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Effect of conjugated linoleic acid mixtures and different edible oils on body composition and lipid regulation in mice

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Abstract

Introduction: **Evidences suggest that commercial and natural conjugated linoleic acids (CLA) differentially affect nutritional status and lipid metabolism.**

Objective: **To investigate the differential effect of two types of CLA preparations supplemented to dietary fats containing different proportions of n-9, n-6 and n-3 fatty acids (FA) on body composition, triacylglycerol (TG) levels and lipid metabolism in mice.**

Methods: **Growing mice were fed diets containing olive, maize and rapeseed oils supplemented with an equimolecular mixture of CLA (mix-CLA) or a rumenic acid (RA)-rich oil for 30 days. Body weight gain, carcass composition, tissue weights, plasma and tissue TG levels, and lipid regulation parameters were evaluated.**

Results: **Independently of the dietary fats, mix-CLA decreased body weight gain and fat depots related to lower energy efficiency, hepatomegaly, increase of serum TG and decrease of muscle TG. Rapeseed oil prevented the hepatic steatosis observed with mix-CLA supplementation to olive and maize oils by increasing TG secretion. RA-rich oil supplementation decreased fat depots without hepatomegaly, hepatic steatosis and hypertriglyceridemia. Olive oil, by an equilibrium between FA uptake/oxidation, prevented the increase of muscle TG induced by the RA-rich oil supplementation to maize and rapeseed oils.**

Discussion and conclusion: **The proportions of dietary unsaturated FA modulated the different mix-CLA and RA-rich oil response to lipid metabolism in mice. Finally, rapeseed oil prevented the hepatic steatosis induced by mix-CLA, and the most beneficial effects of RA-rich oil were observed when supplemented to olive oil, due to the reduced lipid accretion without changes in TG levels.**

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EFECTOS DE MEZCLAS DE CONJUGADOS DEL ÁCIDO LINOLÉICO Y DIFERENTES ACEITES COMESTIBLES SOBRE LA COMPOSICIÓN CORPORAL Y REGULACIÓN DE LÍPIDOS EN RATONES

Resumen

Introducción: **Las evidencias sugieren que las mezclas de Ácido Linoleico Conjugado (ALC) de origen comercial o natural diferencialmente afectan diferencialment al estado nutricional y al metabolismo lipídico.**

Objetivo: **Investigar el efecto de dos preparaciones de ALC como complemento de grasas dietarias con diferentes proporciones de ácidos grasos (AG) n-9, n-6 y n-3 sobre composición corporal, niveles de triacilglicéridos (TG) y metabolismo lipídico en ratones.**

Métodos: **Se alimentó a ratones en crecimiento con dietas con aceite de oliva, maíz y canola, o colza suplementadas con una mezcla equimolecular de ALC (mezcla-ALC) o aceites ricos en ácido ruménico (AR) por 30 días. Se evaluó: ganancia de peso, composición corporal, peso de tejidos, niveles de TG plasmáticos y séricos, y parámetros de regulación lipídica.**

Resultados: **Independientemente de las grasas dietarias, la mezcla-ALC redujo el peso corporal y depósitos grasos relacionados con hepatomegalia, incremento de TG séricos y descenso de TG musculares. El aceite de canola previno la esteatosis hepática producida por la mezcla-ALC a aceites de oliva y maíz por incremento de la secreción de TG. AR decreció los depósitos grasos sin hepatomegalia, esteatosis hepática e hipertrigliceridemia. Aceite de oliva previno el incremento de TG musculares inducidos por suplementación con AR al aceite de maíz y canola.**

Discusión y conclusión: **Las proporciones de AG insaturados dietarios modularon la respuesta de mezcla-ALC y AR al metabolismo lipídico en ratones. Finalmente, aceite de canola previno la esteatosis hepática inducida por mezcla-ALC, y los efectos benéficos más notorios fueron observados cuando aceite de oliva fue suplementado con AR, debido a la reducida acreción de lípidos sin cambios en los niveles de TG.**

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Palabras clave: *Ácido linoléico conjugado. Ácido ruménico. Estado nutricional. Metabolismo de lípidos.*

Abbreviations

AIN: American Institute of Nutrition.
ALA: α-linolenic acid.
ALP: alkaline phosphatase.
ALT: alanine transaminase.
ARA: arachidonic acid.
AST: aspartate transaminase.
BW: body weight.
*c*9,*t*11: *cis*-9,*trans*-11.
CLA: conjugated linoleic acid.
CPT-I: carnitine palmitoyltranferase-I.
DHA: docosahexaenoic acid.
EE: energetic efficiency.
EI: energy intake.
EPA: eicosapentaenoic acid.
EWAT: epididymal white adipose tissue.
FA: fatty acids.
FAS: fatty acid synthase.
G6PDH: glucose-6-phosphate dehydrogenase.
LA: linoleic acid.
LPL: lipoprotein lipase.
M: maize.
ME: malic enzyme.
O: olive.
OA: oleic acid.
PPARγ: peroxisomal proliferator activated receptor-γ.
PUFA: polyunsaturated fatty acid.
R: rapeseed.
RA: rumenic acid.
SREBP-1c: transcription factor sterol regulatory element binding protein-1c.
*t*10,*c*12: *trans*-10,*cis*-12.
TG: triacylglycerol.
TG-SR: triacylglycerol-secretion rate.
U: units.

Introduction

Conjugated linoleic acid (CLA) is a group of positional and geometrical isomers of linoleic acid (LA) that are interesting due to their functional properties[1,2]. The major contribution of natural CLA is supplied by dairy products and meats where *cis*-9,*trans*-11 (*c*9,*t*11)-CLA, known as rumenic acid (RA), represents 80-90% of total CLA. On the other hand, industrially synthesized commercial CLA mainly contains a mixture of equimolecular amounts of *c*9,*t*11-CLA and *t*10,*c*12-CLA (mix-CLA) (approximately 37-40% each).

