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Freeze-Dried Watermelon Supplementation Has Modest Effects on Bone and Lipid Parameters of Ovariectomized Mice.

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ABSTRACT: This study investigated the effects of two doses of freeze-dried watermelon (WM) on bone and lipid parameters in ovariectomized (OVX) mice, a model of post-menopausal osteoporosis. Three-month-old C57BL/6 female mice (n=46) were sham-operated (SHAM) or OVX and randomly assigned to the control or WM diets for 12 weeks: SHAM-control, OVX-control, OVX+1%, or 10% (wt/wt) freeze-dried WM. All diets were isocaloric and isonitrogenous, and had the same calcium and phosphorus concentrations. Freeze-dried WM supplementation was not able to prevent the decrease in whole body, tibial, and lumbar bone mineral density due to estrogen deficiency. Micro-computed tomography analyses showed that WM was also not able to modulate changes in tibial trabecular and cortical bone microarchitecture due to ovariectomy. However, the lumbar trabecular micro-architecture analyses revealed that the WM-10% group had a similar connectivity density, trabecular number, trabecular separation, and structure model index as the SHAM group. Supplementation with 10% WM reduced plasma cholesterol and total liver lipids to the level of the SHAM group but was still similar to that of the OVX-control group. Supplementation with 10% WM increased liver catalase (CAT) mRNA levels but had no effects on mRNA levels of glutathione peroxidase (GPX) and the pro-inflammatory cytokine interleukin-6. There were no differences in plasma activity of the antioxidant enzymes GPX and CAT between all treatment groups. Our findings demonstrate some positive effects of watermelon for modulating lipids and attenuating lumbar vertebral bone loss arising from ovarian hormone deficiency.

Keywords: dyslipidemia, estrogen deficiency, menopause, osteoporosis, watermelon

INTRODUCTION

The decline in sex hormones that occurs during menopause results in unwanted side effects, including increased risk for osteoporosis and cardiovascular disease. Osteoporosis is a medical condition arising from loss of bone tissue that causes the bone to become fragile and prone to fracture or breaking. Over 10 million Americans currently suffer from osteoporosis and more than 43 million have low bone mass (i.e., osteopenia) (Wright et al., 2014). If appropriate measures are not taken, the prevalence of osteoporosis and osteopenia among older adults is projected to increase three-fold by the year 2030 (Wright et al., 2014). Furthermore, the average cost of osteoporosis treatments and injury management can be extremely expensive depending on the type of treatment (Drake et al., 2015). Therefore, strategies are needed to prevent or delay the development of osteoporosis.

Postmenopausal osteoporosis accounts for approximately 80% of osteoporosis cases that develop (Wright et al., 2014). The risk for osteoporosis increases when women enter menopause due to the decrease in production of the sex hormone, estrogen. There are many physiological changes that affect bone due to estrogen deficiency. Changes in calcium balance, increased production of inflammatory factors and reactive oxygen species, increased activity of bone-breaking cells (i.e. osteoclasts), and decreased activity of bone-forming cells (i.e. osteoblasts) all occur due to estrogen deficiency and all compromise bone quality (Pacifici, 1998). In addition to osteoporosis, certain risk factors for cardiovascular disease such as dyslipidemia also increase during menopause (Pacifici, 1998).

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Pharmacological options for osteoporosis either inhibit bone breakdown (anti-resorptive medications) or increase the rate of bone formation (Tosteson et al., 2003; Drake et al., 2015). For example, the drug alendronate is used to inhibit bone breakdown and increase bone mass, which can reduce the risk of bone fracture. However, low compliance rates and adverse side effects, such as joint or muscle pain and gastrointestinal complications (e.g. nausea, heartburn, abdominal pain, and ulcers), are associated with these medications (Tosteson et al., 2003; Drake et al., 2015). Therefore, interest in seeking natural alternatives for the prevention or treatment of osteoporosis has recently gained popularity.

One alternative approach for maintaining skeletal health that can delay or even prevent postmenopausal osteoporosis is consumption of a diet rich in fruits and vegetables (Tucker et al., 1999; Deyhim et al., 2005; Devareddy et al., 2008; Boeing et al., 2012). Studies have suggested fruits such as blueberries and plums are effective in improving bone mineral density and microarchitecture in postmenopausal women and animal models of postmenopausal osteoporosis (Deyhim et al., 2005; Devareddy et al., 2008). Another fruit that may have a potential in sustaining skeletal health is watermelon (WM). WM contains many bioactive compounds that are shown to be important in skeletal health, including vitamin C, β-carotene, potassium, phenolic compounds (e.g. flavanoids, carotenoids, and triterpenoids), lycopene, arginine, and citrulline (Liu, 2003; Sahni et al., 2009; Mackinnon et al., 2011; Shanely et al., 2016). However, despite WM containing these various bioactive components, we are not aware of any studies investigating the effects of freeze-dried WM on skeletal health and lipid parameters in ovarian hormone deficiency. Therefore, the objective of this study was to determine the extent to which WM is able to prevent bone loss and attenuate lipid parameters in ovariectomized (OVX) mice, an animal model for postmenopausal osteoporosis. We hypothesized that due to the many bioactive compounds in WM with antioxidant and anti-inflammatory properties, supplementation with freeze-dried WM should improve bone and lipid parameters that are compromised by ovarian hormone deficiency.

