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Original article

The Effect of conjugated linoleic acid (CLA) on inflammatory factors in Non-Alcoholic Fatty Liver Disease (NAFLD): A randomized controlled clinical trial

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Summary. The worldwide alarming increase of non-alcoholic fatty liver disease (NAFLD), leads to advent of wide range of therapeutic strategies. As a functional supplement, conjugated linoleic acid (CLA) is related to weight loss and improves histology parameters of inflammatory markers patients with NAFLD. The aims of this study were to investigate the effects of CLA in company with vitamin E on body composition as well as inflammation by evaluating anthropometric parameters and the serum level of liver enzymes and inflammatory factors in NAFLD patients. In a randomized single-blind controlled trial with two subgroups, 54 obese patients with NAFLD randomized to consume supplement with either three softgel capsules of CLA (80% softgel ,1000mg) in company with one softgel capsule of vitamin E (400 IU) (CLA group) or one softgel capsule of Vitamin E (control group), daily for eight weeks. All the participants were asked to maintain their prescribed diet and usual lifestyle habits. Body composition was assessed at the beginning and end of the study using bioelectrical impedance analysis (BIA). At the end of the study, fat mass (P=0.001), muscle mass (P=0.023) and total body water (P=0.004) were significantly better in CLA group. The mean values of tumor necrosis factor (TNF)-α, interleukin (IL)-6, high sensitivity C-reactive protein (hs-CRP) and serum alanine aminotransferase/aspartate aminotransferase (ALT/AST) ratio were significantly decreased in CLA group. Also; ALT/AST ratio (P=0.046) and TNF- α (P=0.001) showed a statistically significant decrease compared to the control group. Besides, the CLA group demonstrated significantly increased levels of interleukin (IL)-10. Considering the vital role of inflammation in progression of NAFLD, our results suggest that CLA could be a promising supplement in NAFLD treatment.

Keywords: Conjugated Linoleic Acids, Inflammation, non-alcoholic fatty liver disease

Introduction

The worldwide increase in the prevalence of obesity has resulted in an alarming advance (14%-30%) (1) of non-alcoholic fatty liver disease (NAFLD) which is the most common liver disease among adults (2). NAFLD is characterized by benign hepatic steatosis and the progressive inflammation of liver, which leads to the more severe form, non-alcoholic steatohepatitis (NASH) (3). The presence of chronic low-grade systemic or hepatic inflammation may lead to NAFLD (4). Researches indicated that altered signaling pathways have been shown to be involved in this disorder. Generally, obesity activates the Iκκ-β/NF-κB signaling pathway, which leads to the activation of inflammatory cells including resident liver macrophages or Kupffer cells (KCs). It also induces the expression of hepatic and systemic pro-inflammatory cytokines such

as tumor necrosis factor (TNF)-α, interleukin (IL)-6 and interleukin (IL)-1β. Subsequently, via complicate pathways, insulin resistance and apoptosis take place and in this way inflammation plays its role in liver failure (5;6).

New studies show the histological presentations of NASH without steatosis, proving that inflammation can take place first and may trigger subsequent steatosis, in contrast with common NASH which begins with steatosis. It demonstrates the important role of inflammation in the development of NAFLD (7).

Currently, there are variety of treatment modalities in order to inhibit NAFLD including lifestyle interventions, surgery and pharmacological approach, the first-choice improving at all ages is lifestyle modification, aimed at obesity treatment and increasing physical activity (8). Due to low compliance and unsatisfactory results by diet and exercise (9), it is believed that dietary supplements and other management strategies such as targeting the components of the metabolic syndrome and liver-directed pharmacotherapy in the case of high-risk patients has become necessary (10).

