Effect of anthocyanin-rich bilberry extract on bone metabolism in ovariectomized rats

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Received November 28, 2017; Accepted December 12, 2017

DOI: 10.3892/br.2017.1029

Abstract. Menopause is associated with increased oxidative stress, which serves a role, in part, in the pathogenesis of postmenopausal bone loss. Fruits and vegetables are rich in antioxidative nutrients and phytochemicals. Berries are a natural source of anthocyanins, and their intake may improve bone health. The aim of the present study was to determine the effect of an anthocyanin-rich bilberry extract (VME) on bone metabolism in an ovariectomized (Ovx) rat. Female Sprague-Dawley rats (12 weeks old) were randomly divided into the following four groups: Baseline, Sham, Ovx and Ovx+VME (n=8-12 rats per group). Rats in the Baseline group were sacrificed immediately, while those in the other groups were subjected to either sham operation (Sham) or bilateral Ovx (Ovx and Ovx+VME). Rats in the Ovx+VME group were administered VME daily at a dose of 500 mg/kg body weight. At 8 weeks after surgery, bone mass and bone histomorphometry were evaluated. The femur bone mineral density (BMD) in the Ovx group was significantly lower than that in the Sham group ($P<0.01$). Supplementation of VME in the Ovx rats did not result in an increase in BMD. Histomorphometric analyses revealed that Ovx resulted in decreased measures of bone volume and trabecular number and increased measures of osteoid volume, mineralizing surface and bone formation rates (all $P<0.01$), whereas VME had no significant effects on these parameters. The present findings indicate that VME did not alter bone metabolism in Ovx rats, suggesting that consumption of VME may not be helpful in preventing postmenopausal bone loss.

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Key words: anthocyanin, bilberry, bone histomorphometry, bone mineral density, menopause, osteoporosis, ovariectomy, rat

Introduction

Menopause is a major health concern for women, as it increases susceptibility to various chronic diseases, including cardiovascular diseases (CVDs), osteoporosis, arthritis, Alzheimer's disease, obesity, age-related eye diseases and cancer (1,2). Menopause is associated with an increase in oxidative stress, resulting from an imbalance between reactive oxygen species (ROS) and the antioxidant system (3-5). Previous studies have demonstrated that oxidative stress is implicated in the pathogenesis of various chronic diseases (6-9). Osteoporosis is a skeletal disorder characterized by compromised bone strength, predisposing patients to increased risk of fracture (10). Although osteoporosis is a multifactorial disorder, estrogen deficiency following menopause serves a critical role in the development of osteoporosis in women (11). There are multiple mechanisms underlying the rapid resorption of bone and loss of bone density due to estrogen deficiency, including direct effects on osteoblastic and osteoclastic cell lineages and the interaction of systemic hormones, local cytokines [including tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6], growth factors, and transcription factors (11,12). Additionally, increased ROS may also decrease bone mineral density (BMD) by inducing TNF-α expression (13).

Hormone replacement therapy (HRT) is established to prevent bone loss following menopause (14,15). Previous results have indeed demonstrated that HRT reduces the risk of fractures, even among women with low fracture risk (16). However, findings from Women's Health Initiative trials in 2002 indicated that the risks associated with HRT outweighed the benefits (17,18). Consequently, various groups have recommended limiting the use of HRT in postmenopausal women at risk of facture and women younger than 60 years or within 10 years of menopause (19). Alternatives to estrogen for the treatment of postmenopausal osteoporosis as recommended by the U.S. Food and Drug Administration (FDA) include bisphosphonates, raloxifene, calcitonin and denosumab; however, these drugs have been reported to exert drug-specific adverse effects (20). In turn, lifestyle modifications have been undertaken, such as changes in exercise and diet. A number of epidemiological studies have identified that higher fruit and vegetable intake was associated with higher BMD (21-25) and a lower fracture risk (26) in postmenopausal women. While the mechanisms underlying these bone-protective effects are

yet to be fully elucidated, antioxidative nutrients and phytochemicals, including vitamin C, carotenoids and polyphenols, which are contained in fruits and vegetables, may improve bone health by scavenging ROS (27).

Anthocyanins are a class of natural polyphenol compounds responsible for the colors of flowers and fruits (28). The positive health effects of foods rich in anthocyanins include CVD prevention and anticancer, anti-inflammatory, antioxidative, anti-obesity, anti-diabetic and neuroprotective activities (29,30). Additionally, Welch *et al* (31) suggested an anti-osteoporotic effect of anthocyanins in a cohort study of twins, in which the differences between the highest and lowest fifths of anthocyanin intake were associated with a 3.4% higher BMD at the spine and a 3.1% higher BMD at the hip. Berries contain abundant anthocyanins and have been recognized as valuable sources of natural medicines and dietary supplements (29,32). Various studies have demonstrated that berry intake may increase antioxidant status and reduce inflammatory biomarker levels *in vivo* (29). Based on these findings, our group hypothesized that berry consumption may be helpful in alleviating bone resorption and bone density loss following menopause. However, a limited number of studies have investigated the impact of berry intake on bone metabolism in animals and humans (29,32). Additionally, berries are a natural source of not only anthocyanins but also vitamin C, a potent antioxidant that may also have a positive effect on bone (33,34), making it difficult to determine whether the antioxidant effects of berries are due to anthocyanins, vitamin C or both. Therefore, in the present study, the effects of an anthocyanin-rich bilberry (Vaccinium myrtillus) extract (VME) on bone metabolism were investigated in ovariectomized (Ovx) rats. Bilberry, a member of the Ericaceous family, is a low-growing shrub native to Europe and North America and is related to, while distinct from, varieties of the North American blueberry (V. corymbosum) (35). The anthocyanin-rich VME has been utilized in the treatment of various eye conditions, including cataracts and glaucoma, as well as for enhancing night vision, due to its proposed anti-inflammatory and antioxidant effects (35,36). The Ovx rat model is the current FDA-approved model for the investigation of menopausal bone changes (37). Ovx has also been demonstrated to induce oxidative stress and impair antioxidant systems in rat bone (38).

