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Blueberry prevents bone loss in ovariectomized rat model of postmenopausal osteoporosis

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Abstract

The objective of the present study was to explore the bone protective role of blueberry in an ovariectomized rat model. Thirty 6-month-old female Sprague–Dawley rats were either sham-operated (Sham) or ovariectomized (Ovx) and divided into three groups: Sham, Ovx (control), Ovx+blueberry (5% blueberry w/w). After 100 days of treatment, rats were euthanized, and blood and tissues were collected. Bone mineral density (BMD) and content of whole body, right tibia, right femur and fourth lumbar vertebra were assessed via dual-energy X-ray absorptiometry. As expected, Ovx resulted in loss of whole-body, tibial, femoral, and 4th lumbar BMD by approximately 6%. Blueberry treatment was able to prevent the loss of whole-body BMD and had an intermediary effect on prevention of tibial and femoral BMD when compared to either Sham or Ovx controls. The bone-protective effects of blueberry may be due to suppression of Ovx-induced increase in bone turnover, as evident by lowered femoral mRNA levels of alkaline phosphatase, collagen type I and tartrate-resistant acid phosphatase to the Sham levels. Similarly, serum osteocalcein levels were also lower in the blueberry group when compared to the Ovx control group, albeit not significantly. In summary, our findings indicate that blueberry can prevent bone loss as seen by the increases in BMD and favorable changes in biomarkers of bone metabolism.

Keywords: Estrogen; Phenolic compounds; Osteopenia; Polyphenols

1. Introduction

Postmenopausal osteoporosis is by far the most common cause of age-related bone loss. It is well known that ovarian hormone deficiency is a major risk factor for osteoporosis. Aside from existing drug therapies, certain lifestyle and nutritional factors are known to reduce the risk of osteoporosis. A number of population-based studies have demonstrated the beneficial effects of fruits and vegetables intakes on indices of bone health in humans [1,2]. Some phytochemicals such as polyphenols present in these fruits and vegetables are responsible for their bone-protective effects [3]. However, the effectiveness of these polyphenols may vary depending on their chemical compositions. For instance, a study by Muhlbauer et al. [3] examined the effects of numerous fruits and vegetables on bone and concluded that consumption of select fruits and vegetables is beneficial to bone, albeit with varying intensity. They have also [3] demonstrated that these bone protective effects are likely due to their phytochemical contents.

Blueberry contains a group of phytochemicals that have been implicated as a mediator of cardiovascular protection. The phytochemicals present in blueberry include phenolic acids (e.g., gallic acid, p-hydroxybenzoic acid, chlorogenic, p-coumaric, caffeic, ferulic and ellagic acids) and flavonoids (anthocyanins, catechin, epichatechin, quercetin, kaempferol and myrecetin) [4–6]. Our recent findings suggest that dried plum, which shares some of the same phenolic and flavonoid compounds with blueberry, exhibits strong bone protective properties in both female [7,8] and male [9] rat models of osteoporosis. There are also a number of animal [10,11] and human studies [12,13] that suggest isoflavones, a subclass of

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flavonoids, prevent bone loss due to ovarian hormone deficiency and aging per se [14]. Hence, it is expected that blueberry with its phenolic compounds can prevent bone loss in ovarian hormone deficiency. Therefore, the present study was designed to examine the extent to which blueberry prevents bone loss in an Ovx rat model.

2. Materials and methods

2.1. Animal care and diets

Thirty, 6-month old female Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) were housed in an environmentally controlled laboratory upon arrival and acclimatized for 5 days. The animals were either ovariectomized (Ovx) or sham-operated (Sham) and divided into three groups: Sham, Ovx (controls) and Ovx + blueberry (5% blueberry w/w). The blueberry powder used in this study was generously donated by US Highbush Blueberry Council (Folsom, CA, USA). The dried blueberry powdered was kept frozen until the time of diet formulation and the diets were stored at −20°C until use. The diets were adjusted to be isonitrogenous and isocaloric. The proximate analyses of the diets confirmed that they were similar in macronutrients, calcium and phosphorus contents. Rats were pair-fed to the average food intake of Sham group and had free access to deionized water. Food intake was recorded every 3 days, and body weights were measured weekly. All conditions and handling of animals were approved by the Institutional Animal Care and Use Committee. At the end of a 100-day treatment period, rats were anesthetized with a mixture of ketamine and xylazine (70 mg and 3 mg/kg body weight, respectively) to measure whole-body bone mineral density (BMD) and bone mineral content (BMC) using dual-energy X-ray absorptiometry (DXA; QDR-4500A Elite; Hologic, Waltham, MA, USA) and then sacrificed. Blood and tissues were collected for further analyses.

