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ORIGINAL CONTRIBUTION

Effect of CLA isomers and their mixture on aging C57Bl/6J mice

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Abstract
Background Dietary supplements containing conjugated linoleic acid (CLA) are widely promoted for weight loss management over the counter. Recently, FDA approved the CLA as Generally Recognized as Safe category so that it can be used in various food and beverages. The combined effect of CLA isomers have been studied extensively in animals and humans, however, the role of individual isomers remains unraveled.
Aim The present investigation addresses the effects of CLA isomers on body composition and body weight as well as safety using female C57Bl/6J aging mice.
Methods Two main CLA isomers and their mixture were fed to 12-months-old female C57Bl/6J mice. Ten percent corn oil (CO) based fat diet supplemented with 0.5% purified *cis* 9 *trans* 11 (c9,t11) CLA or *trans* 10 *cis* 12 (t10,c12) CLA or their mixture (CLA mix, 50:50) for 6 months. The lean mass, fat mass, glucose, non-esterified fatty acids, and insulin were examined at the end of study.
Results As a result of 6 months dietary intervention, both t10,c12 CLA and CLA mix groups showed increased lean mass and reduced fat mass compared to that of c9,t11 CLA and CO group. However, insulin resistance and liver hypertrophy were observed in t10,c12 CLA and CLA mix groups based on the results of homeostasis model assessment, revised quantitative insulin-sensitivity check index (R-QUICKI), intravenous glucose tolerance test, and liver histology. Liver histology revealed that increased liver weight was due to hypertrophy.
Conclusion In conclusion, the major CLA isomers have a distinct effect on fat mass, glucose, and insulin metabolism. The t10,c12 isomer was found to reduce the fat mass and to increase the lean mass but significantly contributed to increase insulin resistance and liver hypertrophy, whereas c9,t11 isomer prevented the insulin resistance. Between the two major CLA isomers, the t10,c12 was attributed to reduce fat mass whereas, c9,t11 improves the insulin sensitivity.

Keywords Obesity · Conjugated linoleic acid · Fat mass · Glucose · Insulin

Introduction

Over the past 3 decades, there has been a marked increase in the prevalence of overweight and obesity, which continues to rise regardless of gender, socio-demographic status, or geographic region. The obesity epidemic imposes a significant threat to the health of the U.S. population, despite the many advancements made in medical and public health interventions [48]. It is an utmost importance to develop a strategy to control this adiposity and subsequent numerous complications like diabetes hypertension and cancer. Recently, conjugated linoleic acid (CLA) has been known to promote loss of body fat and weight. CLA represents a group of positional and geometric isomers of conjugated dienoic derivatives of linoleic acid. The major dietary source of CLA for humans is ruminant meats; such as beef, lamb, and dairy products, including milk and

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cheese [25]. The major isomer of CLA in natural foods is *cis*-9, *trans*-11 (c9,t11) [17, 20]. CLA is marketed commercially in the USA in products such as Natrol®, Your Life®, Vitamin World®, Nature's Way®, and Nature's Plus®. These products are available over the counter in supermarkets, drug stores, and health food stores, and can be bought from wholesalers throughout the USA. CLA is also sold in Asia, Canada, Europe, and Japan. Most of the CLA products sold as dietary supplements for human consumption contain 60–90% CLA in the form of either free fatty acids or triglycerides, and they usually contain a mixture of isomers, predominantly c9,t11 and *trans* 10, *cis* 12 (t10,c12) [22]. Health benefits and biological functions of CLA date back to the 1980s, when Ha et al. [12] reported inhibition of chemically induced skin neoplasia in mice pretreated with CLA. A number of beneficial effects of CLA from different aspects including cancer [13], immune function [30], atherosclerosis [24], weight gain [34], food/energy intake, as well as body composition and bone loss [3–5, 37] have been published. The antiobesity effects of CLA have been supported by studies in animals [8, 33–35, 46, 49, 50] and humans [23, 51]. However, a number of adverse effects, including insulin resistance, and increase in fasting glucose have also been reported in animal [32] and human studies [23]. Recently, the FDA has approved the CLA as a 'Generally Recognized as Safe' (GRAS) category [15] (http://www.nutraingredients-usa.com), which will encourage additional commercial production of food and beverages with CLA supplementation. However, some recent studies have questioned the safety of long term supplementation with CLA. Therefore, it is very important to determine isomer specific effects of CLA in health and diseases. This is paradoxical, because CLA-mediated hyperinsulinemia have been observed in several studies in mice [8, 40, 46] and humans [38, 39]. These paradoxical findings may arise from the differential effects of c9,t11 CLA and t10,c12 CLA, the different ratio of the two isomers used, the different levels or doses of CLA used, species variation, or metabolic status of the experimental animal. Most of these studies used synthetically prepared CLA, a mixture of c9,t11 CLA and t10,c12 CLA isomers. There is growing evidence that individual isomers of CLA have specific physiological functions. These diverse findings of CLA led us to hypothesize that individual CLA isomers may have a specific effect on health in aging mice. Hence, the aim of this present study is to unravel the isomer-specific effect of CLA in aging C57BL/6J mice, which on a high fat diet, develop obesity, and insulin resistance [45, 52]. In this investigation, we have delineated that t10,c12 isomer is found to reduce the body fat mass and increase the body lean mass, but significantly contributed to the increase of insulin resistance and liver hypertrophy, whereas c9,t11 isomer improved insulin sensitivity. Our primary goal was to differentiate between the isomer-specific effects of CLA in the prevention of age-associated muscle loss in C57Bl/6J mice, as muscle loss is the major health concern in the geriatric population, leading to falls and prevention of early morbidity and mortality.

