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ORIGINAL CONTRIBUTION

Eight weeks of conjugated linoleic acid supplementation has no effect on antioxidant status in healthy overweight/obese Korean individuals

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Abstract
Purpose The aim of this study was to evaluate the effect of CLA supplementation on antioxidant metabolism in healthy overweight/obese Korean individuals.
Methods We performed a randomized, double-blind, placebo-controlled trial, where 29 healthy overweight/obese (BMI $\geq$ 23 kg/m^2) participants (2 men and 27 women) were randomly selected to receive placebo ($n = 15$, 2.4 g olive oil/day) or 2.4 g/day CLA mixture ($n = 14$, 36.9% of cis-9, trans-11 and 37.9% of trans-10, cis-12) for 8 weeks.
Results There were no significant differences in plasma total radical-trapping antioxidant potential (TRAP), lipid peroxidation (conjugated dienes), lipid-soluble antioxidant vitamin concentration, erythrocyte antioxidant enzyme (superoxide dismutase, catalase, glutathione peroxidase) activities, and leukocyte DNA damage between the CLA and placebo groups.
Conclusions The data suggest that short-term supplementation (8 weeks) with CLA (2.4 g/day) might have no significant effects on lipid peroxidation and antioxidant metabolism.

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Keywords Conjugated linoleic acid · Overweight/obese · Antioxidant status

Introduction

The incidence of obesity increased steadily from 1998 to 2005 in Korea [1], and 1 in every 3 individuals was diagnosed as obese in 2008 [2]. However, in 2007, Korea had the lowest obesity rate among the other 30 member nations of the Organization for Economic Cooperation and Development (OECD). Obesity increases the risk of many degenerative diseases, particularly diabetes, heart disease, stroke, and certain types of cancer, and it also reduces life expectancy by 6–7 years on average [3]. Increasing data show that obesity is a state of chronic oxidative stress—an imbalance between tissues free radicals, reactive oxygen species (ROS), and antioxidants [4]. Several possible mechanisms have been reported for the generation of oxidative stress in obesity, including elevated tissue lipid levels [5, 6], inadequate antioxidant defenses [7, 8], and increased rates of free radical formation [9]. Therefore, identifying compounds that enable weight loss, have antioxidant effects, or have a positive effect on oxidative stress is considered as an important approach towards treating obesity. In this regard, human studies have confirmed that conjugated linoleic acid (CLA) is one such compound that enables weight loss [10, 11]. We previously observed that 8 weeks of CLA supplementation significantly decreased body weight (−0.75 kg), BMI (−0.27 kg/m^2), and hip circumference (−1.11 cm) in overweight/obese Korean individuals [12].

In Korea, CLA is receiving substantial attention after its approval as a functional health food for weight control by the Korean Food and Drug Administration in 2005 [13].

The term CLA refers to several positional and geometric conjugated dienoic isomers of linoleic acid, and it was first discovered by Pariza and Hargreaves [14]. At various positions along the 18-carbon chain of linoleic acid, double bonds formed by a chemical reaction or a microbial reaction by *Butyrinvibrio fibrisolvens* and other anaerobic bacteria found in ruminant animals were observed [15]. Among several CLA isomers, the 2 isomers with known biological activity are *cis*-9, *trans*-11 (c9t11) and *trans*-10, *cis*-12 (t10c12) CLA [16]. Ever since Ha et al. [17] reported that CLA was an effective inhibitor of benzopyrene-initiated mouse epidermal neoplasia in 1987, various studies have revealed the antiatherogenic [18, 19] and antiinflammatory [20, 21] properties of CLA in several animal models. The effects of CLA on the anti/pro-oxidative status in cells [22–24], animal models [25–28], and humans have also been reported [29–36]. However, the results of these studies are contradictory and the true effect remains.

Therefore, we systematically evaluated the effects of 8-week CLA supplementation on lipid peroxidation and antioxidant metabolism in healthy overweight/obese Korean participants, which were extended from the previous study [12].

