Journal of Pre-Clinical and Clinical Research, 2010, Vol 4, No 2, 118-121
www.jpccr.eu

ORIGINAL ARTICLE

Conjugated linoleic acid has no effects on atherosclerosis but induces liver steatosis in apoE/LDLR $^{-/-}$ mice fed a fructose-rich diet

Renata B. Kostogrys[1], Magdalena Franczyk-Żarów[1], Edyta Maślak[1], Mariusz Gajda[2], Łukasz Mateuszuk[3], Stefan Chłopicki[3]

[1] *Department of Human Nutrition, Faculty of Food Technology, Agricultural University, Cracow, Poland*
[2] *Department of Histology, Jagiellonian University Medical College, Cracow, Poland*
[3] *Department of Experimental Pharmacology, Jagiellonian University Medical College, Cracow, Poland*

Abstract: The current study was designed to investigate the effects of CLA on several known risk factors of atherosclerosis in apoE/LDLR$^{-/-}$ mice fed fructose diet. The mice at the age of 2 months were divided into 2 experimental groups and fed the following diets: I- AIN-93G + 60% fructose (Fructose) and II- AIN-93G + 60% fructose + 0.5% CLA (Fructose+CLA). After 2 months of feeding, plasma lipid profile, glucose concentration, liver fatty acid composition, and quantification of atherosclerosis were analyzed. There were no differences in body and kidney weight. Liver weight was significantly increased in the CLA-treatment group which was related to higher liver lipid concentration. No changes in plasma lipid profile nor liver fatty acid composition were observed between experimental groups. These results indicate that CLA increased liver lipid accumulation. It may cause liver steatosis in experimental mice.

Key words: CLA, fructose, liver steatosis, atherosclerosis

INTRODUCTION

Atherosclerosis is a progressive disease of the medium and large arteries, characterized by the accumulation of lipids in inflammatory cells leading to foam-cell formation. It is the primary cause of heart disease and stroke. In westernized societies, it may be the indirect cause of approximately 50% of all deaths. The western diet contains not only a large amount of fat, but also sugar, such as fructose, the intake of which has increased steadily during the past 2 decades. Moreover, this type of diet frequently causes non-alcoholic fatty liver disease (NAFLD) [1].

In a relatively short period of time, the dietary consumption of fructose has increased several folds above the amount present in natural food, caused by the use of high fructose corn sweeteners and sucrose in manufactured food. In human diets, approximately one –third of dietary fructose originates from fruit, vegetables, and other natural sources, and two-thirds was added to beverages and food in the diet (e.g. soft drinks, fruit-flavored drinks, sweets, jams, syrups, and bakery products).

Although there is little evidence that modest amounts of fructose have detrimental effects on carbohydrate and lipid metabolism, larger doses have been associated with numerous metabolic abnormalities in laboratory animals and humans, suggesting that high fructose consumption adversely affects health.

Conjugated linoleic acid (CLA) is a term that refers to a collection of positional and geometric isomers of linoleic acid (LA)- 18:2 n-6 with conjugated double bonds. These fatty acids are a natural food component occurring in the lipid fraction of ruminant meat, milk, and other dairy products. The *cis*-9, *trans*-11 CLA isomer (c9,t11-CLA) is the principal dietary form of CLA, but lower levels of *trans*-10, *cis*-12 (t10,c12-CLA) and other isomers are also present in these food sources [2]. It has been largely demonstrated that CLA has positive effects in cancer [3-5], cardiovascular disease [6-9], diabetes [10-12], obesity [13-15] and liver steatosis [16].

The aim of the study was to investigate effects of CLA on fructose-induced atherosclerosis risk factors in apoE/LDLR $^{-/-}$ mice.

