

## Dietary conjugated linoleic acids promote fatty streak formation in the C57BL/6 mouse atherosclerosis model

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Conjugated linoleic acids (CLA) are positional isomers of linoleic acid which have been suggested by some to possess antiatherosclerotic properties. To test this hypothesis, three groups of twenty C57BL/6 mice were fed on atherogenic diets containing: 5 g CLA/kg, 2.5 g CLA + 2.5 g linoleic acid/kg or 5 g linoleic acid/kg. All diets were fed for 15 weeks and contained (g/kg): triacylglycerol 145, free fatty acids 5, cholesterol 10 and cholic acid 5. At the completion of the experimental period, when data from both groups fed on CLA were combined, dietary CLA did not produce significant differences in body weight, serum total cholesterol concentration or serum HDL-cholesterol concentration. However, mice receiving CLA developed a significantly higher serum HDL-cholesterol : total cholesterol ratio and a significantly lower serum triacylglycerol concentration than controls. Despite causing a serum lipoprotein profile considered to be less atherogenic, the addition of CLA to the atherogenic diet increased the development of aortic fatty streaks. Considering the increased atherogenesis associated with dietary CLA in the present study, and the failure to demonstrate a significant beneficial effect of CLA in other animal studies, there is currently no conclusive evidence to support the hypothesis that CLA protect against atherogenesis.

### Atherosclerosis: Conjugated linoleic acids: Dietary fat

Conjugated linoleic acids (CLA) are positional isomers of linoleic acid which have been shown to have anticarcinogenic properties (Parodi, 1997) and have been suggested to reduce atherogenesis (Haumann, 1996; Nicolosi *et al.* 1997). CLA are produced by bacteria and are found at highest concentrations in food products derived from ruminants (Haumann, 1996). Meat from ruminants contains approximately 5.5 mg CLA/g fat while milk fat contains about 7 mg CLA/g (Chin *et al.* 1992). Fats from vegetables and non-ruminant animals contain a concentration of approximately 0.7 mg CLA/g (Chin *et al.* 1992). Dietary supplements which contain CLA are also commercially available.

Two animal experiments investigating the effect of dietary CLA on atherosclerosis have been reported. In a study using hamsters, atherogenic diets containing CLA produced significantly smaller aortic fatty streaks (thought to be an early lesion of atherosclerosis; Fuster, 1994) than diets containing no free fatty acids (Nicolosi *et al.* 1997). However, no significant differences were observed when fatty streak formation in CLA-fed hamsters was compared with that in hamsters fed on diets containing linoleic acid (Nicolosi *et al.* 1997). Supplementation of cholesterol-fed rabbits with 0.5 g CLA/d did not significantly reduce fatty

streak lesion development (Lee *et al.* 1994). Although it was initially thought that CLA may be able to protect LDL particles from oxidation (Ha *et al.* 1990; Ip *et al.* 1991; Lee *et al.* 1994; Nicolosi *et al.* 1997), a recent *in vitro* investigation (van den Berg *et al.* 1995) showed no antioxidant properties attributable to these fatty acids.

When fed on an atherogenic diet, C57BL/6 mice develop fatty streak lesions in the intima of the aortic sinus within 15 weeks. This enables trials involving relatively large numbers of animals without excessive cost, and, as these mice are inbred, they possess little genetic variation. Limitations of mice as a model for atherosclerosis development include the absence of lipoprotein(a) and cholesteryl ester transfer protein, both of which are known to influence human atherogenesis (Breslow *et al.* 1996).

The purpose of the present study was to investigate the effect of dietary CLA on early atherogenesis in C57BL/6 mice.

### Methods

#### *Experimental animals*

Sixty, 6-week-old, female C57BL/6 mice were obtained from the Animal Breeding Station, Department of Laboratory

**Abbreviations:** CLA, conjugated linoleic acids.

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Animal Sciences, University of Otago, New Zealand. Mice were housed in groups of five in solid-floored cages and kept in a room maintained at  $22 \pm 1^\circ$ , humidity  $60 \pm 5\%$ , air exchange 12 times/h and on a 12 h light–dark cycle. The mice were acclimatized for 2 weeks, during which time they were fed on a commercial mouse diet. At the start of the trial the mean mouse weight was 20.6 g.

#### Experimental design

Twenty mice were randomly allocated into each of the three dietary groups. Two treatment groups received atherogenic diets containing either 5 or 2.5 g CLA/kg. To ensure all diets contained equal amounts of free fatty acids, the diet containing 2.5 g CLA/kg also contained 2.5 g linoleic acid/kg. Mice in the control group were fed on a diet containing 5 g linoleic acid/kg.

