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Canola and hydrogenated soybean oils accelerate ectopic bone formation induced by implantation of bone morphogenetic protein in mice

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ABSTRACT

Canola oil (Can) and hydrogenated soybean oil (H2-Soy) are commonly used edible oils. However, in contrast to soybean oil (Soy), they shorten the survival of stroke-prone spontaneously hypertensive (SHRSP) rats. It has been proposed that the adverse effects of these oils on the kidney and testis are caused at least in part by dihydro-vitamin K (VK) 1 in H2-Soy and unidentified component(s) in Can. Increased intake of dihydro-VK1 is associated with decreased tissue VK2 levels and bone mineral density in rats and humans, respectively. The aim of the present study was to determine the effects of these oils on bone morphogenetic protein (BMP)-induced ectopic bone formation, which is promoted by VK2 deficiency, in relation to the role of VK in the γ-carboxylation of osteocalcin and matrix Gla protein. A crude extract of BMPs was implanted into a gap in the fascia of the femoral muscle in 5-week-old mice maintained on a Soy, Can, or H2-Soy diet. Newly formed bone volume, assessed by three-dimensional X-ray micro-computed tomography and three-dimensional reconstruction imaging for bone, was 4-fold greater in the Can and H2-Soy groups than in the Soy group. The plasma carboxylated osteocalcin (Gla-OC) and total OC (Gla-OC plus undercarboxylated osteocalcin [Glu-OC]) levels were significantly lower in the Can group than in the Soy group ($p < 0.05$). However, these levels did not significantly differ between the H2-Soy and Soy groups. The plasma Gla-OC/Glu-OC ratio in the Can and H2-Soy groups was significantly lower (in Can; $p = 0.044$) or was almost significantly lower (in H2-Soy;

Abbreviations: BMP, bone morphogenetic protein; Can, canola oil; cMGP, carboxylated matrix Gla protein; dihydro-VK1, 2′, 3′-dihydro-vitamin K1; Gla, carboxyglutamic acid; Gla-OC, carboxylated osteocalcin; Glu-OC, undercarboxylated osteocalcin; G6PDH, glucose-6-phosphate dehydrogenase; H2-Soy, hydrogenated soybean oil; mCT, micro-computed tomography; SHRSP rat, stroke-prone spontaneously hypertensive rat; Soy, soybean oil; TRI/3D-BON, three-dimensional reconstruction imaging for bone; ucMGP, undercarboxylated MGP; VK, vitamin K.

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Osteocalcin
Vitamin K
Bone morphogenetic protein

$p = 0.053$) than that in the Soy group. In conclusion, Can and H2-Soy accelerated BMP-induced bone formation in mice to a greater extent than Soy. Further research is required to evaluate whether the difference in accelerated ectopic bone formation is associated with altered levels of VK2 and VK-dependent protein(s) among the three dietary groups.

1. Introduction

Canola oil (Can) and hydrogenated soybean oil (H2-Soy) are widely consumed by humans. However, the survival of stroke-prone spontaneously hypertensive (SHRSP) rats is significantly shortened when they are fed a diet containing Can, H2-Soy, high-oleic safflower oil, high-oleic sunflower oil, olive oil, or evening primrose oil compared to soybean oil (Soy), perilla oil, flaxseed oil, fish oil, lard, or butter fats [1–6]. A diet containing 10% Can increases blood pressure [7,8], decreases platelet counts [8], enhances blood coagulation [9], increases hepatic glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) activity [10], alters Na^+/K^+ ATPase activity [7], decreases antioxidant enzyme activity [10], increases plasma aldosterone levels while decreasing plasma and testicular testosterone levels [11], and decreases the activities of antioxidant enzymes in red blood cells [6,12]. In 2000, a soybean phytosterol fraction was identified as a probable cause for the shortened survival of SHRSP rats [13]. However, free fatty acid fractions from Can containing similar amounts of phytosterols did not exhibit survival-shortening effects on SHRSP rats [14]. Therefore, we hypothesized that the presence of factor(s) other than the fatty acids and phytosterols in these oils might affect the survival of SHRSP rats [5,14].