MATERIALS AND METHODS

Animals and diets. The apoE/LDLR $^{-/-}$ mice used in study were obtained from the Cardiovascular Research Institute Maastricht, Maastricht University (The Netherlands), and bred in animal house in the Medical Research Centre at the Polish Academy of Science in Warsaw. The animals were housed in colony cages in a temperature-controlled environment (22-25°C) with a 12-h light cycle. They had free access to food and water. All procedures involving animals were conducted according to the Guidelines for Animal Care and Treatment of the European Union and were approved by the Local Animal Ethics Commission.

Up to the age of 2 months, the mice were fed a commercial, cholesterol-free, pellet diet. Diet and water, consumed *ad libitum*, were regularly checked and provided daily. At the age of 2 month, the mice (n=12) were divided into 2 experimental groups and fed the following diets for the next 2 months: **I** – AIN-93G + 60% fructose (**Fructose**) and **II** – AIN-93G + 60% fructose + 0.5% CLA (**Fructose+CLA**). The composition of the diets, based on Reeves at al. [17], is shown in Table 1. After 2 months of feeding, the mice were injected with heparin,

Corresponding author: Dr. Renata B. Kostogrys, Department of Human Nutrition, Faculty of Food Technology, Agricultural University, Balicka 122, 30-149 Cracow, Poland.

Received: 4 November 2010; accepted: 19 December 2010

Table 1 Composition of experimental diet (%).

	I. Fructose	II. Fructose+CLA
Corn starch	–	–
Fructose	63.2486	63.2486
Caseine	20	20
Sucrose	–	–
Soybean oil	7	6.167
Celulose powder	5	5
Mineral mixture[a]	3.5	3.5
Witamin mixture[b]	1	1
Choline	0.25	0.25
Tert-butylhydrochinon	0.0014	0.0014
CLA[c]	-	0.833

[a] AIN-93G mineral mixture
[b] AIN-93G vitamin mixture
[c] CLA The CLA oil (Luta-CLA® 60), obtained from BASF, Ludwigshafen, Germany. contained 600 g CLA/kg, with equal representation of two major CLA isomers (cis-9, trans-11 and trans-10, cis-12).

anesthetized with sodium thiopental, and finally sacrificed by cervical translocation.

Sample collection and analysis. The liver and kidney were dissected and snap-frozen. The samples were stored at -80°C until analysis. The fat from liver were extracted using LECO and analyzed using GC-MS analyzer (Shimadzu GC-MS, Model QP 5050A).

Blood samples were centrifuged (4,000 *g,* 4 min) to obtain plasma samples, and then analyzed using commercially available kits for total cholesterol (TC; Liquick Cor-Chol 60 no 2-204; Cormay, Lublin, Poland), and triacylglycerols (TAG; Liquick Cor-TG 30 no 2-262; Cormay, Lublin, Poland).

Quantification of atherosclerosis in aortic roots (cross-section analysis). In the anesthetized mice, the thorax was longitudinally opened, the right atrium incised, and the heart was perfused by phosphate-buffered saline (PBS, pH=7.4) through the apex of the left ventricle at a constant pressure of approximately 100 mm Hg. Next, the heart and the ascending aorta were dissected. The excised heart and ascending aorta were embedded in OCT compound (CellPath, Oxford, UK) and snap-frozen. 10 µm-thick cryosections were cut from the aortic root using a standardized protocol [18, 19]. Serial sections were cut from the proximal 1 mm of the aortic root. Eight sections were collected at 100-µm intervals, starting at a distance of 100-µm from the appearance of the aortic valves. Sections were thaw-mounted on poly-L-lysine coated slides and air dried. After fixation in 4% paraformaldehyde (pH=7), sections were stained with Meyer's hematoxylin and oil red-O (Sigma-Aldrich, St. Louis, MO, USA) [19]. Oil red-O-stained sections were examined under an Olympus BX50 (Olympus, Tokyo, Japan) microscope and used for quantitative evaluation. Images of the aorta were recorded using an Olympus Camedia 5050 digital camera, and stored as TIFF files of resolution 1024 × 768 pixels. The total area of the lesion was measured semiautomatically in each slide using LSM Image Browser 3 software (Zeiss, Jena, Germany). For each animal, a mean lesion area was calculated from 8 sections, reflecting the cross-section area covered by atherosclerosis [20].