Materials and methods

Animals

Eleven-months-old female C57Bl6/J mice, weighing 23–24 g, were purchased from Jackson Laboratories (Bar Harbor, Maine 04609 USA) and fed a standard diet (Harlan Teklad LM-485) for 1 month. At 12 months of age, weight matched mice were divided into four groups. Each group consisted of 20 mice (5 mice/cage) and fed an experimental diet for 6 months. The animals were maintained in a 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 at San Antonio (San Antonio, TX, USA). The mice were fed the American Institute of Nutrition (AIN)-93 diet containing CLA isomer in 10% corn oil (CO) as a high fat diet ad libitum for 6 months. Body weights were measured weekly.

Experimental diet preparation

The diets were supplemented with 10% CO as a high fat diet, 0.5% c9,t11 CLA, 0.5% t10,c12 CLA and a mixture of c9,t11 CLA and t10,c12 CLA (CLA mix). The mixture of c9,t11 CLA and t10,c12 CLA isomers were in equal amounts, i.e., 0.25:0.25%. The CLA isomers were supplied by Lipid Nutrition, Channahon, IL, USA. The c9,t11 CLA enriched diet contained approximately 61% of c9,t11 CLA isomer and t10,c12 CLA contained 71% of t10,c12 CLA. The composition of the semipurified diet per 100 g of diet is presented in Table 1. Fresh diet was provided everyday in the afternoon (between 1:00 and 2:00 pm).and leftover food was removed daily to prevent rancidity. Animals were fed 5 g diet/mouse.

Measurement of body composition

At 12 and 18 months of age, (pre and post) body composition of mice in each experimental group were determined using dual-energy X-ray absorptiometry (DXA) Lunar

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 or CO + CLA[b,c,d]	10.00

[a] All diet ingredients were purchased from MP Biomedicals (Irvine, CA, USA)

[b,c,d] Diets consisted of 10% CO or 9.5% CO + 0.5% c9,t11 CLA, 0.5% t10,c12 CLA and mixture of 0.25% c9,t11 CLA and 0.25% t10t12 CLA, respectively

PIXImus (GE, Madison, WI, USA) and data was analyzed with PIXImus software as described previously [4, 5, 44].

Serum metabolites and cytokines

The intravenous glucose (1 g/kg) tolerance test (IVGTT) was performed after 20 weeks after the start of the experimental diets, using five mice from each dietary group. One week prior to sacrifice, blood samples were taken from the intraorbital, retrobulbar plexus from anesthetized mice to measure fasting glucose, insulin, and non-esterified fatty acid (NEFA) in serum. Glucose was analyzed spectrophotometrically using Glucose Colorimetric Assay Kit, (QuantiChrom, Hayward, CA, USA). Insulin was analyzed using a rat/mouse Ultra sensitive rat insulin ELISA kit (Crystal Chem Inc., IL, USA). Insulin-like growth facor-1 (IGF-1) and adiponectin was assayed using mouse IGF-1 and adiponectin Quantikine immunoassay kit, respectively (R&D Systems, MN, USA). Leptin was assayed using an active murine leptin kit (Diagnostic Systems Laboratories, TX, USA). Triglycerides were analyzed spectrophotometrically using Triglycerides colorimetric Assay Kit (Cayman Inc, IL, USA). TNF-α and IL-6 were measured by using ELISA kits (eBiosciences, CA, USA). All the serum metabolites and cytokines were measured following the instructions provided by the manufacturers. Insulin resistance was calculated by using HOMA [28]; however, it has not been validated for use in animal models [47]. Further, insulin sensitivity, another index of insulin resistance, was determined using R-QUICKI, (i.e., the revised quantitative insulin sensitivity check index) [36].

