

Research Article

Inhibition of Bone Loss by *Cissus quadrangularis* in Mice: A Preliminary Report

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Women drastically lose bone during and after menopause leading to osteoporosis, a disease characterized by low bone mass increasing the risk of fractures with minor trauma. Existing therapies mainly reduce bone resorption, however, all existing drugs have severe side effects. Recently, the focus is to identify alternative medicines that can prevent and treat osteoporosis with minimal or no side effects. We used *Cissus quadrangularis* (CQ), a medicinal herb, to determine its effects on bone loss after ovariectomy in C57BL/6 mice. Two-month old mice were either sham operated or ovariectomized and fed CQ diet. After eleven weeks, mice were sacrificed and the long bones scanned using pQCT and μ CT. In the distal femoral metaphysis, femoral diaphysis, and proximal tibia, control mice had decreased cancellous and cortical bone, while CQ-fed mice showed no significant differences in the trabecular number, thickness, and connectivity density, between Sham and OVX mice, except for cortical bone mineral content in the proximal tibia. There were no changes in the bone at the tibio-fibular junction between groups. We conclude that CQ effectively inhibited bone loss in the cancellous and cortical bones of femur and proximal tibia in these mice.

1. Introduction

Osteoporosis is a disease associated with aging that causes fragility of bones making them susceptible to fractures with minor trauma. Bone is a dynamic organ that undergoes lifelong changes by bone remodeling using specialized cells and is the predominant process after attaining peak bone mass around the third decade. Remodeling is an essential process for maintaining the skeleton by repairing any damaged portions and removal of old bone as well as for discharging calcium and phosphorus from bone stores to maintain ionic homeostasis in the body. An imbalance in the process of bone remodeling where there is increased bone resorption alone or in combination with decreased bone formation results in net loss of bone. After attaining peak bone mass during the third decade, humans start losing bones at the rate of 0.6 to 1% every year for the rest of their lives. In case of women, during menopausal age, there is drastic loss of bone.

Treating and/or preventing bone loss can be focused on overall reduction of bone resorption and/or increasing bone formation. Currently, there are several different groups of agents that are used to treat and prevent osteoporosis. But the risks of side effects caused by these drugs are severe [1–4]. This has, recently, led to the search for alternative medicines to treat and prevent osteoporosis.

Different cultures around the world have used herbs for thousands of years to treat several health conditions. One of the herbs that have shown beneficial effects on bone belongs to the *Cissus* family of plants. *Cissus quadrangularis* (CQ) is a medicinal herb used in Siddha and Ayurvedic medicine since ancient times in Asia, as a general tonic and analgesic, especially for bone fracture healing [5]. Recently, CQ has been linked to several health benefits such as antiobesity [6], reduction of proinflammatory cytokines [7], anti-inflammatory [8], antioxidant [9], antigluco-corticoid [10], and antidiabetic properties [11].

As early as in the 1960's, CQ was used to determine its beneficial effects on bone fracture healing in young rats and it has been reported that CQ significantly enhances fracture healing process [12]. The study has further demonstrated that in the presence of CQ, bone mineralization takes place much earlier, when compared to that seen in its absence [13]. During bone mineralization, accumulation of mucopolysaccharides precedes the actual mineralization process and CQ increased mucopolysaccharides at the site of fracture [14]. Udupa and Prasad [15] have reported that CQ hastens fractures by reducing the total convalescent period by 33% in experimental animals when compared to those of the controls. CQ also increased calcium uptake and mechanical properties of bone in rats when compared to that of the controls [15]. More recently, Shirwaikar et al. [16] have demonstrated that the mechanical strength of bones in ovariectomized rats increased, significantly, in the long bones and lumbar vertebra. Petroleum ether extracts of CQ stimulated osteoblastogenesis and mineralization in bone marrow mesenchymal cells and murine osteoblastic cell lines [17, 18]. In the present study, we determined the effects of CQ on postmenopausal bone loss in the long bones of female mice. We tested the bones using peripheral quantitative computed tomography (pQCT) and microcomputed tomography (μ CT). With pQCT, we determined the effects of CQ on the two envelopes (periosteal and endocortical) and the BMD and BMC measurements, while with μ CT, we determined the microarchitecture of the cancellous bone in the distal femoral metaphysis. Both techniques give different measurements and they complement each other. We also tested some bone biochemical markers and proinflammatory cytokines in the serum.

2. Materials and Methods

2.1. Animals. Weanling C57BL/6 female mice were obtained from Jackson Laboratory (Bar Harbor, ME) and maintained in our laboratory animal facility. When mice were eight weeks of age, they were either sham operated or ovariectomized. After one month, mice were divided into the following groups: Group (1) Lab chow sham (LC S), ($n = 10$); Group (2) Lab chow ovariectomy (LC O), ($n = 11$); Group (3) *Cissus quadrangularis* sham (CQ S), ($n = 11$); Group (4) CQ ovariectomy (CQ O), ($n = 11$) and fed the respective diets. Mice were maintained in the dietary regimens for eleven weeks and sacrificed. CQ was purchased from 1fast400 (Northborough, MA) in powder form and mixed with modified AIN-93 diet at a concentration of 500 mg/kg b wt. Mice were weighed regularly. Blood was collected by retro-orbital bleeding from anesthetized mice and tibia and femur were removed and stored for pQCT and μ CT densitometry. All animal procedures were done according to the UT Health Science Center at San Antonio IACUC guidelines.

2.2. Measurement of Body and Organ Weights. Mice were weight matched at the beginning of the treatment using a CS 200 (Ohaus, Pine Brook, NJ, USA) balance. At

the time of sacrifice, body weight was recorded. Uterus, peritoneal adipose tissue, liver, spleen, and kidneys were carefully dissected out and weighed using a Mettler Balance (Columbus, OH, USA).

