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Conjugated linoleic acid protects against age-associated bone loss in C57BL/6 female mice☆,☆☆

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Abstract

Osteoporosis is one of the major causes of morbidity in the elderly. Inflammation exerts a significant influence on bone turnover, inducing the chronic form of osteoporosis. Dietary nutrition has the capacity to modulate inflammatory response. Therefore, nutritional strategies and lifestyle changes may prevent age-related osteoporosis, thereby improving the quality of life of the elderly population. Conjugated linoleic acid (CLA) has been shown to positively influence calcium and bone metabolism. Hence, this study was undertaken to examine the effect of CLA on bone mineral density (BMD) in middle-aged C57BL/6 female mice. After 10 weeks on diet, CLA-fed mice (14 months) maintained a higher BMD in different bone regions than corn oil (CO)-fed mice. The increased BMD was accompanied by a decreased activity of proinflammatory cytokines (such as tumor necrosis factor α, interleukin-6 and the receptor activator of NF-κB ligand) and decreased osteoclast function. Furthermore, a significant decrease in fat mass and an increase in muscle mass were also observed in CLA-fed mice compared to CO-fed mice. In conclusion, these findings suggest that CLA may prevent the loss of bone and muscle mass by modulating markers of inflammation and osteoclastogenic factors.

Keywords: Conjugated linoleic acid; Cytokines; Inflammation; Aging; Bone

1. Introduction

Nearly 200 million people worldwide, including approximately 25 million Americans, suffer from osteoporosis — a condition characterized by low bone mass and increased bone fragility that leads to osteoporotic fracture, which is largely a problem of the elderly, particularly elderly women [1]. After attaining peak bone mass between the ages of 20 and 30 years, both men and women start losing bone at a rate of about 0.5–1% yearly [2]. The reasons for the age-associated increase in fractures is not clear, but loss of bone mass appears to be a major factor [3]. Bone mineral density (BMD) appears to decline with increasing age [4]. Decline in BMD and increased fracture risk are an inevitable part of the aging process and require new strategies for its prevention. Osteoporosis is easier to prevent than to treat. In general, once bone loss has occurred, it cannot be replaced. Dietary therapy and/or lifestyle changes are considered as viable alternatives to minimize bone loss and to decrease the necessity for osteoporosis-preventing drug therapy. It is well established that dietary patterns modulate BMD in the elderly [5]. Our recent studies with $n-3$ fatty acids (FAs) showed that they prevent bone loss compared to $n-6$ FAs in MRL/lpr mice and in ovariectomized Balb C mice [6,7]. Our very recent study with conjugated linoleic acid (CLA) showed that it increases bone mass in both cancellous and cortical bones in young male Balb/C mice [8].

Considerable attention over the last several years has been focused on the possible beneficial effects of dietary CLA [9], including antiobesity actions in animal [10] and human [11,12] models; anticarcinogenic and antitumorigenic effects [13]; reduction in the risk of atherosclerosis, hypertension and diabetes; improvement in feed efficiency; promotion of energy metabolism; and positive effect on immune function [14,15]. CLA is a collective term used to refer to positional and geometric isomers of linoleic acid (C18:2) with a conjugated double bond [16]. Two predominant isomers of CLA — *cis*-9,*trans*-11 (c9t11) and *trans*-

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10,*cis*-12 (t10c12) — are naturally found in dairy products and ruminant meats, with c9t11 CLA being the most abundant isomer (e.g., 80% c9t11 CLA and 10% t10c12 CLA). CLA is also available commercially as a dietary supplement for weight loss, with both isomers reported to be present at equal amounts (e.g., 35% each).

Recently, focus has been directed on the effects of CLA on skeletal health. Very recently, it has been reported that dietary CLA may positively benefit BMD in postmenopausal women [17]. Increased whole-body ash in young mice fed a diet with CLA supplementation suggests that CLA may enhance bone mineralization and protect against bone loss [18]. CLA increased the bone formation markers osteocalcin and alkaline phosphatase in a murine osteoblastic cell line [19]. Anhydrous butterfat, a rich natural source of CLA, also stimulated the rate of bone formation in young growing chicks by modulating prostaglandin (PG) E_2, which plays an important role in the local regulation of bone formation and bone resorption [20]. However, while CLA has been shown to increase bone mass, ash and/or mineral content in some studies with young, growing, experimental animals (mice [18] and pigs [21]), others have reported a lack of effect in rats [22,23] and pigs [24]. In light of the PGE_2-lowering ability of CLA, it is speculated that CLA may have a beneficial effect on age-associated bone loss. However, the effect of CLA on age-associated bone loss has not been investigated so far. Hence, this study was designed to examine the effect of corn oil (CO; $n-6$ FA) as control and of CLA on BMD in 12-month-old female C57BL6 mice fed for 10 weeks. We used c9t11 and t10c12 CLA isomers in an equal ratio in this study.

