Effect of β-Crytoxanthin on Circulating Bone Metabolic Markers: Intake of Juice (*Citrus Unshiu*) Supplemented with β-Cryptoxanthin Has an Effect in Menopausal Women

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The effects of prolonged intake of juice prepared from Satsuma mandarin (*Citrus unshiu* MARC.) containing β-cryptoxanthin on circulating biochemical markers of bone metabolism in subjects, including menopausal woman, were investigated. Ninety volunteers, aged 27–65 years (19 men and 71 women), were enrolled in this study. The 71 females included 35 premenopausal women (ages, 27–50 years) and 36 postmenopausal women (ages, 46–65 years). Volunteers were divided into four groups; placebo juice without β-cyptoxanthin (5 men and 19 women), juice containing β-cyptoxanthin at 1.5 mg/200 ml of juice/day (4 men and 17 women), 3.0 mg/day (5 men and 17 women), and 6.0 mg/day (5 men and 18 women). Placebo or juice (200 ml) was ingested once a day for 28 or 56 days. Serum β-cryptoxanthin concentrations were significantly increased after the intake of juice containing β-cryptoxanthin (1.5, 3.0, or 6.0 mg/day) for 28 or 56 days, and the increases were dose-dependent. Bone-specific alkaline phosphatase and γ-carboxylated osteocalcin are serum bone markers of bone formation, and bone tartrate-resistant acid phosphatase (TRACP) and N-telopeptides of type I collagen are markers of bone resorption. Bone-specific alkaline phosphatase activity was significantly increased after the intake of juice containing β-cryptoxanthin (3.0 or 6.0 mg/day) for 56 days as compared with the value obtained before intake. γ-Carboxylated osteocalcin concentration was significantly increased after the intake of juice containing β-cryptoxanthin (3.0 or 6.0 mg/day) for 28 or 56 days as compared with the value obtained before intake or after the intake of placebo juice. Serum TRACP activity and type I collagen N-telopeptide concentration were significantly decreased after the intake of juice containing β-cryptoxanthin (3.0 or 6.0 mg/day) for 28 or 56 days as compared with the value obtained before intake or after intake of placebo juice, and significant decreases were also seen after the intake of 1.5 mg/day β-cryptoxanthin as compared with the value obtained before intake. In menopausal women, bone-specific alkaline phosphatase activity and γ-carboxylated osteocalcin concentration were significantly increased after the intake of juice containing β-cryptoxanthin (3.0 or 6.0 mg/day) for 56 days as compared with the value obtained after placebo intake. Also, this intake caused a significant decrease in bone TRACP activity. Meanwhile, serum calcium, inorganic phosphorous, and parathyroid hormone (intact) were not changed after the intake of β-cryptoxanthin-containing juice for 28 or 56 days. This study demonstrates that the prolonged intake of juice fortified with β-cryptoxanthin has stimulatory effects on bone formation and inhibitory effects on bone resorption in humans, and that the intake has an effect in menopausal women.

Key words —— β-cryptoxanthin, bone metabolic marker, bone formation, bone resorption, osteoporosis, menopause

INTRODUCTION

Aging induces a decrease in bone mass in both men and women. This decrease is due to increased bone resorption and to decreased bone formation. In women, ovarian hormone deficiency at meno-

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pause stimulates bone loss.[1,2] Osteoporosis with its accompanying decrease in bone mass is widely recognized as a major public health problem.[3] A decrease in bone mass leads to bone fractures. Pharmacologic and nutritional factors may help to prevent bone loss with aging.[4,5] Recent studies have shown that isoflavones (including genistein and daidzein), which are contained in soybeans,[6–9] and menaguinone-7, an analogue of vitamin K_2 which is abundant in fermented soybeans,[10–12] have stimulatory effects on osteoblastic bone formation and inhibitory effects on osteoclastic bone resorption *in vitro*, thereby increasing bone mass.[13,14] Food factors may have a role in the prevention of bone loss with increasing age.

β-Cryptoxanthin is a carotenoid that is abundant in Satsuma mandarin oranges (*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 *in vitro*.[15] β-Cryptoxanthin has stimulatory effects on bone formation and inhibitory effects on bone resorption in rat femoral tissue culture *in vitro*.[16] β-Cryptoxanthin can stimulate cell proliferation and mineralization in osteoblastic cells *in vitro*.[17,18] The carotenoid can inhibit osteoclast-like cell formation induced by various bone-resorbing factors in mouse marrow cultures *in vitro*,[19] and it stimulates apoptotic cell death and has suppressive effects on cell function in mature osteoclastic cells *in vitro*.[20] Thus β-cryptoxanthin has been demonstrated to have stimulatory effects on osteoblastic bone formation and inhibitory effects on osteoclastic bone resorption *in vitro*.

Oral administration of β-cryptoxanthin has been shown to have an anabolic effect on bone components in young and aged rats *in vivo*.[21,22] Oral administration of β-cryptoxanthin has preventive effects on bone loss in streptozotocin-diabetic rats and ovariectomized rats which are animal models of osteoporosis.[23,24] Supplementation of β-cryptoxanthin has been demonstrated to have preventive effects on bone loss due to increasing age.

The intake of β-cryptoxanthin-reinforced juice has been shown to have stimulatory effects on bone formation and inhibitory effects on bone resorption in healthy individuals as estimated based on serum biochemical markers of bone metabolism *in vivo*,[25,26] suggesting that the intake of dietary β-cryptoxanthin has preventive effects on osteoporosis.

This study was undertaken to determine whether the prolonged intake of juice (*Citrus unshiu*) fortified with increased β-cryptoxanthin content has an effect on bone metabolism in menopausal women, premenopausal women, and men.

MATERIALS AND METHODS

Materials —— Juice prepared from Satsuma mandarin oranges (*Citrus unshiu* MARC.) was supplied by Ehime Beverage, Inc. (Matsuyama, Japan). Reinforced juice with increased β-cryptoxanthin content was prepared by supplementation with β-cryptoxanthin isolated from Satsuma mandarin. The content of β-cryptoxanthin in the reinforced juice was 1.5, 3.0, or 6.0 mg/200 milliliter (ml) of juice. Placebo juice did not contain β-cryptoxanthin. The nutritional composition of juice with or without β-cryptoxanthin is shown in Table l.

Experimental Procedures —— Ninety adults, aged 27–65 years (19 men and 71 women), who were judged to be healthy with no abnormal liver or kidney function as assessed by standard clinical and biochemical data, were enrolled as volunteers in this study. Informed consent was obtained from all before enrollment. The intake of other foods with an abundance of β-cryptoxanthin was prohibited during the experimental period.