2.2. Urine and blood collection

One day prior to the termination of the study, rats were placed in metabolic cages, and urine was collected from 6:00 p.m. to 6:00 a.m. During this period, rats had no access to food, but they were given deionized water. Urine was collected in acid-washed tubes and was frozen at −20°C until required for analysis. Serum was separated by centrifugation at 4000 rpm for 20 min at 4°C. Aliquots of serum were frozen and kept at −20°C for later analyses.

2.3. Serum and urine markers of bone metabolism

Serum alkaline phosphatase (ALP) activity, an indicator of bone formation, was measured using a Cobas Fara II Clinical Analyzer (Montclair, NJ, USA). The intra- and interassay coefficients of this process were 4.4 % and 5.7 %, respectively. Serum osteocalcin was assessed using radioimmunoassay kits from Immutopics (San Clemente, CA, USA) following the manufacturer's instructions. The intra- and interassay coefficients of variation (CV) of this procedure were 2.7% and 5.8%, respectively. Urinary creatinine was measured colorimetrically with a commercially available kit from Roche Diagnostics (Sommerville, NJ) using a Cobas Fara II Clinical Analyzer (Montclair, NJ). Urinary deoxypyridinoline (Dpd) was measured by a competitive enzyme immunoassay in a microassay stripwell format (Quidel, Mountain View, CA, USA). The intra- and interassay CVs were 1.7% and 6.3% and 4.3% and 4.6% for creatinine and Dpd, respectively.

2.4. Bone density assessment

To assess bone loss due to Ovx and treatment, rats were anesthetized and scanned before surgery (baseline) and at the end of treatment using DXA equipped with appropriate software for BMD (CV=1.4%) and BMC (CV=4.6%) assessment in small laboratory animals. Additionally, upon sacrifice, individual bones were isolated, cleaned, and kept at −20°C for later analyses. The right tibia and femur and fourth lumbar vertebra were later scanned using DXA to determine BMC and BMD.

2.5. RNA extraction and Northern blot analysis

Four bone specimens from each treatment group were randomly selected for Northern blot analyses. The distal femur which had been stored at −80°C was pulverized using Spex 6700 freezer mill (Metuchan, NJ, USA), and total cellular RNA from right femur was extracted as described elsewhere [15] and quantified by ultraviolet absorbance. For Northern blot analysis, RNA samples were denatured and were electrophoretically fractionated on 1% agarose gel. After electrophoresis, the RNA in the gel was transferred overnight to membrane (Hybond-N+, Amersham Biosciences, Piscataway, NJ, USA) by capillary action. Transferred total RNAs to the membrane were stained with methylene blue and visualized using a VersaDoc imaging system (Bio-Rad, Hercules, CA, USA) to confirm the integrity of RNA and ensure equal loading. After autocross-linking and washing, the membrane was prehybridized to remove nonspecific background. Hybridizations were separately performed with [^{32}P]-labeled cDNA probes of ALP, collagen type I (COL), tartrate-resistant acid phosphatase (TRAP) and 18S at 65°C for 2 h. [^{32}P]-labeled cDNAs were prepared with [^{32}P]dCTP (Amersham Biosciences) by Megaprimer DNA labeling system (Amersham Biosciences) and purified by Nick column (Amersham Biosciences). The blots were washed twice and placed on a phosphor screen (Bio-Rad, Hercules, CA, USA) in cassette and kept at room temperature overnight. The levels of ALP, COL, TRAP and 18S mRNAs were detected using Molecular Image FX (Bio-Rad) and quantified using a Quantity One soft program (Bio-Rad). cDNA of 18S ribosomal RNA (Ambion, Austin, TX, USA) was used as an internal control to normalize gene expression. cDNA probes of ALP (2.5 kb) and COL

(1.6 kb) were generously provided by Dr. Tobias (University of Bristol Division of Medicine, Bristol, UK). TRAP cDNA probe was obtained using the method of Battaglino et al. [16].

2.6. Statistical analyses

The data analyses involved estimation of means and S.E.M. using InStat Version 3.0 (GraphPad Software, San Diego, CA). Analysis of variance (ANOVA) was performed to determine whether there were statistically significant ($P<.05$) differences among the groups. When ANOVA indicated any significant differences among the means, Student–Newman–Keuls multiple comparison test was used to determine which means were significantly different ($P<.05$).

