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Cis-9, *trans*-11 and *trans*-10, *cis*-12 CLA Mixture does not Change Body Composition, Induces Insulin Resistance and Increases Serum HDL Cholesterol Level in Rats

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Abstract: Synthetic supplements of conjugated linoleic acid (CLA) containing 50:50 mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers have been commercialized in some places for reducing body fat. However the safety of this CLA mixture is controversial and in some countries the CLA usage as food supplement is not authorized. Changes in insulinemic control and serum lipids profile are potential negative effects related to consumption of CLA mixture. The present study aimed to evaluate the effects of a diet containing mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA on prevention of obesity risk as well as on potential side effects such as insulin resistance and dyslipidemia in Wistar rats. Thirty male Wistar rats were randomly assigned to the following dietary treatments (n=10/group), for 60 days: Normolipidic Control (NC), diet containing 4.0% soybean oil (SO); High Fat-Control (HF-C), diet containing 24.0% SO; High Fat-synthetic CLA (HF-CLA), diet containing 1.5% of an isomeric CLA mixture (Luta-CLA 60) and 22.5% SO. Luta-CLA 60 (BASF) contained nearly 60% of CLA (*cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA at 50:50 ratio). The HF-CLA diet contained 0.3% of each CLA isomer. HF-CLA diet had no effect on dietary intake and body composition. HF-CLA-fed rats had lower levels of PPARγ protein in retroperitoneal adipose tissue, hyperinsulinemia compared to HF-C-fed rats, hyperglycemia compared to NC-fed rats while no differences in glycemia were observed between NC and HF-C groups, increased HOMA index and higher levels of serum HDL cholesterol. Thus, feeding rats with a high fat diet containing equal parts of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers had no effect on body composition and induced insulin resistance. Despite HF-CLA-fed rats had increased serum HDL cholesterol levels, caution should be taken before synthetic supplements containing *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA are recommended as a nutritional strategy for weight management.

Key words: *cis*-9, *trans*-11 CLA; *trans*-10, *cis*-12 CLA; obesity; insulin resistance; dyslipidemia

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1 INTRODUCTION

The prevalence of overweight and obesity is increasing at an alarming rate[1]. According to World Health Organization[2], once considered a problem only in high income countries, overweight and obesity are now dramatically on the rise in low- and middle-income countries. In 2008 a total of more than half a billion adults were obese worldwide[3] and it is projected that this number will rise to 1.12 billion by 2030[1]. Furthermore, overweight and obesity are major risk factors for a number of chronic diseases, since visceral adipose tissue is an independent risk factor for insulin resistance, type 2 diabetes mellitus[4], hypertension, subclinical inflammation and dyslipidemia, all factors leading to atherosclerosis[5].

There is compelling evidence that diet plays an important role in the prevention of a number of non-communicable diseases, including obesity and type-2 diabetes[6]. In this context, conjugated linoleic acid (CLA) has attracted considerable attention in the scientific community due to its health-promoting properties reported in a number of *in vitro* and animal studies[7]. CLA refers to the positional and geometric conjugated dienoic isomers of linoleic acid (C18:2 n-6)[8] which are predominantly found in the lipid fraction of meat, milk and other dairy products from ruminant[9]. Although nearly twenty isomers have been identified in ruminant products[10], *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA are the more biologically active[11] and they are the major CLA isomers in the commercial preparations[12].

Diet containing mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA or containing *trans*-10, *cis*-12 CLA alone have been shown as having anti-obesity properties[8, 13], such as decreased body fat mass, increased lean body mass[12] and reduction of energy intake[11]. Synthetic supplements of CLA containing 50:50 mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers have been commercialized in some places for reducing body fat[14]. However the safety of CLA isomer mixture to assist in weight loss is not unanimity in the scientific community and the CLA isomer mixture usage as food supplement is not authorized in some countries[15, 16]. Hyperinsulinemia associated with insulin resistance[8] which may be responsible for increasing type 2 diabetes risk, decreased serum HDL cholesterol and increased serum LDL cholesterol, are side effects which have been already reported as related to CLA isomer mixture consumption[15].

Thus, use of mixture of active CLA isomers *cis*-9, *trans*-11 and *trans*-10, *cis*-12 as dietary supplement is controversial and mechanisms of action for CLA effects are not yet fully understood[11]. More studies are needed to elucidate these mechanisms of action, providing valuable information on the efficacy, specificity, and potential side effects of CLA isomer mixture, before its use as a dietary strategy for weight loss. In light of potential anti-obesity properties and side effects of conjugated linoleic acid, we investigated the effects of a diet containing mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA on preventing obesity risk as well as on insulin resistance and dyslipidemia in Wistar rats.

2 EXPERIMENTAL

2.1 Ethics statement

This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals[17]. All procedures with animals were approved by the Ethic Committee on Animal Experimentation of Federal University of Juiz de Fora at Minas Gerais, Brazil, protocol number 053/2012.

