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Refeeding with conjugated linoleic acid increases serum cholesterol and modifies the fatty acid profile after 48 hours of fasting in rats

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Abstract

There is no consensus about the effects of conjugated linoleic acid (CLA) on lipid metabolism, especially in animals fed a high-fat diet. Therefore, the objective of the present study was to evaluate the incorporation of CLA isomers into serum, liver and adipose tissue, as well as the oxidative stress generated in rats refed with high-fat diets after a 48 hour fast. Rats were refed with diets containing soybean oil, rich in linoleic acid [7% (Control Group - C) or 20% (LA Group)], CLA [CLA Group – 20% CLA mixture (39.32 mole% *c*9,*t*11-CLA and 40.59 mole% *t*10,*c*12-CLA)], soybean oil + CLA (LA+CLA Group - 15.4% soybean oil and 4.6% CLA) or animal fat (AF, 20% lard). The CLA group showed lower weight gain and liver weight after refeeding, as well as increased serum cholesterol. The high dietary fat intake induced fat accumulation and an increase in α-tocopherol in the liver, which were not observed in the CLA group. Circulating α-tocopherol was increased in the CLA and CLA+LA groups. The high-fat diets reduced liver catalase activity. CLA isomers were incorporated into serum and tissues. In this short-term refeeding experimental model, CLA prevented hepatic fat accumulation, although it produced an increase in serum cholesterol.

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Key words: *Conjugated linoleic acid. High-fat diet. Fasting. Refeeding. Fatty acids.*

REALIMENTACIÓN CON ÁCIDO LINOLEICO CONJUGADO AUMENTA COLESTEROL SÉRICO Y ALTERA EL PERFIL DE ÁCIDOS GRASOS DESPUÉS DE 48 HORAS DE AYUNO EN RATAS

Resumen

No hay consenso acerca de los efectos del ácido linoleico conjugado (CLA) sobre el metabolismo lipídico, especialmente en animales alimentados con una dieta alta en grasa. Por lo tanto, el objetivo del presente estudio fue evaluar la incorporación de isómeros de CLA en el suero, hígado y tejido adiposo, así como el estrés oxidativo generado en ratas realimentadas con dietas altas en grasa después de 48 horas de ayuno. Los animales fueron realimentados con dietas que contenían aceite de soja, rico en ácido linoleico [7% (Groupo Control - C)], o 20% (Groupo LA)], CLA [Groupo CLA - 20% de mezcla de CLA (39,32% moles del *c*9,*t*11-CLA y 40.59% moles del *t*10,*c*12-CLA)], aceite de soja + CLA (Grupo LA+-CLA - 15.4 % de aceite de soja y 4,6% de CLA) o grasa animal (Grupo AF, 20% de manteca de cerdo). El grupo CLA tuvo menor aumento de peso y menor peso hepático después de la realimentación, así como aumento del colesterol total em el suero. La dieta alta en grasa indujo la acumulación de grasa y un aumento de α-tocoferol en el hígado, que no se observaron en el grupo CLA. El α-tocoferol serico fue mayor en los grupos CLA y LA+CLA. Las dietas altas en grasa redujeron la actividad de la catalasa hepática. Isómeros de CLA fueron incorporados em el suero y tejidos. En este modelo de realimentación de corto prlazo, el CLA ha impedido la acumulación de grasa hepática, aunque genero un aumento del colesterol total sérico.

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Palabras clave: *Acido linoleico conjugado. Dieta rica en grasa. Ayuno. Realimentación. Ácidos grasos.*

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Abbreviations

CLA: conjugated linoleic acid
GSH: reduced glutathione.
HDL: high density lipoprotein cholesterol.
MUFAs: monounsaturated fatty acids.
PUFAs: polyunsaturated fatty acids.
SFAs: saturated fatty acids.
TAG: triacylglycerol.
TBARS: thiobarbituric acid-reactive species.
TNF-α: tumor necrosis factor-α.

Introduction

Conjugated linoleic acid (CLA) can be naturally found in ruminant meat and dairy products. Cis-9, trans-11 (*c*9, *t*11)-CLA is the most abundant isomer in these sources[1]. Several foods fortified with synthetic CLA might also display functional properties. These products contain equimolecular proportions of *c*9, *t*11-CLA and *t*10, *c*12-CLA isomers.

The use of CLA supplements have shown potential beneficial effects, such as the ability to inhibit carcinogenesis, to decrease the risk of mammary cancer by 50%[2], to reduce atherosclerosis[3] and diabetes risk[4], and to decrease body fat accumulation in humans and animals[5,6].

Treatment with a 50:50 mixture of *c*9, *t*11-CLA and *t*10, *c*12-CLA isomers is able to generate a small increase in fat-free mass in humans[7] and also body fat reduction in overweight or obese individuals consuming CLA[8]. CLA intake has promoted lower body weight gain and lower white fat pad weight in male rats fed a high-fat diet[9], and also a decrease in fat mass and an increase in lean mass in C57Bl/6J female mice[10].

Prolonged fasting causes metabolic and hormonal changes related to lipid metabolism, triggers lipolysis and the release of free fatty acids from adipose tissue into the bloodstream, supplying fatty acids for hepatic uptake[11]. This process is necessary for blood glucose maintenance. The expression of enzymes participating in fatty acid oxidation are increased under fasting conditions[12], whereas the activity of enzymes involved in lipogenesis are decreased[13].

