α-Linolenic acid but not conjugated linolenic acid is hypocholesterolaemic in hamsters

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Conjugated linolenic acid (CLN) refers to a group of octadecatrienoic acid isomers that have three double bonds in conjugation. Both pomegranate and tung seed oils are rich in CLN but the major isomer in the former is cis9,trans11,cis13 while in the latter it is cis9,trans11,trans13. The present study examined the effects of CLN, isolated from either pomegranate seed oil or tung seed oil, and α-linolenic acid (LN), isolated from flaxseed oil, on serum cholesterol levels in male hamsters (body weight 105 g; age 10 weeks) fed a 0.1 % cholesterol and 10 % lard diet, for a period of 6 weeks. All hamsters were allowed free access to food and fluid. The blood samples were taken by bleeding from the retro-orbital sinus into a heparinized capillary tube under light ether anaesthesia after overnight fasting at weeks 2, 4 and 6. It was found that supplementation of CLN at levels of 12.2–12.7 g/kg diet exhibited no significant effect on serum cholesterol level while LN at a similar level of supplementation had serum cholesterol reduced by 17–21 % compared with the control diet containing no LN and CLN. Supplementation of CLN and LN significantly decreased hepatic cholesterol but no effect was observed on heart and kidney cholesterol levels. It was concluded that LN possessed hypocholesterolaemic activity while CLN had no effect on blood cholesterol, at least in hamsters.

Cholesterol: Conjugated linolenic acids: Octadecatrienoic acid: Pomegranate seed oil: Tung seed oil

Conjugated linolenic acid (CLN) is a generic term used to describe a group of positional and geometric isomers of octadecatrienoic acids that contains three double bonds in conjugation. Dietary intake of CLN by humans is currently unknown. Dietary CLN is quantitatively minor in the vegetable oils, accounting for up to 0.2 % by weight (Yurawecz et al. 1993). The amount of CLN could be increased in partially hydrogenated soyabean oil up to 0.2 % by weight (Yurawecz et al. 1993). The amount of CLN is significantly higher than those fed an α-linolenic acid (LN)–enriched linseed oil diet. Similarly, Koba et al. (2002) found that supplementation of CLN in the free fatty acid form increased serum TG level compared with LN in rats. In recent years, hamsters have been more commonly used than rats as a model to study lipoprotein metabolism because, like humans, the major blood cholesterol carrier in hamsters is LDL, whereas in rats, most of its blood cholesterol occurs in HDL (Nistor et al. 1987; Lehmann et al. 1993). The present study was therefore carried out to examine further the effect of pure free CLN, isolated from either pomegranate seed oil or tung seed oil, on serum cholesterol levels, compared with that of LN, isolated from flaxseed oil, in hamsters.

Abbreviations: CLA, conjugated linoleic acid; CLN, conjugated linolenic acid; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; LN, α-linolenic acid; TC, total cholesterol; TG, triacylglycerols.

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Materials and methods

Isolation of conjugated linolenic acid from pomegranate and tung seed oils

Pomegranate seeds and tung seed oil were purchased from a local store at Xinxiang City, Henan, China. Pomegranate seeds were crushed into fine particles in a grinder and the oils were extracted using n-hexane followed by the removal of hexane in a rotary evaporator. Both pomegranate seed oil and tung oil were saponified in 1 M-KOH solution at 95°C under nitrogen. The saponified substances were saved and acidified to pH 1-0 using 1 M-H2SO4. The top layer containing free fatty acids was collected for further purification. CLN present in the total free fatty acids after saponification was purified by crystallization. The total free fatty acid fraction was warmed at 50°C and dissolved into methanol in a ratio of 1:3 (v/v) in a flask. After flushing with nitrogen, the flask was placed into a 0–4°C refrigerator for 8 h. The yellowish needle-shaped crystals containing mainly CLN were filtered and saved. The process was repeated twice until the CLN mixture reached 70% purity.

