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Combination of conjugated linoleic acid with fish oil prevents age-associated bone marrow adiposity in C57Bl/6J mice☆

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Abstract

The inverse relationship between fat in bone marrow and bone mass in the skeleton of aging subjects is well known. However, there is no precise therapy for the treatment of bone marrow adiposity. We investigated the ability of conjugated linoleic acid (CLA) and fish oil (FO), alone or in combination, to modulate bone loss using 12 months old C57Bl/6J mice fed 10% corn oil diet as control or supplemented with 0.5% CLA or 5% FO or 0.5% CLA+5% FO for 6 months. We found, CLA-fed mice exhibited reduced body weight, body fat mass (BFM) and enhanced hind leg lean mass (HLLM) and bone mineral density (BMD) in different regions measured by dual energy x-ray absorptiometry (DXA); however, associated with fatty liver and increased insulin resistance; whereas, FO fed mice exhibited enhanced BMD, improved insulin sensitivity, with no changes in BFM and HLLM. Interestingly, CLA+FO fed mice exhibited reduced body weight, BFM, peroxisome proliferator-activated receptor gamma and cathepsin K expression in bone marrow with enhanced BMD and HLLM. Moreover, CLA+FO supplementation reduced liver hypertrophy and improved insulin sensitivity with remarkable attenuation of bone marrow adiposity, inflammation and oxidative stress in aging mice. Therefore, CLA with FO combination might be a novel dietary supplement to reduce fat mass and improve BMD.

Keywords: Conjugated linoleic acid; Bone adiposity; Fat mass; Fish oil; Obesity

1. Introduction

An enhanced adipogenesis in the bone marrow (BM) is inversely correlated to trabecular bone volume during aging and in patients with osteoporosis [1]. Osteoporosis is a major public health concern characterized by excessive skeletal fragility in the aging population. The direct cost associated with obesity in the United States is ~$100 billion and for osteoporosis, it has risen rapidly and reached ~17.5 billion dollars per year [2]. Bone tissue undergoes remodeling throughout life, balancing between bone resorption and bone formation. Imbalances of bone remodeling can result in gross perturbations in skeletal structure and function, and potentially to morbidity and shortening of lifespan [3]. Currently, there is no precise therapy to treat age-associated BM adiposity. Recently, there is an extensive interest in the potential health benefits of dietary supplementation with conjugated linoleic acid (CLA), including anti-carcinogenic and anti-tumorogenic effects [4], reduction in the risk of atherosclerosis, hypertension, and diabetes, improvement in food efficiency, promotion of energy metabolism, anti-osteoporotic and positive effect on immune function [5]. On the other hand, the beneficial effect of FO has been demonstrated in several human diseases, including cardiovascular diseases [6], autoimmune inflammatory diseases, rheumatoid arthritis, inflammatory bowel disease, osteoporosis [7,8], sepsis, vascular compliance, blood pressure [9], diabetes [10], etc. In view of its beneficial effects, the FO formulation OMACOR (Currently marketed as LOVAZA, by GSK, Research Triangle Park, NC, USA) has been approved by the Food and Drug Administration (FDA) as an adjunct to diet in patients with high triglycerides. Similarly, CLA has achieved the Generally Recognized As Safe status by the FDA for its use in certain foods and beverages [11].

We and others have shown that CLA profoundly reduces body fat mass and is popularly used as a weight loss management strategy [5,12]. We have also reported that CLA is anti-osteoclastogenic and is able to prevent age-associated bone loss in mice [13]. However, CLA has been shown to exhibit some adverse effects [14] including reduction in insulin sensitivity in subjects with Type 2 diabetes [15] and augmentation of the pre-existing insulin resistance [16,17]. Additionally, long-term studies in rodents have demonstrated a lipodystrophic effect of CLA [18] associated with decreases plasma adiponectin and leptin levels and increased insulin resistance [19]. There are paradoxical findings that CLA causes liver enlargement [20] accompanied by increase in tissue lipid content in mice [14], whereas in obese insulin-resistant rats, it reduced the hepatic steatosis, improved liver function and favorably modified lipid metabolism [21]. On the other hand, FO has been shown to have beneficial effects on glucose and lipid metabolism in rodent studies, improve insulin sensitivity [22,23], reduce triglyceride [24] and to improve bone

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mineral density (BMD) in ovariectomized mice [7]. Thus, although CLA supplementation reduces body fat and osteoporotic bone loss, it is associated with hepatomegaly and insulin resistance, whereas FO is known to reduce triglycerides (TGs), improve insulin sensitivity, and enhance BMD. Therefore, we speculate that the combining CLA with FO could be an excellent strategy in the management of fat mass reduction and osteoporotic bone loss, circumventing the CLA-induced hepatomegaly and insulin resistance. In the present study, aging C57Bl/6J mice were fed American Institute of Nutrition (AIN) 93 diet, containing 10% corn oil (CO) as a dietary fat source, which promotes osteoporosis and obesity [25], glucose intolerance and insulin resistance thus representing a useful model for studying the early stages in the development of obesity [25,26] and age-related bone loss [25,27]. The combined supplementation of CLA and FO showed reduced fat mass, reduced BM adiposity, improved hind leg lean mass (HLLM), improved BMD, along with no hepatomegaly and insulin resistance in C57Bl/6J aging mice.

2. Research design and methods

2.1. Animals

Eleven month-old female C57Bl/6J mice, weighing 24–26 g were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and provided free access to water and standard chow AIN93G, which is the growth diet recommended by AIN for acclimatization for a period of one month. At twelve months, weight matched animals were divided into four groups containing 10 in each. Subsequently, the animals were housed in a standard controlled animal care facility in cages (5 mice/cage) and fed a diet containing CO, FO, CLA and combination of CLA and FO ad libitum for 6 months. The animals were maintained in temperature controlled room (22–25°C, 45% humidity) on a 12:12-h dark-light cycle. National Institutes of Health guidelines were strictly followed, and all the studies were approved by the Institutional Laboratory Animal Care and Use Committee of the University of Texas Health Science Center, San Antonio, TX, USA. Body weight was measured weekly.

2.2. Diet preparation

The 4 different experimental diets (Table 1) were prepared using AIN93G, supplemented with CO, FO, and CLA as follows,

a) Corn oil (CO) diet: supplemented with 10% corn oil,
b) FO diet: Supplemented with 5% FO [(eicosapentaenoic acid (EPA)/docosahexaenoic acid (DHA)=30/20)] with 5% CO,
c) CLA diet: Supplemented with 0.5% Clarinol-80 (containing 50:50 of *cis* 9 *trans* 11 CLA and *trans* 10 *cis* 12 CLA) in 9.5% CO (CLA),
d) CLA+FO diet: Supplemented with 0.5% CLA in 4.5%CO with 5% FO (CLA+FO).

Table 1
Composition of semi-purified experimental diets

Ingredients[a]	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
CO[b], FO[c], CLA[d], FO+CLA[e]	10.00

[a]All diet ingredients were purchased from MP Biomedicals (Irvine, CA, USA); CLA, from Lipid Nutrition, Channahon, IL, USA and Fish oil (EPA/DHA=30/20) from Ocean Nutrition Canada.
[b–e]Diets consisted of 10% CO or 5% CO with 5% FO or 0.5% CLA in 9.5% CO or 0.5 % CLA in 4.5% CO with 5% FO respectively.