Materials and methods

Anthocyanin-rich VME. The anthocyanin-rich VME (containing ~39% anthocyanins) used in the current study was provided in powder form by Wakasa Seikatsu Co., Ltd. (Kyoto, Japan). VME contains a total of 15 anthocyanins in all possible combinations of 5 anthocyanidins (cyanidin, delphinidin, peonidin, petunidin and malvidin) containing 3 types of sugar moieties (3-O-arabinosides, 3-O-glucosides and 3-O-galactosides) (35). The powder was stored at -20˚C until use and dissolved in distilled water (DW) to a concentration of 50 mg/ml (5%) prior to administration.

Animals and diets. The experimental protocol was approved by the Animal Ethical Committee at Aichi Medical University (Nagakute, Japan; approval no.: 2013-61). A total of 44 female Sprague-Dawley rats, at 10 weeks of age, were purchased from Charles River Laboratories Japan, Inc. (Hino, Shiga, Japan). Upon arrival, they were housed in a temperature (23±1˚C)- and humidity (55±5%)-controlled room under a 12-h light/dark cycle and provided standard rodent chow (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and water via an automatic watering system ad libitum. After a 2-week acclimation period, the rats were randomly divided into four groups: Baseline (n=8), Sham (n=12), Ovx (n=12) and Ovx+VME (n=12). Rats in the Baseline group were anesthetized by intraperitoneal injection of medetomidine hydrochloride (Nippon Zenyaku Kogyo Co., Ltd., Koriyama, Japan; 0.15 mg/kg body weight), midazolam (Astellas Pharma, Inc., Tokyo, Japan; 2 mg/kg body weight) and butorphanol tartrate (Meiji Seika Pharma Co., Ltd., Tokyo, Japan; 2.5 mg/kg body weight), and were immediately sacrificed by cardiac puncture. The inclusion of the Baseline group provided initial values of skeletal measures, thereby allowing for the determination of changes in skeletal tissue resulting from surgery and aging (39). Rats in the remaining three groups were anesthetized and subjected either to sham (Sham) or Ovx (Ovx and Ovx+VME) surgeries. At 2 days after surgery, rats in the Ovx+VME group were administered VME by gavage at a dose of 500 mg/kg body weight (equivalent to 10 ml/kg body weight of 5% VME) daily for 8 weeks. The dose of VME was determined based on previous studies in rodents (40,41). Rats in the other two groups were given DW by gavage at a dose of 10 ml/kg body weight daily for 8 weeks. A total of 3 rats in the Sham group (6.8% of the total) were lost as a consequence of accidental fatality at 6 days post-surgery. At 8 weeks after surgery, the rats in all three groups were sacrificed. The rats were administered intraperitoneal injections of the fluorochrome markers tetracycline-HCl (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany; 20 mg/kg body weight) and calcein (Sigma-Aldrich; Merck KGaA; 10 mg/kg body weight) at 5 and 2 days before necropsy, respectively, for the evaluation of bone dynamics by histomorphometry. At necropsy, the uterus was resected and weighed to determine whether the Ovx surgery had been successful. The right femurs from all rats were wrapped in saline-soaked gauze and stored at -20˚C for subsequent densitometry. The right tibiae were cleaned of soft tissue and fixed in 70% ethanol for bone histomorphometry.

Bone densitometry. The right femurs were thawed at room temperature. Bone mineral content (BMC) and BMD were determined by dual-energy X-ray absorptiometry (DXA; QDR-Discovery A; Hologic, Inc., Marlborough, MA, USA) using the QDR-Discovery A high-resolution scanning software (version 13.3; Hologic, Inc.) designed for the measurement of small animal bones. Additionally, BMD and BMC in the proximal, mid-diaphyseal and distal parts of the femur were determined by dividing the femur into three equal parts according to length. The coefficients of variation for repeated scans on the same bone were <1.0%. Prior to measurements, a tissue calibration scan was performed with the Hologic small animal phantom.

Peripheral quantitative computed tomography (pQCT). Following DXA measurements, pQCT was performed using an XCT Research SA+ (Stratec Biomedical AG, Birkenfeld, Germany). The right femurs were placed in a polypropylene

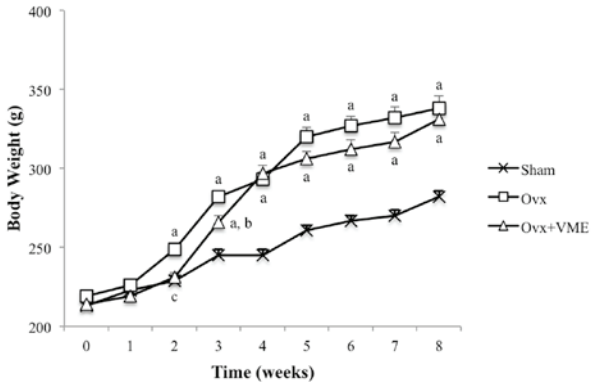

Figure 1. Mean body weights of sham-operated (Sham) and ovariectomized rats supplemented with (Ovx+VME) or without (Ovx) an anthocyanin-rich bilberry extract for 8 weeks (n=9-12/group). The values represent the mean ± standard error of the mean. [a]P<0.01 vs. Sham; [b]P<0.05, [c]P<0.01 vs. Ovx (Sham vs. Ovx vs. Ovx+VME, analysis of variance with Tukey's honest significant difference test). VME, anthocyanin-rich bilberry extract; Ovx, bilateral ovariectomy.

