

Oral administration of β -cryptoxanthin prevents bone loss in ovariectomized rats

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Abstract. The effect of β -cryptoxanthin, a kind of carotenoid, on ovariectomy-induced bone loss was investigated. β -Cryptoxanthin was isolated from Satsuma mandarin (*Citrus unshu*, MARC). β -Cryptoxanthin (5 or 10 μ g/100 g body weight) was orally administered once daily for 3 months to ovariectomized (OVX) rats. OVX induced a significant increase in body weight and a significant decrease in serum calcium and inorganic phosphorus concentrations as compared with those of sham-operated (control) rats. These alterations induced by OVX were significantly prevented by the administration of β -cryptoxanthin (5 or 10 μ g/100 g). The analysis using a peripheral quantitative computed tomography (pQCT) showed that OVX induced a significant decrease in mineral content and mineral density in the femoral-diaphyseal and -metaphyseal tissues and polar strength strain index in the metaphyseal tissues. These decreases were significantly prevented by the administration of β -cryptoxanthin (5 or 10 μ g/100 g). Moreover, OVX induced a significant decrease in calcium content and alkaline phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues and deoxyribonucleic acid (DNA) content in the metaphyseal tissues. These decreases were significantly prevented by the administration of β -cryptoxanthin (5 or 10 μ g/100 g). This study demonstrates that β -cryptoxanthin has a preventive effect on OVX-induced bone loss *in vivo*.

Introduction

Bone loss with aging induces osteoporosis, which is widely recognized as a major public health problem (1-3). The decrease

in bone mass leads to bone fracture. Bone loss may be due to decreased bone formation and increased bone resorption. Pharmacological and nutritional factors are needed to prevent bone loss with increasing age. Chemical factors in food may help to prevent bone loss with aging (4). The chemical compounds in food that act on bone metabolism, however, are poorly understood.

Retinal (vitamin A) is known to have a detrimental effect on bone at high doses. In laboratory animals, high levels of vitamin A lead to accelerated bone resorption, bone fractures, and osteoporotic bone lesions (5-7). The effects of carotenoids on bone metabolism, however, have not been fully clarified. Carotenoids are present in fruit and vegetables. More recently, it has been shown that β -cryptoxanthin has a unique anabolic effect on bone calcification *in vitro* (8). Lutein, lycopene, and β -carotene do not have an effect on bone calcification *in vitro* (8,9). β -Cryptoxanthin has a direct stimulatory effect on bone formation and an inhibitory effect on bone resorption in cultured bone tissue *in vitro* (9).

β -Cryptoxanthin has been shown to have a stimulatory effect on proliferation, differentiation and mineralization due to enhancing the gene expression of proteins [including Runx2 type 1 and type 2, alkaline phosphatase and α 1 (I) collagen], which are involved in bone formation in osteoblastic MC3T3-E1 cells (10,11). Moreover, β -cryptoxanthin has been demonstrated to have a potent inhibitory effect on osteoclast-like cell formation in mouse marrow culture *in vitro* (12). The inhibitory action of β -cryptoxanthin on osteoclastic bone resorption may partly involve in a newly synthesized protein component that is related to receptor activator of NF- κ B ligand (RANKL) stimulation in osteoclastogenesis (12). Thus, the cellular mechanism by which β -cryptoxanthin has a stimulatory effect on osteoblastic bone formation and an inhibitory effect on osteoclastic bone resorption has been clarified.

The oral administration of β -cryptoxanthin has been shown to induce an anabolic effect on bone components in the femoral-diaphyseal (cortical bone) and -metaphyseal (trabecular bone) tissues in young and aged rats *in vivo* (13,14). Moreover, it has been found that streptozotocin-induced bone loss in rats with a diabetic state is prevented by the oral administration of β -cryptoxanthin *in vivo* (15). The prolonged intake of dietary β -cryptoxanthin has been shown to have a stimulatory effect on bone formation and an inhibitory effect on bone resorption due to estimating serum biochemical markers of

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bone metabolism in normal individuals (16). β -Cryptoxanthin may have a preventive effect on osteoporosis with increasing age.

The present study was undertaken to determine the preventive effect of oral administration of β -cryptoxanthin on induced bone loss in OVX rats. We found that OVX-induced bone loss is prevented by oral administration of β -cryptoxanthin once daily for 3 months.