The Clarinol-80 was supplied by Lipid Nutrition, Channahon, IL, USA. FO (EPA/DHA=30/20) was obtained from Ocean Nutrition Canada. The mice were provided with fresh food everyday between 1 and 2 p.m. Diets were prepared each week, purged with nitrogen and frozen in daily portions in sealed polyethylene bags to minimize the oxidation of fatty acids. The composition of semipurified diets is presented in Table 1.

2.3. Measurement of BMD, total body fat mass and hind leg lean mass by dual energy x-ray absorptiometry (DXA)

Region specific BMD, total body fat mass (BFM), and hind leg lean mass (HLLM) were measured by DXA before start of the AIN93 diet and after 6 months on the diets, and data were analyzed using Lunar PIXImus mouse software [7,25]. Prior to scanning, mice were anesthetized with an intramuscular injection of 0.1 mL/100 g body weight of mouse cocktail containing ketamine/xylazine/phosphate-buffered saline (PBS) (3:2:5, by vol). Upon completion of scanning, BMD was determined in the following bone areas using the PIXImus software, version 2.1: distal femoral metaphysis (DFM) (knee joint) to include cancellous (trabecular) bone, proximal tibial metaphysis (PTM) and femoral diaphysis (FD). Intra-scan coefficients of variation were 0.79%, 3.30% and 1.35% for DFM, PTM and FD respectively; interscan coefficients of variation were 5.47%, 3.86% and 5.12% for DFM, PTM and FD respectively. The coefficients of variation are in agreement with studies examining the precision and accuracy of the PIXImus densitometer [28].

2.4. Intravenous glucose tolerance test

Intravenous glucose tolerance test (IVGTT) was performed after 20 weeks using 6 mice from each dietary group. For the IVGTT, 6-h-fasting mice were anesthetized with an intramuscular injection of 0.1 ml/100 g body weight of mouse cocktail containing ketamine/xylazine/PBS (3:2:5, by vol). Blood sample was drawn from the retrobulbar, intraorbital, capillary plexus followed by intravenous injection of D-glucose (1 g/kg) into the tail vein (volume load 10 μl/g). Blood samples were again drawn 5, 10, 20, 50 and 75 min after the glucose injection. After immediate centrifugation at 4°C, serum was collected and stored at −80°C.

2.5. Blood and tissue collection for biochemical and histological analysis

After 6 months on the experimental diet, the animals were sacrificed. One week prior to sacrifice, mice were fasted for 6–8 h, blood samples were taken from the intraorbital, retrobulbar plexus from anesthetized mice to measure fasting glucose, insulin and nonesterified fatty acid in serum (NEFA). At the end of 6 months, the mice were anaesthetized and blood was obtained by intraorbital capillary plexus. Serum was collected and stored at −80°C. Liver, gastrocnemius and quadriceps muscles were collected, weighed and frozen in liquid nitrogen and stored in −80°C. Spleen, tibia and femur were processed for subsequent splenocyte culture and BM culture respectively. Right side of complete hind leg was fixed in 4% formalin and processed for Oil Red O staining to measure BM adiposity after dietary intervention.

2.6. Measurement of serum metabolites

Serum glucose (QuantiChrom, Hayward, CA, USA), TGs (Cayman Chemical, Ann Arbor, MI, USA), NEFA (Wako Pure Industries, Ltd. Osaka,

Japan), catalase (CAT) and malondialdehyde (MDA), (Cell Biolabs, Inc., San Diego, CA, USA) were analyzed spectrophotometrically using Colorimetric Assay Kits following manufacturers' protocol. Insulin was analyzed using a rat/mouse Ultra sensitive rat insulin ELISA kit (Crystal Chem, Research, Downers Grove, IL, USA). Adiponectin and leptin were analyzed by using ELISA kits (R&D systems, Minneapolis, MN, USA) following manufacturer's protocol.

2.7. Splenocytes preparation and culture

Spleen was aseptically removed and placed in 5 ml of Roswell Park Memorial Institute medium (RPMI) 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 25 mmol/L HEPES, 2 mmol/L glutamine, 100 000 U/L penicillin and 100 mg/L streptomycin (Gibco, Grand Island, NY, USA). Single-cell suspensions were made by teasing spleens between frosted ends of two sterile glass slides. After a 5-min centrifugation at 1000 rpm to separate cells from debris, the cells were washed twice in RPMI medium. Splenic lymphocytes were isolated by layering over Histopaque (Sigma, St. Louis, MO, USA), centrifuging at 1000 rpm for 15 min at 22°C followed by washing twice in RPMI 1640 complete medium. Cells were counted, and viability was determined by trypan blue exclusion method [25].

2.8. Isolation of whole BM cells and culture

Whole BM cells were aseptically isolated as described [25]. In brief, cells were counted and viability was determined by trypan blue exclusion method. Cells (10×10^6/well) were plated in 12-well plates and bacterial lipopolysaccharide (LPS) was added at the concentration of 5.0 μg/ml for 24 h at 37°C in a humidified atmosphere of air/CO_2 95:5 (mol%). After 24 h, cells and culture medium were collected together and centrifuged at 2000 rpm for 5 min. The pellets were stored at −80°C for gene expression assays and supernatants were analyzed for tumor necrosis factor α (TNF-α) and interleukin (IL)-6. BM cells cytosolic extract were used to measure the levels of MDA, CAT and superoxide dismutase (SOD).

2.9. Hematoxylin and eosin staining of liver tissue

After animal sacrifice, liver tissues were excised and fixed in 10% formalin for 24 hrs at room temperature (RT). Tissue was then embedded in paraffin following conventional methods. 5 μm sections were made and stained with hematoxylin and eosin (H&E) staining procedure for subsequent histological analysis.

2.10. Cathepsin K and peroxisome proliferator-activated receptor gene expression in BM cells by real time reverse transcriptase-polymerase chain reaction (RT-PCR)

mRNA expression for genes encoding cathepsin K (*ctsk*) and peroxisome proliferator-activated receptor gamma ($PPAR_\gamma$) were measured using real time RT-PCR. Frozen bacterial LPS-stimulated BM cells were vortexed in lysis buffer and RNA was isolated using RNeasy Mini Kit following the manufacturer's instructions (Qiagen, Valencia, CA, USA). Total RNA concentration was assessed in NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Real-time RT-PCR was carried out using TaqMan RNA-to-C_T one-step kit (Applied Biosystems, Foster City, CA, USA) in an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) using fluorescent TaqMan methodology. Real-time quantitative PCR was performed for each of the following genes, using ready-to-use primer and probe sets predeveloped by Applied Biosystems (TaqMan Gene Expression Assays) were used to quantify *ctsk* (*ctsk*, Mm00484036_m1), $PPAR_\gamma$ (Mm01184321_m1) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Mm99999915_g1) as an endogenous control. mRNA Ct values for these genes were normalized to the house-keeping gene *GAPDH* and expressed as relative increase or decrease compared to the CO group.