Tissue collection for biochemical analysis and liver histology

After 6 months on the experimental diet, the mice were anaesthetized and blood was obtained by intraorbital capillary plexus. Serum was collected and stored at −80°C. Liver, spleen, gastrocnemius, and quadriceps muscles were collected, weighed and frozen in liquid nitrogen and stored in −80°C Liver tissues were fixed in 4% formaldehyde. Sections of liver were embedded in paraffin and stained with hematoxylin and eosin to identify steatosis and changes in morphology.

Statistical analysis

Data are presented as mean values ± SEM. Differences among the groups (CO, c9,t11 CLA, t10,c12 CLA and CLA mix) were tested by one-way analysis of variance (ANOVA) followed by Newman–Keuls post hoc test. A P value ≤ 0.05 was considered statistically significant. The analyses were performed using Graphpad prism for Windows (La Jolla, CA, USA).

Results

Food intake, body weights, and organ weights

The baseline body weight values were 24.03 ± 0.2, 24.28 ± 0.35, 23.46 ± 0.34, and 23.81 ± 0.45 g for CO, c9,t11 CLA, t10,c12 CLA and CLA mix groups, respectively. As shown in the Fig. 1a, 6 months administration of CLA mix caused a significant decrease ($P \leq 0.05$) in body weight gain (26%) when compared to CO (58%). The t10,c12 CLA group showed highly pronounced difference in weight gain (10%) compared to CO (58%) group. In contrast, there was no significant change in body weight gain (52%) in the c9t11 CLA mix fed group relative to the CO (58%) group. As observed in our previous study with CLA feeding [5], this study also did not show any significant difference in food intake among different dietary groups (Table 3).

The administration of t10,c12 CLA and CLA mix to middle aged mice showed significant increase in liver weight compared to CO group. The CO group liver weight was 1.51 ± 0.06 g, however, in t10,c12 CLA and CLA mix fed mice, liver weighed 2.26 ± 0.25 and 2.01 ± 0.09 g, respectively, which is 96% more in t10,12 CLA and 74% more in CLA mix fed mice, as shown in Table 2. Interestingly, the skeletal muscle quadriceps and gastrocnemius muscle weights were moderately increased (not significant) in t10,c12 CLA and CLA mix fed mice. The increased weight of the skeletal muscle in t10,c12 CLA and CLA mix fed mice showed by DXA analysis (Table 3),