The mice were fed *ad libitum* for 15 weeks. They were then fasted for between 8 and 12 h, and killed by CO<sub>2</sub> inhalation.

All procedures involving animals in this study were conducted under guidelines established by the Massey University Animal Ethics Committee and with their prior approval.

#### Experimental diets

The atherogenic diets used were similar to those reported by Nishina *et al.* (1990) and are illustrated in Table 1. All diets contained (g/kg): triacylglycerol 145, free fatty acids 5, cholesterol 10 and cholic acid 5. The vitamin and mineral mixes were formulated according to guidelines established by the US National Research Council (1995). Solutions containing either > 95% CLA or > 99% linoleic acid were purchased from Nu-Chek-Prep Inc., Elysian, MN, USA. All other chemicals were purchased from either BDH Chemicals New Zealand Ltd, Palmerston North, New Zealand or Sigma Chemical Co., St Louis, MO, USA.

After the dry ingredients were mixed, water was added to form a dough which was then cut into strips and dried for 3 d at  $29^\circ$ . Once dry, the diets were stored in a freezer at  $-20^\circ$ . One week's supply of diet was removed from the freezer

and kept refrigerated at  $4^\circ$  until fed. Mice were given fresh diet from the refrigerator each day.

#### Sample collection and processing

Immediately after killing, between 0.5 and 1.0 ml blood was collected from each mouse by cardiac puncture. The blood was transferred to a test tube and centrifuged at 1500 g for 15 min to separate serum. Serum cholesterol and triacylglycerol concentrations were determined using a Hitachi 704 autoanalyser (Boehringer Mannheim GmbH, Mannheim, Germany). Serum HDL-cholesterol concentrations were determined after selective precipitation of apolipoprotein B-containing lipoproteins using PEG 6000 (Izzo *et al.* 1981).

The liver from one mouse in each cage was fixed in neutral buffered formalin, embedded in paraffin, sectioned, and then stained with haematoxylin and eosin for routine histological examination.

#### Morphological evaluation of fatty streaks

The method used to quantify the aortic fatty streak lesions was based on that of Paigen *et al.* (1987b) and has been described in detail in a previous publication (Munday *et al.* 1998). Briefly, after formalin fixation, the hearts were passed through ascending concentrations of gelatin, frozen, and then sectioned using a cryostat. Once the aortic sinus was reached, twenty-four consecutive 10 µm sections were taken and the cross-sectional area of fatty streak lesions in every second section was measured. All lesion assessment was performed blind by the same researcher.

#### Statistical analysis

Differences among dietary groups were investigated using ANOVA techniques. Relationships between serum lipids, body weight, and fatty streak formation were analysed using linear regression. All statistics were calculated using the Statistical Analysis Systems statistics package (SAS Institute Inc., Cary, NC, USA).

## Results

A summary of the measurements made at the completion of the trial is presented in Table 2. During the trial, four mice were removed from each dietary group. In all instances, removal was due to weight loss resulting from the failure of mice to adapt to the experimental diet. Although accurate measurement of food intake is not practicable using this model, no differences in food disappearance rates were noticed and no significant differences in body weight were observed among the treatment groups.

No significant differences in mean serum total cholesterol concentration, or in mean serum HDL-cholesterol concentration, were observed among the dietary groups. Compared with controls, feeding 5 g CLA/kg diet significantly ( $P = 0.008$ ) increased the serum HDL-cholesterol: total cholesterol ratio. Although it approached significance ( $P = 0.09$ ), the ratio observed in mice fed on 2.5 g CLA/kg diet was not significantly different from that of controls. When data from

**Table 1.** Composition of the experimental diets containing different levels of conjugated linoleic acids (CLA)

Diet ...	Control	2.5 g CLA/kg	5 g CLA/kg
Ingredients (g/kg as fed)			
Sucrose	480.64	480.64	480.64
Casein	200	200	200
Maize oil	45	45	45
Olive oil	50	50	50
Anhydrous milk fat	50	50	50
Cellulose	50	50	50
Salt mix*	50	50	50
Vitamin mix*	50	50	50
DL-Methionine	3	3	3
DL- $\alpha$ -Tocopherol	1.36	1.36	1.36
CLA	0	2.5	5
Linoleic acid	5	2.5	0
Cholesterol	10	10	10
Cholic acid	5	5	5

\* Formulated according to National Research Council (1995) guidelines.