Statistical analysis. Results are expressed as means ± S.E.M. Where appropriate, the data were examined by Student's t test using STATISTICA 6.1 package (StatSoft Inc., USA). The differences between treatments means were considered significant at $p<0.05$. The data resulting from quantification of atherosclerosis were analyzed by the non-parametric Mann-Whitney test and differences between treatments means were considered significant at $p<0.05$.

RESULTS

Effect of CLA on body and organs weight. It was shown that CLA significantly increased liver weight and fat concentration in Fructose+CLA group. No significant effects of CLA on body and kidney weight between experimental groups were observed (Table 2).

Table 2 Metabolic and plasma parameters in apoE/LDLR $^{-/-}$ mice fed I – Fructose and II – Fructose+CLA diet.

	Fructose	Fructose+CLA
Initial body weight [g]	24.01±1.0	23.87±0.7
Final body weight [g]	26.39±0.7	25.27±0.5
Liver weight [% bw]	4.81±0.2 ^{A}	6.17±0.2 ^{B}
Liver fat concentration [% of fat]	6.17±0.8 ^{A}	11.10±1.7 ^{B}
Kidney weight [% bw]	1.59±0.04	1.70±0.06
Total cholesterol [mmol/l]	25.87±2.2	31.06±1.8
Triacylglycerol [mmol/l]	1.09±0.1	1.48±0.2
Glucose [mg/dl]	188.38±35.2	156.43±36.8

A, B – significant difference p < 0.05

Effect of CLA on plasma lipid profile and glucose. CLA had no effects on plasma total cholesterol and triacylglycerol concentration in mice. Also, the glucose level did not differ between the experimental groups (Table 2).

Effect of CLA on fatty acid composition in liver. Dietary treatment had no significant effect on the proportions of SFA and MUFA in liver. However, SFA tended to increase in the CLA group. In contrast, PUFA significantly decreased after CLA feeding. Although both CLA isomers were ingested at equivalent amounts, *trans*-10, *cis*-12 was incorporated in significantly lower proportion than *cis*-9 *trans*-11 into lipids (Table 3).

Table 3 Fatty acid composition of liver (wt.%).

	I. Fructose	II. Fructose+CLA
SFA	28.81±3.7	35.45±4.5
MUFA	34.13±15.0	38.03±17.3
PUFA	37.13±4.7^{A}	26.70±2.8^{B}
C14:0	0.48±0.05	0.4±0.04
C15:0	0.1±0.02	0.2±0.03
C16:0	23.2±0.72	27.9±3.4
C16:1	2.05±0.3	1.75±0.21
C17:0	0.2±0.02	0.3±0.03
C18:0	4.0±0.48	5.5±1.69
C18:1 n-9	32.1±0.97	36.3±1.87
C18:2 n-6	33.1±1.12	22.8±3.45
C18:3 n-6	0.5±0.09	0.3±0.06
C20:1	0.9±0.09	1.3±0.07
C18:3	1.0±0.17	0.8±0.0
C18:2c9t11	0^{A}	0.7±0.13^{C}
C18:2t10c12	0^{A}	0.2±0.03^{B}
C20:2 n-6	0.28±0.05	0.3±0.0
C20:3 n-6	0.5±0.07	0.57±0.03
C20:4 n-6	1.75±0.34	1.78±0.62

A, B, C – significant difference p < 0.05

Effect of CLA on development of atherosclerosis. CLA had no effect on the development of atherosclerosis in mice. Area of plaques as measured in aortic roots (cross-section) did not differ significantly between the experimental groups.