Many investigations[1,2] have shown that CLA may have beneficial effects on cancer, obesity, inflammatory response, atherosclerosis and glucose and lipid metabolism. However, controversial and detrimental health effects have also been reported[2-4]. In addition, it has been demonstrated that RA has a mechanism of action on lipid metabolism different from *trans*-10,*cis*-12 (t10,c12)-CLA(mix-CLA), resulting in dissimilar body fat accretion[3-5]. The conflicting results may be related to several factors such as comparison of humans with studies of experimental animals, species of animals, physiological conditions, type and level of CLA and dietary fat, and time of feeding. A number of studies have shown that α-linolenic acid (ALA)[6], γ-linolenic acid[7], docosahexaenoic acid (DHA)[8] and arachidonic acid (ARA)[9] rich oils might attenuate or prevent insulin resistance and fatty liver induced by *t*10,*c*12-CLA or mix-CLA in mice. These effects might be related to the incorporation of specific fatty acids (FA) into the biological membranes and eicosanoid production derived from the different FA. Therefore, we hypothesized that the extension and type of the beneficial or negative effects of oils containing different relative amounts of the individual CLA on lipid metabolism are related not only to the presence of certain FA, but also to the proportion of the n-9, n-6 and n-3 FA present in the diet. Specifically, at least to our knowledge, there are no systematic studies dealing with the effects on nutritional parameters and lipid metabolism of commercial and natural-like CLA added to diets containing different proportions of unsaturated FA. Thus, the aim of this work was to investigate the differential effect of mix-CLA and RA-rich oil supplemented to dietary fats containing different proportions of n-9, n-6 and n-3 FA on body composition, TG levels and some regulatory mechanisms involved lipid metabolism in mice.

Methods

Materials

Most nutrients were chemical grade or better, with the exception of olive (O) oil (Nucete, La Rioja, Argentina), maize (M) oil (Arcor, Córdoba, Argentina), rapeseed (R) oil (Krol, Entre Ríos, Argentina), sucrose, cellulose, and maize starch, which were obtained from local sources. Mix-CLA and RA-rich oils were kindly provided by Lipid Nutrition B.V. (Wormerveer, The Netherlands). Standard chow for the adaptation period of animals was from Grupo Pilar® (Pilar, Córdoba, Argentina). FA standards were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All solvents and reagents used for the FA quantification were chromatography grade, and all the other chemicals used were at least ACS degree. Plasma TG, alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) kits were purchased from Wiener Co. (Rosario, Argentina).

Diets

The diet compositions (table I) were based on the American Institute of Nutrition *ad hoc* writing committee recommendation (AIN-93G diet)[10]. All diets were isoenergetic (16.5 MJ/kg), exceeded the essential FA recommendations, and differed either in 1) dietary

Table I
Composition of experimental diets (g/kg of dry diet)

Ingredient	*O*	*O+RA*	*O+mix-CLA*	*M*	*M+RA*	*M+mix-CLA*	*R*	*R+RA*	*R+mix-CLA*
Maize starch	529.5	529.5	529.5	529.5	529.5	529.5	529.5	529.5	529.5
Casein	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0
Sucrose	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Olive oil	70.0	60.0	60.0	–	–	–	–	–	–
Maize oil	–	–	–	70.0	60.0	60.0	–	–	–
Rapeseed oil	–	–	–	–	–	–	70.0	60.0	60.0
RA-rich oil	–	10.0	–	–	10.0	–	–	10.0	–
Mix-CLA oil	–	–	10.0	–	–	10.0	–	–	10.0
Fibre	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Mineral mixture*	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0
Vitamin mixture*	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
L-cystine-L-metionine	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Choline	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Total energy (MJ/kg)	16.5	16.5	16.5	16.5	16.5	16.5	16.5	16.5	16.5

*Vitamin and mineral mixtures were formulated according to Reeves et al.[10]
O, olive; RA, rumenic acid; mix-CLA, *c*9,*t*11-CLA and *t*10,*c*12-CLA in equimolecular amounts; CLA, conjugated linoleic acid; M, maize; R, rapeseed.

fat source: O, M or R oils, and 2) absence or presence of 1 g of mix-CLA or RA-rich oil/100 g diet. The combination of these 2 variables allowed us to create the following diets: O; O+RA; O+mix-CLA; M; M+RA; M+mix-CLA; R; R+RA and R+mix-CLA containing 7 g of total fat/100 g diet. The FA composition of dietary fats, as well as the OA/LA/ALA proportions are shown in table II. These FA compositions as methyl esters were determined by gas chromatography using the equipment and conditions previously reported[11].

Animals and treatments

The experimental procedures were approved by the Ethics Committee of our School of Biochemistry and compiled according to the Guide for the Care and Use of Laboratory Animals[12]. Male CF1 mice were obtained at the age of three weeks from the facilities at our University. The mice were housed in animal quarters under controlled conditions (23 ± 2ºC and 12 h light-dark cycle) in individual stainless steel metabolic cages. After two weeks of adaptation period, mice (~22 g)

Table II
*Fatty acid composition of dietary fats**

Fatty acid	*Olive oil*	*Maize oil*	*Rapeseed oil*	*RA-rich oil*	*mix-CLA oil*
14:0	0.0	0.0	0.1	0.0	0.0
16:0	17.1	12.2	4.0	3.9	5.9
*c*9-16:1	2.0	0.1	0.2	0.0	0.0
17:0	0.1	0.0	0.0	0.0	0.0
18:0	1.6	1.9	2.2	1.0	1.2
*c*9-18:1	55.2	32.0	61.1	13.4	9.1
*c*11-18:1	4.8	0.5	3.5	0.5	0.4
*c*9,*c*12-18:2	17.2	51.3	18.4	1.5	1.1
*c*9,*t*11-18:2	0.0	0.0	0.0	60.5	39.0
*t*10,*c*12-18:2	0.0	0.0	0.0	17.8	38.8
20:0	0.3	0.5	0.5	0.0	0.0
*c*11-20:1	0.2	0.3	0.9	0.0	0.0
*c*9,*c*12,*c*15-18:3	0.7	0.9	8.6	0.0	0.0
22:0	0.1	0.2	0.2	0.1	0.0
24:0	0.0	0.2	0.0	0.0	0.0
Others	0.7	0.0	0.2	1.2	4.7
OA/LA/ALA proportions	55.2/17.2/0.7	32.0/51.3/0.9	61.1/18.4/8.6		

