a CH diet revealed a 3.5-fold increase in serum 1.25(OH)2D3 (Table 5) and a remarkable increase in VDR and CaBP D9K mRNA (Fig. 3). These findings might support an explanation for why intestinal Ca absorption was not depressed by chitosan feeding.

The effects of vitamin C supplementation on Ca and P balance and bone mass were also examined in OVX SHRSP rats fed on 10% chitosan diet. In the rats fed a diet containing chitosan and 1.5% vitamin C, femoral Ca and P contents increased by approximately by 5 and 3%, respectively, as compared to those of rats fed chitosan alone. Moreover, the BMD and strength of L4 were also elevated to the control (CE) level. Vitamin C, however, didn’t exert a positive effect on femoral BMD and strength. Vitamin C has been reported to stimulate intestinal Ca absorption because of the chelating action of ascorbate with calcium ion (23, 24). And Deuchi et al. (8) found that, in rats fed a diet containing 5% chitosan, 1.5% sodium ascorbate supplementation could increase Ca absorption. In the present study, there was no significant difference in Ca absorption between the CH and CHVC groups. These findings are in accordance with previous results that Ca and P balances in pigs on a diet supplemented with vitamin C (1,000 mg/kg) did not differ from those of pigs fed a control diet, and also that vitamin C supplementation had no effect on the plasma concentration of Ca, IP or 1,25(OH)2D3 (25). Thus, decreased serum Ca and increased serum P as well as the 1.25(OH)2D3 level in the CHVC group were most likely induced not by vitamin C supplementation but by chitosan ingestion.

The present study also showed that urinary Ca excretion was lower by approximately 30% in the CHVC group than in the CH group, thus showing an improvement in the Ca retention rate. Therefore, vitamin C supplementation is thought to prevent bone loss by reducing Ca mobilization from the bone, which was brought on by chitosan ingestion. Vitamin C is known to stimulate type I collagen matrix synthesis, osteocalcin accumulation and matrix mineralization in osteoblast cultures (26). Since osteocalcin and carboxyterminal propeptide of type I collagen (PICP) were not assessed in the current study, we are unable to prove whether or not the increased bone mass in L4 caused by vitamin C supplementation was due to increased synthesis of the bone matrix. Further investigation will be required in this respect.

In conclusion, the results obtained from the present investigation indicate that dietary chitosan with a low-Ca intake can probably induce bone loss by enhancing urinary Ca excretion rather than inhibiting Ca absorption. Vitamin C supplementation may help to prevent bone loss of the fourth lumbar vertebrae through the increment of retained Ca followed by the suppression of urinary Ca excretion. Chitosan is being advertised as a diet supplement that can prevent obesity. From the findings shown above, attention must be given to whether habitual massive chitosan dosages are used with a low-Ca diet, especially in the case of postmenopausal women.

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