

MATERNAL CONJUGATED LINOLEIC ACID CONSUMPTION PREVENTED TRIACYLGLYCEROL ALTERATIONS INDUCED BY A HIGH-FAT DIET IN MALE ADULT RAT OFFSPRING.

Original Research

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Abbreviations:

ACC: acetyl-CoA carboxylase; AIN: American Institute of Nutrition; C: soybean oil; CLA: conjugated linoleic acid; CPT-Ia: carnitine palmitoyltransferase Ia; EWAT: epididymal white adipose tissue. FA: fatty acid; FAME: fatty acid methyl esters; FAS: fatty acid synthase; G6PDH: glucose 6 phosphate dehydrogenase; GM: gastrocnemius muscle; HPRT1: hypoxanthine phosphoribosyltransferase 1; LC-PUFA: long chain-polyunsaturated fatty acid; LPL: lipoprotein lipase; PPAR α : peroxisome proliferator-activated receptor α ; SCD-1: stearoyl-CoA desaturase-1; SREBP-1c: sterol regulatory element-binding protein; TAG: triacylglycerol; TAG-SR: triacylglycerol-secretion rate; UBC: ubiquitin C.

Abstract:

Maternal nutritional programming by a high-fat (HF) diet is related to hepatic lipid accumulation and steatosis in offspring. Conjugated linoleic acid (CLA) might ameliorate impaired hepatic lipid homeostasis; therefore, the aim was to investigate the potential preventive effect of maternal CLA consumption on triacylglycerol (TAG) metabolism alterations induced by HF diets in adult male rat offspring receiving or not receiving CLA.

Female Wistar rats were fed a control (C) diet, HF diet or HF diet supplemented with CLA (HF+CLA) for 4 weeks before mating and throughout pregnancy and lactation. After weaning, for 9 weeks, male offspring of C or HF rats continued with the same diets as their mothers (C/C or HF/HF groups, respectively), and male offspring of HF+CLA rats were fed HF or HF+CLA diets (HF+CLA/HF or HF+CLA/HF+CLA groups, respectively). Nutritional parameters, serum and liver TAG levels, the TAG secretion rate, and the activities as well as gene expression of key hepatic enzymes involved in TAG regulation were assessed.

The most interesting results were that maternal CLA decreased epididymal white adipose tissue (EWAT) weight and prevented serum and liver TAG accumulation induced by a HF diet in adult male offspring receiving or not receiving CLA. The prevention of liver steatosis in HF+CLA/HF+CLA and HF+CLA/HF offspring was associated with an increased hepatic TAG secretion. Overall, this study provides evidence that maternal CLA consumption programs TAG regulation and in this way contributes to lowering lipid levels in tissues and preventing liver steatosis in particular.

Introduction

Conjugated linoleic acid (CLA) is the general name of a mixture of positional and geometric isomers of linoleic acid with conjugated unsaturated double bonds. The major natural sources of CLA are meat and dairy products from ruminant animals, where CLA is synthesized as an intermediate fatty acid (FA) during the biohydrogenation of linoleic acid to stearic acid, but it can also be synthetically produced^(1,2) for commercial purposes by different techniques⁽³⁾. Many investigations have shown that CLA may have beneficial effects on cancer, obesity, the inflammatory response, atherosclerosis and glucose and lipid metabolism^(4,5). However, controversial and detrimental health effects have also been reported⁽⁶⁾, probably due to, among other differences, the different levels, proportions and biochemical properties of the two major isomers *c9,t11*-CLA and *t10,c12*-CLA of natural and commercial CLA and to the species-specific effects^(7,8).

On the other hand, maternal obesity in humans is associated with an increased risk of obesity and metabolic-related disorders in adult offspring^(9,10). Maternal overnutrition generates changes in the uterine milieu during pregnancy, leading to developmental alterations and defects in organ function and metabolism in offspring⁽¹¹⁾. Thus, maternal nutritional programming by exposure to hypercaloric diets, such as a HF diet, is closely related to hepatic lipid accumulation, insulin resistance⁽¹²⁾, cardiovascular diseases, non-alcoholic liver diseases and steatohepatitis in offspring^(13,14). Both overnutrition and undernutrition during pregnancy and infancy result in higher susceptibility to obesity and poor metabolic health in adult life⁽¹⁵⁾. Regardless of the amount of energetic nutrients, FA composition has been suggested to be a crucial factor in the nutritional programming of metabolic phenotypes in adults⁽¹⁶⁾. Foetal exposure to various indicators of maternal diet such as increased pre- or early pregnancy body mass index, gestational weight gain, or gestational diabetes, increases the risk of offspring obesity and metabolic syndrome in humans and animals^(17,18). Different studies have demonstrated the importance of the type of dietary FA consumption in rats on the reproductive performance and metabolic parameters of their offspring. Thus, CLA supplementation during critical windows of development may represent a therapeutic strategy in the prevention of early-life programming of metabolic and reproductive dysfunction⁽¹⁹⁻²¹⁾. Previously, in adult offspring rats fed normo-energetic diets, we demonstrated that a programming effect of CLA on lipid metabolic pathways leads to a preventive effect on triacylglyceride (TAG) accretion in adipose tissue and liver⁽²²⁾. Therefore, the aim of this study was to investigate the preventive effect of maternal CLA consumption on TAG metabolism alterations induced by HF diets in adult male offspring receiving or not receiving CLA.

Methods

Chemicals and reagents

Nutrients for diet preparation were of analytical grade, with the exception of soybean oil (Cada Día, Córdoba, Argentina), sucrose, and corn starch, which were food grade and obtained from local sources. CLA oil was obtained from Lipid Nutrition B.V. (Wormerveer, The Netherlands) and consisted of an equimolecular mixture of *c*9,*t*11-CLA (38.99% -w/w-) and *t*10,*c*12-CLA (38.76% -w/w-). All solvents and reagents used for the FA quantification were of chromatography grade and were purchased from Merck (Darmstadt, Germany). GLC-463 standards containing 52 FAME mixtures (purity >99%) were purchased from Nu-Chek Prep, Inc. (Elysian, MN). CLA, cis/trans mix (Catalogue #05507) was purchased from Sigma-Aldrich Inc. (St Louis, MO, USA). The International CYTED Net (208RT0343) provided other FAME standards. Primers used for PCR analysis were synthesized by Invitrogen (Carlsbad, California, USA). For the enzyme assays, the materials were from Sigma-Aldrich Inc. (St Louis, MO, USA). All the other chemicals and reagents utilized were at least American Chemical Society (ACS) degree or molecular grade and were acquired from Merck (Darmstadt, Germany), Invitrogen (Carlsbad, California, USA) and Applied Biosystems (Foster City, California, USA). The TAG test kit was obtained from the Sociedad de Bioquímicos (Santa Fe, Argentina).