MATERIALS AND METHODS

Diet formulation and preparation

WM was obtained from a local supermarket, the rind removed, and the remaining pulp freeze-dried. The macronutrient, fiber, calcium, and phosphorus content of freeze-dried WM were analyzed by NP Analytical Laboratories (St. Louis, MO, USA) and the lycopene and citrulline+arginine contents were analyzed as previously published (Fall et al., 2019). The freeze-dried WM pulp was incortrol) formulation at a ratio of 1% and 10% (wt/wt). The macronutrient, fiber, calcium, and phosphorus contributions of WM were accounted for such that all diets were isocaloric and isonitrogenous, and contained the same calcium, phosphorus, and fiber contents (Table 1). Diets were prepared at Harlan Laboratories (Madison, WI, USA).

Animal care and necropsy

Eight-week-old C57BL/6 female mice were purchased

Table 1. Diet composition (unit: g/kg diet)

Ingredients	AIN-93M (control)[1)]	1% Watermelon[2)]	10% Watermelon[2)]
Watermelon	0	10	100
Cornstarch	466	456.4	377.6
Sucrose	100	107.3	107.3
Dextrinized cornstarch	155	155	155
Casein	140	139.14	131.39
Soybean oil	40	39.77	37.68
Cellulose	50	49.90	48.97
Vitamin mix (AIN 93VX)	10	10	10
Calcium and phosphorus deficient mineral mix (TD 98057)	13.4	13.4	13.4
Calcium carbonate	12.5	6.090	6.440
Calcium phosphate, dibasic	2.4	8.67	7.91
Sucrose	6.7	6.840	7.250
Choline bitartrate	2.5	2.5	2.5
L-cysteine	1.8	1.8	1.8
tert-Butyl-hydroquinone	0.008	0.008	0.008

[1)]Based on the AIN-93M formulation from Reeves et al. (1993).
[2)]All diets were isocaloric and isonitrogenous, and had the same calcium, phosphorus, and fiber content and prepared at Harlan Laboratories (Madison, WI, USA).
Watermelon was added wt/wt basis and provided 8.61% protein, 2.32% fat, 1.03% fiber, 0.0914% calcium, and 0.253% phosphorus as analyzed by NP Analytical Laboratories (St. Louis, MO, USA). Freeze-dried watermelon contained 568 mg/kg and 34 g/kg of lycopene and citrulline + arginine, respectively.

from Charles River Laboratories (Chicago, IL, USA). Mice were housed in plastic cages and kept on a 12-h light/dark cycle in a temperature- and humidity-controlled room. Mice were allowed to acclimate to this environment for one week prior to treatment. During the acclimation period, mice were given *ad libitum* access to the AIN-93M control diet and deionized water. After the acclimation period, mice were weighed and were either OVX or sham-operated (SHAM), and then randomly assigned to one of four treatment groups (n=9～13/group): SHAM-control, OVX-control, OVX-1% WM, or OVX-10% WM. Animals were group housed (3～5 mice/cage) and fed their respective diets for 12 weeks. Food intake was measured every three days and body weight were monitored weekly. Feces were collected at the end of the treatment for lipid analyses. Guidelines for the ethical care and treatment of animals from the Animal Care and Use Committee at Oklahoma State University were strictly followed (Protocol number: HS 12-2).

After 12 weeks of dietary treatment, mice were sacrificed and specific tissues were collected. The mice were anesthetized with ketamine/xylazine cocktail (100.0/10.0 mg/kg body weight) and body composition was assessed by GE Lunar Piximus with Series Software version 1.4x (GE Lunar Corporation, Madison, WI, USA). Blood samples were obtained from the carotid artery and placed into ethylenediaminetetraacetic acid coated tubes. Plasma was separated by centrifugation at 1,500 *g* for 20 min at 4°C. Aliquots of plasma were frozen and kept at −80°C for later analyses of lipids and antioxidant enzymes. The visceral adipose tissue, uterus, and liver were collected, weighed, and snap-frozen in liquid nitrogen.