One of the promising dietary supplements known as conjugated linoleic acid (CLA) is a mixture of unsaturated isomers of octadecadienoic acid with variety of beneficial biological activities. As a functional food, interest in CLA increased considerably following the observation of anti-obesity, anti-diabetic, anti-atherogenic and anti-inflammatory effects in vitro as well as in certain animal models. Recently, various researches have reported the effectiveness of CLA in human (11;12). Even though the mechanism action of CLA is not completely clear, the potential mechanisms were suggested. First, CLA regulates energy metabolism by decreasing the energy intake and increasing the energy expenditure. Secondly, it modulates adipocyte metabolism by inhibiting adipogenesis and reducing the adipose cell number as well as increasing fatty acid β-oxidation (13). In some studies, supplementation of CLA lead to the decrease of the level of pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β and adipokines and also increase of the level of IL-10 as an anti-inflammatory cytokine, results in modulating the inflammation (14;15). The aims of this study were to investigate the effects of CLA in company with vitamin E on body composition and inflammation by evaluating the serum levels of inflammatory factors in patients with NAFLD.

Methods

Study Design

This randomized single-blind controlled clinical trial was aimed to determine the effect of CLA on body composition and inflammatory factors among obese NAFLD patients. All the study protocol was approved by the Ethics Committee of Tabriz University of Medical Sciences, Tabriz, Iran and was registered in the Iranian Registry of Clinical Trials website (IRCT2014020516491N1). Participants who referred to the specialized and sub-specialized clinics of Tabriz University of Medical Sciences and underwent ultrasonography for the diagnosis of fatty liver between January 2014 and March 2015 were identified for this study. All the ultrasonography tests were performed by the same highly-skilled sonographer. Hepatic steatosis is classified into three mild, moderate and severe groups based on Saverymuttuscoring system (16). The inclusion criteria were as follows: age between 20-50 years old, body mass index (BMI) between 30-40 kg/m^2. Fifty four patients were confirmed as NAFLD patients based on the mentioned inclusion criteria. Subjects with pregnancy or lactation, being post-menopause and athletic, those with alcohol consumption; inflammatory conditions such as infection, hypertension, family history of hyperlipidemia, cardiovascular diseases, lung, renal or liver diseases, liver transplantation, biliary disease, known autoimmune disease, cancer, perceived diseases such as burns or injury during the study, surgery in the last three months, medications such as anti-hypersensitive, insulin sensitivity enhancer, hepatotoxic drugs, statins, contraceptive pills and estrogens as well as vitamin and mineral supplements, antioxidant supplementation in the last two months were excluded. All the patients received 400 IU/day vitamin E as their usual treatment during the study. Personal characteristics including demographics and disease history were obtained. Patients with NAFLD were randomly divided into two "CLA" and "Control" groups. Subjects in "CLA" group received CLA 80% soft gel 1000 mg supplied by NutriFit (NutriCentury,

Canada;containing both cis-9, Trans-11,trans-10 and cis-12 type CLAs in equal proportion) three times with a meal daily associated with weight loss diet and those in the control group only followed a weight loss diet for eight weeks (Figure 1).

Each participant was called every two weeks during the study in order to receive their supplements in four batches. A food frequency questionnaire (FFQ) for assessing habitual diet was completed at the beginning of the study. The energy of the prescribed diet was defined 700 kcal less than the calculated amount per person based on the height, sex and current weight, using Harris-Benedict formula (17). Patients consumed a diet consisting of 55:30:15 percent energy from carbohydrate, fat, and protein (C: F: P), respectively. All the participants were asked to maintain their prescribed diet and usual lifestyle habits.

Dietary Assessment

Mean daily dietary intake was assessed using three-day food record set in three times and was immediately completed at the beginning of the study, the fourth week, and at the end of the study. Home measurements were used to quantify portion sizes. Energy and macro-nutrients intakes were analyzed using Nutritionist IV software. (Ver.3.5.2, San Bruno,CA).

Physical Activity Measurement

Three physical activity questionnaires Metabolic Equivalent of Task (MET) were completed at the beginning, at the fourth week, and at the end of the intervention and reported as MET per day.

Anthropometric and Body Composition Measurements

Weight and height of patients were measured with Seca scale (Hamburg, Germany) and tape to the nearest 100 g and 0.5 cm, respectively. BMI was calculated and then waist and hip circumferences were measured after expiration at the midpoint between the lowest rib and the iliac crest and at the widest point between hip and buttock to the nearest 0.5 cm. Waist-to-hip and weight-to-height ratios were evaluated. Body composition was assessed at the beginning and end of the study using bioelectrical impedance analysis (BIA).