2.3. Collection of Blood Serum Biochemical Markers, Proinflammatory Cytokine Assays and Leptin. Blood was collected by retro-orbital bleeding from anesthetized mice and serum was obtained by centrifugation at $300 \times g$ for 15 minutes at 4°C . Procollagen type 1 amino terminal propeptide (PINP), Tartrate Resistant Acid Phosphatase (Trap5b) and ALP levels in the serum were measured using Rat/Mouse PINP EIA kit (IDS, Fountain Hills, CA, USA), Mouse Trap EIA kit (IDS, Fountain Hills, CA, USA), and Quantichrome ALP assay kit (Bioassay systems, Hayward, CA, USA), respectively. Osteocalcin was measured using IRMA kit (Alpco Diagnostics, Salem, NH, USA). TNF- α , IL-1, and IL-6 were assayed using OptiEIA kits (BD Biosciences Pharmingen, San Diego, CA, USA). Serum leptin was measured using ELISA kit (Diagnostic systems laboratory, Webster, TX, USA).

2.4. Peripheral Quantitative Computerized Tomography Densitometry (pQCT). Cortical and cancellous bones of distal femoral metaphysis (DFM), proximal tibial metaphysis (PTM), and pure cortical bone at the femoral diaphysis (FD) and tibia fibula junction (TF) were analyzed by pQCT densitometry, using an XCT research M system (Norland Stratec, Birkenfeld, Germany) as described previously [19, 20]. In the proximal tibial metaphysis (PTM) and distal femoral metaphysis (DFM), both cancellous and cortical bone surrounding the cancellous bone were scanned and analyzed. At the PTM and DFM, 5 slices were scanned including the growth plate and one slice, 1 mm distal to the knee joint (PTM) and 1 mm proximal to the knee joint (DFM) was analyzed. The following parameters were determined for both sites: cancellous bone mineral content (Cn BMC), cancellous bone mineral density (Cn BMD), cortical bone area (Ct Ar), cortical BMC (Ct BMC), cortical BMD (Ct BMD), cortical thickness (Ct Th), periosteal perimeter (Peri PM), and endocortical perimeter (Endo PM).

One slice was scanned at the FD (mid-diaphysis) and at the TF junction for measuring pure cortical bone and the following parameters determined: Ct B Ar, Ct BMC, Ct BMD, Ct Th, Peri PM, and Endo PM.

2.5. Micro Computerized Tomography (μ CT). Scans of the DFM was done using a high-resolution Xradia μ CT 200 scanner (Xradia, Inc. Concord, CA) at 20 microns. All images were acquired using standard parameters, X-ray source of 90 KV, power of 8.0 W and current of $4.0 \mu\text{A}$. Each scan consisted of 181 slices with an exposure time of 30 seconds per slice. The scans were analyzed using Tri/3D Bon (Ratoc System Engineering Co., Ltd., Tokyo, Japan) for the parameters including total volume (TV), bone volume (BV), BV/TV, trabecular number (Tb N), trabecular thickness (Tb Th), trabecular separation (Tb S) and connectivity density (Conn Den).

TABLE 1: Effects of *Cissus quadrangularis* on the body weight and weight of different organs of female ovariectomized C57BL/6 mice.

Parameters/Groups	LC Sham	LC OVX	% difference (LC Sham versus LC OVX)	CQ Sham	CQ OVX	% difference (CQ Sham versus CQ OVX)
Initial body weight (g)	19.17 ± 0.42	19.26 ± 0.41		19.15 ± 0.45	19.25 ± 0.34	
Final body weight (g)	22.86 ± 0.86 ^a	27.42 ± 1.19	20↑	26.41 ± 0.65 ^b	29.73 ± 0.66 ^a	12↑
Adipose tissue weight (g)	0.45 ± 0.11 ^a	1.14 ± 0.18	153↑	1.12 ± 0.09 ^b	1.50 ± 0.10	34*↑
Uterus weight (g)	0.142 ± 0.011 ^a	0.072 ± 0.011	51↓	0.144 ± 0.015	0.043 ± 0.008 ^c	30*↓
Liver weight (g)	1.088 ± 0.043 ^a	1.318 ± 0.050	21↑	1.211 ± 0.043	1.330 ± 0.050	
Spleen weight (g)	0.086 ± 0.006 ^a	0.150 ± 0.018	74↑	0.116 ± 0.011	0.096 ± 0.005 ^d	
Kidney weight (g)	0.267 ± 0.012	0.303 ± 0.015		0.275 ± 0.014	0.253 ± 0.009	

Data are Mean ± SE. ^a*P* < 0.05 versus LC O; ^b*P* < 0.05 versus LC S; ^c*P* < 0.05 versus CQ S; ^d* = *P* < 0.05 versus LC; ↑ = increase; ↓ = decrease.

2.6. Statistical Analysis. Results are expressed as Mean ± SE. Data was analyzed with one-way ANOVA and unpaired *t*-test using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA, USA). *P* < 0.05 was considered to be significant. Newman-Keuls multiple comparison test was used to analyze the differences between groups for significance.

3. Results

3.1. Effects on Body Weights and Organ Weights

Effects of CQ. Body weight (16%) and adipose tissue weight (148%) significantly increased in CQ S mice, when compared to that of LC S mice (Table 1). Uterus weight did not change between CQ S and LC S mice (Table 1). Body weight (8%) of CQ O mice increased significantly when compared to that of LC O mice. Liver weight increased in CQ S mice but this was not statistically significant when compared to that of LC S mice (Table 1). No significant changes were seen in the weights of the spleen and kidney (Table 1).

Effects of Ovariectomy

Lab chow groups. Body weight (20%) and adipose tissue weight (153%) increased significantly in LC O mice, while uterus weight (51%) decreased significantly in LC O mice, when compared to that of LC S mice (Table 1). Liver and spleen weights significantly increased in the LC O mice by 21% and 74% respectively, when compared to that of LC S mice (Table 1).

CQ groups. Body weight significantly increased in the CQ O group although no significant differences were observed in the adipose tissue weight between CQ S and CQ O mice (Table 1). Uterus weight (30%) decreased significantly in CQ O mice, when compared to that of CQ S mice (Table 1). No significant differences were observed in the liver, spleen and kidneys weights (Table 1). Uterus was carefully examined for any changes and no visible changes were seen.