Bone collection and analyses

The spine, tibia, and femur were collected and cleaned of adhering tissue for dual energy x-ray absorptiometry (DXA) and micro-computed tomography (μCT) analyses. Bone mineral density (BMD), area (BMA), and content (BMC) of the tibia and lumbar vertebra were assessed using GE Lunar Piximus with Series Software version 1.4x (GE Lunar Corporation).

The microarchitectural parameters of the tibia and L4 vertebra were assessed using μCT (MicroCT40, Scanco Holding AG, Wangen-Brüttisellen, Switzerland). The proximal tibial metaphysis and mid-diaphysis were used to analyze trabecular and cortical bone, respectively. Scans of the tibial metaphysis were performed at a resolution of 2,048×2,048 pixels (i.e. 6 μm each slice). Semi-automated contours were placed beginning at 60 μm (10 slices) distal to the proximal growth plate and the volume of interest (VOI) was 882 μm (147 slices). The images were analyzed at a threshold of 325 with a sigma and support of 1.2 and 2.0, respectively. The trabecular parameters evaluated included bone volume (BV), total bone volume (TV), bone volume expressed per unit of total volume (BV/TV), trabecular number (TbN), trabecular thickness (TbTh), trabecular separation (TbSp), connectivity density (ConnDens), structural model index (SMI), and degree of anisotropy (DA). Analysis of cortical bone was evaluated by analyzing a 52 slice VOI at the mid-point of the tibia. Assessment of cortical bone included cortical porosity, thickness, area, and medullary area of the tibial mid-diaphysis. The images were analyzed at a threshold of 260 with a sigma and support of 0.8 and 1.0, respectively.

Analyses of the microarchitectural parameters of the L4 vertebra were performed by examining images at a resolution of 1,024×1,024 pixels, 80 μm from the dorsal and caudal growth plates. Similar for the tibial analysis, semi-automated contours were placed to assess secondary spongiosa within the VOI. The images were analyzed at a threshold of 325 with a sigma and support of 1.2 and 2.0, respectively.

Liver RNA extraction and quantitative real-time polymerase chain reaction (PCR)

RNA was extracted from the liver (n=6/group) using STAT60 (Tel-Test Inc., Friendswood, TX, USA) according to the manufacturer's instructions. RNA concentrations and quality were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The quality of the RNA was further assessed by agarose gel electrophoresis and by examining the integrity of 18S and 28S rRNA. RNA samples were stored at −80°C until use in synthesis of cDNA and real-time PCR.

Quantitative real-time PCR was used to determine mRNA expression of the anti-oxidant enzymes catalase (CAT) and glutathione peroxidase (GPX) and the pro-inflammatory cytokine interleukin-6 (IL-6) in the liver. All results were calculated by the comparative cycle number at threshold (C_T) method (User Bulletin No. 2; Applied Biosystems, Foster City, CA, USA) using cyclooxygenase (Cyclo) as the invariant control. The primers used for qPCR are shown in Table 2.

Lipid and anti-oxidant enzymes analyses

Plasma concentrations of the non-esterified fatty acids (NEFA), total cholesterol, and triglycerides were determined using the BioLis 24i clinical chemistry analyzer from Carolina Liquid Chemistries Corporation (Brea, CA, USA). All kits were purchased from Carolina Liquid Chemistries Corporation (Brea, CA, USA) except for NEFA, which was purchased from Wako Diagnostics (Richmond, VA, USA); all manufacturer's instructions were strictly followed. Plasma CAT and GPX activity were determined using commercially available kits from Cayman Chemical Company (Ann Arbor, MI, USA) and results were determined using the Synergy HT spectropho-

Table 2. List of primer sequences used in real-time PCR

Symbol	Name		Primer sequence	Accession number
Cyclo	Cyclophilin	Forward	5′-TGG AGA GCA CCA AGA CAG ACA-3′	NM_011149
		Reverse	5′-GCC GGA GTC GAC AAT GAT-3′	
CAT	Catalase	Forward	5′-CCG AGT CTC TCC ATC AGG TTT-3′	NM_009804.2
		Reverse	5′-TCA TGT GCC GGT GAC CAT-3′	
GPX	Glutathione peroxidase	Forward	5′-AAC TCG GAG ATA CTC CCC AGT CT-3′	NM_008161
		Reverse	5′-GCT GGA AAT TAG GCA CAA AGC-3′	
IL-6	Interleukin-6	Forward	5′-GAG GAT ACC ACT CCC AAC AGA CC-3′	NM_031168
		Reverse	5′-AAG TGC ATC ATC GTT GTT CAT ACA-3′	

tometer (BioTek Instruments Inc., Winooski, VT, USA).