Also, fasting is able to induce an increased expression of PPAR-α and β-oxidation genes activated by this transcription factor in healthy men[13]. This situation also results in rapid uptake and use of nutrients, in addition to lower glucose availability and the utilization of fat as the main energy substrate.

Refeeding with a carbohydrate-rich diet after a 48 hour fast causes hepatic steatosis in *Wistar* rats, with an increase in liver volume[14]. Likewise, rats refed with high-fat diet after a 24 hour fast had an increase in serum and hepatic triacylglycerols (TAG)[15], and after 48 hour of fasting also exhibited an increase in oxidative stress markers[16].

A supply of CLA can modify the energy metabolism of mice, increase the energy expenditure and reduce the body lipid reserves without changing diet consumption[17]. The offer of CLA to rodents after prolonged periods of fasting has not been previously described. Therefore, refeeding with a high-fat diet containing CLA represents a valuable model to study the metabolic effects of these isomers in a context of increased nutrient uptake and consumption.

Most studies have offered limited doses of CLA to animals, trying to reproduce human consumption. Thus, there is little information about the effect of high doses of CLA on isomer incorporation into the liver and adipose tissue or about the transport of these fatty acids in large quantities in the postprandial state.

Therefore, the objective of the present study was to analyze the changes in lipid metabolism by determining the incorporation of the main CLA isomers commercially available into serum, liver and adipose tissue, as well as their potential negative effects in rats refed with high-fat diets after 48 hours of fasting.

Materials and Methods

Chemicals

Standard and biochemical reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Chromatography grade chemicals were used for fatty acid (FA) quantification and chemical of ACS purity for other procedures. H_2O_2 was purchased from Merck & Co, Inc. (São Paulo, SP, Brazil).

Diets

All diets were based on the American Institute of Nutrition Ad Hoc Committee recommendation (AIN-93G)[18]. Five different diets were freshly prepared for this experiment: the control (C) diet contained 20% casein as the protein source and 7% soybean oil (20% energy) as the dietary fat source, 5% fiber, 3.5% mineral mix (Premix Mineral AIN-93 G, Rhoster Ltda, Araçoiaba da Serra, Brazil), 1% vitamin mix (Mix Vitamínico AIN-93, Rhoster Ltda, Araçoiaba da Serra, Brazil), 0.3% L-cystine, 0.25% choline, and 0.002% as di-tert-butyl methyl phenol. High-fat diets were prepared by replacing carbohydrate with fat (38.5% energy as fat) while keeping the other components. The linoleic acid (LA) diet contained 20% soybean oil, in which 59% of the FA were LA. The conjugated linoleic acid (CLA) diet contained 20% Tonalin® CLA (Illertissen, Germany), a dietary CLA supplement. The linoleic acid plus conjugated linoleic acid (LA+CLA) diet contained 15.4% soybean oil and 4.6% Tonalin® CLA. The animal fat (AF) diet was prepared using 20% lard.

Tonalin® CLA (Cognis Corporation, La Grange, Illinois, USA) is a dietary CLA supplement commercially

available for human consumption. It is composed of an equimolecular mixture of *c*9,*t*11-CLA (39.32 mole%) and *t*10,*c*12-CLA (40.59 mole%) as the main sources of isomeric fatty acids.

The quantity of CLA offered to the LA+CLA group (4.6%) was chosen in order to determine the use of these isomers as a food supplement, considering that doses offered to humans in clinical studies range from 1.3 to 5.8 g/day[7]. The offer of 20% Tonalin® to the CLA group provided about 16 g CLA per 100 g diet. The objective of the offer of this large quantity was to analyze whether a high dose of CLA would be harmful to rats starved for a long time.

The FA composition of lipid sources as methyl esters was determined by gas chromatography using the procedure indicated below. The soybean oil presented 59.03 moles% as 18:2n-6, 23.47 moles% as 18:1n-9, 13.42 as 16:0. The CLA offered 39.32 moles% as *c*9,*t*11-CLA, 40.59 moles% as *t*10,*c*12-CLA and 15.16 moles% as 18:1n-9. The lard used presented 42.33 moles% as 18:1n-9, 26.40 moles% as 16:0, and 16.27 moles% as 18:2n-6.

Animals

Newly weaned male *Wistar* rats weighing approximately 160 g were obtained from the animal facilities of the Faculty of Medicine of Ribeirão Preto – University of São Paulo. Before the beginning of the study, there was a 7 day acclimatization period, during which all rats were given free access to the standard diet and water. The animals were kept in individual cages on a 12 h light–dark cycle at 22ºC and fed standard rat chow. The study was approved by the Animal Research Committee of the Faculty of Medicine of Ribeirão Preto – University of São Paulo, SP, Brazil. The animals were randomly divided into five groups of 8 animals each. After a 48 h fast, rats were refed for 24 hours with the C, LA, CLA, LA+CLA or AF diets in order to investigate the effects of different FA during a short-term refeeding period.

Next, the animals were sacrificed under anesthesia (1+100 mg of azepromazine and ketamine/kg b.w., respectively) immediately after refeeding, and blood and tissues (liver, adipose tissue and gastrocnemius muscle) were collected. All tissues were weighed and immediately immersed in liquid nitrogen for future analysis.