Vitamin K (VK) 1 is present in high concentrations in vegetable oils, such as Soy and Can, and we believe that it may be one of the factors that affect the survival of SHRSP rats. VK1 contains one double bond at the phytyl side chain. Upon ingestion, the side chain of VK1 is removed enzymatically to form VK3, and then an isoprenyl side chain with 4 double bonds is attached to VK3 to form VK2 [15,16], which has stronger physiological activities, including γ-carboxylation of the Glu residues of osteocalcin (OC; bone Gla protein) and matrix Gla protein (MGP), than VK1 in extrahepatic tissues [17,18]. During the industrial hydrogenation of vegetable oils, the double bond in the side chain of VK1 is hydrogenated to form dihydro-VK1, which is absorbed and delivered to various tissues [19], but is not converted to VK2 [20] (Fig. 1A). Moreover, dietary dihydro-VK1 is reported to decrease tissue VK2 levels, resulting in VK2 deficiency [19].

VK is a cofactor for γ-glutamyl carboxylase, which catalyzes the posttranslational modification of VK-dependent proteins such as coagulation factors (II, VII, IX, and X), MGP, and OC [21]. VK hydroquinone is the active form of VK (Fig. 1B) required for the synthesis of Gla proteins. VK antagonists such as warfarin, which is a plant product, inhibit the carboxylation of VK-dependent proteins by inhibiting the synthesis of VK hydroquinone [22,23] (Fig. 1B). Nicotinamide adenine dinucleotide phosphate (NADPH) [24] required for the production of VK hydroquinone is supplied by G6PDH, which is a cytosolic enzyme in the pentose phosphate pathway of glucose metabolism. Thus, G6PDH activity might serve as a potential indicator of VK hydroquinone production.

MGP is a 14-kDa extracellular matrix protein synthesized by chondrocytes, vascular smooth muscle cells, endothelial cells, and fibroblasts in the heart, lung, kidney, skin, and arterial vessel walls [25]. MGP undergoes two types of posttranslational modifications – glutamate carboxylation and serine phosphorylation [25] – and potently inhibits arterial calcification [26–28] (Fig. 1C). MGP-deficient mice die within 2 months of birth because of extensive arterial calcification leading to blood vessel rupture [27]. MGP modulates the activity of bone morphogenetic protein (BMP; Fig. 1C), which induces osteogenesis in soft as well as in hard tissues [29–31].

Similar to MGP, OC is a calcium-binding protein involved in bone metabolism, and it is carboxylated on its glutamate residues (Fig. 1C). OC-deficient mice exhibit increased bone formation [32]. Gla-OC plays an important role in preventing bone hyperplasia (Fig. 1C).

VK plays a key role in the synthesis of both MGP and OC. Undercarboxylated MGP (ucMGP) and OC (Glu-OC) are indirect markers of VK2 deficiency [34]. Specifically, the plasma level of ucMGP is an indicator of vascular calcification [35]. However, a previous study reported that a phosphorylated $MGP^{3\text{-}15}$ peptide lacking Gla residues inhibited calcification [36]. Therefore, the role of VK in the regulation of the anticalcification activity of MGP has remained controversial.

Plasma dephospho-ucMGP is a marker of vascular calcification in chronic kidney disease [37,38] and is associated with low VK levels [38–40]. Thus, VK deficiency accelerates calcification and inhibits coagulation [28], and optimal VK intake is important in order to reduce the risk of occurrence of these diseases [28].

In this study, the effects of Soy and Can (rich in VK1) and H2-Soy (rich in dihydro-VK1) on BMP-induced ectopic bone formation were examined in relation to the role of VK in the γ-carboxylation of OC and MGP.

2. Materials and methods

2.1. Chemicals

All reagents were of analytical grade or higher quality and were purchased from commercial suppliers. Ethyl esters of linoleic and α-linolenic acids, VK1, VK2, and VK3 were purchased from Wako Pure Chemicals (Osaka, Japan), and dihydro-VK1 was synthesized according to a published method [41]. Soy and Can (rapeseed oil) were purchased at a local market, and H2-Soy for human consumption was obtained from Hamari Chemicals Ltd. (Osaka, Japan).

