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ORIGINAL ARTICLE

Modulation of bone turnover by *Cissus quadrangularis* after ovariectomy in rats

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Abstract

In women, age-related bone loss is associated with increased risk of bone fracture. Existing therapies are associated with severe side effects; thus, there is a need to find alternative medicines with less or optimal side effects. *Cissus quadrangularis* (CQ), an Ayurvedic medicine used to enhance fracture healing, was tested for its bone protective properties and studied to discern the mechanism by which it is beneficial to bone. Female Sprague Dawley rats were either sham operated or ovariectomized and were fed CQ for 3 months. Several biochemical markers, cytokines and hormones were assayed. Femur, tibia and lumbar vertebrae were subjected to pQCT and μCT densitometry. MC3T3 cells were cultured, treated with CQ and used to analyze miRNA content and subjected to qPCR for gene expression analysis related to bone metabolism. CQO rats showed protected bone mass and microarchitecture of trabecular bone in the distal femoral metaphysis and the proximal tibial metaphysis. The lumbar vertebrae, however, showed no significant changes. Serum protein expression levels of P1NP increased and Trap5b and CTX levels decreased with in vivo CQ treatment. Some influence on the anti- and pro-inflammatory markers was also observed. Significantly high level of estradiol in the CQO rats was observed. In vitro expression of a few genes related to bone metabolism showed that osteocalcin increased significantly. The other genes—collagen I expression, SPP1, BMP2, DCAT1—decreased significantly. Certain miRNA that regulate bone turnover using the BMP pathway and Wnt signaling pathways were upregulated by CQ. qPCR after acute treatment with CQ showed significantly increased levels of osteocalcin and decreased levels of Wnt/β catenin antagonist DCAT1. Overall, CQ protected the microarchitecture of the long bones from ovariectomy-induced bone loss. This may be because of decreased inflammation and modulation through the BMP and Wnt signaling pathways. We conclude that CQ is a potential therapeutic agent to treat postmenopausal osteoporosis with no side effects.

Keywords *Cissus quadrangularis* · Postmenopausal bone loss · Static histomorphometry and bone strength · Bone biochemical markers · miRNA and qPCR

Introduction

Age-related decline in bone mass is very well established in both women and men. However, women, during and after menopause, undergo bone loss due to the sudden decline of estrogen in the body [1, 2]. This phenomenon is characterized by loss of trabecular bone in the metaphyses of long bones and the lumbar vertebrae. Loss of estrogen decreases bone mass but also increases the risk of obesity and fat accumulation, diabetes, increased inflammation and other medical conditions [3].

Currently, there are several therapies to reduce bone loss including, but not limited to, hormone replacement therapy (HRT), bisphosphonates, selective estrogen receptor modulators (SERMs), monoclonal antibodies and parathyroid hormone (PTH). However, each of these has demonstrated severe side effects restricting the period of use, and once discontinued the accrued bone is lost [4]. HRT has been linked to causing ovarian and breast cancer, while bisphosphonates and SERMs' can cause brittle bones, bone necrosis, gastrointestinal problems and potentially increase the risk of certain cancers. The immunosuppressive properties of monoclonal antibodies like denosumab increase the

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risk of infections. PTH is the only FDA approved anabolic agent, however, it is also associated with several side effects such as brittle bones, hypercalcemia, headaches, nausea and arthralgia; therefore, FDA has approved PTH treatment for a maximum of 24 months [5, 6]. After withdrawal of PTH, patients start losing bone again unless they are given other medications [7].

In recent times, awareness of drug-related side effects has led people to seek alternative/ indigenous medicine. In the US, the use of alternative medicine is on the rise [8]. Depending on the ethnicity and the disease, it is estimated that 12–26% of the US population uses herbal medicine. The percentage is higher in certain regions of the country and among certain ethnic groups [9]. The use of alternative medicine may also be cost effective. One such alternative medicine used in the realm of Ayurvedic/ indigenous medicine to enhance healing of bone fracture is *Cissus quadrangularis* (CQ) [10]. CQ has been implicated in increasing deposition of mucopolysaccharides at the site of bone fracture and the mobilization of calcium to the protein matrix for enhanced mineralization [11, 12]. Recently, different extracts of CQ have been tested in ovariectomized animal models [13], stem cells [14] and osteoblast like cells [15] to determine the bone protective properties.

In the present study, we determined the effects of CQ on post-menopausal bone loss in the long bones and lumbar vertebrae and determined some information on the mechanism of action in female rats. We examined the bones using peripheral quantitative computed tomography (pQCT) and micro-computed tomography (μCT). In addition, we studied the effects on biochemical markers of bone metabolism, pro-inflammatory cytokines and hormones. We also used CQ-treated MC3T3 cells to determine changes in the expression levels of certain genes as well as microRNA (miRNA) levels related to bone metabolism.

Materials and methods

Animals

Seven-month-old female Sprague Dawley rats were either sham operated or ovariectomized and divided into the following groups: Group 1: baseline (B) ($n=10$); Group 2: control (C) ($n=13$); Group 3: age matched control Sham (CS) ($n=11$); Group 4: age matched control ovariectomy (CO) ($n=16$); Group 5: CQS (2g/kg/b wt) ($n=13$); Group 6: CQO (2g/kg/b wt) ($n=15$). They were either sham operated or ovariectomized and started on the respective diets one week after surgery. Rats were pair fed and maintained on the respective diet for 3 months. Food intake was recorded daily and body weight was recorded weekly. At the time of sacrifice, blood was collected and serum stored at − 80 ºC for the different biochemical, cytokine, and hormone analyzes. The femur, tibia and lumbar vertebrae were removed and stored for pQCT and μCT densitometry. The study was approved by the University IACUC and NIH guidelines were followed for the care and use of animals.

Diet

CQ plant was identified and collected by Dr. N. Loganathan from Tamil Nadu, India. The plants were dried and powdered. Using this powder, Harlan Teklad (Madison, WI) prepared the diets. Control diet was AIN-93 and the CQ diet consisted of AIN-93 + CQ (2g/kg/b wt). The concentration of CQ was selected based on the literature [16].

Measurement of body weight, muscle weights and organ weights

Rats were weight matched at the beginning of the experiment. Body weight was recorded weekly and at the time of sacrifice. During sacrifice, uterus, peritoneal adipose tissue, quadriceps and gastrocnemius muscles, liver, spleen and kidneys were carefully removed and weighed.

Liver and spleen pathology

After weighing, liver and spleen were collected and immersion fixed in 10% phosphate-buffered formalin. After 24 h, tissues were routinely processed through a series of alcohols and embedded in paraffin. Tissue was sectioned at 5–6 μ, and stained with hematoxylin and eosin. Slides were examined using an Olympus BX40 microscope and photographs were taken with an Olympus DP71 camera.

Serum biochemical markers and hormones

Blood was collected by cardiac puncture from anesthetized rats and serum was obtained by centrifugation at 300*g* for 15 min at 4 ˚C. Bone biochemical markers including alkaline phosphatase (ALP), procollagen type 1 amino terminal peptide (P1NP), tartrate-resistant acid phosphatase 5b (Trap5b) and c-terminal telopeptide (CTX) were measured using Rat P1NP EIA, Trap EIA and Ratlaps EIA, respectively (Immuno Diagnostics Systems, Fountain Hills, AZ). Osteocalcin was assayed using osteocalcin IRMA kit (ALPCO, Salem, NH). Other biochemical markers comprising adiponectin and leptin were measured using the respective kits (Abcam, Cambridge, MA). In addition, estradiol (Estradiol EIA kit, Cayman Chemical Company, Ann Arbor, MI), parathyroid hormone (ALPCO immunoassays, Salem, NH), insulin (Sigma-Aldrich Co, St Louis, MO) and insulin-like growth factor-I (Immuno Diagnostics, systems, Fountain Hills, AZ) were also measured.

Serum cytokine assays

Serum cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN-γ, and TNF-α) were measured, using a multiplexed bead-based immunoassay kit on a Bio-Plex Pro wash station and a Bio-Plex 200 reader (Bio-Rad, Hercules, CA). The assay was run according to the manufacturer's instructions and the results were analyzed using Bio-Plex Manager v 5.0 Software (Bio-Rad) with 5PL curve fitting.