* All values are means as weight percentages of total fatty acid methyl esters.
RA, rumenic acid; mix-CLA, *c*9,*t*11-CLA and *t*10,*c*-12-CLA in equimolecular amounts; CLA, conjugated linoleic acid; OA, oleic acid; LA, linoleic acid; ALA, α-linolenic acid.

were randomly divided into ten weight-matched groups (n=6 per group). One group was killed at the start of the experiment with the purpose of determining initial composition and energetic content of carcass. The remaining animals had free access to water and were fed *ad libitum* during 30 d with one of the following diets: O; O+RA; O+mix-CLA; M; M+RA; M+mix-CLA; R; R+RA or R+mix-CLA. Mice were weighed, food intakes were recorded, and total faeces were collected daily during the whole dietary treatment. On day 30 (9.00-11.00 AM), mice were anaesthetized with azepromazine (1 mg/kg) and ketamine (100 mg/kg), their bodies were shaved, the abdomens were cut open and following the removal of the visceral organs, carcasses were weighed, chopped and frozen at -20ºC until the compositional evaluation. In other experiments with the nine experimental groups (n = 6 per group), at the end of the dietary treatments, animals were anaesthetized in order to collect blood samples by cardiac puncture and dissected tissues according to the assay proposed or to perform the *in vivo* hepatic TG secretion rate (TG-SR) test. Blood samples were centrifuged at 4°C, and serum was immediately used or stored at -80ºC until analyzed. Liver, epididymal white adipose tissue (EWAT) and gastrocnemius muscle were frozen, weighed, and stored at -80°C until processed.

Laboratory analysis

– *Carcasses, faeces and food compositions*. The protein, water and total fat contents, in aliquots from the carcass homogenate, faeces, and diets were estimated by Official Methods of Analysis of AOAC International[13].

– *Bioavailability indexes of fat and protein*. The apparent absorption of dietary fat and protein, as bioavailability indexes, were assessed as the percentages of ingested fat or protein that were not excreted in the faeces.

– *Carcass energy retention and energy efficiency*. Carcass energy content was estimated from protein and lipid levels[14]. Carcass energy retention was estimated from the final carcass energy content and the carcass energy content at the start of the experiment in the weight-matched animals killed on day 0. The energy intake (EI) was calculated by multiplying weight of food consumed daily by the number of kJ/g diet. Energetic efficiency (EE) was estimated as percentage of body energy gain (kJ/30 d) divided by EI (kJ/30 d).

– *Serum parameters*. TG concentrations and biomarker enzyme activities of liver damage (ALT, AST and ALP) were carried out in serum by spectrophotometric methods using commercially available test kits.

– *Liver and muscle TG content*. Aliquots of liver or gastrocnemius muscle were powdered and homogenized in a saline solution for TG content quantification by the spectrophotometric method cited in different articles[11,15].

– *In vivo hepatic TG-SR*. Another set of animals submitted to the same dietary treatments was fasted overnight and anaesthetized, as indicated above. Then the *in vivo* hepatic TG-SR was assayed according to the method based on the inhibition of peripheral removal of TG-rich lipoproteins by Triton WR1339, adjusted by our group[11,15].

– *Total Lipoprotein Lipase (LPL) activities*. Homogenates of gastrocnemius muscles and acetone powders of EWAT were prepared according to the procedure previusly reported[11], and the total LPL activities were measured through a fluorimetric method[16]. In the case of EWAT, the results were expressed as, U (units)/g EWAT and U/ whole pad EWAT (U/EWAT), while the total LPL activity of gastrocnemius muscle was expressed as U/g gastrocnemius, considering that 1 U = 1 nmol of fluoresceine/min.

– *Lipogenic enzyme activities*. In liver and EWAT homogenates, fatty acid synthase (FAS; EC 2.3.1.85), malic enzyme (ME; EC 1.1.1.40) and glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) activities were measured according to the methods described by Lynen (1969)[17], Hsu and Lardy (1969)[18] and Kuby and Noltmann (1966)[19], respectively. In liver, enzyme activities were expressed either as nmol NADPH consumed (FAS) or produced (G6PDH and ME) /min/mg of protein (1 mU = 1 nmol NADPH/min). In the case of EWAT, the results were expressed as mU/g EWAT (1 U = 1 mmol of NADPH consumed or produced/min) or per whole EWAT (U/EWAT). Protein content was determined by the Lowry technique[20].

– *Carnitine palmitoyltranferase-I (CPT-I) activities*. CPT-Ia and CPT-Ib (EC 1.3.99.3) activities were assessed in the mitochondrial fraction of liver and muscle, respectively, by the method of Bieber et al.[21] The CPT-I activities were expressed as mU/mg of protein (1 mU = 1 nmol CoA/min).

Statistical analysis

The statistical analysis was performed using SPSS, version 17.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as means ± standard errors of mean values, and were statistically analyzed by 3×3 ANOVA. All post-hoc multiple comparisons were made using Tukey's critical range test. Significant differences were considered at $P < 0.05$.