Biochemical Analysis

Analysis of parameters related to lipid metabolism

Total fat content was quantified in liver and muscle by the method of Bligh and Dyer[19], using 500 mg liver and 1 g muscle. For analysis of the FA profile in liver, adipose tissue and serum, lipids were extracted by the same method[19] and methylated with potassium methoxide. We used 500 mg of liver, 250 mg of adipose tissue and 0.8 ml of serum. The FA determination as methyl esters was carried out by gas chromatography (Shimadzu 2014 chromatograph equipped with a flame ionization detector) using an SP-Sil 88 capillary column (Varian, Darmstadt, Germany) (100 m, 0.25 mm ID, film thickness 0.20 mm). The temperature of both the injector and the detector was 250°C. Nitrogen was used as carrier gas. Injections were performed in the split mode. Fatty acid methyl esters were identified according to retention time using commercial standards (AccuStandard, New Haven, USA and Sigma, St. Louis MO, USA). The chromatographic data were processd using the GC Solutions software.

Total serum cholesterol, high density lipoprotein cholesterol (HDL-C) and TAG levels were determined using commercial kits (Diagnostic Labtest ® SA, Brazil, TAG ref. n° 87-2/100, cholesterol ref. n° 76, and HDL-C ref. n° 13; and SB Laboratory, Santa Fe, Argentina). The same procedure was applied for total cholesterol and TAG from tissues prior to liver and muscle fat extraction. The extracted fat was dissolved in 1 ml of isopropanol and then processed according to the kit protocol.

Analysis of antioxidants and oxidative stress parameters

Hepatic α-tocopherol was determined by HPLC (LC-20A, Shimadzu ®) as described by Arnaud *et al*.[20], using a C-18 (Shimpack CLC-ODS 4.6 x 25 cm) column. Total reduced glutathione in liver was determined by the method of Sedlack and Lindsay[21]. Liver catalase activity was assessed according to the technique proposed by Aebi[22]and was expressed as U (mol/min)/mg protein. Lipid peroxidation was determined based on thiobarbituric acid-reactive species (TBARS) according to the method proposed by Mihara and Uchiyama[23].

Serum analysis

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined using commercial kits (Diagnostic Labtest ® SA, Brazil, AST ref. n° 109 and ALT n° 108). Serum tumor necrosis factor-α (TNF-a) was determined using an enzyme-immunoassay kit (BD Biosciences, San Jose, CA, USA, catalog n° 560479). Glucose levels were determined using a commercial kit (SB Laboratory, Santa Fe, Argentine).

Statistical analysis

Values were expressed as mean ± standard error of the mean of six animals per group. Statistical diffe-

rence between groups was established by ANOVA followed by the Tukey test. In all analyses, the level of significance was set at $P < 0.05$.

Results

Table I shows the general and biochemical parameters of each group. There were no significant differences in body weight before fasting or after refeeding. Weight gain was lower in the CLA group compared to LA, LA+CLA and AF (P <0.01 for LA and P <0.05 for LA and CLA + AF). The group refed with the CLA-rich diet had lower liver weight compared to the other groups (P <0.001). In addition, the percentage of liver weight relative to body weight was lower in the CLA group compared to the other (P <0.001). No differences in adipose tissue weight were found between groups.

Liver fat content was increased by the high dietary fat intake (LA vs C group, P <0.05), but was prevented by CLA. Serum cholesterol level was higher in the CLA group compared to the other groups (C, LA and LA+CLA, P <0.01 for AF and P <0.05). The other parameters determined in serum (HDL-C and glucose) remained unchanged.

There was no significant difference in serum transaminase or TNF-a between groups. With respect to the antioxidant and oxidative stress parameters, the high dietary fat intake induced accumulation of α-tocopherol in liver (LA, LA+CLA and AF vs C, P <0.01), which did not occur in the CLA Group. Regarding serum α-tocopherol, the CLA group showed the highest values compared to the other groups (P <0.001), followed by the LA+CLA group (P <0.001 vs C, LA and AF groups). Liver catalase activity was reduced by the high-fat diet intake, which did not occur in the presence of CLA (P <0.01 vs C for CLA and LA+CLA). Hepatic GSH and TBARS content did not differ between the refeeding protocols.

Figure 1 shows the values of TAG and total cholesterol in liver and muscle. The AF Group had a higher liver TAG content than the C, LA and CLA groups (P <0.05 for LA and C, P <0.001 for CLA). Muscle TAG content was higher in the AF group compared to the LA+CLA group (P <0.05). Cholesterol content in liver or in gastrocnemius muscle was not affected by the dietary treatments.

The serum FA panel (Table II) reflected the FA profile from the diet sources. As expected, the CLA group showed a higher percentage of the *c*9, *t*11-CLA and *t*10,*c*12-CLA isomers than the LA+CLA group (P <0.001 for both).

Regarding the liver FA composition, presented in Table III, the CLA group showed low amounts of saturated FA related to the decreased palmitic acid (16:0) content.

The sum of monounsaturated fatty acids (MUFAs) was higher in the AF group compared to the others (P <0.01 vs C and P <0.001 vs LA, CLA and CLA + LA); the LA, LA+CLA and CLA groups showed lower values than C (P <0.05 for LA, P <0.01 for CLA and P <0.05 for LA+CLA). Palmitoleic acid (16:1) showed the lowest average in the LA, CLA and LA+CLA groups compared with the C group (P <0.001 for LA, LA+CLA and CLA vs C). Oleic acid (18:1) was higher in the AF group compared to the other groups (P <0.001) and lower in the CLA Group than in the C Group (P <0.05).

