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Research Article

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Evidence for Bone Reversal Properties of a Calcium-Collagen Chelate, a Novel Dietary Supplement

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Abstract

Menopause drastically increases the risk of osteoporosis, and although drug therapies are available, having an efficacious dietary supplement as an adjuvant therapy or alternative is desirable. Recent findings suggest that a calcium-collagen chelate (CC) in the form of a dietary supplement is highly effective in improving bone mass in osteopenic rats. Therefore, we hypothesized that the consumption of CC reverses bone loss in postmenopausal women with osteopenia as early as three months. Women 1 to 5 years postmenopausal, not on hormone replacement therapy or any other prescribed medication known to influence bone metabolism were randomized to one of two treatment groups to receive as a dietary supplement intervention daily for three months of either of the following: 500 mg of calcium carbonate and 5 µg vitamin D (control), or 5 g of CC containing 500 mg of calcium carbonate and 5 µg vitamin D. Bone mineral density of lumbar spine and total body were assessed at baseline and at three months using dual-energy X-ray absorptiometry. Blood was collected at baseline and three months to assess bone biomarkers of bone metabolism. CC significantly increased total body bone mineral density when compared to the control group ($P<0.05$). A significant increase ($P<0.05$) in the BAP/TRAP5b ratio percent change was indicated for the CC group. Collectively, these preliminary data suggested that CC enhances bone mass potentially by increasing the rate of bone formation more than bone resorption in the process of bone turnover.

Keywords

Osteoporosis; Estrogen; Collagen; Calcium-chelate; Sclerostin

Introduction

Ovarian hormone deficiency is well-known to be associated with the development of osteoporosis and increased risk for fractures [1,2]. Current drug therapies as well as certain lifestyle and nutritional factors are known to reduce the risk of osteoporosis [3,4]. Despite the availability of drug therapies, a considerable number of women would prefer dietary supplements as an alternative/adjunctive to conventional therapeutic options [5]. The bone matrix is comprised of hydroxyapatite, a calcium phosphate crystal [$Ca_{10}(PO_4)6(OH)_2$], lipids, water, non-collagenous proteins, and collagen. Of the organic bone matrix, type I collagen occupies 90-95% of its organic mass. Type I collagen provides bone its viscoelastic strength, torsional stiffness, and load bearing capability while also nucleating proteins for crystalline deposition [6]. Furthermore, it is well known that bone loss accrues with the loss of collagen [7] and age-related changes in the collagen network lead to reductions in bone mechanical strength and elasticity [8], which contribute to the occurrence of osteoporotic fractures. Although studies investigating the efficacy of hydrolyzed collagen for the prevention and/or treatment of osteoporosis in humans are limited [9-11], animal evidence suggests [12-14] that supplemental collagen can effectively improve bone health. In a twelve-week intervention study, Guillerminet et al. [12] demonstrated that administering hydrolyzed collagen to ovariectomized (OVX) osteopenic mice completely reversed the loss of whole body bone mineral density (BMD). This increase in BMD was suggested to occur because of the ability of collagen to lower plasma concentration of C-telopeptide of type I collagen (C-TX), a marker of bone resorption, while increasing bone-specific ALP (BAP), a marker of bone formation, in comparison with OVX control mice after only four weeks of treatment. Studies using rat models of osteoporosis evaluating the effect of collagen on bone and bone biomarkers have made similar observations. For instance, Han and colleagues [14] tested cod gelatin using 3-month old Sprague-Dawley OVX rats and found both femoral neck BMD and trabecular microarchitectural properties of OVX rats on gelatin diet to be significantly superior to those of OVX controls. In part, these investigators attributed the beneficial effects of gelatin on bone by its ability to significantly reduce urinary excretions of N-telopeptide of type I collagen (N-TX) and deoxypyridinoline (DPD), also markers of bone resorption, perhaps due to suppressed mRNA levels of receptor activated NF-κB ligand (RANKL) in bone. To our knowledge, the effect of collagen on sclerostin, which negatively influences osteogenesis, has not been investigated [15,16]. Sclerostin is widely considered a negative regulator of bone formation, and lower circulating levels are indicative of treatment efficacy.