Animals

Male and virgin mature female Wistar rats were provided by the facilities of our university. The animals were housed in collective cages, with the exception of mating, pregnancy and lactation periods, under controlled conditions (23±2°C and 12-h light–12-h dark) with free access to standard food and water. All the experiments were conducted in compliance with the Guide for the Care and Use of Experimental Laboratory Animals⁽²³⁾ and were approved by the Animal Ethics Committee of the School of Biochemistry (Universidad Nacional del Litoral).

Diet preparation

For all the experiments, three diets (Table 1) were used: control (C), high-fat (HF) and HF+CLA diets. The composition of the C diet was based on the American Institute of Nutrition Ad Hoc Committee recommendation (AIN-93G) formulated for the growth, pregnancy and lactation phases of rodents⁽²⁴⁾, using 7% soybean oil as a source of fat. The HF diet was enriched in fat by substituting 13 g of carbohydrate/100 g of diet with an equal amount of soybean oil, reaching 20 g of fat/100 g of diet. The % of energy provided by fat (38.5%) in the HF and HF+CLA diets slightly exceeds the recommended amount of dietary fat for the healthy adult population (20% to 35% of energy)⁽²⁵⁾; however, it is reasonable for the human population⁽²⁶⁾. The amount of CLA used reflects

the frequently consumed levels of nutritional supplementation. The HF+CLA diet was obtained by substituting of 2.86 % (w/w) of soybean oil in the HF diet with the same amount of CLA oil. The diets were freshly prepared, gassed with nitrogen and stored at 0-4°C. The FA composition of the experimental diets (Table 2) was determined by gas chromatography as indicated below.

Experimental design

After a 1-week adaptation period (Figure 1), 18 female rats (274.1 ± 8.6 g) were randomly divided into three experimental dietary groups (n=6/group), considering that one female rat was housed with one male from the same treatment group. After mating, each female was placed in an individual cage. The rats were fed the C, HF or HF+CLA diet for 4 weeks before mating and throughout pregnancy and lactation. At parturition, litter size was standardized to eight pups per dam in the C, HF and HF+CLA diets. The pups were maintained with their own mothers until weaning. The post-weaning male offspring of mothers fed with the C or HF diet continued with the same diets as their mothers, leading to the formation of the C/C and HF/HF groups (initial weights: 98.6 ± 5.3 g and 97.8 ± 6.1 g, respectively) (n=6 per group); the offspring of mothers fed HF+CLA were divided into two groups fed with HF diet (HF+CLA/HF; initial weights: 98.3 ± 4.2 g), or HF+CLA diet (HF+CLA/HF+CLA; initial weights: 101.2 ± 2.3 g) (n=6 per group). Based on preliminary studies, the time of experimental feeding of the offspring was 9 weeks after weaning. At the end of the dietary treatment, one set of 24 animals (n=6 per group) was fasted overnight and sacrificed (9:00-11:00 AM) under anaesthesia (1 mg azepromazine + 100 mg ketamine/kg body weight) by cardiac exsanguination. Blood was collected, and serum was obtained after centrifugation (1000 g for 10 min at 4°C). The liver, gastrocnemius muscle and epididymal white adipose tissue (EWAT) were dissected, weighed and immediately frozen. All samples were stored at -80°C until analysis. The same experimental protocol of feeding was followed with a second set of 24 male offspring rats divided into 4 experimental groups (n=6 per group) for the hepatic TAG secretion rate experiments, as explained below.

Gas chromatography analysis

The FA composition of the experimental diets, liver, EWAT and serum was determined by gas chromatography using a Shimadzu (GC 2014) chromatograph equipped with a flame ionization detector. Analyses were carried out with a capillary column CP Sil 88 (100 m, 0.25 µm film thickness). The carrier gas was hydrogen with a split ratio of 1:10. The column temperature was held at 75°C for 2 min after injection, then 5°C/min to 170°C, held for 40 min, 5°C/min to 220°C and held for 40 min. The injection volume was 0.5 µL, and the column flow was 0.8 mL/min. Total fats in diets, tissues and serum were extracted using the method described by Bligh and Dyer⁽²⁷⁾.

The fatty acid methyl esters (FAMES) were formed by transesterification with methanolic potassium hydroxide solution as an interim stage before saponification (ISO 5509:2000, Point 5 IUPAC method 2.301). FAMES were identified by comparison of their retention times relative to those of commercial standards. Further GC details could be obtained from previous publications^(28,29). The values of FA content were expressed as the percentage of total FAMES (Table 2). The detection limit for the main FAME identified ranged from 0.01% to 0.03%.

Triacylglycerol levels in serum and liver

TAG levels in serum were determined by spectrophotometric methods using a commercially available test kit (Sociedad de Bioquímicos, Santa Fe). To assess the liver TAG levels, portions of frozen tissue (0.5 g) were powdered and homogenized in saline (10% w/v) for TAG content quantification. Liver TAG levels were determined by the method of Laurell⁽³⁰⁾.

