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Effects of conjugated linoleic acid (CLA) on development of atherosclerosis in ApoE/LDLr$^{-/-}$ mice

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Abstract: The effect of conjugated linoleic acid (CLA) on development of atherosclerosis in genetically modified mice ApoE/LDLr $^{-/-}$ was studied. Animals were divided into 2 groups: control (standard AIN-93G diet) (n=8) and CLA (AIN-93G supplemented with 0.5% CLA) (n=6). After 2 months of feeding plasma lipid profile, glucose concentration, liver fatty acid composition and quantification of atherosclerosis were analyzed. In the CLA group, decreased body mass was observed. Plasma total cholesterol and triacylglycerols levels were significantly higher in animals fed CLA compared to Control. There was no effect of CLA on liver and kidney weights, nor on lipid profiles of adipose tissue. Plasma glucose levels were unchanged after CLA feeding. No effect of CLA on atherosclerosis was observed. These results indicate that dietary CLA reduced body mass and increased plasma TC and TAG level. This might be caused by decrease in adipose tissue mass and increase in liver lipogenesis in ApoE/LDLr$^{-/-}$ mice.

Key words: ApoE/LDLr$^{-/-}$ mice, CLA, atherosclerosis

INTRODUCTION

Atherosclerosis is one of the most common reason of cardiovascular diseases. It is chronic disease which consists of formation changes in endothelium. That changes cause decreasing of blood vessels elasticity and reducing their diameter. It is known that the most important reason of atherosclerosis is hipertriglyceridemia [1]. Overweight and obesity are also very dangerous risk factors of these disease. Therefore some recommendation about healthy diet and life style occurs.

Conjugated linoleic acid (CLA) is a collective term to describe a class of positional and geometric isomers of linoleic acid (*cis*-9, *cis*-12 C18:2 *n*-6) in which the two double bonds are separated by a single C-C bond, but not by a methylene group (-CH_2-). CLA isomers are intermediate products of bacterial biohydrogenation of dietary unsaturated fatty acids in the rumen, and are mainly found in fat of ruminant milk and meat [2].

In the last ten years, extensive research have indicated that CLA isomers, fed to laboratory animals, showed several health-related properties, including anti-adipogenic [3-6], anti-carcinogenic [7-9], anti-inflammatory [10, 11] and anti-atherogenic [12-18] effects.

The objective of this study was to verify anti-atherogenic potency of CLA, fed to apolipoprotein E and low density lipoprotein receptor double knockout mice (apoE/LDLR $^{-/-}$), representing unique and reliable model of atherogenesis [19-21]. Properties of CLA were identified using serum lipid profile, glucose concentration, liver fatty acid composition and development of atherosclerosis.

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MATERIALS AND METHODS

Animal and feeding. The apoE/LDLR$^{-/-}$ mice used in this study were obtained from Cardiovascular Research Institute Maastricht, Maastricht University (The Netherlands) and bred in the animal house in Warszawa (Polish Academy of Science, Medical Research Center). Animals were held in colony cages in a regular temperature environment (22-25°C) with a 12-h light cycle.

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, pelleted diet. Diet and water, consumed *ad libitum*, were regularly checked and provided daily. At the age of 2 months, mice (n=14) were divided into two experimental groups and fed the following diets for the next 2 months: **I** – AIN-93G (**Control**) and **II** – AIN-93G + 0.5% CLA (**Control+CLA**). The diets composition based on Reeves at al. [22] is shown in Table 1.

Body mass of mice was monitored weekly. After 2 months of feeding with respective diets, the mice were deprived of food overnight, injected intraperitoneally with 1000 IU of heparin (Sanofi-Synthelabo; Paris, France), and after 10 min, anesthetized intraperitoneally with 40 mg/kg of sodium thiopental (Biochemie; Vienna, Austria), and finally sacrificed by cervical translocation.

Table 1 Composition of experimental diet (%).

	I Control	II Control+CLA
Corn starch	53.2486	53.2486
Caseine	20	20
Sucrose	10	10
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
CLAc	-	0.833

[a] AIN-93G mineral mixture,
[b] AIN-93G vitamin mixture
[c] CLA – 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).

Blood sampling and plasma lipid profile analyses. Blood samples were taken from the left ventricle of the heart, collected into test tubes and centrifuged (4 000 g, 4 min) to obtain plasma samples. The samples were deep frozen (-80°C) and stored until further analysis. Plasma samples were 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). The results were expressed in mmol/L.