3.2. Effects on Serum Bone Biochemical Parameters, Proinflammatory Cytokines, and Leptin

3.2.1. Bone Biochemical Markers

Effects of CQ

Serum P1NP levels (45%) decreased significantly in CQ S mice, when compared to that of LC S mice (Table 2). No significant differences were observed between CQ and LC mice with respect to Trab5b and ALP levels (Table 2).

Effects of Ovariectomy

Lab chow groups. P1NP (21%) and ALP (48%) levels decreased significantly in LC O mice, when compared to those of LC S mice (Table 2). Trap5b levels were not significantly different between LC S and LC O mice (Table 2).

CQ groups. No significant differences were observed in serum P1NP, ALP and Trap5b levels between CQ S and CQ O mice (Table 2).

3.2.2. Proinflammatory Cytokines

Effects of CQ. TNF- α (48%), IL-1 β (84%) and IL-6 (53%) levels decreased in the CQ S mice when compared to those of LC S mice (Table 2).

Effects of Ovariectomy

Lab chow groups. Although there was an increase in the cytokines that were measured in the LC O mice these increases were not statistically significant between the different groups (Table 2).

CQ groups. No significant differences were observed between CQ S and CQ O mice with respect to pro-inflammatory cytokines (Table 2).

3.2.3. Leptin Levels

Effects of CQ. Leptin levels (450%) increased significantly in CQ S mice when compared to that of LC S mice (Table 2).

TABLE 2: Effects of *Cissus quadrangularis* on the Biochemical markers of bone turnover, pro-inflammatory cytokines and leptin in the serum of female ovariectomized C57BL/6 mice.

Parameters/Groups	LC Sham	LC OVX	% difference (LC Sham versus LC OVX)	CQ Sham	CQ OVX	% difference (CQ Sham versus CQ OVX)
P1NP (ng/mL)	13.23 ± 1.17 ^a	10.44 ± 0.18	21↓	7.29 ± 0.85 ^b	6.55 ± 0.57	10↓
Alkaline phosphatase (U/L)	1.95 ± 0.24 ^a	0.93 ± 0.18	48↓	1.60 ± 0.09	2.09 ± 0.57	6
Osteocalcin (ng/mL)	45.78 ± 1.11	51.82 ± 7.74		40.45 ± 2.27	34.53 ± 4.49	
Trap5b (U/L)	13.53 ± 0.87	12.39 ± 0.65		12.92 ± 0.56	13.23 ± 1.46	
IL-1β (pg/mL)	418 ± 143	580 ± 127	39↑	66 ± 8 ^b	278 ± 30 ^d	322*↑
IL-6 (pg/mL)	818 ± 152	888 ± 202	9↑	387 ± 35 ^b	547 ± 77 ^a	41↑
TNF-α (pg/mL)	1.47 ± 0.43	2.12 ± 0.45	44↑	0.76 ± 0.21	0.69 ± 0.08 ^a	13↓
Leptin (pg/mL)	169 ± 27 ^a	1100 ± 85	550↑	621 ± 115 ^b	1321 ± 147 ^c	112↑

Data are Mean ± SE. ^aP < 0.05 versus LC O; ^bP < 0.05 versus LC S; ^cP < 0.05 versus CQ S; ^dP < 0.08 versus LC O; * = P < 0.05 versus LC; ↑: increase; ↓: decrease.

Effects of Ovariectomy

Lab chow groups. Leptin levels (550%) increased in the LC O mice, when compared to that of LC S mice (Table 2).

CQ groups. Leptin levels (112%) increased significantly in the CQ O mice when compared to that of CQ S mice (Table 2).

3.3. pQCT Densitometry

3.3.1. Distal Femoral Metaphysis (DFM)

Effects of CQ. CQ S did not change any of the parameters studied when compared to those of LC S fed mice in the distal femoral metaphysis (Figure 1). But CQ O mice had significantly higher Cn BMC (8%), Cn BMD (34%), Ct BMC (32%), Ct BMD (8%), and Ct Th (30%) when compared to those of LC O mice (Figures 1(A)–1(D) and 2(A)). Endo PM (4%) decreased significantly in CQ O mice when compared to that of LC O mice (Figure 2(C)).

Effects of Ovariectomy

Lab chow groups. LC O mice had significantly less Cn BMC (34%), Cn BMD (36%), Ct BMC (36%), Ct BMD (4%), and Ct Th (26%), and increased Endo PM (6%), when compared to those of LC S mice (Figures 1(A)–1(D), 2(A) and 2(C)).

CQ groups. No significant differences were observed in Cn BMC, Cn BMD, Ct BMC, Ct BMD, and Ct Th levels between CQ S and CQ O mice (Figures 1(A)–1(D) and 2(A)). Endo PM increased in CQ O mice but this increase was not statistically significant (Figure 2(C)).

3.3.2. Femoral Diaphysis (FD)

Effects of CQ. In FD, CQ did not change any of the parameters studied, when compared to those of LC S mice (Figures 1(C), 1(D) and 2(A)–2(C)). CQ O mice had significantly higher Ct Th, when compared to those of LC O mice (Figure 2(A)).

Effects of Ovariectomy

Lab chow groups. LC O mice had significantly less Ct BMC (19%), Ct BMD (8%), Ct Th (15%), and increased Endo PM (5%), when compared to those of LC S mice (Figures 1(C), 1(D) and 2(A) and 2(C)).

CQ groups. No significant differences were observed in the Ct BMC, Ct BMD, and Ct Th levels between CQ S and CQ O mice (Figures 1(A)–1(D) and 2(A)). Endo PM increased in CQ O mice but this increase was not statistically significant (Figure 2(C)).

3.3.3. Proximal Tibial Metaphysis (PTM)

Effects of CQ. CQ did not change any of the parameters studied when compared to those of LC S fed mice in the proximal tibial metaphysis (Figures 3(A)–3(D) and 4(A)–4(C)). CQ O mice had significantly higher Ct BMC (48%), Ct BMD (2%), and Ct Th (42%) when compared to those of LC O mice (Figures 3(C)–3(D) and 4(A)). Endo PM decreased in CQ O mice when compared to that of LC O mice, but this decrease was not statistically significant (Figure 4(C)).