Materials and methods

Subjects

Thirty apparently healthy subjects aged 19–65 years with a body mass index (BMI) of 23–38 were recruited from Y Fitness center, Seoul, Korea. The study was performed between October 2007 and January 2008 in agreement with the guideline from the Korean Society for the Study of Obesity: normal weight corresponded to $18.5 \leq \text{BMI} < 23$ kg/m^2, overweight to $23 \leq \text{BMI} < 25$ kg/m^2, and obesity was defined by a BMI ≥ 25 kg/m^2. Subjects were excluded from the study if they had a history of diabetes mellitus, renal, liver, pancreatic, or chronic inflammatory or infectious diseases, cardiac failure or malignant tumors. In addition were excluded the cases using drug therapy including antioxidant vitamin supplementation, special diets, or dietary substitutes including CLA supplementation for weight loss, as well as the cases extremely highly consuming the CLA-enriched egg, pork, and milk. Individual characteristics, health status, and smoking habits were also obtained by questionnaire. The procedures were explained in detail to all the volunteers in advance, and all gave their signed informed consent before participating in the study. This study was approved by the Hannam University Ethics Committee and conducted in agreement with the Declaration of Helsinki of 1975 as revised in 1983 and in accordance with the International Conference on Harmonization guidelines.

Study design

The design adopted was a randomized, double-blind, placebo-controlled design. The subjects were assigned at random to the placebo group (2.4 g olive oil/4 capsules, $n = 15$) or CLA supplement group (2.4 g CLA/4 capsules, $n = 14$). The active soft-gel capsule contained 750 mg olive oil of which 78.8% was CLA (Lipozen, Inc., Korea) containing 36.9% of c9t11 and 37.9% of t10c12 and produced by chemical isomerization of linoleic acid from safflower oil. All subjects were on an ad libitum diet and participated in a standard training program offered by local training center 3 days per week. The subjects were instructed to maintain their usual pattern of dietary intake during the study, and the compliance was monitored through biweekly phone calls.

Plasma total radical-trapping antioxidant potential (TRAP)

TRAP was measured using a modification of the photometric method developed by Rice-Evans and Miller [37]. The method for measuring antioxidant activity is predicated on the antioxidant-induced inhibition of the absorbance of the radical cation of 2,2′-azinobis (3-ethylbenzothiazoline 6-sulfonate) ($ABTS^+$). The $ABTS^+$ radical cation is formed by the interaction of $ABTS^+$ (150 μM) with the ferryl-myoglobin radical species, which is, in turn, generated by the activation of metmyoglobin (2.5 μM) by H_2O_2 (75 μM). Ten microliters of sample/buffer/Trolox-standard was added to tubes containing 400 μL of PBS buffer, 20 μL of metmyoglobin, and 400 μL of ABTS and mixed by vortexing. The reaction was initiated by the addition of 170 μL of H_2O_2. After 6 min of incubation, the absorbance was measured at 734 nm using a spectrophotometer. Values are expressed as trolox equivalent antioxidant capacity (TEAC) and defined as the millimolar concentration of the trolox antioxidant capacity of a calibration curve.

Baseline conjugated dienes in LDL

Baseline LDL conjugated diene levels were determined according to the methods outlined by Ahotupa et al., with slight modifications [38]. Plasma (100 μL) was added to 700 μL of heparin citrate buffer (0.064 M trisodium citrate, 50,000 IU/L heparin, pH 5.05), and this suspension was allowed to stand for 10 min at room temperature. The insoluble lipoproteins were then sedimented by centrifugation at 2,500 rpm for 10 min. The pellet was resuspended in 100 μL of Na-phosphate buffer 0.1 M containing

0.9% NaCl (pH 7.4). Lipids were extracted from the 100 μL of LDL suspension with chloroform–methanol (2:1), dried under nitrogen, redissolved in cyclohexane, and analyzed spectrophotometrically at 234 nm. Oxidation during sample preparation was prevented by the addition of EDTA.

