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ORIGINAL CONTRIBUTION

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Effects of *trans*-10,*cis*-12 conjugated linoleic acid on cholesterol metabolism in hypercholesterolaemic hamsters

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Abbreviations: CLA: conjugated linoleic acid, HMGCoAR: 3-hydroxy-3-methylglutaril coenzyme A reductase, ACAT: Acyl coenzyme A:cholesterol acyltransferase, CEH: cholesteryl ester hydrolase

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Abstract *Background* Conjugated linoleic acid (CLA) has received great attention in recent years because of its pleiotropic biological activities, but considerably fewer studies have been published addressing its role in serum lipids and atherosclerosis compared to other topics covered. *Aims of the study* The aim of the present study was to assess the effects of the *trans*-10,*cis*-12 isomer of CLA on cholesterolaemia and on several metabolic pathways involved in cholesterol metabolism in hamsters. *Methods* Animals were fed atherogenic diets supplemented with 0.5% linoleic acid, 0.5% *trans*-10,*cis*-12 CLA or 1.0% *trans*-10,*cis*-12 CLA, for 6 weeks. Serum lipoproteins were separated by FPLC. Cholesterol in serum and liver, as well as triacylglycerols and phospholipids in liver were assessed by spectrophotometry. 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR), acyl-coenzyme A:cholesterol acyltransferase (ACAT) and cholesteryl ester hydrolase (CEH) activities were measured by radiometry, and LDL receptors were determined by Western blot. *Results* *trans*-10,*cis*-12 CLA feeding did not modify food intake nor final body weight. Although serum total cholesterol remained unchanged, when cholesterol fractions were analyzed a significant decrease in VLDL-cholesterol was observed in CLA-fed animals, without changes in HDL-cholesterol or LDL-cholesterol. *trans*-10,*cis*-12 CLA decreased cholesterol ester content and increased free cholesterol in liver. The activity of HMGCoAR was not modified. In contrast, ACAT activity was reduced by both CLA doses and CEH was increased by the high CLA dose. LDL receptors were significantly reduced by *trans*-10,*cis*-12 feeding when expressed as arbitrary units per mg of protein, however, the total receptor mass remained unchanged. *Conclusions* These results suggest that, under the present experimental conditions, *trans*-10,*cis*-12 CLA feeding reduces cholesterol esterification in liver and decreases the minority serum VLDL-cholesterol fraction, but it does not show a hypocholesterolaemic effect. A dose–response effect was not observed.

Key words CLA – cholesterol – hamster – liver

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Introduction

Conjugated linoleic acid (CLA) has received great attention in recent years because of its pleiotropic biological activities [2, 17, 27], but considerably fewer studies have been published addressing its role in serum lipids and atherosclerosis compared to other topics covered, such as obesity or cancer.

The effects of CLA on serum cholesterol in animals and humans are quite controversial and, at the moment, a general consensus has not been achieved [3, 5, 8, 11–13, 20, 21, 23, 31, 32, 37–39, 41]. Moreover, only a few studies have addressed the effects of CLA on different pathways of cholesterol metabolism [37–39].

Although different animal species have been used for this purpose (hamster, mouse, rat, and rabbit), the hamster has been considered as a very suitable model for cholesterol and lipoprotein metabolism studies because it shares some common metabolic features with humans: (a) the rate of hepatic cholesterol synthesis is relatively low as compared with that of the whole animal [10, 34], (b) substantial amounts of cholesterol are transported in the low density lipoproteins when the diet contains cholesterol [24], (c) bile acid synthesis is not activated by dietary cholesterol [10], (d) receptor-dependent and independent uptake of LDL exist [34], and (e) apoB 100 is produced exclusively in the liver [18].

The aim of the present study was to assess the effect of the *trans*-10,*cis*-12 CLA isomer on serum cholesterol in hamsters fed on an atherogenic diet, as well as on various aspects of cholesterol metabolism.

