Original Communication

Vitamin C reverses bone loss in an osteopenic rat model of osteoporosis

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Abstract: Fruits and vegetables are rich in vitamin C with antioxidant properties which are known to influence bone quality. This study evaluated whether vitamin C (1000 mg/L) added to drinking water reverses the bone loss in ovariectomized rats. Ninety-day-old female Sprague-Dawley rats were randomly assigned to either sham (n = 14) or ovariecotmized groups (n = 28). Sixty days after ovariectomy, the treatments were sham, ovariectomy (OVX), OVX + vitamin C (22 mg oral intake daily) for 60 days. Urine was collected for deoxypyridinoline (DPD) evaluation, rats were sacrificed, and antioxidant capacity, osteopontin, alkaline phosphatase (ALP), and bone specific tartrate resistant acid phosphatase (TRAP) were evaluated in the plasma. Right femur and 5th lumbar were evaluated for bone density, strength, ash, Ca, and Mg concentrations. Antioxidant capacity, ALP activity, osteopontin decreased (p-value < 0.05), while TRAP and urinary DPD increased (p-value < 0.05) with ovariectomy. In contrast, vitamin C increased (p-value < 0.05) antioxidant capacity, ALP activity, osteopontin concentration and reduced (p-value < 0.05) TRAP and urinary DPD excretion, respectively. Ovariectomy reduced (p-value < 0.05) bone quality, bone ash, Ca and Mg concentrations. Vitamin C increased (p-value < 0.05) femoral density without affecting (p-value > 0.1) femoral strength, ash, or Ca, and Mg concentrations, while it increased (p-value < 0.05) the 5th lumbar density, ash, and Ca and Mg concentrations. In conclusion, vitamin C increased bone quality and antioxidant capacity in ovariectomized rats.

Keywords: Vitamin C, ovariectomy, bone quality, antioxidant capacity

Introduction

According to the U.S. Office of the Surgeon General, osteoporosis is the most common type of the bone disease of aging and one of the major public health problems [1]. Osteoporosis is characterized by micro-architectural deterioration of bone tissue predisposing humans to increased risk of fracture [2, 3]. Estrogen deficiency in women is one of the leading causes of osteoporosis [4]. It has been reported that during puberty, gonadal hormones stimulate bone formation which leads to increased bone mineral density [5]. In contrast, in middle aged postmenopausal women, gonadal hormones declined to prepubertal levels which increased bone resorption and reduced bone mineral density [6]. In a meta-analysis study, decreased bone mineral density has been observed in postmenopausal women and calcium supplementation delayed the onset of bone mineral loss [7]. Increased bone lose has led to bone fracture [8]. It has been reported that one out of every two Caucasian women and one in five men will experience an osteoporosis-related fracture at some point in their lifetime [1].

Several epidemiological studies have evaluated a positive association between eating diets low in fat but high in fruits and vegetables and health [9, 10]. Improved bone quality is one of the positive health outcomes reported to be achieved by eating diets rich in fruits and vegetables [11]. In both animal and human studies, diets rich in fruits and vegetables positively influenced bone mineral status [12–16]. A recent study linked inadequate intake of fruits and vegetables to higher risk of hip fracture [17].

Protective association of high intake of fruits and vegetables with bone quality is related to quenching free radicals as oxidative stress is implicated in the pathogenesis of osteoporosis [18, 19]. Fruits and vegetables are a rich source of many nutrients and bioactive compounds with antioxidant properties that may protect bones against oxidative stress [20]. In animal studies, eating diets rich in citrus pulp or drinking citrus juice protected bone against oxidation and reduced bone loss [21–23].

