Original paper

Mechanism Underlying the Preventive Effect of Hyuganatsu Orange (*Citrus tamurana* Hort. ex Tanaka) on Osteoporosis

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Here, we examined the preventive effect of Hyuganatsu orange juice (*Citrus tamurana* Hort. ex Tanaka) on osteoporosis in postmenopausal women and evaluated whether the underlying mechanism involves altered calcium absorption in the intestinal tract. Twenty-five postmenopausal healthy women aged 59–64 years were randomly divided into two groups: the control and Hyuganatsu groups. The treatment group consumed Hyuganatsu orange juice daily for 3 months. As a result, decreases in serum levels of intact procollagen type I N-propeptide (IPINP), a parameter of bone formation, and tartrate-resistant acid phosphatase 5b (TRAP5b), a parameter of bone resorption, were observed. No statistically significant differences were observed between the two groups with respect to other serum parameters used for clinical evaluation of osteoporosis and liver function. In addition, we measured the rate of intestinal calcium absorption, a major determinant, using everted sacs of rat intestine *in vitro*, and found that the addition of active components such as water extract (WE) and WE-ultrafiltration (WEU) of Hyuganatsu orange juice to the incubation medium led to a marked increase in the absorption of calcium in the intestinal tract. We also observed that arabinogalactan was the active component of Hyuganatsu orange juice. Together, our results suggest that Hyuganatsu orange is a useful citrus fruit and that daily drinking of juice may be effective in preventing osteoporosis in postmenopausal women.

Keywords: Hyuganatsu orange, osteoporosis, postmenopausal women, calcium absorption, arabinogalactan

Introduction

Postmenopausal osteoporosis is a chronic bone disease characterized by low bone mineral density (BMD) and bone

microarchitectural disruption, leading to bone fragility and increased susceptibility to fractures. This disease has become a major public health issue in aged women in recent years

Abbreviations: BAP, bone alkaline phosphatase; BMD, bone mineral density; IPINP, intact procollagen type I N-propeptide; NTX, type I collagen cross-linked N-telopeptide; TRAP5b, tartrate-resistant acid phosphatase 5b; ucOC, 17-undecarboxylated form of osteocalcin; WE, water extract; WEU, WE-ultrafiltration; AST, aspartate aminotransferase; ALT, alanine transaminase; γ -GTP, γ -glutamyl transpeptidase

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(Granado-Lorencio *et al.*, 2014) and is thought to be associated with estrogen deficiency (Garnero *et al.*, 1996; Jochem *et al.*, 2005). Bone homeostasis is a complex process regulated by bone-resorbing osteoclasts and bone-forming osteoblasts (Karsenty *et al.*, 2009). The imbalance between the regulation of osteoclast and osteoblast activities is known to result in certain metabolic bone diseases including osteoporosis (Feng and McDonald, 2011).

In human and ovariectomized animals, BMD is measured using single X-ray absorptiometry or dual-energy X-ray absorptiometry. On the other hand, the usefulness of serum and urine parameters of bone metabolism has been recently highlighted (Tariq *et al.*, 2016; Watts, 1999). Bone-specific alkaline phosphatase (BAP) and intact procollagen type I N-propeptide (IPINP) are used as serum parameters of bone formation, while tartrate-resistant acid phosphatase 5b (TRAP5b) and type I collagen cross-linked N-telopeptide (NTX) serve as serum parameters of bone resorption (Hallen *et al.*, 2002). In addition, homocysteine and 17- α -undercarboxylated form of osteocalcin (ucOC) are used as parameters of bone extracellular matrix (Ducy *et al.*, 1996; Oury *et al.*, 2013). In the present study, we used these parameters to evaluate metabolism of bone formation, resorption, and bone matrix.

Nutrition is an important factor in the development and maintenance of bone health (Prentice, 2004). Calcium and vitamin D have been identified as important nutritional factors that maintain normal bone metabolism, while other nutrients such as magnesium, zinc, copper, vitamin C, vitamin K, polyphenols, and carotenoids also exert beneficial effects (Deyhim *et al.*, 2006; Liu *et al.*, 2007; Prentice, 2004; Yamaguchi, 2008). Fruits and vegetables are rich sources of these nutrients and epidemiological studies have shown an association between intake of fruits and vegetables and BMD in young and elderly subjects (Sahni *et al.*, 2009; Sugiura *et al.*, 2011). In addition, hesperidin, a citrus flavonoid, and β -cryptoxanthin, a citrus carotenoid, were found to prevent bone loss in ovariectomized rats or mice (Uchiyama and Yamaguchi, 2006; Chiba *et al.*, 2003).