Materials and methods

Chemicals. β -Cryptoxanthin (100% purity) was supplied by Ehime Beverage Inc. (Matsuyama, Japan). All other chemicals were reagent grade from Sigma Chemical (St. Louis, MO, USA) and Wako Pure Chemical Industries (Osaka, Japan). All water used was glass-distilled.

Animals. Female Wistar rats (6 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). The six animals in each group were fed commercial laboratory chow (solid) containing 57.4% carbohydrate, 1.1% calcium, and 1.1% phosphorus at a room temperature of 25°C, and were given distilled water freely. Rats were given a sham OVX or bilateral OVX under ether anesthesia (11). The sham-operated animals were fed matched amounts of chow for 1 week, and then used in the experiment.

Administration procedure. β -Cryptoxanthin was dissolved in corn oil. A concentration of 10 or 20 $\mu\text{g}/0.5\text{ ml}/100\text{ g}$ body weight was orally administered to rats through a stomach tube once daily for 3 months. Control rats received corn oil (0.5 ml/100 g body weight) orally. The animals were sacrificed by cardiac puncture under light ether anesthesia at 24 h after the last administration, and the blood and femur removed immediately.

Peripheral quantitative computed tomography (pQCT) for the femur. The femur was removed, and immediately immersed in 70% ethanol solution. Femoral-diaphyseal and -metaphyseal mineral content, mineral density, polar strength strain index and diaphyseal cortical thickness were measured by using pQCT (XCT Research SA⁺ Stratec Medizintechnik GmbH, Pforzheim, Germany). The measurement with pQCT for femoral metaphysis was carried out at 3.0 mm from the growth plate. The measurement for femoral diaphyseal was carried out at half the femoral length.

Assay of serum and bone biochemical components. Blood samples obtained by cardiac puncture were centrifuged 30 min after collection, and the serum was separated. Serum was frozen at -80°C until assay. Serum calcium and inorganic phosphorus concentrations were determined using an assay kit (Wako Pure Chemical Industries).

The diaphyseal or metaphyseal tissues were dried for 16 h at 110°C. Calcium was determined by atomic absorption spectrophotometry (8). The calcium content in bone tissue was expressed as mg/g of dry bone.

To assay alkaline phosphatase activity, the diaphyseal and metaphyseal tissues were immersed in 3.0 ml of ice-cold barbital buffer 6.6 mM (pH 7.4), cut into small pieces, and

Table I. Change in body weight and serum component levels in ovariectomized (OVX) rats orally administered β -cryptoxanthin.

Treatment	Body weight (g)	Serum level (mg/dl)	
		Calcium	Inorganic phosphorus
Control	207.7 \pm 3.2	10.02 \pm 0.16	4.83 \pm 0.14
OVX	246.3 \pm 5.7 ^a	9.32 \pm 0.10 ^a	4.09 \pm 0.25 ^a
OVX+ β -cryptoxanthin			
5 $\mu\text{g}/100\text{ g}$	233.0 \pm 4.3 ^a	9.41 \pm 0.10 ^a	4.50 \pm 0.23 ^a
10 $\mu\text{g}/100\text{ g}$	214.2 \pm 3.7 ^b	9.87 \pm 0.09 ^b	5.38 \pm 0.19 ^b

Female rats (6 weeks old) were ovariectomized, and 1 week later the animals were orally administered β -cryptoxanthin (5 or 10 $\mu\text{g}/100\text{ g}$ body weight) once a daily for 3 months. Rats were killed by bleeding 24 h after the last administration. Each value is the mean \pm SEM of 6 rats. ^aP<0.01 compared with the control value. ^bP<0.01 compared with the value obtained from OVX.

disrupted for 60 sec with an ultrasonic device. The supernatant centrifuged at 600 x g for 5 min was used to measure enzyme activity. Enzyme assay was carried out under optimal conditions. Alkaline phosphatase activity was determined using the method of Walter and Schutt (17). Enzyme activity was expressed as μmol of liberated p-nitrophenol/min/mg of protein. Protein concentration was determined by the method of Lowry *et al* (18).