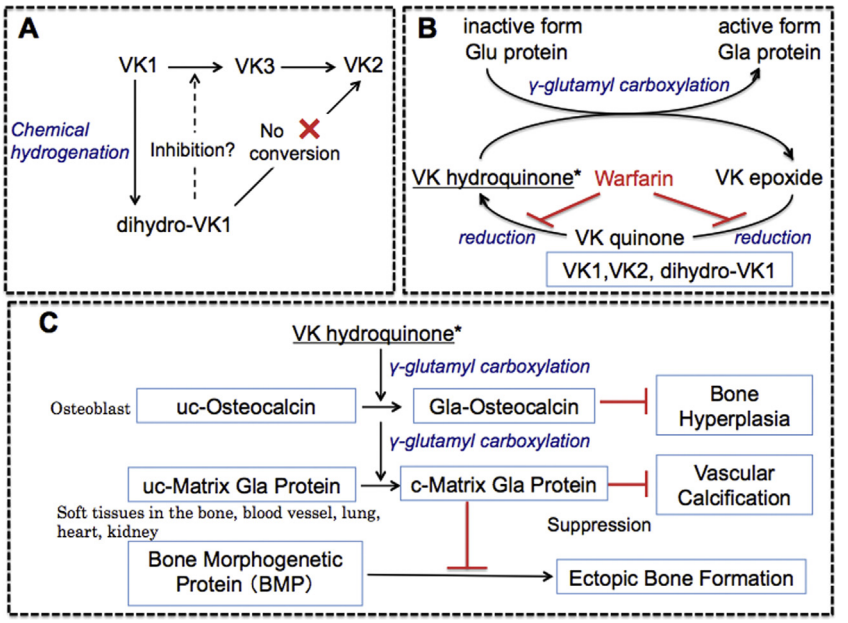

Fig. 1. Conversion of vitamin K (VK) 1 to VK2, production of dihydro-VK1, the VK cycle, and γ-glutamyl carboxylation. (A) VK1, but not dihydro-VK1, is enzymatically converted into VK2 (MK-4) via VK3 [15,16,20]; VK1 is converted to dihydro-VK1 by the partial hydrogenation process [33]. (B) The active form of VK (VK hydroquinone) is synthesized by the reduction of VK quinone (VK1, VK2, and dihydro-VK1) [20]. (C) Gla proteins are synthesized from Glu proteins by γ-glutamyl carboxylase via a VK hydroquinone-dependent synthesis pathway.

Porcine cortical bones were purchased from a slaughterhouse.

2.2. Animals and diets

The basal diet consisted of AIN-93G without added Soy and VK1 (Funabashi Farm Co., Ltd., Chiba, Japan). The Soy and Can diets were prepared by mixing the basal diet and the appropriate oil at a ratio of 93:7. To prevent essential fatty acid deficiency in the mice on the H2-Soy diet, H2-Soy and essential linoleic and α-linolenic acids were added to the basal diet at final concentrations of 5.2%, 0.9%, and 0.9%, respectively. Dietary oils are the sole sources of VK1. The diets were pulverized and stored at −30 °C under hermetic conditions and were used within 3 months of preparation. The Soy diet was used as a control because it does not significantly shorten the survival of SHRSP rats [2–5]. The food provided to the animals was replaced daily.

Male ddY mice (4 weeks old) were obtained from Japan SLC, Inc. (Hamamatsu, Japan) and were randomly assigned to one of three groups ($n = 6$ per group). Mice in each group were housed in the same cage under a 12/12 h light–dark cycle at 23 °C and were fed a diet containing Soy, Can, or H2-Soy throughout the experiment. The study protocol was approved by the Ethics Committee of Aichi-Gakuin University, School of Dentistry (ethical clearance number: AGUD 157).

2.3. Determination of the dihydro-VK1 content of Soy, Can, and H2-Soy

VK homologues were extracted from the oils as follows: the oil (75 μL) was mixed vigorously with methanol (5 mL) for 5 min, and VK homologues were separated by centrifugation at 2000 rpm for 5 min and quantified as described previously [42]. Briefly, high performance liquid chromatography (HPLC) was performed by injecting 50 μL of the sample extract into the column (Nucleosil 100-5C18, 4.6 mm × 150 mm; GL Science, Tokyo, Japan). The sample was eluted with 100% methanol at a flow rate of 1 mL/min at room temperature. The effluent was fed directly into a post-column reduction system (Platinum-Black Column RC-10, 4.0 mm × 30 mm; Shiseido, Tokyo, Japan) with an applied potential of −400 mV to reduce the homologues, which were detected using fluorescence spectrophotometry (FS-8020; Tosoh, Tokyo, Japan) at excitation and emission wavelengths of 320 and 430 nm, respectively. The concentration of the VK homologue was measured using the peak area method and calculated from a calibration curve.