tube filled with saline and were scanned at a 0.46-mm collimation and 0.12-mm voxel size. The scan line was adjusted using the scout view, and transverse sections were recorded at the distal femoral metaphysis [to determine total cross-sectional area (CSA), mm^2; 4.0-mm proximal to the distal growth plate) and at the midshaft (mid-point of the bone length). Analyses were performed using XCT 6.20 software (Stratec Biomedical AG) in contour mode 2 and peel mode 2 (threshold 464 mg/cm^3) for the calculation of trabecular and total bone parameters at the metaphysis, as well as in cortical mode 1 (threshold 690 mg/cm^3) for the determination of cortical bone parameters at the diaphysis. At the femoral metaphysis, trabecular BMC (Tb.BMC; mg), trabecular BMD (Tb.BMD; mg/cm^3) and trabecular cross-sectional area (Tb.CSA; mm^2) were measured. At the midshaft, cortical BMC (Ct.BMC; mg), cortical BMD (Ct.BMD; mg/cm^3), cortical CSA (Ct.CSA; mm^2), cortical thickness (Ct.Th; mm), periosteal circumference (Peri.C; mm) and endosteal circumference (Endo.C; mm) were evaluated.

Bone histomorphometry. The right tibiae were trimmed of soft tissue and fixed at 4˚C with 70% ethanol for 14 days. The proximal one-thirds of the tibiae were stained with Villanueva bone stain (Maruto Instrument Co., Ltd., Tokyo, Japan) at room temperature for 7 days, then embedded undecalcified in methyl methacrylate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) following dehydration in a graded series of ethanol (70, 95, 95 and 100%). Frontal sections (5 μm) were cut using a microtome (RM2255; Leica Microsystems GmbH, Wetzlar, Germany) and mounted on slides. Specimens were examined under a fluorescence microscope (Nikon Corporation, Tokyo, Japan). Structural and dynamic histomorphometric indices were measured in the cancellous bone at 0.435-1.7625 mm distal to the epiphyseal growth plate, which consists of secondary spongiosa, using a semi-automatic image analysis system (Histometry RT Camera; System Supply, Co., Ltd., Nagano, Japan) at a magnification of x250. The primary indices included tissue volume (TV), bone volume (BV), bone surface (BS), osteoid volume (OV), osteoid surface (OS), trabecular thickness (Tb.Th), osteoblast surface (Ob.S), osteoclast surface (Oc.S), eroded surface (ES), single- and double-labeled surfaces (sLS and dLS, respectively) and interlabel width. Calculated from these parameters were the percentages of BV (BV/TV), OV (OV/BV), OS (OS/BS), Ob.S (Ob.S/BS), Oc.S (Oc.S/BS), ES (ES/BS), sLS and dLS (sLS/BS and dLS/BS, respectively). Trabecular number (Tb.N) and mineralizing surface (MS)/BS were calculated as (BV/TV)/Tb.Th and (sLS/2 + dLS)/BS, respectively. Mineral apposition rate (MAR) was calculated from the distance between the labels divided by the time between labels, and was corrected for section obliquity. Bone formation rate (BFR/BS) was calculated by multiplying the MS/BS by the MAR, The histomorphometric nomenclature used in the present study was in accordance with a report of the American Society for Bone and Mineral Research Histomorphometry Nomenclature Committee (42).

Statistical analyses. All data are expressed as the mean ± standard error of the mean (SEM), and all data management and statistical analyses were performed using JMP 9.0.2 (SAS Institute, Inc., Cary, NC, USA). The specific effects of Ovx and VME were examined by comparing values of the Sham, Ovx and Ovx+VME groups with one-way analysis of variance or analysis of covariance using body weight as the covariate (43,44), followed by Tukey's honest significant difference test. Differences were considered significant when P<0.05.

Results

Effects of VME on the uterus and body weight of Ovx rats. The uterus weight of rats in the Ovx group was significantly lower than that of rats in the Baseline and Sham groups (P<0.01; data not shown), confirming that the Ovx was successful. There was no significant difference in uterine weight between the Ovx and Ovx+VME groups, suggesting that VME may lack an estrogenic property. From 2 weeks post-surgery, the body weights of rats in the Ovx group were significantly increased compared with those in the Sham group (P<0.01). Although there was a slight delay in the increase in body weight of the Ovx+VME group, there was no significant difference in body weight between the Ovx and Ovx+VME groups by the end of the experiment (Fig. 1).

DXA of the right femur. The BMD of the right femur of rats in the Ovx group was significantly lower than that in the Sham group (P<0.01). Supplementation of VME in the Ovx rats did not result in an increase in the right femur BMD. When the femur was divided into three equal segments in length, there were significant decreases in the BMD of the Ovx group compared with the Sham group (P<0.01) at the proximal and distal thirds of the femur (sites high in cancellous bone), though not at the middle third (a site high in cortical bone). By contrast, right femur BMC values did not differ significantly between the Sham and Ovx groups. The Ovx rats supplemented with VME (Ovx+VME group) exhibited no significant changes in BMC or BMD compared with the rats in the Ovx group, regardless of the femur site (proximal, diaphyseal or distal; Table I).

pQCT of the right femur metaphysis and diaphysis. The trabecular and cortical parameters of the right femur

Table I. Femur BMC and BMD in the sham-operated (Sham) rats and ovariectomized rats supplemented with (Ovx+VME) or without (Ovx) an anthocyanin-rich bilberry extract.