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

Quantification of atherosclerosis in aortic roots (cross-section analysis). In anesthetized mice, the thorax was longitudinally opened, the right atrium was 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 [23,24]. Serial sections were cut from the proximal 1 mm of the aortic root. Eight sections were collected at 100-µm intervals starting at a 100-µm distance 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) [25]. 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 eight sections, reflecting the cross-section area covered by atherosclerosis [25].

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 treatment groups were considered significant at $p<0.05$. The data resulting from quantification of atherosclerosis were analyzed by the nonparametric 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 decreased body weight in Control+CLA diet (Table 2). No differences in kidney and liver weights between groups were observed. CLA had no effect on lipid accumulation in liver as well as on glucose concentration in plasma.

Table 2 Metabolic and plasma parameters in apoE/LDLR$^{-/-}$ mice fed control and CLA diets.

	I Control	II Control+CLA
Initial body weight	24.93±0.69	23.92±0.68
Final body weight [g]	26.97±1.01^{A}	23.28±0.51^{B}
Liver weight [% bw]	4.26±0.18	5.08±0.23
Liver fat concentration [% of fat]	7.76±1.37	5.91±1.12
Kidney weight [% bw]	1.52±0.09	1.40±0.08
Total cholesterol [mmol/l]	22.37±2.09^{A}	31.4±0.25^{B}
Triacylglycerol [mmol/l]	0.72±0.12^{A}	1.52±0.22^{B}
Glucose [mg/dl]	172.9±39.3	150.8±32.3
Cholesterol in liver [µg/mg]	65.2±23.7	174.6±12.05

Effect of CLA on fatty acid composition in adipose tissue. No effects of dietary treatments on fatty acid composition and proportions of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in adipose tissue were observed (Table 3, Figure 1).

Table 3 Fatty acid composition of adipose tissue (wt.%).

	I Control	II Control+CLA
C14:0	1.0±0.0	0.8±0.1
C15:0	0.1±0.0	0.0
C16:0	16.2±1.4	18.4±0.4
C16:1	3.8±0.4	2.7±0.3
C18:0	1.7±0.2	0.9±0.1
C18:1	29.6±0.7	30.8±0.6
C18:2	44.5±1.9	42.9±0.5
C20:1	0.6±0.1	0.2±0.0
C18:3	2.6±0.3	1.9±0.1
C18:2c9t11	0.0	1.0±0.1
C18:2t10c12	0.0	0.4±0.0

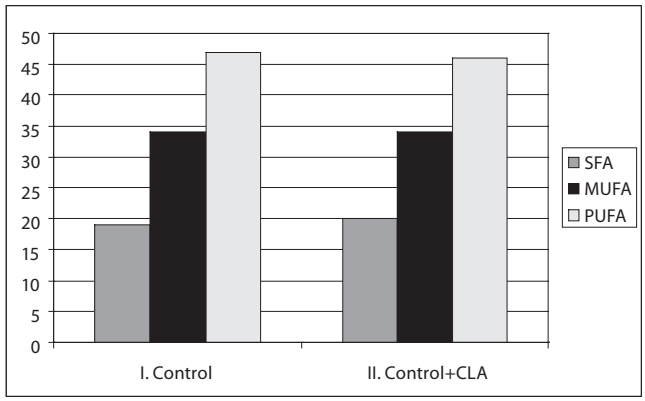

Figure 1 Fatty acid profile in adipose tissue in apoE/LDLR$^{-/-}$ mice fed: I – Control, II – Control+CLA.

Effect of CLA on fatty acid composition in liver. Chromatographic analysis showed that CLA increased the proportion of total saturated and monosaturated fatty acids and decreased the proportion of total polyunsaturated fatty acids in liver. Additionally CLA significantly decreased 16:1 and 18:3 contents in Control+CLA diet (Table 4).

Table 4 Liver fatty acid composition in apoE/LDLR$^{-/-}$ mice fed: I – Control, II – Control+CLA. The means bearing different letters were significantly different ($p< 0.05$; analyzed by Student's t test).