Lipoprotein lipase activity in adipose tissue and gastrocnemius muscle

The removal capability of TAG-rich lipoproteins was evaluated by lipoprotein lipase (LPL) activities in the main tissues responsible for the uptake of TAG: adipose tissue and muscle. The LPL enzyme activity of adipose tissue was quantified in EWAT acetone powder by the fluorometric method of Del Prado *et al.*⁽³¹⁾. Briefly, EWAT samples were delipidated by double extraction with cold acetone followed by double extraction with diethyl ether. The powders obtained were resuspended and incubated in a buffer (25 mM NH₄Cl, pH 8.1 containing 1 IU/mL of heparin). The enzymatic reaction was carried out in a medium containing dibutyl fluorescein (DBF) as the enzyme substrate. The quantification of LPL activity was performed by measuring the increase in fluorescence ($\lambda_{\text{excitation}} = 490 \text{ nm}$; $\lambda_{\text{emission}} = 530 \text{ nm}$). In parallel, an identical assay was carried out in the same samples but in the presence of NaCl during the incubation to inhibit specific enzyme activity. LPL activity was estimated as the difference between non-specific lipolytic and total lipolytic activity. Values are expressed as pmol fluorescein/min/g of tissue and as nmol fluorescein/min/total EWAT. To assess muscle LPL activity, gastrocnemius muscle (GM) samples were homogenized in NH₄Cl/NH₄OH-heparin buffer. Then, the quantification of LPL activity in muscle was performed as previously described for adipose tissue⁽³¹⁾. The measured activity was expressed as nmol fluorescein/min/mg of protein.

Hepatic TAG secretion rate

A second set of animals subjected to the same dietary treatments was fasted overnight and anaesthetized as indicated above. Then, 600 mg/kg of body weight of Triton WR 1339 in saline solution, an agent known to inhibit the peripheral removal of TAG-rich lipoproteins, was injected

intravenously. Blood samples were taken immediately before and 120 min after the injection of the Triton solution for the estimation of TAG accumulation in serum. The hepatic TAG secretion rate (TAG-SR) was estimated based on the serum TAG concentration at 0 and 120 min, plasma volume and body weight. Further details have been previously reported⁽³²⁾.

Liver enzyme activities

Liver samples were homogenized in buffer (pH 7.6) containing 150 mM KCl, 1 mM MgCl₂, 10 mM N-acetyl-cysteine and 0.5 mM dithiothreitol. After centrifugation at 100,000 g for 40 min at 4°C, the supernatant fraction was used for the quantification of enzyme activities. Acetyl-CoA carboxylase (ACC, E.C. 6.4.1.2), fatty acid synthase (FAS, E.C. 2.3.1.85) and glucose-6 phosphate dehydrogenase (G6PDH, E.C. 1.1.1.49) activities were measured following a previously used protocol⁽³³⁾. Enzyme activities were expressed as mU/mg protein, where 1 mU was 1 nmol NADH consumed (ACC), 1 nmol NADPH consumed (FAS) or 1 nmol NADPH produced (G6PDH), per min. Carnitine palmitoyltransferase-Ia (CPT-Ia; EC 1.3.99.3) activity was assessed in the mitochondrial fraction. Liver samples were homogenized in a buffer (pH 7.4) containing 0.25 M sucrose, 1 mM EDTA, and 10 mM Tris-HCl. Homogenates were centrifuged at 700 g for 10 min at 4°C, and supernatant fluid was centrifuged again (12 000 g, 15 min, 4°C). Pellets were resuspended in a buffer (pH 7.4) containing 70 mM sucrose, 220 mM mannitol, 1 mM EDTA, and 2 mM HEPES. CPT-Ia activity was assessed in the pellet by the method of Bieber *et al*⁽³⁴⁾. The pellet protein content was obtained as described above. The CPT-Ia activities were expressed as mU/mg protein (1 mU = 1 nmol CoA/min). The protein content was determined using bovine serum albumin as a standard⁽³⁵⁾.

Extraction and analysis of RNA and quantification by RT-PCR

Total RNA was isolated from the liver using TRIzol (Invitrogen), according to the manufacturer's instructions. RNA samples were then treated with a DNA-free kit (Applied Biosystems) to remove any contamination with genomic DNA. The yield and quality of the RNA were assessed by measuring the absorbance at 260, 270, 280 and 310 nm and by electrophoresis on 1.3% agarose gels. One microgram of total RNA from each sample was reverse transcribed to first-strand complementary DNA (cDNA) using M-MLV RT. Relative mRNA levels were quantified using real-time PCR with a StepOne 18 TM Real-Time PCR Detection System (Applied Biosystems). Sequence-specific commercially synthesized primers (Invitrogen Custom Primers) were used (Genbank: ACC, NM_022193.1; FAS, NM_017332.1; sterol regulatory element-binding protein-1c (SREBP-1c), NM_0012 76708.1; stearoyl-CoA desaturase-1 (SCD-1), NM_139192.2; CPT-Ia,

NM_031559.2; PPAR α , NM_013196.1; β -actin, NM_031144.3; ubiquitin C (UBC), NM_017314.1 and hypoxanthine phosphoribosyltransferase 1 (HPRT1), NM_012583.2), and the sequences were as follows: ACC: 5'-AAC AGT GTA CAG CAT CGC CA-3' (forward), 5'-CAT GCC GTA GTAG GTT GAG GT-3' (reverse); FAS, 5'-CAG AAC TCT TCC AGG ATAG TCA ACA -3' (forward), 5'- GTC GCC CTAG TCA AGG TTC AG -3' (reverse); SREBP-1c, 5'- GGA GCC ATAG GAT TAGC ACA TT-3' (forward), 5'- GCT TCC AGA GAG GAG CCC AG-3' (reverse); SCD-1, 5'-CAC ACG CCG ACC CTC ACA ACT-3' (forward), 5'-TCC GCC CTT CTC TTT GAC AGC C-3' (reverse); CPT-Ia, 5'- ACG TAGA GTAG ACT GGT GGG AAG AAT-3' (forward), 5'-TCT CCA TAGG CGT AGT AGT TAGC TAGT-3' (reverse); PPAR α , 5'- CCC CAC TTAG AAG CAG ATAG ACC-3' (forward), 5'- CCC TAA GTA CTAG GTA GTC CGC-3' (reverse); β -actin 5'-CAT GAA GAT CAA GAT CAT TAGC TCC T-3' (forward), 5'-CTAG CTT GCT GAT CCA CAT CTAG-3' (reverse); UBC 5'- ACACCAAGAAGGTCAAACAGGA-3' (forward), 5'-CA CCTCCCCATCAAACCCAA-3' (reverse); HPRT1 5'-TCCTCCTCA GACCGCTT TTC-3' (forward), 5'-ATCACTAATCACGACGCT GGG-3' (reverse). Standard curves for each primer were generated on separate runs using several serial dilutions (1/10–1/1000) of pooled cDNA samples. The corresponding primer efficiency (E) of one cycle in the exponential phase was calculated according to the equation $E=10(-1/\text{slope})^{(36)}$. All the efficiencies of the primers were 100% (SEM 10%). Target genes were standardized with the geometric mean of three housekeeping genes: β -actin, UBC and HPRT1. Relative expression ratios were calculated using the recommended $2^{-\Delta\Delta Ct}$ method⁽³⁷⁾.