2.11. Histological evaluation of femur head for bone adiposity by Oil Red O staining

Formalin fixed hind leg bones were placed in saturated solution of linoleic acid in ethylene glycol (500 ml of 70% ethylene glycol and 5g of linoleic acid were mixed for 1 h at RT, allowed to stand for several hrs at RT in a separating funnel, the bottom layer drawn off) for 3 days at 56°C. Bones were then sequentially rinsed for 8 h each in several changes of 70% ethanol at RT followed by with several changes of water. The bones were decalcified in 2% chromic acid for 40 h at 4°C followed by rinsing with water for 8 h and incubation with 5% aqueous sodium bicarbonate for 24 h at RT. The bones were finally rinsed with several changes of water for 24–72 h at RT. Bones were then placed in 70% ethanol and processed, embedded in paraffin and stained with 0.5% Oil Red O in isopropanol (v) for 10 min. Lipid vacuole area were assessed using a light microscope with a digitalized camera and a MetaVue image analysis system (Olympus America, Center Valley, PA, USA). The area of lipid vacuoles was calculated from six different fields.

2.12. Measurement of MDA levels, CAT, and SOD activity in BM cells

After 24 hrs BM cells treated with bacterial LPS were used to prepare cytosolic extracts using a Nuclear Extract Kit from Active Motive (Carlsbad, CA), following manufacturer's protocol. Fifty micrograms of cytosolic extracts were used to determine MDA levels, CAT and SOD activities using commercially available kits.

2.13. Cytokines measurement in serum, conditioned medium of cultured splenocytes and BM cells

Isolated BM cells and splenocytes (10×10^6 cells/well) were plated in six-well plates and incubated with 5.0 μg/ml of bacterial LPS for 24 h at 37°C in a humidified atmosphere of air/CO_2 95:5 (mol %). After 24 h, the culture medium was collected and analyzed for TNF-α and IL-6 by ready-set-go ELISA kits (eBioscience, San Diego, CA, USA) following manufacturer's protocol.

2.14. Statistical analysis

Data are presented as mean values±S.E.M. Differences among the groups (CO, FO, CLA and CLA+FO) were tested by one-way analysis of variance (ANOVA) followed by Newman–Keuls multiple comparison post hoc test. $P\leq.05$ was considered statistically significant. The analyses were performed using Graphpad prism for Windows (La Jolla, CA, USA).

3. Results

3.1. Effect of CLA+FO on body weights and CLA-induced liver enlargement

The initial body weights for 12 months old C57Bl/6J mice in CO, FO, CLA and CLA+FO groups were 25.59±0.60, 24.84±0.79, 25.17±0.26 and 24.50±0.61 g, respectively. Chronic 6-month feeding of CLA and CLA+FO caused a significant reduction ($P<.05$) in body weight gain when compared to CO-fed mice (Fig. 1A). The CLA-fed mice showed a 6% weight gain compared to 28% in CO-fed mice, the difference between the two being very highly significant ($P<.01$). Similarly, CLA+FO fed group showed lowered body weight gain (9%) compared to CO fed group (28%). However, no significant change in body weight gain (27%) was observed in mice fed FO alone. The CLA fed mice showed significant increase in liver weight compared to CO, FO and

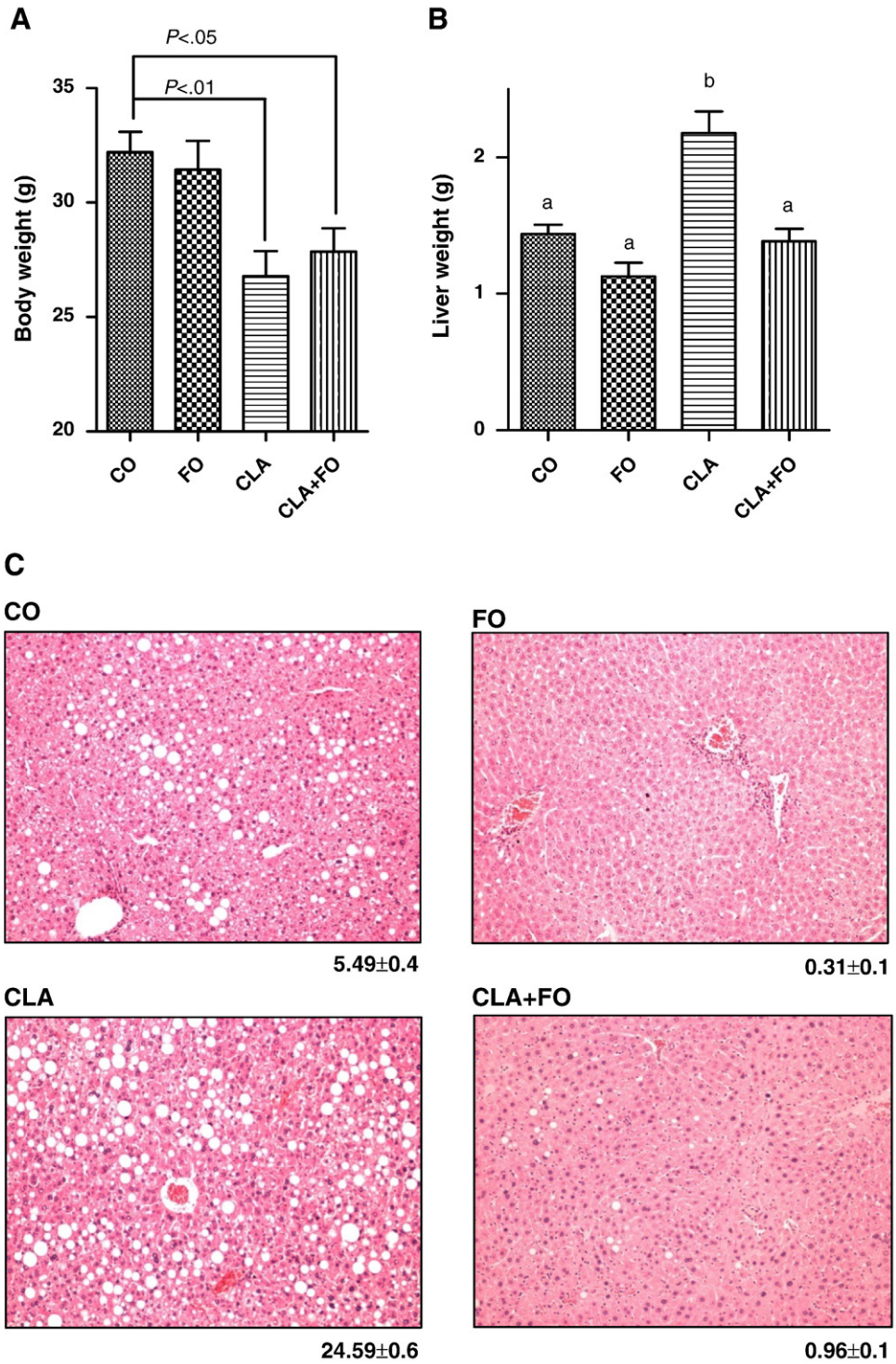

Fig. 1. Effect of CO, FO, CLA and CLA+FO on body weight (A), liver weight (B) and liver histology (C). Twelve-month-old C57Bl/6J mice (n=10) were fed CO, FO, CLA and CLA+FO for 6 months. (C) Panels show H&E-stained liver sections from representative mice (n=6/group). White circular area represents lipid. Values represent total lipid area (μm^2) by histomorphometry. Images are ×200 magnifications. Values with different letters are significantly different by one way ANOVA followed by Newman-Keuls multiple comparison post-hoc test (P<.05).