Citrus is a rich source of bioactive compounds and nutrients. Citrus is well known to be high in vitamin C. Vitamin C is a potent antioxidant with the ability to scavenge superoxide anion and hydrogen peroxide [24, 25]. However, several epidemiologic studies examined the association of vitamin C intake with bone mineral density (BMD) and the reports were not conclusive [26–31]. A few human retrospective studies using the National Health and Nutrition Examination Survey (NHANES III) data, showed that dietary vitamin C intake had inconsistent association with BMD [30] and the effect of dietary and serum ascorbic acid was inconsistent with self-reported fracture among adult participants in NHANES III [31]. In another study subjects who took vitamin C supplements had improved bone mineral density in the hip and of the femoral neck [32]. A study using rats with defective L-gulonolactone oxidase synthesis observed reduced bone formation and decreased bone mechanical properties in these animals [33]. In other animal studies using guinea pigs, vitamin C deficiency also decreased bone density [34, 35]. In vitamin C deficient rats, reduced hydroxylation of lysine and proline, collagen formation, and poor bone mechanical properties was observed [36]. In vitro, vitamin C stimulated osteoblast proliferation and collagen synthesis [37]. In addition, vitamin C was associated with in vitro differentiation of ST2 cells into osteoblast-like cells, formation of type I collagen, with an increased in alkaline phosphatase activity [38].

A few studies found that vitamin C supplementation had no effect on bone mineral content of pigs; however, in guinea pigs, supplemental vitamin C improved trabecular bone formation in femurs [39, 40]. Despite controversies over the impact of vitamin C deficiency on bone mineral density, efficacy of vitamin C supplementation on bone quality is yet understudied. Therefore, the objective of the present study was to determine whether daily addition of vitamin C to drinking water would improve bone quality and antioxidant status in ovariectomized rats.

Materials and methods

Animals and Diets

The husbandry and treatment of the rats were in compliance with the NRC guidelines for laboratory animals [41]. The research study was approved by the Texas A&M University-Kingsville Institutional Review Board (*TAMUK-IRB)* prior to initiating the study. In this study, ninety-day-old female Sprague-Dawley rats (n = 42) from Hilltop Laboratory (Scottdale, PA) were housed individually in an environmentally controlled animal laboratory and acclimated with a semi-purified, powdered casein-based diet, AIN-93M (Teklad, Madison, WI) diet for three days before surgery. At the time of surgery, the rats were weighed randomly and then divided into two groups: a sham-control group (n = 14) and an ovariectomized group (OVX, n = 28) (Figure 1). Detailed ovariectomy procedure is described previously [42]. The rats were housed in individual cages throughout the study and they were pair-fed to the three days average food intake of the sham group for 60 days to ensure bone loss has occurred similar to our previous study [43]. After 60 days post-ovariectomy, the treatments were sham (control, n = 14), OVX group (n = 14), and OVX + vitamin C (n = 14) and rats continued to be housed in individual cages until they were euthanized. There were no complications associated with ovariectomy procedures and rats were active within 24 hours. Rats were continued to be pair-fed to the average three days food intake of the sham group for the 60 days duration of the study. Vitamin C (1000 mg) plus 100 gm table sugar to improve palatability (preliminary study) was added to one liter of distilled and deionized water for the vitamin C group while the sham and ovariectomized rats only received 100 gm table sugar added to 1 L of distilled and deionized water. At the termination of the study, all rats were weighed and sacrificed for sample collection as discussed previously [21–23].

Blood parameters

Two months after drinking vitamin C solution daily, rats were euthanized using ketamine/xylazine and bled from the abdominal aorta. Blood samples were collected in heparinized tubes and centrifuged (4°C) at 1,500 x g for 15 minutes. The plasma was separated and an aliquot was refrigerated for analyzing plasma antioxidant capacity using a commercially available kit (Calbiochem, San Diego, CA, USA) as a quantitative measure of circulating antioxidant status. The plasma alkaline phosphatase was assessed using a commercially available kit (Thermo Electron, Louisville, CO, USA) as an index of bone formation. The bone TRAP activities were evaluated using a commercially available kit (Quidel, San Diego, CA. USA) as an index of bone turnover. The plasma osteopontin was measured to quantitatively assess bone formation using a commercially available protocol (R&D Systems, Minneapolis, MN, USA). All assays were done in duplicates.

