Effects of β-Cryptoxanthin on Bone Metabolism in a Rat Model of Osteoporosis

[1]Noriko Ikeda, [2]Toshie Sugiyama, [3]Toshiko Suzuki, [4]Katsuyuki Mukai and [2]Seiji Kusuhara
[1]Graduate School of Science and Technology, [2]Department of Agrobiology,
Niigata University, 2-8050 Ikarashi, Nishi-Ku, 950-2181 Niigata, Japan
[3]Department of Biotechnology, Toyama Prefectural University,
5180 Kurokawa, Imizu, 939-0398 Toyama, Japan
[4]UNITIKA Ltd., R&D Center, 23 Kozakura, Uji, 611-0021 Kyoto, Japan

Abstract: Osteoporosis is a bone disease in which Bone Mineral Density (BMD) is reduced with a consequent increase in the risk of bone fractures. β-cryptoxanthin (β-CRP) is present in large amounts in Satsuma mandarins and was recently reported to stimulate bone formation. In this study, researchers investigated the effects of β-CRP in Satsuma Mandarin Pulp (SMP; 2 mg g^{-1} β-CRP) on bone metabolism in an Ovariectomized (OVX) rat model of osteoporosis. Female rats (12 weeks of age) were ovariectomized and orally administered vehicle, 0.03 g day^{-1} SMP or 0.3 g day^{-1} SMP for 5 weeks. After that serum concentrations of osteocalcin (an osteoblastic bone formation marker) tended to be higher in the SMP groups than in the OVX vehicle group while those of collagen type I degradation products (an osteoclastic bone resorption marker) tended to be lower in the SMP groups. By bone histomorphometry, bone trabecular volume/tissue volume ratios and trabecular numbers were significantly higher in the SMP groups than in the OVX vehicle group while trabecular separation and osteoclast number/bone surface ratios were significantly lower in the SMP groups. By immunohistochemistry, percentage areas of osteocalcin immunoreactivity on trabecular surface were significantly greater in the SMP groups than in the OVX vehicle group. Dual-energy X-ray absorptiometry analyses revealed that BMDs of the lumbar vertebrae and femora, tibiae tended to increase as the dose of SMP increased in the OVX rats. In conclusion, oral SMP administration stimulated osteoblastic bone formation and inhibited osteoclastic bone resorption in OVX rats, thereby preventing the bone loss associated with osteoporosis.

Key words: Bone metabolism, β-cryptoxanthin, osteoporosis, rat, Satsuma mandarin

INTRODUCTION

Bone is a specialized connective tissue that makes up the skeletal system. This tissue serves three functions; mechanical, providing support and muscle attachment sites for locomotion; protective for vital organs and bone marrow and metabolic as a reservoir of ions, especially calcium and phosphate for the maintenance of serum homeostasis which is essential for life. In human adulthood, bone mass is constantly maintained by a balance between osteoblastic bone formation and osteoclastic bone resorption. Osteoporosis is a major public health problem in women and arises from estrogen deficiency after menopause which leads to bone loss through increased osteoclastic bone resorption (Lorenzo et al., 1998; Jilka et al., 1992; Pacifici et al., 1989). This disease causes a decrease in Bone Mineral Density (BMD) and a consequent increase in the risk of bone fractures. Estrogen Replacement Therapy (ERT) is considered to be the most effective method for reducing the incidence rate of osteoporosis in postmenopausal women (Anderson et al., 2004; Lindsay et al., 1999). However, ERT may be accompanied by disadvantages such as endometrial and breast cancer induction in some women. Therefore, ERT is only recommended for women who have no contraindications. Thus, it would be most helpful to discover natural and safe dietary substances that can minimize bone loss. Recently, many nutritional and pharmacological factors have been explored for their potential to prevent bone loss. Several recent reports have indicated that soy isoflavones may have beneficial effects on bone (Morabito et al., 2002; Anderson et al., 1999). Genistein, one of the plant isoflavones has been shown to stimulate osteoblastic bone formation, inhibit osteoclastic bone resorption and prevent bone loss in Overiectomized (OVX) rats (Anderson et al., 1998). However, some experiments in vitro or in vivo have indicated that isoflavones dose-dependently stimulate the