**Table 2.** Lipid variables in C57BL/6 mice fed on atherogenic diets containing different levels of conjugated linoleic acids (CLA) for 15 weeks\*  
(Mean values and standard deviations for sixteen mice per dietary group)

Diet ...	Control		2.5 g CLA/kg		5 g CLA/kg	
	Mean	SD	Mean	SD	Mean	SD
Total aortic fatty streak area (mm <sup>2</sup> )	0.13 <sup>a</sup>	0.13	0.33 <sup>b</sup>	0.27	0.25 <sup>ab</sup>	0.22
Serum total cholesterol (mmol/l)	4.17 <sup>a</sup>	0.74	3.57 <sup>a</sup>	0.72	3.90 <sup>a</sup>	1.16
Serum HDL-cholesterol (mmol/l)	1.39 <sup>a</sup>	0.21	1.40 <sup>a</sup>	0.34	1.61 <sup>a</sup>	0.31
HDL-cholesterol : total cholesterol	0.34 <sup>a</sup>	0.08	0.40 <sup>ab</sup>	0.09	0.43 <sup>b</sup>	0.10
Serum triacylglycerol (mmol/l)	0.56 <sup>a</sup>	0.13	0.51 <sup>ab</sup>	0.08	0.47 <sup>b</sup>	0.10
Final body weight (g)	22.01 <sup>a</sup>	1.18	21.08 <sup>a</sup>	1.22	21.34 <sup>a</sup>	1.51

<sup>a,b</sup> Mean values within a row not sharing a common superscript letter were significantly different,  $P < 0.05$ .

\*For details of diets and procedures, see Table 1 and p. 252.

both groups of CLA-fed mice were combined, dietary CLA significantly increased the HDL-cholesterol : total cholesterol ratio ( $P = 0.01$ ). Serum triacylglycerol concentrations were significantly higher in controls than in mice receiving 5 g CLA/kg diet ( $P = 0.01$ ), but not 2.5 g/kg ( $P = 0.16$ ). When data from the two CLA-fed groups were combined, the inclusion of CLA into the atherogenic diet significantly lowered serum triacylglycerol concentration ( $P = 0.03$ ).

Lipid-containing lesions were present in the intima of the aortic sinus in all mice. These lesions were most common close to the origins of the coronary arteries and at the base of the aortic valves and consisted of sub-endothelial collections of macrophages containing numerous lipid globules. In some mice, these globules were also found extracellularly around areas of macrophage accumulation and were present in the underlying media. Mice receiving diets containing 2.5 g CLA/kg ( $P = 0.01$ ), but not 5 g CLA/kg ( $P = 0.12$ ), developed a significantly greater area of fatty streaks than controls. When data from both groups of mice receiving CLA were combined, the addition of CLA to the diet significantly increased aortic fatty streak development ( $P = 0.02$ ). In the present study, fatty streak area was not correlated with the serum concentration of total cholesterol ( $P = 0.5$ ), HDL-cholesterol ( $P = 0.4$ ), or triacylglycerol ( $P = 0.3$ ), or with either the HDL-cholesterol : total cholesterol ratio ( $P = 0.9$ ), or body weight ( $P = 0.8$ ).

All livers examined in this study were enlarged and pale tan in colour. Histological examination revealed diffuse hepatic lipidosis which did not differ in severity among groups on the basis of subjective assessment.

## Discussion

In C57BL/6 mice, dietary CLA significantly increased aortic fatty streak formation. This is in contrast to a study by Lee *et al.* (1994) in which no significant differences in fatty streak development were observed between rabbits supplemented daily with either 0.5 g CLA or 0.5 g coconut oil. The results of the present study are also in contrast to a study using hamsters in which an atherogenic diet containing CLA resulted in significantly less atherosclerosis than a control diet containing no free fatty acids. However, this result is difficult to interpret because control hamsters were also significantly heavier, raising the possibility that they may have consumed more of the atherogenic diet (Nicolosi *et al.*

1997). When CLA-fed hamsters were compared with hamsters fed on a control diet containing free linoleic acid, no differences in body weight or fatty streak formation were observed (Nicolosi *et al.* 1997).

In the present study, dietary CLA increased the serum HDL-cholesterol : total cholesterol ratio. As was observed in the study by Lee *et al.* (1994), this increase was produced by non-significant changes in both serum total cholesterol and HDL-cholesterol concentration. In hamsters, dietary CLA significantly lowered serum total cholesterol concentrations, however, HDL-cholesterol concentrations were unaltered by CLA in this model (Nicolosi *et al.* 1997). Hepatic lipid metabolism has been shown to be affected by CLA (Belury & Kempa-Steczko, 1997) which may explain how they produce changes to the serum lipoprotein profile.

