Published in final edited form as:

Lipids. 2014 March ; 49(3): 211–224. doi:10.1007/s11745-013-3872-5.

Conjugated linoleic acid prevents ovariectomy-induced bone loss in mice by modulating both osteoclastogenesis and osteoblastogenesis

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Abstract

Postmenopausal osteoporosis due to estrogen deficiency is associated with severe morbidity and mortality. Beneficial effects of conjugated linoleic acid (CLA) on bone mineral density (BMD) have been reported in mice, rats and humans, but the effect of long term CLA supplementation against ovariectomy-induced bone loss in mice and the mechanisms underlying this effect have not been studied yet. Eight weeks old ovariectomized (Ovx) and sham operated C57BL/6 mice were fed either a diet containing 0.5% safflower oil (SFO) or 0.5% CLA for 24 weeks to examine BMD, bone turn over markers and osteotropic factors. Bone marrow (BM) cells were cultured to determine the effect on inflammation, osteoclastogenesis, and osteoblastogenesis. SFO/Ovx mice had significantly lower femoral, tibial and lumbar BMD compared to SFO/Sham mice; whereas, no difference was found between CLA/OVX and CLA/Sham mice. CLA inhibited bone resorption markers whereas enhanced bone formation markers in Ovx mice as compared to SFO fed mice. RT-PCR and FACS analyses of splenocytes revealed that CLA inhibited pro-osteoclastogenic RANKL and stimulated decoy receptor of RANKL, OPG expression. CLA also inhibited pro-inflammatory cytokine and enhanced anti-inflammatory cytokine production of LPS-stimulated splenocytes and bone marrow cells. Furthermore, CLA inhibited osteoclast differentiation in BM and stimulated osteoblast differentiation in BM stromal cells as confirmed by TRAP and Alizarin Red staining, respectively. In conclusion, CLA may prevent postmenopausal bone loss not only by inhibiting excessive bone resorption due to estrogen deficiency but also by stimulating new bone formation. CLA might be a potential alternative therapy against osteoporotic bone loss.

Keywords

Osteoporosis; ovariectomy; bone mineral density; osteoclastogenesis; osteoblastogenesis; inflammation

Introduction

Estrogen deficiency occurring either naturally or surgically, predisposes women to the development of postmenopausal osteoporosis, a major health problem that leaves patients at risk for osteoporotic fractures which have been associated with severe morbidity and mortality (1). Ovariectomized animals are widely used as a model for this disease (2). Estrogen and/or hormone replacement therapies (ERT and/or HRT) were commonly used to

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Conflict of interest: The authors have no conflict of interest to declare.

prevent bone loss associated with postmenopausal osteoporosis. However, these treatments incurred adverse side-effects such as uterine, ovarian and breast cancer, and increased risk of cardiovascular diseases (3, 4). In order to avoid potential side effects associated with hormone therapies, maintaining dietary therapy and/or lifestyle changes have been considered promising and viable alternatives to minimize and treat bone loss in postmenopausal women.

Over the last several years, considerable attention and research has focused on potential benefits of dietary CLA. For example, it may act as anti-carcinogenic and have anti-tumorogenic effects (5), reduce the risk of atherosclerosis, hypertension and diabetes, promote energy metabolism and body weight management (6-11), as well as improve immune function (12, 13) and musculoskeletal health (14-26). Ruminant food products such as beef, lamb, and dairy products naturally contain CLA isomers (27). It should be noted that the this study makes use of a CLA preparation that combines cis-9,trans-11 (c9t11) and trans-10,cis-12 (t10c12) CLA isomers (50:50 CLA). The cis-9,trans-11 CLA isomer is the most common in nature, and consists of approximately 80–95% of the CLA found in food sources (28). However, when CLA is prepared by chemical processes from linoleic acid, the trans-10,cis-12 isomer is comparatively more present than in nature, and consists of approximately 50% of the CLA. This preparation is referred to as ‘50:50’ CLA mixture, which most CLA studies have used previously (17). Although it has been demonstrated that CLA exerts a beneficial role on bone and muscle health through the trans-10,cis-12 CLA isomer (14, 15), previous studies suggested that the overall bioactivity of CLA might rely on the interaction between both of these isomers (6). It is possible that the cis-9,trans-11 isomer may work as an antagonist to the trans-10,cis-12 CLA isomer which decreases insulin sensitivity (28).

Dietary CLA may help maintain healthy BMD in postmenopausal women (29). Previous studies have noted positive effects in bone health (14, 16-19), and increased whole body-ash in mice fed CLA supplementation (30). CLA treatment is associated with decreased bone resorption markers, osteoclastogenic genes, and signaling molecules in osteoclastic cell line (18, 24), increased markers of bone formation, such as osteocalcin and alkaline phosphatase in murine osteoblastic cell line (31), increased formation of mineralized bone nodules in human osteoblast-like cells (25) and increased osteoblast and decreased adipocyte differentiation in human mesenchymal stem cells (26). Furthermore, young chicks fed anhydrous butterfat, a natural source rich in CLA, showed increased rate of bone formation through reducing prostaglandin (PG) E₂, which mediates bone formation and resorption (32), while CLA is known to increase bone mass in a number of different mammals (mice (30); pigs (33)), others have not been able to confirm this finding (rats (34, 35); pigs (36)). Given that CLA modulates PGE₂ activity, it is possible that CLA supplementation could reduce bone loss in postmenopausal women through this mechanism. The majority of the previous studies are of short term (4-12 weeks) and do not include data on bone loss in ovariectomized mice fed CLA alone (11, 37). There was a report of beneficial effects from short-term treatment of CLA (9 weeks) against ovariectomy-induced bone resorption in rat (21). However, the long-term effects and associated mechanisms have not yet been studied. Hence, this study examines the effect of long-term administration of CLA in a mouse model of postmenopausal bone loss (38) and the mechanisms underlying this effect.