Results

All diets were well accepted showing a similar energy intake, as well as fat and protein apparent absorption associated with a positive body weight gain (table III). Nevertheless, the body weight gain was altered by the source of dietary fats and by the CLA. Specifically, R oil-fed animals increased the body

Table III
Effect of experimental diets on nutritional and body composition parameters

	O	*O+RA*	*O+mix-CLA*	*M*	*M+RA*	*M+mix-CLA*	*R*	*R+RA*	*R+mix-CLA*	*ANOVA*
BW gain (g)	8.3 ± 0.5[a]	8.6 ± 0.6[a]	4.4 ± 0.5[b]	8.5 ± 0.5[a]	8.9 ± 0.6[a]	7.0 ± 0.3[c]	11.5 ± 0.5[d]	7.8 ± 0.5[ac]	6.7 ± 0.5[c]	F, I, F × I
EI (kJ/d)	59.3 ± 4.0	61.6 ± 4.8	60.8 ± 7.1	58.9 ± 4.9	53.2 ± 2.3	56.0 ± 2.9	58.9 ± 3.6	58.8 ± 6.7	57.3 ± 4.2	NS
Apparent absorption (%)										
Fat	97.1 ± 0.6	97.9 ± 0.1	96.8 ± 0.8	95.0 ± 1.3	97.0 ± 0.2	97.0 ± 0.7	96.5 ± 0.4	97.9 ± 0.1	97.9 ± 0.1	NS
Protein	93.2 ± 0.4	93.7 ± 0.3	95.0 ± 0.3	92.8 ± 0.3	93.6 ± 0.5	93.9 ± 0.8	92.8 ± 0.5	93.9 ± 0.7	93.3 ± 0.5	NS
Carcass energy retention (kJ/d)										
Total	2.4 ± 0.2[a]	1.9 ± 0.1[a]	0.2 ± 0.1[b]	2.0 ± 0.2[a]	1.9 ± 0.1[a]	1.0 ± 0.1[c]	3.8 ± 0.1[d]	2.2 ± 0.2[a]	0.4 ± 0.1[bc]	F, I, F × I
Fat	1.3 ± 0.2[a]	0.6 ± 0.0[b]	-0.7 ± 0.1[c]	0.9 ± 0.1[ab]	0.7 ± 0.1[b]	-0.6 ± 0.1[c]	2.5 ± 0.1[d]	1.3 ± 0.2[a]	-0.6 ± 0.0[c]	F, I, F × I
Protein	1.2 ± 0.1[abc]	1.4 ± 0.0[ad]	0.8 ± 0.1[e]	1.1 ± 0.1[bc]	1.2 ± 0.1[abc]	1.5 ± 0.0[d]	1.3 ± 0.1[ab]	1.0 ± 0.0[ce]	1.1 ± 0.1[bce]	F, F × I
EE (%)	4.2 ± 0.2[a]	3.5 ± 0.1[ab]	0.3 ± 0.1[c]	3.2 ± 0.2[b]	3.6 ± 0.3[ab]	1.7 ± 0.1[d]	6.3 ± 0.1[e]	3.7 ± 0.3[ab]	0.8 ± 0.1[c]	F, I, F × I
Carcass composition (g/100g)										
Fat	9.7 ± 1.2[a]	8.0 ± 0.6[a]	4.5 ± 0.4[b]	9.3 ± 0.8[a]	9.3 ± 0.6[a]	3.9 ± 0.3[b]	13.6 ± 0.6[c]	10.4 ± 0.8[a]	4.5 ± 0.2[b]	F, I, F × I
Protein	24.9 ± 0.8	24.1 ± 0.1	25.3 ± 0.5	24.3 ± 0.4	25.1 ± 1.3	27.1 ± 1.0	23.1 ± 0.4	22.7 ± 0.6	24.2 ± 0.3	NS
Water	63.0 ± 1.0	64.5 ± 0.4	66.0 ± 0.3	63.2 ± 1.0	63.7 ± 0.3	67.0 ± 0.3	60.1 ± 0.7	63.2 ± 1.0	66.6 ± 0.3	NS
Tissue weights										
Liver										
g	2.2 ± 0.1[a]	2.4 ± 0.1[a]	3.0 ± 0.2[b]	2.3 ± 0.2[a]	2.4 ± 0.1[a]	2.6 ± 0.2[ab]	2.2 ± 0.1[a]	2.1 ± 0.1[a]	2.8 ± 0.1[b]	I
g/100 g BW	6.7 ± 0.3[a]	6.8 ± 0.2[a]	8.8 ± 0.3[b]	6.5 ± 0.3[a]	7.1 ± 0.1[a]	8.0 ± 0.2[b]	6.7 ± 0.2[a]	6.6 ± 0.2[a]	8.3 ± 0.3[b]	I
EWAT										
g	0.4 ± 0.0[a]	0.3 ± 0.0[b]	0.1 ± 0.0[c]	0.5 ± 0.0[a]	0.3 ± 0.0[b]	0.1 ± 0.0[cd]	0.7 ± 0.0[e]	0.2 ± 0.0[bd]	0.1 ± 0.0[c]	F, I, F × I
g/100 g BW	1.3 ± 0.1[a]	0.9 ± 0.1[b]	0.4 ± 0.0[c]	1.5 ± 0.1[ae]	1.0 ± 0.1[b]	0.4 ± 0.0[cd]	1.8 ± 0.1[e]	0.7 ± 0.1[bd]	0.4 ± 0.1[c]	I, F × I
Gastrocnemius										
g	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	NS
g/100 g BW	0.8 ± 0.1	0.9 ± 0.0	0.9 ± 0.0	0.8 ± 0.0	0.9 ± 0.0	0.9 ± 0.1	0.8 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	NS

The results are expressed as mean values with their standard errors for six animals per group.
[a,b,c,d,e]Mean values within a row with dissimilar superscript letters were significantly different ($P < 0.05$) by Tukey's critical range test after ANOVA (3 × 3). F, effect of dietary fat source; I, effect of absence or presence of CLA isomers; NS, not significant.
O, olive; RA, rumenic acid; mix-CLA, *c*9,*t*11-CLA and *t*10,*c*12-CLA in equimolecular amounts; CLA, conjugated linoleic acid; M, maize; R, rapeseed; BW, body weight; EI, energy intake; EE, energy efficiency; EWAT, epididymal white adipose tissue.

weight gain, and the supplementation with mix-CLA in all groups and RA-rich oil in R-mice diminished this parameter.

