Anabolic Effect of *β*-Cryptoxanthin on Bone Components in the Femoral Tissues of Aged Rats *in Vivo* and *in Vitro*

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The effect of *β*-cryptoxanthin on bone components in the femoral tissues of aged rats was investigated. *β*-Cryptoxanthin was isolated from Satsuma mandarin (*Citrus unshiu* MARC.). *β*-Cryptoxanthin (5, 10, or 20 μg/100 g body weight) was orally administered once daily for 7 days to aged (50 week old) female rats. The administration of *β*-cryptoxanthin (10 or 20 μg/100 g body weight) caused a significant increase in calcium content, alkaline phosphatase activity and deoxyribonucleic acid (DNA) content in the femoral-diaphyseal and -metaphyseal tissues. Femoral-diaphyseal calcium content and alkaline phosphatase activity were significantly increased by the dose of 5 μg/100 g body weight. A significant increase in metaphyseal alkaline phosphatase activity was also seen with the dose of 5 μg *β*-cryptoxanthin/100 g body weight. Moreover, bone tissues were cultured for 48 hr in serum-free Dulbecco's modified Eagle's medium containing either vehicle or *β*-cryptoxanthin (10^{-7} or 10^{-6} M). The presence of *β*-cryptoxanthin (10^{-7} or 10^{-6} M) caused a significant increase in calcium content and alkaline phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues obtained from aged (50 week old) female rats. These increases were completely abolished in the presence of cycloheximide (10^{-6} M), an inhibitor of protein synthesis. This study demonstrated that *β*-cryptoxanthin has an anabolic effect on bone components in aged female rats *in vivo* and *in vitro*.

Key words —— *β*-cryptoxanthin, bone formation, osteoporosis, aged rat femur

INTRODUCTION

Aging induces a decrease in bone mass,[1,2] and osteoporosis with its accompanying decrease in bone mass is widely recognized as a major public health problem. The most dramatic expression of the disease is represented by fractures of the proximal femurs.[3] Bone loss with increasing age may be due to decreased bone formation and increased bone resorption. Pharmacological and nutritional factors may prevent bone loss with aging.[4] We investigate the anabolic effect of various food factors on bone metabolism in preventing osteoporosis.[5–10] Chemical compounds in food that act on bone metabolism, however, are poorly understood.

The anticarcinogenic effects of various micronutrients and phytochemicals found in vegetables and fruits, such as carotenoids, have been demonstrated in laboratory studies.[11] Carotenoids have been shown to play a possible biological role in cancer prevention.[11] This preventive effect on osteoporosis, however, has not been fully clarified. Vitamin A is known to have a detrimental effect on bone at high doses. High levels of vitamin A lead to accelerated bone resorption, bone fractures, and osteoporotic bone lesions in animals.[12–14]

β-Cryptoxanthin is a carotenoid abundant in Satsuma mandarin (*Citrus unshiu* MARC.), and it is enzymatically converted from *β*-carotene (provitamin A) in plants. Of the various carotenoids (including *β*-cryptoxanthin, lutein, lycopene, and *β*-carotene) and rutin (quercetin-3-rutinoside), *β*-cryptoxanthin has been found to have a unique anabolic effect on bone calcification.[15] It has a stimulatory effect on bone formation and an inhibitory effect on bone resorption in rat femoral tissue culture *in vitro*.[16] Moreover, *β*-cryptoxanthin inhibits osteoclast-like cell formation induced by bone-resorbing factors in mouse marrow cultures *in vitro*,[17] suggesting that it inhibits osteoclastic bone resorption.

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A decrease in bone components in the femoral tissues is found in aged rats.[18] This study, therefore, was undertaken to determine whether β-cryptoxanthin has an anabolic effect on these components in aged rats *in vivo*. We found that oral administration of the substance does induce this effect on the femoral tissues.

MATERIALS AND METHODS

Chemicals —— Dulbecco's modified Eagle's medium (DMEM) (high glucose, 4.5 g/dl) and a penicillin-streptomycin solution (5000 units/mg penicillin and 5000 μg/ml streptomycin) were obtained from Gibco Laboratories (Grand Island, NY, U.S.A.). Bovine serum albumin (BSA), cycloheximide, and corn oil were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). β-Cryptoxanthin (100% purity) was supplied by Ehime Beverage Inc. (Matsuyama, Japan). All other chemicals were reagent grade from Wako Pure Chemical Industries (Osaka, Japan). All water used was glass-distilled.

Animals —— Female Wistar rats (50 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). The animals were fed commercial laboratory chow (solid) containing 57.4% Ca and 1.1% P for 7 days at a room temperature of 25°C and had free access to distilled water.