For analyses of fecal and liver lipids, portions of livers and feces were homogenized and extracted with a 2:1 (v/v) chloroform : methanol mixture. After addition of 0.12 mol/L NaCl solution to the extraction solution and separation of phases, the organic phase were poured into a pre-weighed aluminum pan for determination of total lipid content using the Folch gravimetric method (Folch et al., 1957).

Statistical analyses

Statistical analyses involved computation of means and standard error (SE) for each of the treatment groups using SAS version 9.4 (SAS Institute, Cary, NC, USA). Analysis of variance and least square means were calculated using the general linear model procedure and the means were compared using Fisher's least significant difference for comparing groups. Differences were considered significant at $P<0.05$.

RESULTS

Body weight, food intake, and tissue weight

All mice were of similar body weight at the start of the dietary treatment (Table 3). From two weeks and until the end of dietary treatment, all OVX mice had higher body weights than the SHAM mice. Addition of WM was not able to reduce the increase in body weight induced by OVX, despite the lower food and caloric intake of the WM groups.

As expected, uterine weights were highest for the SHAM-control group; all the OVX groups had similar uterine weights (Table 3). There was no significant difference in liver weight between all treatment groups. Consistent with the final body weight results, visceral adipose tissue weight was significantly higher in the OVX mice, with WM supplementation further increasing adipose tissue weight.

Whole body composition, and tibia and lumbar bone parameters

The percent whole body fat content as analyzed by x-ray absorptiometry was consistent with the weight of the isolated visceral adipose tissue (Table 4). The WM fed groups had a higher percent body fat and a lower percent lean mass compared with mice fed the control diet. In addition, mice receiving WM, particularly the at the 1% dose, had lower whole body BMD, BMC, and total area than mice in the SHAM-control group.

Table 3. Food intake, body weight, and relative weights of tissue

Parameters	SHAM-control (n=12)	OVX-control (n=9)	OVX-1% WM (n=12)	OVX-10% WM (n=13)	*P*-value
Food intake					
Grams/mouse/d	3.40±0.04[a]	3.50±0.06[a]	3.30±0.05[b]	3.30±0.05[b]	0.0006
Calories/mouse/d	13.0±0.9[a]	13.3±1.4[a]	11.8±1.1[b]	11.7±1.1[b]	<0.0001
Body weights (g)					
Initial	22.5±0.3	22.3±0.3	22.7±0.3	22.7±0.2	0.802
Final	23.50±0.15[b]	29.60±1.10[a]	30.60±0.52[a]	30.40±0.81[a]	<0.0001
Relative tissue weights (%)					
Uterus	0.31±0.02[a]	0.05±0.01[b]	0.05±0.01[b]	0.06±0.01[b]	<0.0001
Liver	4.06±0.13	3.92±0.20	3.94±0.06	3.68±0.14	0.230
Adipose tissue	1.68±0.12[c]	4.24±0.29[b]	5.45±0.26[a]	5.37±0.29[a]	<0.0001

Values are mean±SE.
In a row, values that do not share the same letter (a-c) are significantly different ($P<0.05$).
SHAM-control, sham-operated mice OVX-control, ovariectomized mice; OVX-1% WM, ovariectomized mice administered 1% freeze-dried watermelon; OVX-10% WM, ovariectomized mice administered 10% freeze-dried watermelon.

Table 4. Whole body composition, tibial, and lumbar bone mineral content (BMC), bone mineral area (BMA), and bone mineral density (BMD)

Parameters	SHAM-control (n=12)	OVX-control (n=9)	OVX-1% WM (n=12)	OVX-10% WM (n=13)	*P*-value
Whole body composition					
% Fat mass	24.9±0.5^{c}	33.2±1.3^{b}	36.4±0.7^{a}	36.7±1.0^{a}	<0.0001
% Lean mass	75.1±0.5^{a}	66.8±1.3^{b}	63.6±0.7^{c}	63.3±1.0^{c}	<0.0001
BMA (cm^2)	12.0±0.2^{a}	10.0±0.2^{b}	9.6±0.1^{b}	9.7±0.2^{b}	<0.0001
BMC (mg)	642.7±16.7^{a}	471.6±20.9^{b}	397.8±21.2^{c}	423.8±22.6^{bc}	<0.0001
BMD (mg/cm^2)	53.6±1.0^{a}	47.1±1.4^{b}	41.2±1.8^{c}	43.7±2.2^{bc}	<0.0001
Tibia					
BMA (cm^2)	0.486±0.006	0.496±0.013	0.505±0.010	0.482±0.005	0.225
BMC (mg)	25.54±0.47^{a}	23.67±0.55^{bc}	24.50±0.54^{ab}	22.58±0.41^{c}	0.001
BMD (mg/cm^2)	52.30±0.44^{a}	47.70±0.84^{b}	48.30±0.43^{b}	47.00±0.54^{b}	<0.0001
Lumbar					
BMA (cm^2)	0.308±0.006	0.311±0.006	0.306±0.007	0.298±0.005	0.503
BMC (mg)	17.75±0.64^{a}	14.22±0.43^{b}	14.50±0.56^{b}	14.54±0.43^{b}	<0.0001
BMD (mg/cm^2)	58.36±1.05^{a}	46.10±0.93^{b}	47.80±1.06^{b}	49.03±1.38^{b}	<0.0001