Materials and Methods

Reagents

Receptor activator of NF- κ B ligand (RANKL) was purchased from Pepro-Tech (London, United Kingdom). Reagents for mouse TNF- α , IL-6, IFN- γ and IL-10 ELISA were purchased from BD Bioscience (San Diego, CA, USA). Corn oil (CO) was from MP

Biomedicals (Aurora, OH, USA). Clarinol A-80, a free fatty acid mixture high in isomers of CLA (the two main isomers present are cis-9, trans-11 (c9,t11) and trans-10, cis-12 (t10,c12) CLA, in a 50:50 ratio) and SFO oil was obtained from Loders-Croklaan, Channahon, USA. RPMI 1640 medium was from Cellgro (Herndon, VA, USA). All other media components were from GIBCO (Invitrogen Corp., Carlsbad, CA, USA). Fatty acids (CLA c9t11, CLA t10c12, LA) were from Cayman Chemicals Company (Ann Arbor, MI, USA). Equal amounts of c9t11, and t10c12 isomers were used for all experimental diets and for *in vitro* cultures. RNA extraction reagent, TRIZOL was from Invitrogen life technologies (Carlsbad, CA, USA). Reverse transcription kit was from Promega Corp. (Madison, WI, USA). TRAP solution (Cat # 387A) and lipopolysaccharide (LPS) from E. Coli 055:B5 (Cat # L2637) were obtained from Sigma Chemical Co. (St Louis, MO, USA).

Animals and experimental diets

Six-week-old C57BL6/J female mice were obtained from Harlan, Indianapolis, Indiana, USA. Weight matched mice were housed in cages (5 mice/cage) in a laboratory animal care facility and fed a standard lab chow diet. At 8 weeks of age, 10 mice were OVX and 10 mice were sham operated and divided into 2 dietary groups provided with semi-purified AIN-93 diets containing either 0.5% SFO or 0.5% CLA (cis-9, trans-11 and trans-10, cis-12 in a 50:50 ratio). The composition of the semipurified diets is shown in Table 1 (10). Mice were fed diet for 24 weeks. Fresh diet was prepared weekly, stored in aliquots at -20°C and provided daily *ad libitum*. This study abided by the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal protocol (10027X) was approved by the Institutional Laboratory Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio.

Measurement of bone mineral density (BMD)

Mice were anesthetized with ketamine/xylazine and dual energy x-ray absorptiometry (DXA) scan was performed as described previously (39, 40) at baseline (8 weeks) (before administration of diet and ovariectomy) and after 24 weeks on experimental diets using a Lunar PIXImus mouse bone densitometer (General Electric). BMD was analyzed manually with PIXImus software as described previously (39, 40).

Collection of tissues and blood serum

After sacrificing the mice serum was collected by centrifuging blood at 3000 rpm for 15 minutes at 4°C and aliquots were stored at -80°C . Spleens were isolated and processed for isolation of splenocytes as described previously (41, 42).

Flow cytometric analysis of RANKL expression in splenocytes

Single cell suspensions of spleen were prepared as described previously (41, 42). Cells were analyzed for the expression of the CD4, CD8, and RANKL cell surface determinants, using Abs conjugated to fluorescein, PE, or PerCP (all from BD Bioscience). The optimal working dilution was standardized for each reagent before use. Before staining for cell surface markers, Fc γ II/III receptors on cells were blocked by incubating the samples with a rat anti-CD32/16 antibody (Fc receptor block, BD Bioscience). Cells were then incubated with the anti-RANKL in the presence or absence of anti-CD4 or anti-CD8 antibodies for 30 min at 4°C . Cells were then washed with wash buffer containing 1% BSA and 0.02% sodium azide. The cells were re-suspended in wash buffer and then analyzed on a FACScan (Becton Dickinson) flow cytometer. Data analysis was performed using Cell Quest Software (version 3.0, BD Bioscience) (42).

RT-PCR analysis of splenocytes

Total RNA was extracted from part of the isolated splenocytes using TRIZOL according to the manufacturer's instructions and RT-PCR analysis was performed as previously described (43). The following primers were used: **RANKL**: 5'-TTT GCA GGA CTC GAC TCT GGA G and 5'-TCC CTC CTT TCA TCA GGT TAT GAG; osteoprotegerin (**OPG**): 5'-ATC ATT GAA TGG ACA ACC CAG G and 5'-TGC GTG GCT TCT CTG TTT CC; **GAPDH**: 5'-GAT CGT GGA AGG GCT AAT GA and 5'-GAC TTT GCC TAC AGC CTT GG. The amplified PCR products were subjected to 1.5% agarose gel electrophoresis and visualized by UV fluorescence after staining with ethidium bromide. Densitometric analysis of bands was done using AlphaImager Imaging System (Alpha Innotech Corp., San Leandro, CA). The same cDNA was amplified using primers specific for GAPDH mRNA as an internal control for RNA quantity.