2.4. Analysis of BMP-induced ectopic bone formation using three-dimensional X-ray micro-computed tomography (3D R_mCT) and three-dimensional reconstruction imaging for bone system (TRI/3D-BON)

A crude extract of BMPs was prepared by freezing and pulverizing fresh porcine cortical bone. The pulverized bone was then demineralized with 0.6 M HCl for 72 h. The preparation was washed with 2 M $CaCl_2$ and then with 0.5 M EDTA, and was extracted with a buffer (6 M urea, 0.5 M $CaCl_2$, 1 mM *N*-ethylmaleimide, and 1 mM benzamidine HCl) [29]. This crude extract of BMPs was used to induce ectopic bone formation in the mice. The mice were anesthetized with isoflurane (Abbott Japan Co., Ltd., Tokyo, Japan) after 1 week of acclimatization, during which they were fed the Soy, Can, or H2-Soy diet; a capsule containing

5 mg of the crude extract of BMPs in #5 gelatin (Dainippon Sumitomo Pharmaceuticals Co., Ltd., Osaka, Japan) was implanted into a gap in the fascia of the right femoral muscle. Ectopic formation of new bone was visualized by scanning with a 3D R_mCT apparatus (Rigaku, Co., Tokyo, Japan) 3 weeks after the operation. The amount of bone was measured using a TRI/3D-BON system (Ratoc System Engineering Co., Ltd., Tokyo, Japan) [43].

2.5. Collection of blood and tissue samples

Mice (age, 9 weeks) were anesthetized with sevoflurane (Mylan Pharmaceuticals, Mylan Inc., Osaka, Japan) and then euthanized by cardiac puncture. Blood was collected in a tube containing 10 μL of 0.5 M ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (pH 7.2). The plasma was separated by centrifugation at 3000 rpm for 15 min at 4 °C and stored at −80 °C. The heart, liver, kidneys, and testes were removed and weighed. The liver sample was stored at −80 °C.

2.6. Determination of G6PDH activity and total antioxidant power in the liver

G6PDH activity was measured using a G6PDH assay kit (colorimetric; ab102529) purchased from Abcam (London, UK). Total antioxidant power was measured using a total antioxidant power kit (TA02) purchased from Oxford biomedical research (Oxford, MI). Both values were measured according to the manufacturers' protocols. Briefly, 10 mg liver samples were homogenized in 0.3 mL of phosphate-buffered saline (pH 7.2), and the supernatants (obtained upon centrifugation at 3000 × *g* for 12 min at 4 °C or 15,000 × *g* for 10 min at 4 °C) were used to measure the total antioxidant power or G6PDH activity, respectively. The protein concentration of the supernatants was determined using the method described by Hartree [44]. Bovine serum albumin (Sigma–Aldrich Co., St. Louis, MO) was used as the standard.

2.7. Determination of cMGP, Gla-OC, and Glu-OC levels in plasma

Plasma levels of cMGP, Gla-OC, and Glu-OC were measured using enzyme-linked immunosorbent assay (ELISA) kits. The CBS-E16540m (Cusabio Biotech Co., Ltd., Hubei, China) and SEB477Mu (Cloud-Clone Corp., Houston, TX, USA) ELISA kits were used to estimate plasma levels of mouse cMGP. Mouse Gla-OC and Glu-OC High-Sensitivity Enzyme Immunoassay Kits (MK127 and MK129, respectively) were purchased from Takara Bio Inc. (Shiga, Japan).

2.8. Statistical analysis

Data are presented as means ± standard errors (SEs). Mean differences were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests using Excel SSR1 2012 for Windows (Social Survey Research Co., Ltd., Tokyo, Japan). Mean differences with respect to the total amount of newly formed bone mass induced by BMP implantation and Gla-OC/Glu-OC ratios were evaluated by one-way ANOVA followed by Dunnett's test (with the Soy group used as the target). Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. VK1 is present in the hydrogenated form in H2-Soy

All three oils contained VK1, but dihydro-VK1 was detected only in H2-Soy (Fig. 2). Ninety-seven percent of VK1 was hydrogenated during the hydrogenation process of Soy. The VK1 content of Soy, Can, and H2-Soy was 0.193 mg%, 0.127 mg%, and 0.006 mg%, respectively. The dihydro-VK1 content of H2-Soy was 0.187 mg%. The total VK content in all three diets exceeded the present recommended dietary allowance for humans [45].