Values are mean±SE.
In a row, values that do not share the same letter (a-c) are significantly different (P<0.05).

Isolated bone (i.e., tibia and lumbar vertebra) was also analyzed by x-ray absorptiometry. Unlike the whole body BMC, the tibial BMC of the OVX-1% WM group was similar to both the SHAM-control and OVX-control groups (Table 4). WM supplementation did not prevent the decrease in the BMD of the tibia and lumbar vertebra induced by OVX. WM treatment did not affect the tibial and lumbar BMA (Table 4).

Proximal tibial metaphysis trabecular BV/TV, TbN, and ConnDens of the OVX animals were significantly lower compared with those of the SHAM animals (Table 5). WM was not able to attenuate the decrease in these trabecular bone parameters induced by OVX. Similarly, OVX mice had higher TbSp than mice in the SHAM-control group and WM was not able to modulate the reduction in TbSp. The same trend was observed for the tibial mid-diaphysis cortical bone parameters, such that all OVX mice had lower cortical thickness and area, and WM did not affect these parameters. There were no significant differences between groups in terms of cortical BV/TV, medullary area and porosity.

Similar to the tibial bone microarchitecture parameters, OVX reduced L4 vertebra BV/TV, TbN, TbTh, and ConnDens, and increased TbSp and SMI. However, unlike for the tibial trabecular bone parameters, WM was able to attenuate some trabecular bone parameters of the L4 vertebra. The 10% WM dose increased TbN and ConnDens, and reduced TbSp and SMI of the L4 vertebra. These parameters were restored to those recorded for the SHAM-control group. There is no statistically significant difference in DA for the L4 trabecular bone between any of the treatment groups.

Lipid parameters and antioxidant enzymes

The total plasma cholesterol concentration was highest in the OVX-1% WM group whereas the OVX-10% WM group has total plasma cholesterols similar to those of the SHAM-control group (Table 6). No significant differences in plasma triglycerides and non-esterified fatty acids were observed between the groups. There were also no significant differences in the activities of the antioxidant enzymes CAT and GPX in the plasma.

All OVX groups have higher levels of total lipids in the liver compared with the SHAM-control group. However, the OVX-10%WM group had total lipid levels in the liver that were statistically similar to that of the SHAM-control group, albeit still statistically similar to the OVX-control. Total lipid levels in fecal material was highest in the OVX-10% WM group and lowest in the OVX-control group.

Relative mRNA abundance of antioxidant enzymes and the inflammatory cytokine IL-6 in the liver

The abundance of mRNA for the antioxidant enzymes CAT and GPX and the inflammatory cytokine IL-6 in the liver were quantified. Unlike in the plasma where there was no difference in the activity of CAT, the OVX-10% WM group had the highest expression of CAT in the liver (Fig. 1). All the other groups have lower CAT gene expression in the liver and are statistically similar to each other. There were no significant differences in the expression of GPX or IL-6 in the liver.

DISCUSSION

The purpose of this study was to determine if freeze-dried

Table 5. Microarchitectural parameters of the lumbar vertebrae, proximal tibial metaphysis, and mid-diaphysis