Serum cytokines measurement

Serum cytokine levels for TNF- α , IL-6, and IFN- γ were measured by standard ELISA techniques as described previously (17).

Splenocyte culture

A portion of the previously isolated splenocytes were used to culture in the presence of LPS. Cells (1×10^6 cells/well) were plated in 96-well plates, and LPS was added at a concentration of 5 μ g/ml for 24 h. Culture media were collected and analyzed for TNF- α , IL-6 and IL-10 as described previously (41).

Serum bone turnover markers measurement

Bone resorption markers, receptor activator of NF- κ B ligand (RANKL), and tartrate resistant acid phosphatase (TRAP)-5b and bone formation marker, N-terminal propeptide of type I procollagen (PINP) were measured in serum samples using mouse free soluble RANKL, mouse OPG, mouse TRAP5b, mouse PINP assay kits from Immunodiagnostic System (IDS) Inc. (Fountain Hills, AZ, USA) and bone formation marker, alkaline phosphatase (ALP) was measured in serum samples using Quantichrome alkaline phosphatase assay kit from Bioassay Systems (Hayward, CA, USA) according to the manufacturer's instructions (17).

Isolation of whole bone marrow (BM) cells and culture

Whole BM cells were aseptically isolated as described previously (43). Cells (5×10^6 /well) were plated in 24 well plates and LPS was added at a concentration of 5.0 μ g/ml for 24 h. Culture media were collected and analyzed for TNF- α , IL-6 and IL-10 as described previously (41, 44).

Cytokine measurement in BM and splenocytes culture supernatants

TNF- α , IL-6, and IL-10 were measured by ELISA using BD OptEIA™ ELISA kits from BD Biosciences Pharmingen (San Diego, CA) according to the manufacturer's instruction.

Osteoclast differentiation in BM cultures

BM cells from the tibias and femurs of 6 weeks old C57BL/6 female mice were collected and cultured as described previously (43). Briefly, cells were suspended in α -MEM containing 15% fetal calf serum (FCS) and cultured in 48-well plates (1×10^6 cells/ml). Osteoclast differentiation was induced in the presence of macrophage colony-stimulating factor (M-CSF) (20 ng/ml) and sRANKL (30 ng/ml) with vehicle control (ethanol) or CLA for 5-days. Concentration of CLA used as an effective dose in this experiment based on

previous publications (18, 24, 26). Fresh media, factors and fatty acid are replaced on Day 3. At the end of the culture, the cells were fixed and then stained with a commercial kit for TRAP (no. 387A; Sigma), a marker enzyme for osteoclast.

Osteoblast differentiation in BM stromal cell cultures

BM stromal cells are generated from whole BM cells isolated from the femurs and tibias of 4-6 week old C57BL/6 female mice obtained from Jackson Laboratories (Bar Harbor, Maine). Cells isolated from two mice were plated in alpha-MEM, supplemented with 10% FCS and 1% penicillin-streptomycin (Sigma Aldrich, St. Louis, MO) in a T₇₅ culture flask, and incubated at 37°C at 5% CO₂. Cells were allowed to grow to confluency and split at 1:2 ratios for minimum of 6 weeks. Remaining viable cells generally are of BM stromal type lineage. BM stromal cells were then plated at a density of 2×10^4 cells per 100ul in a 96 well plate in alpha-MEM, supplemented with 10% FCS. After 24 hours of incubation, media were replaced with osteogenic media (supplemented with 50 µM of Ascorbic Acid and 10 mM of β-Glycerophosphate (Sigma Aldrich, St. Louis, MO)) with vehicle control or CLA and incubated for an additional 7-10 days, with media, factors and fatty acid changes every 2 days. Plates were then washed with PBS and cells were fixed with 10% formalin by incubating the plates at RT for 15 minutes. Cells were then washed with PBS 3x, avoiding to disturb the fragile monolayer of cells. After the final aspiration, 50 µl of Alizarin Red S Solution (Alizarin Red S 2g, distilled water 100ml, adjusted pH 4.1 to 4.3 using 0.5% ammonium hydroxide) (Sigma Aldrich, St. Louis, MO) for 20 minutes at RT. Wells were then carefully washed with distilled water and Alizarin Red S stained OD intensity was determined by histomorphometry using the Metaview Image Analysis System software.

Osteoblast differentiation of C3H10T1/2 Cells

C3H10T1/2 cells purchased from American Type Culture Collection (Rockville, MD) were plated at a density of 2×10^3 cells per 500 µl in a 48 well plate in alpha-MEM, supplemented with 10% FCS. After 24 hours of incubation, media were replaced and the osteogenic differentiation factor (BMP-2 100 ng/ml) (Sigma Aldrich, St. Louis, MO) was added with vehicle or CLA and incubated for an additional 10 days. Media, factors and fatty acid were changed every 3 days. Cells were then washed with cold PBS and fixed with 10% formalin. Cells were again washed with PBS 2x and incubated in 500 µl of Alkaline Phosphatase Solution (1 BCIP/NBT tablet to 10ml distilled water)(Sigma Aldrich, St. Louis, MO) for 10-15 minutes at room temperature, or until desired staining was achieved. Wells were then carefully rinsed with PBS/20mM EDTA and alkaline phosphatase stained OD intensity was analyzed by histomorphometry using the Metaview Image Analysis System.