The sum of polyunsaturated fatty acids (PUFAs) was higher in the CLA group compared to the C group (CLAxC, P <0.001), LA (CLAxLA, P <0.05) and AF (CLAxAF, P <0.001), higher in LA+CLA and LA compared with C Group (LA+CLAxC and LAxC,

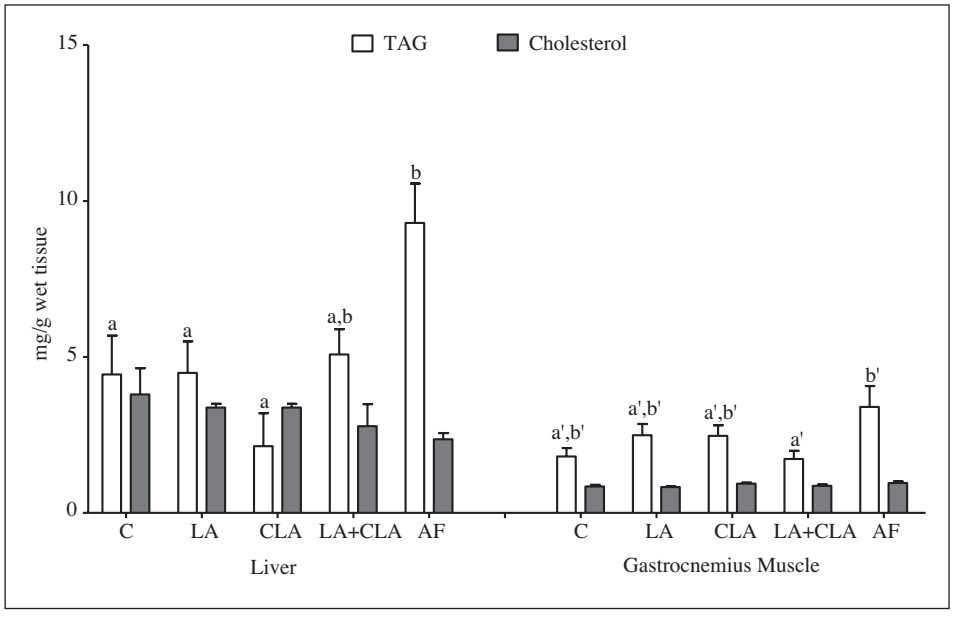

Fig. 1.—Triacylglycerol and cholesterol content in liver and gastrocnemius muscle. [a,b]Bars of the same color and with different letters were significantly different for liver; [a',b']bars of the same color and with different letters were significantly different for gastrocnemius muscle (P <0.05, ANOVA followed by the Tukey post-test). TAG – triacylglycerols; C – Control group; LA – high linoleic acid; CLA – high conjugated linoleic acid; LA+CLA - high linoleic acid plus CLA; AF - high animal fat.

Table I
General, biochemical, antioxidant and oxidative stress parameters

	C	*LA*	*CLA*	*LA+CLA*	*AF*
Weight gain after refeeding (g)	20.00±2.26[a],b	23.00±1.22a	15.20±1.07b	21.40±0.81a	21.10±1.10a
Body weight at sacrifice (g)	153.4±5.70	158.8±4.11	145.8±1.57	153.10±2.00	150.2±4.86
Diet consumption (g/day)	19.10±0.77	18.70±0.73	18.50±3.19	16.90±0.72	16.60±1.30
Diet consumption (kcal/day)	73.15±2.94	86.02±3.37	85.10±14.69	77.74±3.32	76.36±5.99
Liver weight (g)	9.498±0.296a	9.371±0.280a	7.150±0.176b	9.139±0.236a	8.978±0.344a
(% Body weight)	6.214±0.121[a]	5.903±0.115[a]	4.904±0.108b	5.966±0.111[a]	5.981±0.149[a]
Adipose tissues weight* (g)	0.235±0.033	0.295±0.025	0.190±0.081	0.193±0.034	0.167±0.015
Liver fat (mg/g wet tissue)	31.73±2.22a	39.90±1.60b,c	33.65±0.90a,b	40.75±1.71c	39.00±1.93[a],b,c
Muscle fat (mg/g wet tissue)	16.66±2.89	12.89±0.70	14.15±0.49	13.81±0.98	16.34±1.59
Serum Cholesterol (mmol/l)	1.186±0.107a	1.120±0.098a	1.878±0.142b	1.242±0.092a	1.335±0.181a
Serum HDL-C (mmol/l)	0.696±0.029	0.764±0.081	0.600±0.057	0.610±0.035	0.676±0.078
Serum TAG (mmol/l)	0.306±0.036	0.349±0.042	0.482±0.076	0.416±0.102	0.406±0.083
Serum Glucose (mmol/l)	7.401±0.851	8.349±0.585	9.629±0.487	8.745±0.841	8.711±0.602
ALT (U/l)	178.97±13.98	155.98±24.08	168.26±16.46	177.30±10.55	208.14±20.87
AST (U/l)	71.55±4.35	66.51±5.24	66.15±3.40	66.15±1.32	73.82±3.79
AST/ALT	0.40±0.01	0.50±0.09	0.41±0.03	0.38±0.02	0.37±0.03
TNF- α (pg/ml)	6.64±0.32	6.58±0.20	6.63±0.35	6.52±0.19	6.56±0.22
Liver α-Tocopherol (nmol/g wet tissue)	23.35±2.85a	63.71±6.14b	39.82±9.04a	59.84±9.00b	59.82±6.60b
Serum α-Tocopherol (μmol/l)	11.71±1.03a	15.50±1.82a	37.55±2.24b	25.01±1.06c	14.79±1.06a
Liver GSH (nmol/g protein)	122.05±16.47	121.98±15.83	111.65±12.62	127.38±14.68	176.06±20.08
Liver Catalase (U/mg protein)	42.15±3.05a	35.86±3.73a,b	27.07±0.73b	26.65±1.12b	30.75±3.02a,b
Liver TBARS (nmol/g pt)	38.31±3.13	32.72±3.43	35.28±6.24	37.52±4.26	40.24±4.88