Effects of Ovariectomy

Lab chow groups. LC O mice had significantly less Cn BMC (32%), Cn BMD (21%), Ct BMC (59%), Ct BMD (2%), Ct Th (65%), Peri PM (3%) and increased Endo PM (7%), when compared to those of LC S mice (Figures 3(A)–3(D) and 4(A)–4(C)).

CQ groups. Although Cn BMC and Cn BMD decreased in CQ O mice, when compared to those of CQ S mice, these decreases were not statistically significant (Figures 3(A) and 3(B)). Ct BMC (34%), Ct BMD (3%), and Ct Th (30%) levels significantly decreased in CQ O mice when compared to those of CQ S mice (Figures 3(C) and 3(D) and Figure 4(a)). Endo PM increased in CQ O mice but this increase was not statistically significant, when compared to that of LC O (Figure 4(C)).

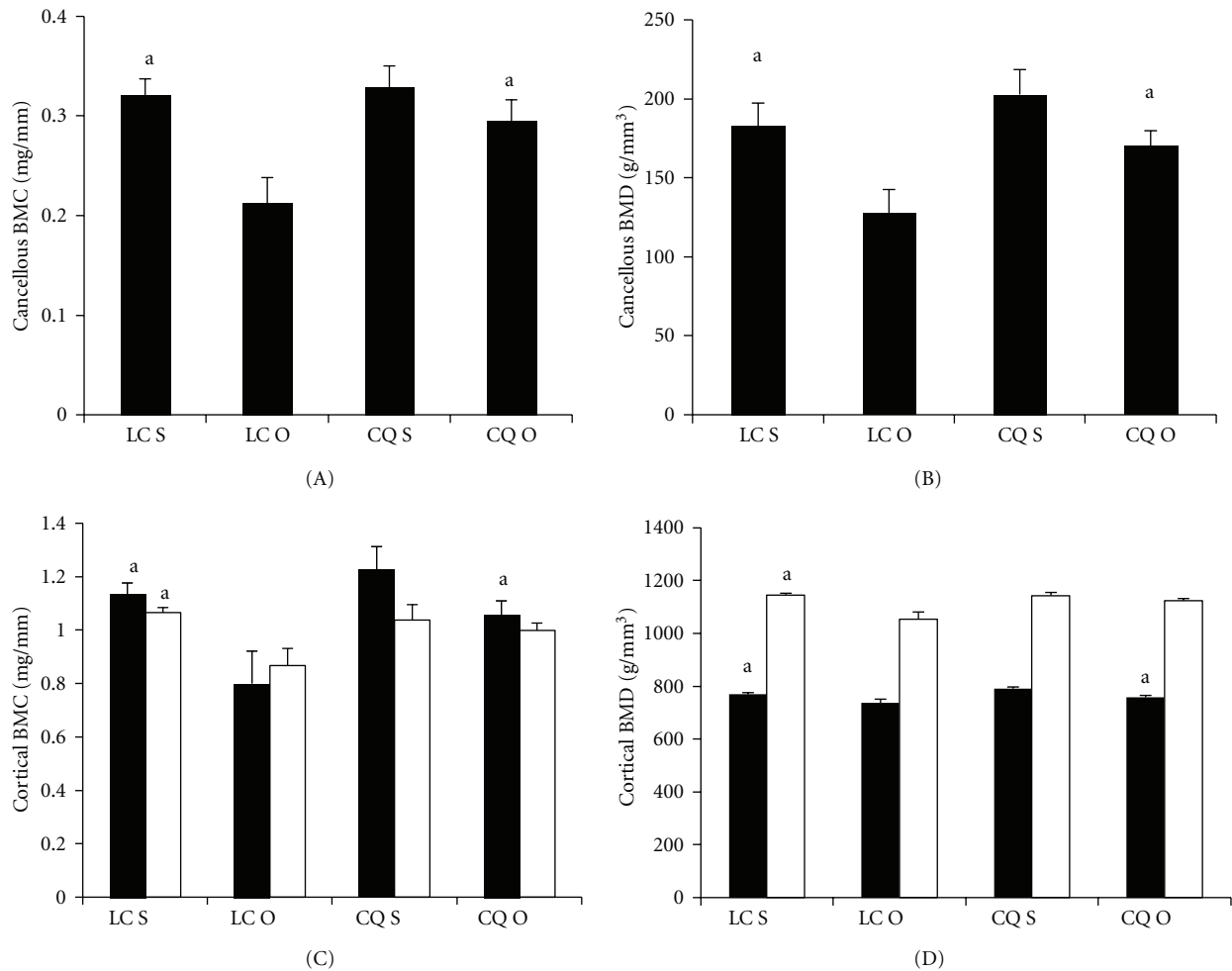


FIGURE 1: Effects of *Cissus quadrangularis* on the cancellous and cortical bone parameters of the distal femoral metaphysis and femoral diaphysis of C57Bl/6 mice after ovariectomy using pQCT. Data are Mean \pm SE. (A) cancellous BMC; (B) cancellous BMD; (C) cortical BMC; (D); Cortical BMD. Black bars represent distal femoral metaphysis; white bars represent femoral diaphysis. LC S: lab chow sham; LC O: lab chow ovariectomy; CQ S: *Cissus quadrangularis* sham; CQ O: *Cissus quadrangularis* ovariectomy. ^a $P < 0.05$ versus LC O.

3.3.4. Tibia Fibular Junction (TF)

Effects of CQ. CQ did not change any of the parameters studied when compared to those of LC fed mice in the tibia fibular junction of sham and ovariectomized mice, except in the Endo PM which significantly decreased in CQ S, when compared to that of LC S mice (Figures 3(C), 3(D), and 4(A)–4(C)).

Effects of Ovariectomy

Lab chow groups. LC O mice had significantly less Ct BMC (13%), Ct BMD (6%) and Ct Th (11%) when compared to those of LC S mice (Figures 3(C), 3(D), and 4(A)). LC O mice showed increased Endo PM (–2%), but this increase was not statistically significant (Figure 4(C)).