Serum lipid profile

Measurements of cholesterol, triglycerides, low-density lipopolysaccharide (LDL), high-density lipopolysaccharide (HDL) and creatinine were obtained using a Beckman Coulter AU480 clinical analyzer, using standard protocols and controls for this instrument.

Peripheral Quantitative Computerized Tomography densitometry (pQCT)

The distal femoral metaphysis (DFM), proximal tibial metaphysis (PTM) and the fourth lumbar vertebra (L_4) were analyzed by pQCT densitometry, using a XCT research M system (Norland Stratec, Birkenfeld, Germany). In all the bones, 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 three slices, 1mm distal to the growth plate (PTM) and 1 mm proximal to the growth plate (DFM) were analyzed. In the fourth lumbar vertebrae (L_4), the cancellous bone between the growth plates was analyzed. The following parameters were determined for all sites: trabecular bone mineral content (Tb BMC), trabecular bone mineral density (Tb BMD), cortical BMC (Ct BMC), cortical BMD (Ct BMD), cortical thickness (Ct Th), periosteal perimeter (Peri PM) and endocortical perimeter (Endo PM).

Micro computerized tomography (μCT)

Scans of the distal femoral metaphysis, proximal tibial metaphysis, and the vertebral body of the fourth lumbar vertebrae were conducted using a high-resolution μCT scanner Xradia μCT 200 (Xradia, Inc. Concord, CA) at 10 microns. All images were acquired using standard parameters, an X-ray source of 90 kV, with power of 8 W and current of 88 μA. Each scan consisted of 500 views with an exposure time of 8 s per slice. The scans were analyzed using Tri/3D Bon (Ratoc System Engineering Co. LTD., Tokyo Japan) for the different parameters including total volume (TV), bone volume (BV), BV/TV, trabecular number (Tb N), trabecular thickness (Tb Th), trabecular width (Tb W), trabecular separation (Tb S), and connectivity density (Conn Den).

MC3T3 cell culture

MC3T3 cells were obtained from ATCC, cultured in α-MEM (Life Technologies, Grand Island, NY) and differentiated using ascorbic acid and β-glycerophosphate. The optimal concentration of CQ and time required for efficient proliferation of the cells were determined earlier and were used to treat the cells with optimal concentration (0.004%) for 48 h.

qPCR for gene expression

Some genes related to bone metabolism—Osteocalcin, osteopontin, BMPr2, Type of collagen I, and DCAT1 (Wnt/β catenin antagonist)—were measured by quantitative PCR (qPCR) using Taqman gene expression system (Applied Biosystems, Carlsbad, CA). **C**ells were homogenized in RNA extraction buffer and the RNA extracted using RNeasy Mini Kit (Qiagen, Valencia, CA). The quality and quantity of the RNA were measured using a Nanodrop spectrophotometer. Reverse transcription was performed to make cDNA using the Advantage RT-for-PCR Kit (Clontech, Mountain View, CA) following manufacturer's instructions. 1 μg of RNA from each sample was used to make cDNA.

Gene expression was measured by quantitative PCR (qPCR) using TaqMan gene expression assays. Amplification efficiencies of all gene expression assays were checked prior to experimentation, with the sample designated as the calibrator, using seven-point dilution series. The Step One Plus Real-Time PCR System (Applied Biosystems, Carlsbad, CA) was used to run the amplification efficiency curves and the experimental plates. Each cDNA sample was run in triplicate and no template control wells were included on each plate for each probe to check for contamination. The qPCR mix consists of sterile water, Universal PCR Master Mix (20 μmol/l) (2X concentration contains AmpliTaq Gold DNA Polymerase, AmpErase UNG, dNTPs with dUTP, ROX passive reference and optimized buffer components) (Applied Biosystems, Carlsbad, CA) and TaqMan gene expression assay (20X mix). The Real-time PCR program consists of a holding stage at 50 ºC for 2 min and 95 ºC for 10 min, and cycling 40 times at 95 ºC for 15 s, and 60 ºC for 1 min. GAPDH was used as the endogenous control gene for normalization [17]. The relative standard curve method was used to quantify gene expression in the samples.

miRNA analysis

RNA was isolated from the control and treated cells using PureZol RNA isolation reagent (Bio-rad Laboratories, Hercules, CA) and sent for miRNA analysis to LC Sciences LLC (Houston, TX) as detailed in Das et al (2013) [18]. Briefly, 2 μg of RNA was 3′ extended with poly (A) and then tagged with fluorescent dye. Overnight hybridization was

performed on a μParaflo microfluidic chip [19]. The different detection probes on the chip have the complementary coding segment to target miRNA (https://microrna.sanger.ac.uk/sequences/). RNA hybridization was done, after melting point temperatures were balanced to the detection probes in 6xSSPE buffer. Following RNA hybridization, chips were exposed to fluorescence tags and images recorded using a laser scanner (GenePix 4000B, Molecular Device) and analyzed using Array-Pro image software (Media Cybernetics) and LOWLESS filter (Locally weighted Regression) [20].

Statistical analysis

Results are expressed as Mean ± SE. Data were analyzed with one-way analysis of variance (ANOVA) and unpaired t test using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA, USA). $p \leq 0.05$ was significant. Newman–Keuls multiple comparison test was used to analyze the differences between groups for significance after ANOVA. Newman–Keuls multiple comparison is a powerful multiple comparison method [21–24]. Moreover, this analysis will give a sequential comparison from the largest to the lowest significance. However, there is a possibility that Type 1 error may occur with this test. In this study, we compare the different bone and biochemical parameters between the control and treated groups to determine the effect of CQ, so a powerful test was used to determine the significance.

Results

Body weight, weight of organs and food intake

Body weight increased significantly in the ovariectomized rats from both the control and CQ-treated groups (Table 1) when compared to the sham rats. CQ consumption did not have any significant effect on the body weight. Weight of the abdominal adipose tissue increased significantly in the CO rats (52%); however, in the CQO rats, it increased only by 6% and was significantly less than that seen in the CO rats. The treatment did not change the overall food intake in any of the groups studied. Similarly, the different organs such as kidney, liver, spleen and the quadriceps and gastrocnemius muscles did not show any significant changes in the weight (Table 1).

Liver and spleen pathology

There were no significant differences in the histological morphology of the liver and spleen cells between the control and CQ-treated groups (data not shown).

Biochemical markers, cytokines, lipid profile and hormones

Bone turnover markers related to bone formation and bone resorption were measured. No significant changes were seen between the control sham and OVX groups as well as the CQ

Table 1 Effects of *Cissus quadrangularis* on the body weight, organ weights and food intake of ovariectomized rats

S. no	Parameters/groups	*B*	*C*	CS	CO	CQS	CQO
1	Initial body weight (g)	274.8 ± 6.6	273.2 ± 5.1	274.1 ± 6.15	276.1 ± 4.47	274.3 ± 6.20	274.7 ± 3.52
2	Final body weight (g)	274.8 ± 6.6	307.5 ± 8.2	289.1 ± 8.72[a]	336.9 ± 6.00	294.8 ± 8.24	334.1 ± 7.82[b]
3	Adipose tissue weight (g)	2.17 ± 0.18	8.02 ± 0.95	6.35 ± 0.88[a]	9.64 ± 0.78	8.13 ± 0.86	8.58 ± 1.05
4	Uterus weight (g)	1.08 ± 0.07	1.22 ± 0.10	1.28 ± 0.07[a]	0.38 ± 0.06	1.14 ± 0.06	0.34 ± 0.05[b]
5	Liver weight (g)	6.74 ± 0.34	8.35 ± 0.32	8.14 ± 0.32	7.86 ± 0.16	8.31 ± 0.32[c]	7.48 ± 0.55
6	Spleen weight (g)	0.57 ± 0.03	0.61 ± 0.02	0.67 ±0.03	0.64 ± 0.02	0.62 ± 0.02	0.700 ± 0.03
7	Kidney weight (g)	1.79 ± 0.04	1.75 ± 0.05	1.70 ± 0.07	1.72 ± 0.05	1.83 ± 0.05	1.70 ± 0.06
8	Wet weight of quadriceps (g)	1.87 ± 0.05	1.92 ± 0.06	1.83 ± 0.08	2.14 ± 0.06	1.92 ± 0.11[c]	1.95 ± 0.09
9	Wet weight of gastrocnemius (g)	1.78 ± 0.05	1.79 ± 0.06	1.82 ± 0.10	2.00 ± 0.09	1.80 ± 0.07	2.03 ± 0.06
10	Food intake (g)	–	13.81 ± 0.25	14.28 ± 0.51	15.06 ± 0.27	13.51 ± 0.34	14.14 ± 0.39