Statistical analysis

Values in Tables 3 to 6 are expressed as the mean with their standard error of the mean (SEM) of six animals per group. The sample size was calculated taking into account a minimum test power of 0.80, at a level of significance of $\alpha=0.05$, and a maximum difference between the response of each variable of the control group with respect to the average of the differences with the experimental groups. The combined standard deviation was estimated from the data to generate the power curve. In all cases, these conditions were met with $n=6^{(38)}$, which did not require further adjustment. The R software package pwr was used.

The statistical analyses displayed in Tables 3 to 6 to compare the treatments for each variable were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) by one-way ANOVA. To determine the differences between the groups, post hoc Tukey's test was used. When there was no homogeneity of variance, the Games-Howell non-parametric test was applied. For the significant differences between the c9,t11-CLA and t10,c12-CLA levels in serum, liver and EWAT (described in Results

Section), Student's t test was performed. For all statistical analyses, significant differences were considered at $p < 0.05$.

Results

During all the experiments in mothers and offspring, the diets were well accepted, and the animals showed a healthy status without any pathological manifestation from the treatments received. The mean food intakes of male offspring of different experimental groups (kJ/d) were C/C: 362.1 ± 23.3 ; HF/HF: 373.9 ± 14.1 ; HF+CLA/HF: 404.4 ± 24.4 and HF+CLA/C+CLA: 398.14 ± 20.1 ($p = 0.08$).

Nutritional parameters

The body and tissue weights of the offspring are shown in Table 3. The body weight of offspring was higher in the HF/HF ($p = 0.001$) and HF+CLA/HF ($p < 0.001$) groups than in the C/C group. However, male offspring of the HF+CLA/HF+CLA group ($p = 0.173$) had a body weight value similar to that of the C/C group. The absolute and relative liver weights were greater in the HF/HF ($p < 0.001$), HF+CLA/HF ($p < 0.001$) and HF+CLA/HF+CLA ($p < 0.001$) groups than those in the C/C group. On the other hand, a significant decrease was observed in absolute and relative EWAT weight in offspring of HF+CLA/HF ($p = 0.01$ and $p < 0.001$) and HF+CLA/HF+CLA ($p < 0.001$ and $p < 0.001$), both when compared to the C/C group and HF/HF groups, respectively.

Incorporation of CLA in liver, epididymal white adipose tissue and serum

The incorporation of CLA isomers in serum and tissues was expressed as the percentage of total FAMES. In all tissues of HF+CLA/HF+CLA offspring, *c9,t11*-CLA showed higher levels vs *t10,c12*-CLA. The *c9,t11*-CLA and *t10,c12*-CLA levels were serum: 1.08 ± 0.08 and 0.31 ± 0.06 , respectively ($p < 0.001$), liver: 1.84 ± 0.04 and 0.72 ± 0.05 , respectively ($p < 0.001$) and EWAT: 4.40 ± 0.11 and 2.80 ± 0.14 , respectively ($p < 0.001$). As reflected, the levels of CLA incorporation in HF+CLA/HF+CLA offspring were EWAT > liver > serum.

Serum and liver TAG regulation

The serum and liver TAG contents and parameters related to their bioregulation are shown in Table 4. Serum and liver TAG levels were increased in the HF/HF ($p < 0.001$ in both tissues) vs the C/C group. In the HF+CLA/HF and HF+CLA/HF+CLA groups, this parameter in both tissues reached similar values to the C/C group. Compared with the C/C group, the hepatic TAG-SR was decreased in HF/HF ($p < 0.001$) but did not change in the two groups of offspring whose mothers were fed the HF+CLA diet. EWAT LPL activity was greatly decreased in all groups fed HF ($p < 0.001$) diets

with or without CLA (HF+CLA/HF, $p < 0.001$ and HF+CLA/HF+CLA, $p = 0.001$). No changes were observed in gastrocnemius muscle LPL activity among the experimental groups.

Hepatic lipogenic and oxidative enzyme activities

Lipogenic and oxidative enzyme activities are shown in Table 5. The hepatic ACC, FAS and G6PDH activities were decreased in the HF/HF, HF+CLA/HF and HF+CLA/HF+CLA groups vs the C/C group ($p < 0.001$). On the other hand, CPT-1a activity was diminished in the two groups of offspring whose mothers were fed the HF+CLA diet (HF+CLA/HF, $p = 0.011$ and HF+CLA/HF+CLA, $p = 0.026$) vs C/C. This parameter was also decreased vs HF/HF in both groups (HF+CLA/HF, $p = 0.009$ and HF+CLA/HF+CLA, $p = 0.023$).

mRNA levels of lipogenic and β -oxidative enzymes and transcription factors

The liver expression of some key enzymes and transcription factor genes related to the synthesis (*Acc*, *Fas*, *Scd-1* and *Srebp-1c*) and β -oxidation (*Cpt-1a* and *Ppara*) of FAs are shown in Table 6. Compared with C/C, the expression of the hepatic FA biosynthesis enzymes, ACC and FAS was diminished in HF/HF ($p = 0.012$ and $p < 0.001$, respectively), HF+CLA/HF ($p < 0.001$ for both enzyme activities) and HF+CLA/HF+CLA ($p < 0.001$ for both enzyme activities). Additionally, SCD-1 mRNA expression was significantly decreased in the HF/HF, HF+CLA/HF and HF+CLA/HF+CLA diets ($p < 0.001$ in all cases), with a greater decrease in HF+CLA/HF ($p = 0.009$) than in HF/HF. The gene expression of the transcription factor *Srebp-1c* did not show any significant change between the experimental groups. Regarding FA oxidation, *CPT-1a* expression did not change in all groups vs the C/C group but was increased in the HF+CLA/HF ($p = 0.021$) group vs HF/HF; however, *PPAR α* was decreased in groups fed HF diets (HF/HF, $p = 0.001$; HF+CLA/HF, $p < 0.001$ and HF+CLA/HF+CLA, $p < 0.001$) vs C/C. The reduction was even more accentuated in HF+CLA/HF+CLA.