2.2 Animals

Thirty (n = 30) male Wistar rats (*Rattus norvegicus Berkenhout, 1769*), 60 days old and weighing 250-300 g, were obtained from the Center of Reproduction Biology of the Federal University of Juiz de Fora (UFJF), Minas Gerais, Brazil. They were kept in a controlled temperature environment (23 ± 2℃) with a photoperiod of 12 hours (7 a.m. to 7 p.m. - light and 7 p.m. to 7 a.m. - dark). Water and the experimental diets were offered on an *ad libitum* basis to the animals throughout the study.

2.3 Dietary treatments and design experimental

After a 7 days acclimatization period in which all animals were fed a commercial chow (Nuvital, Colombo, PR, Brazil), the rats were randomly assigned to three dietary treatments (n = 10/group), for 60 days: Normolipidic Control (NC), diet containing 4.0% soybean oil (SO); High Fat-Control (HF-C), diet containing 24.0% SO; High Fat-enriched synthetic CLA (HF-CLA), diet containing 1.5% Luta-CLA 60 and 22.5% SO. Luta-CLA 60 (BASF AG, São Paulo, Brazil) is composed of 30% *cis*-9, *trans*-11 CLA, 30% *trans*-10, *cis*-12 CLA, 27.4% C18:1 *cis*-9/*trans*-15, 4.06% C16:0, 3.64% C18:0, 1.23% C18:2 *cis*-9, *cis*-12, 0.06% C14:0.

All diets were produced according to the American Institute of Nutrition (AIN-93M)[18]. Ingredients were carefully mixed in order to obtain a homogeneous mass which was used to produce handmade pellets. The pellets were prepared weekly, purged with nitrogen and stored at −20℃ in daily portions in sealed polythene bags to minimize the oxidation of fatty acids. The composition of purified diets is presented in Table 1.

Samples of pellets (50 g) from each diet were randomly collected and analyzed for chemical composition according to reference methods[19, 20]. To determine the fatty acid composition of experimental diets, total lipids were extracted according to Hara and Radin[21]. Fatty acid methyl esters (FAME) were obtained according to Christie[22] with

Table 1 Composition of purified experimental diets.

	NC[1]	HF-C[2]	HF-CLA[3]
	% of the diet (g/100g of total diet)		
Corn starch[4]	46.6	29.1	29.1
Dextronized corn starch[4]	15.5	15.5	15.5
Casein[5]	14.0	17.3	17.3
Sucrose[5]	10.0	10.0	10.0
Cellulose[4]	5.0	5.0	5.0
AIN-93 mineral mix[5]	3.5	3.5	3.5
AIN-93 vitamin mix[5]	1.0	1.0	1.0
L-Cystine[4]	0.18	0.18	0.18
Choline bitartrate[4]	0.25	0.25	0.25
tert-Butylhydroquinone[5]	0.01	0.01	0.01
Soybean oil	4.00	24.00	22.5
CLA mix[6]	–	–	1.50

[1] Normolipidic Control; [2] High Fat-Control; [3] High Fat-CLA; [4, 5] Dietary ingredients were purchased from Farmos (Rio de Janeiro, RJ, Brazil) and Rhoster (Araçoiaba da Serra,SP,Brazil), respectively; [6] Luta-CLA 60 (BASF AG, São Paulo, Brazil) composed of 60% CLA with a 50:50 ratio of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA.

modifications[23] and determined by gas chromatography (model 6890 N; Agilent Technologies Brasil Ltda., Barueri, Brazil) fitted with a flame-ionization detector and equipped with a CP-Sil 88 fused silica capillary column (100 m × 0.25 mm × 0.2 μm film thickness; Varian Inc., Mississauga, ON). Operating conditions included injector and detector temperatures both at 250℃, H_2 as the carrier gas (1 mL/min), and for the flame-ionization detector (35 mL/min), N_2 as the makeup gas (30 mL/min), and purified air (286 mL/min). The FAME were identified by comparison with 4 FAME reference standards (Supelco37 mix #47885-U, linoleic acid isomers mix #47791, CLA isomers mix #05632; Sigma-Aldrich, St. Louis, MO, and Nu-Chek GLC-463); minor *trans*-18:1 isomers were identified according to their elution order reported under the same chromatographic conditions[24, 25]. The fatty acid composition of experimental diets was expressed as a weight percentage of total fatty acids using theoretical relative response factors described by Wolff[26]. The chemical composition and the fatty acid profile of experimental diets are presented in Table 2.

The CLA isomers contents in HF-CLA diet was calculated as follows: (dry matter content of the diet) x (fat content x 0.95) x (Concentration of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA in g/100g of total fatty acids). The 5% discount on fat content was applied to correct for the glycerol concentration in triacylglycerol molecules[27]. Based on the above-mentioned calculations, the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA contents in HF-CLA diet were 0.3% and 0.3%, respectively.

The rats were provided (between 11 a.m. and 12 p.m.) fresh food (F_i) *ad libitum* daily and the refusals were weighed the next day (F_f), immediately before the provision of another F_i. Average food intake (grams/animal) was estimated as follows: (F_i - F_f)/5 (number of animals per cage). Individual body weight was measured every 5 days throughout the treatment period. After the treatment period, the rats were fasted for 12 hours (7 a.m. to 7 p.m.) and blood samples collected from a tail nick for glycemic determinations using the glucose oxidase method[28]. Immediately after glycemic determinations, animals were anesthetized with an intraperitoneal injection of a xylazine (10 mg/Kg)/ketamine (90 mg/Kg) solution, and euthanized by total exsanguination. Glycemic determinations were performed prior to anesthesia as it was shown to induce hyperglycemia[29]. After euthanasia, it was determined the liver weight, blood and adipose tissue samples and carcasses were analyzed for parameters described below.