	I. Control	II. Control+CLA
C14:0	0.6±0.0	0.5±0.1
C15:0	0.2±0.0	0.2±0.0
C16:0	27.5±1.8	29.6±3.1
C16:1	3.0±0.4^a	1.5±0.1^b
C17:0	0.2±0.0	0.3±0.1
C18:0	3.8±0.7	5.4±0.7
C18:1 n-9	31.2±2.5	36.5±1.7
C18:2 n-6	29.4±4.2	21.5±3.5
C18:3 n-6	0.56±0.1^a	0.24±0.1^b
C20:0	0.7±0.1	0.9±0.1
C18:3	1.7±0.1^a	0.64±0.1^b
C18:2c9t11	0.0^a	0.4±0.1^b
C18:2t10c12	0.0^a	0.1±0.0^b
C20:2 n-6	0.2±0.0	0.3±0.0
C20:3 n-6	0.3±0.1	0.5±0.1
C20:4 n-6	1.4±0.2	2.4±0.4

Effect of CLA on lipid profile in plasma and liver. The effect of dietary treatments on plasma lipoprotein concentrations and cholesterol level in liver is shown in Table 2. The evident significant effect of CLA treatments was the increase of plasma TC and TAG in mice fed Control+CLA diet as compared to the Control ($p<0.05$).

Effect of CLA on development of atherosclerosis. Area of plaques as measured in aortic roots (cross-section) did not differ significantly between experimental groups (Figure 2, Figure 3).

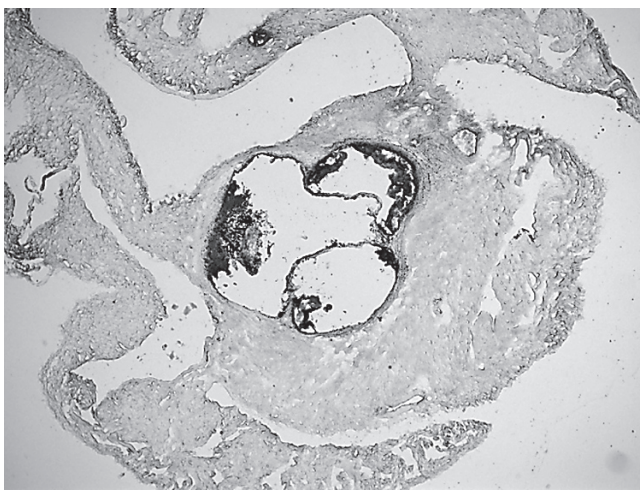

Figure 2 Quantification of cross sections of aortic roots showing amount of aortic plaque in apoE/LDLR$^{-/-}$ mice fed: I – Control diet.

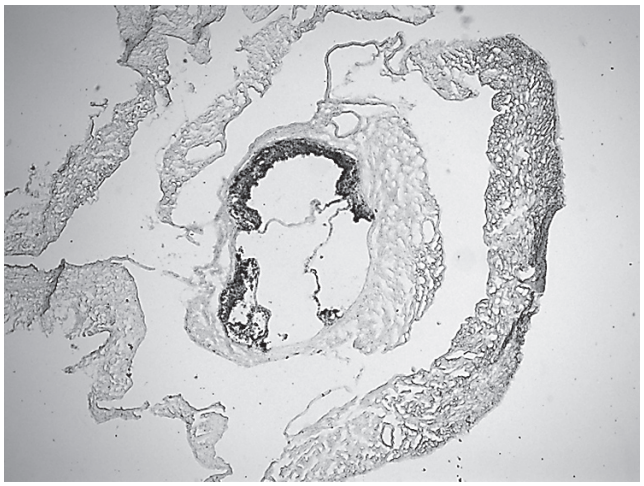

Figure 3 Representative images of cross sections of aortic roots showing aortic plaque in apoE/LDLR$^{-/-}$ mice fed: II –Control+CLA diet.

DISCUSSION

Effect of CLA on body and organs weight. It was observed that CLA significantly decreased body mass in Control+CLA group. Previous studies in animals fed CLA mixture or the t10,c12 isomer showed significantly decreased body weight and fat mass compared to control animals [26-29]. Similar results received Stangl [30]. In rats fed diet with the mixture of CLA isomers (3g/100g diet) body mass decreased compared to control group.

Multiple mechanisms for CLAs effect on body fat reduction have been suggested, i.e. increasing energy expenditure, modulating adipocyte metabolism, modulating adipokines and cytokines, increasing adipocyte lipolysis and fatty acid β-oxidation [31,32]. According to our results it is seemed that responsible for body mass reduction might be decreasing adipose tissue mass and increasing liver lipogenesis showed as significant increase in plasma TC and TAG level.