Bone quality assessment

The right femurs and 5th lumbar were harvested, stripped of soft tissues, and then stored in glass vials at –20°C. Femoral density and 5th lumbar analysis were evaluated as dis-

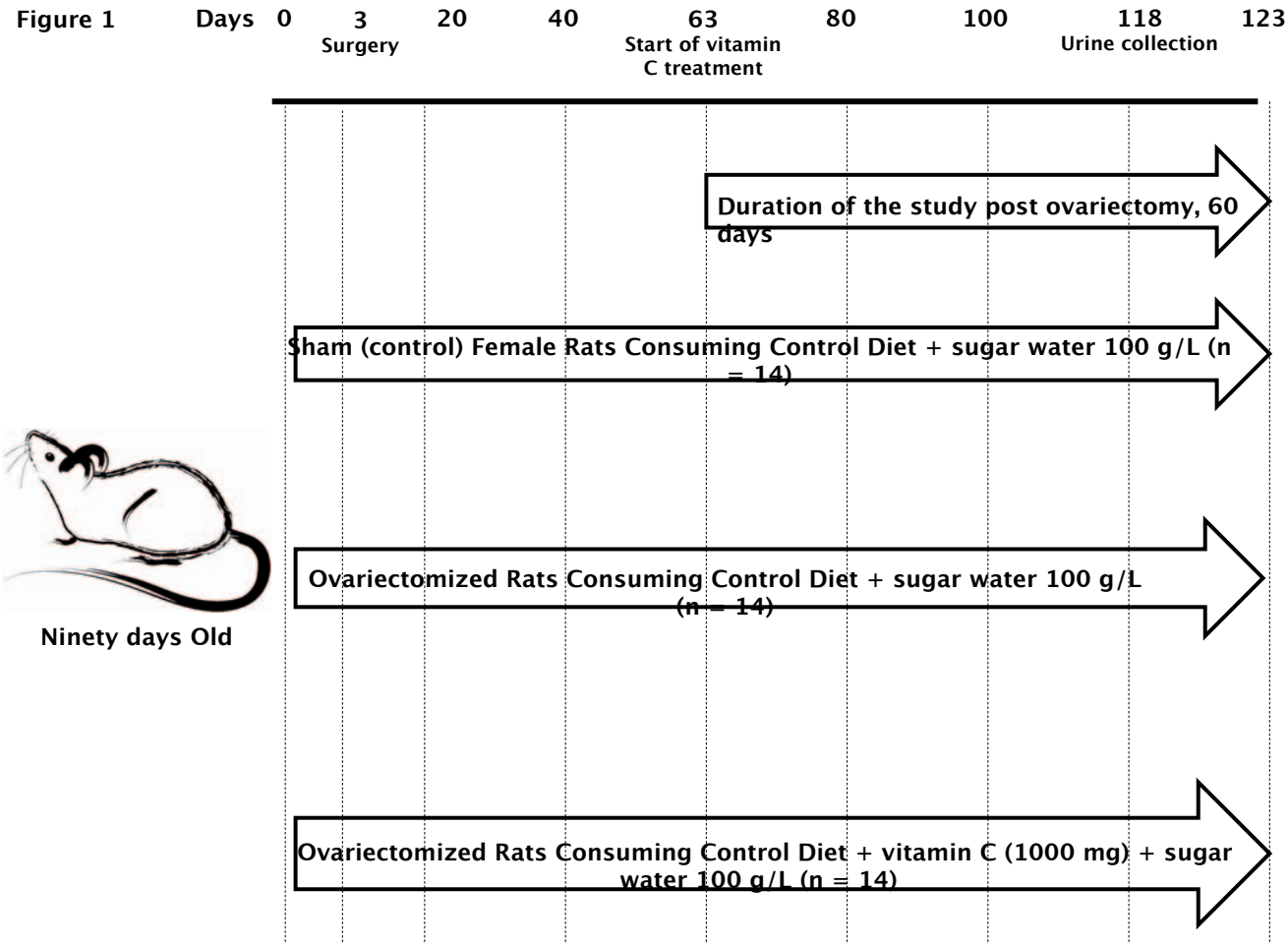

Figure 1. Treatment groups and duration of drinking vitamin C solution in ovariectomized rats.