DISCUSSION

The aim of the present study was to investigate the effects of CLA on the weight of organs, blood lipid profile, and atherosclerosis level in ApoE/LDLR$^{-/-}$ mice fed a fructose diet Such a diet has been used to induce a hyperlipidemic condition in mice. Fructose feeding enhances hepatic secretion of VLDL and may decrease its plasma clearance, which frequently results in modest hypercholesterolemia and hypertriglyceridemia.

In ApoE/LDLR$^{-/-}$ mice, dietary CLA have no effect on body and kidney mass. However, it was shown that there was a significant increase in liver weight in mice fed Fructose+CLA diet, which was related to increased liver lipid accumulation in that group. Several studies in mice have shown that CLA feeding may lead to increased liver weight. Belury et al. [21] showed that mice fed 0.5% CLA mixture for 6 wk, decreased in body weight and increased liver lipid content. DeLany et al. [22] observed liver fat accumulation by histopathologic examination in AKR/J mice fed 1% CLA mixture for 40 days. Javadi et al. [23] also showed that feeding CLA to mice resulted in increased liver weight and induced liver steatosis. They suggest that the increase in liver TAG may be mediated by increased hepatic fatty acid synthesis relative to oxidation. Other authors explain that in the absence of adipose tissue, blood fats have a greater chance to be taken up and stored in the liver. This could also explain the lack of changes in plasma TC and TAG concentration after CLA treatment revealed in our study.

The present study demonstrates that CLA had no impact on the atherosclerosis level in ApoE/LDLR$^{-/-}$ mice. Results from studies about CLA effects on atherosclerosis are also equivocal. While Munday et al. [24] showed that a CLA mixture promoted fatty streak formation in the C57BL/6 mouse atherosclerosis model, most studies indicated anti-atherosclerotic effects of CLA [25-27]. Recent studies have shown the isomer-specific effect of CLA. Results of Arbones-Mainar [28] and De Roos et al. [29] demonstrated that *cis*-9, *trans*-11-CLA impedes, whereas *trans*-10, *cis*-12-CLA promotes atherosclerosis in apolipoprotein E knockout mice. Anti-atherosclerotic effects of *cis*-9, *trans*-11-CLA were also shown by Valeille et al. [30] and Toomey et al. [31], and in their another study the pro-atherosclerotic properties of *trans*-10, *cis*-12-CLA was demonstrated by Arbones-Mainar et al. [32].

In conclusion, the addition of CLA mixture to a high-fructose diet resulted in the development of liver steatosis without altering the plasma lipid profile or level of atherosclerosis in Apolipoprotein E and low density lipoprotein receptor double knockout mice. Although observations made in the mouse model cannot be directly extrapolated to humans, our data should be a warning for people before taking CLA supplementation, until such time as researchers will be in accordance with their opinion about the safety of CLA treatments.

ACKNOWLEDGMENTS

The authors would like to acknowledge the assistance and support of Prof. P. M. Pisulewski, without which this study could not have been realized.