Mineralized bone matrix formation in α-smooth muscle actin (SMA) positive bone marrow stromal cell (BMSC)

α-SMA-BMSC generously donated by Dr. Harris were plated at a density of 200,000 cells per well of 12 well plate in 2 ml of α-MEM medium supplemented with 15% FCS and cultured for 4 days. Medium was then replaced with osteogenic medium (supplemented with 100 µg/ml ascorbic acid, 5 µM β-glycerophosphate and 20 ng/ml BMP-2) and cultured for another 2 weeks in the presence or absence of 50 µM CLA. Cultures were changed with fresh medium, factors and fatty acid every other day. Cells were washed in PBS 2x, then fixed in 10% formalin and again washed 2x in PBS. Cells were then dehydrated serially with 80%, 95% and 100% ethanol and air-dried. Cells were then rehydrated serially with 100%, 95% and 80% ethanol and finally to water. 2% silver nitrate solution were added to the wells and exposed to sunlight for 15 minutes. The plates were then rinsed with water and incubated with 5% sodium thiosulfate for 3 minutes. Plates were again rinsed with water and incubated with acid fuchsin solution for 5 minutes. Plates were then washed with 95% ethanol twice and 100% ethanol twice and air-dried. Histomorphometry was done by

measuring optical density of vonkossa stained area using the Metaview Image Analysis System.

Statistical analysis

Data are presented as mean values $\pm$ S.E.M. Differences among the groups were tested by one-way analysis of variance and student's t-test. Newman-Keuls post hoc test were used followed by ANOVA if multiple correlations were made. Two way ANOVA was used to determine the effect of Diet, surgery and the interactive effect of diet and surgery among the groups. A p-value less than or equal to 0.05 was considered to be statistically significant. The analyses were performed with Graphpad prism for Windows (La Jolla, CA).

Results

Effect of CLA on body weight and BMD

There was no difference in initial average body weights among the groups (data not shown). After 24 weeks on diet body weight of SFO/Sham, SFO/Ovx, CLA/Sham and CLA/Ovx were 50.35 ± 2.74 g, 47.90 ± 1.56 g, 42.94 ± 2.09 g and 41.00 ± 1.78 g respectively. From these data, the body weights of CLA-fed mice were found to be significantly lower compared to SFO fed mice. However, there was no significant increase in body weight due to ovariectomy in either group. Daily food consumption was monitored. There was slight decrease in food consumption in CLA groups but not significant. The food consumption of SFO/Sham, SFO/Ovx, CLA/Sham and CLA/Ovx were 4.1 ± 0.17 g/day, 4.2 ± 0.19 g/day, 3.7 ± 0.09 g/day, 3.9 ± 0.15 g/day respectively. An increase in lean mass was observed in CLA fed mice as compared to SFO fed mice. No differences were observed in baseline BMD of different bone regions prior to Sham and Ovx surgery using DXA (data not shown). However, after 24 weeks of experimental diets, BMD of SFO/OVX mice was significantly lower in femur, tibia and lumbar vertebra compared to SFO/Sham mice (Table 2). On the contrary, no difference in BMD was noted in all of these regions between CLA/OVX and CLA/Sham mice (Table 2). BMD in different regions were significantly higher in CLA/Ovx mice when compared to that of SFO/Ovx mice. Multiple comparisons using two-way ANOVA revealed that CLA had a very significant effect over SFO on BMD loss protection. However, ovariectomy alone showed significant BMD loss only in PTM and Lumbar 2 region. There was no significant interaction between diet and surgery found. The uterine wet weight was measured at the time of sacrifice to confirm estrogen levels of OVX and Sham mice. Uterine weight of both SFO and CLA fed groups was significantly decreased in mice with ovariectomy performed at 8-weeks of age (Data not shown).

Effect of CLA on serum cytokines and bone turnover markers

Serum TNF- α level was significantly lower in CLA fed mice compared to SFO fed mice both in sham and OVX groups (Table 3). No significant difference was noted in serum IL-6 level among the groups. Serum IFN- γ level was significantly higher in CLA/Ovx mice as compared to SFO/Ovx mice. These data indicate that CLA may modulate inflammation. Serum levels of bone resorption markers, RANKL and TRAP5b and bone formation markers, PINP and ALP, were measured. In regards to bone resorption markers, RANKL is one of the most critical osteoclastogenic factors (45, 46), and serum TRAP5b levels are used as indicators of the status of osteoclasts function (14). Interestingly, both serum RANKL and TRAP5b levels were significantly less in CLA/Ovx mice than in SFO/Ovx mice (Figure 1A and 1B). No difference was observed between the sham groups. On the contrary, serum bone formation markers PINP and ALP were significantly higher in CLA/Ovx mice as compared to the SFO/Ovx mice (Figure 1C and D). Furthermore, bone formation markers were slightly higher but non-significant in CLA/Sham mice as compared to SFO/Sham mice. These

results indicate that CLA may prevent BMD loss in Ovx mice by modulating both bone resorption and bone formation.