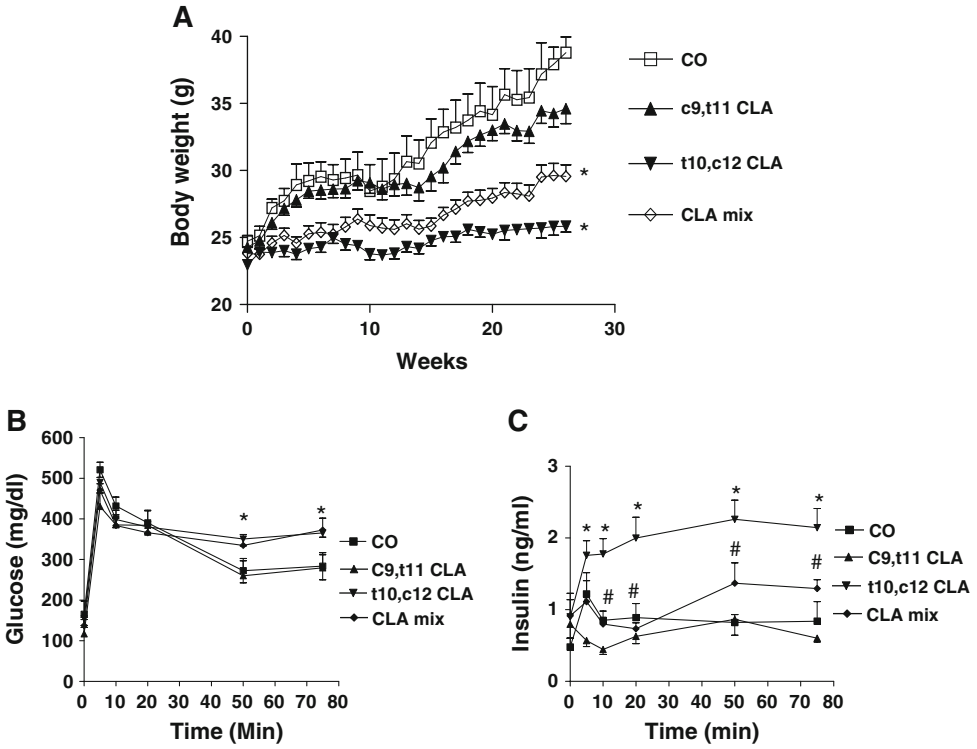

Fig. 1 Body weight and IVGTT of C57Bl/6J mice fed individual isomers of CLA and their mixture for 6 months. **a** Body weight of 18-months-old mice fed 10% corn oil (CO) or supplemented with 0.5% c9,t11 CLA or t10,c12 CLA isomer or CLA mix for 6 months. **b** Fasting serum glucose levels after intravenous glucose administration. **c** Fasting serum insulin levels after intravenous glucose administration. Glucose (1 g/kg) was injected into the tail vein of mice fed with CO, c9, t11 CLA, The t10,c12 CLA and CLA mix. The IVGTT was performed at 5 months after starting the diets. Data are means ± SEM from five independent experiments. Values with *different signs* are significantly different at $P < 0.05$ by Newman–Keuls one way ANOVA

Table 2 Serum metabolites, adipokines, and organ weights in 18-months-old C57Bl/6J mice fed with CLA isomers and their mixture for 6 months. Serum metabolites and adipokines

Parameter	CO	c9,t11 CLA	t10,c12 CLA	CLA mix
Glucose (mg/ml)	223.7 ± 8.473	197.3 ± 13.58	294.0 ± 23.60*	271.8 ± 11.97*
Insulin (ng/ml)	0.41 ± 0.04	0.35 ± 0.10	0.62 ± 0.06*	0.68 ± 0.07*
NEFA (mEq/L)	0.93 ± 0.05	0.82 ± 0.11	1.11 ± 0.06	1.04 ± 0.04
Triglycerides (mg/dl)	62.39 ± 3.60	68.38 ± 2.52	50.17 ± 3.71*	42.94 ± 5.81*
HOMA-IR	4.96 ± 0.56	4.15 ± 0.75	8.50 ± 0.85*	7.15 ± 0.82*
R-QUICKI	0.52 ± 0.17	0.59 ± 0.22	0.44 ± 0.27*	0.45 ± 0.31*
Leptin (μg/ml)	5.30 ± 0.84	3.28 ± 1.32	0.86 ± 0.36*	1.99 ± 0.40*
Adiponectin μg/ml)	2.96 ± 0.02	2.73 ± 0.10	3.06 ± 0.04	2.87 ± 0.05
TNF-α (pg/ml)	60.83 ± 5.28	45.86 ± 4.81	37.45 ± 3.47*	39.98 ± 3.44*
IL-6 (pg/ml)	0.31 ± 0.03	0.30 ± 0.02	0.28 ± 0.01	0.30 ± 0.01
IGF-1 (pg/ml)	17.20 ± 7.40	19.29 ± 5.92	19.85 ± 7.92	21.76 ± 12.44*
Organ weights				
Liver (g)	1.51 ± 0.06	1.62 ± 0.10	2.26 ± 0.25*	2.01 ± 0.09*
Spleen (g)	0.14 ± 0.02	0.12 ± 0.01	0.16 ± 0.02	0.121 ± 0.00
Adipose tissue (g)	2.84 ± 0.33	2.69 ± 0.24	0.76 ± 0.06*	1.35 ± 0.10*
Quadriceps (g)	0.14 ± 0.009	0.15 ± 0.010	0.15 ± 0.012	0.16 ± 0.018
Gastrocnemius (g)	0.16 ± 0.002	0.17 ± 0.003	0.16 ± 0.014	0.19 ± 0.014

Data are means ± SEM, ($n = 17$–20 mice/group)

Values with asterisk are significantly different at $P < 0.05$ by Newman–Keuls one way ANOVA

demonstrated the significant increase in hind leg lean mass compared to CO group.