CQ groups. No significant differences were observed in the Ct BMC, Ct BMD, and Ct Th levels between CQ S and CQ O mice (Figures 3(C), 3(D), and 4(A)). Endo PM increased in CQ O mice but this increase was not statistically significant (Figure 4(C)).

3.4. μ CT Densitometry

Effects of CQ

CQ treatment did not significantly change the trabecular number, trabecular thickness, connectivity density, and BV/TV (Figure 5) but trabecular separation (65%) significantly decreased in CQ S mice when compared to that of LC S mice (Figure 5). There was 45% increase in Tb N and 28% increase in connectivity density in CQ S mice but these increases were not statistically significant, when compared to those of LC S mice (Figure 5). CQ O mice had significantly higher trabecular number (353%) and connectivity density (363%) and lower trabecular separation (28%), when compared to those of LC O mice (Figure 5).

Effects of Ovariectomy

Lab Chow Groups. In LC O mice, Tb N (65%); connectivity density (92%) and BV/TV (69%) decreased significantly, when compared to those of LC S mice

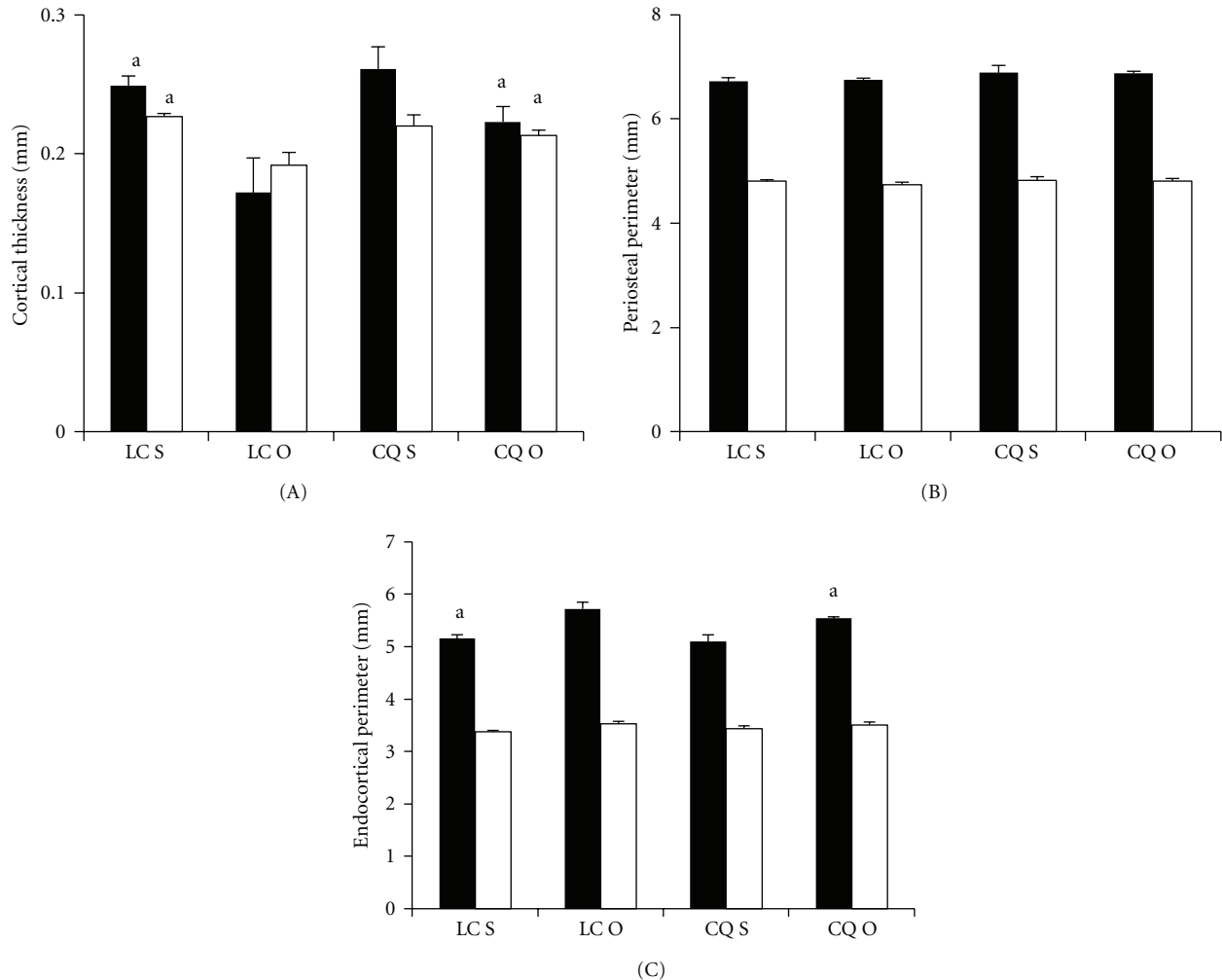


FIGURE 2: Effects of *Cissus quadrangularis* on the cortical bone thickness and perimeters of the distal femoral metaphysis and femoral diaphysis of C57Bl/6 mice after ovariectomy using pQCT. Data are Mean \pm SE. (A) Cortical thickness; (B) periosteal perimeter; (C) Endocortical perimeter. Black bars represent distal femoral metaphysis; white bars represent femoral diaphysis. LC S: lab chow sham; LC O: lab chow ovariectomy; CQ S: *Cissus quadrangularis* sham; CQ O: *Cissus quadrangularis* ovariectomy. ^a $P < 0.05$ versus LC O.

(Figure 5). Although Tb Th decreased by 20%, this decrease was not statistically significant.

CQ groups. In CQ O mice, Tb Sp (73%) significantly increased, and BV/TV (48%) significantly decreased when compared to those of CQ S mice (Figure 5).