3. Results

3.1. Body and organ weights

In spite of pair feeding the animals, the final body weights of Ovx controls were significantly higher than the Sham animals (Table 1). Blueberry was not able to prevent this Ovx-induced weight gain. As expected, Ovx caused atrophy of uterine tissue, indicating the success of the surgical procedure and blueberry treatment did not have any uterotropic effects (Table 1).

3.2. BMC and density

Ovx caused a significant reduction in BMD of whole body, tibia, femur and fourth lumbar vertebra (Table 2). This reduction in whole-body BMD was prevented by 5% blueberry treatment as the mean values were not statistically different from the Sham group but higher than the Ovx control group. The animals in 5% blueberry group also had higher tibia and femoral BMD values, and these were not different from either the Sham or Ovx controls, indicating the modest bone protective properties of blueberry. Blueberry did not have any effect on 4th lumbar BMD and BMC.

3.3. Messenger RNA levels of ALP, COL and TRAP using Northern blot

To explore the role of blueberry on bone at the molecular level, mRNA levels of select bone matrix proteins and TRAP were assessed. As expected, Ovx increased the gene expression of ALP, COL and TRAP by 75%, 137% and 32%, respectively (Figs. 1–3) compared to Sham. This Ovx-induced elevated gene expression was down regulated by blueberry treatment significantly in all of the three mRNA levels assessed. These findings indicated that the bone protective effect of blueberry is due to the suppression of bone turnover.

Table 1
Effects of Ovx and blueberry on food intake and body and organ weights

Blueberry	Sham	Ovx	Ovx+5% blueberry	*P* value
Average food intake (g/day)	15.35±0.30	14.99±0.36	15.10±0.38	.9757
Body weights (g)				
Initial	201±3	199±3	198±4	.8282
Final	241±6^{b}	270±6^{a}	282±9^{a}	.0027
Uterus (g)	0.5558±0.02^{a}	0.0942±0.02^{c}	0.1133±0.03^{c}	<.0001

Values are means±S.E.M. Values that do not share the same superscript letters are significantly ($P<.05$) different from each other; n=6–10 rats per group.

Table 2
Effects of Ovx, and blueberry on bone mineral area, BMC and BMD

Blueberry	Sham	Ovx	Ovx+5% blueberry	*P* value
Whole body				
Area (cm^2)	53.442±0.785^{b}	57.685±0.785^{a}	56.166±1.111^{ab}	.0027
BMC (g)	8.77±0.129	8.94±0.129	8.98±0.183	.5234
BMD (g/cm^2)	0.164±0.001^{a}	0.155±0.001^{b}	0.160±0.001^{a}	.0002
Left tibia				
Area (cm^2)	1.41±0.023	1.478±0.023	1.475±0.033	.1797
BMC (g)	0.303±0.008	0.301±0.008	0.303±0.011	.9768
BMD (g/cm^2)	0.214±0.002^{a}	0.204±0.002^{b}	0.205±0.004^{ab}	.0235
Right tibia				
Area (cm^2)	1.438±0.027^{b}	1.530±0.027^{a}	1.526±0.039^{a}	.0526
BMC (g)	0.301±0.008	0.302±0.008	0.308±0.11	.8510
BMD (g/cm^2)	0.209±0.002^{a}	0.197±0.002^{b}	0.202±0.003^{ab}	.0032
4th Lumber vertebra				
Area (cm^2)	0.550±0.008	0.558±0.008	0.548±0.012	.7221
BMC (g)	0.130±0.003^{a}	0.123±0.003^{a}	0.119±0.004^{ab}	.0622
BMD (g/cm^2)	0.236±0.003^{a}	0.220±0.003^{b}	0.219±0.003^{b}	.0007
Femur				
Area (cm^2)	1.717±0.022	1.745±0.023	1.778±0.031	.2836
BMC (g)	0.410±0.008	0.389±0.008	0.410±0.011	.1378
BMD (g/cm^2)	0.239±0.003^{a}	0.223±0.003^{b}	0.231±0.004^{ab}	.0012

Values are means±S.E.M. Values that do not share the same superscript letters are significantly ($P<.05$) different from each other; n=6–10 rats per group.