Materials and methods

Animals, diets, and experimental design

The experiment was carried out with thirty 9-week-old (105 ± 2 g) male Syrian Golden hamsters, purchased from Harlan Ibérica (Barcelona, Spain), in accordance with the Institution's guide for the care and use of laboratory animals. Hamsters were individually housed in polycarbonate metabolic cages (Techniplast Gazzada, Guguggiate, Italy), placed in an air-conditioned room (22 ± 2°C) with a 12 h light-dark cycle and fed on semi-purified atherogenic diets consisting of 200 g/kg casein (Sigma, St. Louis, MO, USA), 4 g/kg L-methionine (Sigma), 200 g/kg wheat starch (Vencasser, Bilbao, Spain), 404 g/kg sucrose (local market), 100 g/kg palm oil (Agra, Leioa, Spain), 30 g/kg cellulose (Vencasser), 1 g/kg cholesterol (Sigma), 4 g/kg choline·HCl (Sigma). The experimental diets were supplemented with 0.5% linoleic acid (control), 0.5% *trans*-10,*cis*-12 CLA or 1.0% *trans*-10,*cis*-12 CLA (Natural Lipids Ltd., Hovdebygda, Norway). Vitamin (11 g/kg) and mineral (40 g/kg) mixes were formulated according to AIN-93 guidelines [30] and supplied by ICN Pharmaceuticals (Costa Mesa, CA, USA). The diets were freshly prepared once a week, gassed with nitrogen and stored at 0–4°C to avoid rancidity. All animals had free access to food and water. The experiments were initiated after a 6-d adaptation period.

Sampling

At the end of the experimental period blood samples were collected under inhalation anesthesia (diethyl ether) by cardiac puncture, and serum was obtained by centrifugation (1,000*g* for 10 min at 4°C). The liver was excised, weighed, sliced and immediately frozen in liquid N_2. All samples were stored at −80°C until analysis.

Serum analysis

Lipoproteins were separated by a fast phase liquid chromatography (FPLC) system (ÄKTA FPLC, Amersham Biosciences, Uppsala, Sweden) using a Superose 6 10/300 GL column (Amersham Biosciences, Piscataway, NJ, USA) following the method described by Kieft et al. [9], with minor modifications. Elution buffer consisted of 150 mM NaCl and 50 mM Na_2HPO_4, pH 7.4, containing sodium azide (0.02%). Sera were filtered with 0.22 μm filters. After column equilibration, samples (200 μl) were loaded onto the column and eluted at a 0.3 ml/min flow. After discarding the first 2 ml, 60 fractions of 0.3 ml were collected sequentially. The absorbance of the eluent was monitored continuously at an optical density of 280 nm. Three peaks of cholesterol were identifiable for each serum sample, corresponding to VLDL (17–22 fractions), LDL (34–40 fractions) and HDL (41–48 fractions).

Serum total cholesterol as well as cholesterol concentration in each eluted fraction were measured by spectrophotometry at 500 nm using a commercial kit (BioSystems, Barcelona, Spain).

Hepatic lipid analysis

Total lipids were extracted from liver following the method described by Folch et al. [7]. The lipid extract was dissolved in isopropanol. Total, free, and esterified cholesterol were determined as previously described [16]. Triacylglycerols and phospholipids were measured by spectrophotometry using commercial kits (BioSystems, Barcelona, Spain).

Microsome isolation and enzyme activity determinations

Samples of liver were homogenized in phosphate buffer (50 mmol/l KH_2PO_4, 0.1 mol/l sucrose, 50 mM NaCl, 10 mM EDTA and 0.5 mM dithiothreitol, pH 7.4), using a Potter-Elvenjhem homogenizer. The homogenate was centrifuged at 20,000*g* and 4°C for 20 min, and the supernatant was centrifuged 1 h at 100,000*g* at 4°C. The supernatant of this centrifugation was recovered as cytosolic fraction. Pellets were resuspended and centrifuged again 1 h at 100,000*g* at 4°C. Microsomal pellets were resuspended in phosphate buffer. Cytosolic and microsomal protein was determined according to the Bradford method [4].

3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR) activity was measured in microsomal fraction by a radiochemical assay based on the formation of 3-^{14}C mevalonate from 3-^{14}C HMG-CoA [29]. Enzyme activity was expressed as picomols of mevalonate produced per minute per milligram of microsomal protein.

Microsomal acyl-coenzyme A:cholesterol acyltransferase (ACAT) activity was measured in the absence of endogenous cholesterol using the method described by Smith and Lutton [33]. Enzyme activity was expressed as picomols of cholesteryl oleate formed per minute per milligram of microsomal protein.