2.4 Analysis of carcass chemical composition

The carcasses were eviscerated, being the gastrointestinal contents and the brain removed while all fat depots, including the perivisceral fat, remained in the carcasses, which were sliced, stored at −80℃, lyophilized (model Liotop L120; Liobras, São Carlos, Brazil) and minced in a knife-type mill. The carcasses were weighed before and after lyophilization to determine their dry matter content. Moisture, ash, protein and lipid contents were determined according to AOAC[19]. Protein content was quantified using

Table 2 Chemical composition and fatty acid profile of experimental diets.

	NC[1]	HF-C[2]	HF-CLA[3]
	Chemical composition, % of diet dry matter		
Dry matter content (%)	79.1	88.4	88.3
Fat	3.11	21.07	20.9
Crude protein	13.1	14.83	15.2
Ash	2.66	2.95	2.95
Neutral Detergent Fiber (NDF)	2.76	3.89	3.00
Carbohydrate	55.4	42.75	44.5
	Energetic composition		
Carbohidrate Energy (%)	73.4	40.7	41.7
Protein Energy (%)	17.3	14.1	14.3
Fat Energy (%)	9.25	45.2	44.0
Kcal/g	2.39	3.71	3.77
	Fatty acid profile (g/100 g of total fatty acids)		
C14:0	0.14	0.13	0.12
C16:0	10.9	10.8	10.6
C18:0	4.44	4.06	4.06
C18:1 *cis*-9/*trans*-15	23.5	22.4	23.5
C18:2 *cis*-9 *cis*-12	49.8	52.4	49.8
C20:0	0.41	0.35	0.30
C20:1 *cis*-11	0.04	0.03	0.03
C18:3 *cis*-9 *cis*-12 *cis*-15	6.11	6.58	5.81
CLA *cis*-9 *trans*-11	n.d.[4]	n.d.	1.70
CLA *trans*-10 *cis*-12	n.d.	n.d.	1.77
C22:0	0.45	0.30	0.21
C24:0	0.04	0.05	0.07

[1] Normolipidic Control, diet containing 4% of soybean oil (SO); [2] High fat-Control, diet containing 24% SO; [3] High fat-enriched synthetic CLA, diet containing 1.5% Luta-CLA 60 (BASF AG, São Paulo, Brazil) and 22.5% SO. Luta-CLA 60 is composed of 60% CLA with a 50:50 ratio of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA; [4] n.d.: not detected.

the Kjeldahl method[19] with Foss equipment (model Kjeltec 8400, Foss, Hillerød, Denmark) and lipid content was determined using the Ankom procedure with an Ankom extractor (model XT10, Ankom Technology, New York, USA).

2.5 Analysis of PPARγ protein level by Western blot

Retroperitoneal adipose tissue samples were homogenized in a lysis buffer [Tris-HCl: 50 mM, pH 7.4, $Na_4P_2O_7$: 30 mM, NP-40: 1%, Triton (1%), SDS: 0.1%, NaCl: 150 mM, EDTA: 5 mM, NaF: 50 mM, plus Na_3VO_4: 1 mM and protease inhibitor cocktail (Roche Diagnostics, Mannheim, DE)] using an Ultra-Turrax homogenizer (IKA Werke, Staufen, DE). After centrifugation (7500 × g for 5 min), the homogenates were stored at −20℃ until SDS-PAGE assay. The total protein content of homogenate was determined by the BCA protein assay kit (Pierce, Illinois, USA). Contents of peroxisome proliferator-activated receptor (PPAR) γ and β-tubulin (loading control) proteins in the retroperitoneal adipose tissue samples were evaluated by individually incubating monoclonal primary antibodies (anti-PPARγ and anti-β-tubulin; 1:1000; from Abcam, Cambridge, UK) overnight at 4℃, followed by proper secondary antibody (1 hour; 1:7000 antibody from Sigma-Aldrich Co., Missouri, USA) and streptavidin (1 hour; 1:7000; Zymed, California, USA) incubation. The protein bands were visualized by chemiluminescence with Kit ECL Plus (GE Healthcare Life Sciences, Buckinghamshire, UK) followed by exposure in the ImageQuant™ LAS 500 (GE Healthcare Life Sciences).

Area and density of the bands were quantified by Image J software (Media Cybernetics, Maryland, USA). The results were normalized by β-tubulin content and expressed as relative (%) to the normolipidic control group.

2.6 Serum metabolites

Blood samples were collected from euthanized animals by cardiac puncture and centrifuged (5714 × g for 5 min) for serum separation. Serum insulin levels were determined using a rat insulin ELISA kit (Mercodia, Uppsala, Sweden). Serum non-esterified fatty acids (NEFA) levels were analyzed using a colorimetric kit (Randox Laboratories, Antrim, United Kingdom), while leptin was analyzed using a Leptin ELISA kit (R&D Systems, Minneapolis, USA). Serum levels of cholesterol[30], triacylglycerol[31], high-density lipoprotein (HDL) cholesterol[32] and low-density lipoprotein (LDL) cholesterol[33] were determined by colorimetry using the BT 3000 equipment from Wiener laboratories.