Isolation of α-linolenic acid from flaxseed

Flaxseeds were crushed in a coffee grinder and the oils were extracted using a mixture of chloroform and methanol (2:1, v/v). The solvents were removed in a rotary evaporator. The flaxseed oil (130 g) was then saponified in 2 litres of methanol containing 0.3 M-KOH at 90°C under a gentle stream of N2 for 2 h. After the removal of methanol in a rotary evaporator, the mixture was acidified to pH 1-0 using 1 M-H2SO4. The top layer containing free fatty acids was washed three times with the same volume of distilled water. Isolation of LN was carried out using two-step crystallization. The free fatty acid mixture was dissolved into three volumes of methanol. The crystallization was carried out at 4°C to remove the saturated fatty acids and then at −18°C to obtain LN. The process was repeated twice until the LN fraction reached 80% purity.

Fatty acid analysis

LN and CLN were converted to the corresponding fatty acid methyl esters according to Igarashi et al. (2004). In brief, a 5 mg sample was dissolved in 1 ml toluene followed by adding 2 ml 14% boron trifluoride in methanol (Sigma Chemical Co., St Louis, MO, USA). The mixture was flushed with a gentle stream of gas and maintained at room temperature for 30 min, followed by adding 4 ml hexane and 1 ml distilled water. The hexane layer containing fatty acid methyl esters was analysed on a flexible silica capillary column (Innowax 19091N-213, 30 m × 0.32 mm internal diameter; J&W Scientific, Folsom, CA, USA) in a HP 5980 Series II gas–liquid chromatograph equipped with a flame-ionization detector (Hewlett-Packard, Palo Alto, CA, USA). Column temperature was programmed from 180 to 230°C at a rate of 2°C/min and then held for 5 min. Injector and detector temperatures were set at 250 and 300°C, respectively. He gas was used as the carrier at a head pressure of 103 kPa. Identification of each fatty acid methyl ester was made by comparison of retention time of authentic standards (Sigma Chemical Co.). It was found that intra-isomerization of CLN species was minimal (<1%) under the present experimental conditions (Igarashi et al. 2004).

Table 1. Composition of the control diet and the experimental diets supplemented with conjugated linolenic acids (CLN) obtained either from pomegranate seed oil (CLN-P) or tung oil (CLN-T) or with α-linolenic acid purified from flaxseed oil (LN-F)

<table>
<thead>
<tr>
<th>Component (g/kg)</th>
<th>Control</th>
<th>CLN-P</th>
<th>CLN-T</th>
<th>LN-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornstarch</td>
<td>488</td>
<td>478</td>
<td>478</td>
<td>478</td>
</tr>
<tr>
<td>Casein</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Sucrose</td>
<td>150</td>
<td>140</td>
<td>140</td>
<td>140</td>
</tr>
<tr>
<td>Mineral mix (AIN-76)</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Vitamin mix (AIN-76A)</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>α-l-Methionine</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Lard</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>CLN-P</td>
<td>0</td>
<td>20</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CLN-T</td>
<td>0</td>
<td>–</td>
<td>20</td>
<td>–</td>
</tr>
<tr>
<td>LN-F</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>20</td>
</tr>
</tbody>
</table>

Diets

The formula previously described by Zhang et al. (2002) was modified to prepare four diets for hamsters. The control diet was prepared by mixing all powdered ingredients and lard listed in Table 1. The CLN-P diet was prepared by mixing 20 g/kg CLN, isolated from pomegranate seed oil, with the other ingredients. Similarly, the CLN-T diet was prepared except for adding 20 g/kg CLN isolated from tung seed oil while the LN-F diet was prepared by using 20 g/kg LN isolated from flaxseed oil. All four powdered diets were then mixed with a gelatin solution (20 g/l) in a ratio of 200 g diet per litre of solution. Once the gelatin had set, the diets were cut into approximately 20 g cubic portions and stored frozen (−20°C).

Animals

Forty-eight male Golden Syrian hamsters (Mesocricetus auratus, 105 ± 5 g, 10 weeks; Laboratory Animal Service Center, The Chinese University of Hong Kong) were divided into four groups (n = 12) fed one of the four diets. All hamsters were housed (two per cage) in an animal room at 23°C with 12/12 h light–dark cycles. The fresh diets were given daily, and uneaten food was discarded. Food intake was measured daily and body weight was recorded twice a week. The hamsters were given free access to food and fluid. The protocol was reviewed and approved by the Committee of Animal Ethics, The Chinese University of Hong Kong. All the hamsters were bled from the retro-orbital sinus into a heparinized capillary tube under light ether anaesthesia after overnight fasting at weeks 0, 2, 4 and 6 (Chan et al. 1999). After clotting, the blood was centrifuged at 1500g for 10 min and serum was collected. At the end of week 6, all the hamsters were killed; liver, heart, kidney and adipose tissues (epididymal and prerenal pads) were removed, washed in saline, weighed and frozen in liquid N2. All samples were stored frozen at −80°C prior to cholesterol analysis.