Corresponding Author: Toshie Sugiyama, Department of Agrobiology, Niigata University, 2-8050 Ikarashi, Nishi-Ku, 950-2181 Niigata, Japan

proliferation of breast cancer cells (Ju *et al.*, 2001; Hsieh *et al.*, 1998; Welshons *et al.*, 1987). Therefore, excessive intake of isoflavones may be associated with an increased risk of breast cancer in humans. Carotenoids are the most widely occurring group of natural pigments in food and plants. β-Cryptoxanthin (β-CRP), one of the carotenoids is present in large amounts in Satsuma mandarins (Citrus unshiu Marc.) as well as in Satsuma mandarin residue (Yano *et al.*, 2005). β-CRP has antioxidant and anticancer activities (Semba *et al.*, 2007; Nishino *et al.*, 2004; Stahl and Sies, 2005). Moreover, β-CRP has a unique anabolic effect on bone calcification. Other carotenoids such as lutein, lycopene and β-carotene do not have any effects on bone calcification *in vitro* (Yamaguchi and Uchiyama, 2003). Moreover, β-CRP has a stimulatory effect on bone formation and an inhibitory effect on bone resorption in rat femoral tissue *in vitro* (Yamaguchi and Uchiyama, 2004). β-CRP also has stimulatory effects on the proliferation, differentiation and bone mineralization of osteoblastic MC3T3-E1 cells as well as inducing the gene expression of bone formation-related proteins such as Runx2 type 1 and 2, alkaline phosphatase and $\alpha 1$ (I) collagen (Uchiyama and Yamaguchi, 2005a, b). Concomitantly, β-CRP inhibits osteoclastic bone resorption through the reduction of stimulatory proteins for Receptor Activator of NF-κB Ligand (RANKL) in osteoclastogenesis (Uchiyama and Yamaguchi, 2004). It has also been demonstrated that oral administration of β-CRP prevents bone loss in OVX rats *in vivo* (Uchiyama and Yamaguchi, 2006a). However, the effects of β-CRP on osteoporotic bone metabolism are not fully defined. Therefore in this study, researchers investigated the effects of dietary β-CRP supplementation on bone metabolism including BMD, bone formation and bone resorption in an OVX rat model of osteoporosis.

MATERIALS AND METHODS

Animals and experimental design: Female Sprague-Dawley rats (10 weeks of age) were purchased from Charles River Laboratories Japan (Yokohama, Japan). About 12 animals were acclimated in an environmentally controlled animal laboratory and fed a commercial food containing 57.3% carbohydrate, 1.1% calcium and 0.9% phosphorus at room temperature with free access to distilled water for 2 weeks. At 12 weeks of age, the animals were divided into 4 groups. One group of rats was sham-operated (Sham group) and the other three groups were subjected to Ovariectomy (OVX groups). The OVX rats were orally administered 0.3 g day^{-1} Satsuma mandarin pulp (SMP; high SMP group), 0.03 g day^{-1} SMP (low SMP group) or 0 g day^{-1} SMP

(vehicle group) for 5 weeks. SMP contains 2 mg g^{-1} β-CRP. Sham rats were orally administered the vehicle. The daily food intakes were measured every 1 week. The body weights were measured at the start and end of the study. At 24 h after the last administration, the rats were anesthetized with ether and blood samples were collected. After standing for 60 min at room temperature, the blood samples were centrifuged at 4,000×g for 10 min to obtain serum. The sera were stored at -80°C for biochemical analyses. All animal and experimental procedures were conducted with the approval of the Animal Care and Ethics Committee of Niigata University.

Serum analysis: Serum concentrations of calcium and inorganic phosphorus were measured by HPLC. Serum concentrations of estradiol were measured using diagnostic kits (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Serum concentrations of Osteocalcin (OC) and Collagen type 1 C-telopeptide degradation products (CTX) were also measured using diagnostic kits (Nordic bioscience diagnostics A/S, Herlev, Denmark) according to the manufacturer's instructions.

Bone histomorphometric analysis: The right femora were removed and dissected from the adhering connective tissue and muscle. Thereafter, the femora were fixed in 10% buffered formalin, decalcified in 10% EDTA and embedded in paraffin. Sections of 5 μm thickness were subjected to hematoxylin-eosin staining and examined under a light microscope. Bone morphometric analysis was carried out with the Image-pro discovery Version 4.5 for Windows software program (Media Cybernetics Inc., Bethesda, MD, USA). Histomorphometric measurements of bone were taken from a 9 mm^2 area in the central region beginning at 0.2 mm distal to the growth plate. Tissue areas were counted in five regions delineated by a grid. The trabecular Bone Volume/Tissue Volume ratio (BV/TV), Trabecular thickness (Tb.Th), Trabecular Number (Tb.N), Trabecular Separation (Tb.Sp) and osteoclast number/bone surface ratio (mm^{-1}; Oc.N/BS) were calculated.