The activity of cholesteryl ester hydrolase (CEH) was measured in cytosolic and microsomal fractions using the method described by Ochoa et al. [25]. Enzyme activities were expressed as picomols of oleate released per minute per milligram of protein.

Quantitation of LDL receptors

The protein level of LDL receptors was determined by Western blot from liver microsomal fractions as described by Milliat et al. [19]. A polyclonal antibody against LDL receptor (SC-11824, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used. Protein expression was measured by densitometric scanning (Bio-Rad GS-700 Imaging Densitometer) and analyzed with Multi-Analyst/PC 1.1 software (Bio-Rad). Receptor levels were expressed as arbitrary units (AU) per milligram of protein and as total mass (AU/liver). The value 100 was attributed to the control group mean.

Statistical analysis

Results are presented as means ± SEM. Statistical analysis was performed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). Data were analyzed by one-way ANOVA followed by Newman–Keuls post-hoc test. Significance was assessed at the $P < 0.05$ level.

Results

Food intake and body weight

No differences in food intake (6.0 ± 0.1 g/d in the control group, 5.7 ± 0.1 g/d in the 0.5% *trans*-10,*cis*-12 CLA group and 5.7 ± 0.1 g/d in the 1.0% *trans*-10,*cis*-12 CLA group) were observed among the three experimental groups. Although no significant differences in final body weight were found between hamsters fed the CLA diets and the controls (121 ± 3 g in the control group; 119 ± 1 g in the 0.5% *trans*-10,*cis*-12 CLA group and 124 ± 2 g in the 1.0% *trans*-10,*cis*-12 CLA group), those fed the 1% CLA diet showed increased body weight gain compared with the other two experimental groups (15 ± 2 g in the control group, 14 ± 1 g in the 0.5% *trans*-10,*cis*-12 CLA group and 19 ± 1 g in the 1% *trans*-10,*cis*-12 CLA group; $P < 0.05$). This increase was not great enough to induce significantly enhanced final body weight, but it led to lower food conversion factor (g food/g body weight gain) (17.8 ± 1.3 in the control group, 18.9 ± 1.9 in the 0.5% *trans*-10,*cis*-12 CLA group and 14.4 ± 1.1 g in the 1.0% *trans*-10,*cis*-12 CLA group; $P < 0.05$), indicating greater food efficiency. This effect was probably due to the increased skeletal muscle mass found in hamsters fed the high dose of CLA, as we previously reported [45].

Serum parameters

No significant changes in serum total cholesterol were induced by *trans*-10,*cis*-12 CLA feeding (6.04 ± 0.11 nmol/l in control group, 6.35 ± 0.13 nmol/l in 0.5% *trans*-10,*cis*-12 group and 6.52 ± 0.11 nmol/l in 1.0% *trans*-10,*cis*-12 group). When cholesterol fractions were analyzed a significant decrease in VLDL-cholesterol was observed in both CLA-fed groups, without changes in HDL-cholesterol or LDL-cholesterol ($P < 0.001$) (Fig. 1).

Liver weight and lipid content

Animals fed the *trans*-10,*cis*-12 CLA isomer showed significantly greater liver size than the controls ($P < 0.001$). No differences in liver phospholipid content were observed among experimental groups. In hamsters fed the low CLA dose both total cholesterol and triacylglycerols were significantly decreased ($P < 0.01$ and $P < 0.05$, respectively) and in those fed the high CLA dose only total cholesterol was significantly reduced ($P < 0.05$). Different effects on cho-