Site	Baseline (n=8)	Sham (n=9)	Ovx (n=12)	Ovx+VME (n=12)
Femur BMC (g)				
Whole	0.283±0.006	0.357±0.009	0.361±0.005	0.353±0.008
Proximal 1/3	0.104±0.003	0.133±0.003	0.133±0.002	0.130±0.003[a]
Mid 1/3	0.063±0.003	0.082±0.002	0.089±0.002	0.088±0.002
Distal 1/3	0.115±0.003	0.140±0.003	0.141±0.002	0.134±0.004[a]
Femur BMD (g/cm^2)				
Whole	0.194±0.003	0.222±0.003	0.208±0.002[b]	0.207±0.003[b]
Proximal 1/3	0.200±0.004	0.229±0.003	0.214±0.002[b]	0.213±0.003[b]
Mid 1/3	0.157±0.004	0.189±0.003	0.183±0.002	0.185±0.003
Distal 1/3	0.215±0.003	0.240±0.004	0.223±0.002[b]	0.219±0.003[b]

Data are expressed as means ± standard error of the mean. [a]P<0.05, [b]P<0.01 vs. Sham (Sham vs. Ovx vs. Ovx+VME, analysis of covariance with Tukey's honest significant difference test). BMC, bone mineral content; BMD, bone mineral density; Ovx, bilateral ovariectomy; VME, anthocyanin-rich bilberry extract.

Table II. Trabecular and cortical parameters determined by peripheral quantitative computed tomography in the sham-operated (Sham) and ovariectomized rats supplemented with (Ovx+VME) or without (Ovx) an anthocyanin-rich bilberry extract.

Parameter	Baseline (n=8)	Sham (n=9)	Ovx (n=12)	Ovx+VME (n=12)
Distal metaphysis				
T.BMC (mg)	9.85±0.31	10.79±0.46	9.46±0.16	9.25±0.30[a]
T.BMD (mg/cm^3)	683±35	757±63	622±43[a]	604±50[b]
T.CSA (mm^2)	14.4±0.5	14.3±0.5	15.3±0.5	15.3±0.4
Tb.BMC (mg)	1.233±0.067	0.983±0.139	1.414±0.095	1.418±0.083
Tb.BMD (mg/cm^3)	331±12	327±16	193±6[b]	201±8[b]
Tb.CSA (mm^2)	3.78±0.28	3.20±0.52	7.29±0.35[b]	7.11±0.39[b]
Diaphysis				
Ct.BMC (mg)	5.79±0.17	7.24±0.14	7.80±0.12	7.54±0.12
Ct.BMD (mg/cm^3)	1,270±10	1,350±0	1,340±0	1,340±0
Ct.CSA (mm^2)	4.57±0.12	5.38±0.14	5.80±0.07	5.63±0.07
Ct.Th (mm)	0.557±0.015	0.663±0.011	0.678±0.07	0.668±0.011
Peri.C (mm)	9.95±0.12	10.19±0.14	10.70±0.12	10.53±0.11
Endo.C (mm)	6.45±0.15	6.03±0.12	6.44±0.15	6.34±0.15

Data are expressed as means ± standard error of the mean. [a]P<0.05, [b]P<0.01 vs. Sham (Sham vs. Ovx vs. Ovx+VME, analysis of covariance with Tukey's honest significant difference test). Ovx, bilateral ovariectomy; VME, anthocyanin-rich bilberry extract; T, total; Tb, trabecular; Ct, cortical; BMC, bone mineral content; BMD, bone mineral density; CSA, cross-sectional area; Th, thickness; Peri.C, periosteal circumference; Endo.C, endosteal circumference.

determined by pQCT are listed in Table II. There was a significant increase in the Tb.CSA of the distal metaphysis in the Ovx group compared with the Sham group (P<0.01), resulting in a significantly lower Tb.BMD in the Ovx group compared with that in the Sham group (P<0.01). There were no significant differences in Ct.CSA or Ct.BMD between the Sham and Ovx groups at the diaphysis. The administration of VME had no significant effect on any of the parameters evaluated by pQCT.

Bone histomorphometry. The histomorphometric measurements of the cancellous bone indices in the proximal tibiae are summarized in Table III. Rats in the Ovx group exhibited significantly lower structural indices (BV/TV, Tb.Th and Tb.N; P<0.01, P<0.05 and P<0.01, respectively) and higher bone formation indices (OV/BV, OS/BS, dLS/BS, MS/BS and BFR/BS; all P<0.01) compared with those in the Sham group due to estrogen deficiency. These differences were not significantly affected by VME administration.

Table III. Static and dynamic cancellous bone indices in the proximal tibiae in the sham-operated (Sham) and ovariectomized rats supplemented with (Ovx+VME) or without (Ovx) an anthocyanin-rich bilberry extract.