CLA+FO fed mice. The liver weight in CLA fed mice was 2.17±0.10 g, which was significantly higher (P<.001) than that of CO, FO, and CLA+FO fed mice in which livers weighed 1.43±0.06, 1.12±0.1 and 1.38±0.09 g, respectively (Fig. 1B). The data of the present study confirmed that CLA-induced liver enlargement in C57Bl/6J aging mice is prevented by supplementation with FO whereas the FO-induced body weight gain is thwarted by CLA.

3.2. Histological changes in CLA-induced fatty liver by CLA+FO

Histological sections of liver tissue from CO and CLA-fed mice showed predominantly large lipid-filled vacuoles (macrovesicular steatosis) Fig. 1C. Liver sections from FO and CLA+FO group revealed a reduction of lipid accumulation, in the form of lipid droplets, or even small lipid droplets (microvesicular steatosis). The histomorphometric

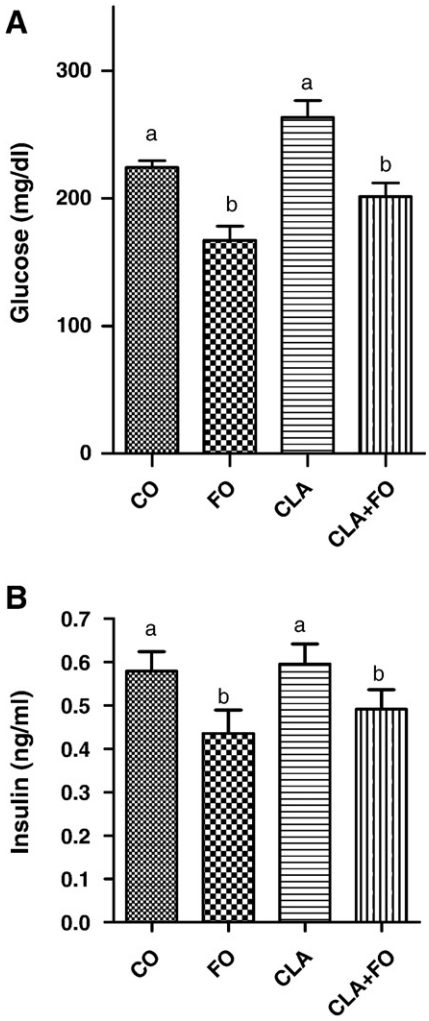

Fig. 2. Effect of CO, FO, CLA and CLA+FO on the fasting glucose (A) and Insulin (B). Twelve-month old C57Bl/6J mice (n=10) were fed with CO, FO, CLA and CLA+FO for 6 months. Values with different letters are significantly different by one way ANOVA followed by Newman-Keuls multiple comparison post hoc test (P<.05).

analysis using microscopy of lipid vacuole area revealed visibly significant differences between the different groups where the lipid vacuole areas in CO, CLA, FO and CLA+FO fed mice livers were 5.49±0.4 μm^2, 24.59±0.6 μm^2, 0.31±0.1 μm^2 and 0.96±0.1 μm^2 respectively (Fig. 1C). The lipid areas were significantly (P<.001) higher and larger in CO and CLA fed mice compared to that of FO and CLA+FO fed mice, confirming that FO prevented CLA-induced liver steostasis.

Table 2
Serum metabolites and cytokines in C57Bl/6J mice fed CO, FO, CLA and CLA+FO for 6 months

Parameter	CO	FO	CLA	CLA+FO
Serum metabolites				
Triglycerides (mg/dl)	70.28±2.15^a	40.66±2.42^b	45.33±6.12^b	41.21±3.05^b
NEFA (mEq/L)	1.24±0.06^a	0.81±0.06^b	1.04±0.03^a	0.88±0.03^b
Serum hormones and adipocyte cytokines				
Leptin (μg/ml)	7.41±0.63^a	5.22±0.60^a	3.38±0.22^b	4.30±0.61^b
Adiponectin (μg/ml)	1.93±0.06^a	2.88±0.07^b	1.16±0.04^a	2.97±0.05^b
TNF-α (pg/ml)	55.12±4.24^a	42.23±2.26^a	37.43±1.44^b	37.22±2.12^b
IL-6 (pg/ml)	120.9±6.30^a	90.0±4.13^a	93.12±7.12^b	80.25±2.36^b

Effect of diet containing CO, FO, CLA and CLA+FO on serum metabolites and cytokines in C57Bl/6J mice fed for six months. Data are means±S.E.M., (n=10 mice/group). Values with different letters significantly different by one way ANOVA followed by Newman–Keuls multiple comparison post hoc test (P<.05).

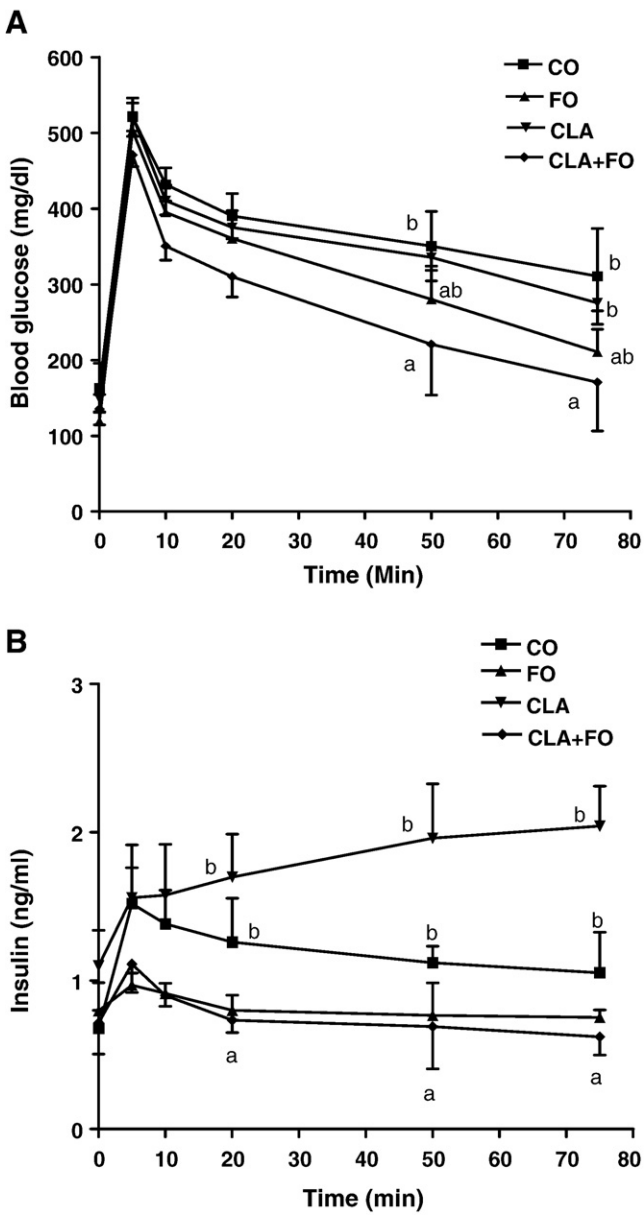

Fig. 3. Plasma levels of glucose (A) and insulin (B) after intravenous glucose administration: 1g/kg glucose was injected into the tail vein of mice fed with CO, FO, CLA and CLA+FO. The IVGTT was performed 5 months after starting the diets. Data are means±S.E.M. from six independent experiments. Values with different letters are significantly different by one way ANOVA followed by Newman-Keuls multiple comparison post-hoc test (P<.05).