Discussion

Human and animal studies have shown that maternal consumption of a HF diet significantly impacts the foetal environment and that offspring could become susceptible to obesity, hyperphagia, hypertension, non-alcoholic fatty liver disease, insulin resistance, glucose intolerance and cardiovascular impairments during adulthood⁽³⁹⁾. CLA has been shown to prevent certain alterations induced by nutritional imbalances^(5,40). In this sense, we have recently demonstrated a programming effect of CLA on lipid metabolic pathways leading to a preventive effect on TAG accretion in

adipose tissue and liver of male adult offspring rats fed normo-energetic diets⁽²²⁾. Thus, the aim of this study was to investigate the potential preventive effect of maternal CLA consumption on TAG metabolism alterations induced by HF diets in adult male offspring receiving or not receiving CLA. Different studies have shown significant evidence that inadequate maternal nutrition, whether undernutrition or overnutrition, results in adverse effects in offspring^(13,14,20). Moreover, it is known that there are key developmental windows during which the adverse effects associated with early-life stressors can be prevented⁽⁴¹⁾, and in this framework, supplementation with a lipid regulatory agent such as CLA could be a useful tool to prevent lipid abnormalities observed in non-communicable chronic diseases. Thus, to the best of our knowledge, this is the first study demonstrating the influence of maternal CLA on lipid accretion in serum, liver and adipose tissue of adult male offspring receiving or not receiving CLA with high levels of dietary fat.

In the present study, using an equimolecular mixture of *c9,t11*-CLA and *t10,c12*-CLA isomers, we found, as expected, that CLA was incorporated in the serum, liver and EWAT of the HF+CLA/HF+CLA group but was not detected in the HF+CLA/HF group, as previously observed⁽²²⁾. In agreement with previous results from our laboratory⁽⁴²⁾ and from other authors^(43,44), the incorporation of *c9,t11*-CLA was higher than that of the *t10,c12*-CLA isomer in EWAT>liver>serum. In HF/HF offspring, we observed an increase in body weight. However, HF+CLA/HF+CLA offspring showed a lower final body weight related to reduced fat depots. The effect of CLA on body weight has been widely investigated in several animal models, including mice and rats^(43,45). Some studies have shown that CLA decreases weight gain^(46,47) and adipose tissue depots⁽⁴⁸⁾, whereas others have shown no effects on these parameters^(49,50). The potential mechanisms by which CLA reduces adipose tissue depots include its impact on energy metabolism, adipogenesis, inflammation, lipid metabolism, and apoptosis⁽⁵⁾ and are mainly related to the action of the *t10,c12*-CLA isomer. Our results showed that in both offspring groups of CLA-fed mothers, a decrease in EWAT weight would be associated with a decrease in LPL enzyme activity. However, in the HF/HF group, even though the LPL enzyme activity decreased, the weight of the EWAT did not change. Thus, there could be other possible mechanisms mediated by CLA that would be involved in the reduction in EWAT, such as the inhibition of SCD-1 activity, enhanced apoptosis of pre-adipocytes and adipocytes and/or lipolysis modulation⁽⁴⁵⁾. In line with some of our results, it has been demonstrated that maternal *c9,t11*-CLA supplementation reversed high-fat diet-induced increases in fat mass, hyperleptinaemia, and dyslipidaemia⁽²⁰⁾, impaired insulin sensitivity and adipocyte hypertrophy⁽²¹⁾, and skeletal muscle atrophy and inflammation⁽⁵¹⁾ in adult male rat

offspring. However, there is no clear explanation of the mechanisms involved in the reduction in adipose tissue by *c9,t11*-CLA.

Fasting serum TAG levels are regulated by the circulating TAG removal rate from adipose tissue and muscle, as well as by the hepatic TAG-SR. Our results showed that the HF/HF group had the highest serum TAG levels despite the reduced hepatic TAG-SR. This result could be explained by the diminished EWAT LPL enzyme activity without changes in the activity of this enzyme in the muscle. CLA feeding of mothers reduced the levels of TAG in the serum of offspring fed HF diets, even when the hepatic TAG-SR remained at normal levels and was higher than that of HF/HF offspring. Similar results were found in hamsters⁽⁵²⁾ and mice⁽⁵³⁾ fed CLA diets. Other authors found no differences in the serum TAG content after CLA supplementation in the diet^(48,54). The dissimilarities in the responses could be attributed to differences in the type of diet and dose levels, meal pattern, age, isomers, species, and animal model, among other causes.

Under our experimental conditions, offspring of the HF/HF group showed a significant increase in hepatic TAG levels. These results are in agreement with those reported by Ferramosca and Zara⁽⁵⁵⁾, supporting that fatty liver is diet-inducible, including HF diets, in rodent animal models. The increased hepatic TAG accretion observed in the HF/HF group of the present study was associated with a lower hepatic TAG-SR. Some studies have demonstrated that a maternal HF diet during pregnancy and lactation alters liver TAG accretion in male offspring but the mechanisms involved in this alteration have not been described^(20,56). Although there is information available about the effect of CLA on lipid accretion, there are no studies on the potential preventive effects of maternal CLA consumption on liver steatosis induced in experimental animal models. In this regard, the liver is an organ that has been reported to be a key target for CLA⁽¹⁾, but the biological effects of CLA isomers on hepatic TAG metabolism differ among species, resulting in particular differences between rats and mice and between the type of isomer. Thus, while *t10,c12*-CLA increased the TAG concentration and induced liver steatosis in mice⁽⁵⁷⁾, data from studies on a model of insulin-resistant rats showed protective effects of *t10,c12*-CLA against hyperlipidaemia and increased liver weight and lipid content⁽⁵⁸⁾. Specifically, in our study, an equimolecular mixture of *c9,t11*-CLA and *t10,c12*-CLA supplementation showed the same levels of hepatic TAG content in both the HF+CLA/HF and HF+CLA/HF+CLA groups as in the C/C group, associated with a normal liver TAG-SR. A very interesting finding of this study was related to the fact that the modulating action of CLA on liver TAG accretion remained even in the absence of CLA in the offspring diet. Thus, taking into account that the same results were observed in both offspring groups, consuming or not consuming CLA after weaning, and that we did not detect CLA in the liver of the HF+CLA/HF

group, we could hypothesize that an early-life CLA programming mechanism that controls liver steatosis may be involved. Similar mechanisms of TAG regulation were previously observed in a study that investigated the influence of maternal CLA on TAG metabolism in adult offspring rats fed a normo-energetic diet⁽²²⁾.