REFERENCES

1. Howard BV, Wylie-Rosett J: Sugar and cardiovascular disease: A statement for healthcare professionals from the Committee on Nutrition of the Council on Nutrition, Physical Activity, and Metabolism of the American Heart Association. *Circulation* 2002**,** **106**(4), 523-527.
2. Kennedy A, Martinem K, Schmidt S, Mandrup S, LaPoint K, McIntosh M: Antiobesity mechanisms of action of conjugated linoleic acid. *J Nutr Biochem* 2010, **3**, 171-179.
3. Belury MA: Inhibition of carcinogenesis by conjugated linoleic acid: potential mechanisms of action. *J Nutr* 2002, **132**, 2995-2998.
4. Field CJ, Schley PD: Evidence for potential mechanisms for the effect of conjugated linoleic acid on tumor metabolism and immune function: lessons from n-3 fatty acids. *Am J Clin Nutr* 2004, **79**, 1190S-1198S.
5. Cho HJ, Kim EJ, Lim SS, Kim MK, Sung MK, Kim JS, Park JH: Trans-10,cis-12, not cis-9,trans-11, conjugated linoleic acid inhibits G1-S progression in HT-29 human colon cancer cells. *J Nutr* 2006, **136**, 893–898.
6. Noone EJ, Roche HM, Nugent AP, Gibney MJ: The effect of dietary supplementation using isomeric blends of conjugated linoleic acid on lipid metabolism in healthy human subjects. *Brit J Nutr* 2002, **88**, 243-251.
7. Tricon S, Burdge GC, Kew S, Banerjee T, Russell JJ, Jones EL, Grimble RF, Williams CM, Yaqoob P, Calder PC: Opposing effects of cis-9,trans-11 and trans-10,cis-12 conjugated linoleic acid on blood lipids in healthy humans. *Am J Clin Nutr* 2004, **80**, 614-620.
8. Valeille K, Gripois D, Blouquit MF, Souidi M, Riottot M, Bouthegourd JC, Serougne C, Martin JC: Lipid atherogenic risk markers can be more favourably influenced by the cis-9 trans-11-octadecadienoate isomer than a conjugated linoleic acid mixture or fish oil in hamsters. *Brit J Nutr* 2004, **91**, 191-199.
9. Arbonés-Mainar JM, Navarro MA, Acín S, Guzmán MA, Arnal C, Surra JC, Carnicer R, Roche HM, Osada J: Trans-10, cis-12- and cis-9, trans-11-Conjugated Linoleic Acid Isomers Selectively Modify HDL-Apolipoprotein Composition in Apolipoprotein E Knockout Mice. *J Nutr* 2006, **136**, 353-359.
10. Ryder JW, Portocarrero CP, Song XM, Cui L, Yu M, Combatsiaris T, Galuska D, Bauman DE, Barbano DM, Charron MJ, Zierath JR, Houseknecht KL: Isomer-specific antidiabetic properties of conjugated linoleic acid - Improved glucose tolerance, skeletal muscle insulin action, and UCP-2 gene expression. *Diabetes* 2001, **50**, 1149-1157.
11. Noto A, Zahradka P, Ryz NR, Yurkova N, Xie XP, Taylor CG: Dietary conjugated linoleic acid preserves pancreatic function and reduces inflammatory markers in obese, insulin-resistant rats. *Metabolism* 2007, **56**(1), 142-151.
12. Mersmann H.J: Mechanism for conjugated linoleic acid-mediated reduction In fat deposition. *J Anim Sci* 2002, **80**(E. Suppl. 2), E126-E134.
13. Evans M, Brown J, McIntosh M: Isomer-specific effects of conjugated linoleic acid (CLA) on adiposity and lipid metabolism. *J Nutr Biochem* 2002, **13**, 508-516.
14. Terpstra AH, Javadi M, Beynen AC, Kocsis S, Lankhorst AE, Lemmens AG, Mohede IC: Dietary conjugated linoleic acids as free fatty acids and triacylglycerols similarly affect body composition and energy balance in mice. *J Nutr* 2003, **133**, 3181-3186.
15. Wang Y, Jones PJH: The role of conjugated linoleic acid in human health. Dietary conjugated linoleic acid and body composition. *American J Clin Nutr* 2004, **79**, 1153S-1158S.
16. Purushotham A, Shrode G, Wendel A, Liu L, Belury M: Conjugated linoleic acid does not reduce body fat but decreases hepatic steatosis in adult Wistar rats. *J Nutr Biochem* 2007, **18**(10), 676-684.
17. Reeves PG, Nielsen FH, Fahey GC: AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* 1993, **123**(11), 1939-1951.
18. Nicoletti A, Kaveri S, Caligiuri G, Bariety J, Hansson GK: Immunoglobulin treatment reduces atherosclerosis in apo E knockout mice. *J Clin Invest* 1998, **102**, 910-918.