The Hyuganatsu orange (*Citrus tamurana* Hort. ex Tanaka) was originally cultivated in Miyazaki Prefecture in Japan. This fruit is unique because its albedo part is sweet and edible. In our previous study, we found that both the homogenate and water-soluble extract from Hyuganatsu orange increased the BMD in ovariectomized rats, an animal model of osteoporosis (Yamaguchi *et al.*, 2012). As little is known about the effects of Hyuganatsu orange on bone metabolism in postmenopausal women, we first examined whether Hyuganatsu orange juice supplementation influences the representative serum parameters, which were affected by the altered bone metabolism in response to ovarian hormone deficiency.

Absorption of intestinal calcium is well-known to be a crucial physiological process, as it determines the input of calcium in the body to maintain bone mineralization and

calcium homeostasis (Diaz de Barboza *et al.*, 2015). The process occurs through transcellular and paracellular pathways that are regulated by hormones, nutrients, and other factors (Alexander *et al.*, 2014). The transcellular pathway implicates calcium movement from the mucosal-to-serosal side of the intestinal barrier against a concentration gradient. It is an active saturable process that predominates in the duodenum and jejunum and is regulated by nutritional and physiological factors, mainly vitamin D. The paracellular mechanism is a non-saturable and passive transport that occurs across the majority of the intestine and is a linear function of luminal calcium concentration. In this experiment, we divided the small intestine into three pieces corresponding to the segments of jejunum and ileum, and measured calcium absorption in these segments *ex vivo*. In addition, to identify the active components of Hyuganatsu orange, the effects of two water-soluble extracts on calcium absorption were evaluated.

Materials and Methods

Preparation of Hyuganatsu orange sample Hyuganatsu orange juice (100% concentration, Brix 11.13, and acidity 1.06) was prepared by performing a three-fold dilution of the concentrated squeezed juice in Miyazakiken Nokyokajyu (Miyazaki). The diluted juice was used for the human study in experiment 1.

In experiment 2, two active components (water extract [WE] and WE-ultrafiltration [WEU]) of Hyuganatsu orange pomace were prepared according to a previously described method; we used orange pomace instead of orange juice for a more effective extraction of the active components. In brief, WE was extracted from the Hyuganatsu pomace in water, centrifuged, and lyophilized following the addition of an equal amount of dextrin. WEU was prepared using WE by ultrafiltration (30 K and 100 K) and lyophilized. Analytical high-performance liquid chromatography (HPLC) was performed using a 300 $\times$ 7.8 mm i.d. TSKgel G5000-PWXL column (Tosoh Bioscience, Tokyo) with phosphate buffer (pH 6.8) at a flow rate of 0.5 mL/min at 40 °C with an RID-10A (Shimadzu Co., Japan). WEU was detected as a single peak, which was then identified as arabinogalactan. The molecular mass of arabinogalactan was 68 kDa, and its arabinogalactan concentration in Hyuganatsu orange juice was 0.19 mg/mL.

Human study This was a randomized, double-blind, cross-over study in healthy postmenopausal women. Twenty-five postmenopausal women aged 59–64 years agreed to participate in the trial. The Research Ethics Committee of Faculty of Medicine, University of Miyazaki approved the study protocol, and completed consent letters were obtained from all participants. The participants were randomly divided into two groups: the control and Hyuganatsu groups. Those from the Hyuganatsu group consumed 125 mL of 100% Hyuganatsu orange juice daily for 3 months; the drinking time was not designated. Participants from the control group received no intervention. Blood was collected from the participants at 0, 1, and 3 months during the study period;

the serum samples were stored at -80°C . Serum parameters of bone metabolism were measured levels of BAP, IPINP, TRAP5b, NTX, homocysteine, ucOC, total protein, albumin, C-reactive protein, urea nitrogen, creatinine, aspartate aminotransferase (AST), alanine transaminase (ALT), and γ -glutamyl transpeptidase (γ -GTP). Measurement of all parameters was consigned to SRL, Inc. (Tokyo).