Table 1. Nutritional Composition of Juice Containing β-Cryptoxanthin

Ingredient	β-Cryptoxanthin-containing juice (mg/200 ml)			
	Placebo	1.5	3.0	6.0
Energy (kcal)	44	43	43	43
Protein (g)	0	0.6	0.6	0.6
Lipid (g)	0	0	0	0
Carbohydrate (g)	10.9	10.2	10.2	10.2
Sodium (mg)	4	1	1	1
β-Cryptoxanthin (mg)	0	1.5	3.0	6.0

The washout and intake periods of each type of juice were 7 and 56 days, respectively. The 90 volunteers were divided into four groups: placebo juice (5 men and 19 women), or reinforced juice containing β-cryptoxanthin at 1.5 mg/200 ml (4 men and 17 women), 3.0 mg/200 ml (22 volunteers; 5 men and 17 women), and 6.0 mg/200 ml (23 volunteers; 5 men and 18 women). Each group was sequentially given 200 ml of juice without β-cryptoxanthin (placebo) or reinforced juice containing β-cryptoxanthin 1.5, 3.0, or 6.0 mg/200 ml once daily for 56 days. Blood samples were collected from each between 9:00 and 11:30 (morning) on the day prior to intake (control), and at 28 and 56 days after the start of intake, and 28 days after the final intake. Serum samples were prepared between 30 and 60 min after blood sampling and then stored at –20°C until assayed.

Analytical Procedures —— Serum β-crypoxanthin concentrations were measured using the procedure of Peng *et al*.[27] To each tube containing 0.25 ml of serum, 250 μl of ethanol solution containing 0.25 ml of serum, 250 μl of ethanol solution containing 1% sodium dodecylsulfate and 0.1% butylhydroxytoluene (BHT), and n-hexane containing 0.1% BHT were added. After the samples were mixed, they were incubated at 37°C for 5 min and then centrifuged for 5 min at 2500 rpm. Samples of the hexane-extracted phase were dried with nitrogen gas, and then mobile phases for the high pressure liquid chromatography (HPLC) system (Amersham Pharmacia Biotechnology, U.S.A.) were added. To separate β-cryptoxanthin, a mobile phase at a flow rate of 1.3 ml/min was used. Mobile phases consisted of acetonitrile, methanol, and 0.1% triethylamine-containing tetrahydrofuran (20 : 75 : 5). Elution was monitored at 451 nm.

The serum γ-carboxylated osteocalcin concentration was assayed using a Gla type osteocalcin (γ-carboxylated osteocalcin) or under carboxylated osteocalcin ELA kit (Takara Shuzo, Shiga, Japan).[28] Serum bone-specific alkaline phosphatase activity was assayed using a METRA BAP EIA kit (Quidel, San Diego, U.S.A.).[29] Serum bone tartrate-resistant acid phosphatase (TRACP) activity was assayed using a Bone TRACP Assay EIA kit (SBA Sciences, Turku, Finland).[30] Serum bone N-telopeptide of type I collagen was measured using an OSTEOMARK NTx Serum EIA kit (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan).[31]

Serum calcium, inorganic phosphorus, albumin, γ-glutamyltransferase (γ-GTP), nitrogen urea, creatinine, glucose, triglyceride, high-density lipoprotein (HDL) cholesterol, and low-density lipoprotein (LDL) cholesterol levels were determined using kits. Serum parathyroid hormone (intact) levels were measured using an EIA kit.

Urine was collected between 9:00 and 11:30 (morning). Urinary deoxypyridinoline, N-telopeptide of type I collagen, and creatinine concentrations were determined using kits.

Statistical Analysis —— Differences in values before and after the intake of each type of juice were estimated using Student's *t*-test. A paired *t*-test was used for differences in values before and after the intake of each juice or between two groups after each intake period. We also used multiple analysis of variance (ANOVA) to compare the treatment groups. Values of *p* less than 0.05 were considered to represent statistically significant differences.

RESULTS

Effects of Intake of Juice Reinforced with β-Cryptoxanthin on Bone Metabolic Markers

Ninety volunteers, aged 27–65 years (19 men and 71 women), were enrolled in this study. The 71 women included 35 (27–50 years) premenopausal women and 36 (46–65 years) menopausal women.

The changes in serum β-cryptoxanthin concentration following the intake of placebo juice or of β-cryptoxanthin-reinforced juice prepared from Satsuma mandarin were examined (Fig. 1). Serum β-cryptoxanthin concentrations increased significantly after the intake of juice containing β-cryptoxanthin for 28 or 56 days as compared with that before intake. This increase was significantly enhanced by the intake of increasing concentrations of β-cryptoxanthin (1.5, 3.0, or 6.0 mg/200 ml of juice/day) for 28 or 56 days. A significant increase in serum β-cryptoxanthin concentration was also observed at 28 days after the final intake of juice containing β-cryptoxanthin.

Serum bone-specific alkaline phosphatase activity was significantly increased after the intake of juice containing β-cryptoxanthin (3.0 or 6.0 mg/day) for 56 days as compared with the value obtained before intake or after the intake of placebo juice (Fig. 2).

Serum γ-carboxylated osteocalcin concentration was significantly increased after the intake of juice containing β-cryptoxanthin (3.0 or 6.0 mg/day) for 28 or 56 days as compared with the value obtained

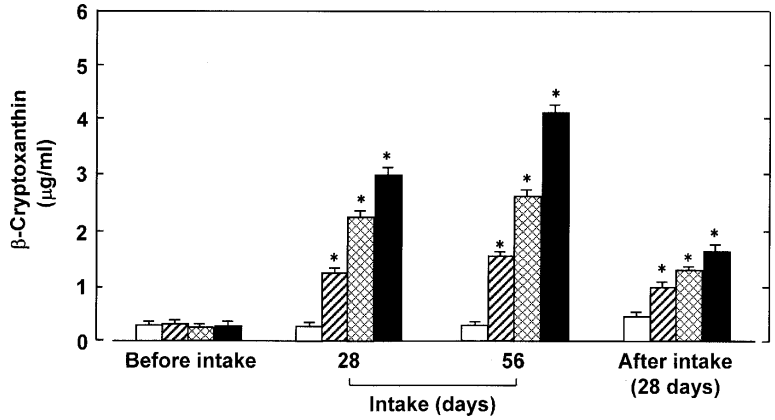

Fig. 1. Changes in Serum β-Cryptoxanthin Concentrations Following Intake of Juice Containing β-Cryptoxanthin

Juice from Satsuma mandarin orange containing β-cryptoxanthin (1.5, 3.0, or 6.0 mg/200 ml of juice/day) was given to volunteers for 28 or 56 days. Ninety subjects (before intake) were divided into four groups of 24, 21, 22, or 23 subjects for the intake of β-cryptoxanthin (1.5, 3.0, or 6.0 mg/day), respectively. Each value is the mean ± S.E.M. of 21 to 24 subjects. *$p < 0.01$ compared with the value obtained before intake of juice or after intake of placebo juice. White bars, placebo juice; hatched bars, juice containing β-cryptoxanthin 1.5 mg/day; double hatched bars, juice containing β-cryptoxanthin 3.0 mg/day; black bars, juice containing β-cryptoxanthin 6.0 mg/day.