3.4. Serum and urinary biomarkers

Serum ALP activity and urinary Dpd were measured to assess the effects on Ovx and blueberry on bone formation

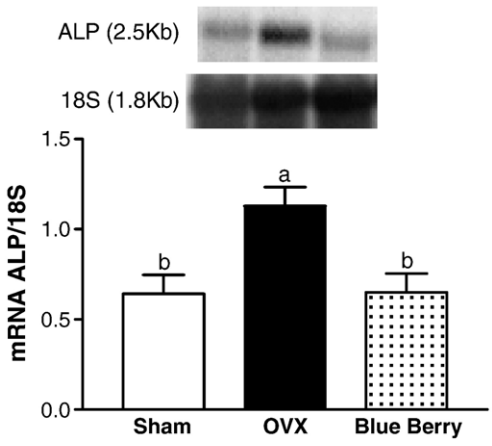

Fig. 1. Effects of Ovx and blueberry on femoral mRNA levels of ALP. Bars are means±S.E.M. Values that do not share the same superscript letters are significantly ($P<.05$) different from each other; n=4 samples per group.

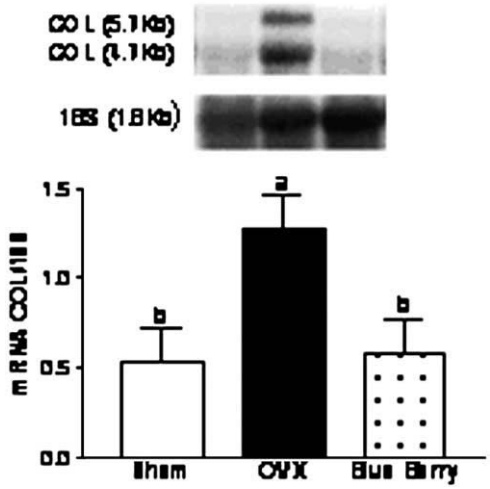

Fig. 2. Effects of Ovx, and blueberry on femoral mRNA levels of COL. Bars are means±S.E.M. Values that do not share the same superscript letters are significantly (P<.05) different from each other; n=4 samples per group.

and bone resorption, respectively (Table 3). Serum osteocalcein, a marker of bone turnover, was also assessed. Serum ALP activity and osteocalcein were increased significantly by Ovx, the increase in ALP activity was further augmented by 5% blueberry treatment. However, the increase in Ovx-induced increase in serum osteocalcein levels were suppressed by blueberry, and the mean values of the blueberry group were not different from either the Sham or Ovx groups. There were no differences in urinary excretion of Dpd among any of the treatment groups.

4. Discussion

The present study reports the findings on the bone-protective properties of blueberry in ovariectomized rat model of postmenopausal osteoporosis. As expected, the findings of the present study indicate that Ovx caused significant bone loss. In humans and other animals including

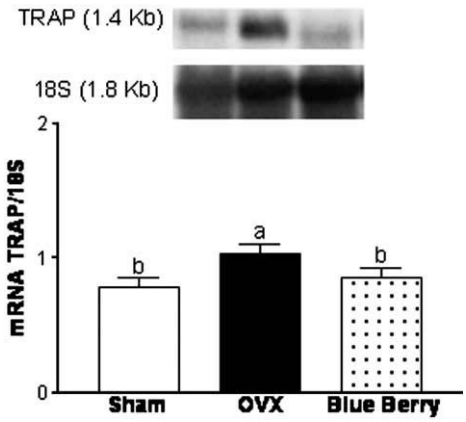

Fig. 3. Effects of Ovx and blueberry on femoral mRNA levels of TRAP. Bars are means±S.E.M. Values that do not share the same superscript letters are significantly (P<.05) different from each other; n=4 samples per group.

Table 3
Effects of Ovx and blueberry on serum and urinary biomarkers of bone metabolism

Blueberry	Sham	Ovx	Ovx+5% blueberry	P value
Serum				
Alkaline Phosphatase activity (U/L)	34±2^{c}	52±3^{b}	63±3^{a}	<.0001
Osteocalcin (ng/mL)	28±1^{b}	34±2^{a}	30±2^{ab}	.0228
Urine				
Deoxypyridinoline (nM/12-h urine)	10.6±0.21	10.09±0.23	9.99±0.23	.9612

Values are means±S.E.M. Values that do not share the same superscript letters are significantly (P<.05) different from each other; n=6–10 rats per group.

rats, ovarian hormone deficiency results in a drastic increase in the rate of bone loss, partly due to a rise in oxygen-derived free radical formation [17,18]. Decreased bone mass is one of the major factors jeopardizing bone integrity, resulting in reduced bone strength and increased susceptibility to fractures. BMD is the gold standard used for the evaluation of individuals at risk of osteoporosis as it best predicts fracture risk in people without previous fractures [19]. A meta-analysis by Johnell et al. [20], which included data from 12 cohort studies of approximately 39,000 men and women, reported that among the different measures studied, BMD assessed using DXA had the strongest predictive ability of fracture risk. The findings of the present study indicate that blueberry protected against the Ovx-induced bone loss as it prevented the loss of whole-body BMD and had an intermediary effect on tibial and femoral BMD. This effect may be due to the components of blueberry which function as free radical scavengers [5,21]. This observation is supported by a report which ranks the blueberry extract to have the highest antioxidant capacity in comparison with other fruits and reference compounds such as vitamin C [6].