3.3. Improvement of insulin resistance by CLA+FO

The higher circulating levels of NEFA in CO fed mice represents insulin resistance, which is strongly associated with obesity, and one of the mechanism may be the generation of metabolic messengers, such as free fatty acids, by adipose tissue that inhibit insulin action on muscle [29]. The fasting serum glucose concentration increased significantly (P<.05) in CO and CLA-fed mice compared to FO and CLA+FO fed mice (Fig. 2A). Contrarily, fasting serum insulin concentration also increased in mice treated with CLA for 6 months, demonstrating hyperinsulinemia which is comparable to CO. However, in CLA+FO fed mice, increased insulin level was associated with reduction in blood glucose concentration suggesting its ability to control hyperglycemia unlike CLA. The serum TGs were

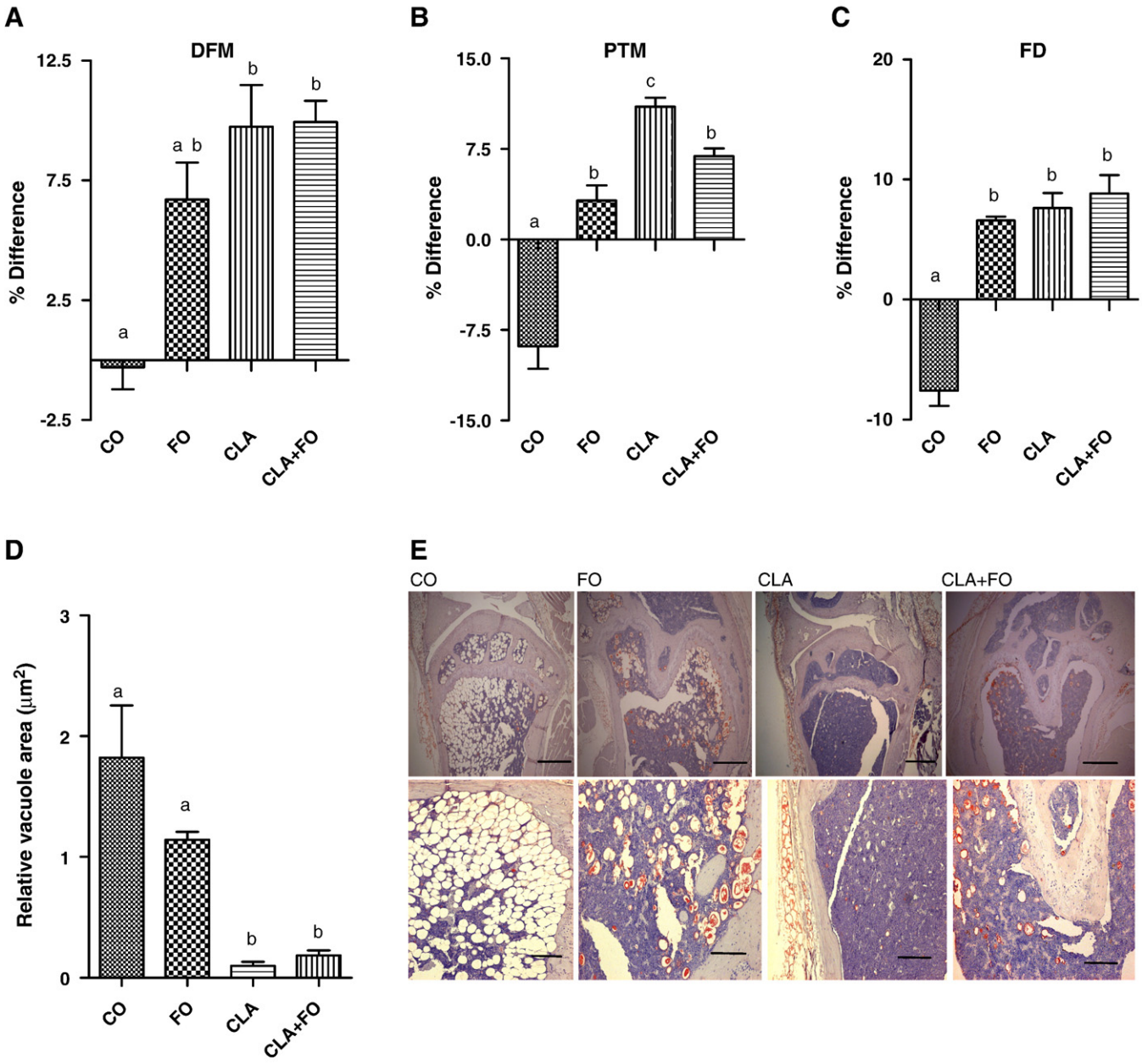

Fig. 4. Effect of CO, FO, CLA and CLA+FO on BMD of (A) DFM, (B) PTM and (C) FD. Values represent percentage of change in BMD from baseline value determined at the beginning of the experiment. (D) Histomophometric evaluation of femoral adipocyte vacuole area (μm^2). (E) Representative photomicrographs of femoral head bone marrow adiposity — femurs were processed after dietary supplementation of CO, FO, CLA and CLA+FO and stained with Oil Red O. Upper panel (4×, scale bar is 500 μm.) demonstrated reduced adiposity in CLA and CLA+FO fed mice than CO and FO fed mice (n=5/group). Lower panel (10×, scale bar is 200 μm.) showed reduced adipocyte cell area in CLA- and CLA+FO-fed mice than CO- and FO-fed mice. 12 month old C57Bl/6J mice (n=10) were fed with CO, FO, CLA and CLA+FO for 6 months. Values with different letters are significantly different by one way ANOVA followed by Newman–Keuls multiple comparison post hoc test (P<.05).

significantly (P<.05) decreased in FO, CLA and CLA+FO compared to CO fed mice (Table 2). Surprisingly, fatty liver was observed in CLA fed mice, despite a reduction in TGs levels compared to CO fed mice. Finally, CLA+FO decreased TG levels, improved insulin levels and reduced glucose levels compared to CO as well as CLA. Fasting serum NEFA was also found to be significantly (P<.05) decreased in FO and CLA+FO fed mice compared to CO group (Table 2). Furthermore, adiponectin levels (Table 2) were significantly (P<.03) increased in CLA+FO fed mice compared to CO fed mice in contrast to leptin levels which were reduced significantly (P<.05) in CLA+FO than CO. These results suggest an affirmative role of CLA+FO in improving CLA-induced insulin resistance and dysmetabolism in high fat fed C57Bl/6J aging mice.