To measure bone DNA content, the diaphyseal and metaphyseal tissues were shaken with 4.0 ml of ice-cold 0.1 N NaOH solution for 24 h after the homogenization of the bone tissue (19). After alkaline extraction, the samples were centrifuged at 1000 μg for 5 min, and the supernatant was determined by the methods of Ceriotti (20) and expressed as the amount of DNA (mg)/g wet weight of bone tissue.

Statistical analysis. The significance of differences between values was estimated by Student's t-test. We also used a multiple ANOVA to compare the treatment groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Change in body weight and serum components in OVX rats administered β -cryptoxanthin. The change in body weight, serum calcium and inorganic phosphorus concentration in OVX rats orally administered β -cryptoxanthin is shown in Table I. β -Cryptoxanthin (5 or 10 $\mu\text{g}/100\text{ g}$ body weight) was orally administered to OVX rats at one week after OVX, and then once daily for 3 months. OVX induced a significant increase in body weight as compared with that of sham-operated (control) rats. This increase was significantly

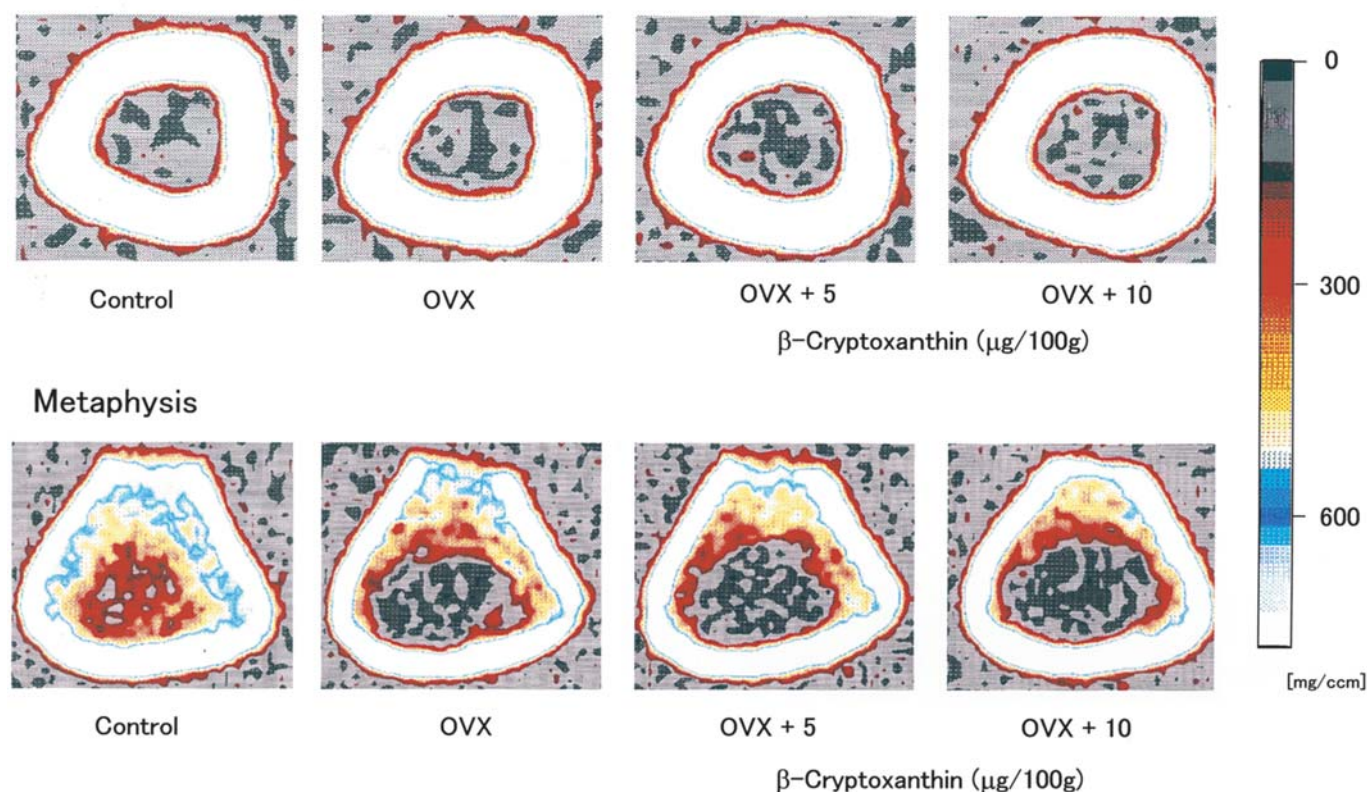


Figure 1. Analysis of bone morphological change in the femoral-diaphyseal and -metaphyseal tissues of ovariectomized (OVX) rats orally administered β -cryptoxanthin. β -Cryptoxanthin (5 or 10 $\mu\text{g}/100\text{ g}$ body weight) was orally administered once daily for 3 months to OVX rats. Peripheral quantitative computed tomography (pQCT) analysis was carried out on the femurs of control (sham operated), OVX or β -cryptoxanthin-administered OVX rats. The figure shows one of six experiments with separate rats.

prevented by oral administration of β -cryptoxanthin (10 $\mu\text{g}/100\text{ g}$).

Serum calcium and inorganic phosphorus concentrations were significantly decreased in OVX rats as compared with that of control (sham operated) rats. This decrease was significantly prevented by oral administration of β -cryptoxanthin (10 $\mu\text{g}/100\text{ g}$) to OVX rats.