The total fat content in the peritoneal abdominal cavity was also assessed. CO fed group showed abdominal fat (2.84 ± 0.33 g) significantly different from t10,c12 CLA (0.76 ± 0.06 g) and CLA mix (1.35 ± 0.10 g) group; however, the total abdominal fat was not changed in c9,t11 CLA (2.69 ± 0.24 g) compared to CO group. These results

Table 3 Effect of CLA isomers on food intake and body composition in 18 months C57Bl/6J mice

Parameter	Diet			
	CO	c9,t11 CLA	t10,c12 CLA	CLA mix
Food intake (g)	3.19 ± 0.06	3.04 ± 0.07	2.99 ± 0.07	3.06 ± 0.05
Body weight (g)				
Baseline	24.03 ± 0.2	24.28 ± 0.35	23.46 ± 0.34	23.81 ± 0.45
Final	40.28 ± 1.07	36.80 ± 1.22	25.69 ± 0.47*	29.56 ± 0.83*
% Difference	58.08 ± 4.33	52.45 ± 4.81	9.84 ± 1.319*	26.22 ± 2.61*
Total body lean mass (g)				
Baseline	17.13 ± 0.53	16.96 ± 0.20	17.42 ± 0.29	16.87 ± 0.27
Final	16.88 ± 0.41	18.26 ± 0.33*	18.65 ± 0.33*	20.05 ± 0.32*
% Difference	−1.12 ± 3.12	9.60 ± 2.16*	7.15 ± 1.61*	15.69 ± 1.61#
Total body fat mass (g)				
Baseline	4.92 ± 0.38	4.86 ± 0.29	4.10 ± 0.23	4.31 ± 0.24
Final	14.92 ± 1.63	14.46 ± 0.98	5.18 ± 0.27*	9.13 ± 0.27#
% Difference	220.8 ± 33.63	195.7 ± 23.15	27.59 ± 6.66*	122.2 ± 19.85#
Ab fat mass (g)				
Baseline	1.26 ± 0.10	1.18 ± 0.12	0.90 ± 0.07	0.98 ± 0.08
Final	9.25 ± 0.44	6.74 ± 0.55	1.74 ± 0.12*	3.75 ± 0.17*
% Difference	590.9 ± 57.09	581.9 ± 71.88	102.9 ± 16.12*	326.2 ± 58.39*
Hind Leg LM (g)				
Baseline	0.66 ± 0.01	0.71 ± 0.01	0.72 ± 0.01	0.69 ± 0.01
Final	0.63 ± 0.02	0.70 ± 0.02	0.84 ± 0.01*	0.80 ± 0.01*
% Difference	−4.80 ± 2.78	−0.37 ± 3.63	19.17 ± 3.35*	14.12 ± 0.16*
Hind Leg FM (g)				
Baseline	0.25 ± 0.02	0.22 ± 0.02	0.15 ± 0.01	0.18 ± 0.02
Final	0.58 ± 0.03	0.57 ± 0.03	0.26 ± 0.01*	0.38 ± 0.01*
% Difference	410.0 ± 78.10	260.4 ± 54.46	101.1 ± 18.93*	172.7 ± 39.90*

Data are means ± SEM, ($n = 17$–20)

Values with different signs are significantly different at $P < 0.05$ by Newman–Keuls one way ANOVA

are supported by the DXA which was performed before starting the diet and finally at the end of study as shown in Table 3.

Serum triglycerides, NEFA, fasting glucose, and insulin determination

Fasting serum NEFA was decreased by 12% in c9,t11 CLA fed mice compared to CO group. However, t10,c12 CLA and CLA mix increased the NEFA 19 and 11%, respectively. The fasting serum glucose concentration was increased significantly in t10,c12 CLA and CLA mix fed mice compared to CO group as shown in Table 2. The fasting serum insulin concentration was significantly increased after 6 months dietary feeding with t10,c12 CLA, demonstrating hyperinsulinemia compared to CO, but in c9,t11 CLA, the insulin levels were unchanged. The t10,c12 CLA and CLA mix diet had shown significant increase in insulin levels compared to CO group. The serum triglycerides were significantly ($P < 0.01$) decreased in t10,c12 CLA and CLA mix group compared to CO control group as shown in Table 2.

Total lean, fat, and abdominal fat mass by DXA

As shown in Table 3, t10,c12 CLA and CLA mix diet fed mice exhibited a significant increase in total body lean mass (BLM) compared to CO group, whereas it was decreased (1%) in the CO fed group. In the c9,t11 CLA fed group, there was no significant change in total BLM. The total body fat mass (BFM) gain was greater in CO mice (220.8 ± 33.63%) compared to that of t10,c12 CLA (27.59 ± 6.66%) and CLA mix (122.2 ± 19.85%) fed mice. Abdominal fat mass (AbFM) was significantly reduced in t10,c12 CLA and CLA mix ($P < 0.05$) fed mice compared to CO. Hind leg lean mass (LM) was significantly increased in t10,c12 CLA and CLA mix ($P < 0.01$), compared to CO. Also there was a significant decrease in

hind leg fat mass (FM) in t10,c12 CLA and CLA mix compared to CO as a control group.