3.2. Ectopic bone formation is enhanced by the Can and H2-Soy diets

Induction of bone formation was assessed 3 weeks after the crude extract of BMPs was implanted in the mice. The total amount of newly formed bone mass in the Soy group was approximately 25% that in the Can and H2-Soy groups

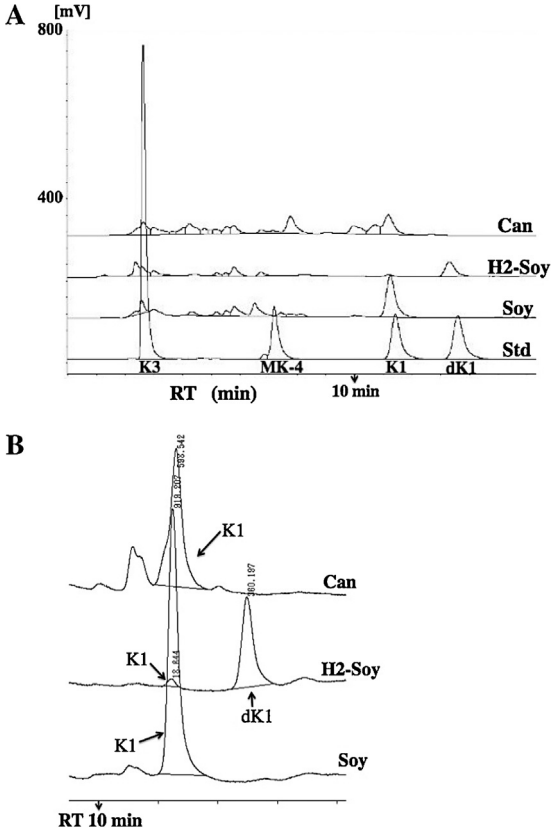

Fig. 2. Determination of the ratio of vitamin K (VK) 1 and dihydro-VK1 in the vegetable oils. (A) High performance liquid chromatography (HPLC) analysis of the standard (Std; 5 ng each) and VK homologues eluted from soybean oil (Soy), hydrogenated soybean oil (H2-Soy), and canola oil (Can). The VK1 and dihydro-VK1 content of H2-Soy was estimated from the ratio of peak areas of VK1 and dihydro-VK1 in the H2-Soy eluted from the column. (B) Exploded view of (A) after 10 min.