Bone morphological change in OVX rats administered β -cryptoxanthin. The morphological change in the femoral-diaphyseal and -metaphyseal tissues of OVX rats was examined using a peripheral quantitative computed tomography (pQCT). Morphological change was observed in the femoral-metaphyseal tissue of OVX rats (Fig. 1). This change was significantly restored by oral administration of β -cryptoxanthin (5 or 10 $\mu\text{g}/100\text{ g}$ body weight) once daily for 3 months. Morphological change was not seen in the femoral-diaphyseal tissue in OVX rats and β -cryptoxanthin-administered OVX rats, as compared with that of control (sham operated) rats.

The change in mineral content, mineral density and polar strength strain index in the femoral-diaphyseal and -metaphyseal tissues of OVX rats was examined by using pQCT analysis. Mineral content in the femoral-diaphyseal and -metaphyseal tissues was significantly decreased in OVX rats (Fig. 2). This decrease was significantly prevented by oral administration of β -cryptoxanthin (5 or 10 $\mu\text{g}/100\text{ g}$) for 3 months.

Mineral density in the femoral-diaphyseal and -metaphyseal tissues was significantly decreased in OVX rats as compared with that of control (sham operated) rats (Fig. 3). This decrease was significantly prevented by oral administration of β -cryptoxanthin (5 or 10 $\mu\text{g}/100\text{ g}$) for 3 months.

The change in femoral polar strength strain index is shown in Fig. 4. The polar strength strain index in femoral-metaphyseal tissue was significantly decreased in OVX rats. Such a decrease was not seen in the femoral-diaphyseal tissue of OVX rats. OVX-induced decrease in the polar strength strain index in femoral-metaphyseal tissue was significantly prevented by oral administration of β -cryptoxanthin (5 or 10 $\mu\text{g}/100\text{ g}$).

Change in bone biochemical components in OVX rats administered β -cryptoxanthin. The change in calcium content, alkaline phosphatase activity, and DNA content in the femoral-diaphyseal and -metaphyseal tissues of OVX rats was examined. Calcium content in the femoral-diaphyseal and -metaphyseal tissues was significantly decreased in OVX rats as compared with that of control (sham operated) rats. This decrease was significantly prevented by oral administration of β -cryptoxanthin (5 or 10 $\mu\text{g}/100\text{ g}$ body weight) for 3 months (Fig. 5).

Alkaline phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues in OVX rats was not significantly changed as compared with that of the control (sham operated)