Parameters	SHAM-control	OVX-control	OVX-1%WM	OVX-10%WM	*P*-value
Proximal tibial metaphysis (trabecular bone)					
BV/TV (%)	7.65±0.21^{a}	5.35±0.24^{b}	4.60±0.30^{b}	4.85±0.35^{b}	<0.0001
TbN (1/mm)	2.62±0.08^{a}	2.25±0.06^{b}	2.19±0.06^{b}	2.24±0.03^{b}	0.0003
TbTh (μm)	55.0±1.5	52.6±2.4	54.8±0.6	53.5±0.9	0.668
TbSp (μm)	378.8±14.3^{b}	448.4±18.8^{a}	462.4±13.3^{a}	446.9±7.0^{a}	0.0019
ConnDens (1/mm^3)	38.0±5.2^{a}	32.2±5.4^{ab}	21.3±2.9^{b}	20.8±4.8^{b}	0.044
SMI	2.00±0.05	2.08±0.09	2.15±0.08	2.33±0.10	0.060
DA	1.80±0.02	1.60±0.09	1.61±0.05	1.65±0.06	0.091
Tibial mid-diaphysis (cortical bone)					
Cortical thickness (μm)	222.3±3.9^{a}	196.3±4.4^{b}	198.0±2.9^{b}	206.2±2.7^{b}	0.0002
Cortical area (mm^2)	681.3±10.9^{a}	635.6±19.2^{b}	618.0±10.4^{b}	637.9±10.6^{b}	0.020
Medullary area (mm^2)	10.84±0.93	11.06±1.30	9.13±0.66	9.78±0.53	0.416
Cortical porosity (%)	1.56±0.13	1.71±0.16	1.48±0.11	1.51±0.08	0.558
Cortical BV/TV (%)	98.40±0.31	98.30±0.39	98.50±0.26	98.50±0.18	0.558
L4 trabecular bone					
BV/TV (%)	11.41±0.52^{a}	5.62±0.44c	5.79±0.55^{c}	8.56±1.50^{b}	0.0004
TbN (1/mm)	3.38±0.07^{a}	2.60±0.07^{bc}	2.57±0.15^{c}	3.03±0.25^{ab}	0.003
TbTh (μm)	43.32±1.33^{a}	35.75±0.91^{b}	36.38±1.30^{b}	37.53±1.06^{b}	0.0006
TbSp (μm)	299.6±6.0^{c}	389.9±11.3^{ab}	399.5±22.3^{a}	342.6±26.2^{bc}	0.003
ConnDens (1/mm^3)	133.2±5.9^{a}	53.3±6.0^{b}	57.7±10.0^{b}	110.5±33.0^{a}	0.010
SMI	1.70±0.06^{b}	2.32±0.12^{a}	2.32±0.06^{a}	2.04±0.22^{ab}	0.012
DA	1.75±0.03	1.74±0.03	1.77±0.02	1.69±0.03	0.283

Values are mean±SE, n=6/group.
In a row, values that do not share the same letter (a-c) are significantly different (P<0.05).
TV, total bone volume; BV, bone volume; ConnDens, connectivity density; SMI, structural model index; TbN, trabecular number; TbTh, trabecular thickness; TbSp, trabecular separation; DA, degree of anisotropy.

Table 6. Lipids and antioxidant enzymes

Parameters	SHAM-control (n=12)	OVX-control (n=9)	OVX-1%WM (n=12)	OVX-10%WM (n=13)	*P*-value
Plasma lipids					
Cholesterol (mmol/L)	2.05±0.05^{b}	2.32±0.17^{ab}	2.59±0.08^{a}	2.07±0.15^{b}	0.002
Triglycerides (mmol/L)	0.37±0.03	0.36±0.04	0.37±0.03	0.36±0.03	0.996
Non-esterified fatty acids (mEq/L)	0.80±0.07	0.98±0.12	0.73±0.09	0.86±0.10	0.359
Plasma antioxidant enzymes					
Catalase activity (nm/mol/min)	10.4±0.5	12.2±0.7	11.5±0.8	11.0±0.5	0.325
Glutathione peroxidase activity (nm/mol/min)	17.5±5.8	29.9±13.2	26.3±11.6	32.7±7.6	0.609
Total lipids					
Liver (mg/g)	72.4±2.9^{b}	94.1±7.4^{a}	102.6±8.8^{a}	87.8±3.6^{ab}	0.006
Feces (mg/g)	11.8±0.3^{bc}	10.7±0.8^{c}	12.8±0.5^{ab}	14.8±0.7^{a}	0.012

Values are mean±SE.
In a row, values that do not share the same letter (a-c) are significantly different (P<0.05).

WM can prevent bone loss and improve lipid parameters in OVX mice. WM was selected for experimentation because it contains many bioactive compounds, including lycopene, vitamin C, β-carotene, arginine, citrulline, and cucurbitacin E; these bioactive compounds have antioxidant and anti-inflammatory activities that have been implicated in improving bone health (Liu, 2003; Sahni et al., 2009; Mackinnon et al., 2011; Shanely et al., 2016). We hypothesized that these components could prevent bone loss and improve lipid profiles in OVX mice. Previous studies have shown that OVX mice are appropriate for studying post-menopausal osteoporosis and have been used to identify effective dietary interventions that can prevent or delay post-menopausal bone loss (Chiba et al., 2003; Mori-Okamoto et al., 2004; Rendina et al., 2012).