In our research plasma glucose level was not significantly different between groups.

Bouthegourd's et al. [33] showed, that hamsters fed diet supplemented with 3,2% mixture of CLA had higher level of plasma glucose compared to Control group. In Nestel et al. [34] there were no differences in plasma glucose level between ApoE mice fed control and c-9, t-11 CLA isomer.

Effect of CLA on fatty acid composition in adipose tissue and liver. In our experiment no effects of dietary treatments on proportions of SFA and MUFA and PUFA in adipose tissue were observed. However, individual isomers of CLA in adipose tissue were identified only in CLA feeding mice.

It was shown that CLA increased the proportion of total saturated fatty acids and decreased the proportion of total polyunsaturated fatty acid in liver. CLA significantly decreased 18:3 content in Control+CLA diet. Belury et al. [35] revealed that dietary CLA was incorporated into neutral and phospholipids at the expense of linoleate. It was shown that oleate was increased and arachidonate was decreased in CLA groups. In addition, increasing dietary CLA was associated with reduced linoleate in hepatic phospholipids.

Effect of CLA on plasma lipid profile. It was observed that the conjugated linoleic acid significantly increased TC and TAG in plasma compared to control diet. This might be the effects of increased liver lipogenesis.

Studies on CLA produced equivocal results. In an early experiment of Lee et al. [36], CLA treatment decreased TC and LDL-C as well as plasma TAG in rabbits. Also, in studies of Nicolosi et al. [37], the hamsters fed CLA showed significantly reduced levels of plasma TC, LDL-+VLDL-C and TAG with no effect on HDL-C. As reported by Munday et al. [38], dietary CLA did not reveal significant differences in serum TC or HDL-C in mice. At the same time, the mice fed CLA showed significantly higher HDL-C:TC ratio and significantly lower TAG concentrations compared to control animals. In contrast to the above findings, increasing dietary CLA in rabbits (0.0-1.0%), led to higher serum TC and TAG levels [12]. Interestingly, the above effects were attenuated at lower dietary CLA concentrations [13]. Additionally, Corino et al. [39] showed that TC and TAG were higher ($p<0.05$) in CLA-fed rabbits. In studies involving healthy humans [14], blends of CLA isomers (cis-9, trans-11: trans-10, cis-12; 50:50 and 80:20) reduced plasma TAG concentrations whereas the 80:20 CLA isomer blend reduced VLDL-C concentrations as well.

Effect of CLA on development of atherosclerosis. ApoE/LDLR-/- mice represent a unique and reliable model of atherogenesis that allows for quantification of anti-atherogenic activities of pharmacological tools [19-21]. Using this model, we assessed the effects of CLA in control diet. It was shown that CLA supplementation had no effect on area of atherosclerosis in ApoE/LDLR-/- mice.

Species differences may account for some of the discrepancies between studies, as the sensitivity to CLA modulation may vary depending on the animal model. However, it appears that even within one model, properties of CLA are not invariable. While Cooper et al. [40] showed that CLA supplementation had no significant effect on the lesion area in either en face preparation of the aorta or in aortic root cross-section, Toomey et al. [18] revealed, that feeding an 80:20 isomeric blend of cis-9, trans-11 and trans-10, cis-12 CLA isomers, at high dietary concentration of 1.0% to apoE-deficient mice, with pre-established atherosclerosis, resulted in its profound resolution. It was accompanied by reduced expression of CD68 within the aortic lesions and down-regulation of the expression of pro-inflammatory genes. Moreover, Arbones-Mainar et al. [17], reported that CLA isomers, as individual compounds have opposite effects. While cis-9 trans-11 CLA showed anti-atherosclerotic properties the trans-10, cis-12 CLA found out to be the proatherogenic isomer.

CONCLUSION

It was observed that the body mass in CLA group was significantly decreased. Plasma total cholesterol and triacylglycerols levels were significantly higher in animals fed CLA compared to Control It might be caused by decreasing adipose tissue mass and increasing liver lipogenesis in ApoE/LDLr$^{-/-}$ mice.

There was no effect of CLA on liver and kidney weights as well as fatty acid composition of adipose tissue. CLA increased amount of SFA and MUFA and decreased amount of PUFA in liver. Plasma glucose levels were unchanged after CLA feeding. No effect of CLA on atherosclerosis was observed.

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