The higher body weight of R oil-mice was associated with a higher fat retention in carcasses, as well as in EWAT pads. The reduced body weight gain by mix-CLA in all dietary sources was mainly associated with losses of fat in carcasses and in fat pads. A differential and slighter effect was produced by RA-rich oil, where only a significant reduction of body weight gain was observed in R+RA animals. In this group, the changes in body weight gain were also related to a reduction in the content of fat carcass and EWAT.

The liver weight was not altered by the dietary fat sources; however, mix-CLA supplementation to diets induced hepatomegaly and these alterations were not associated with changes in biomarkers of hepatic damage (table IV).

In the absence of CLA supplementation, the TG levels in serum, liver and gastrocnemius muscle were not modified by the dietary fat source, but were altered by the type of CLA (table IV). Serum TG were increased in all animals fed diets supplemented with mix-CLA and in those mice fed with the M+RA diet. Hepatic TG levels showed differential effects depending on the fat source considered in mix-CLA supplementation and did not show differences by RA-rich oil supplementation. In this regard, the increase by mix-CLA in hepatic TG content was O+mix-CLA > M+mix-CLA > R+mix-CLA. In this latter group, this increase is not statistically significant when compared with those animals without mix-CLA. The TG content in the gastrocnemius muscles of animals fed diets supplemented with mix-CLA was significantly reduced, while RA-rich oil supplementation increased the TG content in those animals fed with M and R oil diets.

In the absence of CLA, the hepatic TG-SR did not change by the dietary fat source; however, it was highly increased in R+mix-CLA mice and in all animals fed with RA-rich oil supplemented diets. The total LPL activity in EWAT was increased by RA-rich oil and in greater proportion by mix-CLA. However, when the EWAT size was considered, the contribution to the total TG removal by LPL in animals fed diets supplemented with mix-CLA was significantly reduced, as well as in M+AR and R+AR mice compared to their respective controls. The gastrocnemius muscle total LPL activity was increased in M+RA and R+RA mice, and did not change by mix-CLA supplementation independently of dietary fat source. Since no differences were found in gastrocnemius weight, the expression of total LPL activity/total muscle showed the same pattern that per g of muscle (data not shown).

In the absence of CLA, the three hepatic lipogenic enzymes (FAS, ME and G6PDH) activities measured did not show differences by dietary fat source (table V) and, with the exception of R+RA mice in the ME activity, the supplementation with mix-CLA or RA-rich oil to diets increased both FAS and ME activities without changes in G6PDH activity. In EWAT, the activities of these three enzymes (expressed as mU/mg protein) did not change by dietary fat source and were increased by mix-CLA and RA-rich oil supplementation. However, when the contribution of these enzymes to the total lipogenesis in the EWAT is estimated, RA-rich oil supplementation increased the activities of the three enzymes in the animals fed with any fat source, with the exception of R+RA mice in which the observed increase in FAS activity did not reach statistical significance. On the other hand, under this expression, mix-CLA supplementation to fat diets did not modify any of the lipogenic enzyme activities evaluated in EWAT.

The main enzymes involved in the β-oxidation of liver and skeletal muscle are the CPT-Ia y CPT-Ib, respectively. This enzyme increased in the liver of animals fed R and M+RA diets. In the gastrocnemius, the CPT-Ib activity was higher in mice fed M or R *vs* O diets, as well as in those fed with the mix-CLA diet.

Discussion

Several studies have reported the benefits and disadvantages on the nutritional status and lipid metabolism of different types of CLA, as well as of different dietary fat sources. To the best of our knowledge, there are no nutritional investigations dealing with the combination of naturally or commercially obtained CLA and different sources of fats and its effects on parameters related to risk of non-communicable disorders. Therefore, the aim was to investigate the differential effect of two types of CLA preparations supplemented to dietary fats containing different proportions of n-9, n-6 and n-3 FA on body composition, TG levels and lipid metabolism in mice.

In comparison with RA-rich oil, mix-CLA supplementation showed a very high influence on body composition and lipid parameters. The present results confirmed, and also extended to different dietary fats, data reported by other authors[3,22,23], in which the mix-CLA decreased the body weight gain associated with a deep reduction in fat storage and hepatomegaly, while RA-rich oil has a lower but significant reduction of fat pads without negative impact on liver. The alterations observed by mix-CLA have been described in mice[3] as a "lipoatropic syndrome", and is due to the *t*10,*c*12-CLA isomer. Different outcomes have been observed in other species; thus, weak or no effects have been reported in normal rats and other animal models, leading to the conclusion that the species and strain are determinant of the biological response of CLA[24,25]. In vivo and in vitro studies[3,4,26,27] have indicated that the putative mechanism of the *t*10,*c*12-CLA effect might include, reduction of the total LPL activity in adipose tissue, decreased preadipocyte differentiation and proliferation, inhibition of stearoyl coenzyme A desaturase activity, increased apoptosis of adipocytes induced by tumor necrosis factor-α and enhanced

Table IV

Effect of experimental diets on serum biomarker of hepatic damage, TG levels and parameters related to TG regulation

