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Vegetable oils rich in alpha linolenic acid increment hepatic n-3 LCPUFA, modulating the fatty acid metabolism and antioxidant response in rats



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ABSTRACT

Alpha-linolenic acid (C18:3 n-3, ALA) is an essential fatty acid and the metabolic precursor of long-chain polyunsaturated fatty acids (LCPUFA) from the n-3 family with relevant physiological and metabolic roles: eicosapentaenoic acid (C20:5 n-3, EPA) and docosahexaenoic acid (C22:6 n-3, DHA). Western diet lacks of suitable intake of n-3 LCPUFA and there are recommendations to increase the dietary supply of such nutrients. Seed oils rich in ALA such as those from rosa mosqueta (*Rosa rubiginosa*), sacha inchi (*Plukenetia volubilis*) and chia (*Salvia hispanica*) may constitute an alternative that merits research. This study evaluated hepatic and epididymal accretion and biosynthesis of n-3 LCPUFA, the activity and expression of Δ -5 and Δ -6 desaturase enzymes, the expression and DNA-binding activity of PPAR- α and SREBP-1c, oxidative stress parameters and the activity of antioxidative enzymes in rats fed sunflower oil (SFO, 1% ALA) as control group, canola oil (CO, 10% ALA), rosa mosqueta oil (RMO, 33% ALA), sacha inchi oil (SIO, 49% ALA) and chia oil (ChO, 64% ALA) as single lipid source. A larger supply of ALA increased the accretion of n-3 LCPUFA, the activity and expression of desaturases, the antioxidative status, the expression and DNA-binding of PPAR- α , the oxidation of fatty acids and the activity of antioxidant enzymes, whereas the expression and DNA-binding activity of SREBP-1c transcription factor and the biosynthetic activity of fatty acids declined. Results showed that oils rich in ALA such as SIO and ChO may trigger metabolic responses in rats such as those produced by n-3 PUFA.

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1. Introduction

Since the discovery of George and Mildred Burr about the biochemical and physiological importance of lipids in the rat which showed that the lack of fat in the diet produced severe alterations in the development of these animals [1], continued with the work of Hansen et al., which showed the essentiality of linoleic acid (C18:2 n-6, LA) and alpha-linolenic acid (C18:3 n-3, ALA) in children [2], a great bulk of scientific information has been accumulated about the metabolic effects of n-6 and n-3 fatty acids [3,4]. LA and ALA are two polyunsaturated fatty acids (PUFA) essential to human and other mammals because they lack of the necessary enzymes to synthesize them and it is for this reason that

these fatty acids should be incorporated by the diet [5]. LA and ALA have specific metabolic functions (energy supply, regulation of enzymatic activity and regulation of gene expression), but one of their most important functions is to be metabolic precursors of long-chain polyunsaturated fatty acids (LCPUFA) [6]. LA is the precursor of arachidonic acid (C20:4 n-6, AA) and ALA the precursor of eicosapentaenoic acid (C20:5 n-3, EPA) and docosahexaenoic acid (C22:6 n-3, DHA) [6]. This conversion which implies elongation and desaturation processes is catalyzed, among others, by two highly controlled desaturase enzymes, Δ -5 desaturase and Δ -6 desaturase [7]. The activity and the expression of these enzymes are controlled by hormones (insulin and estrogens), the intracellular redox status and the activity of sterol regulatory element binding protein-1c (SREBP-1c) transcription factor [6,8]. The synthesis of n-3 and n-6 LCPUFA occurs mainly in the liver, and to a lesser extent in other tissues such as the brain, testicles and mammary gland [9]. It is relevant that after the increase of tissue levels of AA, EPA and DHA produced by the dietary supply of

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LA and ALA, the synthesis of LCPUFA is decrease as a result of a product/precursor control of Δ -5 desaturase and Δ -6 desaturase activity [10]. In addition there is consensus that, while the formation of n-6 and n-3 LCPUFA from their precursors is low, this conversion is essential to ensure physiological levels of LCPUFA in various tissues, but especially in the nervous tissue [11]. Research on the metabolism of n-6 and n-3 LCPUFA points out the fundamental role of AA in the activity of the immune system and brain development [12,13], while EPA and DHA exert a significant protective effect of cardiovascular health [14]. DHA is a fatty acid particularly relevant to ensure optimal brain and visual development [15], and even in the protection of nervous tissue during aging [16]. EPA and DHA have also a relevant role in the regulation of the lipid metabolism by controlling the activities and gene expression of key enzymes, particularly in the hepatic tissue [8–10]. These fatty acids favor the increase in the oxidation of fatty acids for energy production, together with the regulation of the activity of gene transcription factors, such as peroxisome proliferator-activated receptor α (PPAR- α) transcription factor and of target genes of this transcription factor [17], particularly those related to the oxidation of fatty acids [18], thus directly impacting with a lower accumulation of fat in the visceral tissue [19].