Intravenous glucose tolerance test (IVGTT)

The IVGTT was performed at 20 weeks after the start of the CLA isomers and CO supplementation. At 5, 10, and 20 min the c9,t11 CLA fed mice eliminated glucose faster than the CO, t10,c12 CLA and CLA mix mice. In contrast, the CO, t10,c12 CLA and CLA mix fed mice had lost their enhanced elimination of glucose, at both 50 and 75 time points (Fig. 1b). The 5-min insulin response to intravenous glucose challenge was increased rapidly to 1.22 ± 0.29 ng/ml in CO, whereas 1.75 ± 0.20 and 1.11 ± 0.29 ng/ml in t10,c12 CLA and CLA mix group, respectively, but was apparently insufficient to maintain normal glucose tolerance (Fig. 1c). More pronounced increase in insulin secretion were observed by glucose challenge in the t10,c12 CLA group as shown in Fig. 1c. Thus, taken together, the basal levels of glucose and insulin results from the glucose challenges demonstrated that t10,c12 CLA and CLA mix fed mice display a more extensive impairment of β-cell function and, consequently, exaggerated diabetes compared with c9,t11 CLA.

Serum pro inflammatory cytokines

As shown in Table 2, the serum TNF-α level was significantly decreased in t10,c12 CLA and CLA mix fed mice compared to that of CO fed mice ($P \leq 0.05$). Serum IL-6 levels were also decreased in the t10,c12 CLA and CLA-fed mice compared with CO fed mice. c9,t11 CLA fed mice showed no differences in IL-6 and TNF-α cytokines.

Liver histology

Histological sections of liver tissue from t10,c12 CLA and CLA mix groups showed predominantly large lipid-filled vacuoles (macrovesicular steatosis). Liver sections from CO and c9,t11 CLA group revealed a heterogeneous mixture of large lipid-filled vacuoles and small lipid droplets (microvesicular steatosis). The CO and c9,t11 CLA group exhibited visibly less lipid-filled vacuoles and were distinguishable from t10,c12 CLA and CLA mix groups. The histomorphometric analysis using Metaview Image Analysis system, revealed that the lipid vacuole areas were significantly larger in t10,c12 CLA and CLA mix groups, indicating liver steatosis (Fig. 2). Lipid filled vacuole area in t10,c12 CLA (31.24 ± 0.7 μm^2) and CLA mix (26.33 ± 0.6 μm^2) were significantly higher than that of CO (6.22 ± 0.3 μm^2) and c9,t11 CLA (7.42 ± 0.4 μm^2).

Discussion

Aging related changes in body composition (i.e., muscle decline and fat mass increase) is the principal etiological factor of metabolic syndrome, type 2 diabetes, cardiovascular diseases and sarcopenia [31, 56, 57]. Given the increasing prevalence of obesity (increase fat mass and decrease muscle mass) with aging, it would be valuable to identify potential therapeutic nutrients/functional foods to improve muscle mass, decrease in fat mass and improve glucose and lipid metabolism, within the context of obesity. In the present investigation, we used C57Bl/6J aging mice fed a high fat diet (10% CO) which develops obesity, insulin resistance and significant decrease in muscle mass, and revealed the differential effects of dietary CLA isomers on hind leg muscle mass, fat mass (leg as well as visceral fat mass), glucose, triglycerides, NEFA, insulin, as well as liver hypertrophy, in aging C57Bl/6J mice.

Further, we also found that t10,c12 CLA and CLA mix fed mice showed increased lean mass, decreased body weight, decreased BFM, and decreased AbFM compared to that of CO and c9,t11 CLA fed groups. CO fed mice exhibited significant reduction of muscle mass with age whereas t10,c12 CLA and CLA mix fed mice showed protection against this age-associated muscle loss. Moreover, t10,c12 CLA and CLA mix fed mice exhibited significant gain of muscle mass with age. These results demonstrate that t10,c12 CLA and CLA mix may be novel dietary supplement to prevent/restore age-associated loss of muscle mass.