	O	*O+RA*	*O+mix-CLA*	*M*	*M+RA*	*M+mix-CLA*	*R*	*R+RA*	*R+mix-CLA*	*ANOVA*
Serum activities (U/L)										
ALT	19.7 ± 1.2^{abc}	12.8 ± 1.0^{d}	22.9 ± 1.5^{b}	20.6 ± 0.7^{abc}	15.2 ± 0.7^{ad}	22.0 ± 1.0^{bc}	18.0 ± 0.8^{abcd}	16.8 ± 2.0^{acd}	23.7 ± 1.9^{b}	I
AST	43.3 ± 5.2	39.3 ± 6.6	54.5 ± 5.7	56.5 ± 2.0	45.5 ± 12.1	64.0 ± 5.5	39.3 ± 4.9	31.5 ± 7.5	42.7 ± 0.7	F, I
ALP	159.4 ± 9.0^{ab}	176.7 ± 16.8^{a}	169.9 ± 8.4^{a}	126.8 ± 18.9^{ab}	149.3 ± 11.3^{ab}	104.0 ± 14.2^{b}	156.0 ± 7.8^{ab}	141.3 ± 21.3^{ab}	153.1 ± 10.2^{ab}	F
TG levels										
Serum (mmol/L)	0.7 ± 0.1^{a}	0.7 ± 0.1^{a}	1.3 ± 0.2^{bc}	0.8 ± 0.0^{a}	1.1 ± 0.0^{b}	1.5 ± 0.1^{c}	0.7 ± 0.0^{a}	0.8 ± 0.1^{a}	1.5 ± 0.1^{c}	F, I
Liver (µmol/g)	32.5 ± 1.6^{a}	37.1 ± 2.0^{ab}	55.5 ± 3.4^{c}	32.7 ± 2.2^{a}	31.0 ± 1.9^{a}	43.6 ± 2.7^{b}	34.0 ± 1.9^{ab}	31.6 ± 2.0^{a}	37.9 ± 2.2^{ab}	F, I, F × I
Muscle (µmol/g)	7.0 ± 1.4^{a}	7.2 ± 0.8^{a}	3.0 ± 0.5^{b}	7.3 ± 0.5^{a}	11.1 ± 1.7^{c}	4.2 ± 0.8^{b}	7.3 ± 0.8^{a}	10.4 ± 1.2^{c}	3.9 ± 0.6^{b}	I
Hepatic TG-SR (nmol/ml/min)	173.9 ± 14.8^{a}	331.5 ± 26.6^{bc}	208.8 ± 9.2^{a}	193.3 ± 13.6^{a}	295.0 ± 37.1^{c}	195.0 ± 14.3^{a}	209.7 ± 20.1^{a}	312.7 ± 34.2^{c}	417.8 ± 32.2^{b}	F, I, F × I
Total LPL activities										
U/g EWAT	0.9 ± 0.0^{a}	1.7 ± 0.2^{b}	2.3 ± 0.2^{c}	0.8 ± 0.1^{a}	1.8 ± 0.1^{b}	2.4 ± 0.0^{c}	1.1 ± 0.1^{a}	1.9 ± 0.1^{bc}	2.2 ± 0.1^{bc}	I
U/EWAT	0.5 ± 0.0^{ab}	0.5 ± 0.1^{b}	0.3 ± 0.0^{c}	0.6 ± 0.1^{ad}	0.5 ± 0.0^{b}	0.5 ± 0.0^{b}	0.8 ± 0.1^{d}	0.5 ± 0.1^{bc}	0.3 ± 0.0^{c}	F, I, F × I
U/g muscle	11.5 ± 0.7^{ab}	12.8 ± 0.7^{b}	12.2 ± 0.4^{b}	9.9 ± 0.6^{a}	12.6 ± 0.6^{b}	9.9 ± 0.5^{a}	10.0 ± 0.5^{a}	11.7 ± 0.5^{b}	10.2 ± 0.4^{a}	F, I

The results are expressed as mean values with their standard errors for six animals per group.

^{a,b,c,d}Mean values within a row with dissimilar superscript letters were significantly different ($P < 0.05$) by Tukey's critical range test after ANOVA (3 × 3). F, effect of dietary fat source; I, effect of absence or presence of CLA isomers; NS, not significant.

O, olive; RA, rumenic acid; mix-CLA, *c*9,*t*11-CLA and *t*10,*c*12-CLA in equimolecular amounts; CLA, conjugated linoleic acid; M, maize; R, rapeseed; ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; TG, triacylglycerol; TG-SR, hepatic TG secretion rate; LPL, lipoprotein lipase; EWAT, epididymal white adipose tissue.

Table V
Effect of experimental diets on lipogenic and oxidative enzyme activities