Effect of CLA on osteoclast modulatory gene expression in splenocytes

Two major osteoclastogenic factors, RANKL and OPG were analyzed by RT-PCR in splenocytes from different groups of mice. Significantly decreased mRNA level of RANKL and increased mRNA level of OPG were found in CLA/Ovx mice as compared to SFO/Ovx mice (Figure 2A and B). Similarly, RANKL protein expression in isolated splenocytes by FACS analysis was also significantly lower in CLA/Ovx mice compared to SFO/Ovx mice (Figure 2C). RANKL positive T cell sub-population in isolated splenocytes were also found lower in CLA/Ovx mice compared to SFO/Ovx mice (Figure 2D and 2E). These data suggest that CLA suppresses the expression of RANKL whereas enhance the expression of OPG by which CLA may inhibit osteoclastic bone resorption.

Effect of CLA on LPS stimulated inflammatory cytokine production by bone marrow cells and splenocytes

Pro-inflammatory cytokines such as TNF- α and IL-6 have been shown to increase bone resorption by regulating osteoclastogenic activity (47). Interestingly, there were significantly lower levels of pro-inflammatory, TNF- α and IL-6 cytokines production by LPS stimulated BM and splenocytes collected from CLA/Ovx mice as compared to that of SFO/Ovx mice (Figure 3A, 3D, 3B and 3E). However, in inflammatory disorders, the anti-inflammatory cytokine IL-10 has been reported to inhibit bone resorption (48). Significantly higher level of IL-10 cytokine production by LPS stimulated BM and splenocytes collected from CLA/Ovx mice was observed as compared to that of SFO/Ovx mice (Figure 3C and 3F). These data indicate that CLA probably maintains better BMD by suppressing bone resorption due to reduced production of pro-inflammatory, pro-osteoclastogenic cytokines as well as increased production of anti-inflammatory, anti-osteoclastogenic cytokines.

Effect of CLA on osteoclasts and osteoblast differentiation

CLA dose-dependently inhibited MCSF and RANKL stimulated TRAP positive multinucleated osteoclasts formation in whole bone marrow cell culture (Figure 4A and 4B). This data indicates that CLA may prevent bone loss by inhibiting osteoclastogenesis. CLA dose-dependently enhanced ascorbic acid and β -glycerophosphate stimulated osteoblast formation in BM stromal cell culture confirmed by Alizarin Red staining (Figure 4C and 4D). This data indicates that CLA may prevent bone loss by enhancing osteoblastogenesis. CLA dose-dependently enhanced BMP-2 stimulated osteoblast formation in pre-osteoblastic C3H10T1/2 cell culture confirmed by ALP staining (Figure 4E and 4F). This data indicates that CLA may prevent bone loss by enhancing osteoblastogenesis. CLA enhanced mineralized bone matrix formation in α -SMA positive BMSC in the presence of ascorbic acid, β -glycerophosphate and BMP-2 as confirmed by nodule formation assay (Figure 5A and 5B). Mineralized bone matrix formation (black stain) was observed surrounded by well formed collagen (pink stain) in CLA treated culture (Figure 5A). These data indicate that CLA may prevent bone loss by enhancing bone formation.

Discussion

Estrogen prevents bone loss through a complex set of mechanisms that ultimately reduce osteoclast formation and enhance their apoptosis, thereby reducing the ability of these cells to resorb bone. (47, 49-53). In the present study, CLA prevented ovariectomy-induced bone loss by attenuating osteoclastogenesis (bone resorption) as well as by enhancing osteoblastogenesis (bone formation). This is the first study to demonstrate the beneficial

effect of long-term supplementation of CLA against ovariectomy-induced bone loss in C57BL/6 mice.

CLA is well known for its capacity to reduce fat mass and body weight (10, 11, 54). In this study, CLA fed groups had significantly lower body weights when compared to SFO fed groups. Previous studies suggest that CLA may have a beneficial role in preventing bone loss (17,21,22,55), in part by minimizing bone loss through a prostaglandin-mediated process (32,55) or by enhancing calcium absorption (35). Moreover, CLA supplementation reduced the rate of bone resorption in adult OVX rats (21), and has been shown to improve immunity and bone formation (29). Very recently, Park et al. demonstrated that CLA improved bone health of ovariectomized ICR mice supplemented with calcium (37). However, not all have been able to confirm the beneficial effects of dietary CLA supplementation. For example, Doyle et al. reported that markers of calcium or bone metabolism were not affected by short-term CLA supplementation (56) and Kelly et al. reported that short-term CLA supplementation of omega-3 PUFA-rich diet enhanced calcium absorption in young growing rats, but had no assessable effect on bone metabolism or bone mass (35). In this study, long-term supplementation of CLA prevented the ovariectomy-induced reduction in BMD not only by inhibiting osteoclastogenesis, but also by stimulating osteoblastogenesis.

Both animal and *in vitro* studies have explored the effects of CLA on osteoblastic factors and bone mineralization (25, 31, 35). In a randomized, double-blind, placebo-controlled trial, CLA supplementation exhibited enhanced serum ALP levels (57). In a cross-sectional study, 4 months dietary c9t11-CLA isomer was found positively related to BMD (58). It has been previously demonstrated that CLA inhibited osteoclastogenesis (18). In this study, CLA reduced bone resorption markers as well as enhanced bone formation markers in serum suggesting that CLA might protect Ovx-induced bone loss by modulating both bone resorption and bone formation. *In vitro* studies with whole bone marrow cells for osteoclast differentiation and bone marrow stromal cells for osteoblast differentiation, and mineralized bone matrix formation support these *in vivo* findings suggesting that CLA inhibits bone resorbing osteoclast formation and stimulates bone forming osteoblast differentiation and mineralization. Previous studies demonstrated that t10c12-CLA could attenuate bone marrow adiposity (59, 60). Platt et al demonstrated that t10c12-CLA favors osteoblast differentiation than adipocyte differentiation in human mesenchymal stem cell (26). Further, Kim et al showed that t10c12-CLA could promote bone formation by inhibiting adipogenesis and by directly enhancing osteoblastogenesis from bone marrow mesenchymal stem cells (23). This suggests that CLA may prevent bone loss by stimulating new bone formation as well as inhibiting bone resorption.