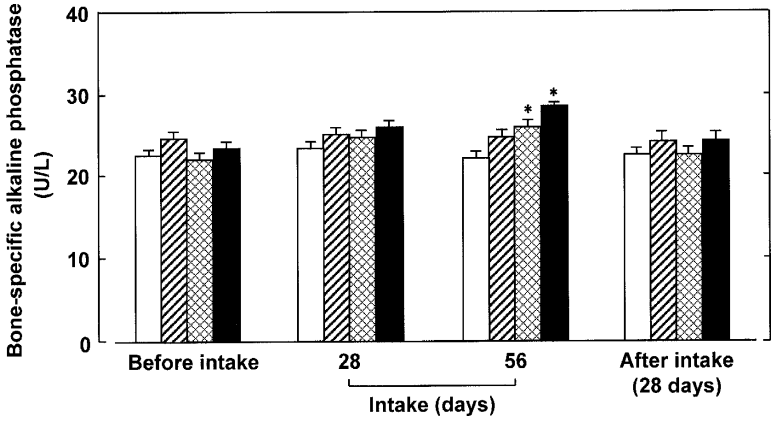

Fig. 2. Changes in Serum Bone-Specific Alkaline Phosphatase Activity Following Intake of Juice Containing β-Cryptoxanthin

The procedure for the intake of juice containing β-cryptoxanthin is described in Fig. 1. Each value is the mean ± S.E.M. of 21 to 24 subjects. *$p < 0.01$ compared with the value obtained before intake of juice or after intake of placebo juice. White bars, placebo juice; hatched bars, juice containing β-cryptoxanthin 1.5 mg/day; double hatched bars; juice containing β-cryptoxanthin 3.0 mg/day; black bars, juice containing β-cryptoxanthin 6.0 mg/day.

before intake or after the intake of placebo juice (Fig. 3). A significant increase in serum γ-carboxylated osteocalcin concentration was also observed at 28 days after the final intake of juice containing β-cryptoxanthin (1.5, 3.0, or 6.0 mg/day) as compared with the value obtained after placebo intake. Meanwhile, the serum undercarboxylated osteocalcin concentration was not significantly changed after the intake of juice containing β-cryptoxanthin (1.5, 3.0, or 6.0 mg/day) for 28 or 56 days (Fig. 4).

Serum bone TRACP activity was significantly decreased after the intake of juice containing β-cryptoxanthin (3.0 or 6.0 mg/day) for 28 or 56 days as compared with the value obtained before intake or placebo intake (Fig. 5). The intake of juice containing β-cryptoxanthin (1.5 mg/day) for 56 days caused a significant decrease in serum TRACP activity as compared with the value obtained before intake.

The serum concentration of N-telopeptide of type I collagen was significantly decreased after the intake of juice containing β-cryptoxanthin (3.0 or 6.0 mg/day) for 28 or 56 days as compared with the value obtained before intake or placebo intake (Fig. 6). This decrease was also observed after the intake of juice containing β-cryptoxanthin (1.5 mg/day) for 56 days.

The urinary concentration of N-telopeptide of

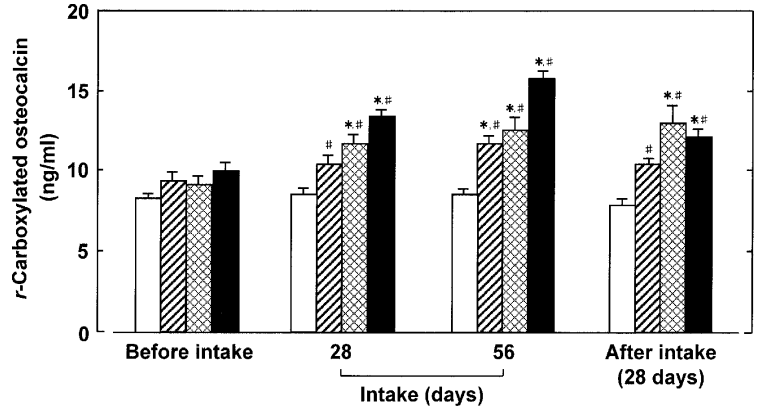

Fig. 3. Changes in Serum γ-Carboxylated Osteocalcin Concentrations Following Intake of Juice Containing β-Cryptoxanthin

The procedure for the intake of juice containing β-cryptoxanthin is described in Fig. 1. Each value is the mean ± S.E.M. of 21 to 24 subjects. *$p < 0.01$ compared with the value obtained before intake of juice. #$p < 0.01$ compared with the value obtained after intake of placebo juice. White bars, placebo juice; hatched bars, juice containing β-cryptoxanthin 1.5 mg/day; double hatched bars; juice containing β-cryptoxanthin 3.0 mg/day; black bars, juice containing β-cryptoxanthin 6.0 mg/day.

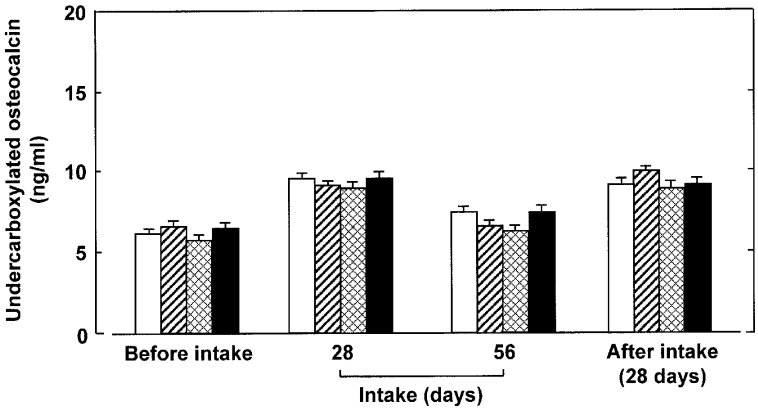

Fig. 4. Changes in Serum Undercarboxylated Osteocalcin Concentrations Following Intake of Juice Containing β-Cryptoxanthin

The procedure for the intake of juice containing β-cryptoxanthin is described in Fig. 1. Each value is the mean ± S.E.M. of 21 to 24 subjects. There were no significant differences among the groups compared with the values obtained after placebo intake. White bars, placebo juice; hatched bars, juice containing β-cryptoxanthin 1.5 mg/day; double hatched bars; juice containing β-cryptoxanthin 3.0 mg/day; black bars, juice containing β-cryptoxanthin 6.0 mg/day.