* Adipose tissues (epididymal and retroperitoneal). a,b,c Values followed by different letters on the same line differed significantly (P <0.05, ANOVA followed by the Tukey post-test). TAG – triacylglycerol; ALT - alanine aminotransferase; AST - aspartate aminotransferase; TNF- α – tumor necrosis factor alpha; GSH – reduced gluthatione; TBARS - thiobarbituric acid-reactive species. C – Control group; LA – high linoleic acid; CLA – high conjugated linoleic acid; LA+CLA - high linoleic acid plus CLA; AF - high animal fat.

Table II
Serum fatty acid composition (mole%) as methyl esters

	C	*LA*	*CLA*	*LA+CLA*	*AF*
14:00	0.87±0.17[a,b]	0.82±0.23[a,b]	0.47±0.04[a]	0.57±0.09[a,b]	1.19±0.21[b]
16:00	30.01±2.75[a,b,c]	26.98±2.46[a,b,c]	18.71±0.84[a]	24.47±1.63[a,c]	33.95±2.45[b]
18:00	8.16±0.71	9.61±1.00	11.75±0.58	11.40±1.58	11.31±0.78
SFA	39.04±2.96[a,b]	37.44±2.71[a,b]	30.93±0.99[a]	36.44±3.15[a,b]	46.49±2.27[b]
16:1n-9	0.26±0.08	0.12±0.08	0.18±0.09	0.03±0.03	0.62±0.34
18:1n- 9	10.88±1.41[a]	10.40±1.09[a]	13.60±0.26[a]	11.10±1.90[a]	18.58±1.78[b]
18:1n-7	1.27±0.16	1.10±0.16	1.21±0.14	1.05±0.18	1.26±0.38
MUFAs	12.83±1.55[a]	11.63±1.23[a]	15.04±0.43[a,b]	12.17±2.01[a]	20.64±2.44[b]
18:2n-6	37.14±1.51[a]	42.06±1.69[b]	9.43±1.06[c]	36.51±0.98[a]	22.24±0.97d
18:3n-3	1.43±0.57	1.40±0.56	0.34±0.09	1.27±0.45	0.13±0.07
c9,t11-CLA	n.d.	n.d.	16.70±0.75[a]	2.94±0.74[b]	n.d.
t10,c12-CLA	n.d.	n.d.	17.16±1.59[a]	3.29±0.51[b]	n.d.
20:4n-6	8.56±1.29	7.18±1.33	8.27±0.78	6.80±1.14	9.49±0.68
22:6n-3	0.47±0.23	0.08±0.08	0.93±0.25	0.46±0.15	0.37±0.19
PUFAs	48.07±1.70[a]	50.86±1.63[a]	54.03±0.80[a]	51.39±1.23[a]	32.74±0.90[b]
Minor/unknown	2.18	0.72	1.33	0.35	0.13

[a,b,c]Values followed by different letters on the same line differed significantly (P <0.05, ANOVA followed by the Tukey post-test). FA - fatty acid, SFAs - saturated FA, MUFAs - monounsaturated FA, PUFAs - polyunsaturated FA; C – Control group; LA – high linoleic acid; CLA – high conjugated linoleic acid; LA+CLA - high linoleic acid plus conjugated linoleic acid; AF - high animal fat.

P<0.001 for both) and AF group (LA+CLAxAF and LAxAF P<0.001 for both) and lower in the AF group compared to the C Group (P<0.05). Linoleic acid (18:2n6) was higher in the LA and LA+CLA groups compared to the C group (LAxC and LA+CLAxC, P <0.01 for both), CLA (LAxCLA and LA+CLAxCLA, P <0.001 for both) and AF (LAxAF and LA+CLAxAF, P <0.001 for both); and CLA Group had a lower mean than the C Group (P <0.05). The content of 18:3n-3 was reduced in the CLA and AF groups vs C group. Arachidonic acid (20:4n-6) levels were higher in the CLA group and lower in the AF group. As expected, the *c*9,*t*11-CLA and *t*10,*c*12-CLA isomers were higher in the CLA group compared to the LA+CLA group (P <0.001 for both).