The *in vivo* evidence supporting the role of collagen supplementation in promoting bone health is convincing. Nonetheless, there is a need for clinical trials to support the *in vivo* findings, which led us to the current study. The purpose of this study was to explore the efficacy of a novel calcium-collagen chelate supplement (CC) in suppressing the progression of bone loss and or stimulating the formation of bone in postmenopausal women with osteopenia. Here, we provide evidence for use of CC for treating osteopenic postmenopausal women, as represented by alterations in biomarkers of bone metabolism as well as whole body BMD.

Materials and Methods

Subjects

For the purpose of the present study, 71 women, one to five years postmenopausal not on hormone therapy for at least three months prior to initiation of the study were recruited with lumbar spine BMD T-scores between 1.0 and 2.5 SD below the reference

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mean [17]. Women were prescreened over the phone and recruited from the greater Tallahassee, FL and surrounding areas using flyers and radio and online listings. Participants treated with calcitonin, bisphosphonates, raloxifene and/or anabolic agents such as parathyroid hormone (PTH) and growth hormone, or steroids for less than three months prior to the start of the study were excluded. In addition, subjects with metabolic bone disease, renal disease, cancer, cardiovascular disease, diabetes mellitus, respiratory disease, gastrointestinal disease, liver disease, or other chronic diseases, heavy smoking (more than 20 cigarettes per day), and current use of any prescription medications known to alter bone and calcium metabolism were excluded. The study protocol was approved by the Institutional Review Board at The Florida State University (FSU), and all experiments were carried out at the. After the initial prescreening, qualified participants were invited to the Human Performance Laboratory of FSU's Department of Nutrition, Food, and Exercise Sciences for the final screening. Subjects signed a consent form after being provided with oral and written descriptions of the study. A complete medical and nutrition history was obtained from all subjects before initiating the treatments. Subjects were advised to maintain their usual physical activity and diet pattern. Those who completed the study were paid a nominal fee for their participation.

Study design

Twenty-nine eligible postmenopausal women out of seventy-one screened volunteers were randomly assigned to one of two treatment groups: 1) control, 2) CC (5 g) as a dietary supplement intervention daily for three months. Additionally, each study participant received 500 mg elemental calcium in the form of calcium carbonate and 5 µg of vitamin D_3 daily. The subjects' compliance was monitored via the following means: the study participants were asked to return any remaining capsules of CC, control and unused calcium/vitamin D pills at their follow-up visit; each individual contacted via telephone on a random date to encourage compliance. Study participants were also provided with a monthly calendar form to record how many CC, control and calcium/vitamin D pills were consumed on a daily basis. The subjects returned their completed calendar forms on the final day of the study.

Dietary and physical activity assessment and anthropometric measurements

For each subject, medical and nutrition histories were obtained at the beginning of the study. Seven-day food frequency questionnaires (FFQ) [18] were used to assess subject diets and dietary supplement intake over the course of one week prior to the baseline and three-month visits. Food Processor SQL Nutrition and Fitness Program software (ESHA Research, Salem, OR) was used to calculate nutrient intake based on data collected from the FFQ. Physical activity patterns, assessed using the Five-City Project Physical Activity Recall [19], and anthropometric data were collected as well. Body mass index (BMI) was calculated as subject weight in kilograms divided by the height in meters squared (kg/m^2) [20].

Bone density assessments

Bone density was assessed at the beginning and at the three-months using dual energy x-ray absorptiometry (iDXA; GE Healthcare Lunar, Madison, WI) equipped with appropriate software for whole body, L1-L4 lumbar spine, and the non-dominant hip BMD. It happens that all subjects in this study were right leg dominant and hence left femoral neck BMD was assessed. Densitometer stability was evaluated by performance of phantom scans on the dates of all data acquisition.

Bone marker measurements

A venous blood sample was obtained after an overnight fast from each subject at the beginning and three-months for bone biomarkers analyses. Blood samples were centrifuged at 3500 g for 15 min at 4°C, serum samples were separated, aliquoted and stored at -80°C until analysis after the study was complete.