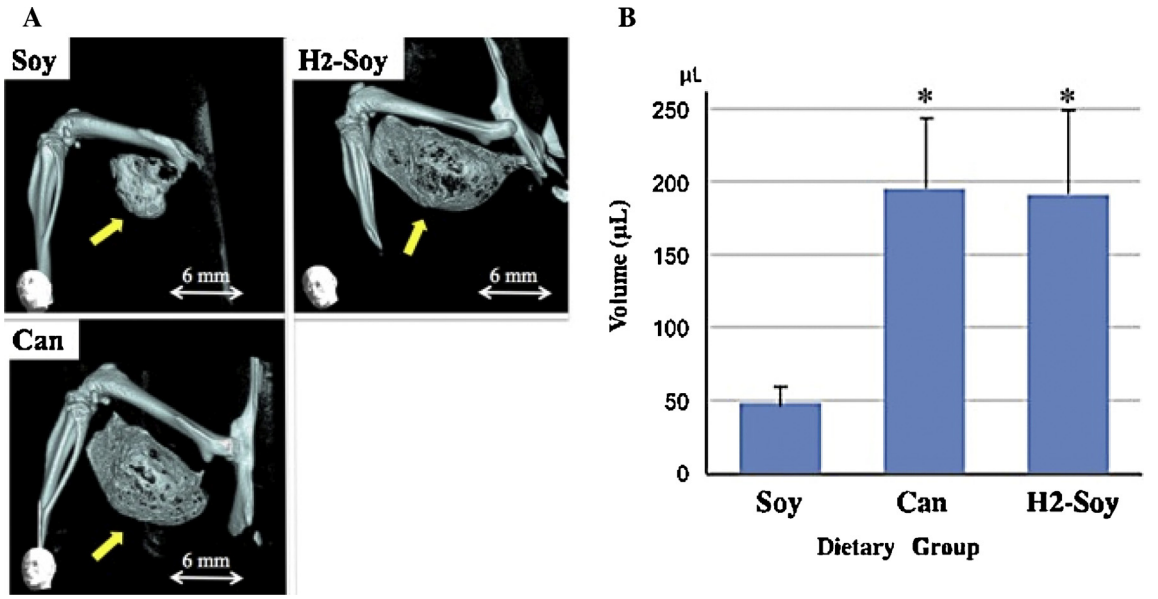

Fig. 3. Newly formed bone in mice fed the soybean oil (Soy), canola oil (Can), or hydrogenated soybean oil (H2-Soy) diet after implantation with a crude extract of bone morphogenetic proteins (BMPs). (A) Representative reconstructed images generated from three-dimensional X-ray micro-computed tomography (3D R_mCT) scans of ectopic newly formed bone (arrow) in the femoral muscle. (B) The volume of newly formed ectopic bone induced by the implantation of the crude extract of BMPs in mice fed the Soy, Can, and H2-Soy diets was calculated from 3D R_mCT scans using three-dimensional reconstruction imaging for bone (TRI/3D-BON).
* $p < 0.05$ vs. Soy.

(Fig. 3); thus, the Can and H2-Soy diets increased bone formation by approximately 4-fold.

3.3. H2-Soy consumption increases the weights of the testis, kidney, and liver

There were no significant differences in the wet weights of the body or heart among the groups (Table 1). However, the testis weight was significantly greater (by factors of 1.35 and 1.29) in the H2-Soy group than in the Soy and Can groups, respectively, whereas the liver and kidney weights were significantly higher in the H2-Soy group than in the Can and Soy groups, respectively. No significant differences in the body and tissue weights were detected between the Soy and Can groups.

3.4. Hepatic G6PDH activity and total antioxidant power

As shown in Table 2, the H2-Soy group had the highest hepatic G6PDH activity and total antioxidant power among the 3 dietary groups. G6PDH activity was significantly higher ($p < 0.05$) in the H2-Soy group than in the Soy and Can groups. In addition, the total antioxidant power was significantly higher ($p < 0.01$) in the H2-Soy group than in the Can group. No significant differences were detected between the Soy and Can groups. G6PDH is known to supply NADPH for the activity of reductases [24], which is required for VK hydroquinone synthesis. The total antioxidant power estimated in this study included the activities of reductases such as superoxide dismutase, catalase, and glutathione peroxidase. These results suggest that, compared to the Soy diet, the Can and H2-Soy diets did not inhibit the production of VK hydroquinone.

3.5. Plasma Gla-OC level, Gla-OC/Glu-OC ratio, and total OC level

The cMGP levels, measured using two different ELISA kits, were not significantly different, and no significant

Table 1
Tissue weights of mice fed the soybean oil (Soy), canola oil (Can), and hydrogenated soybean oil (H2-Soy) diets.[a]

Weight (g)	Soy	Can	H2-Soy
Body	40.2 ± 0.8	40.2 ± 0.7	42.8 ± 1.5
Heart	0.18 ± 0.003	0.18 ± 0.003	0.19 ± 0.007
Liver	1.87 ± 0.10	1.65 ± 0.06**	2.09 ± 0.10
Kidney	0.58 ± 0.02*	0.61 ± 0.03	0.67 ± 0.02
Testis	0.20 ± 0.01*	0.21 ± 0.02*	0.27 ± 0.01

[a] Values are represented as mean ± standard error (SE) ($n = 6$ per group).
* $p < 0.05$ vs. H2-Soy.
** $p < 0.01$ vs. H2-Soy.

Table 2
Hepatic glucose-6-phosphate dehydrogenase (G6PDH) activity and total antioxidant power in mice fed the soybean oil (Soy), canola oil (Can), and hydrogenated soybean oil (H2-Soy) diets.[a]

	Soy	Can	H2-Soy
G6PDH[b]	2.10 ± 0.11*	2.09 ± 0.09*	2.47 ± 0.11
TAOP[c]	57.8 ± 3.92	55.6 ± 0.85**	65.7 ± 2.94

[a] Values are represented as mean ± SE of duplicate assays ($n = 6$ per group).
[b] G6PDH: glucose-6-phosphate dehydrogenase (mU/mg protein).
[c] TAOP: total antioxidant power (uric acid equivalent; pmole/mg protein).
* $p < 0.05$ vs. H2-Soy.
** $p < 0.01$ vs. H2-Soy.