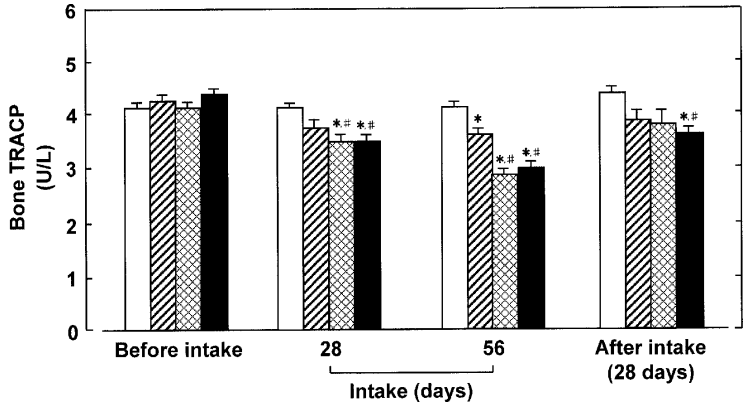

Fig. 5. Changes in Serum Bone TRACP Activity Following Intake of Juice Containing β-Cryptoxanthin

The procedure for the intake of juice containing β-cryptoxanthin is described in Fig. 1. Each value is the mean ± S.E.M. of 21 to 24 subjects. *$p < 0.01$ compared with the value obtained before intake of juice. #$p < 0.01$ compared with the value obtained after intake of placebo juice. White bars, placebo juice; hatched bars, juice containing β-cryptoxanthin 1.5 mg/day; double hatched bars, juice containing β-cryptoxanthin 3.0 mg/day; black bars, juice containing β-cryptoxanthin 6.0 mg/day.

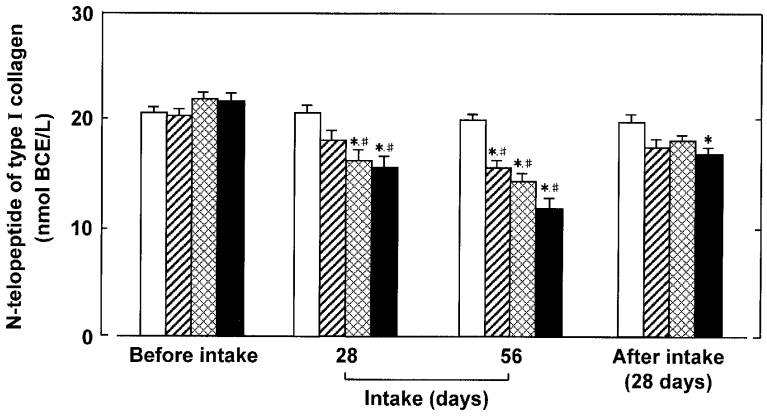

Fig. 6. Changes in Serum Concentration of N-Telopeptide of Type I Collagen Following Intake of Juice Containing β-Cryptoxanthin

The procedure for the intake of juice containing β-cryptoxanthin is described in Fig. 1. Each value is the mean ± S.E.M. of 21 to 24 subjects. *$p < 0.01$ compared with the value obtained before intake of juice. #$p < 0.01$ compared with the value obtained after intake of placebo juice. White bars, placebo juice; hatched bars, juice containing β-cryptoxanthin 1.5 mg/day; double hatched bars, juice containing β-cryptoxanthin 3.0 mg/day; black bars, juice containing β-cryptoxanthin 6.0 mg/day.

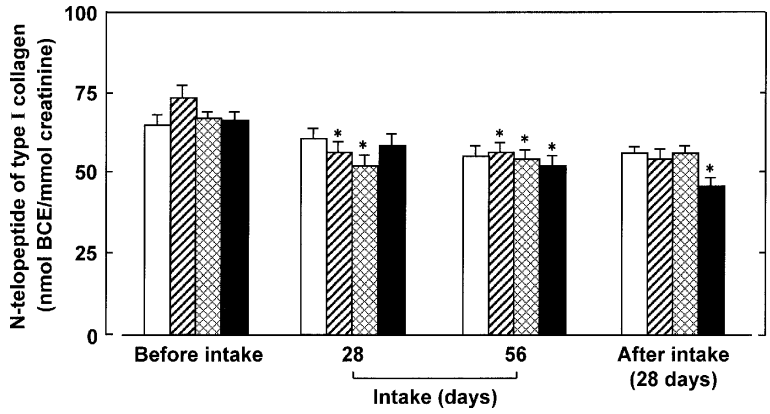

Fig. 7. Changes in Urinary Concentration of N-Telopeptide of Type I Collagen Following Intake of Juice Containing β-Cryptoxanthin

The procedure for the intake of juice containing β-cryptoxanthin is described in Fig. 1. Each value is the mean ± S.E.M. of 21 to 24 subjects. *$p < 0.01$ compared with the value obtained before intake of juice. White bars, placebo juice; hatched bars, juice containing β-cryptoxanthin 1.5 mg/day; double hatched bars; juice containing β-cryptoxanthin 3.0 mg/day; black bars, juice containing β-cryptoxanthin 6.0 mg/day.

type I collagen concentration was significantly decreased after the intake of juice containing β-cryptoxanthin (1.5, 3.0 or 6.0 mg/day) for 56 days as compared with the value obtained before intake (Fig. 7). A significant decrease in urinary N-telopeptide concentration was not seen as compared with the value obtained after placebo intake.

Changes in Bone Metabolic Markers Following the Intake of Juice Reinforced with β-Cryptoxanthin in Men, Women, and Menopausal Women

The data for serum bone metabolic markers obtained after the intake of juice containing β-cryptoxanthin for 28 or 56 days were analyzed in the 19 men, 35 premenopausal women, and 36 menopausal women. In the men, the serum γ-carboxylated osteocalcin concentration was significantly increased after the intake of juice containing β-cryptoxanthin (3.0 or 6.0 mg/day) for 28 or 56 days as compared with the value obtained after placebo intake (Fig. 8). Serum bone TRACP activity in the men was significantly decreased after the intake of juice containing 6.0 mg/dayβ-cryptoxanthin for 28 days or 1.5, 3.0, or 6.0 mg/dayβ-cryptoxanthin for 56 days as compared with the value obtained after placebo intake. The serum concentration of N-telopeptide of type I collagen was significantly decreased after the intake of juice containing β-cryptoxanthin (6.0 mg/day) for 56 days.