3.4. IVGTT

The IVGTT was performed at 20 weeks of feeding of experimental diet. At 5, 10 and 20 minutes after IV injection of glucose, the CLA+FO fed mice eliminated glucose faster than CO and CLA fed mice. In contrast, the CO and CLA fed mice showed lesser elimination of glucose, at both 50- and 75-min time points (Fig. 3A), although a higher concentration of insulin was observed at these points. Thus, taken together, the basal levels of glucose and insulin, as a result of glucose challenge, demonstrated that CLA and CO treated mice display a more extensive impairment of β-cell function and, consequently, an exaggerated insulin resistance compared with

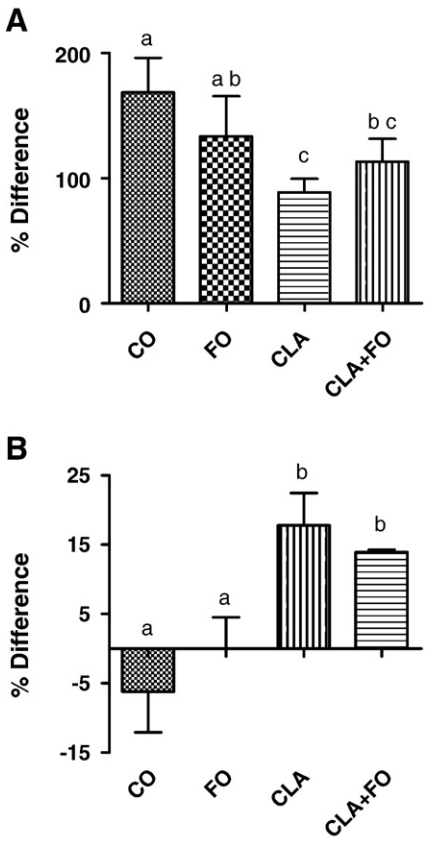

Fig. 5. Effect of CO, FO, CLA and CLA+FO on the total fat mass (A) and hind leg lean mass (B). 12 month old C57Bl/6 mice (n=10) were fed with CO, FO, CLA and CLA+FO for 6 months. Values with different letters are significantly different by one way ANOVA followed by Newman–Keuls multiple comparison post hoc test (P<.05).

CLA+FO fed mice. The 5-min insulin response to intravenous glucose challenge was increased rapidly to 1.63±0.04 ng/ml in CO and 1.78±0.35 ng/ml in CLA fed mice; however, it was apparently insufficient to maintain normal glucose tolerance. A more pronounced and significant (P<.05) increase in insulin secretion was observed by glucose challenge in CLA fed mice demonstrating hyperinsulinemia Fig. 3B, unlike in CLA+FO fed mice.

3.5. CLA+FO increases BMD in femur and tibia regions measured by DXA

Baseline BMD of different bone regions measured prior to the start of the experimental diet showed no differences among the groups. To examine the effect of CLA+FO on age-associated bone loss, we measured the BMD after feeding the experimental diet for 6 months, using DXA. The results are expressed as % difference, as shown in Fig. 4A–C. The BMD in the DFM, PTM and the FD regions of the FO-, CLA- and CLA+FO-fed mice was significantly higher (P<.01) than that of CO-fed mice. These findings indicate that 12-month-old mice, when placed on CO-enriched diet for 6 months, resulted in bone loss [25], which was restored by FO, CLA and CLA+FO dietary intervention.

3.6. Reduction of BM adiposity by CLA+FO

Oil Red O staining of femoral head from 18 months old mice fed an experimental diet for 6 months, revealed a significant (P<.01) increase in vacuole area of adipocytes (Fig. 4D), in CO (1.22±0.7 μm^2)- and FO (1.4±0.6 μm^2)-fed mice when compared to CLA (0.09±0.03 μm^2)- and CLA+FO (0.18±0.04 μm^2)-fed mice. Bone forming osteoblasts and fat forming adipocytes are both derived from mesenchymal stem cells (MSCs) [30], which are found in many tissues and are abundant in the BM stroma [31]. Of note, the fat cell size was also reduced in FO, CLA and CLA+FO, which is important determinant of age-related adiposity [32]. Reduction of BM adiposity in the femur head section revealed the positive role of CLA-, CLA+FO- and FO-fed mice in the prevention of age-associated bone loss (Fig. 4E).

3.7. CLA+FO decreases total body fat mass (BFM) and increases HLLM measured by DXA

Total BFM was decreased (Fig. 5A) and HLLM was significantly (P<.05) increased (Fig. 5B) in CLA and CLA+FO fed mice compared to CO fed mice. Furthermore, we noted the skeletal muscle wet weight of quadriceps and gastrocnemius muscles were moderately increased (not significant, data not shown) in CLA and CLA+FO fed mice. The increased weight of the skeletal muscle, showed by DXA analysis demonstrated a significant (P<.05) increase in HLLM in CLA (9%) and CLA+FO (10%) fed mice compared to that in CO (−10%) fed mice (Fig. 5B).

3.8. Gene expression by LPS-stimulated BM cells

Quantitative real-time RT-PCR further demonstrated that, bacterial LPS-stimulated mRNA expression of *ctsk* was significantly decreased (P<.05) in BM from FO, CLA and CLA+FO groups as compared to that in CO group, demonstrating that FO, CLA or their combination decrease osteoclastogenic bone resorption, thus improving BMD (Fig. 6A). Attempts were made to determine the RUNX2 mRNA expression, however, the levels were not detectable in BM cells (data not shown). Additionally, the PPAR$_\gamma$ expression was significantly (P<.05) decreased in FO-, CLA- and CLA+FO-fed mice compared to that in CO-fed mice (Fig. 6B). An additive inhibitory effect on PPAR$_\gamma$ expression was observed in CLA+FO-fed mice. The inhibitory effects of CLA+FO on *ctsk* and PPAR$_\gamma$ in BM cells confirmed their possible protective effect against bone resorption in aging mice.

3.9. CLA+FO decreases MDA levels and increases CAT activity in BM cells

The CO diet fed mice showed a significant (P<.02) increase in MDA levels compared to FO-, CLA- and CLA+FO-fed mice. In contrast, antioxidant enzyme CAT activity was significantly (P<.001) increased in FO-, CLA- and CLA+FO-fed mice groups when compared to CO fed mice (Fig. 6C and D). Even though there was increase in SOD activity in FO-, CLA- and CLA+FO-fed mice, it was not significantly different from that in CO fed mice. It is established that osteoporotic women have significantly reduced antioxidant enzyme activity and increased lipid peroxidation end-product, like MDA [33]. Previous reports have shown that reactive oxygen species such as superoxide anion are involved in the pathogenesis of bone loss by stimulating osteoclast differentiation and bone resorption [34]. These results indicated that a combination of CLA+FO may suppress osteoclastic bone resorption in mice by inhibiting the reactive free radicals or lipid peroxidation.