Serum lipids

Serum TC and TG levels were determined enzymatically by using commercial kits (Sigma Chemical Co.). High-density lipoprotein cholesterol (HDL-C) was measured after precipitation of LDL and VLDL with phosphotungstic acid and magnesium chloride.
(Sigma Chemical Co.). Non-high-density lipoprotein cholesterol (non-HDL-C) was calculated by deducting HDL-C from the TC.

**Determination of cholesterol in liver, heart and adipose tissues**

Total lipids were extracted from 300 mg of tissue sample with the addition of 1 mg stigmastanol as an internal standard, using 15 ml chloroform–methanol (2:1, v/v). The lipid extracts were then saponified with 6 ml 1 M NaOH in 90 % ethanol at 90°C for 1 h, and the non-saponified substances including cholesterol were then converted to their trimethylsilyl-ether derivatives by a commercial trimethylsilyl reagent (Sigma Chemical Co.). Analysis of the cholesterol trimethylsilyl-ether derivative was performed in a fused silica capillary column (SAC™-5, 30 m x 0.25 mm internal diameter; Supelco Inc., Bellefonte, PA, USA) in a Shimadzu GC-14B GLC equipped with a flame-ionization detector (Shimadzu, Tokyo, Japan). The column temperature was set at 285°C and maintained for 30 min. He gas was used as carrier at a head pressure of 150 kPa. Cholesterol in the tissue sample was calculated according to the amount of internal standard stigmastanol added (Chan et al. 1999).

**Statistics**

Data are expressed as means with their standard deviations. Where applicable, ANOVA was used to evaluate statistically significant differences among the control, CLN-P, CLN-T and LN-F groups using Sigmastat (Jandel Scientific Software, San Rafael, CA, USA). Subsequently, Student’s t test was used to compare the difference between any two groups. Differences were considered significant at $P < 0.05$.

**Results**

**Fatty acid composition of dietary fat**

The fatty acid content was expressed as g/kg diet (Table 2). CLN-P and CLN-T had 12.2–12.7 g CLN/kg diet in contrast to the LN-F diet which had no CLN but had 13.3 g LN/kg diet. The other fatty acids among the two CLN experimental diets were similar except that the major CLN isomer in the CLN-P diet was cis9,trans11,cis13 while CLN-T contained mainly cis9,trans11,trans13.

**Body weight and food intake**

The changes in the body weight and food intake of the hamsters are demonstrated in Table 3. No significant differences in body weight gain were observed although the CLN-P group had a smaller average body weight compared to the other three groups. Similarly, there were no significant differences in food intakes among the four groups but it appeared that the CLN-P group had a food intake slightly lower than the other three groups. The organ weights were similar among the four groups except that the LN-F group had smaller liver and heart than the control group.

**Serum TC, HDL-C, TG and non-HDL-C/HDL-C**

Four groups had similar levels of serum TC, HDL-C and TG at the beginning of the experiment (Table 4). At the end of week 2, the serum TC level of the LN-F group started to be significantly lower compared with those of the other three groups. This was mainly caused by lowering of the non-HDL-C level in the LN-F group, thus leading to a lower ratio of non-HDL-C to HDL-C (Table 4). No significant differences in serum TC, HDL-C, non-HDL-C and TG were observed among the control, CLN-P and CLN-T groups. The serum TG level in hamsters fed the LN-F diet was the lowest during the 6-week feeding. In general, supplementation of CLN either from pomegranate seed oil or tung seed oil did not cause any significant change in serum TC level compared with the control, but addition of LN to the diet led to reduced TC and non-HDL-C levels.

**Liver, heart and kidney cholesterol**

Supplementation with CLN and LN significantly decreased the hepatic cholesterol level but not heart and kidney cholesterol levels (Table 5). However, no significant differences in hepatic cholesterol among the three experiment groups could be observed. The LN-F group had an adipose tissue cholesterol level significantly lower than the other three groups (Table 5). No difference in adipose tissue cholesterol levels was seen among the control, CLN-P and CLN-T groups.