The body weight was recorded at the beginning of the study and at the time of sacrifice. The organ weights were recorded at the time of sacrifice. Food intake was monitored daily

Data are Mean ±SE

B baseline, *C* control, *CS* control Sham, *CO* control ovariectomy, *CQS Cissus quadrangularis* fed Sham, *CQO Cissus quadrangularis* fed Ovariectomy

[a] $p \leq 0.05$ vs CO

[b] $p \leq 0.05$ vs CQS

[c] $p \leq 0.05$ vs B

Table 2 Effects of *Cissus quadrangularis* on biochemical markers levels in ovariectomized rats

Parameters/groups	*B*	*C*	CS	CO	CQ S	CQ O
Bone formation and resorption markers						
Alkaline phosphatase (U/L)	10.40 ± 1.14	24.09 ± 2.98[d]	24.98 ± 2.66[d]	25.59 ± 4.03	7.14 ± 0.87[a,d]	8.24 ± 0.82[b]
P1NP (ng/ml)	1.27 ± 0.03	1.20 ± 0.046[d]	1.18 ± 0.02[b,d]	1.16 ± 0.026	1.56 ± 0.07[a,d]	1.38 ± 0.04[b,c]
CTX (ng/ml)	84.37 ± 6.03	62.12 ± 3.45[d]	66.58 ± 1.20[d]	93.96 ± 13.88	78.53 ± 5.76	67.43 ± 6.74
Trap5b (U/L)	10.16 ± 1.43	12.70 ± 1.29	11.40 ± 0.78	8.59 ± 1.32	5.94 ± 1.48[a]	2.22 ± 0.32[b,c]
Osteocalcin (ng/ml)	130.1 ± 35.38	106.0 ± 22.05	84.05 ± 26.27	73.05 ± 36.76	29.48 ± 2.57[a,d]	37.98 ± 8.97
Lipid profile						
Cholesterol (mg/dL)	131.4 ± 17.72	96.80 ± 7.81	102.4 ± 8.518[b]	133.2 ± 10.44	112.0 ± 13.16	120.4 ± 9.537
Triglycerides (mg/dL)	96.00 ± 7.80	79.60 ± 11.59	72.25 ± 4.39[b,d]	103.6 ± 10.39	92.33 ± 2.40[a]	79.25 ± 10.87
High density lipoprotein (mg/dL)	86.00 ± 11.49	65.40 ± 5.07	67.20 ± 4.76[b]	85.00 ± 5.61	77.00 ± 7.06	74.60 ± 5.91
Low density lipoprotein (mg/dL)	21.40 ± 2.79	14.40 ± 1.36[d]	17.40 ± 1.44[b]	28.40 ± 3.44	19.80 ± 2.71	26.60 ± 2.50

Blood was collected at the time of sacrifice and the different biochemical markers were measured in the serum

Data are Mean ±SE

B baseline, *C* control, *CS* control Sham, *CO* control ovariectomy, *CQS Cissus quadrangularis* fed Sham, *CQO Cissus quadrangularis* fed Ovariectomy

[a] $p \leq 0.05$ vs CS

[b] $p \leq 0.05$ vs CO

[c] $p \leq 0.05$ vs CQS

[d] $p \leq 0.05$ vs B

sham and OVX groups in the ALP levels (Table 2). However, ALP decreased significantly after CQ treatment, when compared to that seen in the control sham rats. There was 71% decrease in ALP levels in the CQS rats when compared to that of the control sham rats. Similarly comparing OVX rats, ALP decreased significantly in the CQO rats by 68% compared to that of the control OVX rats (Table 2). While there were no changes in the osteocalcin levels between C sham and OVX rats. CQS rats showed 65% ($p \leq 0.05$) decrease in osteocalcin levels, when compared to that of control sham rats. CQO rats showed 48% ($p \leq 0.05$) decrease in osteocalcin levels, when compared to that of the control OVX rats (Table 2). There was 12% decrease in osteocalcin levels in the CQO rats compared to that of the control OVX rats (Table 2). There was an increase in the CQ-treated ovariectomized rats in serum osteocalcin levels (29%), when compared to that of the control CQS rats (Table 2). There was no difference in the P1NP levels between the control sham and control OVX rats. CQ, however, increased P1NP levels in both the sham and OVX groups. CQ sham rats showed a 32% ($p \leq 0.05$) increase, when compared to that in the control sham group and CQOVX rats showed an increase of 19% ($p \leq 0.05$) compared to that of control OVX rats. There was no difference in the Trap5b levels in the control sham and OVX rats. However, CQ treatment significantly decreased Trap5b levels in the sham (68%, $p \leq 0.05$) rats, compared to that of the control sham rats. In the CQO rats also, there was significant decrease (76%, $p \leq 0.05$) in

Table 3 Pro-inflammatory cytokine levels in the serum of ovariectomized rats after treatment with *Cissus quadrangularis*

S. no	Parameters/groups	*B*	*C*	CS	CO	CQS	CQO
1	Tumor necrosis factor (pg/ml)	333.7 ± 7.8	279.1 ± 26.1[b]	325.8 ± 14.9	275.8 ± 13.5	262.7 ± 39.7	258.1 ± 29.3
2	GM-CSF (pg/ml)	91.5 ± 10.4	74.1 ± 4.6	58.5 ± 4.4[*,b]	100.9 ± 12.3	67.2 ± 9.1	77.0 ± 7.1
3	Interferon γ 1 (pg/ml)	57.40 ± 14.4	47.54 ± 25.7	27.94 ± 1.7[a]	61.04 ± 9.8	32.99 ± 8.1	34.37 ± 3.8
4	Interleukin 1α (pg/ml)	51.07 ± 12.0	35.73 ± 22.2	29.58 ± 2.0[a]	61.48 ± 10.4	36.30 ± 10.9	44.91 ± 9.4
5	Interleukin 1β (pg/ml)	140.3 ± 47.7	27.8 ± 18.0[b]	43.0 ± 9.3[b]	119.0 ± 31.1	54.3 ± 18.6	149.8 ± 28.8
6	Interleukin 2 (pg/ml)	211.6 ± 23.8	216.7 ± 38.0	164.2 ± 14.5	181.7 ± 24.1	167.1 ± 32.1	164.7 ± 16.8
7	Interleukin 4 (pg/ml)	31.84 ± 4.6	10.79 ± 3.0[b]	15.26 ± 1.7[b]	21.71 ± 3.7	20.91 ± 6.7	20.96 ± 3.8
8	Interleukin 6 (pg/ml)	450.8 ± 26.4	343.2 ± 80.3	446.6 ± 31.1[a]	382.0 ± 19.1	272.3 ± 73.2***,[b]	350.5 ± 32.1**
9	Interleukin 10 (pg/ml)	251.5 ± 41.06	238.5 ± 61.84	190.0 ± 7.0.99	201.7 ± 22.4	189.0 ± 28.6	233.6 ± 31.9

Blood was collected at the time of sacrifice and the levels of cytokines were measured in the serum

Data are Mean ±SE

B baseline, *C* control, *CS* control Sham, *CO* control ovariectomy, *CQS Cissus quadrangularis* fed Sham, *CQO Cissus quadrangularis* fed Ovariectomy