Considering this, background dietary recommendations have been established for the intake of n-6 and n-3 PUFA, whereas the conversion of PUFA to the respective LCPUFA is highly regulated [8–10] and that each fatty acid (i.e. AA, EPA and DHA) has specific tissue functions [6–8]. Western diets unfortunately provides an important imbalance in the intake of PUFA and LCPUFA, observing a high intake of n-6 PUFA (LA) and n-6 LCPUFA (AA), and a very low intake of n-3 PUFA (ALA) and a more restrictive ingestion of n-3 LCPUFA (EPA and DHA) in different groups of the Western population [20]. This dietary situation has been associated with an increase in the risk of developing non-communicable chronic diseases, particularly cardiovascular diseases [20], and most recently non-alcoholic fatty liver disease [21], and even lower levels of DHA in breast milk [22]. In this regard, research is recently focused in the study of new or non-traditional vegetable oils that contain more than 10% of ALA as a source for this essential fatty acid. This is the case of rosa mosqueta oil (33% ALA), sacha inchi oil (48% ALA) and chia oil (63% ALA). It has been demonstrated that consumption of these oils produced increased levels of n-3 LCPUFA and a significant reduction of n-6 LCPUFA in the liver and other tissues [23]. The present study aims to assess the effect of feeding rats with oils having high levels of ALA as the single source of dietary fat, on the hepatic and epididymal n-3 LCPUFA metabolism (fatty acid accretion, activity and expression of key enzymes and transcription factors) along with changes in the oxidative status and the antioxidant response.

2. Material and methods

2.1. Ethic statement

Experimental animal protocols and animal procedures complied with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, NIH Publication 6-23, revised 1985) and were approved by the Bioethics Committee for Research in Animals, Faculty of Medicine, University of Chile (protocol CBA#654 FMUCH).

2.2. Animals and diets

Sixty young male Wistar rats (age 3 weeks) were obtained from the Animal Facility of the Nutrition Department, Faculty of Medicine, University of Chile. Animals were randomly assigned to one

of five experimental groups, all with unrestricted access to the control and experimental diets ($n=12$ per group). Each group was fed an isocaloric diet, with a macronutrient distribution of 20% protein, 10% fat and 70% carbohydrates, and supplemented with micronutrients according to the nutritional requirements for these animals. The total fat in control and experimental groups was exclusively provided as follows: control group, sunflower oil (SFO, <1% ALA); experimental groups: canola oil (CO, 10% ALA); rosa mosqueta oil (RMO, 33% ALA), sacha inchi oil (SIO, 48% ALA) and chia oil (ChO, 63% ALA). The same abbreviations were used to identify the control and experimental groups in results. The complete composition of the diet was previously described [18]. All components for the elaboration of the diets were locally purchased, including SFO and CO. RMO was obtained from COESAM SA (Santiago, Chile); SIO was obtained from Amazonia Agroindustria SA (Lima, Perú) and; ChO was obtained from Benexia SA (Santiago, Chile). Supplementary Table S1 depicts the fatty acid composition of control and experimental diets. All oils were refined oils with a very low amount of antioxidants (tocopherols and polyphenols) (data not shown). The dietary intervention was carried out for 21 days. At the end of the intervention, animals were fasted overnight and anaesthetized by intraperitoneal injection (1 mL kg^{-1}) of zolazepam chlorhydrate (25 mg mL^{-1}) and tiletamine chlorhydrate (25 mg mL^{-1}) mixture (Zoletil 50). Hepatic and epididymal fat tissue samples were collected from each rat, immediately placed into chilled sample vials and frozen at -80°C for subsequent assays.

2.3. Fatty acid profile

Quantitative extraction and separation of total lipids from hepatic and epididymal fat tissue were carried out according to Bligh and Dyer [24] in the presence of butylated hydroxytoluene (BHT) as antioxidant. All tissues samples were homogenized in ice-cold chloroform/methanol (2:1 v/v) containing 0.01% BHT in an Ultraturrax homogenizer (Janke & Kunkel, Stufen, Germany). Total lipids from hepatic and epididymal fat tissue were extracted with chloroform/isopropanol (2:1 v/v). Phospholipids from hepatic and epididymal fat tissue were separated from the total lipid extract by thin layer chromatography (TLC) on silica gel plates (aluminum sheets $20 \times 20 \text{ cm}^2$, silica gel 60 F-254; Merck), using the solvent system hexane/diethyl ether/acetic acid (80:20:1 v/v/v). After the development of plates and solvent evaporation, lipid spots were visualized by exposing the plates to a Camag UV (250 nm) lamp designed for TLC. The solvent system allows the separation of phospholipids, cholesterol, triacylglycerols and cholesterol ester according to their relative mobility. Triacylglycerols spots were removed from the plates with either diethyl ether or chloroform/methanol (2:1 v/v), according to Ruiz-Gutierrez et al. [25].