4. Discussion

Cissus quadrangularis belongs to the vitaceae family and is found in South East Asia where it is edible and is used as a vegetable. This plant has been used from ancient times to enhance fracture healing and has several other health benefits including antiinflammatory [8], antiglucocorticoid [10], antidiabetic [11] antibacterial [5, 21], and antioxidant properties [9]. This plant has triterpenoids [22, 23], steroids [22, 24] stilbenes [25], flavanoids [13], lipids [13], and several catalpols [13]. Slowly, interest in natural products for the treatment and prevention of disease is growing in the quest to minimize severe side effects that existing drugs

can cause and WHO has endorsed the safe and effective use of such medicines [26]. We studied the effects of CQ-dried powder (stems and leaves) in an animal model for postmenopausal bone loss. Although CQ by itself did not increase bone mass we observed that it decreased bone loss in the distal femoral metaphysis and proximal tibial metaphysis regions of the long bones that have both cancellous and cortical bones. Loss of cancellous bone from these regions is typical after ovariectomy and menopause, mainly because endocortical resorption is stimulated. Bone protection in the distal femur and proximal tibia is mainly due to decreased bone resorption at the endocortical bone surface and preservation of trabecular microarchitecture. Cancellous bone at the femur also showed higher trabecular number, connectivity density and BV/TV in CQ O mice suggesting that bone resorption is decreased considerably. This is supported by the well preserved trabecular morphology in the CQ O mice (Figure 5) and data is in line with reports using CQ ethanol extracts. When young Wistar rats

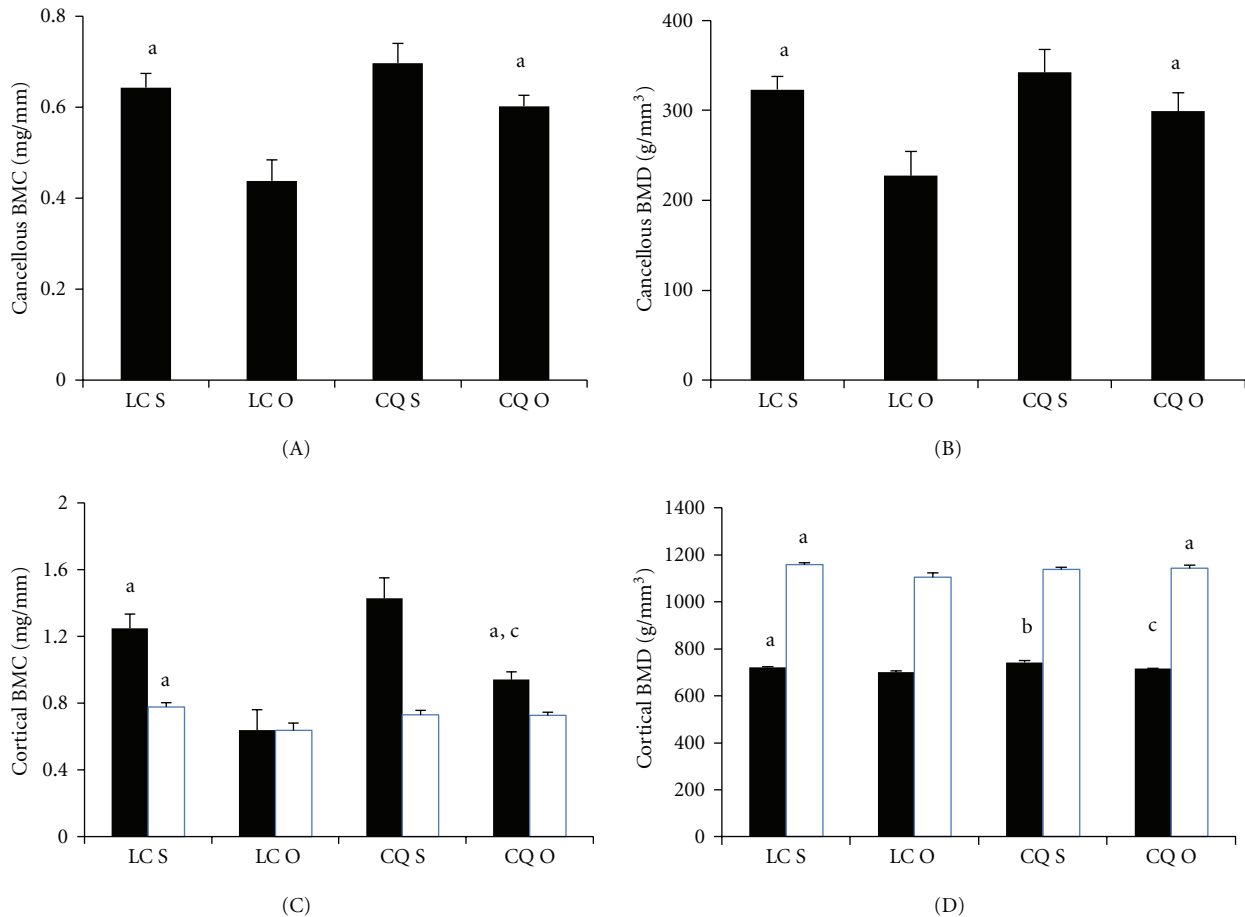


FIGURE 3: Effects of *Cissus quadrangularis* on the cancellous and cortical bone parameters of the proximal tibial metaphysis and tibia fibula junction of C57Bl/6 mice after ovariectomy using pQCT. Data are Mean \pm SE. (A) cancellous BMC; (B) cancellous BMD; (C) cortical BMC; (D) cortical BMD. Black bars represent distal femoral metaphysis; White bars represent femoral diaphysis. LC S: lab chow sham; LC O: lab chow ovariectomy; CQ S: *Cissus quadrangularis* sham; CQ O: *Cissus quadrangularis* ovariectomy. ^a $P < 0.05$ versus LC O; ^b $P < 0.05$ versus CQ S; ^c $P < 0.05$ versus LC S.

were fed CQ ethanol extracts, after ovariectomy for three months, there was restoration of architecture and increased biomechanical properties in the femur [16]. However, this is the first report to show the effects of CQ on bone using densitometric morphometric analyses including actual BMD and BMC values for the different bone sites. Moreover, we have tested several bone sites (femoral and tibial metaphysis as well as femoral and tibial diaphysis) as it is well known that different bone sites do not react to treatment regimens in the same manner [27].