Plasma lipid-soluble vitamins

Plasma concentrations of retinol, carotenoids, lycopene, tocopherol, and coenzyme Q_{10} were determined simultaneously by RP–HPLC (reversed-phase high-pressure liquid chromatography) according to the method of Jakob and Elmadfa [39]. Briefly, plasma proteins were precipitated with ethanol, and lipids were extracted with n-hexane. After evaporation, dry residue was redissolved with 150 μL of methanol–dichlormethane (85:15, v/v) and mixed, and then 100 μL of this solution was injected into a guard-column (Merck LiChrospher 100 RP18 (10 μm), 250 × 4 mm). Samples were run at a flow rate of 1.0 mL/min on a Dionex HPLC system (Summit^{TM} HPLC, USA). Absorption was monitored at 325 nm for retinol, at 295 for tocopherol, at 450 nm for carotenoids and lycopene, and at 270 nm for coenzyme Q_{10}. Concentrations were calculated from areas under the curve using an external calibration curve.

Erythrocytes antioxidant enzyme activities

Glutathione peroxidase activitiy (GSH-Px)

GSH-Px was determined according to the method described by Beutler [40]. Ten microliters of erythrocytic hemolysate was added to 100 μL of 1 M Tris-HCI–5 mM EDTA buffer (pH 8.0), 20 μL of 0.1 M glutathione, 100 μL of 10 U/mL glutathione reductase, and 100 μL of 2 mM NADPH and filled with H_2O to a final volume of 1 mL. After 10 min of incubation at 37 °C, the reaction was initiated by the addition of 10 μL of t-butyl hydroperoxide, and the absorbance was measured at 340 nm. The reaction was run for 90 s, and the loss of NADPH was monitored by the change in $A_{340\ nm}$/min.

Catalase activity (CAT)

Catalase activity was measured according to the method developed by Aebi [41]. In 50 mL of 50 mM phosphate buffer (pH 7) was dissolved 100 μL of erythrocytic hemolysate, and 2 mL of the mixture was added to a cuvette. The reaction was initiated by the addition of 1 mL of 30 nM H_2O_2 at 20 °C. The H_2O_2 decomposition rate was measured at 240 nm for 30 s using a spectrophotometer.

Superoxide dismutase (SOD)

The SOD activity was assayed in erythrocyte suspension by the procedure of Marklund and Marklund [42]. To 500 μL of the hemolysate were added 3.5 mL of water, 1 mL of ethanol, and 0.6 mL chloroform. After centrifugation at 3,000 Unit/min for 2 min, various dilutions were prepared from the supernatant. Twenty microliters of pyrogallol was added to each dilution after incubation at 37 °C for 10 min. The reaction was monitored spectrophotometrically at 320 nm for 2 min. The unit of the enzyme was defined as the amount that inhibits the autoxidation of pyrogallol by 50%.

DNA damage determination by alkaline comet assay

The alkaline comet assay was conducted according to Singh et al. [43]. The isolated leukocytes duplicated from one subject were subjected to oxidative stress by suspension in PBS with 100 μM H_2O_2 for 5 min on ice. The leukocytes were mixed with 75 μL of 0.7% low-melting agarose (LMA) and added to the slides precoated with 0.5% agarose. The slide was then immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, and 1% sodium laurylasarcosine; 1% Triton X-100 and 10% DMSO) for 1 h at 4 °C. The slides were next placed into an electrophoresis tank containing 300 mM NaOH and 10 mM Na_2EDTA (pH 13.0) for 40 min. For electrophoresis of the DNA, an electric current of 25 V/300 ± 3 mA was applied for 20 min at 4 °C. The slides were washed three times with a neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min at 4 °C and then treated with ethanol for another 5 min before staining with 50 μL of ethidium bromide (20 μg/mL). Measurements were taken by image analysis (Komet 4.0; Kinetic Imaging, Liverpool, U.K.) and fluorescence microscope (DMLB; LEICA Wetzlar, Germany), determining tail length (50 cells from each of two replicate slides).