2.4. Synthesis and gas chromatographic analysis of fatty acid methyl esters (FAME)

Triacylglycerols for FAME synthesis were extracted from the silica gel spots with 15 mL of chloroform/methanol/water (10:10:1 v/v/v) and evaporated under nitrogen stream. FAME of triacylglycerols from hepatic and epididymal fat samples were prepared with boron trifluoride (12% methanolic solution) according to Morrison and Smith [26], followed by treatment with methanolic sodium hydroxide (0.5 N). FAME samples were cooled and extracted with 0.5 mL of hexane and subsequently separated and quantified by gas-liquid chromatography in an Agilent Hewlett-Packard equipment (model 7890A, CA, USA) using a capillary column (Agilent HP-88, $100 \text{ m} \times 0.250 \text{ mm}$; I.D. $0.25 \mu\text{m}$) and a flame ionization detector (FID) as previously described [23].

2.5. Determination of hepatic Δ -5 and Δ -6 desaturase activities

Hepatic samples (500 mg) frozen in liquid nitrogen were homogenized in a buffer solution pH 7.9 containing 10 mmol/L HEPES, 1 mmol/L EDTA, 0.6% Nonidet P-40, 150 mmol/L NaCl, and protease inhibitor solution (1 mmol/L phenylmethylsulfonyl fluoride, 1 μ g/mL aprotinin, 1 μ g/L leupeptin, and 1 mmol/L orthovanadate). Hepatic homogenates were centrifuged at 5 °C; first at 10,000g for 30 s, followed by centrifugation of the supernatant at 8,000g for 5 min, and finally at 100,000g for 60 min, to obtain the extracts for desaturases measurement. The activity of Δ -6 desaturase was assayed by measuring the amount of 18:3, n-6 produced from 18:2, n-6, whereas Δ -5 desaturase was determined by the amount of 20:3, n-6 being converted into 20:4, n-6, using albumin-bound fatty acid precursors (18:2, n-6 and 20:3, n-6) [27]. Analytical procedures for the assay of enzymes were carried-out according to Valenzuela et al., [28], and gas-liquid chromatographic analysis was assessed as previously described [23].

2.6. Gene expression assays

Total RNA was isolated from hepatic samples using Trizol (Invitrogen, Paisley, UK), according to the supplier's protocols. Purified RNA (2 mg) was then treated with DNase (DNA free kit; Ambion, Austin, TX, USA) and used to generate first-strand cDNA with M-MLV reverse transcriptase (Invitrogen), utilizing random hexamers (Invitrogen) and dNTP mix (Bioline, London, UK), according to the manufacturer's protocol. The resultant cDNA was amplified with specific primer for rats in a total volume of 10 μ L. Supplementary Table S2 depicts the gene specific primer sequences used in the study. Primers were optimized to yield 95–100% of reaction efficiency with PCR products by agarose gel electrophoresis to verify the correct amplification length. Real Time PCR (qPCR) was performed in a Stratagen Mx3000P System (Agilent Technologies) following the manufacturer's recommendation (Applied Biosystems, Foster City, CA, USA). All the expression levels of the target genes studied were normalized by the expression of glyceraldehyde-3-phosphate dehydrogenase enzyme (GAPDH) as internal control (Applied Biosystems). Fold changes between groups were calculated by the $2^{-\Delta\Delta C_t}$ method.

2.7. Assessment of PPAR- α and SREBP-1c DNA-binding activity

Nuclear extracts from hepatic tissue (left lobe) were obtained using a commercial extraction kit (Cayman Chemical Company, Item 10011223, Ann Arbor, MI, USA). PPAR- α and SREBP-1c DNA-binding activity was assessed with a commercial ELISA kit. (Cayman Chemical Company, Item 10006915 for PPAR- α and 10010854 for SREBP-1c) and according to the manufacturer's instructions. Values were expressed as percentage of PPAR- α and SREBP-1c DNA-binding with respect to a positive control provided by the ELISA kit.