Immunohistochemistry: A mouse monoclonal antibody recognizing rat OC was purchased from Abcam Ltd. (Cambridge, UK). The antibody reactivity against OC was confirmed to be most sensitive at a dilution of 1:100. Paraffin sections prepared as described above were immunohistochemically stained for OC using the Avidin-Biotin-peroxidase Complex (ABC) technique at room temperature. Briefly after blocking the nonspecific immunoreactivity with 1% normal goat serum in 0.01 M Phosphate-buffered Saline (PBS) for 30 min, the

sections were incubated with the primary antibody for 30 min, washed in PBS and incubated with biotinylated goat polyclonal anti-mouse IgG as the secondary antibody (Vector Laboratories Inc., Burlingame, CA, USA) for 30 min. Thereafter, the sections were washed in PBS and reacted with alkaline phosphatase-conjugated ABC (VECTASTAIN ABC-AP kit; Vector Laboratories Inc.) for 30 min. After washing, red coloration to demarcate regions of immunostaining was produced using an Alkaline phosphatase substrate kit (Vector Laboratories Inc.). After washing with distilled water, the sections were faintly counterstained with methyl green. Immunostaining reactions were recorded using a light microscope and processed for image analysis with the above-described Image-pro discovery software program.

Dual-energy X-ray absorptiometry: The left femora, left tibiae and lumbar vertebrae were removed and dissected from the adhering connective tissue and muscle. Thereafter, they were wrapped in plastic film and stored at -20°C until BMD analysis. The BMDs of the femora, tibiae and lumbar vertebrae were measured using dual-energy X-ray absorptiometry (QDR-2000; Hologic, Waltham, MA, USA).

Statistical analysis: The significance of differences between groups was estimated by multiple ANOVA. Values of $p<0.05$ were considered to indicate statistical significance.

RESULTS AND DISCUSSION

Food intakes and changes in body weight: Daily food intakes were higher in the OVX groups than in the sham group (Table 1). However, there were no significant differences in the food intakes among the OVX groups. The initial body weights did not differ among the four groups. At the end of the study, the mean body weights were significantly higher in the OVX groups than in the sham group (Table 1).

Serum analysis: The serum concentrations of calcium and inorganic phosphorus did not differ significantly among the groups (Table 2). On the other hand, the serum concentrations of estradiol were significantly lower in the OVX groups than in the sham group (Table 2).

The serum concentrations of OC, a marker of bone formation, tended to be higher in the SMP groups than in the OVX vehicle group (Table 2). The serum concentrations of CTX, a marker of bone resorption, increase in the OVX groups (Table 2). However, the CTX concentrations tended to be lower in the SMP groups than in the OVX vehicle group. The statistical analyses of the serum concentrations of OC and CTX were not carried out because the numbers of samples for the serum OC and CTX analyses were very few (n = 2).

Bone histomorphometric analysis: Summaries of the structural measurements obtained from the histomorphometry are shown in Table 3. BV/TV and Tb.N were significantly higher in the SMP groups than in the OVX vehicle group. Moreover, Tb.Th also tended to be higher in the SMP groups than in the OVX vehicle group. Tb.Sp was significantly lower in the SMP groups than in the OVX vehicle group. Oc.N/BS, a parameter of bone resorption was also significantly lower in the SMP groups than in the OVX vehicle group.

Table 1: Food intakes and body weights

| | Food intake | Body weight (g) | |
Groups	(g day^{-1})	At start	At end
OVX+high SMP	19.950±0.861^a	268.7±5.487^a	363.7±5.667^{ab}
OVX+low SMP	19.423±0.392^a	267.0±3.512^a	374.0±5.196^a
OVX+vehicle	17.523±0.581^{ab}	255.7±5.925^a	345.0±4.509^b
Sham	15.053±0.543^b	255.0±4.583^a	299.7±5.239^c

Each value is the mean±SEM. Significant difference with different letters among the groups by ANOVA test ($p<0.05$)

Table 2: Serum concentrations of Calcium (Ca), inorganic Phosphorus (iP), Estradiol (E2), Osteocalcin (OC) and type 1 C-telopeptide degradation products (CTX)

Groups	Ca (mg dL^{-1})	iP (mg dL^{-1})	E2 (pg mL^{-1})	OC (ng mL^{-1})	CTX (ng mL^{-1})
OVX+high SMP	10.50±0.06^a	10.20±1.31^a	24.98±13.26^b	20.516	10.462
OVX+low SMP	10.80±0.21^a	11.87±1.88^a	32.43±5.16^b	14.605	9.959
OVX+vehicle	10.37±0.44^a	8.93±0.38^a	37.40±3.62^b	9.589	11.237
Sham	10.47±0.07^a	9.60±1.83^a	89.83±8.73^a	10.848	8.389