We measured levels of several serum biochemical markers to determine the influence of CQ on the state of bone turnover in these mice. P1NP and ALP were decreased in LC O mice as expected. With CQ treatment P1NP was decreased in both sham and OVX mice which suggests that CQ maybe altering the processing of procollagen to collagen. However, there was no difference in P1NP levels between CQ S and CQ O groups. ALP that was measured is the total ALP which is an indirect marker for increased osteoblast activity, and with CQ treatment especially in the OVX group, there was increased activity which is in line with reports that show increased ALP activity in bone marrow cells in rats treated

with CQ [18]. We also measured osteocalcin as a direct marker for osteoblast activity and Trap5b as a marker for osteoclast activity. We observed that there were no statistical differences between the levels, of either of these markers in any of the groups studied; therefore, CQ does not alter the levels of these markers. Based on these results, we suggest that CQ does not change the bone turnover rate in these mice.

The mechanism(s) by which CQ inhibits bone loss is yet to be fully studied. As a preliminary investigation, we measured a few proinflammatory cytokines. Certain proinflammatory cytokines like TNF- α , IL-1, IL-6, and IL-11 play a critical role in the bone remodeling process [28, 29] mainly by activation of osteoclasts and increasing bone resorption [30–32]. While IL-1 activates NF- κ B and MAPKs through TRAF-6, it may also induce PGE₂ and the expression of RANKL in osteoblast [28]. IL-6 is produced by osteoblasts and stimulate the formation of osteoclasts [33]. Interestingly, IL-6 knockout mice do not lose cancellous bone after ovariectomy [33]. In our study, significant decreases in the serum levels of IL-1 (84%), IL-6 (53%), and TNF- α (40%) were observed with CQ diet. Even in CQ O mice the levels of IL-6 and TNF- α were significantly lower than those of