2. Materials and methods

2.1. Animals and experimental diets

Ten-month-old female C57BL/6 mice were obtained from Harlan (Indianapolis, IN). Weight-matched mice were housed in a laboratory animal care facility in cages (5 mice/cage) and fed a standard diet (Harlan Teklad LM-485) for 2 months. At 12 months of age, mice were divided into two dietary groups and fed a semipurified diet with AIN-93M vitamin and mineral mixes [25] containing either 10% CO or 9.5% CO+0.5% CLA (Clarinol Powder; Loders Croklaan). The composition of the semipurified diet is presented in Table 1. Fresh diet was prepared weekly, stored in aliquots at −20°C and provided daily (4 g/mouse). Mice were maintained on a 12-h light/dark cycle in an ambient temperature of 22–25°C at 45% humidity. National Institutes of Health guidelines were strictly followed, and all studies were approved by the Institutional Laboratory Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio (San Antonio, TX). Body weight was measured in all mice biweekly.

Table 1
Composition of experimental diets

Ingredient[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 or CO+CLA[b]	10.00

[a] All diet ingredients were purchased from MP Biomedicals (Irvine, CA).
[b] Diets consisted of 10% CO or 9.5% CO+0.5% CLA.

2.2. Measurement of BMD, lean body mass and body fat mass

BMD, lean body mass and body fat mass were measured by dual-energy X-ray absorptiometry (DEXA) at baseline (12 months) and after 10 weeks on dietary treatment using a Lunar PIXImus mouse bone densitometer (General Electric), and data analysis was carried out manually with PIXImus software [6]. Calibration of the instrument was conducted as suggested by the manufacturer. An aluminum/lucite phantom (TBMD=0.0700 g/cm^2; % fat=14.0) was placed on a specimen tray and measured 25 times without repositioning. Thereafter, the phantom was analyzed daily before animal testing for quality control purposes. Before bone scanning was performed, mice were anesthetized with intramuscular injections of ketamine/Rompun/NaCl (3/2/5). The densitometer was calibrated daily with a phantom supplied by the manufacturer. During measurements, the animals lay in prone position, with posterior legs maintained in external rotation with tape. Hip, knee and ankle articulations were in 90° flexion. Upon completion of scanning, BMD was determined in the following bone areas using the PIXImus software, version 1.46: distal femoral metaphysis (DFM) (knee joint) to include cancellous (trabecular) bone, proximal tibial metaphysis (PTM), femoral diaphysis (FD), tibial diaphysis (TD), lumbar spine vertebra 3 (L_3) and lumbar spine vertebra 4 (L_4). Intrascan coefficients of variation were 0.79%, 3.30%, 1.35%, 3.48%, 1.19% and 1.20% for DFM, PTM, FD, TD, L_3 and L_4, respectively; interscan coefficients of variation were 5.47%, 3.86%, 5.12%, 1.36%, 2.37% and 2.20% for DFM, PTM, FD, TD, L_3 and L_4, respectively. The coefficients of variation are in agreement with studies examining the precision and accuracy of the PIXImus densitometer [26,27].

2.3. Collection of blood serum

Blood was collected by retro-orbital bleeding from anesthetized mice that had been deprived of food overnight, and serum was obtained by centrifugation at 300×g for 15 min at 4°C for the measurement of glucose and insulin. At the termination of the study, mice were killed by cervical dislocation, blood was collected and serum was separated for the measurement of other metabolites.

Table 2
Effect of CLA on body composition in aging C57BL/6 female mice[a]

	CO			CLA			P^b		
	Baseline	10 weeks	Change in weight (g)	Baseline	10 weeks	Change in weight (g)	CLA	Time	CLA×Time
Body weight (g)	31.50±1.27	43.40±1.91	11.90±1.40	31.36±0.93	36.53±0.94	5.133±0.83*	<.05	<.0001	<.05
Lean mass (g)	18.36±0.55	16.84±0.21	−1.518±0.44	18.37±0.64	19.08±0.79	0.7267±1.24	.08	.50	.08
Fat mass (g)	6.94±0.83	21.04±1.66	14.10±1.55	6.76±0.52	12.03±0.43	4.842±0.79*	<.001	<.0001	<.001

[a] Values are presented as mean±S.E.M. ($n=10$).
[b] From two-way ANOVA.
* Significantly different from change in weight in the CO group, by unpaired t test.