cussed previously [43]. Femoral and 5th lumbar biomechanical properties were measured by a three-point bending test (Stable Micro System, Canton, MA, USA). A force versus deformation curve was recorded to measure bending strength of the intact femur and the 5th lumbar as described previously [43].

Urinary Deoxypyridinoline Concentration

Five days before termination of the study, rats were transferred to metabolic cages for 24 hour urine collection. Metabolic cages used in this study was modified cages which were similar in size and dimension with the exception of the slope at the base of the cage for urine collection in a 50 ml plastic tube. Nevertheless, for the duration of the study, all rats walked on a screen mesh to avoid urine contamination with hair and fecal materials. During the urine collection, rats continued drinking the treatment solution and were pair-fed. Urine was collected in acid-washed tubes, the total volume was measured, and samples were stored at –20°C for later analyses. Deoxypyridinoline in the urine was analyzed as an index of bone deterioration as described by the manufacturer assay kit and expressed per milligram concentration of creatinine (Quidel, San Diego, CA. USA). Urine samples for deoxpyridinoline were also analyzed in duplicates.

Bone ash

The right femurs and 5th lumbar from all treatment groups were placed in a drying oven at 100 °C until bones reached complete dryness as discussed previously [21–23]. Samples were then weighed and pulverized in covered crucibles at 600 °C for 16 hours. The ashed samples were diluted with 4N HCl solution and aliquots were taken and re-diluted with 0.5% nitric acid and 0.5% lanthanum chloride solu-

tion. Bone calcium and magnesium were analyzed using flame atomic absorption spectrophotometry (Model 800, Perkin-Elmer, Norwalk, CT).

Statistical analyses

The data were analyzed using the Shapiro–Wilk test to verify the normal distribution [44]. In this study the P-value was greater than 0.05 suggesting normal distributions. Further, the homogeneity of variance was evaluated using Levene's test. Levene's test indicated there were no significant deviation from the variance, then the data were analyzed by Analysis of Variance (ANOVA) using the General Linear Model procedure of SAS[â] (version 7) to determine the effects of vitamin C on bone quality, indices of bone turnover, and calcium and magnesium concentrations in the bones followed by the least square means procedure of SAS[â] for separating means that were significantly (P£ 0.05) different [45].

Results

In the present study, initial weight was not different between treatment groups (Table 1). Drinking vitamin C solutions for 60 days did not (p-value >0.1) affect fluid or food consumption (Table 1). However, ovariectomized rats had higher (p-value <0.05) body weight than the sham (control) group.

Plasma antioxidant capacity decreased (p-value <0.05) with ovariectomy while vitamin C treatment normalized antioxidant capacity in the plasma of ovariectomized rats drinking vitamin C to the level of the sham group (Table 1). Alkaline phosphaste activity and osteopontin decreased (p-value <0.05) with ovariectomy, while drinking vitamin C solution increased (p-value <0.05) alkaline phosphatase activity and osteopontin. In contrast, the plasma TRAP and urinary DPD increased (p-value <0.05) in ovariectomized rats compared to the sham group. Drinking vitamin C substantially (p-value <0.05) reduced the elevated TRAP in the plasma and decreased urinary DPD excretion.