In premenopausal women, the serum γ-carboxylated osteocalcin concentration and bone-specific alkaline phosphatase activity were significantly in-

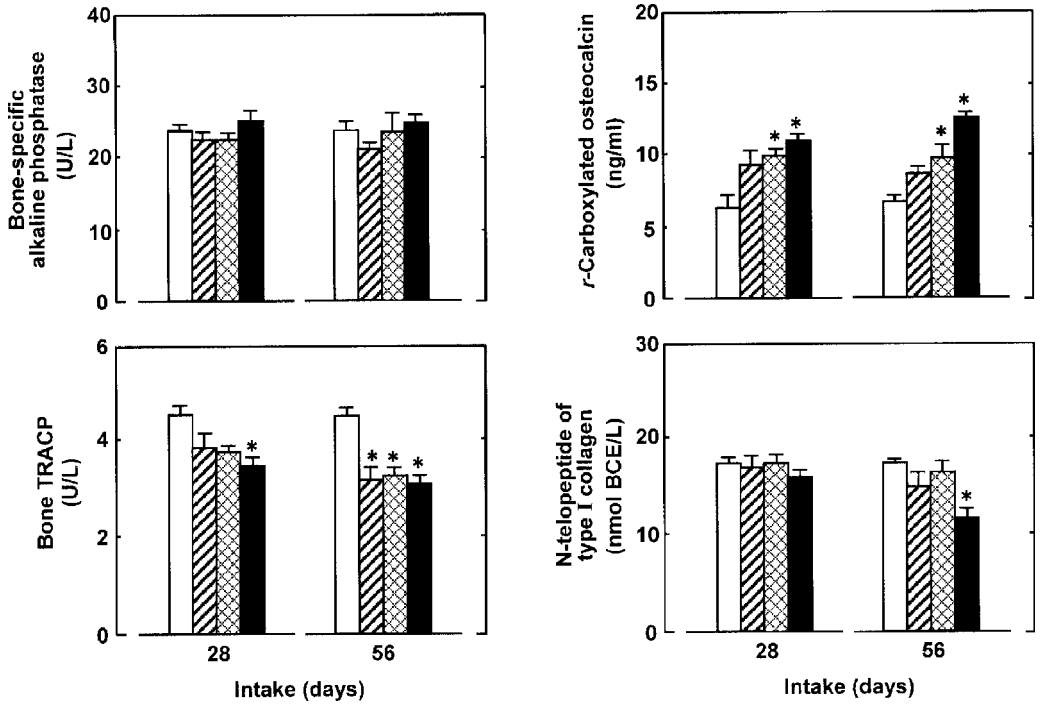

Fig. 8. Changes in Serum Bone Metabolic Markers in Males Following Intake of Juice Containing β-Cryptoxanthin

The procedure for the intake of juice containing β-cryptoxanthin is described in Fig. 1. Nineteen males (aged 32–64 years) were divided into four groups (5, 4, 5, and 5 subjects); the intake of juice (placebo) without β-cryptoxanthin or juice containing β-cryptoxanthin 1.5, 3.0, or 6.0 mg/day, respectively. Each value is the mean ± S.E.M. *$p < 0.01$ compared with the value obtained after intake of placebo juice. White bars, placebo juice; hatched bars, juice containing β-cryptoxanthin 1.5 mg/day; double hatched bars; juice containing β-cryptoxanthin 3.0 mg/day; black bars, juice containing β-cryptoxanthin 6.0 mg/day.

creased after the intake of juice containing β-cryptoxanthin (1.5, 3.0, or 6.0 mg/day) for 56 days as compared with the value obtained after placebo intake (Fig. 9). Serum bone TRACP activity and type I collagen N-telopeptide concentration in premenopausal women were significantly decreased after the intake of juice containing β-cryptoxanthin (3.0 or 6.0 mg/ day) for 28 or 56 days (Fig. 9).

In menopausal women, serum bone-specific alkaline phosphate activity and γ-carboxylated osteocalcin concentration were significantly increased after the intake of juice containing β-cryptoxanthin (3.0 or 6.0 mg/day) for 56 days as compared with the value obtained after placebo intake (Fig. 10). A significant increase in serum γ-carboxylated osteocalcin was also seen after the intake of juice containing β-cryptoxanthin (6.0 mg/day) for 28 days. Serum bone TRACP activity was significantly decreased after the intake of juice containing β-cryptoxanthin (3.0 or 6.0 mg/day) for 56 days. The serum concentration of N-telopeptide of type I collagen was significantly decreased after the intake of juice of β-cryptoxanthin (6.0 mg/day) for 28 or 56 days.

Effects of Intake of Juice Reinforced with β-Cryptoxanthin on Other Markers

Serum biochemical findings after the intake of juice containing β-cryptoxanthin (1.5, 3.0, or 6.0 mg/day) for 56 days are shown in Table 2. Serum biochemical findings were not significantly changed after the intake of juice containing β-cryptoxanthin as compared with the value obtained after placebo intake.

Hematological changes (including the number of leukocytes, red blood cell, and thrombocytes) were not observed after the intake of juice containing β-cryptoxanthin (1.5, 3.0, or 6.0 mg/day) for 56 days (data not shown). Urinary biochemical findings (including calcium, creatinine, protein, and glucose) were not significantly changed after the intake of juice containing β-cryptoxanthin (data not shown). Blood pressure was not significantly changed after the intake of juice containing β-cryptoxanthin (1.5–6.0 mg/day).

The intake of juice reinforced with β-cryptoxanthin did not have any negative effects.

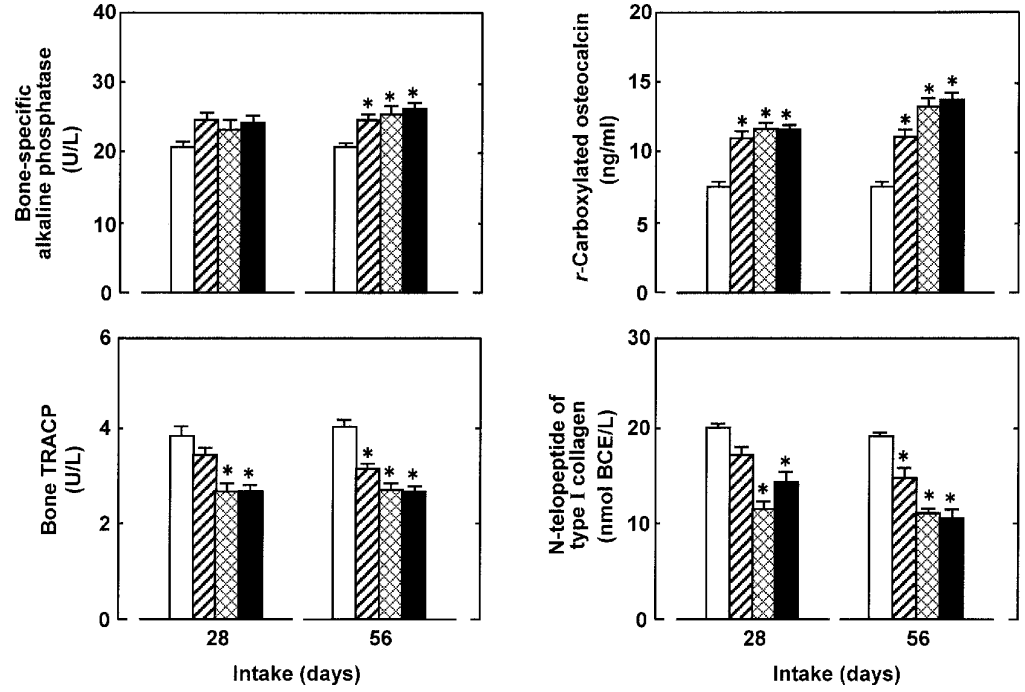

Fig. 9. Changes in Serum Bone Metabolic Markers in Premenopausal Women Following Intake of Juice Containing β-Cryptoxanthin