3.10. Effect of CLA+FO on LPS-stimulated proinflammatory cytokines production by splenocytes and BM cells

We observed protective effect of CLA+FO against age-associated BMD-loss in mice, through the regulation of bone-resorbing, inflammatory cytokines expression. Pro-inflammatory cytokines such as, IL-6 and TNF-α, are key regulators of osteoclastogenic activity and have been shown to increase bone resorption with age in humans [35]. Interestingly, we found significant (P<.04) decrease in IL-6 and TNF-α

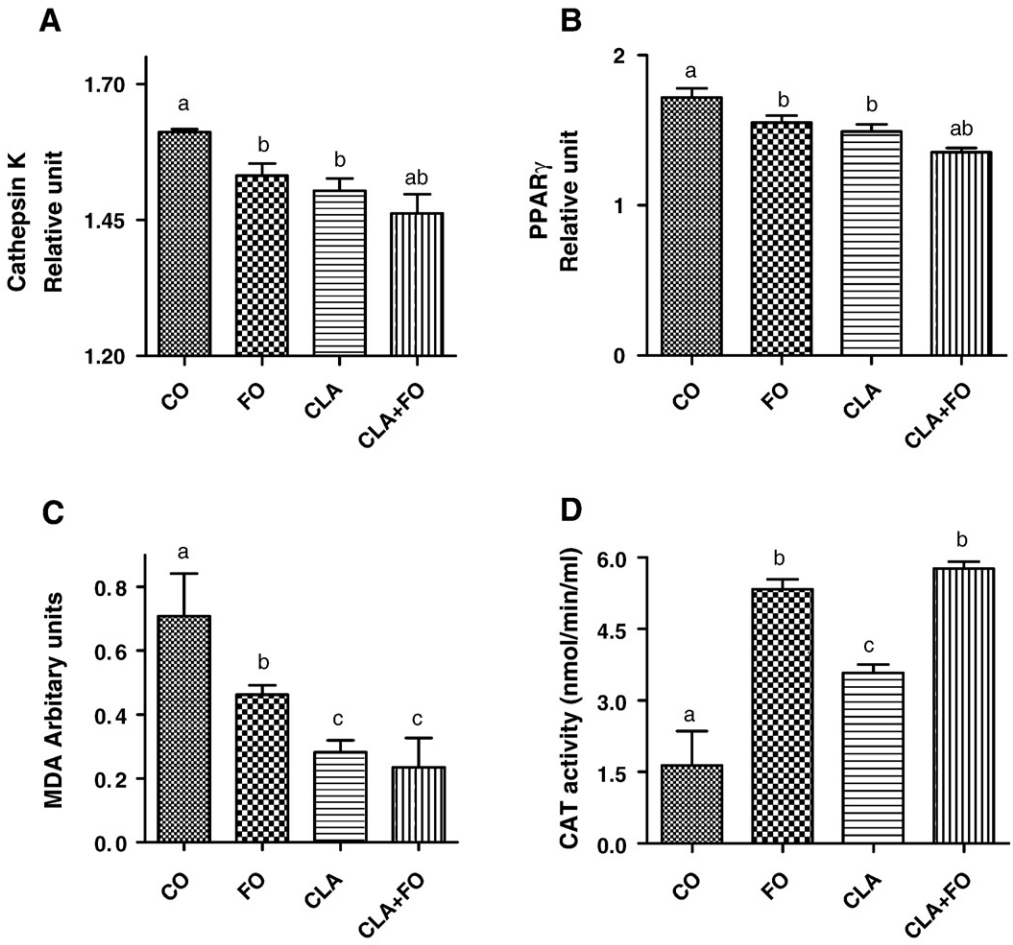

Fig. 6. CLA and CLA+FO down-regulate the expression of *ctsk* (A) and PPAR$_\gamma$ (B). Expression of osteoclast specific gene *ctsk* and adipocyte specific PPAR$_\gamma$ in C57Bl/6J (n=6) aging mice was determined by quantitative real-time RT-PCR in LPS-stimulated bone marrow cells from mice fed either fat-diet CO as a control or diet enriched with FO, CLA and CLA+FO for 6 months. MDA (C) and CAT (D) activity in bone marrow cells after dietary supplementation of CO, FO, CLA and CLA+FO in aging mice. 12 month old C57Bl/6J (n=6) mice were fed with CO, FO, CLA and CLA+FO for 6 months. Results are expressed as means±S.E.M.; Values with different letters are significantly different by one way ANOVA followed by Newman-Keuls multiple comparison post hoc test (P<.05).

production in LPS-treated splenocytes (Fig. 7A and B) and BM cells (Fig. 7C and D) of CLA+FO-fed mice compared to that in CO-fed mice. These results indicate that the reduction of pro-inflammatory cytokines by BM and splenocytes may prevent age-associated bone loss indirectly by inhibiting bone resorbing osteoclastogenesis.

4. Discussion

Given the escalating prevalence of overweight population and osteoporosis worldwide, it would be advantageous to identify potential therapeutic nutrients/functional foods capable of decreasing fat mass as well as preventing bone loss. CLA is widely used in weight loss management and to reduce fat mass [12,36], which is the principal etiological factor of the metabolic syndrome like insulin resistance and Type 2 diabetes. CLA has been known to improve BMD in animals [13]. However, supplementation with CLA or its t10,c12 isomer is associated with induction of insulin resistance and transmigration of lipids to liver resulting in hepatic steatosis [14]. In this investigation, attempts have been made to reduce CLA-induced liver hypertrophy and insulin resistance by incorporating FO in the CLA supplemented diet, as FO is also known to reduce TGs and improve insulin sensitivity, as well as BMD. Additionally, we have ascertained the novel use of CLA with FO in maintaining BMD and muscle mass in high fat diet fed C57Bl/6J aging mice.

In this dietary intervention study, we demonstrated that combination of CLA and FO alleviates CLA-induced insulin resistance and hepatic steatosis in C57Bl/6J aging mice. Importantly, in addition to that, it also helps to prevent age-associated bone loss, decrease fat mass and increase hind leg lean mass. These beneficial effects on age-associated bone loss were associated with increased BMD, decreased BM adiposity and down-regulation of genes involved in osteoclastogenesis, as well as osteotropic factors, such as TNF-α and IL-6. Furthermore, CLA+FO induced the production of potent anti-inflammatory, antisteatotic, insulin-sensitizing adipokine, e.g., adiponectin.

Non-alcoholic fatty liver disease (NAFLD) is distinctive among liver diseases, because its etiology is closely related to the metabolic syndrome [37], observed similarly in CLA [14,38]. Much of the increased prevalence of NAFLD is driven by weight gain particularly in aging [37] in which visceral fat is the most important factor for the development of hepatic steatosis [39] in obese people. Interestingly, CLA reduces visceral fat, but develops insulin resistance, which is commonly observed in CLA-fed mice [14,40] and humans [16,41]. Indeed, it has been reported that, in the absence of obesity, even in patients with total lipodistrophy, insulin resistance leads to hepatic steatosis [42,43]. Although the mechanisms underlying the association of insulin resistance and hepatic steatosis remain unclear, altered insulin sensitivity has been shown to increase hepatic de novo lipogenesis and to induce lipolysis of adipocyte TGs and flux of free