In previous studies, CLA mix or the t10,c12 CLA isomer fed animals exhibited significantly decreased body weight and fat mass compared to control animals [34, 35, 41, 49]. CLA feeding did not affect food intake in this study. Our previous study with CLA [5] and this study, however, were not consistent with some of the other investigators' reports [14, 35], potentially due to their use of essential fatty acids deficient diet. We used a very high level of essential fatty acids, in the form of corn oil, in our diets, which may have decreased the effect of CLA on food intake.

Dietary supplementation with CLA isomers induces an intricate response in the mouse, including marked changes in adipose tissue, liver histology, and profound alterations in several endocrine blood parameters. In the present study, increased glucose and insulin levels were found only in t10,c12 CLA and CLA mix group, demonstrating the hyperinsulinemia followed by glucose intolerance. The changes in glucose, insulin, and NEFA levels were markedly less in c9,t11 CLA compared to CO, and CLA mix. On other hand, t10,c12 CLA isomer increased NEFA and induced a prodiabetic state. CLA mix mediated increases in plasma insulin levels and insulin resistance have already been reported in humans [29, 38]. Shepherd et al. demonstrated that intervention with a c9,t11 CLA enriched diet

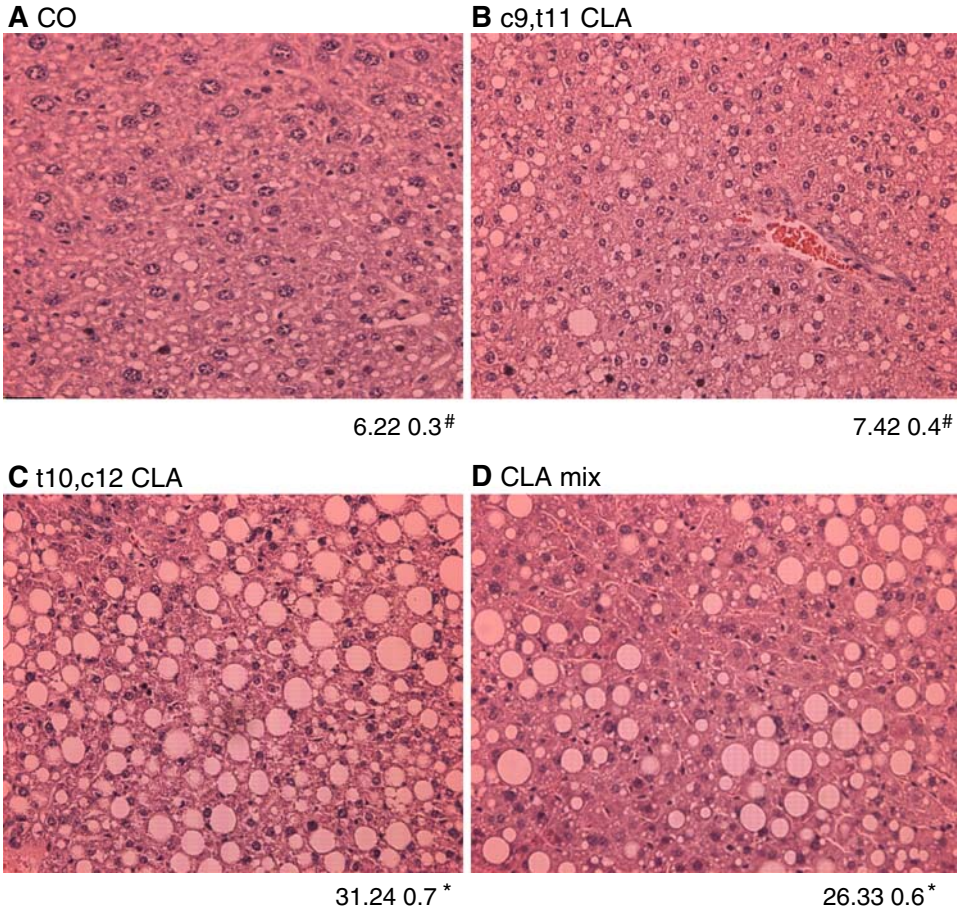

Fig. 2 Liver histology of 18-months-old C57Bl/6J mice fed with individual isomers of CLA and their mixture for 6 months *Panels* show hematoxylin and eosin (H&E) stained representative liver sections from 18-months-old C57Bl/6J mice fed CO, c9,t11 CLA, t10,c12 CLA and CLA mix for 6 months. Circular vacuole area resembles lipid. *Bottom numbers* represent % area containing lipid droplets by histomorphometery using Metaview Image Analysis System. Data are means ± SEM. ($n = 6$–8 per group). Values with *different signs* are significantly different at $P < 0.05$ by Newman–Keuls one way ANOVA. Image magnification 200×

significantly reduced the plasma insulin and glucose concentrations, decreased the HOMA-IR index of insulin resistance, and improved the revised QUICKI indicator of insulin sensitivity in the well-characterized *ob*/*ob* mouse model that displays an obese insulin-resistant phenotype [42]. In this study, we have also noted decreased glucose, HOMA-IR and improved QUICKI in c9,t11 CLA isomer fed mice compared to that of other groups.