Ca, iP and E2 values are the mean±SEM. OC and CTX values are the mean significant difference with different letters among the groups by ANOVA test ($p<0.05$)

Table 3: Bone histomorphometric measures

Groups	BV/TV (%)	Tb.Th (µm)	Tb.N (mm^{-1})	Tb.Sp (µm)	Oc.N/BS (mm^{-1})
OVX+high SMP	29.49±2.40^a	24.24±0.72^a	1.35±0.12^a	737.4±71.3^b	1.12±0.17^b
OVX+low SMP	29.50±2.31^a	23.11±0.59^a	1.44±0.13^a	706.3±61.4^b	1.14±0.06^b
OVX+vehicle	13.62±1.54^b	18.75±3.21^a	0.89±0.05^b	1306.7±86.0^a	2.60±0.28^a

BV/TV: Trabecular Bone Volume/Tissue Volume ratio, Tb.Th: Trabecular Thickness, Tb.N: Trabecular Number, Tb.S: Trabecular Separation, Oc.N/BS: Osteoclast Number/Bone Surface ratio each value is the mean±SEM. Significant difference with different letters among the groups by ANOVA test ($p<0.05$)

Table 4: Osteocalcin (OC) immunostaining areas and Bone Mineralized Desities (BMDs)

Groups	OC immunoreactive area (%)	BMDs (g cm^{-2})		
		Lumbar vertebra	Femur	Tibia
OVX+hhigh SMP	18.754±1.2591[a]	0.2467±0.0030[b]	0.2354±0.0061[a]	0.1973±0.0019[ab]
OVX+low SMP	19.516±1.0268[a]	0.2387±0.0036[b]	0.2284±0.0024[a]	0.1947±0.0012[b]
OVX+vehicle	8.135±0.7260[b]	0.2344±0.0021[b]	0.2242±0.0053[a]	0.1881±0.0039[b]
Sham	No observation	0.2710±0.0035[a]	0.2455±0.0048[a]	0.2087±0.0039[a]

Each value is the mean±SEM. Significant difference with different letters among the groups by ANOVA test ($p<0.05$)

Immunohistochemistry: OC was detected on the trabecular bone surface and its immunostaining was more intense in the SMP groups than in the OVX vehicle group (Fig. 1). The percentage areas of OC immunostaining on the trabecular bone surface were significantly greater in the SMP groups than in the OVX vehicle group (Table 4).

Dual-energy X-ray absorptiometry: The BMDs of the lumbar vertebrae were significantly lower in the OVX groups than in the sham group (Table 4). The BMDs of the femora and tibiae also represented the similar result. In the OVX rats, the BMDs for all bone sites tended to be higher in the SMP groups than the OVX vehicle group but these differences did not reach significance.

Carotenoids in various vegetables and fruits reduce the risks for many chronic diseases such as cancer and heart diseases (Rao, 2002). Satsuma mandarins have been identified as an especially significant source of β-CRP. Recent studies have shown that β-CRP has unique anabolic effects on bone calcification *in vitro* and *in vivo* (Uchiyama and Yamaguchi, 2005a, b, 2006a, b). In the present study, researchers investigated the effects of β-CRP on bone metabolism in an OVX rat model of osteoporosis.