Activated T-cells secrete RANKL, which binds to the RANK receptor and promotes osteoclast formation and activation, thereby prolonging osteoclast survival and suppressing apoptosis (61). RANKL is secreted by activated T-cells and is a key contributor to inflammation-induced bone destruction (62). RANKL and OPG are the master regulators of osteoclast differentiation (63, 64). There are evidences that anti-osteoclastogenic effects of estrogen include its ability to stimulate the production of OPG (65) by blunting the production of TNF- α (47, 66) which stimulate the stromal cell production of M-CSF and RANKL (67, 68). Therefore, altering the RANKL/OPG ratio is a critical mechanism in evaluating the pathogenesis of bone diseases (64, 69). In this study, CLA/Ovx mice showed a reduction of RANKL expression and an enhancement of OPG expression in splenocytes when compared to SFO/Ovx mice. Thus, altering the RANKL/OPG expression ratio may lead to a reduction in osteoclast formation and associated bone destruction, and Ovx-induced BMD loss prevention by CLA might be in part due to this mechanism.

The cytokines IL-6, IL-1 and TNF- α are involved in mechanisms of bone remodeling and these markers of inflammation are involved in the pathogenesis of osteoporosis (70). In addition, many of these effect of cytokine-mediated bone pathology are driven by the impact of these cytokines on differentiation of and activity of osteoclasts (71). Pro-inflammatory markers have been shown to enhance osteoclast-mediated bone resorption by acting on mesenchymal stem cells and osteoclast precursors. These cytokines increase bone resorption, and are key regulators of osteoclastogenic activity (47, 72). Furthermore, these cytokines induce the expression of COX-2 in osteoblastic and stromal cells, which increases production of PGE₂, a potent stimulator of osteoclastogenesis (73). In a recent study, Barbour et al showed that elevated levels of inflammatory markers for all three cytokine-soluble receptors were associated with an increased risk of hip fractures in older women (70). In this study, OvX induced serum TNF- α production was suppressed in CLA supplemented group suggesting potential anti-inflammatory and anti-osteoclastogenic effect of CLA via down-regulating the production of TNF- α . Although there was no significant difference in serum IL-6 levels across groups, lower levels of both TNF- α and IL-6 in LPS-stimulated BM and splenocytes were found in CLA-fed/OVX mice when compared to that of SFO fed OvX mice, which, in part, could explain the mechanisms of preventing BMD loss by CLA in these mice. These data are consistent with previous findings that CLA may decrease levels of these cytokines in vivo and in vitro (17, 18, 74). A decreased activity of IL-6 and TNF- α may also explain the lower level of the bone resorption marker, TRAP in CLA-fed mice observed in this study. Furthermore, these data also show increased LPS-stimulated production of IL-10 in BM cells and splenocytes in CLA-fed mice compared to SFO-fed mice. Very recently, McCarthy et al. demonstrated that the immunoregulatory response of CLA on regression of atherosclerosis is associated with enhanced production of IL-10 (75). The anti-inflammatory cytokine, IL-10 reportedly decreases osteoclastogenesis and plays a critical role in regulation of pro-inflammatory cytokine levels in vivo (76, 77). Furthermore, IL-10 blocks the effects of one of the most important inflammatory signaling regulators, p50 and p65 NF- κ B heterodimers in human monocytes (78). Up-regulation of IL-10 may also be one of the mechanisms by which CLA suppresses bone resorption. IFN- γ derived from CD4⁺ T cells also known to suppress osteoclast differentiation (79). IFN- γ suppresses osteoclast differentiation by incurring a negative feedback loop for RANKL-mediated osteoclast activation (79). LPS-stimulated IFN- γ production in BM cells or splenocytes was not examined in this study. However, the serum IFN- γ data showed that CLA/OvX mice had significantly higher level of IFN- γ as compared to that of SFO/OvX mice. In fact, the data showed lower levels of serum RANKL in CLA/OvX mice, which supports the notion that IFN- γ exerts its anti-osteoclastogenic effect by interfering with RANKL signaling. These findings suggest that CLA prevents OvX-induced bone loss by suppressing osteoclastic bone resorption mainly by modulating inflammatory cytokine production and associated signaling.