Ovariectomy reduced (p-value <0.05) femoral and 5th lumbar density, bone strength, bone ash, and bone Ca and Mg concentrations. Vitamin C increased (p-value <0.05) femoral density without affecting (p-value >0.1) femoral strength, ash, and femoral Ca and Mg concentrations compared to ovariectomized rats. However, the vitamin C treated group had a significant (p-value <0.05) improvement in 5th lumbar density, strength, bone ash, bone Ca and Mg concentrations. Despite significant (p-value <0.05) increases in 5th lumbar strength, bone density, bone ash, and bone Ca and Mg concentrations with drinking vitamin C, the variables monitored were significantly (p-value <0.05) lower than those of the sham group (Table 1).

Discussion

As expected, food and water intake were similar across treatments because all rats were pair-fed to the mean food intake of the sham-control group. Similar to our previous study, increased final weight with ovariectomy is possibly related to metabolic changes and obesity in these animals [43]. Increased risk of obesity has been observed among postmenopausal women [46]. Although, body composition was not evaluated in this study, similar observation of increased body weight with ovariectomy has been observed previously [43, 47].

Fluid intake was similar across treatment groups and all rats consumed similar amount of sugar from drinking water. On an average, the vitamin C treated group consumed 22 mg of vitamin C daily. The amount of vitamin C consumed was 68 mg/Kg body weight. The current U.S. Recommended Daily Allowance (RDA) for vitamin C is between 100–120 mg per day for adults [48]. Adjusting for body surface area, the Human Equivalent Dose (HED) dosage of vitamin C is 12.8 mg/Kg body weight as discussed previously [49]. Therefore, the potential benefit effects of vitamin C on bone quality observed in this study was at a pharmacological dosage.

Oxidative stress is an inability of cellular enzymatic and non-enzymatic antioxidant defense systems to detoxify free radicals efficiently [50]. Our present study shows ovariectomy lowered antioxidant capacity in Sprague-Dawley rats. Results from our study support the notion that gonadal hormones are required for antioxidant capacity to be within normal range. This conclusion is stemmed from two studies indicating that bone remodeling in postmenopausal women were affected by antioxidant enzymes [51, 52]. In this study, drinking vitamin C solutions normalized antioxidant capacity, which suggests that vitamin C supplementation or consuming foods rich in vitamin C can improve antioxidant defense mechanisms despite lacking gonadal function. Potential benefit of vitamin C on bone quality may be mediated by reducing peroxidation as observed in one human study supplemented with 250 mg of vitamin C twice daily as it significantly lowered serum malondialdehyde concentration [53].

It is established that loss of estrogen on bone remodeling is the reason for decreased bone strength observed in the OVX group [43]. Further, reduced bone quality observed in the femurs and the 5th lumbar is related to reduced bone mineral contents and lower bone density, pre-

disposing the bone to micro-architectural *deterioration* and reduced bone strength similar to previous studies [54, 55]. The significant improvement in femoral density with vitamin C treatment suggests that vitamin C plays a significant role in bone quality during postmenopausal states.

Although femoral bone is mostly of a cortical type and response to dietary treatment may take longer than the trabecular bones seen in the 5th lumbar, vitamin C treatment increased bone density, bone strength, and bone Ca and Mg concentration in the 5th lumbar. In our study, mode of action of vitamin C related to bone quality was observed from increased indices of bone formation like alkaline phosphatase and osteopontin and reduced markers of bone resorption like bone specific TRAP and urinary DPD.

It is important to note that in this study we allowed bone loss to occur for 60 days before vitamin C intervention took place. Therefore, this study suggests that vitamin C plays a significant role in modulating indices of bone formation and markers of bone resorption in bones that are more active like trabecular bones. However, there are limitations in our study. In this study we did not have an additional sham control group drinking vitamin C solution. Another limita-

Table 1. The effects of vitamin C supplementation on antioxidant status, alkaline phosphatse, bone tartrate resistant acid phosphatase, urinary deoxypyridinoline, osteopontin, bone strength, bone calcium and magnesium status in ovariectomized rats.