2.7 HOMA and R-QUICKI

Homeostatic Model Assessment (HOMA) index was calculated as follows: [fasting insulin (ng/mL) × fasting glucose (mM)]/22.5. A high HOMA index denotes low insulin sensitivity[34], although it should be acknowledged that the HOMA model has not been validated for use in animal models[35] Revised Quantitative Insulin Sensitivity Check Index (R-QUICKI) is another equation to assess insulin sensitivity[36]. This index was calculated as following: [1/log fasting insulin (mU/mL) + log fasting glucose (mg/dL) + log NEFA (mmol/L)][36].

2.8 Oral glucose tolerance test (OGTT)

After 55 days on the experimental diets, the rats were fasted for 12 hours (7 a.m. to 7 p.m.) and received a 50% glucose solution (2 g/kg body weight) by oral gavage[37−40]. Blood samples were collected from a tail nick for glycemic determinations using the glucose oxidase method[28] at 0, 30, 60, 90, 120 and 240 minutes post gavage. Due to reasons previously described[29], anesthesia was not used in the OGTT. Changes in blood glucose concentration during the oral glucose tolerance test were evaluated by the estimate of the total area under the curve (AUC).

2.9 Statistical analysis

Results were expressed as means ± SEM (standard error mean). The statistical analyses were performed using Prism 5.0 (GraphPad Software, Inc). Data from different dietary groups were analyzed by one-way ANOVA for overall significance followed by Tukey's post-hoc tests to identify differences between treatment groups. Treatment effects and differences between means were considered significant when $p < 0.05$.

3 RESULTS

Food intake of HF-C and HF-CLA diets was 28.55% and 26.69% lower than NC food intake, respectively, while no difference was observed between HF-C and HF-CLA (Table 3). The energy intake observed in rats fed with the HF-C and HF-CLA diets was 11.05% and 15.68% higher than in NC-fed rats, respectively, but there was no difference between HF-C and HF-CLA (Table 3). No differences in initial and final body weight and weight gain (expressed as a percentage of initial weight) were observed among groups (Table 3).

The effect of NC, HF-C and HF-CLA diets on body weight during all experimental period is shown in Fig. 1. On the 10th day, body weight of rats fed with HF-C and HF-CLA diets were both decreased in comparison to those fed with the NC diet (Fig. 1).

According to carcass chemical composition, no differences in moisture, lipid, protein and ash contents was observed among treatment groups (Table 3). PPARγ protein levels in adipose tissue were decreased by 38.06% and 29.80% in HF-CLA-fed rats in comparison to those fed with the NC and HF-C diets, respectively (Fig. 2).

Fasting serum insulin levels were increased by 25.63% in HF-CLA-fed rats in comparison to those fed with HF-C diet, (Fig. 3A). Glucose concentration was increased by 8.90% in HF-CLA-fed rats in comparison to those fed with the NC diet, while no differences in glycemia were observed between NC and HF-C groups (Fig. 3B).

There was no difference in serum NEFA levels between rats fed with the HF-CLA and HF-C diets, but values observed in high fat control group was 21.60% lower than in NC (Table 3). Serum concentrations of leptin did not differ among dietary treatments (Table 3).

HOMA index was increased by 29.85% and 32.74% in HF-CLA-fed rats in comparison to those fed with the NC and HF-C diets, respectively (Fig. 4A). R-QUICKI index was unchanged by the dietary treatments (Fig. 4B). The area under the OGTT glycemic curve (AUC) did not differ among dietary treatments (Table 3).

Serum cholesterol levels of rats fed with the HF-C and HF-CLA diets were both decreased by 18.27% and 11.96% compared to those fed with the NC diet, respectively (Fig. 5A), as well as serum triacylglycerol levels of rats fed with the HF-C and HF-CLA diets were both decreased by 31.29% and 26.73% compared to those fed NC diet (Fig. 5B). Serum levels of HDL cholesterol were increased by 10.08% and 23.29% in HF-CLA-fed rats as compared to those fed with both the NC and HF-C diets, respectively (Fig. 5C). There was no difference in serum LDL cholesterol levels between rats fed with the HF-CLA and HF-C diets, but values observed in these groups were 28.32% and 23.60% lower than in NC group, respectively (Fig. 5D). Liver weights of HF-C and HF-CLA groups was 23.20% and 21.88% lower than NC value, respectively,

Table 3 Metabolic and serum parameters in Wistar rats fed with mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA for 60 days.