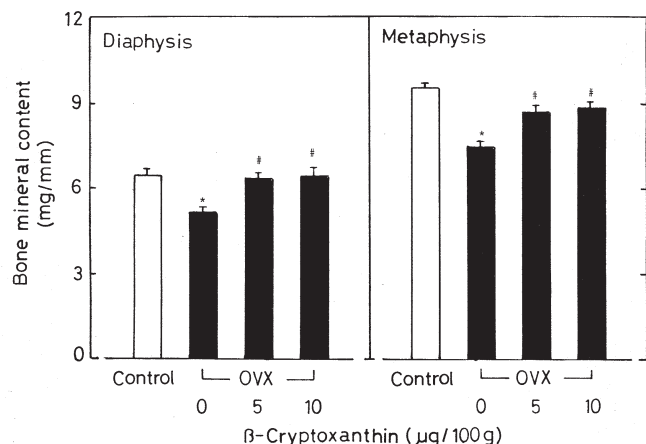


Figure 2. Change in bone mineral content in the femoral-diaphyseal and -metaphyseal tissues of ovariectomized (OVX) rats orally administered β -cryptoxanthin. β -Cryptoxanthin (5 or 10 $\mu\text{g}/100\text{ g}$ body weight) was orally administered once daily for 3 months to OVX rats. Bone mineral content was measured by using pQCT. Each value is the mean \pm SEM of six experiments with separate rats. * $P < 0.01$ compared with the control value. # $P < 0.01$ compared with the value obtained from OVX (control) rats.

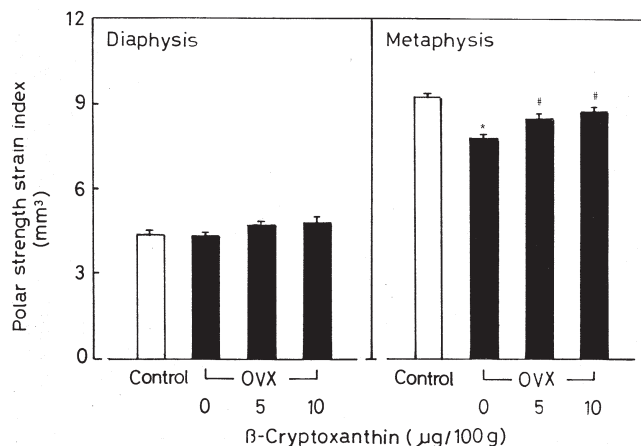


Figure 4. Change in polar strength strain index in the femoral-diaphyseal and -metaphyseal tissues of ovariectomized (OVX) rats orally administered β -cryptoxanthin. β -Cryptoxanthin (5 or 10 $\mu\text{g}/100\text{ g}$) was orally administered once daily for 3 months to OVX rats. Bone polar strength strain index was measured by using pQCT. Each value is the mean \pm SEM of six experiments with separate rats. * $P < 0.01$ compared with the control value. # $P < 0.025$ compared with the value obtained from OVX (control) rats.

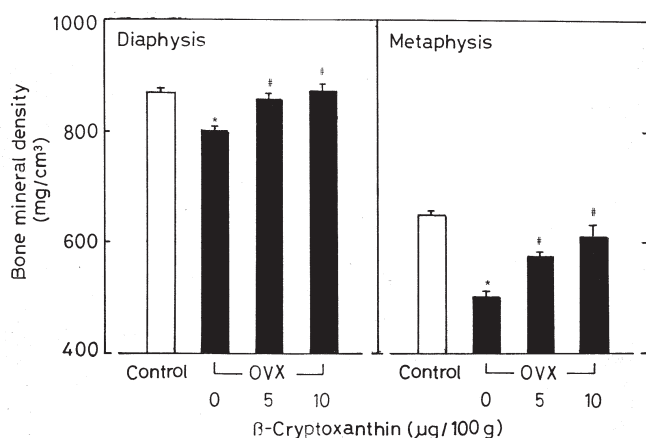


Figure 3. Change in bone mineral density in the femoral-diaphyseal and -metaphyseal tissues of ovariectomized (OVX) rats orally administered β -cryptoxanthin. β -Cryptoxanthin (5 or 10 $\mu\text{g}/100\text{ g}$) was orally administered once daily for 3 months to OVX rats. Bone mineral density was measured by using pQCT. Each value is the mean \pm SEM of six experiments with separate rats. * $P < 0.01$ compared with the control value. # $P < 0.01$ compared with the value obtained from OVX (control) rats.