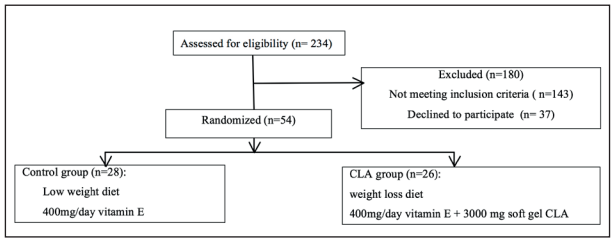

Figure 1. Flow chart of the study

Biochemical Measurements

Following 12 hours of fasting, 10 mL of blood samples was taken from each patient at the beginning and end of the two months trial. Aliquots of serum were collected in micro-tubes and stored at -70◦C until analysis, including serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), IL-6, IL-10, TNF-α, and high sensitivity C-reactive protein (hs-CRP). Liver enzymes were measured by International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) method (Biosystem, Barcelona, Spain) using anauto-analyzer instrument (Hitachi 911, Indonesia). The plasma concentration of hs-CRP was estimated using immunoturbidity. In addition, we measured serum concentrations of TNF-α, IL-6 and IL-10 with commercially available ELISA kits (TNF-α and IL-6 with ORGENIUM, IL-10 with BOSTER).

Statistical Analysis

All the statistical procedures were performed using SPSS software (version 16.0). Normality of continuous variable was tested by Kolmogorov-Smirnov test. Data were expressed as mean ± SD for normally distributed variables. Baseline characteristics as the qualitative data were test by chi-squared test. Independent t-test was used for evaluating the mean data at the beginning of the study in both CLA and control groups. Differences in mean of the continuous variables between the two groups were tested using analysis of covariance (ANCOVA) for covariates and adjusting baseline measurements including weight, waist circumference, hip circumference and daily total intake energy in control group. The assessment changes before and after the intervention was applied using paired t-test in case of normality, otherwise using Wilcoxon rank t-test. A repeated measure analysis was used for assessing dietary intake and physical activity over the study.

Results

Among 54 individuals participated in the current research, 38 (n=19 for each group) completed the study (Figure 1) and dropouts were excluded. Baseline characteristics of the patients are summarized in Table 1.

There were no significant differences at baseline between the two groups in terms of age (36.74± 6.87 years and 38.58±8.24 years in intervention and control groups, respectively), gender, marital status, education level, and severity of fatty liver (P>0.05) which means they could be ignored as a confounder.

Table 2 shows mean daily energy and macronutrient intakes in both groups. Dietary analysis showed no differences in energy and macronutrient intakes between the two groups at baseline, in week four and week eight of the study (P>0.05) except for fat intake in week 8 (P=0.019). As there was a significant change in total energy intake in the control group, it was considered as confounder in advanced analysis. Indeed, no significant changes in physical activity (expressed as MET/day) were observed over the study using repeated measure analysis (P>0.05) (Table 2).

At baseline, mean weight, waist and hip circumferences were significantly different between the two groups (P=0.031, P=0.030 and P=0.015, respectively); thus, they were considered as covariates in the analysis (Table 3). Anthropometric measurements decreased significantly in both groups while there was no difference in changes between the groups (P>0.05). Fat mass, muscle mass and total body water improved significantly over the study in the CLA group compared to the control group.

At the beginning of the study, the groups were similar in the level of inflammatory factors and liver enzymes. IL-6 and hs-CRP concentrations decreased and IL-10 increased, but this change was non-significant after eight weeks. Not only the changes of TNF-α level in the intervention group were significant (P=0.010), but also these changes were significant between both groups after the intervention (P=0.001). At the end of the study, a significant decrease was found in ALT/AST ratio in the CLA group (P=0.025)

Table 1. Summary of baseline characteristics of the patients

Characteristic	Control (n=19) (%)	CLA (n=19) (%)	*P*
Female	84.2	89.5	0.999[a]
Single	89.5	100	0.486[a]
Education level			
High school diploma or below	57.9	57.9	0.999[a]
University graduate	42.1	42.1	
Severity of Fatty liver			
Mild	78.9	68.4	0.461[a]
Moderate and Severe	21.1	31.6	

CLA: conjugated linoleic acid/ [a] Chi-squared test.