**Discussion**

The present study examined the effect of CLN supplementation on the serum lipid profile compared with that of LN in hamsters. The results clearly demonstrated that LN possessed a favourable effect but CLN had no effect on serum lipids. The observation is in agreement with that reported by Dhar et al. (1999), who found that supplementation of 0.5, 2 and 10% CLN derived from karela oil in the diet had no effect on blood TC, HDL-C and non-HDL-C levels, compared with the linoleic acid-enriched sunflower oil diet in rats. Instead, addition of CLN-enriched karela oil in the diet led to increased levels of serum TC, TG, VLDL-C, LDL-C and non-HDL-C in rats if compared with an LN-enriched linseed oil diet (Dhar & Bhattacharyya, 1998). In both tung seed oil and karela seed oil, cis9,trans11,trans13-18:3 is the major isomer. In the present study, it accounted for 10 g/kg diet for hamsters whereas in the study by Dhar et al.
it ranged from 5 to 100 g/kg diet for rats. In this regard, it is clear that no favourable effect on the blood lipoprotein profile is associated with supplementation of CLN in both rats and hamsters. Pomegranate and tung seed oils have different CLN isomer profiles. The former has \(\text{cis}9,\text{trans}11,\text{cis}13\)-18 : 3 dominant whereas in the latter \(\text{cis}9,\text{trans}11,\text{trans}13\)-18 : 3 is predominant. The present study found that both CLN-P and CLN-T groups had similar serum and hepatic cholesterol levels, indicating these two isomers did not have different effects on cholesterol metabolism. Together with the previous studies of Dhar & Bhattacharyya (1998) and Dhar et al. (1999), it can be concluded that CLN is not hypocholesterolaemic, regardless of whether it is present in the form of either triacylglycerol or free fatty acid.

The number of conjugated double bonds may affect significantly the cholesterol-lowering properties of a conjugated fatty acid. This view is best illustrated when the effect of CLN on blood cholesterol was compared with that of conjugated linoleic acids (CLA), a group of conjugated octadecadienoic acid isomers. The former has three double bonds while the latter contains two double bonds in conjugation. CLA was found to possess antiatherosclerotic activity when rabbits and hamsters were fed a high cholesterol diet (Lee et al. 1994; Nicolosi et al. 1997). Like its unconjugated isomer linoleic acid, CLA has also been demonstrated to reduce serum cholesterol levels in hamsters fed a high cholesterol diet (Nicolosi et al. 1997; Yeung et al. 2000). However, this was not true for CLN and LN; the former had no

### Table 3. Body weight, organ weight and food intake in hamsters fed the control diet and the experimental diets supplemented with conjugated linolenic acids (CLN) obtained either from pomegranate seed oil (CLN-P) or tung oil (CLN-T) or with \(\alpha\)-linolenic acid purified from flaxseed oil (LN-F)

(Mean values with their standard deviations)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CLN-P</th>
<th>CLN-T</th>
<th>LN-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body wt (g)</td>
<td>105·5  5·5</td>
<td>106·0 9·7</td>
<td>104·0 5·7</td>
<td>104·2 6·0</td>
</tr>
<tr>
<td>Final body wt (g)</td>
<td>135·4  5·0</td>
<td>128·9 10·2</td>
<td>132·7 8·1</td>
<td>130·7 10·3</td>
</tr>
<tr>
<td>Food intake (g/d)</td>
<td>9·5  0·1</td>
<td>9·0  0·1</td>
<td>10·4 0·6</td>
<td>9·7  0·5</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>5·5(\text{a}^{7}) 0·3</td>
<td>5·2(\text{a}^{7}) 0·8</td>
<td>5·1(\text{a}^{7}) 0·5</td>
<td>5·0(\text{a}^{7}) 0·6</td>
</tr>
<tr>
<td>Heart (g)</td>
<td>0·5(\text{a}^{7}) 0·1</td>
<td>0·5(\text{a}^{7}) 0·1</td>
<td>0·5(\text{a}^{7}) 0·3</td>
<td>0·4(\text{a}^{7}) 0·1</td>
</tr>
<tr>
<td>Kidney (g)</td>
<td>1·1  0·1</td>
<td>1·1  0·1</td>
<td>1·2 0·2</td>
<td>1·1  0·1</td>
</tr>
<tr>
<td>Brain (g)</td>
<td>0·9  0·1</td>
<td>0·9  0·1</td>
<td>0·9  0·1</td>
<td>0·9  0·1</td>
</tr>
</tbody>
</table>

\(*\) Mean values in a row with different letters differ significantly at \(P<0\cdot 05\).
effect while the latter was hypolipidaemic, as shown in the present study. There is no study to date that has investigated why conjugated octadecadienoic acids and conjugated octadecatrienoic acids affect cholesterol metabolism differently.