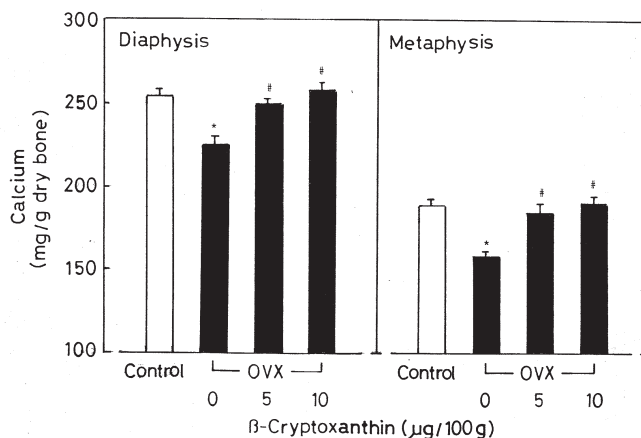


Figure 5. Change in calcium content in the femoral-diaphyseal and -metaphyseal tissues of ovariectomized (OVX) rats orally administered β -cryptoxanthin. β -Cryptoxanthin (5 or 10 $\mu\text{g}/100\text{ g}$ body weight) was orally administered once daily for 3 months to OVX rats. Each value is the mean \pm SEM of six experiments with separate rats. * $P < 0.01$ compared with the control value. # $P < 0.01$ compared with the value obtained from OVX (control) rats.

(Fig. 6). Oral administration of β -cryptoxanthin (10 $\mu\text{g}/100\text{ g}$) for 3 months caused a significant increase in alkaline phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues of OVX rats.

The change in DNA content in the femoral-diaphyseal and -metaphyseal tissues of OVX rats is shown in Fig. 7. OVX caused a significant decrease in DNA content in femoral-metaphyseal tissue as compared with that of the control (sham operated). This decrease was significantly prevented by oral administration of β -cryptoxanthin (5 or 10 $\mu\text{g}/100\text{ g}$). Diaphyseal DNA content was not significantly changed in OVX rats. However, the oral administration of β -cryptoxanthin (5 or 10 $\mu\text{g}/100\text{ g}$) to OVX rats caused a significant increase in DNA content in the femoral-diaphyseal and -metaphyseal tissues as compared with that of control (sham operated) rats.

Discussion

Prolonged oral administration was found to have a preventive effect on the increase in body weight and the decreases in serum calcium and inorganic phosphorus concentrations, bone mineral content, bone mineral density, polar strength strain index and bone biochemical components induced in OVX rats. Moreover, OVX-induced morphological changes were clearly restored by oral administration of β -cryptoxanthin. These observations demonstrate that oral administration of β -cryptoxanthin can prevent bone loss in OVX rats. β -Cryptoxanthin has been shown to have a stimulatory effect on osteoblastic bone formation (8-11) and an inhibitory effect on osteoclastic bone resorption (8,12) *in vitro*. Presumably, the preventive effect of β -cryptoxanthin on OVX-induced bone

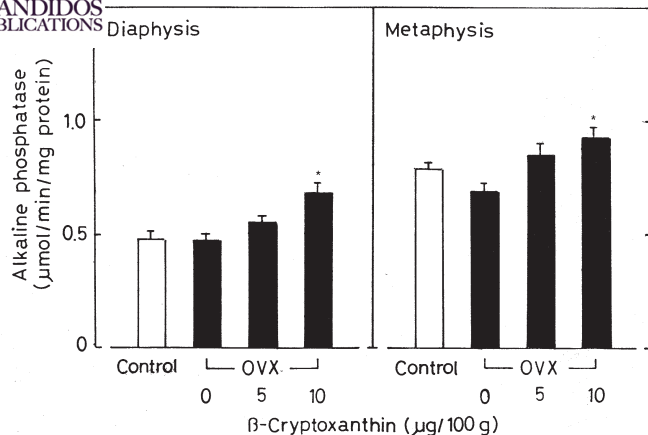


Figure 6. Change in alkaline phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues of ovariectomized (OVX) rats orally administered β -cryptoxanthin. β -Cryptoxanthin (5 or 10 $\mu\text{g}/100\text{ g}$) was orally administered once daily for 3 months to OVX rats. Each value is the mean \pm SEM of six experiments with separate rats. * $P < 0.01$ compared with the control value.