Table 2. Daily Total Energy, Macronutrient Intakes and Physical Activity

Variable	Control n=19 Mean±SD	CLA n=19 Mean±SD	*P*
Energy (kcal)			
Week 0	1272.71±292.33	1120.68±306.25	0.126[a]
Week 4	1124/09±334.92	1154.96±252.81	0.757[a]
Week 8	1157.18±340.92	1171.45±325.60	0.897[a]
P	0.020[b]	0.921[b]	
Carbohydrate (g)			
Week 0	192.73±56.51	161.67±56.57	0.099[a]
Week 4	176.57±59.45	165.09±35.30	0.416[a]
Week 8	182.85±69.23	163.72±42.53	0.321[a]
P	0.441[b]	0.833[b]	
Protein (g)			
Week 0	52.70±14.03	49.96±13.92	0.51[a]
Week 4	51.44±14.21	49.77±12.20	0.708[a]
Week 8	48.35±10.40	48.19±12.55	0.887[a]
P	0.298[b]	0.808[b]	
Fat (g)			
Week 0	30.71±8.09	31.90±9.41	0.680[a]
Week 4	28.20±12.19	35.83±13.26	0.081[a]
Week 8	26.88±7.98	34.90±11.67	0.019[a]
P	0.213[b]	0.435[b]	
Physical activity (MET/day)			
Week 0	36.41±4.20	38.27±2.69	0.117[a]
Week 4	36.06±4.12	38.69±2.61	0.027[a]
Week 8	36.75±3.76	38.28±2.44	0.148[a]
P	0.203[b]	0.439[b]	

CLA: conjugated linoleic acid, Met: metabolic equivalents, SD: standard deviation, / a: independent t-test, b: repeated measure analyses.

Table 3. Anthropometric Measurements and Body Composition Before and After the Intervention

Variable	Control n=19 Mean±SD	CLA n=19 Mean±SD	*P*
Height (cm)	159.24±6.67	158.81±8.93	0.867[b]
Weight (kg)			
Before	89.36±9.34	82.10±10.60	0.031[b]
After	84.84±9.56	77.30±10.45	0.559[c]
MD (CI95%)	4.5(3.71 to 5.28)	4.80(4.10 to 5.50)	
P	<0.001	<0.001	
BMI (kg/m²)			
Before	35.27±3.46	32.72±4.63	0.064[b]
After	33.50±3.63	30.80±4.45	0.46[c]
MD (CI95%)	1.77(1.46 to 2.07)	1.97(1.26 to 2.22)	
P	<0.001	<0.001	
Waist circumference (cm)			
Before	109.28±9.92	103.18±6.34	0.03[b]
After	103.70±.34	97.34±7.01	0.952[c]
MD (CI95%)	5.58(4.63 to 6.53)	5.84(4.72 to 6.96)	
P	<0.001	<0.001	
Hip circumference (cm)			
Before	119.73±7.89	113.68±6.75	0.015[b]
After	116.11±8.19	109.08±7.09	0.342[c]
MD (CI95%)	4.60(3.45 to 5.57)	3.63 (2.69 to 5.56)	
P	<0.001	<0.001	
Waist to hip ratio			
Before	0.91±0.047	0.90±0.047	0.804[b]
After	0.89±0.054	0.89±0.054	0.530[c]
MD (CI95%)	0.02(0.01 to 0.021)	0.01(0.00 to 0.02)	
P	<0.001[a]	<0.001[a]	
Waist to height ratio			
Before	0.68±0.067	0.65±0.053	0.08[b]
After	0.65±0.069	0.61±0.054	0.778[c]
MD (CI95%)	0.03(0.02 to 0.04)	0.03 (0.02 to 0.04)	
P	<0.001[a]	<0.001[a]	
Fat mass (%)			
Before	46.71±6.35	46.05±5.71	0.015[b]
After	45.55±6.69	41.43±6.46	0.001[c]
MD (CI95%)	1.16(0.39 to 1.93)	4.61(2.81 to 6.41)	
P	0.005[a]	<0.001[a]	
Muscle mass (%)			
Before	32.44±4.36	33.92±2.57	0.210[b]
After	33.02±4.34	35.55±2.79	0.023[c]
MD (CI95%)	-0.63(-0.82 to -0.44)	-1.63(-2.39 to -0.88)	
P	<0.001[a]	<0.001[a]	
Total body water			
Before	36.31±4.35	36.04±3.90	0.846[b]
After	38.14±4.59	38.14±4.41	0.004[c]
MD (CI95%)	-0.45(-1.00 to 0.09)	-0.094(-3.02 to -1.161)	
P	0.102[a]	<0.001[a]	