2.4. Serum cytokine measurement

Serum cytokine levels for tumor necrosis factor α (TNF-α) and interleukin (IL) 6 were measured by standard enzyme-linked immunosorbent assay (ELISA) techniques using commercially available BD OptEIA ELISA kits for TNF-α and IL-6 (BD Biosciences, San Diego, CA), as described previously [28]. Each well of flat-bottom 96-well microtiter plates was coated overnight with 100 μl of purified anti-TNF-α and anti-IL-6 antibodies (4 μg/ml in binding solution) at 4°C. The plates were rinsed four times with washing buffer, and then samples were added, followed by incubation for 2 h at room temperature. The plates were washed four times with washing buffer, followed by the addition of biotinylated anticytokine antibodies. The plates were incubated in room temperature for 1 h and then washed four times with washing buffer. Streptavidin–alkaline phosphatase conjugate was added, and the plates were incubated for 30 min at room temperature. The plates were again washed four times with washing buffer, and chromogen substrate was added. The plates were then incubated at room temperature to achieve the desired maximum absorbance and were read at 410 nM in an ELISA reader (Dynex Technologies, UK).

2.5. Serum receptor activator of NF-κB ligand (RANKL) and TRA5b measurement

Serum RANKL and tartrate-resistant acid phosphatase (TRAP) were measured using mouse-free serum RANKL and mouse TRAP5b ELISA assay kits from Immunodiagnostic System (Fountain Hills, AZ), according to the manufacturer's instructions.

2.6. Serum glucose, insulin and leptin measurement

Insulin was analyzed using a rat/mouse insulin ELISA kit from Linco Research (St. Charles, MO). Glucose was analyzed spectrophotometrically using glucose (Trinder; Sigma Diagnostics, St. Louis, MO). Leptin was assayed using an active murine leptin kit from Diagnostic Systems Laboratories (Webster, TX).

2.7. Statistical analysis

Data are expressed as mean±S.E.M. To test the significance of the effects of diet, time and their interaction, data from the CO and CLA groups were analyzed by two-way analysis of variance (ANOVA). When interaction ($P<.05$) existed between diet and time, the significance of differences between CO and CLA groups was further analyzed by Bonferroni's posttest. The significance of differences in body weight, fat mass, lean mass and BMD from baseline to the end of the study between the CO and CLA groups was analyzed by unpaired t test. GraphPad Prism 4.0 was employed for statistical analyses. Differences were considered significant when $P<.05$.

3. Results

3.1. Body weight, lean mass and fat mass

Daily food consumption was monitored. There was no difference in food consumption between the groups (CO, 3.23±0.45 g/day; CLA, 3.45±0.78 g/day). However, body weight gain was significantly higher in the CO-fed group than in the CLA group. We also examined lean mass and fat mass by DEXA analysis. Fat mass gain was significantly

Table 3
Effect of CLA on organ/tissue weights

Organ/tissue	CO	CLA	P
Liver (g)	1.95±0.07	2.57±0.287	.0521
Spleen (g)	0.091±0.005	0.09±0.005	.9176
Total abdominal fat (g)	5.50±0.58	3.74±0.34*	.0467
Gastrocnemius wet weight (g)	0.115±0.004	0.138±0.003*	.0026
Gastrocnemius dry weight (g)	0.044±0.002	0.057±0.006	.0704
Quadriceps wet weight (g)	0.126±0.003	0.170±0.002*	<.0001
Quadriceps dry weight (g)	0.043±0.001	0.056±0.0015*	.0006

Values are presented as mean±S.E.M. ($n=10$).
* Significantly different by unpaired t test.