BAP, a marker of bone formation, and TRAP5b, a marker of bone resorption, were measured using commercially available ELISA kits (Quidel Biosystems, Mountain View, CA). The reference values for BAP and TRAP5b were 14.2–42.7 U/L and 4.3 ± 1.5 U/L, respectively. The limit of detection (LoD) for the BAP and TRAP5b assays were 0.7 U/L and 0.2 U/L, respectively. Blood levels of calcium were assessed using atomic absorption spectrometry (Perkin Elmer Analyst 100; San Jose, CA) with reference values of 8.5-10.5 mg/dL and LoD of 0.82 mg/dL. Circulating 25(OH)-vitamin D was assessed using a sandwich ELISA (Alpco, Salem, NH) at baseline and three-months. The reference values and LoD for the 25(OH)-vitamin D assay were 20-30 ng/mL and 1.28 ng/mL, respectively.

Serum levels of sclerostin were measured using a human sclerostin EIA kit (TECOmedical; Quidel Corporation, San Diego, CA), following the manufacturer's procedure. Briefly, 25 µL of serum was loaded to each assay well, incubated for five minutes with shaking followed by 24 hours of incubation at 4°C. The optical density was read at 450 nm with a reference wavelength of 590 nm, and values are reported as ng/mL. The reference value for sclerostin in this cohort was 0.69 ± 0.20 ng/mL and LoD for this assay was 0.015 ng/mL.

Statistical analyses

Data were analyzed using ANOVA methods with PROC MIXED in PC SAS (Version 9.1, SAS Institute, Cary, NC) analyzing the main and interaction effects of the two factors, treatment (control or CC) and time (baseline or after treatment). The mean changes in each time point for the control and CC treatment groups were compared by analyzing interaction effects of the two factors, treatment and time, using the SLICE option in an LSMEANS statement. Data are reported as least square mean ± standard error (SE), unless stated otherwise, $P<0.05$ was regarded as statistically significant.

Results

Baseline characteristic and anthropometric measurements

Twenty women (10 controls and 10 CC) completed the study. Figure 1 shows 29 women were randomly assigned to control or CC. The attrition rates were not significantly different between the two treatment groups (approximately 30% in each group). The most common reasons for attrition included noncompliance with study protocol, claims of medical and health related conditions that prevented continued inclusion in the study and personal reasons. Baseline characteristic data for women who completed the study are presented in table 1. Age, years since menopause, height, body mass index and L1-L4 BMD (T-score) were similar at baseline among the treatment groups. The 20 participants who remained in the study adhered to the regimens, as indicated by self-monitoring checklist provided to them on a monthly basis.

Bone mineral density

In terms of BMD, participants who received CC not only did not lose further bone, but rather significantly gained 1% whole body BMD (Figure 2) from baseline. Although CC was able to prevent bone loss in lumbar spine (L1-L4), the percent changes (0.93%) from baseline did not reach the significant level after three months (Table 2). In contrast to the other sites, left femoral neck BMD was not affected by either treatment, but non significant decreases (0.01%) were found in both groups.

Serum biomarkers of bone metabolism

Intake of the CC resulted in no changes versus baseline value for serum BAP levels while in the control group serum BAP levels decreased after three months (Table 3). TRAP5b levels decreased in the CC group at 3 months and the changes versus baseline was more than 1% (Table 3). However, none of the biomarker levels changes reached the significant level. Interestingly, when the ratios of BAP/TRAP5b were compared between these two groups, the CC group showed a significant increase in BAP/TRAP5b ratio changes as compared to the control group (P<0.05, Figure 3). Serum calcium levels and vitamin D levels were also examined before and after supplementation. Neither the CC nor placebo influenced serum calcium and vitamin D concentrations. Figure 4 represents serum concentrations of sclerostin of both groups. No significant differences in values were found between treatments or time; however, percent changes sclerostin of the control group were only 0.7% as compared to the CC group (11%; P=0.367).