Administration Procedures —— β-Crytoxanthin was dissolved in corn oil at a concentration of (5, 10, or 20 μg/ml/100 g body weight) and orally administered to rats through a stomach tube once daily for 7 days. Control rats received corn oil (1 ml/100 g body weight) orally. The animals were killed 24 hr after the last administration by cardiac puncture under light ether anesthesia, and the blood and femur were removed immediately.

Bone Culture —— The femurs, which were obtained from aged (50 week old) female rats, were removed aseptically after exsanguinations and soaked in ice-cold 0.25 M sucrose solution. The femur was cleaned of soft tissue and marrow, and the diaphysis and metaphysis (not containing epiphyseal tissue) were separated. The femoral-diaphyseal and -metaphyseal tissues were cut into small pieces. Femoral-diaphyseal or -metaphyseal fragments were cultured for 48 hr in 35-mm dishes in 2.0 ml of medium consisting of DMEM (high glucose, 4.5 g/dl supplemented with 0.25% BSA) plus antibiotics (100 units of penicillin and 100 μg of streptomycin/ ml medium).[19] In the experiments, bone tissue was cultured for 48 hr in a medium containing either vehicle or compound (including 0.1% ethanol). Cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO_2 and 95% air.

Analytical Procedures —— Blood samples were centrifuged for 30 min after collection, and the serum was separated. Serum calcium levels were was determined using the method of Willis.[20] Serum inorganic phosphorus levels were measured using the method of Taussky and Shon.[21]

The diaphyseal and metaphyseal tissues were dried for 16 hr at 110°C. Calcium content was determined by atomic absorption spectrophotometry.[21] Calcium content in bone tissues is expressed as milligrams/gram of dry bone.

To assay alkaline phosphatase activity, the diaphyseal and metaphyseal tissues were immersed in 3.0 ml of ice-cold 6.5 mM barbital buffer (pH 7.4), cut into small pieces, homogenized with a physcotron homogenizer, and disrupted for 60 sec with an ultrasonic device. The supernatant centrifuged at $600 \times g$ 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.[22] Enzyme activity was expressed as μmol of p-nitrophenol liberated/minute/milligram of protein. Protein concentration was determined using the method of Lowry *et al.*[23]

To measure bone DNA content, the diaphyseal and metaphyseal tissues were shaken with 4.0 ml of ice-cold 0.1 M NaOH solution for 24 after homogenization of the bone tissues.[24] After alkali extraction, the samples were centrifuged at $10000 \times g$ for 5 min, and the supernatant was collected. DNA content in the supernatant was determined using the method of Ceriotti.[25] and expressed as the amount of DNA (mg)/g wet weight of bone tissue.

Statistical Analysis —— The significance of difference between values was estimated using Student's *t*-test. *p*-Values of less than 0.05 were considered to indicate statistically significant differences.

RESULTS

Effect of Administration of β-Cryptoxanthin on Bone Component in Aged Rats *in Vivo*

The body weight of aged female rats was not significantly altered by the oral administration of β-cryptoxanthin (5, 10, or 20 μg/100 g body weight)

Table 1. Body Weight and Serum Components in Aged Female Rats Orally Administered β-Cryptoxanthin

Dose (μg/100 g body weight)	Body weight (g)	Serum concentration (mg/dl)	
		Calcium	Inorganic phosphorus
Control	253.0 ± 5.9	9.52 ± 0.69	5.02 ± 0.19
5	251.0 ± 3.3	9.49 ± 0.32	5.51 ± 0.34
10	252.2 ± 1.7	9.12 ± 0.16	4.53 ± 0.13
20	248.6 ± 9.8	8.59 ± 0.13	5.27 ± 0.22

Rats were orally administered β-cryptoxanthin (5, 10, or 20 μg/100 g body weight) once daily for 7 days. Each value is the mean ± S.E.M. of six rats. Data were not significant as compared with the control value.
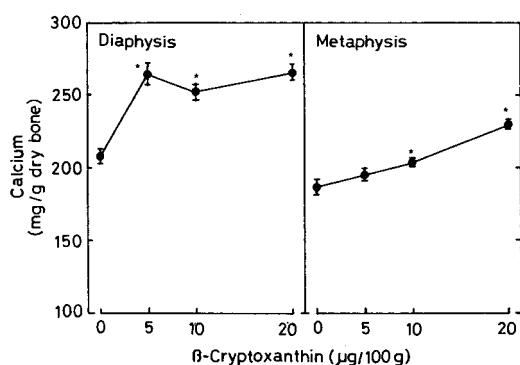
Fig. 1. Effect of Administration of β-Cryptoxanthin on Calcium Content in Femoral-Diaphyseal and -Metaphyseal Tissues of Aged Rats *in Vivo*