The 16:1/ 16:0 and 18:1/ 18:0 ratios allow us to analyze the conversion of the palmitic and stearic saturated fatty acids (SFAs) to the oleic and palmitoleic monounsaturated fatty acids (MUFAs) by the enzyme Δ9 desaturase. The 16:1/ 16:0 ratio was lower in the LA, CLA, LA+CLA and AF groups compared to the C Group (P <0.001). The 18:1/ 18:0 ratio was also reduced in the C, LA, CLA and LA+CLA groups vs AF group (P <0.05).

The 20:4n-6 / 18:2n-6 ratios are used to evaluate the conversion rate by elongases and desaturases regarding n-6 FA, and the 22:6n-3 /18:3n-3, ratios are used to track the conversion of n-3 FA. The CLA group showed the highest 20:4n-6/18:2n-6 and 22:6n-3 /18:3n-3 ratios compared to the other groups (P <0.05).

In epididymal adipose tissue (Table IV), the sum of SFAs was reduced in the CLA Group, consequent to the reduction of palmitic acid (16:0). In addition, the sum of MUFAs was higher in the AF group due to the elevated content of oleic acid (18:1) and cis-monoenoic acid (20:1). The sum of PUFAs was higher in the LA and LA+CLA groups compared to the C group (P <0.05), and the AF group had the lowest values compared to the C group (P <0.01). Linoleic and linolenic acid content was reduced in the CLA and AF groups. The *c*9,*t*11-CLA and *t*10,*c*12-CLA isomers were higher in the CLA Group compared to the LA+CLA group (P <0.05 for both).

Discussion

The liver fat content was increased by the high dietary fat intake, a fact that was not observed in the CLA group. Although the effects of CLA on liver metabolism are controversial, especially regarding the increase of liver size and fat accumulation, in the present study the supply of CLA after a prolonged fast did not cause hepatic abnormalities such as fatty liver disease.

The offer of large quantities of soybean oil (LA and LA+CLA Groups) led to an increase in hepatic fat which was not saw in the CLA group. In adult male *Wistar* rats fed a high-fat diet with 20% lard and 10%

Table III
Liver fatty acid composition (mole%) as methyl esters

	C	*LA*	*CLA*	*LA+CLA*	*AF*
14:00	0.44±0.04[a]	0.26±0.01[b]	0.32±0.04[b]	0.26±0.02[b,c]	0.40±0.04[a,b]
16:00	31.02±1.28[a]	24.48±0.77[b]	19.08±0.78[c]	24.33±0.54[b]	29.18±0.76[a]
18:00	12.94±0.99[a]	17.21±1.22[b,c]	19.61±0.71[c]	16.78±1.04[a,b,c]	12.59±1.18[a,b]
24:00:00	0.32±0.02[a,b]	0.36±0.02[a]	0.32±0.02[a,b]	0.27±0.01[b]	0.35±0.03[a,b]
SFAs	44.73±0.51[a]	42.32±0.87[a,b]	39.32±0.94[b]	41.63±0.84[a,b]	42.51±0.46[a]
16:01	3.06±0.44[a]	0.47±0.07[b,c]	0.26±0.04[b]	0.26±0.04[b]	1.49±0.28[c]
18:1n-9c	14.72±1.50[a]	10.56±1.18[a,b]	7.51±0.30[b]	10.75±0.96[a,b]	25.25±2.40[c]
20:1n-9	0.15±0.01[a]	0.15±0.02[a]	0.19±0.04[a]	0.15±0.01[a]	0.33±0.04[b]
24:01:00	0.67±0.04[a,b]	0.63±0.08[a,b]	0.58±0.05[a,b]	0.45±0.05[a]	0.93±0.22[b]
MUFAs	18.64±1.92[a]	11.84±1.19[b]	8.59±0.29[b]	11.61±0.94[b]	28.01±2.56[c]
18:2n6c	16.10±0.95[a]	22.08±1.26[b]	11.08±0.84[c]	22.15±0.77[b]	13.18±0.63[a,c]
c9,t11-CLA	n.d.	n.d.	8.16±1.70[a]	2.59±0.34[b]	n.d.
t10,c12-CLA	n.d.	n.d.	6.91±1.06[a]	1.28±0.12[b]	n.d.
18:3n-3	0.33±0.04[a]	0.61±0.06[b]	0.13±0.02[a]	0.64±0.08[b]	0.14±0.02[a]
20:3n-3	0.75±0.06[a]	0.52±0.03[b]	0.45±0.04[b]	0.47±0.02[b]	0.59±0.06[b]
20:4n-6	14.45±1.24[a,b]	16.66±1.23[a,b]	18.49±0.95[a]	14.40±0.99[a,b]	11.95±1.24[b]
22:6n-3	5.00±0.46[a,b]	5.98±0.46[a]	6.88±0.52[a]	5.22±0.40[a,b]	3.61±0.57[b]
PUFAs	36.63±2.23[a]	45.84±0.65[b]	52.09±0.71[c]	46.75±0.58[b,c]	29.48±2.14d
16:1/16:0	0.096±0.011[a]	0.019±0.002[b]	0.013±0.002[b]	0.010±0.001[b]	0.050±0.008[c]
18:1n-9/18:0	1.228±0.197[a]	0.676±0.128[a]	0.387±0.029[a]	0.678±0.101[a]	2.166±0.427[b]
20:4n-6/18:2n-6c	0.909±0.083[a]	0.792±0.097[a]	1.684±0.058[b]	0.663±0.067[a]	0.906±0.084[a]
22:6n-3/18:3n-3	18.58±4.72[a,b]	11.14±1.79[a,b]	55.18±5.86[c]	9.39±1.78[b]	27.18±5.38[a]
Minor/unknown	1.64	1.64	1.31	1.48	1.42