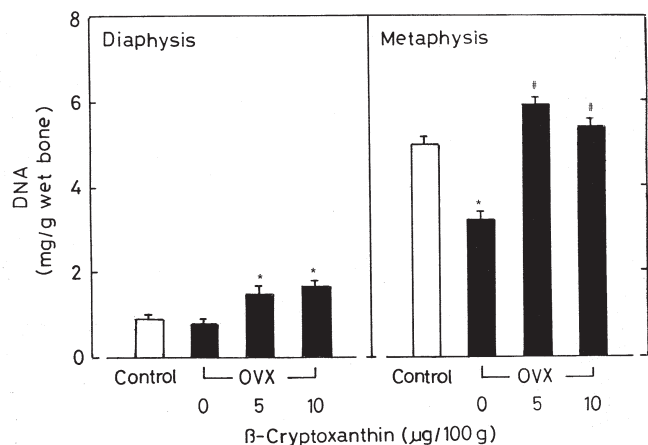


Figure 7. Change in DNA content in the femoral-diaphyseal and -metaphyseal tissues of ovariectomized (OVX) rats orally administered β -Cryptoxanthin. β -cryptoxanthin (5 or 10 $\mu\text{g}/100\text{ g}$) was orally administered once daily for 3 months to OVX rats. Each value is the mean \pm SEM of six experiments with separate rats. * $P < 0.01$ compared with the control value. # $P < 0.01$ compared with the value obtained from OVX (control) rats.

loss results from increased bone formation and decreased bone resorption by β -cryptoxanthin.

Bone loss was remarkable in the femoral-metaphyseal (trabecular bone) tissue of OVX rats. Biochemical components (including calcium, alkaline phosphatase and DNA) in the femoral-metaphyseal tissue were significantly decreased in OVX rats. These decreases were clearly restored by oral administration of β -cryptoxanthin. β -Cryptoxanthin (10^{-7} or 10^{-6} M) has been shown to enhance the expression of Runx2 (type I and type II), alkaline phosphatase and $\alpha 1$ (I) collagen mRNAs in osteoblastic MC3T3-E1 cells *in vitro* (11). Moreover, β -cryptoxanthin (10^{-7} or 10^{-6} M) can stimulate the expression of insulin-like growth factor-I (IGF-I) and transforming growth factor- $\beta 1$ (TGF- $\beta 1$) mRNAs in osteoblastic MC3T3-E1 cells *in vitro* (10). As a result, β -cryptoxanthin stimulates proliferation, differentiation and mineralization

in osteoblastic MC3T3-E1 cells (10,11). It is possible that β -cryptoxanthin stimulates osteoblastic bone formation in OVX rats.

The decrease in serum calcium and inorganic phosphorus concentration was observed in OVX rats. OVX-induced hypocalcemia may cause acceleration of bone resorption which is involved in secretion of the parathyroid hormone. These decreases were significantly restored by the oral administration of β -cryptoxanthin to OVX rats. β -Cryptoxanthin has been shown to inhibit osteoclastogenesis in the bone marrow culture system *in vitro* (12). Also, β -cryptoxanthin has an inhibitory effect on parathyroid hormone (10^{-7} M)-induced decrease in calcium content in rat femoral-diaphyseal and -metaphyseal tissues in the tissue culture system *in vitro* (9). Presumably, β -cryptoxanthin reveals an inhibitory effect on osteoclastic bone resorption in OVX rats.

The preventive effect of β -cryptoxanthin on OVX-induced bone loss was seen by oral administration for 3 months with a dose of 5 or 10 $\mu\text{g}/100\text{ g}$ body weight of β -cryptoxanthin, although the serum concentration of the carotenoid was not determined. The dose may have a physiological significance. It has been reported that the serum concentration of β -cryptoxanthin increases due to consumption of vegetable juice in women to the range of 1.3×10^{-7} to 5.3×10^{-7} M (21). The anabolic effect of β -cryptoxanthin on osteoblastic bone formation and osteoclastic bone resorption was observed at 10^{-7} and 10^{-6} M *in vitro* (8-12). Presumably, the supplemental intake of β -cryptoxanthin has a preventive effect on bone loss with aging.

Interestingly, it was found that the increase in body weight in OVX rats was clearly restored by the prolonged oral administration of β -cryptoxanthin (10 $\mu\text{g}/100\text{ g}$ body weight). This result suggests that OVX-induced fat growth is prevented by the intake of β -cryptoxanthin. It is speculated that β -cryptoxanthin has an effect on lipid metabolism with OVX.

In conclusion, it has been demonstrated that the prolonged oral administration of β -cryptoxanthin has a preventive effect on bone loss induced in OVX rats *in vivo*.

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