To further elucidate the mechanism involved in hepatic TAG accretion, the potential participation of lipogenesis and β -oxidation of FAs was studied. The elevated hepatic TAG content in the HF/HF group was accompanied by a reduction in lipogenesis without changes in β -oxidation. Specifically, the reduced ACC, FAS and G6PDH activities and mRNA levels of *Acc*, *Fas*, *Scd-1* and *Srebp-1c*, with normal CPT-1a activity, clearly demonstrated an imbalance between lipogenesis and β -oxidation. These effects might be explained by the fact that a HF diet suppresses de novo lipogenesis in rodents⁽⁵⁹⁾. In addition, CLA has the capability of acting as a potent ligand and activator of the PPAR group of nuclear receptors that regulate the gene expression of enzymes involved in lipogenesis and lipid oxidation⁽⁶⁾, and these mechanisms attenuate the hepatic steatosis observed in adult Wistar rats⁽⁴²⁾. In our results, the normalization of the hepatic TAG levels in offspring whose mothers were fed HF+CLA diets was not related to an accentuated decrease in lipogenesis or increase in β -oxidation. In fact, the enzyme activities of ACC, FAS and G6PDH in HF+CLA/HF and HF+CLA/HF+CLA and the gene expression of *Acc*, *Fas* and *Scd-1* remained strongly inhibited by HF diets, as shown by the similar pattern of changes observed in the HF/HF group. The expression of lipogenic enzymes is regulated by SREBP-1⁽⁶⁰⁾, among other factors. However, the mRNA abundance of this protein did not show a decrease with CLA feeding. In agreement with our results, decreased FAS⁽⁶¹⁾ activity and *Fas* and *Scd-1*⁽⁴⁰⁾ mRNA levels in the liver were observed in CLA-fed rats. Regarding FA oxidation, the mRNA levels of *Cpt-1a* and *Ppara* did not change in the two groups of offspring whose mothers were fed HF+CLA diets, in agreement with the results observed by Park *et al.*⁽⁴⁹⁾ in mice. However, increased⁽⁶²⁾ and reduced⁽⁶³⁾ FA oxidation induced by CLA feeding have been reported. In agreement with previous findings reported by Reynolds *et al.*⁽²⁰⁾, we found that maternal consumption of a HF diet during pregnancy and lactation results in increased lipid accretion, associated with several metabolic disorders. Moreover, our results showed a similar reduction in adipose tissue lipid accretion and normal TAG levels in the plasma and liver of both offspring groups of CLA-fed mothers, indicating that CLA prevented the alterations induced by a HF diet through programming mechanisms, at least through the modulation of the gene expression and activities of key enzymes, as well as by the liver TAG secretion rate. These results reveal emerging evidence that early-life interventions may prevent detrimental effects of maternal diet-induced programming. Our results are in agreement with the

findings of Segovia *et al.*⁽⁶⁴⁾, who demonstrated that there are critical windows of developmental plasticity in which the effects of an adverse early-life environment can be reversed by maternal dietary interventions. Additionally, Gray *et al.*⁽⁶⁵⁾ reported that a maternal HF diet causes developmental programming of endothelial dysfunction and hypertension in male offspring, which can be partially improved by maternal CLA supplementation, independent of offspring body weight. In our study, we specifically highlighted that maternal CLA supplementation prevented alterations produced by a HF diet on TAG accretion and metabolism in adult offspring receiving or not receiving CLA. Thus, we hypothesize that an early-life CLA programming mechanism that controls liver and EWAT TAG accretion may be involved. While it might have been interesting to study the effects of CLA intake on offspring not exposed to CLA in the uterus, our goal focused on the potential preventive effect of maternal CLA consumption in the development of hepatic steatosis of adult male offspring receiving a HF diet alone or supplemented with CLA for 9 weeks after weaning. Thus, this study emphasizes the importance of balanced nutrition during pregnancy and demonstrates the efficacy of nutritional supplements with CLA as novel therapeutic agents during critical windows of development. Furthermore, the data provided by this study could be very important in the development of dietary strategies that allow for reducing or preventing obesity and fatty liver disease in humans consuming high-fat or unbalanced diets.

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Conflict of interest

The authors have no conflicts of interest to disclose.

Authorship:

Bernal C.A. designed and planned the study as well as analysed the results, interpreted the findings, and wrote the manuscript; González M.A. contributed to the design and planning of the study, interpretation of the findings and writing of the manuscript; Lavandera J.V. performed the sample analysis, analysed the data, interpreted findings, and prepared the manuscript; Gerstner C.D.; Fariña A.C. and Sain J. carried out data collection and analytical determinations and assisted in the maintenance and sacrifice of rats.