In the current study, we demonstrated that our OVX mice had increased bone loss, as shown by decreased BMD and compromised bone microarchitecture. We did not observe any significant differences between the tibial and lumbar BMD between mice in the OVX-control and WM fed groups. Additionally, the whole-body BMD values were significantly lower in the OVX-1% WM group

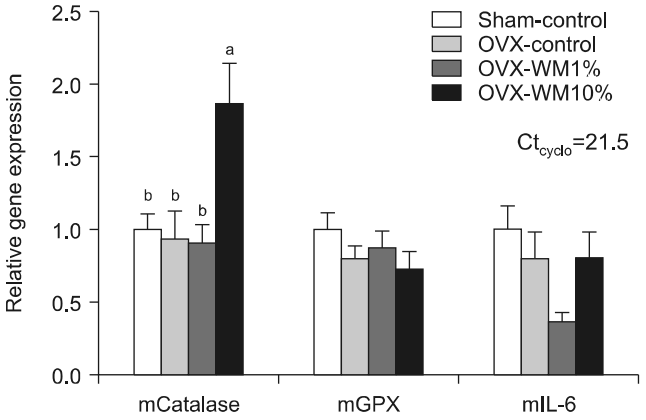

Fig. 1. Comparison of liver relative mRNA abundance of antioxidant enzymes and inflammatory cytokines in sham-operated (SHAM) and ovariectomized (OVX) mice fed control and two doses of watermelon (WM) diets (OVX-WM1% and OVX-WM10%). Bars are relative to SHAM-control group (set at 1.0). Cyclophilin was used as the invariant control and had a CT value of 21.5. Values are mean±SE. Bars that do not share the same letter (a,b) are significantly different (P<0.05). GPX, glutathione peroxidase; IL-6, interleukin 6; Cyclo, cyclophilin.

compared with the OVX-control group, but mice in the OVX-10% WM showed comparable results to those in the OVX-control group. The BMD values for the tibia, lumbar, and whole body suggest there is no benefit of WM on this parameter.

Analyses of bone trabecular parameters by μCT also showed a similar trend; the findings from DXA showed some positive effects of WM on a few of the bone parameters, particularly those of the L4 vertebra. Previous research has shown that the tibia and lumbar are practical sites to assess changes in bone microarchitectural parameters using μCT (Rendina et al., 2012; Smith et al., 2014). We observed a slight positive outcome in the lumbar trabecular bone of mice in the OVX-10% WM group, although there was no effect on the tibial trabecular and cortical bones. In women with osteoporosis, vertebral fractures are very common and generally occur below the mid-thoracic region (Chen et al., 2013). Nearly 3/4 of all vertebral fractures occur during routine daily activities, such as bending over and lifting objects, and only about 1/3 of those come to medical attention and prompt preventative treatments (Cummings and Melton, 2002). Analyses of the L4 region of the vertebra showed that mice in the OVX-10% WM group are able to restore connectivity density to the levels of the SHAM-control group, despite the increase in body weight and body fat. The OVX-10% WM group had comparable BV/TV, SMI, and TbSp parameters as the SHAM-control group; however, these parameters were also comparable to those of the OVX-control group. These results suggests that the 10% dose of WM had an intermediate effect in restoring these parameters and may be able to restore some of the bone parameters that are lost during estrogen deficiency.

In order to understand the mechanism by which WM positively affects bone, inflammatory and antioxidant markers were assessed in both the plasma and liver. Increased inflammation and oxidative stress have both been associated with the onset and progression of postmenopausal osteoporosis (Manolagas, 2010; Trouvin and Goëb, 2010). In this study, IL-6 concentrations in the liver was chosen as the inflammatory marker because of its role in osteoclastogenesis (Trouvin and Goëb, 2010). IL-6 has receptors on both osteoblasts and osteoclasts but its main physiological effects occur through stimulatory effects of other pro-inflammatory cytokines (Kwan Tat et al., 2004). We did not observe any significant changes in abundance of IL-6 mRNA in the liver. However, other studies have reported a reduction in other inflammatory markers, such as C-reactive protein, monocyte chemoattractant protein-1, and interferon-γ, and increased concentrations of the anti-inflammatory cytokine IL-10 (Poduri et al., 2013; Hong et al., 2015; Hong et al., 2018). Since these parameters were not assessed in this study, it is unknown if they may affect the bone.