The procedure for the intake of juice containing β-cryptoxanthin is described in Fig. 1. Thirty five females (aged 27–50 years) were divided into four groups (10, 9, 8, and 8 subjects); the intake of juice (placebo) without β-cryptoxanthin or juice containing β-cryptoxanthin 1.5, 3.0, or 6.0 mg/day, respectively. Each value is the mean ± S.E.M. $*p < 0.01$ compared with the value obtained after intake of placebo juice. White bars, placebo juice; hatched bars, juice containing β-cryptoxanthin 1.5 mg/day; double hatched bars; juice containing β-cryptoxanthin 3.0 mg/day; black bars, juice containing β-cryptoxanthin 6.0 mg/day.

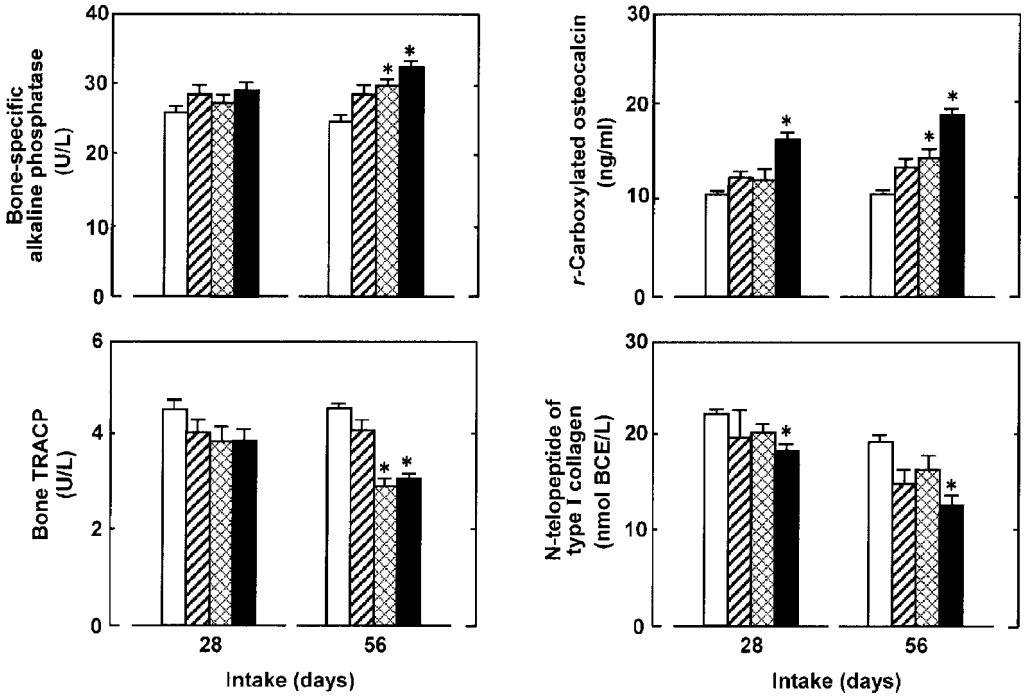

Fig. 10. Changes in Serum Bone Metabolic Markers in Menopousal Women Following Intake of Juice Containing β-Cryptoxanthin

The procedure of the intake of juice containing β-cryptoxanthin is described in Fig. 1. Thirty six females (aged 46–65 years) were divided into four groups (9, 8, 9, and 10 subjects; the intake of juice (placebo) without β-cryptoxanthin or juice containing β-cryptoxanthin 1.5, 3.0, or 6.0 mg/day, respectively. Each value is the mean ± S.E.M. $*p < 0.01$ compared with the value obtained after intake of placebo juice. White bars, placebo juice; hatched bars, juice containing β-cryptoxanthin 1.5 mg/day; double hatched bars; juice containing β-cryptoxanthin 3.0 mg/day; black bars, juice containing β-cryptoxanthin 6.0 mg/day.

Table 2. Serum Metabolic Findings Following Intake of Juice Supplemented with β-Cryptoxanthin

Serum level	β-Cryptoxanthin (mg/day)			
	Placebo	1.5	3.0	6.0
Calcium (mg/dl)	9.39 ± 0.05	9.36 ± 0.08	9.30 ± 0.06	9.31 ± 0.06
Inorganic phosphorus (mg/dl)	3.1 ± 0.08	3.1 ± 0.09	3.4 ± 0.09	3.2 ± 0.08
Parathyroid hormone (pg/ml)	37.0 ± 2.12	40.0 ± 2.66	39.4 ± 3.41	37.3 ± 2.58
Albumin (g/dl)	4.5 ± 0.04	4.5 ± 0.04	4.5 ± 0.04	4.5 ± 0.04
γ-GTP (U/L)	23.0 ± 2.76	19.8 ± 3.49	25.1 ± 3.75	18.9 ± 1.38
Nitrogen urea (mg/dl)	13.4 ± 0.59	13.1 ± 0.78	13.5 ± 0.68	14.2 ± 0.57
Creatinine (mg/dl)	0.63 ± 0.03	0.66 ± 0.02	0.64 ± 0.03	0.64 ± 0.02
Glucose (mg/dl)	88.6 ± 1.20	91.4 ± 1.35	90.5 ± 0.87	89.3 ± 1.06
Triglyceride (mg/dl)	70.0 ± 6.57	53.4 ± 3.60	64.5 ± 4.86	66.0 ± 4.05
HDL cholesterol (mg/dl)	69.6 ± 2.47	68.4 ± 2.71	70.5 ± 3.22	69.7 ± 2.56
LDL cholesterol (mg/dl)	112.4 ± 5.35	113.0 ± 7.72	110.2 ± 5.50	114.9 ± 6.38

Juice from Satsuma mandarin oranges supplemented with β-cryptoxanthin was given to volunteers for 56 days. Ninety subjects were divided into four groups of 24, 21, 22, and 23 subjects. Each value is the mean ± S.E.M. Data were not significant as compared with that of placebo.