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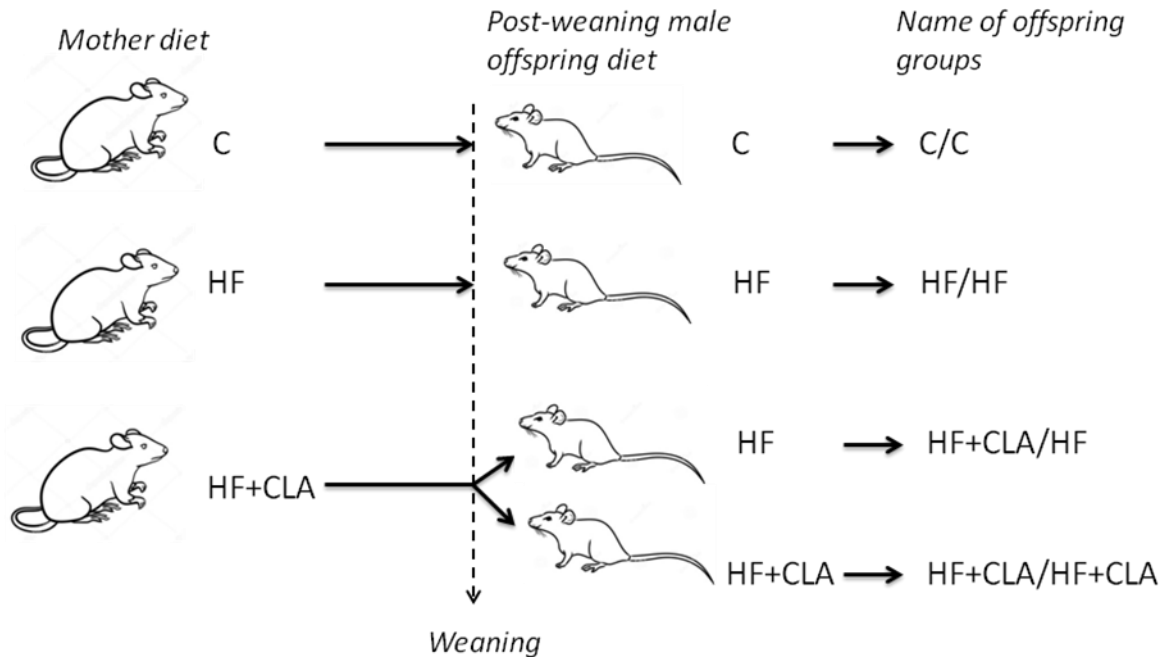
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Figure 1: Experimental design



C, control diet; HF, high-fat diet; HF+CLA, high-fat diet supplemented with conjugated linoleic acid. Mother diet: for 4 weeks before mating and throughout pregnancy and lactation, post-weaning male offspring diet: for 9 weeks after weaning.

Name of offspring groups: C/C, HF/HF, HF+CLA/HF, HF+CLA/HF+CLA. In all cases, the denomination to the left of the dash corresponds to the mother's feeding, and the denomination to the right of the dash corresponds to the offspring's feeding.

Table 1: Composition of experimental diets (g/100 g dry diet)

	C	HF	HF+CLA
Soybean oil	7.0	20.0	17.14
CLA oil	-		2.86
Protein	20.0	20.0	20.0
Corn starch	52.95	39.95	39.95
Sucrose	10.0	10.0	10.0
Cellulose	5.0	5.0	5.0
Vitamin mixture	1.0	1.0	1.0
Mineral mixture	3.50	3.50	3.50
L-Cystine-L-Methionine-Choline	0.55	0.55	0.55

C, control diet; HF, high-fat diet; HF+CLA, high-fat diet supplemented with conjugated linoleic acid.

C: based on the American Institute of Nutrition Ad-Hoc Committee recommendation (AIN-93G) formulated for growth, pregnancy and lactation phases of rodents²⁴.

Vitamin mixture (per kg of diet): nicotinic acid, 30.0 mg; pantothenate, 15.0 mg; pyridoxine, 6.0 mg; thiamin, 5.0 mg; riboflavin, 6.0 mg; folic acid, 2.0 mg; vitamin K, 750.0 µg; D-biotin, 200.0 µg; vitamin B12, 24.0 µg; retinyl acetate, 1.2 mg; cholecalciferol, 0.025 mg; DL- α -tocopheryl acetate, 50 mg.

Mineral mixture (mg/kg of diet): Ca, 5000.0; P, 1561.0; K, 3600.0; S, 300.0; Na 1019.0; Cl, 1571.0; Mg, 507.0; Fe, 35.0; Zn, 30.0; Mn, 10.0; Cu, 6.0; iodine, 0.2; Mo 0.15; Se, 0.15; Si, 5.0; Cr 1.0; F, 1.0; Ni, 0.5; B, 0.5; Li, 0.1; and V, 0.1.

Table 2: Fatty acid composition of experimental diets

Fatty acid	C	HF	HF+CLA
SFAs			
16:0	10·84	10·84	10·12
18:0	4·50	4·50	4·01
24:0	0·11	0·11	0·09
Total	15·45	15·45	14·22
MUFAs			
<i>c</i> 9-18:1	17·6	17·6	16·27
<i>c</i> 11-18:1	4·10	4·10	3·56
<i>c</i> 5-20:1	0·16	0·16	0·13
<i>c</i> 11-20:1	0·14	0·14	0·12
Total	21·90	21·90	20·08
PUFAs			
<i>c</i> 9, <i>t</i> 11-CLA	ND	ND	5·57
<i>t</i> 10, <i>c</i> 12-CLA	ND	ND	5·53
<i>c</i> 9, <i>c</i> 12-18:2	54·30	54·30	46·53
<i>c</i> 6, <i>c</i> 9, <i>c</i> 12-18:3	0·31	0·31	0·26
<i>c</i> 9, <i>c</i> 12, <i>c</i> 15-18:3	7·42	7·42	6·36
<i>c</i> 11, <i>c</i> 14, <i>c</i> 17-20:3	0·39	0·39	0·33
Total	62·40	62·40	64·58

Values are expressed as the mean (% of total FAMES); C, control diet; HF, high-fat diet; HF+CLA, HF diet supplemented with conjugated linoleic acid; ND, not detected; SFAs: saturated fatty acids; MUFAs: monounsaturated fatty acids; PUFAs: polyunsaturated fatty acids; CLA: conjugated linoleic acid. A mixture of CLA was used as the source of CLA isomers and had an equimolecular amount of *c*9,*t*11-CLA (38·99%) and *t*10,*c*12-CLA (38·76%).

Table 3: Body and tissue weights of offspring

	C/C		HF/HF		HF+CLA/HF		HFCLA/HF+CLA		ANOVA
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	p-value
Final BW (g)	322.3 ^a	1.2	337.1 ^b	3.3	343.3 ^b	3.54	315.3 ^a	2.8	< 0.001
Liver weight (g)	8.64 ^a	0.07	10.31 ^b	0.25	10.69 ^b	0.17	10.34 ^b	0.18	< 0.001
Liver weight (g/100g BW)	2.68 ^a	0.22	3.06 ^b	0.09	3.11 ^b	0.07	3.28 ^b	0.07	< 0.001
EWAT (g)	3.48 ^a	0.06	3.11 ^a	0.13	2.40 ^b	0.28	2.14 ^b	0.11	< 0.001
EWAT (g/100g BW)	1.07 ^a	0.02	0.92 ^a	0.02	0.70 ^b	0.05	0.68 ^b	0.02	< 0.001

C, control diet; HF, high-fat diet; HF+CLA, HF diet supplemented with conjugated linoleic acid; BW, body weight; EWAT, epididymal white adipose tissue.