Table 4
Effect of CLA on BMD (g/cm^2) in aging C57BL/6 female mice[a]

Bone regions	CO			CLA			P[b]		
	Baseline	10 weeks	Change in BMD (mg/cm^2)	Baseline	10 weeks	Change in BMD (mg/cm^2)	CLA	Time	CLA×Time
DFM	89.24±2.16	80.52±2.94	−8.72±2.27	87.34±2.30	95.93±2.19	8.73±4.91*	<.05	.83	<.01
PTM	69.16±1.50	70.42±2.37	1.26±0.94	71.12±3.21	79.13±0.27	9.17±3.77*	<.01	<.05	.12
FD	61.28±1.94	60.04±1.30	−1.24±2.46	62.62±1.38	70.53±1.60	8.90±2.06*	<.01	<.05	<.01
TD	46.72±1.53	50.44±1.33	3.72±1.50	42.92±1.75	48.83±0.80	7.10±2.44	.13	<.05	.36
L_3	59.94±9.08	50.43±2.92	−12.3±9.74	53.86±4.93	66.93±2.80	20.47±3.74*	.15	.68	<.01
L_4	50.94±3.10	32.63±3.92	−16.38±4.89	53.76±5.37	53.53±6.03	3.05±6.75*	<.01	<.05	<.05

[a] Values are presented as mean±S.E.M. (n=10).
[b] From two-way ANOVA.
* Significantly different from change in BMD in the CO group, by unpaired t test.

higher in CO-fed mice than in CLA-fed mice. The results of body composition are shown in Table 2.

3.2. Effect of CLA on different organs and tissues

As bone loss is associated with muscle loss with age, we examined if CLA can prevent age-associated muscle loss. At the end of dietary treatment, mice were sacrificed, and the gastrocnemius and quadricep muscles were collected and weighed (wet weight). The results are shown in Table 3. The muscles were then dried at 80°C for 48 h for the complete removal of water and weighed (dry weight) again. Interestingly, the age-associated loss of quadriceps and gastrocnemius muscles was dramatically prevented by CLA treatment.

3.3. Effect of dietary fat on BMD

BMD was measured at 12 months (baseline) and after 10 weeks of dietary treatment. The results are shown in Table 4. BMD significantly increased in DFM, PTM and FD in CLA-fed mice, whereas BMD decreased or increased minimally in CO-fed mice. BMD increased in TD in both CO- and CLA-fed mice, but the increase was higher in CLA-fed mice. BMD was also significantly higher in lumbar regions in CLA-fed mice compared to CO-fed mice. These results suggest that CLA-fed aging mice maintain higher cancellous and cortical BMD in major bone compartments compared to CO-fed mice.

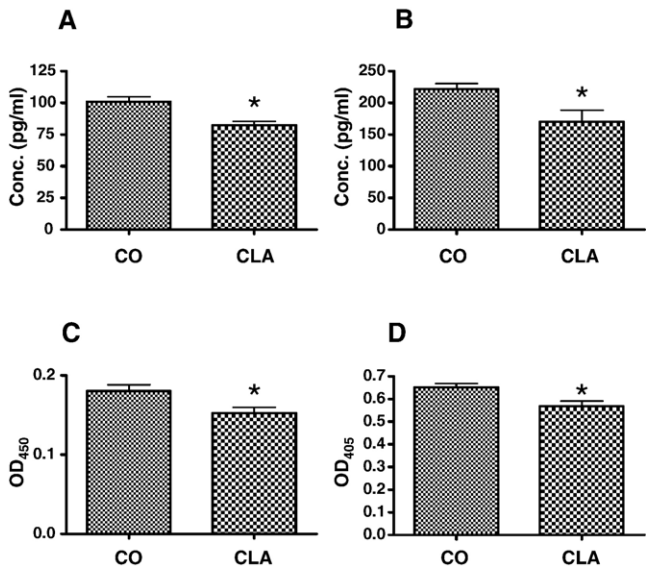

Fig. 1. After 10 weeks on diet, CLA- and CO-fed mice (10 mice/group) were sacrificed and serum was collected. Serum was analyzed for (A) IL-6, (B) TNF-α, (C) free serum RANKL and (D) TRAP5b levels using standard ELISA kits. Each bar represents the mean±S.E.M. of seven duplicate samples. *Significantly different by unpaired t test.

3.4. Effect of CLA on serum cytokines

To examine if CLA has any effect on osteoclastogenic factors (proinflammatory cytokines), we determined the serum IL-6 and TNF-α levels in CLA- and CO-fed mice. Serum TNF-α and IL-6 levels were decreased significantly in CLA-fed mice compared to CO-fed mice (Fig. 1A and B). The results indicate that CLA inhibits the production of the osteoclastogenic proinflammatory cytokines IL-6 and TNF-α.