19. Elhage R, Jawien J, Rudling M, Ljunggren HG, Takeda KS, Akira S, Bayard F, Hansson GK: Reduced atherosclerosis in interleukin-18 deficient apolipoprotein E – knockout mice. *Cardiovasc Res* 2003, **59**, 234-240.
20. Robertson AK, Rudling M, Zhou X, Gorelik L, Flavell RA, Hansson GK: Disruption of TGF-beta signaling in T cells accelerates atherosclerosis. *J Clinical Invest* 2003, **112**, 1342-1350.
21. Belury MA, Kempa-Steczko A: Conjugated linoleic acid modulates hepatic lipid composition in mice. *Lipids* 1997, **32**, 199-204.
22. DeLany JP, Blohm F, Truett AA, Scimeca JA, West DB: Conjugated linoleic acid rapidly reduces body fat content in mice without affecting energy intake. *Am J Physiol* 1999, **276**, 1172-1179.
23. Javadi M, Beynen AC, Hovenier R, Lankhorst A, Lemmens AG, Terpstra AH, Geelen MJ: Prolonged feeding of mice with conjugated linoleic acid increases hepatic fatty acid synthesis relative to oxidation. *J Nutr Biochem* 2004, **15**(11), 680-687.
24. Munday JS, Thompson KG, James KA: Dietary conjugated linoleic acids promote fatty streak formation in the C57BL/6 mouse atherosclerosis model. *Brit J Nutr* 1999, **81**, 251-255.
25. Kritchevsky D, Tepper SA, Wright S, Tso P, Czarnecki SK: Influence of conjugated linoleic acid (CLA) on establishment and progression of atherosclerosis in rabbits. *J Am* Coll Nutr 2000, **19**, 472S-477S.
26. Kritchevsky D, Tepper SA, Wright S, Czarnecki SK: Influence of graded levels of conjugated linoleic acid (CLA) on experimental atherosclerosis in rabbits. *Nutr Res* 2002, **22**, 1275-1279.
27. Wilson TA, Nicolosi RJ, Chrysam M, Kritchevsky D: Conjugated linoleic acid reduces early aortic atherosclerosis greater than linoleic acid in hypercholesterolemic hamsters. *Nutr Res* 2000, **20**, 1795-1805.
28. Arbones-Mainar JM, Navarro MA, Guzman MA, Arnal C, Surra JC, Acin S, Carnicer R, Osada J, Roche HM: Selective effect of conjugated linoleic acid isomers on atherosclerotic lesion development in apolipoprotein E knockout mice. *Atherosclerosis* 2006**, 189**(2), 318-327.
29. De Roos B, Rucklidge G, Reid M, Pickard K, Navarro MA, Arbone´s-Mainar JM, Guzman-Garcia MA, Osada J, Browne J, Loscher CE, Roche HM: Divergent mechanisms of cis9, trans11- and trans10, cis12-conjugated linoleic acid affecting insulin resistance and inflammation in apolipoprotein E knockout mice: a proteomics approach. *FASEB J* 2005, **19**, 1746-1748.
30. Valeille K, Ferezou J, Amsler G, Quignard-Boulange A, Parquet M, Gripois D, Dorovska-Taran V, Martin JC: A cis-9,trans-11-conjugated linoleic acid-rich oil reduces the outcome of atherogenic process in hyperlipidemic hamster. *Am J Physiol-Heart C* 2005, **289**(2), H652-H659.
31. Toomey S, Roche H, Fitzgerald D, Belton O: Regression of pre-established atherosclerosis in the apoE(-/-) mouse by conjugated linoleic acid. *Biochem* Soc *T* 2003, **31**, 1075-1079.
32. Arbones-Mainar JM, Navarro MA, Acin S, Guzman MA, Arnal C, Surra JC, Carnicer R, Roche HM, Osada J. Trans-10, cis-12-and cis-9, trans-11-conjugated linoleic acid isomers selectively modify HDL-apolipoprotein composition in apolipoprotein E knockout mice. *J Nutr* 2006, **136**(2), 353-359.