Ex vivo calcium absorption study The modified everted sac technique was employed to measure the absorption of calcium in the intestinal tract, as described by Parsons *et al* (1984). In brief, 8-week-old male Sprague-Dawley rats were fed with commercial pellets (CE-2, Clea Japan, Tokyo) for 2 days and allowed free access to water. Rats were sacrificed by decapitation and the whole intestine was excised and washed with cold saline. The intestine was divided into three parts and 12-cm lengths of upper (corresponding to jejunum fraction), middle, and lower (corresponding to ileum fraction) intestine were cut, rinsed, everted, and ligated into a closed sac using surgical thread. A total of 1.0 mL saline (serosal fluid) was injected into the sac, which was then transferred to a 20-mL conical flask containing 10 mL of 16.9 mM calcium chloride (CaCl_2), 5.8 mM monopotassium phosphate (KH_2PO_4), and 0.9% sodium chloride (NaCl ; pH 6.6) with either 0.2% WE, 0.2% WEU, or 0.1% dextrin. The flask was shaken in a water bath at 37°C for 40 min. We confirmed that the rate of apparent absorption of calcium in these three intestinal segments was linear as a function of incubation time for more than 40 min, as described by Saito *et al* (1999). At the end of the incubation, the serosal fluid in the sac was collected from the different segments and calcium concentration was determined using an Aqua-auto Kainos calcium test kit (Kainos Laboratories, Inc., Tokyo).

Statistical analysis Data are expressed as mean $\pm$ standard error (SE) and were analyzed with a Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

Results

Prior to the inception of the human intervention study, we confirmed that all serum parameters of liver function and bone metabolism in these postmenopausal women were within the reference levels, as indicated in the clinical instruction manual, except for the ucOC level, which was higher than the reference range for all of these women. All data were comparable between the two groups at time zero, as shown in Table 1. Under these conditions, serum parameters were expressed as zero in all groups (Fig. 1).

We measured serum IPINP and BAP, as typical markers of bone formation. The levels of IPINP in the control group remained unchanged for 3 months, while that in the Hyuganatsu group gradually decreased; after 3 months, the values were significantly lower in the Hyuganatsu group than the control group. On the other hand, BAP level, which is known to increase after menopause, showed a gradual increase

over 3 months; however, the effect of Hyuganatsu orange juice on this parameter was negligible. We also evaluated markers of serum resorption such as TRAP5b and NTX. Compared with the control group, the Hyuganatsu group showed a significant reduction in the level of TRAP5b after 3 months, but not after 1 month (Fig. 1). On the other hand, no difference in the level of NTX was observed between the two groups during the study period. Analysis revealed no significant differences in the levels of the bone matrix-related parameters ucOC and homocysteine between the two groups after 3 months of study.

No significant difference was observed in the degree of calcium absorption between the three intestinal segments in the control group (4.82 ± 0.37 , 3.37 ± 0.13 , and 5.53 ± 0.33 mg/g for the upper, middle, and lower segments, respectively), as shown in Fig. 2. On the other hand, the addition of both WE and WEU to the incubation medium significantly and drastically increased calcium absorption in all three segments. However, in both the WE and WEU groups, serosal calcium content in the middle segment of the intestine tract was higher than that in the other two segments (Fig. 2).

Discussion

Serum parameters of liver function such as AST, ALT, and γ -GTP levels were within the normal range at 0 months in the control and test subjects, and the consumption of Hyuganatsu orange juice for 3 months had no influence on these parameters (data not shown), suggesting that the orange juice had no unfavorable effects on liver function during the study period.

Hyuganatsu orange juice consumption for 3 months favorably altered some parameters of bone metabolism, including bone formation and bone resorption, in postmenopausal women (Fig. 1). A biochemical marker of bone turnover that reflects bone changes faster than BMD is available for measuring serum and urine. High bone turnover associated with increased bone formation and resorption was observed in postmenopausal women (Gossiel *et al.*, 2018). Therefore, we measured the level of IPINP, a marker of not only bone formation but also osteoporosis risk. The IPINP level observed after 3 months of Hyuganatsu orange juice consumption was significantly lower than that observed at 0 months. In addition, serum IPINP levels were significantly lower in the group that consumed Hyuganatsu orange juice for 3 months than in the control group, suggesting that the presence of active components in Hyuganatsu orange juice reduced the risk of postmenopausal osteoporosis.

Next, we measured the level of serum TRAP5b, a marker of bone resorption that has been used to estimate bone metabolism in osteoporosis patients (Halleen *et al.*, 2001), and found that daily intake of Hyuganatsu orange juice significantly lowered serum levels of TRAP5b after 3 months compared to that at 0 months. We previously observed that WE of Hyuganatsu orange decreased the number of TRAP-positive cells among rat pre-osteoclasts and increased rat osteoblast cell numbers *in vitro*. These observations suggest that Hyuganatsu

Table 1. Physical and clinical characteristics of the study groups.