*$p = 0.07$ vs C

**$p = 0.06$ vs CO

***$p = 0.06$ vs CS

[a]$p \leq 0.05$ vs CO

[b]$p = 0.05$ vs B

Table 4 Effects of *Cissus quadrangularis* on hormone levels in ovariectomized rats

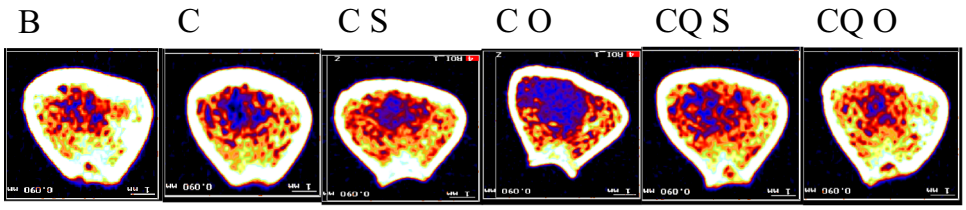
S. no	Hormone	*C*	CS	CO	CQS	CQO
1.	Adiponectin (ng/ml)	15.10 ± 1.39	17.54 ± 0.79	16.06 ± 1.02	14.25 ± 1.10	14.89 ± 2.24
2.	Leptin (pg/ml)	147.2 ± 71.7	34.07 ± 7.26[a]	245.4 ± 46.02	112.6 ± 24.12	194.6 ± 25.12
3.	Estradiol (pg/ml)	32.60 ± 4.20	29.93 ± 4.64[a]	10.07 ± 3.40	31.04 ± 3.18	22.63 ± 3.60[a]
4.	Parathyroid Hormone (pg/ml)	5.10 ± 1.19	3.98 ± 1.54	4.81 ± 0.80	4.65 ± 0.58	3.48 ± 0.61
5.	Insulin (ng/ml)	0.366 ± 0.036	0.341 ± 0.068	0.267 ± 0.023	0.298 ± 0.104	0.330 ± 0.031
6.	Insulin-like growth factor-I (ng/ml)	1077 ± 205.9	1431 ± 264.3[a]	1708 ± 147.7	1796 ± 298.8[a]	1686 ± 312.4

Blood was collected at the time of sacrifice and the hormones were measured in the serum

Data are Mean ±SE

B baseline, *C* control, *CS* control Sham, *CO* control ovariectomy, *CQS Cissus quadrangularis* fed Sham, *CQO Cissus quadrangularis* fed Ovariectomy

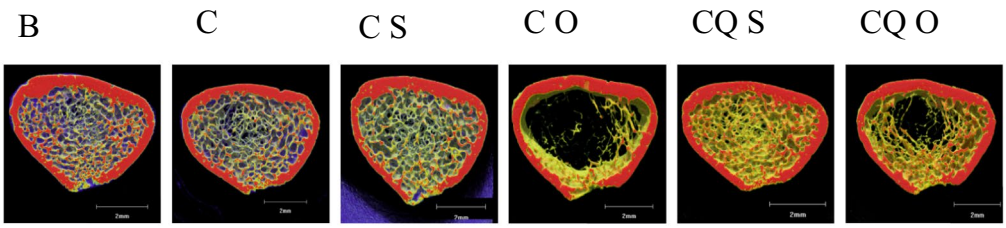
[a]$p \leq 0.05$ vs CO



Parameters	B	C	C S	C O	CQ S	CQ O
Trabecular BMC (mg/mm)	1.61±0.18	1.23±0.19	1.25±0.13	0.33±0.07[a,b]	1.21±0.16	0.90±0.20
BMD (mg/mm^3)	236.0±20.1	167.1±20.93	197.8±17.07	39.51±6.94[a,b]	172.3±19.14	114.2±27.73
	0.707±0.021	0.61±0.03	0.58±0.01	0.50±0.01[a]	0.57±0.02	0.52±0.02
BMC (mg/mm)	8.94±0.19	8.29±0.31	7.71±0.20	7.31±0.13	7.70±0.18	7.14±0.24
^{3})	1128±22	1147±21	1179±16	1190±15	1131±21	1128±22
Periosteal perimeter (mm)	13.53±0.22	13.94±0.29	13.08±0.20	13.83±0.23	13.70±0.23	14.09±0.25
Endocortical perimeter (mm)	9.08±0.17	10.11±0.32	9.41±0.22	10.65±0.30[a]	10.12±0.25	10.85±0.31

The distal femoral metaphysis was scanned using a pQCT densitometer to get the cortical and trabecular parameters from ovariectomized rats fed, control or Cissus quadrangularis (CQ) (2g/Kg body weight) for three months. B = Baseline, C = Control, C S = Control Sham, C O = control Ovariectomy, CQ S = *Cissus quadrangularis* Sham, CQ O = *Cissus quadrangularis* Ovariectomy. [a]p<0.05 vs C S; [b]p<0.05 vs CQ S

Fig. 1 Effects of *Cissus quadrangularis* on the distal femoral metaphysis trabecular and cortical parameters from ovariectomized rats. *B* baseline, *C* control, *CS* control sham, *CO* control ovariectomized, *CQSCissus quadrangularis* sham, *CQO* CQ ovariectomized. *$p \leq 0.05$ vs CO; **$p \leq 0.05$ vs B



Parameters	B	C	C S	C O	CQ S	CQ O
Trabecular thickness (mm)	84.55±1.10	78.84±2.37	76.73±1.67[a]	71.7±0.98	76.86±1.87	74.83±2.25
Trabecular number (1/mm)	2.02±0.01	1.72±0.0.7	1.77±0.07[a]	0.52±0.06	1.75±0.05	0.79±0.17[a,b]
Trabecular width (mm)	156.7±4.7	161.7±8.5	152.4±3.8[a]	97.9±2.4	150.4±3.5	116.4±6.6[a,b]
Trabecular separation (mm)	186.2±7.9	233.5±7.3	225.3±7.8[a]	395.5±39.5	222.5±5.0	333.8±19.6[a,b]
Connectivity density (1/mm^3)	77.14±6.01	49.82±3.14	54.77±3.20[a]	8.81±1.63	54.43±2.07	18.50±7.03[a]
BV/TV	30.43±2.04	23.41±1.78	23.23±1.29[a]	5.44±0.78	23.33±1.084	9.90±2.79[a,b]

Static histomorphometry of the distal femoral metaphysis was analyzed using a µCT densitometer to get the trabecular parameters from ovariectomized rats, fed control or Cissus quadrangularis (CQ) (2g/Kg body weight) for three months. B = Baseline, C = Control, C S = Control Sham, C O = Control Ovariectomy, CQ S = *Cissus quadrangularis* Sham, CQ O = *Cissus quadrangularis* Ovariectomy. [a]p<0.05 vs C O; [b]p<0.05 vs CQ S

Fig. 2 Effects of *Cissus quadrangularis* on the static histomorphometric parameters of the distal femoral metaphysis from ovariectomized rats. *B* baseline, *C* control, *CS* control sham, *CO* control ovariectomized, *CQS Cissus quadrangularis* sham, *CQO* CQ ovariectomized. *$p \leq 0.05$ vs CO; **$p \leq 0.05$ vs CQS; ***$p \leq 0.05$ vs B

Trap5b levels when compared to that of the control OVX rats (Table 2). Levels of CTX increased in the control OVX rats by 36%, when compared to that of control sham rats. Again, CQO rats showed a 28% decrease in CTX levels when compared to that of the control OVX rats. There was no difference in CTX levels in the sham rats between the control and CQ-treated groups (Table 2).

Lipid profile

Ovariectomy increased cholesterol (39%, $p \leq 0.05$), triglycerides (43%, $p \leq 0.05$), HDL (27%, $p \leq 0.05$) and LDL (63%, $p \leq 0.05$) significantly in control rats (Table 2). CQ increased the different lipids that were measured, but this increase was not statistically significant when compared to those of CS rats except for triglyceride levels (28%, $p \leq 0.05$) (Table 2). No significant differences were observed in the lipids measured between the CQS and CQO rats (Table 2).