3.5. Effect of CLA on serum RANKL and TRAP5b

To examine if CLA has any effect on the most pivotal osteoclastogenic factor RANKL [29,30], we examined the level of serum RANKL by ELISA. RANKL level was significantly less in CLA-fed mice than in CO-fed mice (Fig. 1C). The results indicate that the key bone-resorbing osteoclastogenic factor is reduced by CLA treatment. As serum TRAP5b [31,32] level indicates the current status of osteoclast function (i.e., TRAP activity), we determined the serum TRAP5b level using mouse TRAP5b ELISA kit. We found a significant reduction of TRAP activity in CLA-fed mice (Fig. 1D), which supports earlier findings that CLA inhibits the production of osteoclastogenic factors.

3.6. Serum biochemistry

As hyperinsulinemia and hyperglycemia are major concerns with CLA treatment, we examined if CLA induces

Table 5
Effect of CLA on serum leptin, glucose and insulin

	CO	CLA	P
Leptin (ng/ml)	34.25±1.91	26.64±2.59*	.0460
Glucose (mg/dl)	159.2±8.38	132.1±11.90	.1120
Insulin (ng/ml)	1.778±0.15	1.303±0.032*	.0213

Values are presented as mean±S.E.M. (n=7).
* Significantly different by unpaired t test.

hyperinsulinemia and hyperglycemia in this study. We did not find hyperinsulinemia and hyperglycemia in our experimental animal model. Serum leptin and insulin levels were significantly less in CLA-fed mice than in CO-fed mice. The results are shown in Table 5.

4. Discussion

In the present investigation, we have shown a novel action of CLA in the prevention of age-associated bone loss in a mouse model. We measured BMD in vivo using DEXA in the cancellous and cortical bones of the femur, tibia and lumbar spine in middle-aged C57BL/6 female mice. When 12-month-old mice were fed either CO or CO+CLA for 10 weeks, CLA-fed mice were found to maintain a higher BMD in pure cortical and cancellous bones. These findings correlated with a decreased activity of proinflammatory cytokines such as TNF-α, IL-6 and RANKL in serum. In spite of rapid advances in the treatment of osteoporosis, some of these therapies, however, were accompanied by adverse side effects, such as uterine, ovarian and breast cancers and an increased risk of cardiovascular diseases [33,34]. The importance of FAs in bone metabolism was indeed demonstrated in the past when Kruger and Horrobin [35] found that essential FA-deficient animals develop osteoporosis, together with increased renal and arterial calcification. They have also indicated the importance of FA in the regulation of Ca metabolism [35,36]. In the past two decades, CLA had been determined to have many potential health benefits [14]. Recently, CLA has been reported to enhance immunity and bone formation [17]. Current evidence suggests that CLA may help to decrease bone loss by reducing PGs in bone tissues [20,37] or by enhancing calcium absorption [38]. Our recent findings also showed higher BMD in young Balb/C male mice fed CLA than in those fed LA-enriched safflower oil [8]. Our present findings indicate that CLA has the potential to be a safe and economically feasible dietary supplement that could serve as an alternative medical approach to preventing bone loss associated with inflammation during aging.

Osteoporosis is characterized by the progressive loss of bone mass resulting from excess osteoclastic bone resorption relative to osteoblastic bone formation. The effect of CLA on osteoblastic factors has been explored in animal studies and in vitro [19,38]. However, no study has been conducted to measure the effect of CLA on osteoclastogenic factors. It is well established that aging is associated with an increase in proinflammatory cytokines such as IL-1β, IL-6 and TNF-α [39–43], and these cytokines are key regulators of osteoclastogenic activity and have been shown to increase bone resorption [44–48]. Furthermore, these cytokines induce the expression of COX-2 in osteoblastic and stromal cells, resulting in an increased production of PGE_2, which is also an essential factor in osteoclastogenesis [49–51]. Our present study found a lower activity of TNF-α and IL-6 in CLA-fed mice, which, in part, could explain the maintenance of higher BMD in these mice. In light of the PGE_2-lowering ability of CLA [20], it is possible that CLA may have a beneficial effect on age-associated bone loss. Although we did not measure PGE_2 in bone organ cultures in the present study, it is likely that a decreased activity of proinflammatory cytokines may have lowered PGE_2 levels in these mice, which could play an additional role in the beneficial effect of CLA on BMD.