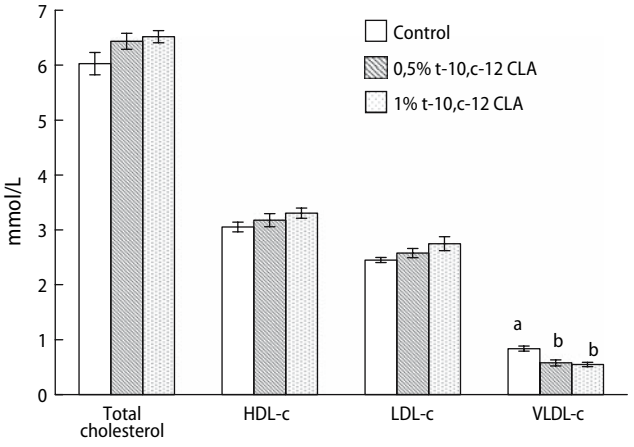

Fig. 1 Distribution of cholesterol in serum lipoprotein fractions measured by FPLC in Syrian Golden hamsters (n = 10/group) fed atherogenic diets (100 g/kg palm oil, 1 g/kg cholesterol and 404 g/kg sucrose) supplemented with 0.5% linoleic acid, 0.5% *trans*-10,*cis*-12 CLA or 1.0% *trans*-10,*cis*-12 CLA, for 6 weeks. Values are means with the standard errors of the means, shown by vertical bars. Values with different superscript letters were significantly different ($P < 0.05$)

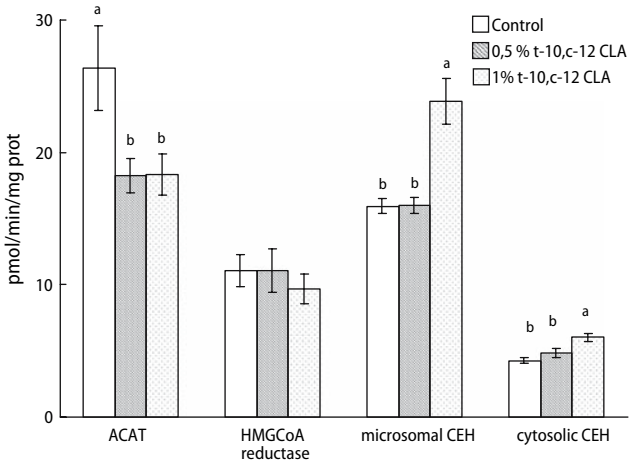

Fig. 2 Activity of cholesterol metabolism enzymes, measured by radiometric techniques, in liver from Syrian Golden hamsters (n = 10/group) fed atherogenic diets (100 g/kg palm oil, 1 g/kg cholesterol and 404 g/kg sucrose) supplemented with 0.5% linoleic acid, 0.5% *trans*-10,*cis*-12 CLA or 1.0% *trans*-10,*cis*-12 CLA, for 6 weeks. Values are means with the standard errors of the means, shown by vertical bars. Values with different superscript letters were significantly different ($P < 0.05$)

lesterol pools were observed: whereas free cholesterol was significantly increased ($P < 0.01$), esterified cholesterol was significantly decreased ($P < 0.01$), in both CLA-fed groups when compared with the controls. This led to a significant reduction in the percentage of cholesterol esterification ($P < 0.001$) (Table 1).

Enzyme activities

As shown in Fig. 2, the activity of HMGCoAR was not modified. In contrast, ACAT activity was reduced in both CLA-fed groups ($P < 0.05$). With regard to CEH, both microsomal and cytosolic activities were increased by the high *trans*-10,*cis*-12 CLA dose ($P < 0.001$). Consequently, the microsomal CEH/ACAT ratio was significantly increased (0.69 ± 0.09 in the control group, 0.88 ± 0.06 in the 0.5% *trans*-10,*cis*-12 CLA group and 1.46 ± 0.17 in the 1.0% *trans*-10,*cis*-12 CLA group; $P < 0.01$).

Table 1 Liver weight and lipid content in hamsters fed on the experimental diets

	Control	0.5% *t*-10, *c*-12 CLA	1% *t*-10, *c*-12 CLA
Weight (g)	6.01 ± 0.14[b]	7.26 ± 0.20[a]	7.68 ± 0.26[a]
(% Body weight)	4.95 ± 0.13[b]	6.12 ± 0.15[a]	6.17 ± 0.18[a]
Triacylglycerols (mg/g)	5.02 ± 0.28[a]	4.14 ± 0.20[b]	5.67 ± 0.28[a]
Phospholipids (mg/g)	12.20 ± 0.50	12.10 ± 0.70	13.4 ± 0.6
Cholesterol (mg/g)			
Total	7.39 ± 0.36[a]	5.89 ± 0.33 [b]	6.30 ± 0.22[b]
Free	1.43 ± 0.05[b]	1.77 ± 0.10[a]	1.84 ± 0.07[a]
Esterified	5.96 ± 0.34[a]	4.05 ± 0.24[b]	4.46 ± 0.16[b]
Cholesterol esterification (%)	81.20 ± 0.70[a]	70.20 ± 0.90[b]	70.7 ± 0.5[b]

Values are means ± SEM (n = 10)
[a,b,c] Values in the same row with different subscript were significantly different ($P < 0.001$)

LDL receptors

LDL receptors were significantly reduced by *trans*-10,*cis*-12 feeding when expressed as arbitrary units per mg of protein ($P < 0.05$). However, the total receptor mass (AU/liver) remained unchanged (Table 2).