Index	Baseline (n=8)	Sham (n=9)	Ovx (n=12)	Ovx+VME (n=12)
Static indices				
BV/TV (%)	23.3±1.2	33.5±2.0	15.2±0.7[b]	14.7±1.0[b]
Tb.Th (μm)	64.9±1.9	66.8±1.2	58.9±1.5[a]	61.5±1.2
Tb.N (/mm)	3.59±0.12	5.02±0.29	2.57±0.09[b]	2.38±0.15[b]
OV/BV (%)	3.20±0.33	1.18±0.20	4.28±0.30[b]	4.67±0.49[b]
OS/BS (%)	24.8±2.5	11.0±1.7	28.7±1.7[b]	28.9±2.2[b]
Ob.S/BS (%)	1.40±0.28	0.72±0.22	2.86±0.61	4.70±0.97
ES/BS (%)	3.06±0.38	2.08±0.32	5.29±0.46	6.22±0.77[a]
Oc.S/BS (%)	1.35±0.26	0.85±0.22	1.99±0.32	2.66±0.46
Dynamic indices				
sLS/BS (%)	38.0±0.9	33.6±2.0	35.3±1.1	34.5±1.1
dLS/BS (%)	18.9±2.2	5.9±1.0	18.2±1.2[b]	17.1±1.6[b]
MS/BS (%)	37.9±2.6	22.7±1.5	35.9±1.2[b]	34.4±1.5[b]
MAR (μm/day)	1.69±0.05	1.12±0.02	1.44±0.03	1.52±0.06[b]
BFR/BS (mm^3/mm^2/year)	0.236±0.022	0.093±0.007	0.188±0.008[b]	0.193±0.015[b]

Data are expressed as means ± standard error of the mean. [a]P<0.05, [b]P<0.01 vs. Sham (Sham vs. Ovx vs. Ovx+VME, analysis of covariance with Tukey's honest significant difference test). Ovx, bilateral ovariectomy; VME, anthocyanin-rich bilberry extract; BV/TV, bone volume; Tb.Th, trabecular thickness; Tb.N, trabecular number; OV/BV, osteoid volume; OS/BS, osteoid surface; Ob.S/BS, osteoblast surface; ES/BS, eroded surface; Oc.S/BS, osteoclast surface; sLS/BS, single-labeled surface; dLS/BS, double-labeled surface; MS/BS, mineralizing surface; MAR, mineral apposition rate; BFR/BS, bone formation rate.

Discussion

Estrogen deficiency is associated with an imbalance in bone metabolism, involving a net increase in bone resorption over formation, leading to excessive and sustained bone loss (11). The increase in bone resorption is the result of increased osteoclastogenesis and decreased osteoclast apoptosis (11,12). ROS may promote osteoclast resorption directly, by stimulating signaling associated with osteoclast differentiation and receptor activator of nuclear factor (NF)-κB (RANK), or indirectly, by stimulating osteoblast/osteoclast coupling and subsequent osteoclast differentiation through RANK ligand (RANKL) (32). A number of studies have demonstrated that berry extracts may reduce oxidative stress (45-47). Karlsen *et al* (48) demonstrated that anthocyanins isolated from bilberries and black currants efficiently suppressed LPS-induced activation of NF-κB in cultured monocytes. Tanabe *et al* (49) reported that cranberry extract inhibited RANKL-dependent differentiation of human pre-osteoclasts and bone resorption activity of osteoclasts. Furthermore, a recent study by Moriwaki *et al* (50) demonstrated that anthocyanin compounds extracted from bilberry and black currant inhibited osteoclast formation from osteoclast precursor RAW264.7 cells. Collectively these findings indicate anthocyanin extracted from berry fruits may alleviate bone resorption and bone density loss following menopause in women. In the present study, an anthocyanin-rich bilberry extract was administered to Ovx rats (Ovx+VME group) for 8 weeks, and BMD was measured using DXA and pQCT. In the VME-treated rats, there were no significant changes in BMD or BMC, even in trabecular or cortical BMD at the metaphyseal and diaphyseal sites, compared with those in rats in the Ovx group. These results were supported by the bone histomorphometry results (BV/TV, Tb.Th and Tb.N). Although these parameters remained stable when the rate of bone resorption was equal to that of bone formation, bone histomorphometry also revealed that the anthocyanin-rich bilberry extract did not affect bone formation (OV/BV, OS/BS, dLS/BS, MS/BS and BFR/BS) or resorption (Oc.S/BS and ES/BS). These results suggested that the anthocyanin-rich bilberry extract may not mitigate the bone losses observed in postmenopausal women.

There have been a limited number of studies that have investigated the effects of consuming berry fruits or their extracts on bone metabolism in Ovx-induced bone loss animal models. Devareddy *et al* (51) demonstrated that Ovx rats (6 months old) fed a diet supplemented with blueberry powder (5% w/w) for 4 months had a higher overall BMD of the whole body but not at the tibia, femur or fourth lumbar vertebra compared with rats in an Ovx group. Additionally, they identified that the supplement treatment down-regulated Ovx-induced elevation of alkaline phosphatase, collagen and tartrate-resistant acid phosphatase (TRAP) gene expression, suggesting that the bone-protective effect of blueberries may be due to the suppression of bone turnover (51). More recently, Zheng *et al* (52) identified that Ovx mice (14 weeks old) given a diet containing 1% anthocyanin-rich blackcurrant extract for 12 weeks had significantly greater femur BMD compared with Ovx control mice. Notably, they demonstrated that the extract reduced the number of TRAP-positive osteoclast-like cells and bone resorption activity, and concluded that the extract

may alleviate bone loss by suppressing osteoclastogenesis and osteoclast function.