DISCUSSION

Bone loss with aging may be due to decreased bone formation and increased bone resorption. Chemical factors in food and plants may help to prevent bone loss due to increasing age. β-Cryptoxanthin, which is present in large amounts in Satsuma mandarin oranges (*Citrus unshiu*), has been demonstrated to have stimulatory effects on osteoblastic bone formation and inhibitory effects on osteoclastic bone resorption *in vitro*.[15–20] The oral administration of β-cryptoxanthin has preventive effects on bone loss induced in animal models of osteoporosis,[23,24] suggesting that the intake of juice containing β-cryptoxanthin may play a role in the prevention of bone loss with osteoporosis.

The prolonged intake of juice containing β-cryptoxanthin has been shown to cause a significant increase in serum markers of bone formation and a corresponding decrease in serum markers of bone resorption in 21 healthy individuals aged 23–47 years (10 males and 11 females).[25] A relationship between serum β-cryptoxanthin and circulating bone metabolic markers has been found in healthy individuals with the intake of juice containing β-cryptoxanthin.[26]

The present double blind study was undertaken to determine whether the prolonged intake of juice reinforced with increased β-cryptoxanthin content had an effect on serum bone metabolic markers in 90 adults (ages, 27–65 years), including 36 menopausal women (ages, 46–65 years). We found that the intake of juice reinforced with β-cryptoxanthin causes a significant increase in circulating bone formation markers and a significant decrease in bone resorption markers in men, premenopausal women, and menopausal women. This supports the view that the intake of juice reinforced with β-cryptoxanthin has a preventive effect on bone loss due to osteoporosis.

Serum β-cryptoxanthin concentration was significantly increased after the intake of juice reinforced with increased β-cryptoxanthin content, and the increase was dose-dependent. A significant increase in serum β-cryptoxanthin concentration was also observed at 28 days at the end of intake, indicating that the carotenoid is stable in the serum. Serum β-cryptoxanthin concentration was in the range of 4.20×10^{-7} M to 4.89×10^{-7} M in the placebo groups. The intake of juice reinforced with β-cryptoxanthin at doses of 1.5, 3.0, or 6.0 mg/day significantly increased the serum concentration to 2.43×10^{-6}, 4.06×10^{-6}, or 5.38×10^{-6} M, respectively. These increases were about 5- or 10-fold as compared with the value obtained before intake or after placebo intake. It has been reported that the serum concentration of β-cryptoxanthin increased, due to the consumption of vegetable juice in women to 1.3×10^{-7} to 5.3×10^{-7} M.[32]

Serum bone-specific alkaline phosphatase and γ-carboxylated osteocalcin are expressed in osteoblastic cells,[33,34] which stimulate bone formation. These markers were significantly increased after the intake of juice reinforced with β-cryptoxanthin. The increase in serum γ-carboxylated osteocalcin was as large as that of serum bone-specific alkaline phosphatase activity, suggesting that β-cryptoxanthin has

a potent effect on the production of γ-carboxylated osteocalcin in osteoblastic cells. The intake of β-cryptoxanthin-containing juice may stimulate osteoblastic bone formation. β-Cryptoxanthin has been demonstrated to stimulate cell proliferation and mineralization in osteoblastic cells *in vitro*.[19,20]

Serum bone TRACP is a specific marker enzyme in osteoclasts,[28] and N-telopeptide of type I collagen is specifically formed following the stimulation of bone resorption.[29] The intake of juice reinforced with β-cryptoxanthin caused a significant decrease in serum bone TRACP activity and the concentration of N-telopeptide, indicating that the carotenoid inhibits osteoclastic bone resorption. β-Cryptoxanthin has been shown to suppress osteoclast-like cell formation and cell function in mature osteoclastic cells *in vitro*.[19,20]

The intake of juice reinforced with β-cryptoxanthin (3.0 or 6.0 mg/day) had a significant effect on circulating bone metabolic markers in men, premenopausal women, and menopausal women. This indicates that the effects of β-cryptoxanthin in stimulating bone formation and inhibiting bone resorption are present in both sexes. Interestingly, the intake of juice reinforced with β-cryptoxanthin (3.0 or 6.0 mg/day) was found to have effects on circulating bone metabolic markers in menopausal women. This indicates that the supplementation of β-cryptoxanthin has preventive effects on bone loss due to osteoporosis in menopausal women. This preventive effect was obvious at a dose of β-cryptoxanthin of 3.0 mg/day in menopausal women. This dose may be suitable in the prevention of osteoporosis.

In conclusion, we have demonstrated that the intake of reinforced juice, which contains more β-cryptoxanthin than regular juice, has a preventive effect on bone loss that accompanies an increase in age.