Statistical analysis

Data were analyzed using the SPSS 14.0 package for Windows. Values were expressed as mean ± SE unless stated otherwise. Statistical differences between groups and within groups were considered significant at $p < 0.05$ by Student's t test and paired t test. Categorical variables were analyzed by using the χ^2 test.

Results

No significant differences were found between the CLA and the placebo groups in demographic variables such as

gender, age, smoking habits, alcohol consumption, and exercise habits, and baseline height measurements (Table 1). In our previous report [12], the body weight and BMI were significantly reduced in the 8-week CLA supplementation group, but not in the placebo group. However, the reduced amounts of body weight, BMI, body fat mass, and lean body mass between the two groups were not statistically significantly different.

Effect of CLA on the markers of oxidative stress and antioxidant status

CLA supplementation for 8 weeks had a nonsignificant effect on TRAP, conjugated dienes (CD), activities of erythrocyte antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), and concentrations of serum lipid-soluble antioxidant vitamin concentrations (retinol, α-, γ-tocopherol, α-, β-carotene, lycopene, coenzyme Q_{10}) (Table 2). Further, no leukocyte protective effects of CLA supplementation against H_2O_2-induced DNA damage were observed.

Discussion

This study aimed to evaluate the effect of CLA supplementation on antioxidant metabolism in healthy overweight/obese Korean individuals. The data suggested that short-term supplementation of CLA (2.4 g/day for 8 weeks) might have no significant effects on lipid peroxidation and antioxidant metabolism.

Reactive oxygen species (ROS), which are constantly formed in the human body, are suggested to contribute to pathological processes during aging and in many diseases such as diabetes, atherosclerosis, and cancer [44]. In healthy individuals, a balance between ROS and the antioxidant defense system is maintained through antioxidative enzymes such as SOD, CAT, and GSH-Px in the erythrocyte or antioxidant vitamins such as ascorbic acid, coenzyme Q_{10}, and tocopherols. SOD converts the dangerous superoxide radicals to hydrogen peroxide, which is further converted to the nontoxic water and oxygen by CAT. Thus, the formation of carbon dioxide bubbles in the blood is prevented [45]. Obesity, on the other hand, is associated with increased systemic oxidative stress, which may result from a combination of adipokine imbalance and reduced antioxidant defenses [46]. Our overweight/obese subjects showed lower levels of antioxidant status (i.e., SOD, GSH-Px, antioxidant vitamins) and higher levels of oxidative damage (DNA damage) at baseline than healthy Koreans [47, 48]. Additionally, we found a positive correlation between body mass index (BMI) and leukocyte DNA tail length, a sensitive biomarker for oxidative stress in overweight/obese participants (BMI $\geq$ 23 kg/m^2) in our study (Fig. 1).

The antioxidative effect of CLA was confirmed in animal studies: chronically ethanol-treated rats showed increased antioxidant enzyme activity [25], and benzo (a) pyrene-treated [28] or dimethylhydrazine (DMH)-treated animals [27] showed decreased tumor incidence. Ha [28] suggested that the β-hydroxyl acrolein moiety in CLA acts as an antioxidant through 2 possible mechanisms: resonance enolization of the β-hydroxy acrolein moiety or the chelation of iron by β-hydroxy acrolein moiety, which would in turn lead to the inhibition of the Fenton reaction. On the other hand, Basu and coworkers reported that CLA caused marked lipid peroxidation in humans when administered via capsules [29–34] or through a CLA-rich diet [35, 36]. Moreover, Risérus et al. [31] found that the oxidative stress caused by CLA was dependent on its isomer. Purified t10c12 CLA (3.4 g/day) supplementation augmented lipid peroxidation by a greater degree than a CLA isomer mixture (equal amount of t10c12 and c9t11). Supplementation with purified c9t11 CLA (3 g/day), which is the predominant CLA isomer in foods and dietary weight loss products, also led to increased lipid peroxidation [34].