In human epidemiological studies and in previous experiments using the C57BL/6 mouse model, atherosclerosis has been found to be inversely correlated with the serum HDL-cholesterol : total cholesterol ratio (Paigen *et al.* 1987a; Luria *et al.* 1991; Stampfer *et al.* 1991; Nishina *et al.* 1993). In the present trial, lesion development was not correlated with this ratio or with any other variable measured. Therefore, CLA probably increased fatty streak formation by a mechanism independent of the serum lipoprotein profile, triacylglycerols or body weight.

It is possible that CLA promote atherosclerosis by stimulation of the immune system. Interleukin-2 production (Wong *et al.* 1997), lymphocyte proliferation (Miller *et al.* 1994), and macrophage phagocytic activity (Cook *et al.* 1993) are all increased in cells cultured from animals supplemented with CLA. In C57BL/6 mice, foam cells are the predominant cell type within the fatty streaks (Stewart-Phillips & Lough, 1991). Foam cells are produced when macrophages become engorged with lipid (Aviram, 1996) suggesting that promotion of the immune system, especially macrophage phagocytosis, could be expected to increase fatty streak formation. CLA may also promote atherosclerosis by their ability to, depending on the concentration, both decrease and increase lipoprotein lipase (EC 3.1.1.34) activity (Park *et al.* 1997). Lipoprotein lipase has been suggested to be an important factor in determining early atherosclerosis (Williams & Tabas, 1995) so that alteration of enzyme activity may influence atherosclerosis.

It is interesting to note that CLA did not promote atherosclerosis in either the cholesterol-fed rabbit or hamster

atherogenesis models (Lee *et al.* 1994; Nicolosi *et al.* 1997). This suggests that the mechanism by which CLA increase fatty streak development in C57BL/6 mice may be unique to this model. The most important question raised from this observation is whether or not CLA promote atherogenesis in man.

The addition of CLA to the diet resulted in a significant decrease in serum triacylglycerol concentration. This is in contrast to previous animal studies where no reduction in triacylglycerol concentrations were observed in rabbits supplemented with CLA (Lee *et al.* 1994) or when CLA-fed hamsters were compared with hamsters fed on linoleic acid (Nicolosi *et al.* 1997).

When the two groups of mice fed on CLA are considered separately, only the group receiving 5 g CLA/kg diet developed an HDL-cholesterol : total cholesterol ratio and a serum triacylglycerol concentration significantly different from that in controls. The addition of 2.5 g CLA/kg to the atherogenic diet resulted in changes which approached, but did not attain statistical significance, suggesting that dietary CLA may have a dose-dependent effect on both these lipid variables. However, fatty streak development was significantly greater than in controls only in mice receiving diets containing 2.5 g CLA/kg. It is possible therefore, that dietary CLA at a level of both 5 and 2.5 g/kg promoted atherogenesis, but that lesion formation was ameliorated in mice receiving 5 g CLA/kg by the development of a less atherogenic serum lipoprotein profile.

No significant differences among the three dietary groups were observed in the mean mouse body weight. During preliminary studies, it was observed that diets containing either CLA or linoleic acid appeared to be less palatable than diets containing triacylglycerol. Free fatty acids have a 'soapy' taste and differences in feed intake (Park *et al.* 1997) and weight gain (Belury & Kempa-Steczko, 1997; Nicolosi *et al.* 1997) were observed when animals fed on diets containing CLA were compared with those fed on diets containing triacylglycerol. Therefore, when using animals to investigate the physiological effects of CLA, it is essential that all the experimental diets contain equal quantities of free fatty acid. If this is not done, it becomes impossible to differentiate between the effects of CLA and the effects of differences in food intake.

In conclusion, the addition of CLA to an atherogenic diet fed to C57BL/6 mice resulted in the development of a serum lipoprotein profile considered to be less atherogenic. If human supplementation with CLA produces a less atherogenic lipoprotein profile without promoting atherogenesis, then CLA may be useful in preventing atherosclerotic disease. However, in the present study, dietary CLA increased the development of aortic fatty streaks and the possibility that dietary CLA also promote atherogenesis in human subjects requires further investigation. Considering the increased atherogenesis observed in this trial and the lack of significant atherosclerosis reduction seen in previous animal studies (Lee *et al.* 1994; Nicolosi *et al.* 1997), CLA cannot currently be regarded as antiatherogenic.

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