REFERENCES

1) Albright, F., Smith, P. H. and Richardson, A. M. (1941) Postmenopausal osteoporosis: its clinical features. *J. Am. Med. Assoc.*, **116**, 2465–2474.
2) Riggs, B. L., Jowsey, J., Kelly, P. J., Jones, J. D. and Maker, F. I. (1969) Effect of sex hormones on bone in primary osteoporosis. *J. Clin. Invest.*, **48**, 1065–1072.
3) Cooper, C. and Melton, J., III (1992) Epidemiology of osteoporosis. *Trends Endocrinol. Metab.*, **3**, 224–229.
4) Bonjour, J.-P., Schurch, M.-A. and Rissori, R. (1996) Nutritional aspects of hip fractures. *Bone*, **18**, 1395–1445.
5) Yamaguchi, M. (2002) Isoflavone and bone metabolism: Its cellular mechanism and preventive role in bone loss. *J. Health Sci.*, **48**, 209–222.
6) Yamaguchi, M. and Gao, Y. H. (1998) Anabolic effect of genistein and genistin on bone metabolism in the femoral-metaphyseal tissues of elderly rats: The genistein effect is enhanced by zinc. *Mol. Cell. Biochem.*, **178**, 377–382.
7) Sugimoto, E. and Yamaguchi, M. (2000) Stimulatory effect of daidzein in osteoblastic MC3T3-E1 cells. *Biochem. Pharmacol.*, **59**, 471–475.
8) Yamaguchi, M. and Gao, Y. H. (1998) Inhibitory effect of genistein on bone resorption in tissue culture. *Biochem. Pharmacol.*, **55**, 71–76.
9) Gao, Y. H. and Yamaguchi, M. (1999) Inhibitory effect of genistein on osteoclast-like cell formation in mouse marrow cultures. *Biochem. Pharmacol.*, **58**, 767–772.
10) Yamaguchi, M., Sugimoto, H. and Hachiya, S. (2001) Stimulatory effect of menaquinone-7 (vitamin K_2) on osteoblastic bone formation *in vitro*. *Mol. Cell. Biochem.*, **223**, 131–137.
11) Yamaguchi, M., Kakuda, H., Gao, Y. H. and Tsukamoto, Y. (2000) Prolonged intake of fermented soybean (*natto*) diets containing vitamin K_2 (menaquinone-7) prevents bone loss in ovariectomized rats. *J. Bone Miner. Metab.*, **18**, 71–76.
12) Tsukamoto, Y., Ichise, M. and Yamaguchi, M. (2000) Prolonged intake of dietary fermented soybean (*natto*) with the reinforced vitamin K_2 (menaquinone-7) enhanced circulating γ-carboxylated osteocalcin concentration in normal individuals. *J. Health Sci.*, **46**, 317–321.
13) Ono, R., Ma, Z. J. and Yamaguchi, M. (2000) Prolonged intake of fermented soybean diets with supplementation of isoflavone and saponin prevents bone loss in ovariectomized rats. *J. Health Sci.*, **46**, 70–74.
14) Yamaguchi, M., Taguchi, H., Gao, Y. H., Igarashi, A. and Tsukamoto, Y. (1999) Effect of vitamin K_2 (menaquinone-7) in fermented soybean (*natto*) on bone loss in ovarientomized rats. *J. Bone Miner. Metab.*, **17**, 23–29.
15) Yamaguchi, M. and Uchiyama, S. (2003) Effect of carotenoid on calcium content and alkaline phosphatase activity in rat femoral tissues *in vitro*: The unique anabolic effect of β-cryptoxanthin. *Biol. Pharm. Bull.*, **26**, 1188–1191.
16) Yamaguchi, M. and Uchiyama, S. (2004) β-Cryptoxanthin stimulates bone formation and inhibits bone resorption in tissue culture *in vitro*. *Mol. Cell. Biochem.*, **258**, 137–144.
17) Uchiyama, S. and Yamaguchi, M. (2005) β-Cryp-

toxanthin stimulates cell proliferation and transcriptional activity in osteoblastic MC3T3-E1 cells. *Int. J. Mol. Med.*, **15**, 675–681.
18) Uchiyama, S. and Yamaguchi, M. (2005) β-Cryptoxanthin stimulates cell differentiation and mineralization in osteoblastic MC3T3-E1 cells. *J. Cell. Biochem.*, **95**, 1224–1234.
19) Uchiyama, S. and Yamaguchi, M. (2004) Inhibitory effect of β-cryptoxanthin on osteoclast-like cell formation in mouse marrow cultures. *Biochem. Pharmacol.*, **67**, 1297–1305.
20) Uchiyama, S. and Yamaguchi, M. (2006) β-Cryptoxanthin stimulates apoptotic cell death and suppresses cell function in osteoclastic cells: Change in their related gene expression. *J. Cell. Biochem.*, **98**, 1185–1195.
21) Uchiyama, S., Sumida, T. and Yamaguchi, M. (2004) Oral administration of β-cryptoxanthin induces anabolic effects on bone components in the femoral tissues of rats *in vivo*. *Biol. Pharm. Bull.*, **27**, 232–235.
22) Uchiyama, S., Sumida, T. and Yamaguchi, M. (2004) Anabolic effect of β-cryptoxanthin on bone components in the femoral tissues of aged rats *in vivo* and *in vitro*. *J. Health Sci.*, **50**, 491–496.
23) Uchiyama, S. and Yamaguchi, M. (2005) Oral administration of β-cryptoxanthin prevents bone loss in streptozotocin-diabetic rats *in vivo*. *Biol. Pharm. Bull.*, **28**, 1766–1769.
24) Uchiyama, S. and Yamaguchi, M. (2006) Oral administration of β-cryptoxanthin prevents bone loss in ovariectomized rats. *Int. J. Mol. Med.*, **17**, 15–20.
25) Yamaguchi, M., Igarashi, A., Uchiyama, S., Morita, S., Sugawara, K. and Sumida, T. (2004) Prolonged intake of juice (*Citrus unshiu*) reinforced with β-cryptoxanthin has an effect on circulating bone biochemical markers in normal individuals. *J. Health Sci.*, **50**, 619–624.
26) Yamaguchi, M., Igarashi, A., Morita, S., Sumida, T. and Sugawara, K. (2005) Relationship between serum β-cryptoxanthin and circulating bone metabolic markers in healthy individuals with the intake of juice (*Citrus unshiu*) containing β-cryptoxanthin. *J. Health Sci.*, **51**, 738–743.
27) Peng, Y.-M., Peng, Y.-S., Lin, Y., Moon, T., Roe, D. J. and Ritenbaugh, C. (1995) Concentrations and plasma-tissue-diet relationships of carotenoids, retinoids and tocopherols in humans. *Nutr. Cancer*, **23**, 233–246.
28) Koyama, N., Ohara, K., Yokota, H., Kurome, T., Katayama, M., Hino, F., Kato, I. and Aki, T. (1991) A one step sandwich enzyme immunoassay for antibodies. *J. Immunol. Methods*, **139**, 17–23.
29) Gomez, B., Ardakani, S., Ju, J., Jenkins, D., Cerelli, M. J., Daniloff, G. Y. and Kung, V. T. (1995) Monoclonal antibody assay for measuring bone-specific alkaline phosphatase activity in serum. *Clin. Chem.*, **41**, 1560–1566.
30) Hallen, J. M., Alatalo, S. L., Suminen, H., Cheng, S., Janckila, A. J. and Vaananen, H. K. (2000) Tartrate-resistant acid phosphatase 5b: A novel serum marker of none resorption. *J. Biol. Miner. Res.*, **15**, 1337–1345.
31) Clements, J. D., Herrich, M. V., Singer, F. R. and Eyre, D. R. (1997) Evidence that serum NTx (collagen-type I N-telopeptides) can act as an immunochemical marker of bone resorption. *Clin. Chem.*, **43**, 2058–2063.
32) McElgot, A. J., Rock, C. L., Shanks, T. G., Flatt, A. W., Newman, V., Faerber, S. and Pierce, J. P. (1999) Comparison of serum carotenoid response between women consuming vegetable juice and woman consuming raw or cooked vegetable. *Cancer Epidemiol. Biomarkers Prev.*, **8**, 227–231.
33) Price, P. A. (1985) Vitamin K-dependent formation of bone gla protein (osteocalcin) and its function. *Vitam. Horm.*, **42**, 65–108.
34) Levy, J. R., Murray, E., Manolagass, S. and Olefsky, J. M. (1986) Demonstration of insulin receptors and modulation of alkaline phosphatase activity by insulin in rat osteoblastic cells. *Endocrinology*, **119**, 1786–1792.