C/C, HF/HF, HF+CLA/HF, HF+CLA/HF+CLA: in all cases, the denomination to the left of the dash corresponds to the mother feeding, and the denomination to the right of the dash corresponds to the offspring feeding.

Data represent mean values with their standard errors; n = 6 animals/groups.

Statistical analyses between groups were established by one-way ANOVA, followed by Tukey's post hoc test to determine the critical differences between the groups.

^{a,b} Mean values within a row with unlike superscript letters were significantly different ($p < 0.05$).

Table 4: Serum and liver TAG levels, hepatic TAG-SR and lipoprotein lipase activities in EWAT and muscle of offspring

	C/C		HF/HF		HF+CLA/HF		HF+CLA/HF+CLA		ANOVA
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	p-value
TAG levels									
Serum (mmol/L)	0.98 ^a	0.06	1.27 ^b	0.04	0.98 ^a	0.04	0.99 ^a	0.08	< 0.001
Liver (μmol/g)	10.73 ^a	0.40	17.51 ^b	0.79	12.48 ^a	1.31	13.17 ^a	1.22	< 0.001
Hepatic TAG-SR (nmol/100 g/min)	210.05 ^a	5.01	138.9 ^b	7.52	189.0 ^a	6.72	194.05 ^a	5.16	< 0.001
EWAT LPL activity (nmol/min per EWAT)	958.05 ^a	89.86	452.99 ^b	14.21	327.85 ^c	33.67	556.46 ^b	67.92	< 0.001
GM LPL activity (nmol/min per GM)	2.02	0.12	1.96	0.04	2.18	0.15	2.18	0.15	= 0.378

C, control diet; HF, high-fat diet; HF+CLA, HF diet supplemented with conjugated linoleic acid; EWAT, epididymal white adipose tissue; TAG-SR, triacylglycerol secretion rate; GM, gastrocnemius muscle; LPL, lipoprotein lipase.

C/C, HF/HF, HF+CLA/HF, HF+CLA/HF+CLA: in all cases, the denomination to the left of the dash corresponds to the mother feeding and the denomination to the right of the dash corresponds to the offspring feeding.

Data represent mean values with their standard errors; n = 6 animals/groups.

Statistical analyses between groups were established by one-way ANOVA, followed by Tukey's post hoc test to determine the critical differences between the groups.

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($p < 0.05$).

Table 5: Activities of lipogenic and oxidative enzymes in the livers of offspring

Enzyme activities (mU/mg protein)	C/C		HF/HF		HF+CLA/HF		HF+CLA/HF+CLA		ANOVA
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	p-value
ACC	80.06 ^a	4.83	51.47 ^b	3.52	48.29 ^b	3.48	53.33 ^b	4.29	< 0.001
FAS	5.54 ^a	0.42	0.75 ^{b,c}	0.04	0.66 ^b	0.06	0.88 ^c	0.07	< 0.001
G6PDH	58.23 ^a	3.54	14.69 ^b	1.17	13.74 ^b	0.99	16.60 ^b	1.49	< 0.001
CPT-1a	1.38 ^a	0.15	1.39 ^a	0.08	1.07 ^b	0.05	1.11 ^b	0.03	= 0.004

C, control diet; HF, high-fat diet; HF+CLA, HF diet supplemented with conjugated linoleic acid; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; G6PDH, glucose-6-phosphate dehydrogenase; CPT-1a, carnitine palmitoyltransferase-1a. 1 mU = 1 nmol NADH consumed/min (ACC); 1 nmol NADPH consumed/min (FAS); 1 nmol NADPH produced/min (G6PDH) and 1 nmol CoA/min (CPT-1a).

C/C, HF/HF, HF+CLA/HF, HF+CLA/HF+CLA: in all cases, the denomination to the left of the dash corresponds to the mother feeding, and the denomination to the right of the dash corresponds to the offspring feeding.

Data represent mean values with their standard errors; n = 6 animals/groups.

Statistical analyses between groups were established by one-way ANOVA, followed by Tukey's post hoc test to determine the critical differences between the groups.

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($p < 0.05$).

Table 6: Hepatic mRNA levels of lipogenic and β -oxidative enzymes and transcription factors of offspring

	C/C		HF/HF		HF+CLA/HF		HF+CLA/HF+CLA		ANOVA
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	p-value
ACC	1.00 ^a	0.09	0.72 ^b	0.06	0.42 ^c	0.07	0.51 ^{b,c}	0.07	< 0.001
FAS	1.00 ^a	0.07	0.41 ^{b,c}	0.04	0.29 ^b	0.03	0.55 ^c	0.03	< 0.001
SCD-1	1.00 ^a	0.01	0.43 ^b	0.04	0.15 ^c	0.01	0.45 ^b	0.13	< 0.001
SREBP-1c	1.00	0.03	0.91	0.04	1.11	0.24	1.12	0.24	= 0.679
CPT-Ia	1.00 ^{a,b}	0.02	0.78 ^a	0.11	1.11 ^b	0.07	1.01 ^{a,b}	0.07	= 0.028
PPAR α	1.00 ^a	0.05	0.63 ^b	0.12	0.45 ^b	0.02	0.23 ^c	0.02	< 0.001

C, control diet; HF, high-fat diet; HF+CLA, HF diet supplemented with conjugated linoleic acid; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; SCD-1, stearoyl-CoA desaturase-1; SREBP-1c, sterol regulatory element-binding protein 1c; CPT-Ia, carnitine palmitoyltransferase-Ia and PPAR α , peroxisome proliferator-activated receptor- α .

C/C, HF/HF, HF+CLA/HF, HF+CLA/HF+CLA: in all cases, the denomination to the left of the dash corresponds to the mother feeding, and the denomination to the right of the dash corresponds to the offspring feeding.

Data represent mean values with their standard errors; n = 6 animals/groups.

Statistical analyses between groups were established by one-way ANOVA, followed by Tukey's post hoc test to determine the critical differences between the groups.

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($p < 0.05$).