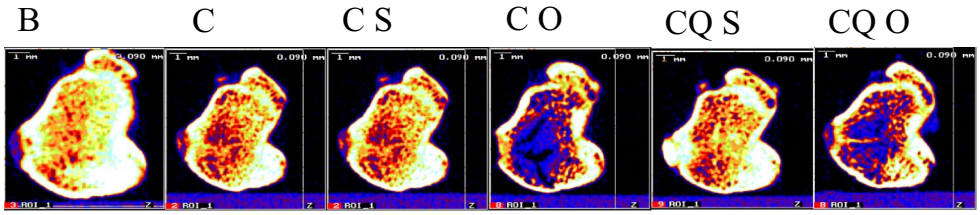
Cytokines

Higher levels of several cytokines were seen in control OVX rats. Significantly higher IFN γ1 (118%, $p \leq 0.05$) and IL 1α (108%, $p \leq 0.05$) were observed in the CO rats (Table 3). Interestingly, IL-6 (15%, $p \leq 0.05$) decreased significantly in the control OVX rats when compared to that of the control sham rats. There was no significant difference between the levels of the different cytokines studied in the CQS and CQO rats (Table 3). IL-6 levels were decreased in CQS (39%, $p = 0.08$) when compared to that of CS and in CQO (8%, $p \leq 0.05$) compared to that of the control OVX rats (Table 3). But significantly higher triglycerides were seen in CQS compared to that of the control sham rats.

Hormones

Other biochemical markers measured included adiponectin and leptin. Significantly decreased levels of adiponectin were seen in the CQS rats when compared to that of control sham rats (Table 4). Leptin levels increased significantly after ovariectomy in the control rats by 86% ($p \leq 0.05$). There was no significant difference between the sham and ovariectomy rats fed CQ (Table 4).

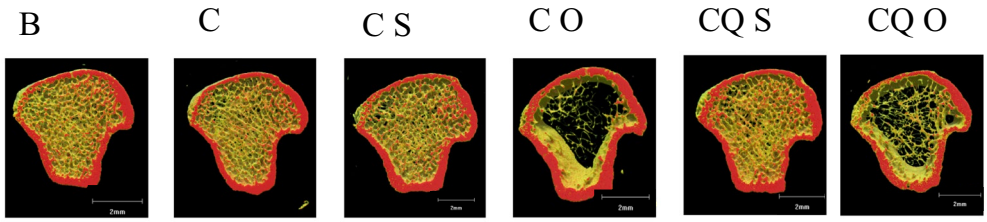
After ovariectomy, estradiol (66%, $p \leq 0.05$) significantly decreased in the control animals (Table 4). There was 27% decrease in estradiol in the CQO group of rats compared to that of the CQS rats, but this was not statistically significant (Table 4)**.** There was no difference between the control sham and CQS groups of rats in the estradiol levels (Table 4). But there was 125% increase in the estradiol levels in the CQO rats, when compared to that of the control OVX rats. PTH levels did not change significantly between the different groups studied (Table 4). However, it increased (21%) in CO rats when compared to that of the CS rats, while CQO rats showed a 25% decrease in the



Parameters	B	C	C S	C O	CQ S	CQ O
Trabecular BMC (mg/mm)	5.10±0.34	3.98±0.30	4.13±0.25[a]	0.91±0.15	3.60±0.24	1.86±0.37[a,b]
Trabecular BMD (mg/mm^3)	423.7±19.4	319.9±24.6	334.4±19.8[a]	60.4±10.1	288.4±18.3	139.1±27.3[a,b]
Cortical thickness (mm)	1.025±0.076	0.819±0.055	0.858±0.040[a]	0.466±0.020	0.747±0.044	0.603±0.052[a,b]
Cortical BMC (mg/mm)	12.73±0.78	10.79±0.68	11.23±0.49[a]	6.79±0.26	9.88±0.53	8.50±0.69[a,b]
Cortical BMD (mg/mm^3)	832.5±16.6	822.5±6.3	825.2±6.7	843.2±9.5	813.1±11.3	814.3±6.6
Periosteal perimeter (mm)	18.27±0.33	18.65±0.214	18.6±0.13	18.77±0.11	18.66±0.11	19.27±0.10
Endocortical perimeter (mm)	11.83±0.38	13.50±0.40	13.21±0.27[a]	15.84±0.13	13.96±0.25	15.48±0.30[b]

The proximal tibial metaphysis was scanned using a pQCT densitometer to get the cortical and trabecular parameters from ovariectomized rats, fed control or Cissus quadrangularis (CQ) (2g/Kg body weight) for three months. B = Baseline, C = Control, C S = Control Sham, C O = control Ovariectomy, CQ S = *Cissus quadrangularis* Sham, CQ O = *Cissus quadrangularis* Ovariectomy.
[a]p<0.05 vs C S; [b]p<0.05 vs CQ S

Fig. 3 Effects of *Cissus quadrangularis* on the trabecular and cortical bone parameters of the proximal tibial metaphysis from ovariectomized rats. *B* baseline, *C* control, *CS* control sham. *CO* control ovariectomized, *CQS Cissus quadrangularis* sham, *CQO* CQ ovariectomized. *$p \leq 0.05$ vs CO; **$p \leq 0.05$ vs CQS; ***$p \leq 0.05$ vs B



Parameters	B	C	C S	C O	CQ S	CQ O
Trabecular thickness (mm)	80.85±0.86	75.93±1.99	75.16±1.52	76.60±1.35	75.26±1.32	80.52±1.94
Trabecular number (1/mm)	2.30±0.08	1.64±0.0.09	1.92±0.08[a]	0.47±0.07	1.88±0.06	0.91±0.14[a,b]
Trabecular width (mm)	130.8±2.7	127.7±5.6	126.2±3.0[a]	100.9±2.0	129.1±3.0	108.9±3.3[b]
Trabecular separation (mm)	156.2±4.8	203.6±9.6	180.7±5.0[a]	451.10±63.66	193.9±6.9	305.8±19.7[a,b]
Connectivity density (1/mm^3)	93.94±5.45	52.46±5.36	68.89±5.03[a]	6.58±1.31	62.11±3.77	20.87±6.35[a]
BV/TV	30.21±1.43	221.19±1.75	23.16±1.14[a]	5.19±0.69	22.72±1.19	9.78±1.76[a,b]

Static histomorphometry of the proximal tibial metaphysis was analyzed using a mCT densitometer to get the trabecular parameters from ovariectomized rats, fed control or Cissus quadrangularis (CQ) (2g/Kg body weight) for three months. B = Baseline, C = Control, C S = Control Sham, C O = Control Ovariectomy, CQ S = *Cissus quadrangularis* Sham, CQ O = *Cissus quadrangularis* Ovariectomy. [a]p<0.05 vs C O; [b]p<0.05 vs CQ S

Fig. 4 Effects of *Cissus quadrangularis* on the static histomorphometric parameters of the proximal tibial metaphysis from ovariectomized rats. *B* baseline, *C* control, *CS* control sham, *CO* control ovariectomized, *CQS Cissus quadrangularis* sham, *CQO* CQ ovariectomized. *$p \leq 0.05$ vs CO; **$p \leq 0.05$ vs CQS; ***$p \leq 0.05$ vs B

PTH levels, when compared to that of the CQ-treated sham rats. Insulin levels did not change significantly in any of the groups studied. Notably, CQ treatment decreased insulin levels but the change was not statistically significant. IGF-1 levels were also not significantly different among the groups studied, although there was a slight increase (19%, $p \leq 0.05$) after ovariectomy in the control animals (Table 4). CQ treatment showed significantly elevated (26%, $p \leq 0.05$) levels of IGF-1 in the sham rats, compared to the CS rats (Table 4); but there was a slight decrease (3%) in the CQO rats when compared to CO rats.