	Control	Hyuganatsu	Reference range (Postmenopausal*)
Subject (n)	12	13	
Age (y)	62.1 ± 2.3	62.5 ± 1.5	
Menarche (y)	13.3 ± 0.3	13.5 ± 0.3	
Menopause (y)	49.1 ± 1.8	49.1 ± 1.5	
Height (cm)	155 ± 2	155 ± 2	
Body weight (kg)	54.3 ± 1.9	57.0 ± 4.0	
BMI (kg/m ²)	22.3 ± 0.5	24.9 ± 2.1	
Blood pressure (mmHg)			
Systolic	135 ± 4	135 ± 5	
Diastolic	78 ± 4	83 ± 4	
Serum parameters			
BAP (U/L)	15.9 ± 1.4	16.0 ± 1.4	3.8–22.6 ¹ (↑)
IPINP (ng/mL)	62.9 ± 7.6	59.6 ± 5.8	26.4–98.2 ¹ (↑)
TRAP5b (mU/dL)	406 ± 53	398 ± 42	120–420 ² (↑)
NTX (nmol BCE/L)	19.9 ± 1.3	18.6 ± 1.7	14.3–89.0 (↑)
Homocysteine (μmol/L)	8.46 ± 0.87	7.29 ± 0.31	3.7–13.5 (↑)
ucOC (ng/mL)	5.87 ± 1.00	6.50 ± 1.02	<4.50 (↑)
Total protein (g/dL)	7.11 ± 0.08	7.35 ± 0.12	6.7–8.3
Albumin (g/dL)	4.59 ± 0.05	4.52 ± 0.1	3.8–5.2
C-reactive protein (mg/dL)	0.10 ± 0.03	0.07 ± 0.02	≤0.30
Urea nitrogen (mg/dL)	14.4 ± 0.9	15.9 ± 1.2	8.0–22.0
Creatinine (mg/dL)	0.63 ± 0.02	0.66 ± 0.03	0.47–0.79 ²
AST (IU/L)	19.7 ± 1.26	21.1 ± 1.03	10–40
ALT (IU/L)	16.3 ± 1.6	17.8 ± 1.38	5–40
Alkaline phosphatase (IU/L)	233 ± 14	240 ± 13	115–359
γ-GTP (IU/L)	23.4 ± 3.5	19.8 ± 1.9	≤30 ²

Data are expressed as mean ± SE (n=12–13).

BAP: bone specific alkaline phosphatase; IPINP: intact procollagen type I N-propeptide;

TRAP5b: tartrate-resistant acid phosphatase 5b; NTX: type I collagen cross linked

N-telopeptide; ucOC: undercarboxylated osteocalcin; AST: aspartate aminotransferase;

ALT: alanine transaminase; γ-GTP: γ-glytamyl transpeptidase

¹Reference range of postmenopausal women.

²Reference range of women.

*Change of postmenopausal

oranges may influence the number or activity of osteoclasts as well as the activity of osteoblasts. In addition to enhancing calcium absorption, as observed in the rat study, Hyuganatsu orange juice intake might also suppress bone loss in postmenopausal women. Indeed, ovariectomized rats treated with Hyuganatsu orange homogenate showed increased BMD in the femur bone.

Serum ucOC and homocysteine levels are known to increase during the postmenopausal period; thus, the mean values of these parameters in both groups were higher than the reference range. Hyuganatsu orange juice had no influence on these parameters. Serum ucOC level was reported to be inversely associated with BMD and is related to vitamin K

status (Nishizawa *et al.*, 2013). In this study, it was suggested that Hyuganatsu orange juice did not show an ameliorative effect on serum ucOC compared to dietary factors such as vitamin K intake. In addition, serum levels of ucOC in both the control and Hyuganatsu groups were slightly higher than the reference range. This might affect the results of our study. Further studies are needed to elucidate this point.

In our previous experiment, we observed using X-ray absorptiometry that treatment with WE of Hyuganatsu orange pomace for 8 weeks increased BMD in the femoral bone of 10-week-old female ovariectomized rats. (Yamaguchi *et al.*, 2012). In other *in vitro* experiments with rat pre-osteoclasts, we found that WE, but not an acetic acid extract, prepared from

Bone formation

Bone resorption

Bone extracellular matrix

Fig. 1. Change in serum parameters involved in bone turnover.