Conclusions

This study demonstrates the novel action of CLA as a modulator for both osteoclastogenesis and osteoblastogenesis in order to combat estrogen deficiency induced bone loss. In addition to the beneficial effect of bone, CLA supplementation reduced body fat mass and improved muscle mass. The currently available anti-osteoporotic drugs act mainly as anti-bone resorptive agents without affecting new bone formation. These drugs are able to inhibit bone resorption without inducing new bone formation, thereby increasing the bone mass mainly of old un-resorbed bone which may not be strong enough against fragile fracture. Very recently, strange fractures are reported in long-term bisphosphonate treated postmenopausal women, which thus raised the question as to whether these adverse effects are due to the presence of un-resorbed old bone in the bone mass (80, 81). Bone mass with a huge amount of old bone may be brittle or more prone to fracture. Therefore, CLA could be a promising

natural alternative in maintaining healthy bone mineral density during aging because of its capacity not only to inhibit bone resorption, but also to stimulate new bone formation. Bone mass with sufficient new bone might be stronger to prevent falls and fractures. These results open up an additional avenue to search for natural alternatives to drugs not only for the treatment of postmenopausal osteoporosis but also for other inflammatory or obesity related bone diseases. These findings provide substantial evidence that CLA has promise to be a novel dietary therapeutic agent against bone diseases as well as obesity. Further pre-clinical studies are required to determine the bone density and rigidity in CLA supplemented animals.

Acknowledgments

We acknowledge Emily Molina for her kind review of our manuscript for grammatical corrections. We are grateful to Dr. Harris for providing α -SMA positive BM stromal cells. This study was supported by NIH grant numbers, NIH-AG034233 (M.M.R.).

List of abbreviations

CLA	conjugated linoleic acid
BMD	bone mineral density
DXA	dual energy x-ray absorptiometry
Ovx	ovariectomized
BM	bone marrow
SFO	safflower oil
RANKL	receptor activator of NF- κ B
OPG	osteoprotegerin
LPS	lipopolysaccharide
TRAP	tartrate resistant acid phosphatase
RT-PCR	reverse transcriptase polymerase chain reaction
FACS	fluorescence activated cell sorting
ERT	estrogen replacement therapy
HRT	hormone replacement therapy
c9t11	cis-9,trans-11
t10c12	trans-10,cis-12
CO	corn oil
ELISA	enzyme linked immunosorbent assay
TNF	tumor necrosis factor
IL	interleukin
PINP	N-terminal propeptide of type I procollagen
ALP	alkaline phosphatase
M-CSF	macrophage colony-stimulating factor
FCS	fetal calf serum

BMP	bone morphogenetic protein
PBS	phosphate buffer saline
SMA	α -smooth muscle actin

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Figure 1. CLA modulates serum bone turnover markers

Eight weeks old C57BL/6 mice were ovariectomized or sham operated and fed experimental diets for 24 weeks. Thirty two weeks old mice were then sacrificed and serum was collected and analyzed for bone resorption marker, TRAP 5b, osteotropic factors, RANKL and bone formation marker, PINP, and ALP by standard ELISA techniques. n=5 mice per group. Each bar represents the mean $\pm$ SEM of five duplicate samples. Value with different superscripts are significantly different at $P < 0.05$ by Newman Keuls one way ANOVA with multiple comparison test.

Figure 2. CLA modulated RANKL and OPG expression in splenocytes

Eight weeks old C57BL/6 mice were ovariectomized or sham operated and fed experimental diets for 24 weeks. Thirty two weeks old mice were then sacrificed and splenocytes were isolated from spleen. (A) RNA was prepared, reverse transcribed and amplified by PCR using primers designed for genes of RANKL, OPG and GAPDH and visualized on agarose gels with ethidium bromide. (B) Relative expression of RANKL and OPG is shown. The intensity of the bands was determined by densitometry and normalized by the level of GAPDH. $n=3$ mice per group. $P < 0.05$ was considered significant. Flow cytometric analysis of RANKL expression in splenocytes (C, D and E). RANKL positive total splenocytes (C), RANKL positive CD8 T cells (D) and RANKL positive CD4 T cells (E) cells in splenocytes were analyzed for the expression of the cell surface determinants for RANKL only, CD8/RANKL and CD4/RANKL respectively. Before staining, Fc γ II/III receptors on cells were blocked by pre-incubation of the samples with a rat anti-CD32/16 Ab. Cells were then incubated with the anti-RANKL with or without anti-CD8 or anti-CD4 antibodies for 30 min at 4 °C. The cells were re-suspended in wash buffer and analyzed on a FACScan flow cytometer. $n=3$ mice per group. P value <0.05 was considered significant by student's t test.

Figure 3. CLA modulates LPS stimulated cytokines production in splenocytes and bone marrow cultures

Splenocytes were isolated from mice fed different experimental diets and 1×10^6 cells were plated per well in 96-well plates, and LPS was added at a concentration of $5 \mu\text{g/ml}$ for 24 h and media were collected for analysis (A, B and C). Bone marrow cells were isolated from mice fed different experimental diets and 5×10^6 cells were plated per well in 24-well plates, and LPS was added at a concentration of $5 \mu\text{g/ml}$ for 24 h and media were collected for analysis (E, F and G). Culture media were analyzed for TNF- α , IL-6, and IL-10 by standard ELISA techniques. $n=3$ mice per group. Each bar represents the mean $\pm$ SEM of three triplicate cultures. p value <0.05 was considered significant by student's t test.