The loss of bone mass and the deterioration of bone microstructure have been linked to an imbalance between bone formation and bone resorption [22,23]. For bone formation, mature osteoblasts synthesize bone matrix proteins including bone-specific ALP and COL [24]. Therefore, ALP and COL are assessed as markers of bone formation. As a part of the process of bone resorption, mature osteoclasts synthesize TRAP, which is released with degradation products of bone matrix [25], and the levels of TRAP are indicative of bone resorption. The serum osteocalcein and urinary Dpd assessed in the present study as well as mRNA levels of select bone matrix proteins indicate that blueberry prevents bone loss by suppression of bone turnover that is increased as a result of Ovx. Ovx increased the mRNA levels of ALP, COL and TRAP by 75%, 137% and 32%, respectively, and this Ovx-induced increase was suppressed by blueberry treatment. Based on the findings of the present study, we can hypothesize that blueberry prevents bone loss in a manner similar to soy isoflavones, which have been shown to effectively lower the

number of mature osteoclasts by inducing osteoclast apoptosis [26] and suppressing osteoclastogenesis [27], thereby reducing bone resorption. Furthermore, the mechanism by which blueberry prevents bone loss is different than that of other fruits examined by our group such as dried plum. Our earlier findings suggest that dried plum protects against bone loss by increasing bone formation, serum bone-specific ALP and IGF-I in postmenopausal women [28] and Ovx rats [29]. However, further studies are needed to elucidate the mechanisms by which blueberry prevents bone loss over time.

An epidemiological study identified that diets rich in fruits and vegetables resulted in greater bone density in a cohort of 1164 elderly men and women [30]. The compounds in fruits and vegetables that are protective against bone loss have not been identified. There is little research on the impact of phenolic compounds found in fruits and vegetables on bone metabolism. Rutin, a flavanoid, was reported to inhibit Ovx-induced bone loss in a female rat model [31]. Resveratrol, a phenolic compound extracted from grapes, increased femoral bone strength in Ovx rat models [32]. Although blueberry does not contain resveratrol or rutin, they do contain phenolic compounds that are structurally similar. Blueberry is primarily comprised of one-half anthocyanin compounds and the rest as phenolic acids and flavanoids [6,33]. On a dry weight basis, total phenolic content is about 2500 mg/100 g and is one of the highest among fruits and vegetables [6]. These limited studies bring forth evidence that blueberry is a rich source of phenolic compounds, and these compounds may play an important role in protecting skeletal health.

Blueberry consists of the flavanoid compounds catechin, quercitin, epicatechin as well as phenolic acids gallic and caffeic acids [5]. The strong antioxidant activity of these compounds is related to the number and their unique location of hydroxyl groups, the presence of a 4-oxo function and of a 2-3 double bond in their structure [34]. The composition and interaction of polyphenols present in blueberry makes it an excellent source of stable free radical scavengers unique among polyphenol containing foods [6,34].

Oxygen-derived free radicals are formed by a number of phagocytic cells including monocytes, macrophages and neutrophils. In chronic inflammatory diseases, these cells gather on nearby bone surfaces [35]. Therefore, it is conceivable that they stimulate osteoclast formation or activation and potentiate the osteoclastic bone resorption. The in vivo and in vitro findings by Garrett et al. [14] indicated that free radicals generated in the bone environment enhanced osteoclast formation and bone resorption, reducing bone mineralization. A study conducted at Tufts University found that anthocyanins from blueberry show antioxidative and anti-inflammatory properties in treating human microvascular endothelial cells [36].

In summary, blueberry is effective in preventing bone loss caused by ovarian hormone deficiency as seen from whole-body, tibial and femoral BMD values. The effects of blueberry on BMD of tibia and femur would have been more pronounced if the number of animals in the blueberry group were equal to the number in the Sham and Ovx groups. Future studies are needed to identify the bioactive component (s) of blueberry, the lowest effective dose of the compound and the mechanisms of action.

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