CLA: conjugated linoleic acid, SD: standard deviation, MD: mean difference, CI: confidence interval/ [a]: paired t- test, [b]: independent t-test, [c]: ANCOVA adjusted for baseline values and energy intake.

Table 4. Metabolic Variables Before and After the Intervention

Variable	Control n=19 Mean±SD	CLA n=19 Mean±SD	*P*
IL-6 (ng/L)			
Before	23.105±16.795	16.184±5.956	0.104[b]
After	22.100±15.781	15.268±5.505	0.739[c]
MD (CI95%)	0.020(-0.010 to 0.051)	0.023(0.000 to 0.046)	
P	0.610[a]	0.042[a]	
IL-10 (ng/L)			
Before	4.252±3.379	6.863±7.064	0.551[b]
After	4.973±3.808	9.00±9.468	0.801[c]
MD (CI95%)	-0.082(-0.139 to -0.024)	-0.092(-0.265 to 0.080)	
P	0.008[a]	0.005[a]	
TNF-α(ng/L)			
Before	26.594±23.282	64.252±94.179	0.099[b]
After	25.568±22.831	37.531±.51.133	0.001[c]
MD (CI95%)	0.021(0.003 to 0.039)	0.204(0.154 to 0.254)	
P	0.020[a]	0.010[a]	
Hs-CRP (mg/dL)			
Before	4.108±4.595	3.612±4.622	0.742[b]
After	3.411±4.127	2.605±3.791	0.105[c]
MD (CI95%)	11.78(6.26 to 17.31)	9.737(1.75 to 17.21)	
P	0.008[c]	0.001[a]	
AST (IU/L)			
Before	18.26±3.94	19.89±17.36	0.405[b]
After	18.05±3.14	17.33±4.21	0.375[c]
MD (CI95%)	0.21(-1.50 to 1.93)	2.55(-0.41 to 5.52)	
P	0.800[a]	0.087[a]	
ALT (IU/L)			
Before	22.37±9.04	24.82±23.01	0.798[b]
After	31.74±28.24	17.83±10.40	0.086[c]
MD (CI95%)	-8.36(-22.04 to 5.30)	7(-1.16 to 15.16)	
P	0.81[a]	0.215[a]	
ALT/AST			
Before	1.24±0.31	1.12±0.44	0.340[b]
After	1.81±1.95	0.98±0.39	0.046[c]
P	0.159[d]	0.025[d]	

CLA: conjugated linoleic acid, IL-6: interleukin-6, IL-10: interleukin-10, TNF-α tumor necrosis factor-α hs-CRP: high sensitivity C-reactive protein, AST: aspartate aminotransferase, ALT: alanine aminotransferase, SD: standard deviation, MD: mean difference, CI: confidence interval/ [a]:paired t- test, [b]: independent t-test, [c]: ANCOVA adjusted for baseline values, energy intake, weight circumference and hip circumference, [d]: Mann-Whitney u test, [e]: Wilcoxon rank t-test.

and the difference in this ratio between the groups was statistically significant (*P*=0.046).

Discussion

According to a practice guideline, current NAFLD therapies include lifestyle modifications (diet, exercise, bariatric surgery) and pharmacotherapy including antioxidants, insulin-sensitizing agents, and hepatoprotective and miscellaneous agents (18). Successful weight loss demonstrated to improve liver metabolic and histology parameters including inflammatory markers associated with NAFLD (19). Regular exercise can also suppress low-grade systemic inflammation (20). Although lifestyle modification has the potential to become a modality for NAFLD treatment (8), it is not effective due to its inadequate compliance and unsatisfactory results (9). Besides, extreme weight loss via starvation or sudden change in body mass by bariatric surgery could be harmful (21). On the other side, pharmacotherapy is an appropriate alternative for NAFLD management. In most cases, insulin resistance is the major target for NAFLD treatment using Insulin-sensitizing agents such as thiazolidinediones, which have recently failed. The long-term safety and efficacy issues have limited its use. Several studies have shown that they could not lead to an improvement in liver histology parameters (22). Nowadays, as stated by the practice guideline, vitamin E (α-tocopherol) medication is considered as a first-line pharmacotherapy in NAFLD treatment and could positively impact steatosis, inflammation and liver histology in patients with NAFLD (18). In our study, all the patients of both groups received vitamin E due to its beneficial effects.