The reasons underlying the inconsistent results between the present study and previous studies are unknown. A previous randomized prospective study designed to examine whether the consumption of freeze-dried blackberries or blueberries (45 g daily) could prevent smoking-induced bone loss in postmenopausal women demonstrated that the loss of total body BMD was significant in women who consumed blueberries but not blackberries for 9 months, despite the higher content of anthocyanins in blueberries compared with blackberries (652.2 vs. 284.1 mg per 45 g freeze-dried berry) (53). Notably, 87% (247.1 mg) of the anthocyanins in the freeze-dried blackberries was cyanidin-3-glucoside, which was a greater percentage than the 1% (6.6 mg) in the freeze-dried blueberries. These findings suggested that the profile or ingredients, and not the total amount of anthocyanins, may be responsible for the discrepancies between previous studies (53), although the active ingredients in the anthocyanins that may prevent bone loss are yet to be identified. Kaume *et al* (54) fed 5% and 10% (w/w) blackberry diets rich in cyanidin-3-glucoside for 100 days to Ovx rats (9 months old) and demonstrated that the 5% (but not 10%) blackberry diet prevented a loss of BMD at the tibia, femur and fourth lumbar vertebra. However, they failed to identify any significant changes in bone formation and resorption markers following diet supplementation, and thus were unable to conclude whether the results were due to the suppression of bone resorption or acceleration of bone formation. Moriwaki *et al* (50) demonstrated that delphinidin, one of the aglycone nuclei of anthocyanins, prevented bone loss in Ovx mice (7 weeks old). They also observed a significant decrease in osteoclast number in delphinidin-treated, soluble RANKL-induced osteoporotic mice, and assumed that delphinidin may prevent bone loss by suppressing bone resorption. Although cyanidin and delphinidin are major anthocyanidins retained in bilberry (55), the current study identified no substantial effects on bone mass, bone formation or bone resorption parameters on examination by bone histomorphometry. Further studies are required to determine which active ingredients have bone-protective effects in berry-extracted anthocyanins.

Differences in the experimental conditions may have also resulted in discrepancies with previous studies. Zhang *et al* (56) fed a diet supplemented with 10% freeze-dried blueberry powder to pre-pubertal rats between postnatal day 20 (PND20) and PND34, after which the diets were either continued (long-term feeding) or switched to a control casein diet (short-term feeding). Rats were then Ovx on PND60 and sacrificed 1 or 3 weeks thereafter, and bone parameters were investigated. The results indicated that the short- or long-term blueberry diet prevented Ovx-induced bone loss at the tibia; bone histomorphometry revealed that the rats fed either short- or long-term blueberry diet had a higher BV/TV, osteoblast number and BFR/BS compared with those in rats in control groups. They also observed that the bone-protective effect of the blueberry diet was exerted through the suppression of osteoblastic cell senescence associated with acute loss of myosin expression following Ovx. The results from their study were in contrast with those from previous *in vitro* and *in vivo* studies (29,32). These other studies suggested that the effects of berry fruits or their extract on bone metabolism were more likely to be exerted through the suppression of bone resorption. Although previous *in vivo* studies (51,52) used mature Ovx rats, the rats used in the study by Zhang *et al* (56) were young Ovx rats in pre-pubertal growth stage. The ages of rats and the time after Ovx are important factors that may influence bone response following Ovx (39). In the present study, 3-month-old Ovx rat models were used to induce bone loss. Although 3-month-old rats are regarded as mature, their bone growth slows but has not stopped (39). Therefore, a favorable effect of VME on bone, if any, may have been overwhelmed by the age of the rats and/or substantial changes associated with Ovx.

In conclusion, supplementation of Ovx rats with an anthocyanin-rich bilberry extract did not prevent Ovx-induced bone loss, at least under the experimental conditions of the present study. As there has been discrepancies between results from previous studies on the effects of berry fruits or their extracts on bone metabolism, further investigations are warranted to determine whether the consumption of berry fruits or anthocyanins extracted from berry fruits may be beneficial in mitigating bone loss in postmenopausal women.

References

1. Greendale GA, Lee NP and Arriola ER: The menopause. Lancet 353: 571-580, 1999.
2. Lobo RA, Davis SR, De Villiers TJ, Gompel A, Henderson VW, Hodis HN, Lumsden MA, Mack WJ, Shapiro S and Baber RJ: Prevention of diseases after menopause. Climacteric 17: 540-556, 2014.
3. Sánchez-Rodríguez MA, Zacarías-Flores M, Arronte-Rosales A, Correa-Muñoz E and Mendoza-Núñez VM: Menopause as risk factor for oxidative stress. Menopause 19: 361-367, 2012.
4. Kolesnikova L, Semenova N, Madaeva I, Suturina L, Solodova E, Grebenkina L and Darenskaya M: Antioxidant status in peri- and postmenopausal women. Maturitas 81: 83-87, 2015.
5. Taleb-Belkadi O, Chaib H, Zemour L, Fatah A, Chafi B and Mekki K: Lipid profile, inflammation, and oxidative status in peri- and postmenopausal women. Gynecol Endocrinol 32: 982-985, 2016.
6. Li H, Horke S and Förstermann U: Vascular oxidative stress, nitric oxide and atherosclerosis. Atherosclerosis 237: 208-219, 2014.
7. Chang YT, Chang WN, Tsai NW, Huang CC, Kung CT, Su YJ, Lin WC, Cheng BC, Su CM, Chiang YF, *et al*: The roles of biomarkers of oxidative stress and antioxidant in Alzheimer's disease: A systematic review. BioMed Res Int 2014: 182303, 2014.
8. Zetterberg M: Age-related eye disease and gender. Maturitas 83: 19-26, 2016.
9. Reuter S, Gupta SC, Chaturvedi MM and Aggarwal BB: Oxidative stress, inflammation, and cancer: How are they linked? Free Radic Biol Med 49: 1603-1616, 2010.
10. NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis, and Therapy: Osteoporosis prevention, diagnosis, and therapy. JAMA 285: 785-795, 2001.
11. Raisz LG: Pathogenesis of osteoporosis: Concepts, conflicts, and prospects. J Clin Invest 115: 3318-3325, 2005.
12. Reid IR: Menopause. In: Primer on the metabolic bone diseases and disorders of mineral metabolism. John Wiley & Sons, Inc., pp165-170, 2013.
13. Lean JM, Jagger CJ, Kirstein B, Fuller K and Chambers TJ: Hydrogen peroxide is essential for estrogen-deficiency bone loss and osteoclast formation. Endocrinology 146: 728-735, 2005.
14. Wells G, Tugwell -P, Shea B, Guyatt G, Peterson J, Zytaruk N, Robinson V, Henry D, O'Connell D and Cranney A; Osteoporosis Methodology Group and The Osteoporosis Research Advisory Group: Meta-analyses of therapies for postmenopausal osteoporosis. V. Meta-analysis of the efficacy of hormone replacement therapy in treating and preventing osteoporosis in postmenopausal women. Endocr Rev 23: 529-539, 2002.
15. Dören M, Nilsson JA and Johnell O: Effects of specific post-menopausal hormone therapies on bone mineral density in post-menopausal women: A meta-analysis. Hum Reprod 18: 1737-1746, 2003.
16. Torgerson DJ and Bell-Syer SE: Hormone replacement therapy and prevention of nonvertebral fractures: A meta-analysis of randomized trials. JAMA 285: 2891-2897, 2001.

17. Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, *et al*; Writing Group for the Women's Health Initiative Investigators: Risks and benefits of estrogen plus progestin in healthy postmenopausal women: Principal results From the Women's Health Initiative randomized controlled trial. JAMA 288: 321-333, 2002.
18. Cauley JA, Robbins J, Chen Z, Cummings SR, Jackson RD, LaCroix AZ, LeBoff M, Lewis CE, McGowan J, Neuner J, *et al*; Women's Health Initiative Investigators: Effects of estrogen plus progestin on risk of fracture and bone mineral density: The Women's Health Initiative randomized trial. JAMA 290: 1729-1738, 2003.
19. Baber RJ, Panay N, Fenton A and Group IMSW; IMS Writing Group: 2016 IMS Recommendations on women's midlife health and menopause hormone therapy. Climacteric 19: 109-150, 2016.
20. Camacho PM, Petak SM, Binkley N, Clarke BL, Harris ST, Hurley DL, Kleerekoper M, Lewiecki EM, Miller PD, Narula HS, *et al*: American association of clinical endocrinologists and american college of endocrinology clinical practice guidelines for the diagnosis and treatment of postmenopausal osteoporosis - 2016. Endocr Pract 22 (Suppl 4): 1-42, 2016.
21. Tucker KL, Hannan MT, Chen H, Cupples LA, Wilson PW and Kiel DP: Potassium, magnesium, and fruit and vegetable intakes are associated with greater bone mineral density in elderly men and women. Am J Clin Nutr 69: 727-736, 1999.
22. Chen YM, Ho SC and Woo JL: Greater fruit and vegetable intake is associated with increased bone mass among postmenopausal Chinese women. Br J Nutr 96: 745-751, 2006.
23. Prynne CJ, Mishra GD, O'Connell MA, Muniz G, Laskey MA, Yan L, Prentice A and Ginty F: Fruit and vegetable intakes and bone mineral status: A cross sectional study in 5 age and sex cohorts. Am J Clin Nutr 83: 1420-1428, 2006.
24. Zalloua PA, Hsu YH, Terwedow H, Zang T, Wu D, Tang G, Li Z, Hong X, Azar ST, Wang B, *et al*: Impact of seafood and fruit consumption on bone mineral density. Maturitas 56: 1-11, 2007.
25. Li JJ, Huang ZW, Wang RQ, Ma XM, Zhang ZQ, Liu Z, Chen YM and Su YX: Fruit and vegetable intake and bone mass in Chinese adolescents, young and postmenopausal women. Public Health Nutr 16: 78-86, 2013.
26. Xu L, Dibley M, D'Este C, Phillips M, Porteous J and Attia J: Food groups and risk of forearm fractures in postmenopausal women in Chengdu, China. Climacteric 12: 222-229, 2009.
27. Qiu R, Cao WT, Tian HY, He J, Chen GD and Chen YM: Greater intake of fruit and vegetables is associated with greater bone mineral density and lower osteoporosis risk in middle-aged and elderly adults. PLoS One 12: e0168906, 2017.
28. Chen L, Xin X, Yuan Q, Su D and Liu W: Phytochemical properties and antioxidant capacities of various colored berries. J Sci Food Agric 94: 180-188, 2014.
29. Hubert PA, Lee SG, Lee SK and Chun OK: Dietary polyphenols, berries, and age-related bone loss: A review based on human, animal, and cell studies. Antioxidants 3: 144-158, 2014.
30. Li D, Wang P, Luo Y, Zhao M and Chen F: Health benefits of anthocyanins and molecular mechanisms: Update from recent decade. Crit Rev Food Sci Nutr 57: 1729-1741, 2017.
31. Welch A, MacGregor A, Jennings A, Fairweather-Tait S, Spector T and Cassidy A: Habitual flavonoid intakes are positively associated with bone mineral density in women. J Bone Miner Res 27: 1872-1878, 2012.
32. Đudarić L, Fužinac-Smojver A, Muhvić D and Giacometti J: The role of polyphenols on bone metabolism in osteoporosis. Food Res Int 77: 290-298, 2015.
33. Morton DJ, Barrett-Connor EL and Schneider DL: Vitamin C supplement use and bone mineral density in postmenopausal women. J Bone Miner Res 16: 135-140, 2001.
34. Sahni S, Hannan MT, Gagnon D, Blumberg J, Cupples LA, Kiel DP and Tucker KL: Protective effect of total and supplemental vitamin C intake on the risk of hip fracture - a 17-year follow-up from the Framingham Osteoporosis Study. Osteoporos Int 20: 1853-1861, 2009.
35. Canter PH and Ernst E: Anthocyanosides of Vaccinium myrtillus (bilberry) for night vision - a systematic review of placebo-controlled trials. Surv Ophthalmol 49: 38-50, 2004.
36. Head KA: Natural therapies for ocular disorders, part two: Cataracts and glaucoma. Altern Med Rev 6: 141-166, 2001.
37. Calciolari E, Donos N and Mardas N: Osteoporotic Animal Models of Bone Healing: Advantages and Pitfalls. J Invest Surg 30: 342-350, 2017.