	Sham	OVX	OVX + vitamin C	
Weight, food and water				
Initial body weight (g)	186 ± 13	186 ± 10	188 ± 10	
Final body weight (g)		275 ± 10^{b}	319 ± 10^{a}	323 ± 11^{a}
Food intake (g)	14 ± 0.43	13 ± 0.37	14 ± 0.43	
Water intake (ml)	21± 3		22 ± 3	22 ± 3
Plasma				
Antioxidant capacity *(mM)*	1.33 ± 0.04^{a}	1.11 ± 0.04^{b}	1.32± 0.04^{a}	
ALP (U/L)	80 ± 3^{a}		45 ± 2^{c}	67 ± 3^{b}
Osteopontin (pg/ml)	55.93 ± 4^{c}		25.15 ± 4^{a}	44.23 ±^{b}
TRAP (U/ml)		2.94 ± 0.1^{c}	6.15 ± 0.1^{a}	4.23 ± 0.04^{b}
Femoral quality				
Density (g/cm^{3})	1.504 ± 0.005^{a}	1.420 ± 0.005^{c}		1.460 ± 0.006^{b}
Strength *(Kg)*		23^{a}	20^{b}	20^{b}
Ash (%)	68^{a}	64^{b}	64^{b}	
Calcium (mg/g DM)	404.00 ± 21^{a}	282.73 ± 21^{b}	320.99 ± 22^{b}	
Magnesium (mg/g DM)	43.25 ± 1.45^{a}	37.37 ± 1.45^{b}		39.08 ± 1.05^{b}
5th lumbar quality				
Density (g/cm^{3})	1.293 ± 0.008^{a}	1.223 ± 0.008^{c}		1.238 ± 0.008^{b}
Strength *(Kg)*		22^{a}	16^{c}	18^{b}
Ash (%)	60^{a}	50^{c}	53^{b}	
Calcium (mg/g DM)	294 ± 11^{a}	259 ± 11^{b}	302 ± 11^{a}	
Magnesium (mg/g DM)	23.85 ± 0.4^{a}	21.15 ± 0.04^{b}	20.88 ± 0.04^{b}	
Urinary				
DPD *(nmol/mg creatinine)*	0.42 ± 0.12^{c}	1.45 ± 0.16^{a}	0.99 ± 0.12^{b}	

$^{a\text{-}c}$Means with unlike superscripts within a row are significantly different ($P < 0.05$).
Number of rats per treatment group, $n = 14$; Alkaline phosphatase (ALP); Bone specific tartrate resistant acid phosphatase (TRAP); Dry matter (DM); deoxypyridinoline (DPD); Gram (g); Kilogram (Kg); Milligram (mg); Centimeter (cm).

tion was providing one dosage of vitamin C to ovariectomized rats. A study of longer duration should be conducted to determine if vitamin C supplementation longer than 60 days will have a similar efficacy on cortical bones as it showed on trabecular bones. Furthermore, a study should be conducted to address if bone loss in ovariectomized rats can be prevented when providing vitamin C supplementation is given immediately after ovariectomy.

In conclusion, the potential benefit of vitamin C observed in this study could possibly be related to improved antioxidant capacity and possibly to *bone hemodynamic* responses to vitamin C. Further, this study suggest that imbalance between antioxidant and oxidant status affect bone quality and antioxidant-based diet may positively affect bone quality and may be an approach to osteoporosis prevention and treatment.

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Author's contribution

Kimberly Strong, Niaz Deyhim, Sarvenaz Vandyousefi, **Alexis Stamatikos** coordinated and implemented the study, conducted laboratory analysis, and helped to draft the manuscript. Kimberly Strong, Niaz Deyhim, Sarvenaz Vandyousefi conducted laboratory analysis. Kimberly Strong drafted the manuscript. Bahram Faraji designed the study and drafted the manuscript. Farzad Deyhim designed, coordinated the study, performed the statistical analyses, and helped to draft the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest.

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