The mechanism by which LN but not CLN is hypcholesterolaemic remains poorly understood. Longer chain n-3 fatty acids do not usually lower serum cholesterol (Harris, 1997; Minihane et al. 2000; Theobald et al. 2004) but LN has been reported to do so (Bjerke et al. 1989; Chan et al. 1991). The present study confirmed the hypcholesterolaemic activity of LN (Table 4). The cholesterol-lowering effect of LN is most likely mediated by suppression on cholesterologenesis in inhibiting both enzymatic activity and mRNA expression of hepatic 3-hydroxy-3-methylglutaryl CoA reductase (Ihara-Watanabe et al. 1998). Although feeding n-3 fatty acids derived from fish oil was associated with an increase in LDL receptor activity (Ventura et al. 1989; Spady, 1993), incorporation of LN in the diet appeared to have no such suppressive effect on mRNA of the LDL receptor (Spady, 1993; Fukushima et al. 2001; Morise et al. 2004). It will be interesting if future studies can investigate the mechanism of why non-conjugated octadecatrienoic acid is hypolipidaemic but conjugated octadecatrienoic acid has no such activity by examining specifically any difference in the effect of dietary CLN and LN on the LDL receptor and 3-hydroxy-3-methylglutaryl CoA reductase.

CLN did not affect the serum cholesterol level but it decreased the hepatic cholesterol level (Table 5). In contrast, LN decreased not only serum TC but also the hepatic cholesterol level (Table 5). The effect of LN was similar to that of CLA, which had been shown to decrease cholesterol levels in both serum and liver (Yeung et al. 2000). CLA also increased the cholesterol level in adipose tissue (Yeung et al. 2000) while no change in the cholesterol level of adipose tissue was observed in the present study when CLN was added to the diet (Table 4). More interesting was that LN reduced the cholesterol level in adipose tissue (Table 4), suggesting that interactions of dietary CLN, LN and CLA with cholesterol metabolism are different in many ways and deserve further investigation.

Intake of dietary CLN is mainly from consumption of the processed vegetable oils. CLN is formed during the processing of vegetable oils as result of the dehydration of secondary oxidation products of linoleic acid (Yurawecz et al. 1993). When twenty-seven vegetable oils were analysed, it was found that the level of CLN ranged from not being detected (<0.001 %) to 0.2 % by weight (Yurawecz et al. 1993). It was also reported that CLN could be produced by isomerization of its non-conjugated isomer, LN, when LN-containing vegetable oils were partially hydrogenated in the production of shortenings and margarines (Mossoba et al. 1991). To date, there is no report that has estimated the current intake of CLN by humans and it is also not certain if CLN has health benefits similar to those of CLA and LN.

Acknowledgement

We thank the Hong Kong Research Grant Council for supporting this research.

References


Table 5. Cholesterol content (mg/g) of the liver, heart and kidney in hamsters fed the experimental diets supplemented with conjugated linolenic acids (CLN) obtained either from pomegranate seed oil (CLN-P) or tung oil (CLN-T) or with α-linolenic acid purified from flaxseed oil (LN-F) (Mean values and their standard deviations)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CLN-P</th>
<th>CLN-T</th>
<th>LN-F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Liver</td>
<td>50.2a</td>
<td>3.2</td>
<td>30.4b</td>
<td>4.6</td>
</tr>
<tr>
<td>Heart</td>
<td>2.8</td>
<td>1.0</td>
<td>3.8</td>
<td>0.4</td>
</tr>
<tr>
<td>Kidney</td>
<td>3.7</td>
<td>0.3</td>
<td>4.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Adipose tissue</td>
<td>0.9a</td>
<td>0.1</td>
<td>0.9a</td>
<td>0.1</td>
</tr>
</tbody>
</table>

a,b Mean values in a row with different letters differ significantly at P < 0.05.