	Dietary Treatments		
	NC	HF-C	HF-CLA
	Dietary intake and body and liver weights		
Intake (g/day/rat)	26.45 ± 1.06	18.90 ± 0.51***	19.39 ± 0.36***
Intake (Kcal/day/rat)	63.19 ± 2.52	70.17 ± 1.89*	73.10 ± 1.34**
Initial body weight (g)	274.50 ± 5.18	264.30 ± 4.75	259.20 ± 5.67
Final body weight (g)	433.90 ± 10.11	421.00 ± 12.17	425.10 ± 9.61
Weight gain (%)	62.15 ± 1.90	59.30 ± 3.54	64.05 ± 1.83
Liver weight (g)	17.50 ± 0.39	13.44 ± 0.52***	13.67 ± 0.44***
	Body composition		
Moisture (%)	50.10 ± 1.05	50.83 ± 1.18	49.76 ± 0.82
Lipid (%)	29.41 ± 1.39	26.14 ± 1.09	28.46 ± 0.48
Protein (%)	17.76 ± 0.32	17.57 ± 0.49	18.21 ± 0.36
Ash (%)	3.23 ± 0.09	3.87 ± 0.38	3.43 ± 0.16
	Serum metabolites and AUC		
NEFA (mmol/L)	0.375 ± 0.023	0.294 ± 0.025*	0.328 ± 0.021
Leptin (ng/mL)	2.21 ± 0.21	1.98 ± 0.19	1.80 ± 0.16
AUC	13180 ± 1505	14610 ± 1021	17400 ± 2007

Data are presented as mean values ± S.E.M (n=10 rats/group). Statistically significant differences were determined by Anova followed by Tukey. Asterisk denotes statistically significant differences compared to NC (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). [1] Normolipidic Control (NC), diet containing 4.0% soybean oil (SO); [2] High Fat-Control (HF-C), diet containing 24% SO; [3] High Fat-CLA (HF-CLA), diet containing 1.5% Luta-CLA 60 (BASF AG, São Paulo, Brazil) and 22.5% SO. Luta-CLA 60 is composed of 60% CLA with a 50:50 ratio of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA.

while no difference was observed between liver weights of high fat diets (Table 3).

4 DISCUSSION

Due to the substantial rise in obesity prevalence and chronic diseases related to overweight condition, such as type 2 diabetes, it would be advantageous to identify potential therapeutic nutrients to assist in obesity prevention[12]. In this context interest in CLA has been increasing due to its potential anti-obesity effects[12]. However the usage of 50:50 mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA as food supplement is not consensus and more studies are necessary to investigate the role of CLA isomers in obesity prevention and side effects related to CLA consumption, mainly associated with insulin resistance and dyslipidemia[15, 16]. Thus, in this paper, we have demonstrated dietary effects of a diet containing mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA in 60-day-old Wistar rats on prevention of obesity risk, as well as insulin sensitivity and profile of serum lipids.

In this study, there was no difference in dietary intake between rats fed with the HF-CLA diet and those fed with the HF-C diet. Effect of CLA on dietary intake remains controversial[41]. Several studies reported that CLA had little or no effect on food intake[42–44] while others have reported a reduction in food intake[45, 46]. Food and energy intake of HF-C and HF-CLA groups differed from the values of the NC group due to the high fat content of HF-C and HF-CLA diets. HF-C and HF-CLA-fed rats adapted to the higher energy density of HF-CLA and HF-C diets by reducing their daily food intake compared to the NC group, an effect that has been previously reported[47]. Daily energy intake was higher in HF-C and HF-CLA-fed rats than in the NC group, which may be attributed to the increased palatability of high fat diets, which is directly related to higher energetic intake[48]. High fat diets are more palatable because fat content is one of the factors that contribute to food palatability[48].

No differences among groups of dietary treatments were observed for body weight gain. In another study, an equi-

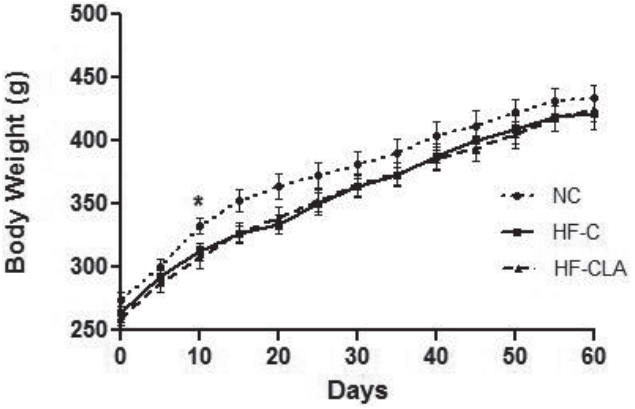

Fig. 1 Effect of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA mixture on body weight. Male Wistar rats fed the following dietary treatments for 60 days: Normolipidic Control (NC): diet containing 4.0% soybean oil (SO); High Fat-Control (HF-C): diet containing 24% SO; and High Fat-enriched synthetic CLA (HF-CLA): diet containing 1.5% Luta-CLA 60 (60% CLA with a 50:50 ratio of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA) and 22.5% SO. All data are presented as mean values ± S.E.M (n=10 rats/group). Statistically significant differences were determined by Anova followed by Tukey. *p<0.05.

molecular mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA was added to the diet of Wistar rats to reach 0.5% of active isomer *trans*-10, *cis*-12 CLA, and no differences in final body weight after 6 weeks were found between control and CLA-treated rats[49]. Similarly, no differences in body weight gain was observed between control group and animals fed with a diet containing 0.25% *cis*-9, *trans*-11 and 0.25% *trans*-10, *cis*-12 CLA[12]. In the present investigation, there was no effect of the dietary treatments on body composition. Similar results were observed in a previous study in which Wistar rats were fed with a diet containing 1% each CLA isomer[50].