[a,b,c]Values followed by different letters on the same line differed significantly (P <0.05, ANOVA followed by the Tukey post-test). FA - fatty acid, SFAs - saturated FA, MUFAs - monounsaturated FA, PUFAs - polyunsaturated FA; C – Control group; LA – high linoleic acid; CLA – high conjugated linoleic acid; LA+CLA - high linoleic acid plus conjugated linoleic acid; AF - high animal fat.

soybean oil for 4 weeks followed by a low-fat diet (6.5%) with 1.5% CLA (39.2% *c*9,*t*11-CLA and 38.5% *t*10,*c*12-CLA), there was a reduction in the liver TAG content and an increased expression of genes related to lipid oxidation, peroxisome proliferator-activated receptor-α and acetyl-CoA oxidase in the CLA-fed group[24]. CLA down regulates the expression of Δ9 desaturase and fatty acid synthase (FAS), enzymes that catalyze rate-limiting steps in lipogenesis[24].

In addition, the AF group showed the greatest accumulation of liver and muscle TAG. According to Sanchez *et al*.[15], a 24 hour fast followed by refeeding with a diet containing 65.2% fat (bacon) causes increased liver TAG accumulation, higher serum triglycerides levels and increased free FA levels in rats[15]. In the present study, the large supply of dietary FA and the possible higher amount of free FA secreted probably induced TAG accumulation in the liver of the AF Group.

Refeeding rats with a diet containing the *c*9,*t*11-CLA and *t*10,*c*12-CLA isomers as the lipid source resulted in increased serum cholesterol levels with no HDL-C change. Bissonauth *et al*.[25] found an increase in low-density lipoprotein cholesterol in hamsters fed the *t*10,*c*12-CLA isomer, while Navarro *et al*.[26] reported no difference in serum total cholesterol of hamsters fed an atherogenic diet supplemented with 0.5% or 1% of the *t*10,*c*12-CLA isomer. However, a decrease of liver cholesterol content was found in these animals and when the fractions were analyzed, there was a decrease of esterified cholesterol percentage[25].

The increase in total serum cholesterol associated with a tendency to increase serum TAG (~57% more compared to the C Group) without a concomitant increase in total liver cholesterol may indicate that CLA enhanced the hepatic secretion of cholesteryl ester and cholesterol. CLA supply has been reported to increase the expression of HMG-CoA reductase, responsible for the synthesis of endogenous cholesterol[27]. Large

Table IV
Epididymal adipose tissue FA composition (mole%) as methyl esters

	C	*LA*	*CLA*	*LA+CLA*	*AF*
10:00	0.47±0.09	0.63±0.07	0.90±0.22	0.64±0.25	0.63±0.11
12:00	0.94±0.18	1.25±0.13	1.76±0.41	1.28±0.46	1.17±0.21
14:00	2.20±0.17	2.33±0.18	2.71±0.27	2.29±0.35	2.45±0.17
16:00	25.86±0.48[a,b]	23.85±0.65[b]	20.19±0.88[c]	22.43±0.45[c]	26.41±0.29[a]
17:00	0.25±0.01	0.25±0.01	0.29±0.07	0.26±0.03	0.32±0.01
18:00	4.29±0.17[a]	4.46±0.25[a]	4.59±0.07[a]	4.78±0.28[a]	5.76±0.30[b]
24:00:00	0.11±0.01	0.11±0.01	0.19±0.07	0.11±0.03	0.15±0.01
SFAs	33.40±0.56[a]	32.00±0.70[a,b]	29.46±1.05[b]	30.89±0.82[a,b]	36.03±0.42[c]
16:01	4.01±0.30	2.93±0.44	2.96±0.37	2.63±0.47	3.45±0.18
18:1n-9c	28.20±0.46[a]	27.82±0.63[a]	28.12±0.79[a]	26.70±0.41[a]	32.39±0.37[b]
20:1n-9	0.23±0.02[a]	0.22±0.02[a]	0.32±0.03[a,b]	0.24±0.02[a]	0.35±0.03[b]
MUFAs	32.45±0.57[a]	30.97±0.99[a]	31.43±1.08[a]	29.57±0.83[a]	36.19±0.35[b]
18:2n-6c	31.89±0.72[a]	34.79±1.22[a]	27.90±0.98[b]	32.56±0.46[a]	26.52±0.48[b]
18:3n-6	0.12±0.02	0.19±0.06	0.40±0.23	0.10±0.02	0.25±0.12
18:3n-3	2.04±0.06[a]	1.16±0.22[a]	1.16±0.22[b]	2.18±0.06[a]	1.12±0.12[b]
c9,t11-CLA	n.d.	n.d.	5.61±1.29[a]	2.56±0.38[b]	n.d.
t10,c12-CLA	n.d.	n.d.	4.88±1.08[a]	2.29±0.35[b]	n.d.
20:4n-6	0.68±0.05	0.60±0.04	0.47±0.10	0.66±0.05	0.68±0.04
20:5n-3	0.02±0.002	0.03±0.01	0.09±0.03	0.03±0.01	0.02±0.001
22:6n-3	0.09±0.01	0.13±0.01	0.12±0.02	0.18±0.03	0.12±0.01
PUFAs	34.84±0.75[a]	38.03±1.45[a,b]	40.61±1.73[b]	40.56±0.94[b]	28.72±0.53[c]
Minor/unknown	0.59	0.60	0.68	0.73	0.76