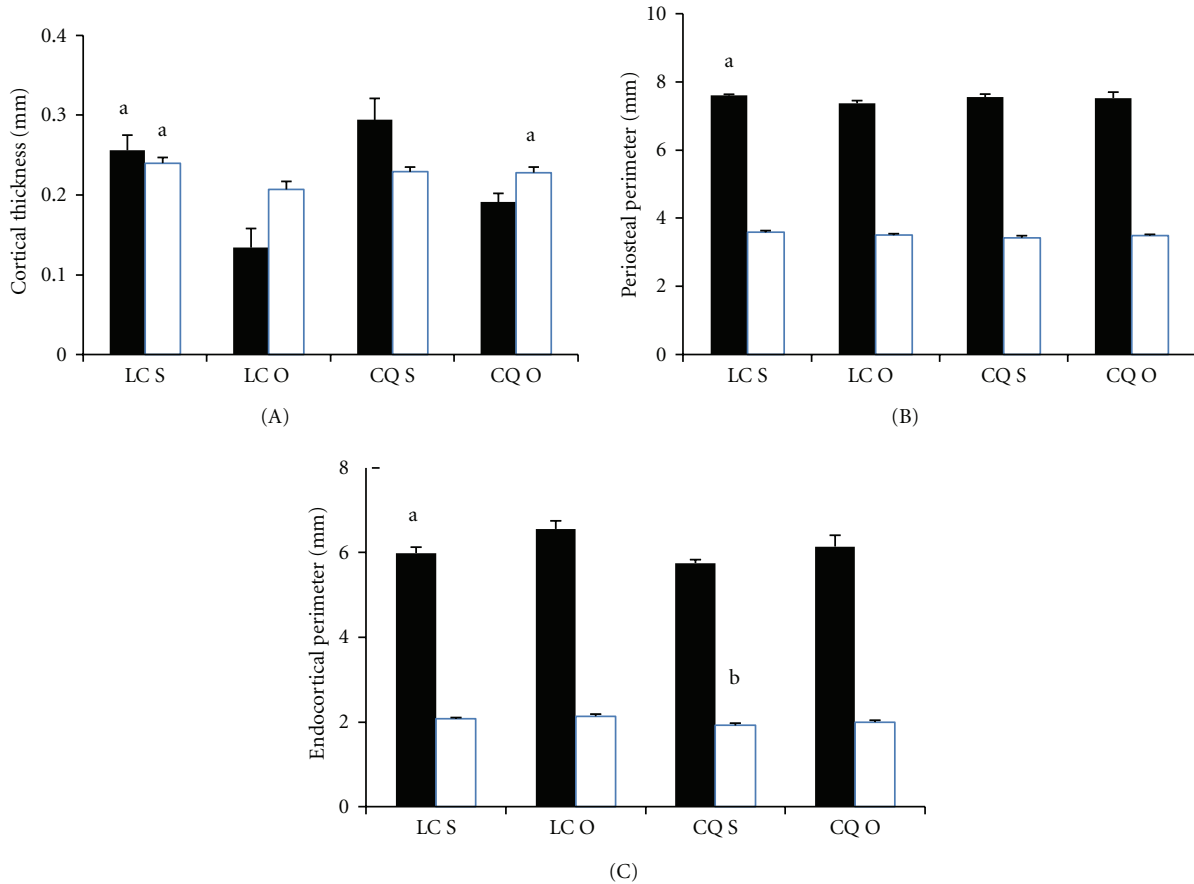


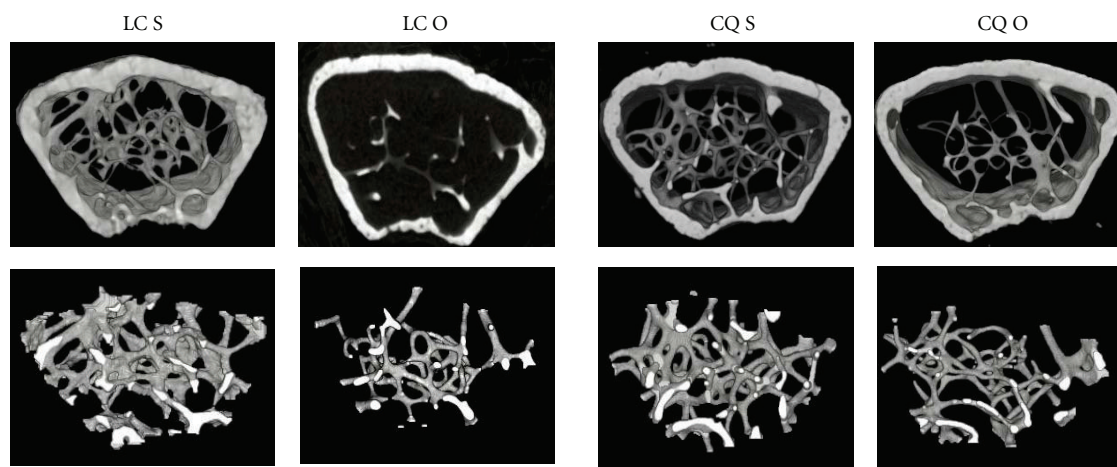
FIGURE 4: Effects of *Cissus quadrangularis* on the cortical bone thickness and perimeters of the proximal tibial metaphysis and tibia fibula junction of C57Bl/6 mice after ovariectomy using pQCT. Data are Mean \pm SE. (A) Cortical thickness; (B) periosteal perimeter; (C) Endocortical perimeter. Black bars represent proximal tibial metaphysis; white bars represent tibia fibula junction. LC S: lab chow sham; LC O: lab chow ovariectomy; CQ S: *Cissus quadrangularis* sham; CQ O: *Cissus quadrangularis* ovariectomy. ^a $P < 0.05$ versus LC O; ^b $P < 0.05$ versus LC S.

the LC O mice and IL-1 levels decreased by 52%. Our results clearly show that CQ alters proinflammatory cytokines and maybe one of the major pathways used to reduce bone resorption—the characteristic response to ovariectomy.

Based on the literature, CQ was also used to induce weight loss [6, 34], but in our mice, we noticed that there was increased body weight and peritoneal fat. Both the studies that reported weight reducing benefits of CQ were in obese humans. The major difference between these reports and our study is that they were using already obese patients while the mice in our study were not obese to begin with. So, it may be that a stimulus (obesity-linked proteins) is required for CQ to reduce fat mass or the increase in fat mass is specific to mice and may not affect humans. We measured the levels of leptin, a hormone derived from the adipose tissue which plays a key role in regulating energy intake and expenditure and also influences bone formation as well as bone resorption [35]. At lower than physiological concentrations leptin stimulates bone formation and probably can induce apoptosis of osteoclasts [36]. We were not surprised to find increased circulating levels of leptin and there was bone loss in the LC O mice, but CQ could protect bone in the long bones suggesting that

CQ blocks the bone resorption action of leptin. It will be interesting to see if CQ has any influence on the adipocytes found in the bone marrow. If CQ reduces the adipocytes in the bone marrow then the bone resorbing properties of leptin will be reduced as local leptin concentrations produced by increased adipocytes in the bone marrow will increase bone loss [37]. Therefore, leptin CQ interaction needs to be further studied to determine the mechanism by which CQ beneficially influences bone through leptin.

CQ may primarily attenuate bone resorption in OVX mice through the downregulation of proinflammatory cytokines but it does not rule out the possibility that it may also act through other pathways. There are reports that CQ also enhances bone mineralization by accumulating mucopolysaccharides at the site of bone formation [14]. Moreover, CQ is reported to increase calcium uptake and mechanical properties of bone in rats [15]. Phytochemical analyses of CQ show the presence of high levels of calcium, vitamin C, β -carotene [38, 39], and flavanoids [25] some of these substances have established beneficial properties on bone. *In vitro* studies have shown that ethanolic extracts of CQ increased mRNA and proteins related to the bone formation pathway and IGF-I, IGF-II, and IGF binding



Parameters/groups	LC S		LC O		Difference (%) (LC S vs LCO)	CQ S		CQ O		Difference (%) (CQ S vs CQ O)
Trabecular number (1/mm)	0.396	0.086 ^a	0.079	0.021	65↓	0.576	0.044	0.358	0.032 ^a	36* ↓
Trabecular thickness (μm)	50.52	4.69	55.41	6.20		43.08	2.56	41.03	1.13	
Trabecular separation (μm)	348	42 ^a	499	96	20↑	192	35 ^b	332	13 ^{a,c}	73* ↑
Connectivity density (1/mm ³)	33.71	2.93 ^a	2.67	2.67		43.27	8.86	12.36	1.52 ^a	
BV/TV (%)	3.74	0.54 ^a	0.763	0.29	69↓	3.86	0.67	2.21	0.17 ^{a,c}	48* ↓

FIGURE 5: Effects of *Cissus quadrangularis* on the static histomorphometry parameters of the distal femoral metaphysis of C57Bl/6 mice after ovariectomy using CT. Data are Mean \pm SE. LC S: lab chow sham; LC O: lab chow ovariectomy; CQ S: *Cissus quadrangularis* sham; CQ O: *Cissus quadrangularis* ovariectomy. ^a $P < 0.05$ versus LC O; ^b $P < 0.05$ versus LC S; ^c $P < 0.05$ versus CQ S, * $P < 0.05$ versus LC. The figures are representative of the mean values that were obtained.

protein [40, 41]. More investigations are necessary to elucidate the mechanism(s) by which CQ influences bone metabolism. However, it is very encouraging to note that in studies done with CQ using very high doses (5000 mg/kg body weight) [9] have not reported any toxic side effects. In the present study, we have used only 500 mg/kg body weight of CQ and observed that the liver, spleen, and kidney weights were not altered significantly, suggesting that CQ may not have any severe side-effects.

5. Conclusions

We conclude that CQ can reduce OVX induced bone loss and it does this in the long bones in a site-specific manner with more effects on the cancellous bone of femur followed by tibia. CQ probably reduces bone resorption primarily by downregulating proinflammatory cytokines that are often increased after ovariectomy. The beneficial effects of CQ are probably due to the flavanoids present. Although the mechanism(s) by which CQ attenuates ovariectomy-induced bone loss has to be studied, CQ being an edible plant and with a history of medicinal effects, especially in healing bone fractures, may be a good supplement to existing medication for the reversal of postmenopausal bone loss.

Conflict of Interests

None of the authors have competing interests to declare.

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