β-Cryptoxanthin (5, 10, or 20 μg/100 g body weight) was orally administered once daily for 7 days to rats. Each value is the mean ± S.E.M. of six rats. *$p < 0.01$ compared with the control (vehicle) value.
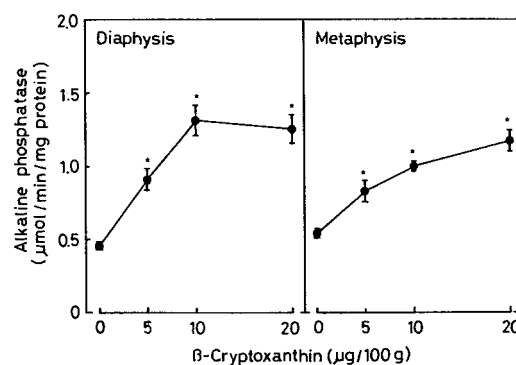
Fig. 2. Effect of Administration of β-Cryptoxanthin on Alkaline Phosphatase Activity in Femoral-Diaphyseal and -Metaphyseal Tissues of Aged Female Rats *in Vivo*

β-Cryptoxanthin (5, 10, or 20 μg/100 g body weight) was orally administered once daily for 7 days to rats. Each value is the mean ± S.E.M. of six rats. *$p < 0.01$ compared with the control (vehicle) value.

for 7 days (Table 1). Serum calcium and inorganic phosphorus concentrations were also not significantly changed by such administration (Table 1). This oral administration did, however, cause a significant increase in calcium content in the femoral-diaphyseal tissues (Fig. 1), and the increase in metaphyseal calcium content was particularly significant with the dose of 10 or 20 μg/100 g body weight.

Alkaline phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues was significantly increased by the oral administration of β-cryptoxxanthin (5, 10, or 20 μg/100 g body weight) to aged female rats for 7 days (Fig. 2), and 10 or 20 μg/100 g body weight for the same period also significantly raised DNA content (Fig. 3).

Effect of β-Cryptoxanthin on Bone Components in Tissue Culture *in Vitro*

Femoral-diaphyseal and -metaphyseal tissues obtained from aged female rats were cultured for 48 hr in a medium containing either vehicle or β-cryptoxanthin (10^{-7} or 10^{-6} M) in the presence or ab-

sence of cycloheximide (10^{-6} M). The presence of β-cryptoxanthin (10^{-7} or 10^{-6} M) caused a significant increase in calcium content (Fig. 4) and alkaline phosphatase activity (Fig. 5) in these tissues. These increases were completely prevented in the presence of cycloheximide (10^{-6} M), an inhibitor of protein synthesis.

DISCUSSION

Previous studies showed that synthetic β-cryptoxanthin has a unique anabolic effect on bone formation and bone calcification *in vitro*,[15] and the compound has been shown to have an inhibitory effect on osteoclastic bone resorption induced by various bone-resorbing factors *in vitro*.[16,17] β-Cryptoxanthin may have a stimulatory effect on bone mass, and it may also have a preventive effect on bone loss.

Bone components are decreased with age.[18] The oral administration of β-cryptoxanthin to aged female rats for 7 days was found to induce an ana-
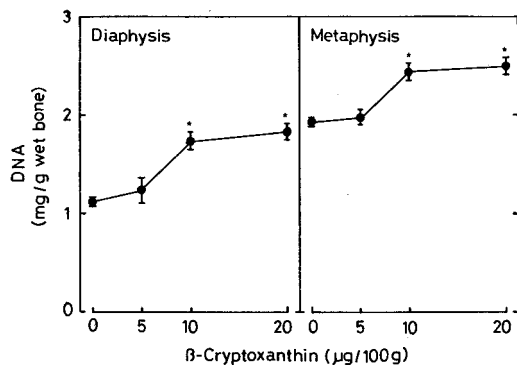
Fig. 3. Effect of Administration of β-Crytoxanthin on DNA in Femoral-Diaphyseal and -Metaphyseal Tissues of Aged Female Rats *in Vivo*