Static histomorphometry and bone parameters

Distal Femoral Metaphysis (DFM)

Trabecular bone mass of the DFM decreased significantly in the control OVX rats, when compared to those of the CS rats (Figs. 1, 2). Tb BMC (26%, $p \leq 0.05$) and Tb BMD (34%, $p \leq 0.05$) decreased significantly in the control OVX rats, when compared to those of control sham rats (Fig. 1). In addition, cortical parameters such as Crt BMC and Crt Th also decreased by 74% ($p \leq 0.05$) and 14% ($p \leq 0.05$) after ovariectomy in the control group (Fig. 1). Ovariectomy, as expected, significantly decreased trabecular bone parameters in the control rats. Tb Th (7%, $p \leq 0.05$), Tb N (71%, $p \leq 0.05$), Tb W (36%, $p \leq 0.05$), BV/TV (77%, $p \leq 0.05$) and Conn Den (84%, $p \leq 0.05$), in the control OVX rats (Fig. 2). The Tb Sp (70%, $p \leq 0.05$) and Endo PM (13%, $p \leq 0.05$) increased significantly in the control OVX rats compared to those of control sham rats (Figs. 1, 2).

With CQ treatment, the ovariectomized rats showed significant decrease in the Tb N (55%, $p \leq 0.05$), Tb W (23%, $p \leq 0.05$), Conn Den (66%, $p \leq 0.05$) and BV/TV (58%, $p \leq 0.05$), when compared to those of the CQS rats (Fig. 2). However, the CQO rats showed significantly higher Tb N (52%, $p \leq 0.05$) and Tb W (19%, $p \leq 0.05$) when compared to those of control OVX rats (Fig. 2). Tb Sp (50%, $p \leq 0.05$) increased in the CQO rats, when compared to that of the CQS rats, but was lower by 16% ($p \leq 0.05$), when compared to those of the control OVX rats (Fig. 2).

PTM

In the PTM of control OVX rats, all the trabecular and cortical bone parameters were significantly decreased in the control OVX rats, except for the Tb Th, Crt BMD and Peri PM. Tb Sp (150%, $p \leq 0.05$) and Endo PM (19%, $p \leq 0.05$) significantly increased in the control OVX rats, when compared to those of the control sham rats (Figs. 3, 4).

CQ treatment had limited benefits on the trabecular and cortical parameters at the PTM. There was significant decrease

Table 5 Effects of *Cissus quadrangularis* on the trabecular and cortical bone parameters of the fourth lumbar vertebra from ovariectomized rats

Parameters/groups	*B*	*C*	CS	CO	CQS	CQO
Trabecular bone mineral content (mg/mm)	1.17 ± 0.11	0.740 ± 0.13[c]	1.19 ± 0.29[a,d]	0.47 ± 0.19	0.70 ± 0.11	0.50 ± 0.16
Trabecular bone mineral density (mg/mm^3)	97.8 ± 8.9	62.1 ± 9.4[c]	106.2 ± 25.0[a]	36.0 ± 15.7	81.47 ± 9.57	29.9 ± 12.0
Trabecular thickness (mm)	89.05 ± 1.16	84.53 ± 1.57	86.65 ± 1.29[a]	80.87 ± 0.84	85.85 ± 1.45	82.78 ± 1.25[b]
Trabecular number (1/mm)	2.18 ± 0.04	2.08 ± 0.04	2.13 ± 0.02[a]	1.73 ± 0.05	2.07 ± 0.05	1.83 ± 0.05
Trabecular width (μm)	348.8 ± 8.8	356.5 ± 9.5	360.9 ± 8.5	278.5 ± 8.3	346.5 ± 8.8	305.7 ± 8.7[a,b]
Trabecular separation (μm)	179.4 ± 6.1	207.3 ± 10.2	196.5 ± 6.0	253.2 ± 7.38	213.8 ± 9.8[c]	245.0 ± 7.5[b]
Connectivity density (1/mm^3)	45.89 ± 1.87	37.13 ± 1.23[c]	39.85 ± 1.71[a,c]	31.71 ± 1.24	39.75 ± 2.10[c]	33.25 ± 1.48[b]
BV/TV	44.83 ± 1.11	39.34 ± 1.24[c]	41.47 ± 1.06[a]	28.23 ± 1.08	40.06 ± 0.94[c]	30.79 ± 1.26[b]
Cortical bone mineral content (mg/mm)	14.83 ± 0.51	13.00 ± 0.58[c]	12.18 ± 0.77[a,c]	9.64 ± 0.41	12.42 ± 0.36[c]	10.31 ± 0.33[b]
Cortical bone mineral density (mg/mm^3)	1010 ± 6	996 ± 5	1006 ± 10	967 ± 5	998 ± 5	976 ± 4[b]
Cortical thickness (mm)	0.961 ± 0.027	0.834 ± 0.031[c]	0.817 ± 0.049[a,c]	0.641 ± 0.028	0.823 ± 0.026[c]	0.666 ± 0.024[b]
Periosteal perimeter (mm)	16.29 ± 0.26	17.43 ± 0.73	17.38 ± 0.2	17.58 ± 0.10	17.75 ± 0.13	17.98 ± 0.14
Endocortical perimeter (mm)	12.25 ± 0.20	12.19 ± 0.63	12.25 ± 0.32	13.55 ± 0.20	12.58 ± 0.23	17.96 ± 0.14[a,b]

Fourth lumbar vertebrae were scanned using pQCT and μCT densitometer to measure the cortical and trabecular parameters

Data are Mean ±SE

B baseline, *C* control, *CS* control Sham, *CO* control ovariectomy, *CQS Cissus quadrangularis* fed Sham, *CQO Cissus quadrangularis* fed Ovariectomy

[a] $p \leq 0.05$ vs CO

[b] $p \leq 0.05$ vs CQS

[c] $p \leq 0.05$ vs B

in Tb BMC (48%, $p \leq 0.05$), Tb BMD (52%, $p \leq 0.05$), Crt BMC (14%, $p \leq 0.05$), and Crt Th (19%, $p \leq 0.05$) in the CQO rats, when compared to those of the CQS rats (Fig. 4). However, comparisons between the control OVX and CQO rats showed that Tb N (94%, $p \leq 0.05$), BV/TV (88%, $p \leq 0.05$), Tb BMC (104%, $p \leq 0.05$), Tb BMD (130%, $p \leq 0.05$), Crt Th (29%, $p \leq 0.05$), Ct BMC (25%, $p \leq 0.05$) and Ct Th (29%, $p \leq 0.05$) were higher in the CQO rats (Figs. 3, 4). Moreover, Tb Sp (32%, $p \leq 0.05$) decreased significantly in the CQO rats, when compared to that of CO rats (Figs. 3, 4).

Lumbar vertebrae

The lumbar vertebra showed significantly decreased trabecular and cortical parameters except for Tb BMD, Ct BMD and Peri PM in the control OVX groups (Table 5). Treatment with CQ did not change the trabecular architecture and both trabecular and cortical bone decreased significantly in the CQO rats, when compared to those of the CQ sham rats (Table 5).