Table 3
Plasma levels of VK-dependent proteins (cMGP, OC, and OC derivatives) in mice fed the soybean oil (Soy), canola oil (Can), and hydrogenated soybean oil (H2-Soy) diets.[a]

	Soy	Can	H2-Soy
cMGP (ng/mL)[b]	113.0 ± 4.9	114.9 ± 3.6	110.9 ± 4.9
cMGP (ng/mL)[c]	88.6 ± 11.2	101.3 ± 18.4	78.7 ± 12.0
Gla-OC (ng/mL)	89.3 ± 7.9	59.0 ± 7.6*	73.8 ± 8.0
Glu-OC (ng/mL)	7.00 ± 0.81	6.55 ± 0.64	7.83 ± 0.82
Total OC (ng/mL)[d]	96.3 ± 8.0	65.5 ± 7.6*	81.6 ± 8.5
Gla-OC/total OC	0.93 ± 0.01	0.89 ± 0.01	0.90 ± 0.01
Gla-OC/Glu-OC	13.8 ± 1.96	9.4 ± 1.34*,**	9.6 ± 0.73***

cMGP, carboxylated MGP; Gla-OC, carboxylated osteocalcin; Glu-OC, undercarboxylated osteocalcin; OC, osteocalcin.

[a] Values are represented as mean ± SE of duplicate assays ($n = 6$ per group).

[b] Measured using a CBS-E16540m kit (Cusabio Biotech Co., Ltd., Hubei, China).

[c] Measured using a SEB477Mu kit (Cloud-Clone Corp., Houston, TX, USA).

[d] Total OC represents the combined levels of Gla-OC and Glu-OC.

* $p < 0.05$ vs. Soy.

** $p = 0.044$ vs. Soy.

*** $p = 0.053$ vs. Soy.

differences were detected in the plasma cMGP levels among the three experimental groups (Table 3). The Gla-OC and the total OC (Gla-OC plus Glu-OC) levels were the lowest in the Can group among the 3 dietary groups; these levels were significantly lower in the Can group than in the Soy group. These levels were lower in the H2-Soy group than in the Soy group, although the differences did not reach statistically significant levels. The Gla-OC/Glu-OC ratio of the Can group was significantly lower than that of the Soy group ($p = 0.044$), and the ratio of the H2-Soy group was almost significantly lower than that of the Soy group ($p = 0.053$).

4. Discussion

Newly formed bone mass induced by the implantation of the crude extract of BMPs was greater by a factor of 4 in the Can and H2-Soy groups than in the Soy group (Fig. 3). Plasma calcium ion levels in the three groups were not measured in the present study because no variations were anticipated [9]. Moreover, the plasma Gla-OC and total OC levels were significantly lower in the Can group than in the Soy group (Table 3). The lower Gla-OC/Glu-OC ratio in the Can and H2-Soy groups compared with the Soy group suggests the presence of differences in VK cofactor activities among the dietary groups (Table 3). The Can diet significantly suppressed the production of total OC compared with the Soy diet ($p < 0.05$; Table 3). The Gla-OC and total OC levels in the Can group were 66% and 68% of those in the Soy group, respectively. The low level of Gla-OC in the Can group may be attributed partially to the low level of total OC. In addition, it has been reported that OC is important for glucose and lipid metabolisms as well as bone metabolism [46]. Reduced total OC levels in the serum are associated with metabolic syndrome [47], the severity of coronary artery disease, and the risk of coronary heart disease in Chinese adults [46].

Although MGP is known to play a role in tissue calcification, no significant differences were observed in the cMGP levels among the dietary groups (Table 3). This may be because the levels of cMGP were insufficient to inhibit ectopic bone formation, or there might have been differences in the phosphorylation level of cMGP, which could not be estimated accurately by the methods used in the current study. In another experiment that used miniature pigs, we found that a 10 w/w% Can diet suppressed the mRNA level of MGP by a factor of 0.66 compared with a 10 w/w% Soy diet, as assessed by DNA microarray analysis (Miyazawa et al., manuscript in preparation). Therefore, the Can diet might suppress the expression of MGP mRNA in mice as well.