However, in the present study, 8 weeks of CLA supplementation did not change the levels of anti/pro-oxidative biomarkers such as TRAP, CD, CAT, SOD, GSH-Px, antioxidant vitamins, and leukocyte DNA damage in the healthy overweight/obese Korean participants. The difference between the results of our human study and the previous animal model studies may be ascribed to the dose of CLA administered. The total amount of CLA administered to mice in which antioxidative properties were observed was up to 13 times higher (26 g/kg) [28] than that administered to our participants (2.0 g/kg). Meanwhile, the discrepancies between our study and Basu's studies [29–36] may be attributed to 2 different factors: The first

Table 1 Characteristics of the study subjects

	CLA	Placebo
Sex		
Men/women (n)	1/13	1/14
Age (years)[a]	39.4 ± 4.4	40.7 ± 4.0
Weight (kg)	65.3 ± 2.4	65.5 ± 2.6
Height (cm)	160.9 ± 1.8	158.8 ± 1.0
BMI (kg/m^2)	25.2 ± 0.7	26.3 ± 1.0
Alcohol use (%)[b]	50.0	53.3
Exercise (%)[c]	85.7	86.7

[a] Values for age, weight, height, and BMI are given as mean ± SE

[b] Alcohol use was expressed as the percentage of the subjects who answered positively to these questions (% yes)

[c] Exercise is expressed as the percentage of subjects who answered "do exercise regularly"

Table 2 Changes of antioxidant status and H_2O_2-induced DNA damage after CLA supplementation

	CLA ($n = 14$)		Placebo ($n = 15$)	
	Week 0	Week 8	Week 0	Week 8
Antioxidant enzymes in erythrocyte				
SOD (U/gHb)	2,102 ± 179 ns[a, b]	1,831 ± 108	2,068 ± 127	2,316 ± 159
CAT (K/gHb)	98.3 ± 5.9	100.5 ± 5.1	82.4 ± 5.4	92.4 ± 5.0
GSH-Px (U/gHb)	21.4 ± 2.5	25.0 ± 3.1	23.4 ± 3.5	25.1 ± 3.0
Serum lipid antioxidant vitamin and vitamin precursor concentrations				
Retinol/T-chol[c]	0.43 ± 0.07	0.40 ± 0.04	0.49 ± 0.05	0.45 ± 0.00
γ–tocopherol/T-chol	1.02 ± 0.08	1.05 ± 0.16	1.24 ± 0.53	1.00 ± 0.36
α–tocopherol/T-chol	6.28 ± 0.67	6.35 ± 0.48	6.63 ± 1.55	6.41 ± 1.05
Lycopene/T-chol	0.16 ± 0.06	0.19 ± 0.09	0.15 ± 0.11	0.22 ± 0.12
α–carotene/T-chol	0.07 ± 0.01	0.08 ± 0.01	0.10 ± 0.03	0.08 ± 0.01
β–carotene/T-chol	0.35 ± 0.07	0.40 ± 0.04	0.45 ± 0.12	0.37 ± 0.11
Coenzyme Q10/T-chol	0.62 ± 0.15	0.69 ± 0.22	0.52 ± 0.10	0.51 ± 0.19
Antioxidant activity and lipid peroxidation status in plasma				
Trap (mM)	1.23 ± 0.09	1.26 ± 0.05	1.30 ± 0.04	1.26 ± 0.05
CD (μM)	4.23 ± 0.39	6.48 ± 1.18	4.10 ± 0.36	6.15 ± 1.51
H_2O_2-induced DNA damage of leukocytes				
Tail DNA (%)	17.6 ± 1.0	16.4 ± 1.5	18.0 ± 0.9	16.8 ± 1.1
Tail moment	22.6 ± 2.7	20.5 ± 4.0	21.7 ± 2.7	20.2 ± 3.2
Tail length (μm)	70.8 ± 4.9	64.7 ± 8.5	65.8 ± 5.0	64.2 ± 6.7