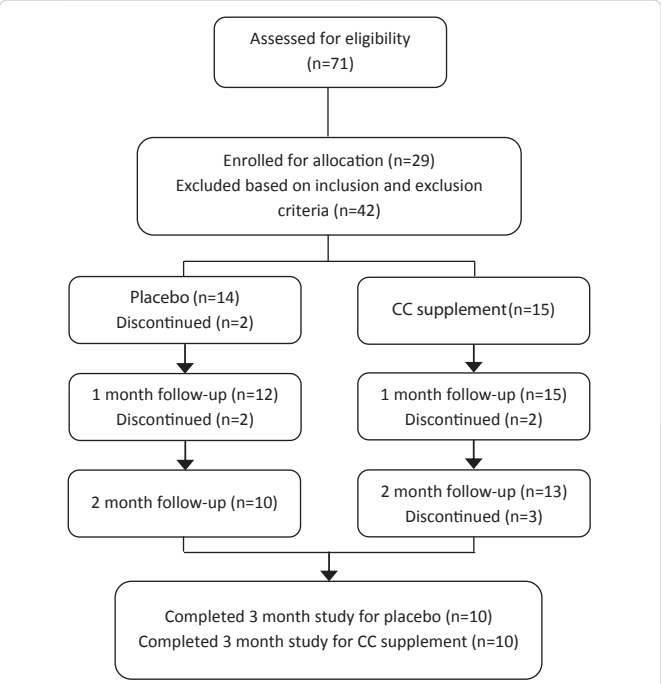

Figure 1: Study flow chart. CC (calcium-collagen chelate).

Table 1: Baseline subject characteristics.

	Control		CC		
	Means	SD	Means	SD	*P*
Age (years)	54.3	1.7	54.9	1.7	0.44
Years since menopause	5.1	1.1	4.4	1.2	0.35
Height (cm)	166.1	7.5	164.9	4.3	0.66
BMI (kg/m^2)	22.3	3.0	24.0	3.6	0.26
Bone mineral density (T-score) L1-L4	-1.6	0.2	-1.5	0.1	0.96

All data are mean ± standard deviation. No significant difference (P<0.05) between groups was observed for any item. The n = 10 for both groups. CC, calcium-collagen chelate; L1-L4, lumbar spine.

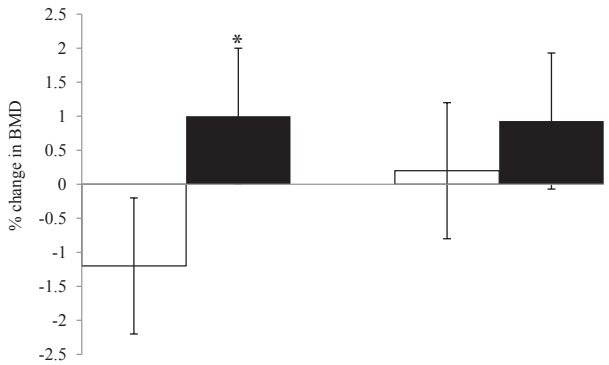

Figure 2: Mean percent changes from baseline values in bone mineral density (BMD) of the whole body and lumbar (L1-L4) after 3 months of supplementation with control (white) or calcium-collagen chelate (black). Bar represent mean ± SE. Asterisk indicates mean values were significantly different between the treatment groups (P<0.05).

Table 2: Effects of calcium-collagen chelate supplementation on bone mineral density.

	Control				CC				
	Baseline		Final		Baseline		Final		
	Means	SE	Means	SE	Means	SE	Means	SE	*P*
L1-L4	0.99	0.01	1.00	0.02	0.99	0.01	1.00	0.01	0.80
Neck left	0.80	0.02	0.80	0.02	0.87	0.01	0.86	0.20	0.08
Total body	1.03	0.01	1.02	0.01	1.08^a	0.02	1.09^b	0.02	0.04

Values represent group bone mineral density (g/cm^2) means ± SE for both groups. Within each group and parameter, values that do not share the same superscript letters are significantly (P<0.05) different from each other. The n = 10 for both groups. CC, calcium-collagen chelate; L1-L4, Lumbar spine; Neck left, left femoral neck.

Discussion

The infrastructure of bone, especially in the trabeculae, is rapidly depleted following five to seven years post-menopause. Our overall findings suggest that supplementing the diets of osteopenic postmenopausal women with this calcium-chelated form of hydrolyzed collagen can improve whole body BMD in a period as short as three months. The data from this study indicate that three months' intake of CC not only retains the bone matrix, but may also suppresses further bone loss. Similar results have been reported in *in vivo* studies using ovariectomized animal models [12-14,21,22]. A recent pilot study with a similar hypothesis on postmenopausal women with osteopenia [9] utilized a bovine, gelatin protein extract (10 g/day) and reported no changes in lumbar BMD after six months, which partially agrees with that of the current study, albeit there are differences in duration. Adam et al. [10] administered a collagen hydrolysate alone, or in combination with calcitonin for 6 months. These investigators reported that collagen may suppress bone resorption by reducing

Table 3: Effects of calcium-collagen chelate supplementation on serum markers of bone metabolism.