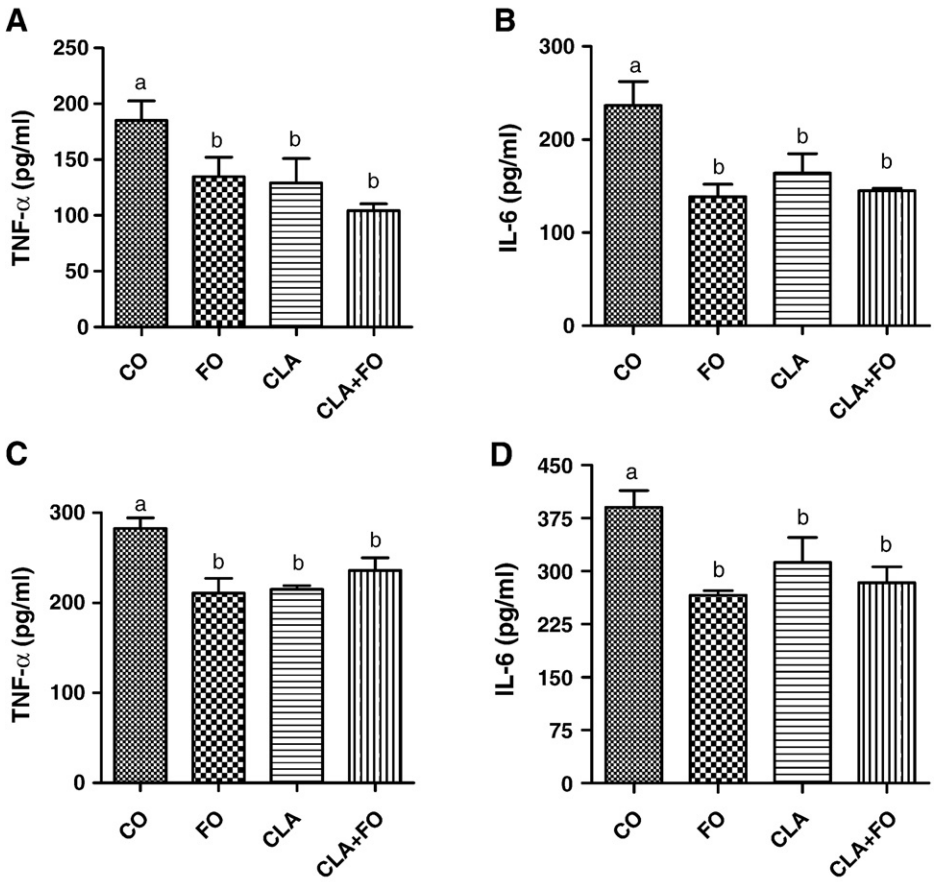

Fig. 7. Effect of CO, FO, CLA and CLA+FO on LPS-stimulated TNF-α and IL-6 secretion by splenocytes (A and B) and bone marrow cells (C and D). Twelve-month-old C57Bl/6J (n=6) mice were fed with CO, FO, CLA and CLA+FO for 6 months and used for splenocytes and bone marrow cells culture. Values with different letters are significantly different by one way ANOVA followed by Newman–Keuls multiple comparison post hoc test (P<.05).

fatty acids to the liver [44]. Our results showed a significant improvement in the glucose tolerance test curves in high fat diet fed C57B6J aging mice supplemented with CLA+FO, presumably by decreasing TGs levels, and by inducing glucose uptake and insulin-signaling components which in turn contributes to the antisteatotic actions of FO+CLA. It is established by a number of investigators that FO supplementation increases adiponetin levels [45,46] and up-regulates PPARα, a key gene involved in the control of hepatic peroxisomal β-oxidation of fatty acids [47]. DHA, a strong $PPAR_{\gamma}$ activator, arouses further interest in developing FO derivatives as a potent antidiabetic drug, without producing undesirable effects, such as obesity and hepatotoxicity [48].

It was previously believed that obesity and osteoporosis were two unrelated diseases. But recent studies have shown that both diseases share several common genetic and environmental factors [2,25]. The common precursor MSCs [30] that lead to the differentiation of both adipocytes and osteoblasts, as well the secretion of adipocyte-derived hormones that affect bone metabolism, may partially explain these associations. Recently Naveiras et al. suggested that adipocytes in BM alter the microenvironment and may have negative consequences for the skeleton. Fat overload would replace functional hematopoietic and/or osteogenic cells from the BM cavity [49]. Reduction of adipose tissue in femur head bone sections suggests the possible role of CLA +FO in osteoblast formation. Number of studies on adipocyte function has revealed that adipose tissue is not just an inert organ for energy storage, but acts as an endocrine organ [50] capable of expression and secretion of a variety of biologically active molecules, such as estrogen, resistin, leptin, adiponectin, IL-6, TNF-α, etc. [50]. These molecules affect human energy homeostasis and also may be involved in bone metabolism, which may contribute to the complex relationship between fat mass and bone. Body fat mass, a component of body weight, is one of the most important indices of obesity, and a substantial body of evidence indicates that fat mass may have beneficial effects on bone [2]. However, in our studies CO fed obese mice exhibited reduced BMD compared to FO-, CLA- and FO+CLA-fed mice. Based on our current state of knowledge, it is unclear whether fat has beneficial effects on bone. We anticipate that this will be an active and fruitful focus of research in the coming years.

Previous work in our laboratory has demonstrated a reduction in ovariectomy-induced BMD loss in mice by dietary FO, accompanied by decreased osteoclastogenesis [7], and also revealed this molecular mechanism by using a *fat-1* transgenic mouse model which has the inherent ability to produce n-3 fatty acids from n-6 fatty acids [51]. Importantly, our group also showed the beneficial effect of FO on bone mass during aging, by modulating bone formation and bone resorption factors [52]. Furthermore, feeding CLA diet in middle-age mice suggests that the loss of bone and muscle may be prevented by modulating the markers of inflammation and osteoclastogenic factors [13]. In this current study, we revealed that *ctsk*, which is abundant in osteoclast, plays a vital role in bone resorption [53] and was down-regulated in CLA+FO fed mice, demonstrating that prevention of age-associated bone loss might be related to bone resorption. It is established that oxidative stress is negatively associated with bone loss [54]. Decreased oxidative stress, as measured by MDA levels and increased CAT activity, explains partly the mechanism of bone loss prevention by CLA+FO supplementation.

The results of the current study strongly support the concept that an increased intake of FO would contribute to the prevention of age-associated bone loss and to the prevention of CLA-induced insulin resistance and metabolic liver disease [55], similar to the improved outcomes reported in cardiovascular disease, arthritis, cystic fibrosis, IgA nephropathy, diabetes, ulcerative colitis, Crohn's disease, asthma and sepsis [24]. Our results are consistent with previous investigations reporting that CLA-induced hepatomegaly can be prevented using FO [56,57] and flaxseed oil [55]. Additionally, our studies revealed that, CLA+FO increases BMD by decreasing the BM adiposity in the femur bone; FO, in combination with CLA, decreases the inflammation status by reducing the proinflammatory cytokines (TNF-α, and IL-6) and, eventually, reduction in oxidative stress which partially explain the mechanism of bone loss prevention by CLA+FO.

In summary, age-associated protection of bone loss by individual fatty acids such as CLA [13], FO [52] and also combination of FO with CLA is associated with decreased total fat mass in mice [57] in agreement with human studies [56] further confirming the benefits of FO supplementation in CLA-induced liver hypertrophy and insulin resistance. Additionally, CLA+FO significantly contributed to the prevention of BM adiposity and increased hind leg lean mass in aging mice. In conclusion, this finding provides a strong and compelling rationale for dietary supplementation of FO during clinical trial with CLA in obese, insulin-resistant aging patients in order to prevent osteoporosis. Additional studies in higher species and humans are still needed before the use of CLA+FO, as a dietary supplement to reduce obesity and osteoporosis in humans.

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