	O	*O+RA*	*O+mix-CLA*	*M*	*M+RA*	*M+mix-CLA*	*R*	*R+RA*	*R+mix-CLA*	*ANOVA*
Lipogenic enzyme activities										
Liver (mU/mg protein)										
FAS	29.7 ± 5.5^{a}	59.5 ± 7.1^{bc}	82.5 ± 2.6^{d}	31.0 ± 4.2^{a}	72.9 ± 3.4^{cd}	50.3 ± 2.5^{b}	33.3 ± 3.8^{a}	49.3 ± 4.3^{b}	62.5 ± 5.7^{bc}	I, F × I
ME	88.5 ± 1.5^{ab}	197.3 ± 6.5^{c}	328.8 ± 5.6^{d}	67.9 ± 5.6^{a}	137.4 ± 13.3^{e}	180.4 ± 3.2^{c}	102.2 ± 7.0^{abe}	122.1 ± 8.4^{be}	207.1 ± 12.6^{c}	F, I, F × I
G6PDH	12.6 ± 1.9	8.8 ± 2.2	10.0 ± 0.9	10.4 ± 1.3	8.2 ± 2.1	8.1 ± 1.6	9.4 ± 2.5	8.4 ± 1.6	6.7 ± 0.8	NS
EWAT (mU/mg protein)										
FAS	25.9 ± 2.1^{a}	65.0 ± 5.9^{b}	55.3 ± 1.6^{b}	25.6 ± 3.6^{a}	56.4 ± 6.1^{b}	51.7 ± 9.0^{bc}	28.2 ± 1.3^{ac}	60.4 ± 5.2^{b}	54.5 ± 6.3^{b}	I
ME	61.8 ± 3.8^{a}	248.6 ± 13.2^{b}	211.9 ± 14.0^{bc}	52.8 ± 4.7^{a}	199.6 ± 12.9^{bc}	165.3 ± 5.3^{c}	54.4 ± 6.3^{a}	231.9 ± 16.3^{b}	222.5 ± 12.2^{b}	F, I
G6PDH	182.3 ± 14.6^{a}	654.3 ± 11.1^{b}	625.1 ± 20.6^{b}	150.2 ± 12.6^{a}	506.9 ± 76.7^{bc}	427.3 ± 50.1^{c}	155.5 ± 18.9^{a}	577.3 ± 40.0^{bc}	598.1 ± 30.5^{bc}	F,I
EWAT (U/EWAT)										
FAS	0.2 ± 0.0^{a}	0.4 ± 0.0^{b}	0.1 ± 0.0^{a}	0.2 ± 0.0^{a}	0.4 ± 0.1^{b}	0.2 ± 0.0^{a}	0.3 ± 0.0^{ab}	0.4 ± 0.1^{b}	0.2 ± 0.0^{a}	I
ME	0.6 ± 0.1^{ab}	1.3 ± 0.1^{c}	0.5 ± 0.1^{a}	0.5 ± 0.1^{a}	1.2 ± 0.0^{c}	0.5 ± 0.0^{a}	0.5 ± 0.1^{ab}	1.3 ± 0.0^{c}	0.8 ± 0.0^{b}	F, I, F × I
G6PDH	1.3 ± 0.2^{a}	3.7 ± 0.3^{b}	1.2 ± 0.2^{a}	1.1 ± 0.1^{a}	3.2 ± 0.2^{b}	1.0 ± 0.1^{a}	1.3 ± 0.2^{a}	3.6 ± 0.4^{b}	1.2 ± 0.2^{a}	I
*CPT-I activities (mU/mg protein)**										
Liver	4.2 ± 0.4^{ab}	4.4 ± 1.1^{abc}	5.7 ± 0.9^{abc}	3.3 ± 0.7^{a}	7.7 ± 0.7^{c}	3.3 ± 0.5^{a}	6.7 ± 0.2^{c}	7.6 ± 0.8^{bc}	6.8 ± 0.4^{bc}	F, I, F × I
Muscle	1.1 ± 0.1^{a}	2.0 ± 0.4^{ab}	5.5 ± 0.2^{cd}	2.9 ± 0.6^{be}	3.5 ± 0.2^{be}	6.6 ± 0.3^{d}	4.0 ± 0.2^{ce}	3.0 ± 0.7^{be}	6.4 ± 0.3^{d}	F, I

The results are expressed as mean values with their standard errors for six animals per group.
^{a,b,c,d,e}Mean values within a row with dissimilar superscript letters were significantly different ($P < 0.05$) by Tukey's critical range test after ANOVA (3 × 3). F, effect of dietary fat source; I, effect of absence or presence of CLA isomers; NS, not significant.
O, olive; RA, rumenic acid; mix-CLA, *c*9,*t*11-CLA and *t*10,*c*12-CLA in equimolecular amounts; CLA, conjugated linoleic acid; M, maize; R, rapeseed; FAS, fatty acid synthase; ME, malic enzyme; G6PDH, glucose-6-phosphate dehydrogenase; EWAT, epididymal white adipose tissue; CPT-I, carnitine palmitoyltransferase-I.
*CPT-Ia in liver, CPT-Ib in muscle.

energy expenditure via the family of uncoupling proteins.

Different sources of dietary fats had a dissimilar impact on the body composition of the animals. Strikingly, mice fed the R diet, showed an increased body weight gain associated with an enhanced energy efficiency and fat accretion. Even though it is generally known that n-3 PUFA reduce adiposity, most of the beneficial effects of n-3 PUFA are attributed to EPA and DHA. However, there is not enough evidence supporting that ALA-rich oils provide the same physiological effects[28]. Thus, in agreement with our results but not with other results from animals fed with ALA-rich diets, Sealls et al.[29] in mice fed diets containing lard (low in n-3 PUFA), rapeseed oil or DHA+EPA-rich oil showed a larger size of the EWAT in the rapeseed oil fed animals. These results were explained by the activation of both transcription factor sterol regulatory element binding protein-1c (SREBP-1c) and peroxisomal proliferator activated receptor-γ (PPARγ) when ALA levels were high, raising the lipogenesis in adipose tissue. In the animals fed with the R diet, the RA-rich oil supplementation decreased body weight gain associated with lower fat depots. The magnitude of the lowering effect of RA-rich oil on body weight gain was significantly lower than that observed by mix-CLA supplementation, but interestingly no hepatomegaly or other adverse effects were present in RA-rich oil fed mice. Independently of the effect on body weight gain, a reduction of the EWAT was found in all animals fed diets supplemented with RA-rich oil. The results of individual RA (purity: 90%) on body and adipose tissue weight are controversial[30], and the biochemical mechanisms proposed are unclear. However, in our experimental conditions the fat reduction induced by RA-rich oil could be related to lower total LPL enzyme activities observed in M+RA and R+RA, but not in O+RA groups. Akahoshi et al.[31] did not find changes in body weight gain of RA (purity: 80%) fed rats for 26 d; however, they found a differential response depending on the adipose tissue considered. In this regard, they showed a lower perirenal adipose tissue weight associated with a tendency of lower epididymal fat pads and a raised brown adipose tissue. Lopes et al.,[30] demonstrated that RA (purity: 90%) fed rats had larger adipocytes than those fed *t*10,*c*12-CLA (purity: 90%), without any differences in the final fat pad weights.