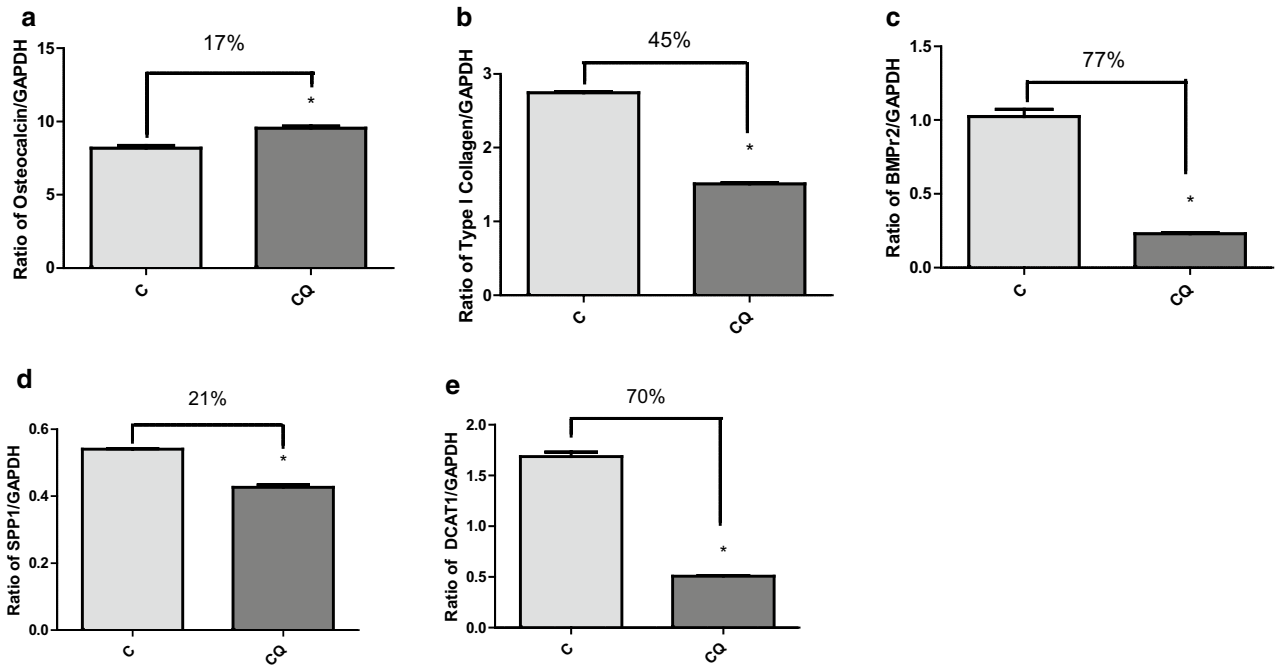
The lumbar vertebra showed significantly decreased trabecular parameters in the control OVX groups (Table 5). Treatment with CQ did not change the trabecular microarchitecture and CQO rats also showed significantly decreased trabecular parameters, when compared to those of the CQS rats (Table 5).

qPCR measurements

The expression of osteocalcin, Type collagen I, BMPr2, osteopontin (SPP1), and DCAT1 was measured after treating differentiated MC3T3 with CQ. Expression of osteocalcin significantly increased (17%) in the CQ-treated cells (Fig. 5a). Acute treatment with CQ also significantly reduced the expression of Type I Collagen (45%), BMPr2 (77%), osteopontin (SPP1) (21%), and DCAT1 (70%) (Fig. 5).

miRNA analysis

miRNA related to bone metabolism was analyzed in MC3T3 cells treated with CQ. The results are presented in Table 6. Several miRNA associated with BMP pathway induced osteogenesis including miR133b-5, miR135a-2-3p, miR138-2-3p and miR181 d-5p were upregulated (Table 6). Osteogenesis promoting miRNA—miR20-5b, miR29c-3p, miR138-2-3p and miR181-d-5p were also upregulated (Table 6). Among those miRNA that are known to be upregulated during osteogenesis miR211-3p, miR335-5p, miR133b-5p, 135a-2-3p were also upregulated in the presence of CQ. Some miRNA that are usually downregulated during osteogenesis including miR138-3p, miR21b and miRNA levels were analyzed. miR21c also were found to increase after CQ treatment (Table 6).



Data are Mean ± SE. C = control, CQ = *Cissus quadrangularis*. a = osteocalcin, b = collagen I, c = BMPr2 (bone morphogenetic protein receptor type 2), d = SPP1 (Secreted phosphoprotein 1 or osteopontin), e = DACT1 (dishevelled binding antagonist of beta catenin 1). *=p≤0.05 vs control.

Fig. 5 Effects of Cissus quadrangularis on the expression of genes related to bone metabolism in MC3T3 cells using qPCR analysis of gene expression

Table 6 miRNA upregulation by *Cissus quadrangularis* in differentiated MC3T3 cells

	Fold change
BMP pathway induced osteogenesis	
miR133b-5p	2
miR135a-2-3p	2
miR 138-2-3p	4
miR181 d-3p	3
Osteogenesis promotion	
miR20-b-5b	2
miR29c-3p	2
Upregulated during osteogenesis	
miR211-3p	2
miR335-5p	2
miR133b-5p	2
135a-2-3p	2
Downregulated during osteogenesis	
miR138-3p	4
miR21b	2
miR21c	4

MC3T3 cells were differentiated and treated with *Cissus quadrangularis* (0.004%)

Discussion

CQ has been implicated in bone healing and improving bone health. We had previously demonstrated that CQ protected bone from ovariectomy induced bone loss in mice [25]. In this paper, we describe the effects of CQ (plant dried and powdered) on bone and report various pathways that are modulated by CQ. The in vivo part of this study showed that CQ-treated OVX rats decreased bone loss in the different skeletal sites of the long bones that were analyzed as observed previously in mice [17]. A significant loss of trabecular bone in the metaphysis and the lumbar vertebrae was seen in untreated OVX rats. Such heavy loss of trabecular bone is the hallmark of decrease in estrogen levels due to OVX or menopause. CQ treatment in the ovariectomized rats reduced the loss of trabecular bone by maintaining the mineral content and density suggesting that there was decreased bone resorption. This is further supported by the observations that the trabecular number and trabecular width were significantly higher in the CQO rats. Moreover, there was significant decrease in the Tb Sp in the CQO rats. Such protection was seen in the long bones, more so in the DFM than the PTM. Surprisingly, in the lumbar vertebrae, CQ-treated OVX rats showed no bone protection. Bone strength

also increased with CQ in the DFM (data not shown). Historically, certain compounds and conditions may increase the density but the bones are still fragile due to faulty protein matrix. The fact that CQ-treated animals can increase bone strength is noteworthy.

In this study, we have used the whole CQ plant powder to make the food pellets for the rats. In India, the whole plant is used as a vegetable for consumption and the ground paste is applied to fracture sites to enhance the healing process and is consumed for several gastrointestinal ailments [26]. These results are in line with several reports [10–13, 19] that have used compounds extracted from CQ, with various solvents, and showed bone protective and bone strengthening properties. A phytoestrogen-rich ethanol extract of CQ when fed to OVX rats showed significantly increased BMD and bone strength in the femur and vertebrae [27]. The same study also reported restoration of bone architecture in the femur [27]. Petroleum ether extracts of CQ in OVX rats increased the biomechanical strength and thickness of both cortical and trabecular bone in the femur [14]. CQ demonstrated anti-osteoclastic activity by reducing TRAP in the femur [13] of OVX rats similar to the observation in this study demonstrating that circulating Trap5b was decreased significantly. Human SaOS-2 cells (human osteoblast like cells) and primary rat calvarial culture increased proliferation, differentiation and mineralization when treated with ethanol extracts of CQ [15]. In another study, using petroleum ether extract of CQ on mesenchymal stem cells from the long bones of male Wistar rats showed enhanced differentiation into osteoblasts and increased extracellular matrix calcification [14]. Therefore, the beneficial properties of CQ to bone health are probably not restricted to a single chemical ingredient as several different solvents extracts, that would separate constituents differentially, have shown similar effects.

There are several reports on the chemical analysis of CQ. The stem of CQ has been reported to have alkaloids, flavonoids, cardiac glycosides and triterpenes [28–30]. The stem also contains phytosterols and stilbenes like resveratrol, piceatannol and pallidol as well as quercetin and kaempferol [31–34]. In addition to these compounds, CQ plant also contains vitamin C and minerals, such as calcium and phosphorus in the stem [30]. Flavonoids and triterpenoids are active ingredients in the stem of CQ with respect to pharmacological applications [31, 35]. It is believed that the presence of flavonoids, stilbenes and calcium in CQ may be the major contributors to the bone protective properties.