The volume of ectopic bone formation induced by the implantation of the crude extract of BMPs was similar between the Can and H2-Soy groups (Fig. 3). However, significant differences were detected in the liver and testis weights (Table 1), hepatic total antioxidant power, and G6PDH activity between the two groups (Table 2). These results suggest that the mechanism underlying the accelerated bone formation might be different in the two groups.

In the H2-Soy group, 97% of VK1 was present as dihydro-VK1 (Fig. 2). In dihydro-VK1, the single 2′, 3′ double bond in the side chain is saturated and the naphthoquinone ring, which is the active site for the carboxylation reaction, is unaltered. This influences the function of VK-dependent proteins [33]. In rats, dihydro-VK1 is reported to be absorbed efficiently, and its biological activity is similar to that of VK1 [20]. In contrast, the intestinal absorption and biological activity of dihydro-VK1 are reported to be lower than those of VK1 in humans [33]. The present study suggests that this cofactor activity of VK was slightly lower in the Can and H2-Soy groups than that in the Soy group.

In the case of the H2-Soy diet, this lower activity may be attributed to dihydro-VK1, because (i) dihydro-VK1 is not converted to VK2 [20], (ii) the cofactor activity of VK2 is stronger than that of VK1 in extrahepatic tissues [17,18], and (iii) intake of dihydro-VK1 reduces tissue VK2 levels [48]. In the Can diet, the VK1 content matched dietary requirements; however, the Gla-OC level in this group was the lowest among the three groups. This may be attributed to the low level of total OC in this group.

VK2 plays important roles in bone homeostasis related to gene transcription through steroid and xenobiotic receptor (SXR; an orphan nuclear receptor) activation [49,50], and in testosterone production in the testis [51,52]. Dihydro-VK1 is crucial for bone homeostasis and testosterone production, because it does not serve as a ligand for SXR and is not converted to VK2 [20], as mentioned above. Suppression of osteocalcin production by the Can diet is crucial for not only glucose, lipid, and bone metabolisms [46] but also steroid hormone metabolism [53]. Osteocalcin promotes testosterone biosynthesis in the mouse testis and modulates reproductive function in humans [53]. A previous study showed that serum and testis testosterone levels were lower in SHRSP rats on the Can and H2-Soy diets than in SHRSP rats on the Soy diet [11]. This is in agreement with our results. The increased weight of the testis in the H2-Soy group might be due to hyperplasia caused by decreased testosterone levels.

In this context, it is important to note that warfarin, a VK antagonist, is widely used to treat individuals with a high

risk of developing thrombosis. The 4-hydroxycoumarins (e.g., warfarin, acenocoumarol, and phenprocoumon) bind to VK reductases and inhibit VK recycling [22,23]. The ensuing inhibition of the carboxylation of coagulation factors results in the formation of inactive non-carboxylated species (including ucMGP and Glu-OC). Blood clotting factors require lower levels of VK for complete γ-carboxylation in the hepatic tissues, whereas higher levels are required for γ-carboxylation of MGP and OC in the extrahepatic tissues [34]. Patients who have atrial fibrillation and use VK antagonists exhibit increased levels of coronary calcification despite a low vascular risk [54]. Moreover, warfarin use is associated with increased mineralization of arterial blood vessels and cardiac valves as well as inactivation of MGP and subsequent vascular calcification in rats [55]. Long-term use of coumarins is associated with enhanced extracoronary vascular calcification, possibly through the inhibition of MGP carboxylation [56] (Fig. 1C). Thus, the present data suggest that if patients using VK antagonists such as warfarin routinely consume Can or H2-Soy, they may be at an increased risk of calcification in their arterial blood vessels and cardiac valves. Our results suggest that the underlying mechanisms responsible for these effects of Can and H2-Soy might be different. They also indicate that these mechanisms might be different from that underlying warfarin-induced calcification.

5. Conclusion

Our studies revealed that dietary Can and H2-Soy enhanced BMP-induced ectopic bone formation by about 4-fold compared with dietary Soy in mouse models. Possible differences in the levels of VK2 status in the H2-Soy group and the suppressed levels of VK-dependent Gla protein(s) in the Can group might explain the differences observed in ectopic bone formation among the three dietary groups. However, further research is required to explain the increase in bone formation by Can and H2-Soy and to understand their potential role in cardiac valve calcification and arteriosclerosis.

Conflict of interest

None declared.

Transparency document

The Transparency document associated with this article can be found in the online version.

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