	Control					CC					
	Baseline		Final			Baseline		Final			
	Means	SE	Means	SE	*P*	Means	SE	Means	SE	*P*	**P*
BAP (U/L)	29.3	2.18	27.0	1.71	0.16	30.3	1.73	29.6	1.00	0.69	0.47
TRAP5b (U/L)	3.5	0.34	3.2	0.30	0.26	3.5	0.45	3.0	0.31	0.08	0.61
Calcium (mg/dL)	10.7	0.44	9.7	0.32	0.09	9.6	0.04	9.7	0.16	0.67	0.06
Vitamin D (ng/mL)	21.9	3.80	23.1	3.02	0.58	28.6	4.36	25.2	3.08	0.14	0.16

Values are mean ± SE. BAP = bone specific alkaline phosphatase; TRAP5b = tartrate resistant acid phosphatase 5b. The *n* = 10 for both groups. CC, calcium-collagen chelate. **P* represents *P* value between groups.

urinary pyridinoline (PD) and DPD concentrations ($P \leq 0.05$). In OVX mice, three months of 25 g/kg body weight hydrolyzed collagen augmented total body BMD and significantly increased the external diameters of femoral cortical bone [12] via stimulating osteoblast activity. Han et al. [14] reported that 3 g/kg/day of cod bone gelatin for three months increased ($P<0.05$) femoral neck and proximal tibial BMD in OVX rats. Furthermore, animals in the treatment group (3 g/kg/day) had a 30.4% higher ($P<0.05$) tibial BV/TV than OVX control animals. These studies provide clear evidence that hydrolyzed collagen improves BMD and are supportive of our findings that CC can rapidly increase bone mass.

A unique component of this study was the observation of improvements over a three-month period. One may question the actuality of changes in bone after only three months of treatment; however, data from a recent study on bed-rest in women suggest that changes in trabecular density and cortical thickness can be detected as early as ninety days due to certain treatments [23]. Similarly, Rizzoli et al. [24] demonstrated that three-month treatment of strontium ranelate, a prescribed dual-action bone agent, can significantly improve cortical thickness and trabecular bone volume, as measured by high-resolution peripheral quantitative-CT. Although CC may not be as potent as teriparatide [rhPTH (1-34)] [25], an approved bone forming medication, it was able to increase total body BMD (Figure 2) after three months. This bone forming ability of CC surpasses that of the combination of calcium and vitamin D. In the present study, women receiving calcium and vitamin D not only did not gain bone, but rather lost more than 1% whole body BMD. These data suggest a rapid effect on bone resulting from CC supplementation and supports the notion of a synergistic mechanism between calcium, vitamin D, and protein, in which consumption of these three nutrients together exerts a positive stimulatory effect on bone [26]. Another factor that might have added to the potency of CC may be due to its unique formulation by chelating calcium to collagen.

High circulating BAP is indicative of bone formation [27], while elevated TRAP5b levels correspond to the increased activity of osteoclasts resulting in bone resorption. The BAP to TRAP5b ratio changes were significantly greater by 10% in women taking CC, depicting a greater rate of bone formation in the treatment group. Yet, this preparation may be acting uniquely on bone, as the effect of collagen hydrolysates alone may not be as significant. For instance, Cuneo et al. [9] gave 10 g/day collagen hydrolysate for six months but found no alterations in serum BAP, C-TX, and osteocalcin, or BMD. The authors suggested that a reason for this lack of effect could be due to inadequate calcium intake by subjects. CC provides calcium and collagen at the same time in a unique bioavailable form. The supplement may allow a synergistic effect that hydrolyzed collagen alone does not. This may explain why CC improved BMD and BAP/TRAP5b ratios at 5 g/day in this study, while hydrolyzed collagen failed to do at 10 g/day. The bone protective effect of CC in part may stem from the suppression of the production of sclerostin; however, the present data are unable to fully support this argument due to a lack of statistical significance in the study comparison (Figure 4). There are a number of reports [28,29] suggesting that sclerostin impairs bone formation, and it may correlate with advancing osteoporosis. The specific mode of action of sclerostin is largely inconclusive, but it is believed that it may serve, at least in part, as a Wingless-