Serum biochemical markers for bone formation and bone resorption were assayed. Bone forming markers such as ALP, OC and P1NP usually increase during bone formation. ALP is present in many tissues and the total ALP levels in the serum are contributed by the intestine, liver, kidney and bone [36]. In the present study, we measured only the total ALP which could have been originated from several organs. Under suitable conditions, osteoblasts secrete ALP while mineralization occurs in the protein matrix. However in our study, we observed that ALP levels were decreased with CQ. Measuring bone-specific ALP may give precise information about the effects of CQ on osteoblast-related ALP secretion. Another marker for bone formation that was measured was osteocalcin (OC), which also decreased with CQ treatment in the serum. However, the CQO rats showed higher levels of circulating OC (27%). OC bonds to the mineralized matrix and manages crystal alignment and maturation [37, 38]. Moreover, circulating levels of OC are now implicated in diabetes, energy metabolism and fat accumulation [39]. But acute treatment of osteoblastic cells with CQ increased (17%) in osteocalcin expression. This also suggests that post-translational modification of OC or modulation of the carboxylation levels of OC by CQ may be the factors affecting OC activity in vivo rather than expression levels at least in the long term. We measured only the total OC. Further studies are required to see if CQ interferes with the carboxylation of OC as decreased carboxylation modulates the activity of OC [40]. The bone forming marker, P1NP, released when procollagen type I is formed was increased significantly with CQ treatment in serum. This suggests that CQ may enhance the formation of procollagen required to form the protein matrix in the bone. An increase in the bone resorption markers such as Trap5b and CTX has been reported after ovariectomy and in postmenopausal women [41]. Trap5b is associated with osteoclast differentiation and number, while CTX is the degradation product of type I collagen [34, 35]. In this study, Trap5b and CTX increased after ovariectomy and CQ treatment reduced these markers suggesting that it controls bone resorption. When cells were acutely treated with CQ, there was a significant reduction in the Type I collagen levels between the control and CQ-treated cells. It is plausible that osteogenesis as measured by gene expression of Type I collagen, in acutely treated bone cells (in vitro), will differ from measures of total serum P1NP (a marker for procollagen formation) from 3-month CQ treated in vivo model.

After ovariectomy, there are increased levels of several pro-inflammatory cytokines [42]. Among the anti-inflammatory cytokines, IL-4, a cytokine that enhances the activation of both B and T cells and decreases inflammation, increased with CQ treatment in this study. IL-6 is another inflammatory cytokine that is increased after ovariectomy and menopause [43–45]. In the present study, IL-6 levels decreased with CQ treatment suggesting that the treatment decreases inflammation. As IL-10 levels did not change, it is more likely that CQ uses IL-4 and IL-6 pathways to reduce inflammation as has been reported [46–48].

Pro-inflammatory cytokines such as TNF α decreased after CQ treatment but IL-1α and β increased in the CQS group. Significant decrease in TNF α has been also reported

in male rats [16] after they were fed methanol-extracted fraction of CQ [13]. They further reported a decrease in IL-β, but to a lesser extent than TNF α. However, we did not see any significant changes on IL-β.

The major hormone that is significantly decreased after ovariectomy and in postmenopausal women is estrogen. CQ treatment increased estrogen levels significantly in OVX rats. Such effects of CQ on estrogen have been reported in OVX rats [49]. The increase in estrogen levels are attributed to the phytoestrogens that are present in CQ. It is well established that loss of estrogen leads to bone loss and supplementation with estrogen or phytoestrogens has a bone protective effect [50–53].

One of the major impacts of ovariectomy is obesity due to accumulation of adipose tissue. Estrogen levels are closely associated with circulating leptin levels as estrogen enhances leptin secretion [54]. Soon after estrogen levels dropped, there was a decrease in circulating leptin levels and by 13 weeks after ovariectomy, there was a significant increase in the circulating leptin levels [55]. Leptin levels increased significantly in the CQ-treated group compared to that of the control group rats. We also observed that there was an increase in body weight after OVX, and CQ did not have an effect on the increase in body weight. But CQ did, however, increase the estrogen levels significantly in the CQO rats, which coincides with increased leptin secretion.

CQ did not have any beneficial effects on the lipid markers that were analyzed. There are reports showing that CQ decreased lipid profile parameters in humans. A formulation of CQ (CDE-300) increased the HDL levels [56] in infants. We used aqueous plant extract which may attribute to the difference in the benefits observed. Another important factor could also be that more concentrated CQ may show significant benefits. However, CQ does not appear to be detrimental to the lipid profile.

Several serum markers assayed are involved in energy metabolism, inflammation and fat accumulation. As osteoblasts and adipocytes differentiate from mesenchymal stem cells (MSC) [57], there is bound to be a close association between the molecules that stimulate the differentiation process. Energy metabolism by OC is through the adiponectin. In the present 3 month in vivo study, there was a trend towards increase in OC expression in CQO vs CQS animals. In the in vitro acute treatment study, OC gene expression actually increased as expected. OC can also influence inflammatory markers and it was observed that IL-1 and IL-10 were not affected. Adiponectin regulates and maintains energy homeostasis [58]. Just like leptin, adiponectin can be both beneficial and detrimental to bone formation. In vitro studies have shown that adiponectin increases osteoblast proliferation, but other studies have shown that serum adiponectin levels are inversely related to BMD [59–61]. In mice overexpressing adiponectin, Abbott et al. have also reported bone site-specific effects of adiponectin [62], which may contribute to the site-specific differences seen in the present study. In the present study, it appears that CQ modulates adiponectin levels and reduces ovariectomized bone loss.

Some miRNA are implicated in bone turnover. CQ elevated the expression of certain miRNA associated with osteogenesis. Many BMP-induced osteogenesis miRNA were upregulated. Level of BMPr2 gene expression is significantly reduced after acute treatment of MC3T3 cells with CQ. Therefore, it may be possible that CQ acts through other BMP's to modulate bone formation. More detailed studies on the expression of several BMP's and other proteins in the BMP signaling pathway may elucidate the action of CQ. There was also upregulation of certain miRNA that promote osteogenesis after CQ treatment. CQ may also enhance the Wnt signaling pathway by repressing inhibitors of this pathway and decreasing adipogenesis at the mesenchymal stem cells. Dishevelled binding antagonist of β catenin 1 (DACT1) promotes the degradation of β catenin 1 [63, 64]. In the present in vitro study, expression of DCAT1 was significantly decreased suggesting that CQ influences the Wnt/b catenin pathway by downregulating the antagonist and influencing bone formation. This study reports only preliminary data with reference to miRNA modulation by CQ. However, this is the first report to show that CQ modulates miRNA and gene expression of several genes promoting osteogenesis by influencing different pathways in combination with in vivo circulating levels of several proteins. More elaborate studies have to be conducted to pinpoint and decipher the specific details about the effects of CQ on miRNA and gene expression.

The most important observation about CQ is that, at the dose used, it appears to be safe and does not trigger clinical signs or histopathology in the liver, spleen and uterus. This is in line with other reports that have shown no side effects with double the concentration of CQ in both animal and humans studies [65, 66].

In conclusion, CQ protected bone in ovariectomized rats at different levels in the long bones studied. In the sham animals, no differences were observed between the CQ-treated and control animals; therefore, CQ does not impact bone mass under normal conditions, implying that physiological changes trigger the influence of CQ on bone turnover. The maximum effect of CQ osteo-protection was seen in the distal femur followed by the proximal tibia. The trabecular thickness, number, BMC, BMD and connectivity density were protected in ovariectomized rats after CQ treatment. CQ likely increases bone mineralization, maintains connectivity amongst trabeculae and reduces bone resorption. Several pathways are affected by CQ: it acts as an anti-inflammatory agent, may reduce resorption by protecting the collagen from breaking down, and may

also enhance bone formation by stimulating the formation of the protein matrix. Some of this can be attributed to the increase in estrogen levels after CQ treatment without affecting PTH levels. Our preliminary evidence in the acutely treated cell culture models also points towards CQ modulating certain miRNA and genes associated with the BMP and Wnt signaling pathways as well as other genes related to bone metabolism such as osteocalcin, osteopontin, Type I collagen and BMPr2. Therefore, CQ may be beneficial to bone maintenance by influencing more than one metabolic pathway. It is interesting that the tribal people who consume CQ regularly have reported a delayed menopause (personal communication, Dr. N. Loganathan). So far no adverse side effects are reported in humans and animal models, which make it a potential safe alternative medicine that can be used to manage bone loss during and after menopause.

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Compliance with ethical standards

Conflict of interest The authors have no disclosures to declare.

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