β-Crytoxanthin (5, 10, or 20 μg/100 g body weight) was orally administered once daily for 7 days to rats. Each value is the mean ± S.E.M. of six rats. *$p < 0.01$ compared with the control (vehicle) value.
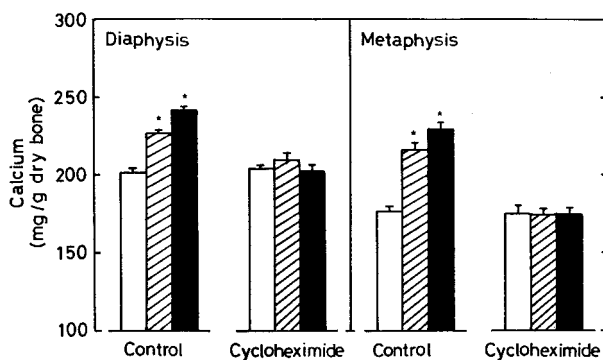

Fig. 4. Effect of Administration of β-Cryptoxanthin on Calcium Content in Femoral-Diaphyseal and -Metaphyseal Tissues obtained from Aged Female Rats *in Vitro*

Bone tissues were cultured for 48 hr in a serum-free medium containing either vehicle of β-cryptoxanthin (10^{-7} or 10^{-6} M) in the presence or absence of cycloheximide (10^{-6} M). Each value represents the mean ± S.E.M. of five experiments with separate rats. *$p < 0.01$ compared with the control (none) value. White bars, control (vehicle); hatched bars, β-crytoxanthin (10^{-7} M); black bars, β-crytoxanthin (10^{-6} M).

Fig. 5. Effect of Administration of β-Cryptoxanthin on Alkaline Phosphatase Activity in Femoral-Diaphyseal and -Metaphyseal Tissues obtained from Aged Female Rats *in Vitro*

Bone tissues were cultured for 48 hr in a serum-free medium containing either vehicle of β-cryptoxanthin (10^{-7} or 10^{-6} M) in the presence or absence of cycloheximide (10^{-6} M). Each value represents the mean ± S.E.M. of five experiments with separate rats. *$p < 0.01$ compared with the control (none) value. White bars, control (vehicle); hatched bars, β-cryptoxanthin (10^{-7} M); black bars, β-cryptoxanthin (10^{-6} M).

bolic effect on bone components *in vivo*. Its administration caused a significant increase in calcium content, alkaline phosphatase activity, and DNA content in the femoral-diaphyseal (cortical bone) and -metaphyseal (trabecular bone) tissues of aged rats. Alkaline phosphatase is an enzyme maker of osteoblasts, and the enzyme participates in bone mineralization.[26] DNA content in bone tissues is an index of the number of bone cells.[27] The administration of β-cryptoxanthin may have a stimulatory effect on osteoblastic bone formation in the femoral tissues of aged female rats *in vivo*. Its anabolic effect was
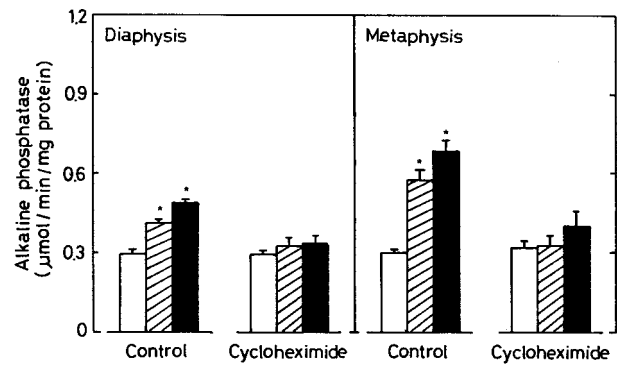
induced by oral administration for 7 days at the lowest dose of 5 μg/100 g body weight, and the substance may also exert potent stimulation bone mass in these rats.

The presence of β-cryptoxanthin (10^{-7} or 10^{-6} M) in culture medium caused a significant increase in calcium content and alkaline phasphatase activity in the femoral-diaphyseal and-metaphyseal tissues obtained from aged female rats *in vitro*. This effect was abolished in the presence of cycloheximide, an inhibitor of protein synthesis, suggesting that newly synthesized protein components are needed for the anabolic effect of β-cryptoxanthin. The carotenoid may have a direct stimulatory effect on bone formation in the femoral tissues of aging rats. Meanwhile, it has been reported that the serum concentration of β-cryptoxanthin increases with the consumption of vegetable juice by women to the range of 1.3×10^{-7} to 5.3×10^{-7} M.[28] Presumably, β-cryptoxanthin has a physiologic role in stimulating bone components.

In conclusion, it has been demonstrated that the oral administration of β-cryptoxanthin induces an anabolic effect on bone components in the femoral tissues of aged female rats *in vivo*. Further studies are in progress to determine the preventive effect of this substance on osteoporosis.

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