It is very well known that dietary fats, as well as CLA might differentially alter the lipid metabolism, changing the levels of TG in the plasma and tissues of animals[15,23]. Besides, there is no systematic study dealing with dietary fats, representing different proportions of n-9, n-6 and n-3 FA, supplemented with diverse types or preparations of CLA on TG regulation. The serum TG levels are regulated, among other mechanisms, by the liver TG-SR and by the peripheral TG removal in the white adipose tissue and skeletal muscle. Mix-CLA increased the serum TG in animals fed with different fat sources which could be explained by a lower adipose tissue total LPL activity and in the R+mix-CLA group, additionally, by a higher hepatic TG-SR. On the other hand, RA-rich oil increased the serum TG levels only in animals fed the M+RA diet and this effect could be due to the higher hepatic TG-SR associated with a lower adipose tissue total LPL enzyme activity. The same effect of RA-rich oil on liver TG-SR and total LPL activity was observed in animals fed the R+RA diet; however, no changes in plasma TG concentrations were demonstrated. At least to our knowledge, there is no study on the physiological mechanisms that could explain this regulatory state observed in the R+RA group, but it could be hypothesized that the composition of the TG-rich lipoprotein of the animals fed the M+RA diet might have a lower affinity for the LPL enzyme than for those lipoproteins of the animals fed the R+RA diet. This hypothesis does not preclude that other biological mechanisms could be implicated. Similar results on serum TG concentration have been observed by de Deckere et al.[32] in hamsters fed with mix-CLA and with individual isomers: *t*10,*c*12-CLA and *c*9,*t*11-CLA. However, a different response was obtained in hamsters by other authors[5]. The lowering effect of mix-CLA has also been observed by our group[15] in mice fed hyperlipidaemic diets. Therefore, there is controversial response of serum TG by CLA and this effect could be related to species, animal model, isomer and type of dietary fat, among other factors.

The increased hepatic TG induced by mix-CLA in O+mix-CLA and M+mix-CLA groups, were clearly related with an unbalance between lipogenesis/β-oxidation that was not compensated with a higher hepatic TG-SR. Specifically, we observed a stimulation of hepatic lipogenic enzyme activities: FAS and ME without changes in CPT-Ia activities in all mix-CLA-fed animals. Surprisingly, we found a differential effect in the R+mix-CLA group, where the liver TG-SR was notoriously increased, leading to an enhanced TG output that prevented the hepatic steatosis. The supplementation with RA-rich oil to the three dietary fats also raised the FAS and ME, without changes in CPT-Ia activity; but in all these groups the increased hepatic TG-SR could counteract and avoid the TG accretion in liver. The hepatic steatosis observed in mice fed O+mix-CLA and M+mix-CLA diets have been discussed in different conditions by other authors[3,15,23] mainly by the same biological mechanisms. In contrast, little information has been reported on hepatic TG levels in animals fed the *c*9,*t*11-CLA isomer but there is consensus on the absence of changes[22,23] in liver TG content. According to Clément et al.[22] hepatic steatosis induced by the pure *t*10,*c*12-CLA isomer, specifically in mice, is secondary to hyperinsulinemia, which causes an increased FA uptake and synthesis, while hepatic steatosis was not noted in pure *c*9,*t*11-CLA-fed mice, which remained normoin-

sulinemic. In addition, Degrace et al.[23] proposed that pure *t*10,*c*12-CLA enhanced the malonyl-CoA generation and thus inhibited the CPT-I and FA oxidation. This mechanism together with an increased lipoprotein uptake and lipogenesis could explain the hepatic steatosis of *t*10,*c*12-CLA fed mice not induced by *c*9,*t*11-CLA.

White adipose tissue and muscle are key regulatory tissues for lipid metabolism and mobilization. Our results of the decreased EWAT weight in mix-CLA-fed mice are in agreement with the reduction of total LPL activity (expressed per total EWAT), and also with the results of other authors[33]. The lower availability of FA in adipose tissue could explain the increased lipogenic enzyme activities observed in this study and in others[34], attempting to compensate the lower TG depots. However, comparing with mix-CLA, RA-rich oil supplementation decreases the total LPL enzyme activity to a lower extent, which seems to be not compensated by the increased lipogenesis in the adipocytes leading to a minor reduction of EWAT weights. Interestingly, we could not discard the possibility that other mechanisms might explain the reduction of EWAT, because in the O+RA group the reduction in EWAT was present parallel to normal total LPL activity. The TG content in muscle were decreased by mix-CLA and, at least in part, could be associated with a high CPT-Ib activity and not with a lower uptake by muscle total LPL activity. The CPT-I enzyme plays a key role in the transport of FA across the mitochondrial membrane; thus, FA might be oxidized obtaining acetyl CoA and energy. These results support further evidence showing that mix-CLA supplementation increases the β-oxidation in skeletal muscle[35] A very different effect was observed by RA-rich oil and depended on the fat source. Thus, RA-rich oil supplementation increased the muscle TG depots in M+RA and R+RA, but not in O+RA groups, and this fat accretion correlated with a raised muscle total LPL activity, without changes in CPT-Ib activity. Further studies on the comparison between the supplementation of mix-CLA or RA-rich oil to dietary fats are needed to elucidate the biological mechanisms involved in muscle TG regulation.

In brief, CLA modified the TG metabolism having different effects depending on the composition of CLA preparation, proportions of dietary unsaturated FA and tissue. The magnitude of reduction of fat in carcass and EWAT was higher in the mix-CLA than in the RA-rich oil supplemented diets, independently of the proportions of n-9, n-6 and n-3 FA presented in dietary fats. However, RA-rich oil showed beneficial effects without hepatomegaly, steatosis, or hypertriglyceridaemia. As a consequence of interactions between CLA and dietary fats, it is highlighted that rapeseed oil prevented the hepatic steatosis observed in mice fed olive and maize oils supplemented with mix-CLA by increasing TG-SR; and olive oil, by an equilibrium between FA uptake/oxidation prevented the increase of muscle TG induced by the RA-rich oil supplementation to maize and rapeseed oils. Thus, the proportions of dietary unsaturated FA modulated the different mix-CLA and RA-rich oil response to lipid metabolism in mice.

Even though the experimental results in animal models, and specifically in mice, cannot be directly extrapolated to humans, knowledge of the mechanisms involved in the benefical effects of natural and commercial CLA when ingested with different edible oils might be useful for the development of functional foods effective to prevent some metabolic disorders observed in human non-communicable chronic diseases.

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