38. Muthusami S, Ramachandran I, Muthusamy B, Vasudevan G, Prabhu V, Subramaniam V, Jagadeesan A and Narasimhan S: Ovariectomy induces oxidative stress and impairs bone antioxidant system in adult rats. Clinica chimica acta; international journal of clinical chemistry 360: 81-86, 2005.
39. Kalu DN: The ovariectomized rat model of postmenopausal bone loss. Bone Miner 15: 175-191, 1991.
40. Sakakibara H, Ogawa T, Koyanagi A, Kobayashi S, Goda T, Kumazawa S, Kobayashi H and Shimoi K: Distribution and excretion of bilberry anthocyanins [corrected] in mice. J Agric Food Chem 57: 7681-7686, 2009.
41. Miyake S, Takahashi N, Sasaki M, Kobayashi S, Tsubota K and Ozawa Y: Vision preservation during retinal inflammation by anthocyanin-rich bilberry extract: Cellular and molecular mechanism. Lab Invest 92: 102-109, 2012.
42. Dempster DW, Compston JE, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR and Parfitt AM: Standardized nomenclature, symbols, and units for bone histomorphometry: A 2012 update of the report of the ASBMR Histomorphometry Nomenclature Committee. J Bone Miner Res 28: 2-17, 2013.
43. Lane MA, Black A, Handy AM, Shapses SA, Tilmont EM, Kiefer TL, Ingram DK and Roth GS: Energy restriction does not alter bone mineral metabolism or reproductive cycling and hormones in female rhesus monkeys. J Nutr 131: 820-827, 2001.
44. Matsushita H, Minami A, Kanazawa H, Suzuki T, Subhadhirasakul S, Watanabe K and Wakatsuki A: Long-term supplementation with young coconut juice does not prevent bone loss but rather alleviates body weight gain in ovariectomized rats. Biomed Rep 6: 585-591, 2017.
45. Jakesevic M, Aaby K, Borge GI, Jeppsson B, Ahrné S and Molin G: Antioxidative protection of dietary bilberry, chokeberry and Lactobacillus plantarum HEAL19 in mice subjected to intestinal oxidative stress by ischemia-reperfusion. BMC Complement Altern Med 11: 8, 2011.
46. Mane C, Loonis M, Juhel C, Dufour C and Malien-Aubert C: Food grade lingonberry extract: Polyphenolic composition and *in vivo* protective effect against oxidative stress. J Agric Food Chem 59: 3330-3339, 2011.
47. Kim B, Ku CS, Pham TX, Park Y, Martin DA, Xie L, Taheri R, Lee J and Bolling BW: Aronia melanocarpa (chokeberry) polyphenol-rich extract improves antioxidant function and reduces total plasma cholesterol in apolipoprotein E knockout mice. Nutr Res 33: 406-413, 2013.
48. Karlsen A, Retterstøl L, Laake P, Paur I, Bøhn SK, Sandvik L and Blomhoff R: Anthocyanins inhibit nuclear factor-kappaB activation in monocytes and reduce plasma concentrations of pro-inflammatory mediators in healthy adults. J Nutr 137: 1951-1954, 2007.
49. Tanabe S, Santos J, La VD, Howell AB and Grenier D: A-type cranberry proanthocyanidins inhibit the RANKL-dependent differentiation and function of human osteoclasts. Molecules 16: 2365-2374, 2011.
50. Moriwaki S, Suzuki K, Muramatsu M, Nomura A, Inoue F, Into T, Yoshiko Y and Niida S: Delphinidin, one of the major anthocyanidins, prevents bone loss through the inhibition of excessive osteoclastogenesis in osteoporosis model mice. PLoS One 9: e97177, 2014.
51. Devareddy L, Hooshmand S, Collins JK, Lucas EA, Chai SC and Arjmandi BH: Blueberry prevents bone loss in ovariectomized rat model of postmenopausal osteoporosis. J Nutr Biochem 19: 694-699, 2008.
52. Zheng X, Mun S, Lee SG, Vance TM, Hubert P, Koo SI, Lee SK and Chun OK: Anthocyanin-rich blackcurrant extract attenuates ovariectomy-induced bone loss in mice. J Med Food 19: 390-397, 2016.
53. Kaume L, Gbur EE, DiBrezzo R, Howard LR and Devareddy L: Antioxidant-rich berries exert modest bone protective effects in postmenopausal smokers without improving biomarkers of bone metabolism. J Funct Foods 9: 202-210, 2014.
54. Kaume L, Gilbert W, Smith BJ and Devareddy L: Cyanidin 3-o-beta-d-glucoside improves bone indices. J Med Food 18: 690-697, 2015.
55. Joseph SV, Edirisinghe I and Burton-Freeman BM: Berries: Anti-inflammatory effects in humans. J Agric Food Chem 62: 3886-3903, 2014.
56. Zhang J, Lazarenko OP, Blackburn ML, Shankar K, Badger TM, Ronis MJ and Chen JR: Feeding blueberry diets in early life prevent senescence of osteoblasts and bone loss in ovariectomized adult female rats. PLoS One 6: e24486, 2011.