Figure 4. CLA modulates osteoclastogenesis and osteoblastogenesis

Bone marrow cells were isolated from C57BL/6 mice and cultured α -MEM supplemented with 10% fetal calf serum in the presence of RANKL and MCSF with vehicle or different concentrations of CLA for osteoclast differentiation (A and B). Bone marrow cells were cultured in α -MEM supplemented with 10% fetal calf serum to generate bone marrow stromal cells and then bone marrow stromal cells were cultured in the presence of ascorbic acid and β -glycerophosphate with vehicle or different concentrations of CLA for osteoblast differentiation (C and D). C3H10T1/2 Cells were cultured α -MEM supplemented with 10% fetal calf serum in the presence of BMP-2 for osteoblast differentiation (E and F). For osteoclast differentiation, after 5 days of culture, cells were fixed and stained for TRAP (A). For the osteoblast differentiation in bone marrow stromal cells, after 10 days of culture, cells were fixed and stained for Alizarin Red (C). For the osteoblast differentiation in C3H10T1/2 cells, after 7 days of culture, cells were fixed and stained for alkaline phosphatase (E). OD intensity was determined by histomorphometry using the Metaview Image Analysis System software for TRAP positive area (B), Alizarin Red positive area (D) and alkaline phosphatase positive area (F). Each bar represents the mean $\pm$ SEM of two independent triplicate cultures. Value with different superscripts are significantly different at $P < 0.05$ by Newman Keuls one way ANOVA with multiple comparison test.

Figure 5. CLA stimulates mineralized bone formation in α -SMA positive bone marrow stromal cells

α -SMA positive bone marrow stromal cells were culture in α -MEM supplemented with 15% fetal calf serum (FCS) for few days and then replaced with osteogenic medium (α -MEM supplemented with 15% FCS with ascorbic acid and β -glycerophosphate and BMP-2) for another two weeks with or without 50 μ M of CLA. Cells were fixed and analyzed for nodule formation by vonkossa staining (A). OD intensity was determined by histomorphometry using the Metaview Image Analysis System software for vonkossa positive area (B). Data represents the mean $\pm$ SEM of quadruplicate cultures. p value <0.05 was considered significant by student's t test.

Table 1

Composition of the experimental diets.

Ingredient ^I	Percent
Casein	14.00%
Corn starch	42.43%
Dextronized corn starch	14.50%
Sucrose	9.00%
Cellulose	5.00%
AIN-93 mineral mix	3.50%
AIN-93 vitamin mix	1.00%
L-cystine	0.18%
Choline bitartrate	0.25%
TBHQ	0.10%
Vitamin E	0.04%
Corn oil (CO)	9.5%
SFO or CLA (50:50 c9t11 and t10c12)	0.5%

SFO, safflower oil; CLA, conjugated linoleic acid.

^I All diet ingredients purchased from MP Biomedicals, Irvine, CA.

Table 2

Composition of the experimental diets.

	SFO/Sham	SFO/Ovx	% change	CLA/Sham	CLA/Ovx	% change	P Value ^b		
							Diet	Surgery	Diet X Surgery
DFM	0.086±0.002 ^a	0.072±0.003 ^b	-16.3	0.093±0.002 ^a	0.088±0.003 ^a	-5.4	<0.001	0.58	0.80
PTM	0.086±0.002 ^c	0.067±0.001 ^a	-22.1	0.083±0.003 ^b	0.076±0.002 ^b	-8.4	0.0007	0.05	0.94
FD	0.071±0.002 ^a	0.061±0.002 ^b	-14.1	0.069±0.002 ^{ab}	0.068±0.001 ^{ab}	-1.4	0.008	0.94	0.64
TD	0.059±0.002 ^a	0.050±0.001 ^b	-15.3	0.056±0.002 ^{ab}	0.052±0.001 ^{ab}	-7.1	0.02	0.26	0.55
L3	0.054±0.006 ^a	0.031±0.006 ^b	-42.6	0.052±0.008 ^a	0.045±0.005 ^a	-13.5	0.05	0.09	0.46
L4	0.050±0.003 ^a	0.023±0.004 ^b	-54.0	0.050±0.008 ^a	0.040±0.006 ^a	-20.0	0.05	0.05	0.83

BMD is expressed as g/cm². Data are expressed as mean ± SEM of n = 5 mice/group. Means in the same row with different superscripts are significantly different at p<0.05 by One-way ANOVA.

SFO, safflower oil; CLA, conjugated linoleic acid; DFM: Distal femoral metaphysis; PTM: Proximal tibial metaphysis; FD: Femoral diaphysis; TD: Tibial diaphysis; L3: Lumbar vertebra 3; L4: Lumbar vertebra 4;

^b From two way ANOVA. p<0.05 was considered significant.

Table 3

Serum cytokine levels in C57BL6 Sham and Ovx mice after 24 weeks of experimental diets with SFO control or CLA.

	SFO/Sham	SFO/Ovx	CLA/Sham	CLA/Ovx
TNF- α (pg/ml)	54.50 $\pm$ 2.94 ^a	59.05 $\pm$ 2.83 ^a	40.05 $\pm$ 1.45 ^b	43.58 $\pm$ 4.49 ^b
IL-6 (pg/ml)	37.82 $\pm$ 4.27	40.18 $\pm$ 3.15	41.75 $\pm$ 5.45	42.05 $\pm$ 2.58
IFN- γ (pg/ml)	41.53 $\pm$ 8.96	25.80 $\pm$ 3.53	38.00 $\pm$ 4.65	35.32 $\pm$ 2.39*

Data are expressed as mean $\pm$ SEM of n = 5 mice/group. Means in the same row with different superscripts are significantly different at p<0.05 by One-way ANOVA. * p<0.05 as compared to SFO/Ovx by students t test.

SFO, safflower oil; CLA, conjugated linoleic acid.