[a,b,c]Values followed by different letters on the same line differed significantly (P <0.05, ANOVA followed by the Tukey post-test). FA - fatty acid, SFAs - saturated FA, MUFAs - monounsaturated FA, PUFAs - polyunsaturated FA; C – Control group; LA – high linoleic acid; CLA – high conjugated linoleic acid; LA+CLA - high linoleic acid plus conjugated linoleic acid; AF - high animal fat.

quantities of FA rise the hepatic secretion of very-low density lipoprotein (VLDL), which transports TAG and cholesterol[28].

It has been reported that prolonged fasting causes a reduction of liver mass[12,15]. After fasting, the animals refed with CLA showed a lower liver weight. Nevertheless, this fact does not seem to be linked to liver injury, since there were no differences in transaminases, hepatic TBARS or TNF-a.

The liver a-tocopherol content was enlarged in the AF, LA and LA+CLA groups, a results that seems to be due to fat accumulation in liver. However, the CLA Group showed the highest serum a-tocopherol levels, which confirms the hypothesis of augmented secretion of VLDL by the liver, since this lipoprotein also transports vitamin E[29]. Furthermore, a-tocopherol transfer protein is reported to be increased by the ingestion of CLA[30].

The low liver catalase activity observed in both CLA-fed groups suggests that the supply of *c*9,*t*11-CLA and *t*10,*c*12-CLA isomers led to a lower formation of H_2O_2 with a consequent decrease in activity of this enzyme. In agreement with our results, Cantwell *et al.*[31] reported that catalase activity was reduced in cultured hepatocytes exposed to large amounts of CLA (20 ppm). In addition, CLA supplementation is known to inhibit PPAR-g expression[32] which is directly related to catalase activity[33].

In this animal model, hepatic GSH concentration was not changed by any dietary treatment. This fact disagrees with other studies[5,34], which suggest an increased synthesis of GSH resulting from CLA consumption. The short-term refeeding may be related to the lack of changes observed.

The serum FA profile reflects the type of diet consumed. The highest concentration of CLA appears to be

dose-dependent and seems to occur similar incorporation of the isomers into cholesterol esters and phosphatidylcholine[35].

Regarding the FA composition of the liver, the CLA group showed a low saturated FA content, related to the decreased content of palmitic acid. There was also an increased stearic acid content in this group. Concerning the MUFAs, oleic acid concentration was reduced in the CLA group although it was present in the diet. Sébédio *et al*.[36] found decreased hepatic oleic acid and increased stearic acid in rats fed only the isomer *t*10,*c*12-CLA. Several authors have reported inhibition of Δ9 desaturase activity by CLA[37,38] which was also observed in our study, since the CLA group had the lowest 18:1/18:0 ratio. The decrease in Δ9 desaturase activity has been reported to be related to a higher secretion of VLDL by the liver and decreased liver TAG content[36].

As expected, there was a higher incorporation of saturated and monounsaturated FA into epididymal adipose tissue in the AF Group. Concerning the incorporation of CLA in the different tissues analyzed, the CLA group showed similar hepatic incorporation of both isomers. However, in the LA+CLA Group, the *c*9,*t*11-CLA level was twice higher than the *t*10,*c*12-CLA level. This may have been related to a higher incorporation of *c*9,*t*11-CLA or a faster metabolic rate of *t*10,*c*12-CLA. In serum and epididymal adipose tissue, there was no difference for the isomers in the CLA and LA+CLA groups despite the greater amount offered to the CLA group.

Refeeding with lard led to TAG and cholesterol accumulation in liver and gastrocnemius muscle and also decreased the amount of PUFAs in liver and adipose tissue and enhanced hepatic α-tocopherol. The LA group showed elevated liver fat, liver PUFAs and α-tocopherol. The CLA group showed serum cholesterol raise, with no liver fat accumulation and a reduction of hepatic catalase activity. In contrast, the LA+CLA Group showed the highest amount of liver fat and α-tocopherol, demonstrating that CLA associated with soybean oil in a high-fat diet did not result in major benefits.

Although, in the context of this study, large amounts of CLA may have generated positive effects, it is not possible to establish the long-term consequences produced by the large incorporation of these FA isomers, considering that CLA plays a role in cell signaling, activating transcriptions factors and thereby regulating the enzymes of lipid metabolism transcription.

In conclusion, refeeding with high-fat diets altered the liver fat and α-tocopherol contents, as well as the serum and liver FA profile. In this acute situation, there was no change in adipose tissue mass or in oxidative stress and antioxidant response. Despite the short refeeding period, CLA was widely incorporated into the serum and tissues analyzed and some changes in FA composition were observed, with an increase in stearic acid and a decrease in palmitic, oleic and linoleic acids. There was a decrease in saturated FA in the liver, especially in the group refed the highest amount of CLA. Acutely, large CLA quantities caused an increase in serum cholesterol concentration, but at the same time prevented fat accumulation in the liver.

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