Food intakes and body weights were higher in the OVX groups than in the sham group. These changes in body weight are known to be due to the effects of OVX. However, there were no significant differences among the OVX groups. These results suggest that SMP has no effect on weight gain. The serum concentrations of calcium and phosphorus did not differ significantly among the groups. On the other hand, the serum concentrations of estradiol were significantly lower in the OVX groups than in the sham group. SMP had no effects on the serum calcium, phosphorus and estradiol levels in OVX rats. The serum concentrations of OC tended to be higher in the SMP groups than in the OVX vehicle group. OC, a protein secreted by mature osteoblasts has a high affinity for hydroxyapatite crystals and plays an important role in bone mineralization. Serum levels of OC have been used clinically to assess bone metabolism and are primarily correlated with the levels of bone formation (Riggs *et al.*, 1986; Brown *et al.*, 1984; Price *et al.*, 1981). Therefore, the present results suggest that oral administration of SMP stimulates bone formation in OVX
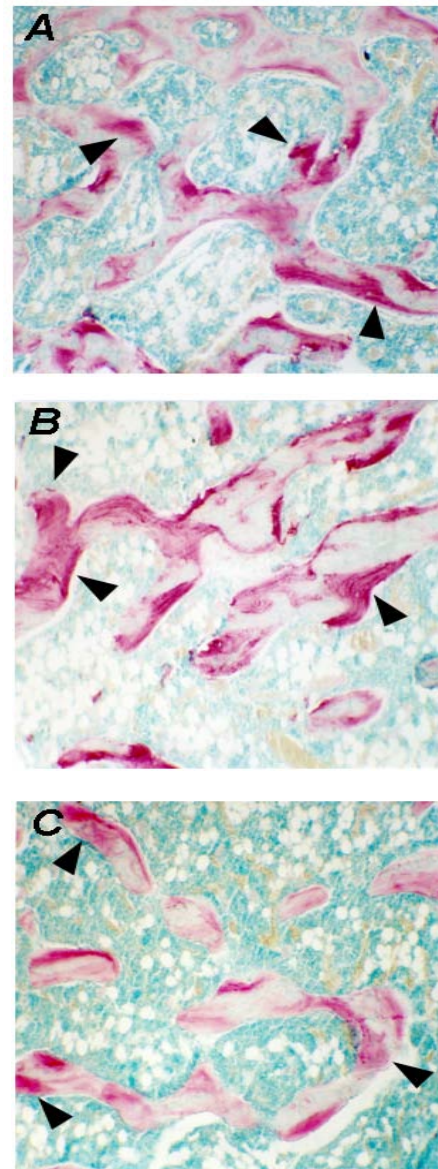

Fig. 1: Osteocalcin localization, images of osteocalcin immunostaining (arrowheads) in decalcified paraffin-embedded femoral sections from ovariectomized rats in ; A) high SMP; B) low SMP and C) vehicle groups are shown. Sections are counterstained with methyl green (original magnification, ×100)

rats. Conversely, the serum concentrations of CTX tended to be lower in the SMP groups than in the OVX vehicle group. CTX are derived from osteoclastic degradation of bone-specific type 1 collagen and considered to be a specific marker for bone resorption (Srivastava *et al.*, 2000). An elevated CTX level was observed in the OVX vehicle group compared with the SMP groups, suggesting that oral administration of SMP inhibits bone resorption in OVX rats.

By bone histomorphometry, BV/TV, Tb.Th and Tb.N were higher in the SMP groups than in the OVX vehicle group. Moreover, Tb.Sp was significantly lower in the SMP groups than in the OVX vehicle group. In rats, trabecular bone volume declines and metaphyseal bone structure deteriorates early after OVX. Trabecular bone strength depends not only on bone volume but also on bone structure (Lesclous *et al.*, 2004; Mellish *et al.*, 1991). Trabecular bone loss in OVX animals is brought about by a decrease in the number and thickness of trabeculae, resulting in increased trabecular separation and a consequent deterioration in trabecular connectivity (Abe *et al.*, 1999; Dempster *et al.*, 1995). As mentioned before, bone loss at 5 weeks after OVX is due to an increase in bone resorption, especially in the metaphysis and SMP is therefore considered to exert an inhibitory effect on bone loss through the inhibition of bone resorption. Moreover, the parameter for bone resorption, Oc.N/BS was significantly lower in the SMP groups than in the OVX vehicle group. Similarly, β-CRP was recently reported to exhibit suppressive effects on TRAP activity and gene expression of enzymes involved in the bone-resorbing activity of osteoclasts (Uchiyama and Yamaguchi, 2006b). Taken together, the present results suggest that SMP precisely inhibits osteoclastic bone resorption.

By immunohistochemistry, the OC staining intensities were stronger in the SMP groups than in the OVX vehicle group. The image analysis further revealed that the percentage areas of OC-positive bone matrix were significantly higher in the SMP groups than in the OVX vehicle group. These results suggest that SMP stimulates osteoblastic bone formation.

At 5 weeks after OVX, the BMD of the lumbar vertebrae of the OVX vehicle group was decreased to 86.5% of the BMD of the sham group. However, the BMDs of the lumbar vertebrae of the SMP groups were higher than that of the OVX vehicle group. Furthermore, the BMD of the lumbar vertebrae of the high SMP group was higher than that of the low SMP group. This preventive effect of oral SMP administration on OVX-induced bone loss was also seen in the femora and tibiae. A recent study showed that β-CRP induces apoptotic cell death of osteoclasts (Uchiyama and Yamaguchi, 2006b). Therefore, it is suggested that β-CRP has direct inhibitory effects on bone resorption.

CONCLUSION

In this study, β-CRP stimulates bone formation and inhibits bone resorption in OVX rats thereby preventing the bone loss associated with osteoporosis.

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