Despite the lack of CLA effects on body composition, the levels in adipose tissue of PPARγ, the master adipogenic regulator[11], was lower in HF-CLA group than in NC and HF-C groups, which at first may seem contradictory since body composition was unchanged. However, differences in adipose tissue PPARγ levels is not necessarily associated with changes in body composition since PPARγ is not the only protein involved in adipogenesis[11] and, interconnected to the PPARγ role in adipocyte differentiation, this protein also regulates insulin sensitivity by transcriptionally activating genes involved in insulin signaling, glucose uptake, and fatty acid uptake and storage[51]. It has been demonstrated that depletion of PPARγ in adipose tissue causes insulin resistance, since PPARγ decreased action in mature adipocytes, leads to reduced expression of key genes required for insulin signaling in adipocytes[52]. In fact, the anti-diabetic drug family known as thiazolidinediones, mediate their insulin-sensitizing effects by directly activating PPARγ[51]. It was previously shown that adipocyte-specific constitutive activation of PPARγ in mature adipocytes can regulate whole body insulin sensitivity[53]. Effects of conjugated linoleic acid on PPARγ has been shown to be isomer-specific, with *trans*-10, *cis*-12 CLA down-regulating and *cis*-9, *trans*-11 CLA up-regulating its expression in the adipose tissue[54]. Therefore, the present study suggests that the effect on PPARγ of *trans*-10, *cis*-12 CLA prevailed over the effect of *cis*-9, *trans*-11 CLA, consequently HF-

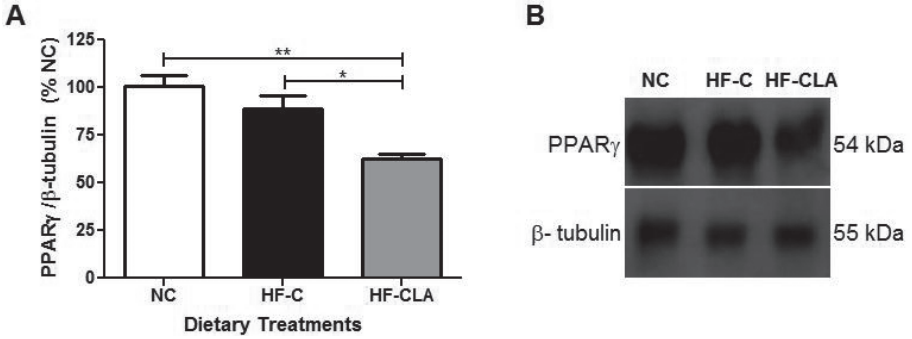

Fig. 2 Analysis of PPARγ protein level in retroperitoneal adipose tissue. PPARγ levels (A) and representative blot for PPARγ and β-tubulin (loading control) (B) of male Wistar rats fed the following dietary treatments for 60 days: Normolipidic Control (NC): diet containing 4.0% soybean oil (SO); High Fat-Control (HF-C): diet containing 24% SO; and High Fat-enriched synthetic CLA (HF-CLA): diet containing 1.5% Luta-CLA 60 (60% CLA with a 50:50 ratio of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA) and 22.5% SO. All data are presented as mean values ± S.E.M (n=10 rats/group). Statistically significant differences were determined by Anova followed by Tukey. *p<0.05, **p<0.01.

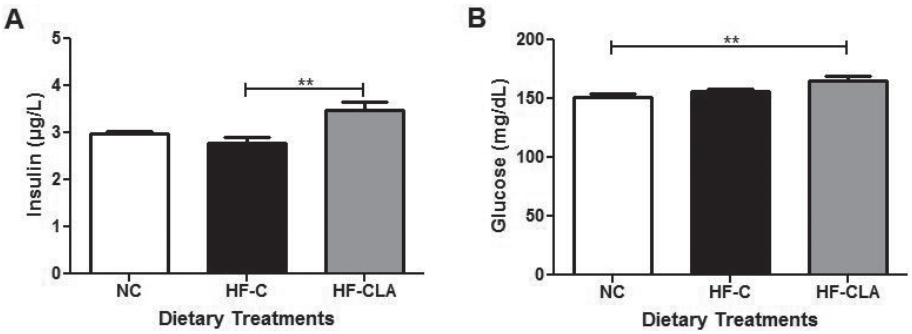

Fig. 3 Effects of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA mixture on serum metabolites. Insulin (A), glucose (B) of male Wistar rats fed the following dietary treatments for 60 days: Normolipidic Control (NC): diet containing 4.0% soybean oil (SO); High Fat-Control (HF-C): diet containing 24% SO; and High Fat-enriched synthetic CLA (HF-CLA): diet containing 1.5% Luta-CLA 60 (60% CLA with a 50:50 ratio of *cis*-9, *trans*-11 and *trans*-10 *cis*-12 CLA) and 22.5% SO. All data are presented as mean values ± S.E.M (n=10 rats/group). Statistically significant differences were determined by Anova followed by Tukey. ** $p<0.01$.