As a new strategy for NAFLD treatment, CLA is identified as a potential dietary substance, acting through a variety of mechanisms to prevent and improve NAFLD. CLA refers to geometric and positional isomers of linoleic acid (13). In the current study, the daily intake of 3g of CLA softgel capsules for eight weeks resulted significant positive changes in fat mass, muscle mass and total body water. This result may indicate the positive effect of CLA on anthropometric indices and body composition. Our results support a previous observation that this reference CLA improved BMI in children (23).

We also evaluated the ALT/AST ratio in both CLA and control groups. Results showed that the ALT/AST ratio markedly decreased in the CLA group (*P*=0.025) and slightly increased in control group (*P*=0.159). The difference between ratio of both groups was statistically significant (*P*=0.046). However, to our knowledge, limited studies have examined the effect of CLA mixture on liver function. In contrast with our results, one study found that the effect of CLA and placebo on serum levels of ALT in healthy non-obese sedentary women did not differ (24). Despite liver function investigations, many studies have examined the effect of CLA on inflammatory factors both in healthy people (25) and in patients with different kinds of diseases such as hyperlipidemia (26), cardiovascular and atherosclerosis diseases (27), rectal cancer (28), and diabetes (29); but no study examined the effect of CLA on patients with NAFLD. To the best of our knowledge, our study was the only study found to investigate the effects of CLA on patients with NAFLD. In the current work, we studied the physiological effect of CLA isomers (a 50:50 mixture of c9t11 and t10c12) on NAFLD patients with low-calorie diets in company with vitamin E and evaluated the molecular markers of inflammation focusing on IL-6, IL-10, TNF-α and also hs-CRP which is a marker of systemic inflammation. In two studies on healthy people, Raff found that CLA had no effect on inflammatory markers, but increase lipid peroxidation (25), while another study by Song showed a decrease in the level of the pro-inflammatory cytokines and TNF-α and increase in level of IL-10 (30). These results may indicate the probable effect of CLA on NAFLD treatment due to its effect on weight and inflammation. Another study on patients with rectal cancer showed that CLA may provide a new complementary treatment by reducing tumor invasion and improving inflammatory factors such as IL-6, TNF-α and hs-CRP (31). In the case of cardiovascular disease, Eftekhari investigated the effect of CLA on atherosclerosis and indicated that CLA decrease the level of IL-6 and hs-CRP (27). In our study, CLA consumption in the form of softgel capsule decreased the level of IL-6 pro-inflammatory factor and hs-CRP as a marker of systemic inflammation and increased the IL-10 anti-inflammatory factor, but there was no significant difference in

these factors among both groups after eight weeks. On the other hand, compared with the control group, significant reductions were found in TNF-α level in both groups (P=0.001), which would be the promising result for consuming CLA in NAFLD treatment. These results are supported by similar experimental studies, as mentioned before. Our results are also in line with the results of two other eight-week studies which investigated the effect of CLA on overweight patients with hyperlipidemia and diabetes, distinctively. They suggested that it may require longer time to realize the CLA effects more significantly (26;29). We also suggest that the low level of CRP might be related to vitamin E, which has a strong anti-oxidative property and known synergistic action with CLA in inhibiting oxidation (31;32).

Further long term clinical trials with bigger sample size are required to study the effect of CLA supplementation. The other limitation of this study was that the analyzed inflammatory parameters were come from serum but not only directly liver tissues. Besides, the estimation of dietary intake as well as determining serum CLA concentration was not possible. Additional study on human cell culture because of vague molecular mechanisms of CLA in humans are also suggested.

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