We also assessed antioxidant enzyme activity in the plasma, and gene expression of these enzymes in the liver. Previous study by Lean et al. (2003) demonstrated that administration of antioxidants, ascorbate or N-acetyl-cysteine (precursor to glutathione) prevents OVX-induced bone loss in mice. Similarly, Rao et al. (2007) found that lycopene intake in postmenopausal women decreases protein oxidation and lipid peroxidation while also decreasing bone resorption. CAT and GPX are both responsible for neutralizing reactive oxygen species, specifically hydrogen peroxide, which is involved in osteoclastogenesis and bone loss caused by estrogen deficiency (Lean et al., 2005). Although we did not observe any significant differences in CAT and GPX activity in the plasma, liver CAT mRNA was significantly higher in mice in the OVX-10% WM group compared with the other groups. These results are consistent with results from our bone analyses, suggesting that 10% WM may be a suitable dose for preventing bone loss by increasing antioxidant activity of CAT. Whether doses of WM higher than 10% are more effective in increasing anti-oxidant activity and preventing bone loss needs to be investigated in future studies.

In addition to changes in the bone, the OVX mice also had an increased body weight. This finding is supported by other studies using OVX models (Roesch, 2006; Rendina et al., 2012). In the present study, WM could not attenuate the increase in body weight induced by ovariectomy despite a lower food intake in the WM groups. All diets were adjusted to be isocaloric and itrogenous, and to have the same calcium and phosphorus concentrations. Possible explanations for this change in body composition could be due to changes in physical activity or basal

metabolic rate, or be related to the components in the WM altering the gut microbiota, thus promoting weight gain. Greiner and Bäckhed (2011) demonstrated that alterations in gut microbiota can increase obesity and promote insulin resistance. Additionally, Laparra and Sanz (2010) discussed that phytochemicals from functional foods used by bacteria to produce secondary metabolites are also able to influence growth and activity of specific intestinal bacteria. This leads to the possibility that the bioactive components of WM, especially those that exist in large quantity such as lycopene, alter the gut bacteria in C57BL/6 female mice. A study by Kim et al. (1997) observed similar effects and showed that increased doses of lycopene increased weight gain in female B6C3F1 mice, a cross between C57BL/6 and C3H mice; however, this was a chemoprevention study rather than in an OVX model. These explanations for the effect of WM on body weight and composition are all speculative and warrant further investigation.

The weight gain observed in the WM-fed mice prompted us to examine lipid parameters. As expected, all mice in the OVX groups had increased proportions of fat in the liver. The SHAM-control mice had the lowest proportions of liver fat and this proportion was comparable to mice in the WM-10% group. This suggests that WM prevents accumulation of fat in the liver. Mice in the WM groups had the highest fecal fat content. Previous research has shown that ovariectomy causes hyperlipidemia (Lucas et al., 2004); however, we did not observe similar results in our study during analyses of plasma lipids. Although there were no significant differences, cholesterol tended to be higher in OVX-control and WM-1% groups, and WM-10% reduced cholesterol to similar levels as mice in the SHAM-control group. A few studies have demonstrated the hypocholesterolemic effects of freeze-dried WM or WM extracts in both animal models and humans (Poduri et al., 2013; Hong et al., 2015; Massa et al., 2016; Hong et al., 2018). The arginine content of WM has been shown to contribute to the hypocholesterolemic effect of WM (Hong et al., 2018).

Although we have shown WM-10% may be promising in attenuating vertebral bone loss, it is necessary to consider the limitations of this study. Bones could have also been used to assess antioxidant enzymes and inflammatory markers to observe a more local effect of the WM rather than examining these parameters in the plasma or liver. Lean et al. (2003) measured glutathione and glutathione reductase in OVX mice, and showed that there were no changes in their levels in the livers and spleens compared with SHAM-control mice, however these parameters were decreased in the bone marrow; this may explain why we did not observe differences in GPX liver mRNA expression or plasma activity. In addition, pro- and anti-inflammatory markers other than IL-6 should also be considered. Finally, data obtained from animal models of human disease should be interpreted with caution as it may not be translatable to humans and requires further testing.

In conclusion, our data demonstrates that WM has positive effects on vertebral bone microarchitecture and lipid parameters in ovariectomized mice. More research is needed in this area to establish WM as a dietary approach for preventing osteoporosis and dyslipidemia due to ovarian hormone deficiency.

AUTHORS' CONTRIBUTIONS

EAL, BJS, and PP-V contributed to the experimental design; PP-V prepared the freeze-dried watermelon powder; MY, KW, SP, MB, and SP conducted study procedures and laboratory analyses; MP conducted the statistical analyses. EAL and MY prepared the first draft of the manuscript; EAL, MY, KW, PP-V, MB, BJS, and PP-V contributed to the final version of paper.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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