[a] Values are mean ± SE

[b] *ns* not significant

[c] Corrected by plasma total cholesterol

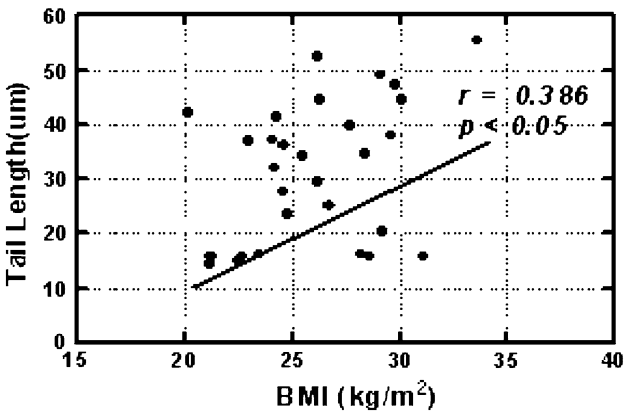

Fig. 1 Correlation between BMI and DNA damage, which was measured in terms of tail length

is the dosage of CLA. In Basu's studies, which show the pro-oxidant effects of CLA, the range of CLA concentrations administered was between 3 and 4.2 g/day. This dosage was much higher than that administered to our participants (2.4 g/day). The second factor is the indicators used to study lipid peroxidation. To investigate the change of lipid peroxidation, Basu et al. measured the levels of 8-iso-prostaglandin $F_{2\alpha}$ (8-Iso-$PGF_{2\alpha}$) and 15-keto-13,14-dihydro-prostaglandin $F_{2\alpha}$ (15-keto-dihydro-$PGF_{2\alpha}$), which are isomeric structures, formed from isoprostanes that are produced by the peroxidation of arachidonic acid in the membrane. On the other hand, we spectrophotometrically determined the baseline values of diene conjugation in LDL, an event that occurs early in the lipid peroxidation reaction, and is an important step for monitoring LDL oxidation [49]. Therefore, these 2 indicators may reflect different lipid peroxidation pathways. To test this hypothesis, a study that investigated lipid peroxidation after dietary supplementation of CLA (mixed isomers of CLA 4.2 g/day) in healthy human subjects for 3 months was conducted. A significant increase in both urinary 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ levels was observed, although no significant difference in the concentration of plasma malondialdehyde (MDA), a reactive aldehyde end product of the lipid peroxidation pathway, was found [29].

Our study design has several limitations. First, detailed dietary intake information was not obtained even though lifestyle habits such as drug therapy, special diets, or dietary substitutes including CLA supplementation for weight loss as well as extremely high consumption of the CLA-enriched egg, pork, and milk were examined and were excluded. Second, given the restricted study population, the results may not be applicable to other ethnic groups or subjects with different health status (i.e., severity of

obesity, disease status, or metabolic syndrome), because their clinical and biochemical characteristics are different from our study subjects. In addition, regarding the discrepancy among the results in human subjects and animal or in vitro experimental models, we might have to concern the efficacy and safety of CLA supplementation. According to the meta-analysis data analyzed by Larsen et al. [50], CLA appears to attenuate increases in body weight and body fat in several animal models, but CLA isomers sold as dietary supplements were not effective as weight loss agents in humans and might actually have adverse effects on human health. It needs to be elucidated with further studies like CLA dose-dependent and long-term supplementations.

Despite these limitations, the present study was the first to systematically confirm the effect of CLA on antioxidant metabolism. It suggested that supplementation with CLA (2.4 g/day) for 8 weeks did not increase lipid peroxidation and had no significant effect on the antioxidant status in the overweight/obese participants. Further human intervention studies, for example, which include extremely obese people or metabolic syndrome and which are performed for much longer periods (i.e., for 6 months and longer), should be conducted to determine the exact effect of CLA supplementation on antioxidant metabolisms and its mechanism, because the production and consumption of CLA-enriched products (i.e., CLA-enriched egg, pork, and milk) and commercial supplements for weight loss have been increasing in the global market.

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