Data are expressed as mean±SE (n=12–13). * $P < 0.05$ for 0 month. # $P < 0.05$ for control. BAP: bone specific alkaline phosphatase; IPINP: intact procollagen type I N-propeptide; TRAP5b: tartrate-resistant acid phosphatase 5b; NTX: type I collagen cross-linked N-telopeptide; ucOC: undercarboxylated osteocalcin.

freeze-dried Hyuganatsu oranges significantly decreased the number of TRAP-positive cells, a parameter of bone formation, and that treatment of the cells with the same extract resulted in increased number of rat osteoblasts. In the present study, we observed alterations in several serum parameters involved in

bone metabolism, including bone formation and bone resorption. However, no changes were observed in bone matrix parameters. To the best of our knowledge, this is the first report to describe the effectiveness of daily consumption of Hyuganatsu orange juice in preventing the incidence and

Fig. 2. Effect of Hyuganatsu orange juice on calcium absorption in everted rat intestine. Data are expressed as mean $\pm$ SE (n=5–6). * P < 0.05 for control. WE: water extract; WEU: WE-ultrafiltration.

progression of osteoporosis in postmenopausal women. Our study results demonstrated that the alterations in serum parameters are favorable for improving the imbalance in bone metabolism during osteoporosis in postmenopausal women.

It is known that calcium absorption is a critical determinant of the balance between bone formation and bone absorption, and plays an important role in maintaining normal bone mass and density. In the present study, a rat everted intestinal model was employed to measure the rate of calcium absorption in the intestinal tract. As shown in Fig. 2, we isolated WE and WEU from Hyuganatsu orange juice and used these fractions in the *ex vivo* intestinal calcium absorption experiment.

Various citrus fruits contain biologically active substances that prevent osteoporosis. For example, treatment of 7-week-old ovariectomized Wistar rats with β -cryptoxanthin once daily for 3 months was shown to increase BMD and mineral content in the diaphysis and metaphysis of the femur to levels observed in the sham-operated animals (Uchiyama and Yamaguchi, 2006). In a human study, carotenoid and flavonoid compounds such as hesperidin, derived from Hyuganatsu orange and extracted with hexane and methanol, were thought to partially influence serum TRAP5b and IPINP levels, thereby suggesting that both β -cryptoxanthin and hesperidin are active components that may ameliorate bone metabolism. Furthermore, we aimed to investigate the active component(s) that ameliorate bone

metabolism, including bone formation and resorption. Our previous data confirmed that WE, but not an acetic acid extract, of Hyuganatsu orange juice contained a predominant active component that was revealed to be the polysaccharide arabinogalactan. These observations are in accordance with those previously reported, as described above.

In another study, we aimed to isolate the active component of Hyuganatsu orange, and tentatively identified arabinogalactan as the bioactive compound in WE and WEU. This arabinogalactan is a water-soluble molecule with a molecular mass of 10 kDa (in preparation for submission). Hata *et al.* (2015) reported that this water-soluble substance inhibits rat osteoclast formation. There are no reports on the effects of arabinogalactan on the prevention of osteoporosis; however, some other polysaccharides and soluble corn fiber have been shown to influence bone turnover (Slevin *et al.*, 2014; Tousein *et al.*, 2013; Jakeman *et al.*, 2016). Thus, arabinogalactan from Hyuganatsu orange is an active substance that enhances calcium absorption and, therefore, alleviates bone turnover. We are currently in the process of confirming the active component in our laboratory. In addition, our present study demonstrated that both WE and WEU enhanced calcium absorption in all three intestinal segments, especially the middle segment. These results suggested that WE of Hyuganatsu and its active substance enhanced calcium absorption through transcellular and paracellular pathways, resulting in alterations of bone

metabolism including bone formation and resorption. In a proteome analysis of everted-sacs incubated with WEU, WEU influenced protein levels related to calcium metabolism and glucose transport (unpublished data). It has been reported that soluble fiber from the ingestion of guar gum hydrolysate, a combination of galacto-oligosaccharide and fructo-oligosaccharide prebiotic mixture, soluble maize fiber, and galacto-oligosaccharides increased calcium absorption (Hara *et al.*, 1999; Bryk *et al.*, 2015; Whisner *et al.*, 2013; Whisner *et al.*, 2014). Yang *et al.* reported that an aqueous extract of *Anoectochilus formosanus* increased calcium absorption, and type II arabinogalactan was isolated as the active component (Yang *et al.*, 2012). This arabinogalactan increases the expression of ABC transporter, which is related to nutrient uptake. In the present study, Hyuganatsu arabinogalactan or its hydrolysate may influence intestinal calcium absorption. The mechanisms by which WEU facilitated calcium absorption need to be further investigated. Taken together, Hyuganatsu orange juice ameliorated the incidence and/or progression of osteoporosis in postmenopausal women.

In conclusion, our results suggested that Hyuganatsu orange juice may serve as a useful functional food for ameliorating the incidence and/or progression of osteoporosis caused by ovarian hormone deficiency in postmenopausal women. The active component responsible for the observed alteration in bone metabolism was proposed to be arabinogalactan.

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