Discussion

The effects of CLA on body fat reduction have been widely demonstrated in rodents [40]. In several studies, animals fed diets supplemented with CLA have also shown greater lean body mass, but the magnitude of the reduction in body fat, relative to the controls was considerably greater than the magnitude of the enhancement in whole body protein [6, 28, 35]. In the present study, *trans*-10,*cis*-12 CLA did not induce significant differences in food intake, nor in final

Table 2 LDL receptor protein expression by Western Blot

	Control	0.5 % *t*-10,*c*-12 CLA	1 % *t*-10,*c*-12 CLA
AU/mg prot	100 ± 9[a]	77 ± 8[b]	63 ± 4[b]
AU/liver	100 ± 9	95 ± 11	87 ± 18

Values are means ± SEM (n = 10)
[a,b] Values in the same row with different subscript were significantly different ($P < 0.05$)

body weight, but hamsters fed the high dose (1%) showed significantly increased food efficiency. This can be related to the fact that, as we previously reported, whereas both doses of *trans*-10,*cis*-12 CLA (0.5 and 1%) induced a body fat-lowering effect, only the high dose (1%) produced a lean body mass-enhancing effect [45]. Despite this change in food efficiency, whole lipid metabolism seems not to be affected by this fact because no significant differences in adipose tissue reduction were observed between both CLA-fed groups [45].

Other potential benefit of CLA on health refers to its effects on serum lipids. Thus, several studies have been performed, using hamsters, to determine the effects of CLA on serum cholesterol, but very few of them have analyzed the influence of CLA on cholesterol metabolism.

These results are in good accordance with some studies published by other authors when hamsters fed on atherogenic diets supplemented with either a CLA mixture or the *trans*-10,*cis*-12 isomer were compared with hamsters fed on the same atherogenic diets supplemented with linoleic acid [37, 41, 42]. Nevertheless, the results obtained by other authors are different from those reported in the present study. Thus, Bissonauth et al. [3] reported a significant increase in LDL-cholesterol induced by *trans*-10,*cis*-12 CLA, but not by *cis*-9,*trans*-11 CLA. Moreover, in some studies, in which lipoproteins were not separated, significant decreases in nonHDL-cholesterol were produced by a CLA mixture [8, 23]. Concerning HDL-cholesterol, although most of the studies did not find significant changes in this lipoprotein, Mitchell et al. [20] observed an increase induced by *trans*-10,*cis*-12 CLA. It should be pointed out that in some studies the *trans*-10,*cis*-12 isomer or CLA mixtures reduced nonHDL-cholesterol when comparisons were done between animals fed on atherogenic diets enriched with CLA and animals fed on atherogenic diets non-supplemented with linoleic acid [5, 41, 42].

The discrepancies are also related to the active isomer. Thus, whereas in the studies published by De Deckere et al. [5] and Bissonauth et al. [3] only the *trans*-10,*cis*-12 induced changes in serum lipids, Valeille et al. [38, 39] observed a significant increase in HDL-cholesterol and a reduction in the nonHDL to HDL-ratio induced by *cis*-9,*trans*-11 CLA and Wilson et al. [42] observed similar decreases in nonHDL-cholesterol and HDL-cholesterol in hamsters fed either *trans*-10,*cis*-12 CLA or the *cis*-9,*trans*-11 CLA. In these three studies, hamsters fed on atherogenic CLA-enriched diets were compared with hamsters fed on atherogenic diets non-supplemented with linoleic acid.