There are number of studies showing that abdominal, particularly visceral obesity (the so called abdominal or central obesity syndrome) is associated with insulin resistance [9, 21]. Rodents fed a high fat diet also rapidly develop an increase in visceral fat [45]. In this investigation, mice fed with CO diet exhibit significantly higher total visceral fat weight (retroperitoneal and mesenteric fat deposits) than that of t10,c12 CLA and CLA mix fed mice after 6 months on experimental diets.

Leptin, a product of the obese gene, is secreted primarily by adipocytes and plays an important role in food intake and regulating energy balance. Circulating leptin is highly correlated with general adiposity in obese rodents [27, 43] and humans [27, 43]. Decreased leptin levels in CLA mix fed mice were correlated with decrease in fat mass in the present study, suggesting that this may be one of the possible mechanisms involved in this present observation. Adiponectin is an adipokine exclusively produced by adipocytes, which decreases the hepatic lipogenesis and increases the FFA oxidation, and hepatic insulin sensitivity in mice [54]. However, we did not find any significant difference in adiponectin levels between the CLA isomers. Interestingly, t10,c12 CLA and CLA mix fed mice showed a fatty liver, which may be one of the possible causes of insulin resistance in these mice. The liver histology revealed large lipid filled vacuoles (macrovesicular steatosis) in the liver of t10c12 CLA and CLA mix groups. In the present study, t10,c12 CLA and CLA mix induced hepatomegaly, accompanied by the accumulation of lipids in the liver. In this context, it seemed possible that the induction of insulin resistance in these mice could be mediated either by liver hypertrophy or by changes in receptor function of membrane composition [11, 26].

In this present investigation, we also noted decreased TNF-α and IL-6 production in the serum of t10,c12 CLA and CLA mix fed mice compared to that of CO fed mice. In our previous study, we showed that CLA fed mice expressed less TNF-α mRNA in peritoneal fat as compared to that of control group [5]. Decreased TNF-α and IL-6 in serum, and macrophages derived from CLA fed rats and

mice have already been reported [1, 16, 53, 55]. However, some studies have reported either no effect or significant elevation of these cytokines in cultured cells from mice and humans [18, 19]. It has been established that IL-6 and TNF-α are negatively associated with muscle loss [7, 10, 31], therefore decreased levels of pro-inflammatory cytokines (IL-6 and TNF-α) by t10,c12 CLA and CLA partially explain the role of these fatty acids in the prevention of muscle loss in high fat diet fed obese C57Bl/6J aging mice.

The present data also suggests that t10,c12 CLA and CLA mix have positive effects in decreasing fat mass, TNF-α and IL-6, thus maintaining lean body mass in high-fat diet-fed mice, which, in part, could explain the anti-inflammatory properties of CLA in these mice. Our study confirms that only t10,c12 CLA isomer contributes to a significant change of body composition in C57Bl/6J mice. The anti-obesity effect of CLA mix has been ascribed to reduced adipocyte size [2, 6], reduced adipocyte proliferation [6], increased adipocyte lipolysis [8] apoptosis [46] and greater fatty acid oxidation and energy expenditure [49] which is predominantly the effect of t10,c12 CLA.

Conclusion

In conclusion, most previous CLA studies have been of short duration and inconclusive. To our knowledge, only two long term CLA studies in human have been conducted [23, 51], however, no CLA isomers specific long term study have been reported particularly with a specific focus on muscle loss in obese aging animals or humans. In these studies, fatty liver formation due to CLA has not been reported in human as seen in mice. This is the first study in which CLA isomers were administered for 6 months in old mice to examine the isomer-specific effects of CLA on muscle mass, which is reported to be reduced during aging in humans as well as in animals. As CLA has been approved by FDA in GRAS category, considering the possible adverse effects of long term use of CLA, further additional isomers specific studies in animals and humans are urgently needed before the CLA isomers usage as a dietary supplement to reduce obesity and to improve muscle mass in humans.

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