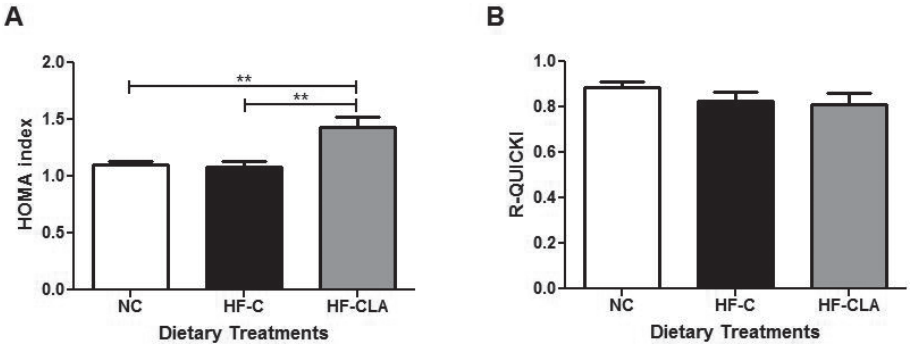

Fig. 4 Effects of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA mixture on indexes of insulin sensibility. HOMA index (A) and R-QUICKI (B) of male Wistar rats fed the following dietary treatments for 60 days: Normolipidic Control (NC): diet containing 4.0% soybean oil (SO); High Fat-Control (HF-C): diet containing 24% SO; and High Fat-enriched synthetic CLA (HF-CLA): diet containing 1.5% Luta-CLA 60 (60% CLA with a 50:50 ratio of *cis*-9, *trans*-11 and *trans*-10 *cis*-12 CLA) and 22.5% SO. All data are presented as mean values ± S.E.M (n=10 rats/group). Statistically significant differences were determined by Anova followed by Tukey. ** $p<0.01$.

CLA-fed rats presented reduced PPARγ levels in adipose tissue.

Rats fed with the diet containing mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA had higher fasting serum insulin levels than rats fed with the high fat control diet. The fasting hyperinsulinemia is an important parameter since it was demonstrated that a gradual increase in serum insulin in the fasting state reflects decreased insulin sensitivity[55]. HF-CLA-fed rats presented hyperglycemia compared to NC-fed rats, while no differences in glycemia were observed between NC and HF-C groups, and higher HOMA index than rats fed with the NC or HF-C diets, which denotes low insulin sensitivity in the HF-CLA group[34]. These results are according to previous study which showed that animals fed with diet containing the mixture of 0.25% *cis*-9, *trans*-11 and 0.25% *trans*-10, *cis*-12 CLA had hyperinsulinemia and insulin resistance, demonstrated by increased HOMA index[12]. On the other hand areas under the curves of oral glucose tolerance tests did not differ among groups of dietary treatments. Thus it is possible to hypothesize that despite the same amount of glucose was presented by NC, HF-C and HF-CLA groups during the OGTT, higher insulin levels may have been required by HF-CLA-fed rats[12, 56].

Concerning the R-QUICK, which also denotes insulin sensitivity[36], there were no differences among groups of dietary treatments. Possibly it occurred because this index considers serum NEFA levels, which was unchanged by HF-CLA diet compared to NC or HF-C diets. Similarly, it was shown in previous study that mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA did not modify serum NEFA levels[12]. Serum NEFA concentration is a risk factor for type 2 diabetes because the combination of excessive levels of non-esterified fatty acids and glucose leads to decreased insulin secretion, impairments in insulin gene expression and beta-cell death by apoptosis[57]. Serum NEFA