All together, these studies reveal the enormous variability concerning the active CLA isomer, the type of lipoprotein affected and the precise effect (increase or decrease) produced. In order to justify these discrepancies, it should be pointed out that important differences in the experimental design, in terms of basal lipoprotein profile, amount of CLA, length of the feeding period, selected control diet (supplemented or not with linoleic acid, non-purified or semi-purified), housing conditions, as well as hamster strain, are observed among the published studies. With regard to diet composition, considerable differences in several key aspects in the control of serum lipids, such as the amount and type of fat, the amount of cholesterol and the type of protein (mainly from vegetable sources in chow diets vs. casein, a more hypercholesterolaemic protein, in semi-purified diets) are found. This makes it very difficult to compare the results.

In the present study the effects of *trans*-10,*cis*-12 CLA on the activity of three hepatic enzymes involved in cholesterol metabolism were analyzed. The lack of changes in the activity of HMGCoAR demonstrated that, under our experimental conditions, this CLA isomer did not modify endogenous cholesterol synthesis. With regard to ACAT and CEH, two enzymes that regulate the balance between hepatic cholesterol pools, ACAT activity was reduced in both CLA-fed groups but CEH activities (cytosolic and mitochondrial) were only increased by the high *trans*-10,*cis*-12 CLA dose. The reduction in hepatic ACAT activity is in line with the decrease in intestinal ACAT activity observed by Thomas Yeung et al. [37] when hamsters were fed a CLA mixture.

These effects are in good accordance with changes observed in liver cholesterol contents. *trans*-10,*cis*-12 CLA significantly decreased total amount of liver cholesterol, but different effects were observed in cholesterol pools. Whereas free cholesterol was significantly increased, esterified cholesterol was significantly decreased in CLA-fed animals when compared with the controls. Similar effects on cholesterol esters were observed by Sher et al. [32] by feeding hamsters with a diet providing 0.3% cholesterol and 1% of a CLA mixture.

The reduction in cholesterol ester pool size in the liver of hamsters fed the *trans*-10,*cis*-12 CLA can be related to the reduction in serum VLDL-cholesterol observed in these animals. This metabolic feature has been shown to be associated with a significant decrease in apoB production and, therefore, in VLDL production and secretion [1, 22]. Reductions in VLDL production have been reported in HepG2 cells incubated with either a CLA mixture [14, 26] or the *trans*-10,*cis*-12 CLA isomer [36, 43], as well as in some in vivo studies [20, 35].

A relevant effect of *trans*-10,*cis*-12 CLA feeding, which is in line with other published studies [3, 5], was the increase in liver weight that, in contrast to that found in the mouse, was not related to fat

accumulation. In a previous study from our laboratory we demonstrated that this effect was due to an increase in the number of hepatocytes and thus, that hyperplasia is involved in hepatomegaly [15].

Although in the present study LDL-cholesterol was not modified by CLA feeding, taking into account that some authors have found significant increases in LDL-receptor mass in isolated hepatocytes [44], we were interested in the study of the effects of *trans*-10,*cis*-12 CLA on LDL-receptor under in vivo conditions. *trans*-10,*cis*-12 CLA significantly reduced the amount of LDL-receptors when expressed as arbitrary units per milligram of protein. However, due to the increased liver size induced by CLA feeding, the total LDL-receptor mass remained unchanged, leading to similar LDL clearance in both CLA-fed groups and in the control group. This result is in good accordance with the lack of effect of CLA-feeding on serum LDL concentration. The reduction in LDL receptor may be related to the significant increase in the free cholesterol pool size induced by *trans*-10,*cis*-12 CLA.

The discrepancy between our results and those found by Yu-Poth et al. [44] may be due to the fact that, under in vitro conditions, the influence of some important conditions such as hormonal environment cannot be taken into account. Moreover, the CLA mixture used in the experiment published by Yu-Poth et al. [44] also contains 8% of 20:4 *n*-6 and 1% of 22:6 *n*-3 and thus, a potential influence of these fatty acids cannot be discarded. In the present study these fatty acids are not present in the CLA oil used for diet supplementation.

The present results showed no dose–response effect of *trans*-10,*cis*-12 CLA in cholesterol metabolism because, with the exception of CEH activities, no differences between both CLA-fed groups were observed.

In summary, under the present experimental conditions, *trans*-10,*cis*-12 CLA isomer reduces cholesterol esterification in liver and decreases the minor serum VLDL-cholesterol fraction, but it does not elicit a hypocholesterolaemic effect. Further research is needed to better understand variations in the results among studies and to determine the conditions that provide the best potential for CLA effects.

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