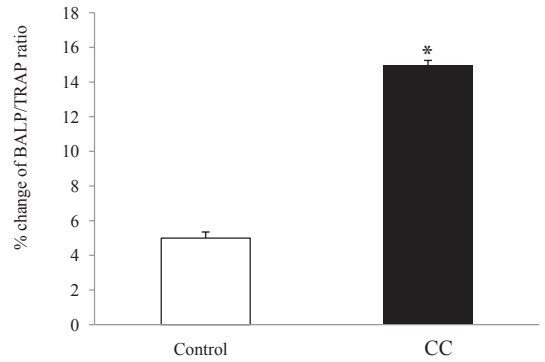

Figure 3: Changes in BAP/TRAP5b ratio (mean ± SE) relative to baseline. Asterisk indicates significant main effect of CC (calcium-collagen chelate) (black), on change in BAP/TRAP5b ratio after 3 months ($P=0.03$) in comparison with control (white). Bone alkaline phosphatase (BAP), tartrate-resistant acid phosphatase (TRAP5b).

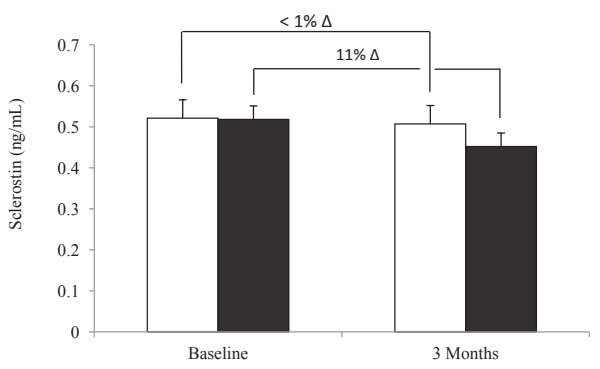

Figure 4: Sclerostin concentrations in serum (mean ± SE) at baseline and after 3-month's treatment with the calcium-collagen chelate (black). Control (white), Δ symbolizes the change following three months.

Int/β-catenin signaling antagonist [15,16], and a master regulator of inhibitory, mineralizing glycoproteins such as matrix extracellular phosphoglycoprotein [30]. Consequently, greater apoptosis of osteoblasts and reduced ALP activity and less bone mass occur [31]. Thus, this calcium-collagen product seems to be more efficient for improving bone parameters in our study; however, further testing is needed to confirm our findings.

The mechanism by which CC promotes bone health is unknown, but in terms of its collagen portion, a trend in the former and current literature imply that from a macro viewpoint, hydrolyzed collagen of different derivations primarily increase osteoblastic activity e.g. greater local ALP [14,21] and type I collagen production [22], but not osteocalcin, a marker of bone turnover [11,14]. From a molecular view, one study indicated that hydrolyzed collagen may down-regulate, whether directly or indirectly, RANKL [14], which is responsible for induction of osteoclast differentiation followed by bone degradation [32]. A potentially more plausible postulate was presented in a recent study which indicated that hydrolyzed collagen, specifically which of low molecular weight, may act by significantly enhancing the organic substance content of bone [33]. In fact, though the greatest amounts accumulated in the liver and kidney, the distribution of radioactive hydrolyzed collagen appeared high in rat femora and tibiae, peaking at six hours following ingestion. These findings agree with those of a 14-day co-culture analysis of osteoblasts and osteoclasts in which hydrolyzed collagen of bovine, porcine, and fish origin stimulated osteoblast activity [12]. Concentrations of 0.5–1.0 mg/mL of all three collagen types at molecular weights of 2 kDa significantly ($P<0.05$) enhanced ALP activity compared to the same collagens at 5 kDa and control. The researchers suggested that smaller collagen peptides may readily interact with bone cells. Pro-Hyp and other Hyp-containing peptides, essential in type I collagen composition, were suggested to remain intact even through the gastrointestinal tract following ingestion [34,35], thus leaving it available for potential activity with bone. *In vitro* and *in vivo* studies are warranted to elucidate CC's mode of action.

Conclusions

The results of this study indicate that CC supplementation has the potential to reverse bone loss in osteopenic postmenopausal women. However, the questions remain unanswered as to how long this bone forming ability of CC will continue, when its effect will plateau, and how long the gain in BMD last after the cessation of taking the CC supplement. Studies are also needed to evaluate the mechanisms of action by which CC increases bone mass.

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