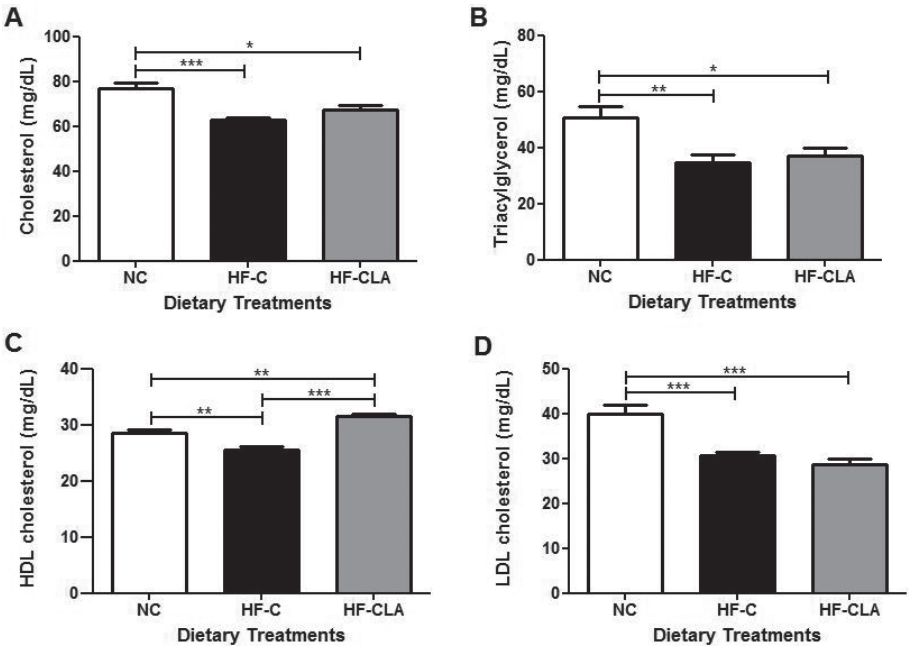

Fig. 5 Effects of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA mixture on serum lipids. Cholesterol (A), triacylglycerol (B), HDL cholesterol (C) LDL cholesterol (D) of male Wistar rats fed the following dietary treatments for 60 days Normolipidic Control (NC): diet containing 4.0% soybean oil (SO); High Fat-Control (HF-C): diet containing 24% SO; and High Fat-enriched synthetic CLA (HF-CLA): diet containing 1.5% Luta-CLA 60 (60% CLA with a 50:50 ratio of *cis*-9, *trans*-11 and *trans*-10 *cis*-12 CLA) and 22.5% SO. All data are presented as mean values ± S.E.M (n=10 rats/group). Statistically significant differences were determined by Anova followed by Tukey. **p*<0.05, ** *p*<0.01, *** *p*<0.01.

levels increased in NC-fed rats may be related to high levels of total cholesterol and triacylglycerol in this group, as was previously demonstrated[58]. Leptin is an adipokine that plays a role in glucose metabolism and insulin sensitivity[59]. In the present study there were no differences in serum leptin level among groups. Probably because body fat was unchanged among experimental groups and circulating leptin is highly correlated with adiposity, therefore individuals exhibiting higher serum leptin were indeed found to be more obese[12]. Similarly, it was shown previously that mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA did not modify plasma leptin level[60].

Concerning the serum lipid concentration, HF-C and HF-CLA-fed rats presented lower levels of total cholesterol, triacylglycerol and LDL cholesterol compared to NC-fed rats. It may be due to high levels of carbohydrate (73.39% of energy) in NC diet, since it was demonstrated that when dietary carbohydrate was increased from 50% to 67% of energy, the fasting triacylglycerol level rose[61], which is commonly related to increased precursors of LDL cholesterol in the blood, the very-low-density lipoproteins, and consequently increased LDL cholesterol levels[62]. All these changes in NC-fed rats contributed to high total cholesterol demonstrated by this group. Rats fed with the diet containing the mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA had favorable changes in serum lipoprotein profile compared to HF-C-fed rats, since the diet containing CLA isomers was responsible for high levels of HDL cholesterol while serum total cholesterol, triacylglycerol and LDL cholesterol concentrations were not modified compared to high fat control diet. Similarly, it was shown that animals supplemented with a diet containing 1% mixture with a 50:50 ratio of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA, presented plasma total cholesterol and LDL cholesterol unchanged compared to those values of control group[63]. Concerning the effect of CLA on the triacylglycerol level, previous studies in animals showed that triacylglycerol concentration was not modified by *cis*-9, *trans*-11 CLA (64-66) or by *trans*-10, *cis*-12 CLA supplementation[66]. The high level of HDL cholesterol in HF-CLA-fed rats may be attributed to *cis*-9, *trans*-11 CLA, as also reported by a previous study[67]. The increased HDL cholesterol level is potentially a beneficial result for two main reasons. Firstly, it possesses properties that have the potential to inhibit the development of atherosclerosis and thus reduce the risk of having a cardiovascular event[68]. Secondly, HDL cholesterol also increases the uptake of glucose by skeletal muscle[69] and stimulates the synthesis and secretion of insulin from pancreatic β cells[70] and may thus have a beneficial effect on glycemic control[68]. However, despite these

beneficial properties of HDL cholesterol, the increase of this molecule in HF-CLA-fed rats was not capable of preventing hyperinsulinemia, hyperglycemia or insulin resistance in this group. Thus, it is possible to hypothesize that the negative effects on insulin metabolism from decreasing PPARγ level in adipose tissue prevail over the potential positive effects related to increased HDL cholesterol level on glycemic and insulinemic control.

The liver weight of HF-CLA-fed rats did not differ of HF-C-fed rats liver weight. Similarly, it was previously demonstrated that liver weight was unchanged by *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA mixture[71]. The high liver weight of NC-fed rats may be an indicative of hepatic fat deposition due to high levels of carbohydrate in this diet. Similarly, it was previously reported that a carbohydrate-rich diet induced hepatic fat deposition[72].

The action mechanisms involved in effects of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA is not well understood yet[73]. The contradictory findings among rodent studies may be due to differences in experimental design, such as CLA isomer combination versus individual isomers, CLA dose and duration of treatment, gender, weight, age and metabolic status of the animals.

5 CONCLUSION

In conclusion, the present investigation suggests that a 60 day feeding of a diet containing mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA to 60-day-old male Wistar rats has effects on insulin, glucose and cholesterol metabolisms. Although diet containing *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA significantly raised serum HDL cholesterol, the mixture of CLA isomers was found not to change body composition, which demonstrates that this diet was useless in preventing the obesity risk, besides it was also found to cause fasting hyperinsulinemia and insulin resistance. These results suggest that caution should be taken before synthetic supplements containing *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA are recommended as a nutritional strategy for weight management.

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