There are evidences that antiosteoclastogenic drugs protect bone loss by blunting the production of IL-1, IL-6 and TNF-α [47,52], which stimulate the stromal cell production of RANKL and macrophage colony-stimulating factor — the sole regulators of osteoclastogenesis [53–55]. Rahman et al. [56] reported that CLA inhibits lipopolysaccharide-induced inflammatory events in the macrophage cell line RAW 264.7, an osteoclast precursor cell, by negatively regulating inflammatory mediators and NF-κB activation [57,58]. We also found significantly lower levels of serum RANKL, as well as TNF-α and IL-6, in CLA-fed mice than in CO-fed mice.

TRAP is primarily a cytochemical marker of macrophages, osteoclasts and dendritic cells [59]. Although osteoclasts contain abundant TRAP and are responsible for bone resorption, the total TRAP activities in the serum, as measured by colorimetric methods, however, minimally reflect bone turnover [60]. TRAP5 is further separated into 5a and 5b by electrophoresis. TRAP5b is considered a prominent product of osteoclasts. Thus, serum TRAP5b levels would reflect the status of bone resorption [60]. Interestingly, we found a significant reduction of TRAP5b activity in CLA-fed mice, which reflects the reduction of bone-resorbing activity. These findings indicate that the possible mechanisms of this age-associated bone loss protection by CLA may be the modulation of osteoclastogenic bone resorption by altering osteoclastogenic factors.

Concomitant losses of skeletal muscle (sarcopenia) and bone mass (osteopenia), along with the gradual accretion and centralization of adipose tissues, typify usual human aging. This increase in fat mass is accompanied by decreases in both muscle and bone compartments of lean tissues, beginning in midlife and continuing into extreme old age [61,62]. Muscle loss is strongly associated with bone loss [63,64]. Loss of muscle mass poses significant health risks to the elderly [65,66]. Therefore, we examined the effect of CLA on the accretion of adipose tissues and on loss of muscle mass. We found a significant reduction of fat mass and protection of muscle loss in CLA-fed mice. Moreover, an increase in fat mass and a decrease in muscle mass are closely linked to the increased activation of proinflammatory cytokines such as TNF-α and IL-6 [67–69]. Thus, CLA may have a protective role in aging by preventing an increase in fat mass and a decrease in lean and bone mass.

CLA has previously been shown to induce hyperglycemia and hyperinsulinemia in rodent models [70], especially in C57BL/6 mice [71], and in obese humans [72]. CLA has also been reported to induce insulin resistance in rodents [73,74]. In our present study, we measured serum levels of glucose and insulin to determine if the beneficial effect of

CLA on bone is associated with adverse effects on insulin sensitivity. Interestingly, CLA decreased both glucose and insulin levels. It has been also shown that, although the short-term administration of CLA induces insulin resistance, prolonged treatment improves glucose tolerance and decreases insulin levels in C57BL/6 mice [75]. Furthermore, obese subjects were reported to have higher plasma leptin levels [76]. We have previously reported that serum leptin levels and mRNA expression in peritoneal fat pads decreased in parallel with the decrease in fat mass in CLA-fed mice on a high-fat diet [25]. Since we observed a significant lowering of fat mass with CLA intake in the present study, we measured serum leptin levels and found it to be significantly lower in CLA-fed mice.

Very recently, it was reported that, among the most abundant isomers of CLA, t10c12 is responsible for the enlargement of the liver (liver hypertrophy) [12,77]. We have also observed a notable enlargement of the liver in CLA-fed mice compared to CO-fed mice. However, the difference was not significant. This liver hypertrophy might be minimized by using different ratios of c9t11 and t10c12 CLA isomers. Future studies using different ratios of these two isomers are needed to determine the appropriate ratio to minimize liver hypertrophy.

In summary, our data demonstrate that the prevention of age-associated bone loss by CLA is closely linked to a decrease in markers of inflammation and osteoclastogenic factors. By inhibiting osteoclastogenic proinflammatory cytokines, CLA not only prevents bone loss due to excessive osteoclastogenesis but also prevents accretion of fat mass and loss of muscle mass during aging. Indeed, our data warrant a clinical trial to show the efficacy of CLA on bone loss protection in aging people. Thus, CLA might become an efficient dietary therapy, improving the quality of life of aging people after successful clinical trials. More studies with different ratios of individual CLA isomers are necessary to establish the safety and the mechanisms of action of the modulation of bone, muscle and fat mass during aging.

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