2.8. Assay of lipolytic and lipogenic enzymatic activity in hepatic and epididymal tissue

Lipolytic activity was determined assessing Acetyl CoA carboxylase (ACC) activity according to the method of Zimmermann et al. [29]. ACC activity was measured using an NADH-linked assay [30]. Fatty acid synthase (FAS) activity in hepatic cytosolic fractions was assessed by measuring malonyl CoA-dependent NADPH oxidation at 37 °C as described by Halestrap et al. [31]. Carnitine-palmitoyl transferase-1 (CPT-1) activity was spectrophotometrically determined using the method described by Karlic et al. [32]. Epididymal G-6-PDH activity was assessed following the increase of NADPH absorption at 340 nm according to method of Cohen et al. [30] as

previously described [33]. For malic enzyme (ME) assessment epididymal adipose tissue samples were homogenized as described for ACC and measured according to Wise and Ball [34].

2.9. Assays of hepatic and plasma oxidative stress-related parameters

Anesthetized rats were subjected to in situ hepatic perfusion with a cold saline solution (KCl 150 mM, Tris 5 mM, pH 7.4) to remove blood and for glutathione and protein carbonylation assessment. Reduced glutathione (GSH) and glutathione disulfide (GSSG) contents were measured through the enzymatic recycling method [35]. Hepatic and plasma content of protein carbonyls, F-2 isoprostanes, and thiobarbituric acid reactant substances (TBARs) and the antioxidant capacity of plasma were measured using specific kits, according to the manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI, USA).

2.10. Assay of hepatic superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase activities

Hepatic samples were homogenized in three volumes of 30 mmol/L phosphate buffer, pH 7.4, containing 1 mmol/L EDTA and 250 mmol/L sucrose. After centrifugation at 750g for 10 min at 4 °C, one aliquot of the supernatant was used for the determination of superoxide dismutase (SOD) and catalase (CAT) activities. The remnant supernatant was centrifuged at 100,000g for 60 min at 4 °C for glutathione peroxidase (GPX) and glutathione reductase (GR) assays according to Chow et al. [36]. SOD was assessed through a commercial assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions. CAT activity was measured according to the method described by Lück [37]. GPX activity was determined according to the method of Paglia and Valentine [38] and GR activity was determined by the method of Horn [39].

2.11. Statistical analysis

Statistical analysis was performed with GraphPad Prism 6.0 software (GraphPad Prism Software, Inc. San Diego, USA). Values shown represent the mean \pm SEM for each experimental group. Evaluations of normality data distribution were performed using the Shapiro Wilk test. Assessment of the statistical significance of differences between mean values was performed by one-way ANOVA and Newman-Keuls' test. A p -value < 0.05 was considered as significant. Analysis of association between different variables was carried-out using the Pearson correlation coefficient.

3. Results

3.1. Fatty acid composition of hepatic triacylglycerols

Table 1 shows the fatty acid composition of hepatic triacylglycerol obtained from the different experimental groups. Increased ALA consumption produced an increase in the hepatic content of n-3 PUFA (ALA) and n-3 LCPUFA (EPA and DHA), and a consequent decrease of n-6 fatty acids (LA and AA) in all experimental groups. The higher tissue accretion of n-3 LCPUFA and the reduction of deposition of n-6 LCPUFA also reflected in the low ratios n-6/n-3 LCPUFA, being remarkable this reduction for SIO and ChO groups.

3.2. Fatty acid composition of epididymal triacylglycerols

Fatty acid composition of triacylglycerols obtained from epididymal adipose tissue is shown in Table 2. The modification of the

Table 1
Fatty acid composition of hepatic triacylglycerols obtained from experimental groups SFO, CO, RMO, SIO and ChO.

	Fatty acid composition (g per 100 g FAME)				
	Groups				
	(a) SFO	(b) CO	(c) RMO	(d) SIO	(e) ChO
Fatty acid					
18:2, n-6 (LA)	27.5 ± 2.1 ^{b,c,d,e}	12.2 ± 1.6 ^a	15.8 ± 1.8 ^a	13.1 ± 1.8 ^a	11.2 ± 1.7 ^a
18:3, n-3 (ALA)	0.34 ± 0.05 ^{b,c,d,e}	3.42 ± 0.4 ^{a,c,d,e}	4.62 ± 0.5 ^{a,b,d,e}	14.6 ± 1.7 ^{a,b,c}	14.5 ± 1.5 ^{a,b,c}
20:4, n-6 (AA)	13.8 ± 0.8 ^{b,d,e}	1.58 ± 0.1 ^{a,c}	8.81 ± 0.9 ^{b,d,e}	2.51 ± 0.3 ^{a,c}	1.85 ± 0.5 ^{a,c}
20:5, n-3 (EPA)	0.42 ± 0.05 ^{b,c,d,e}	0.76 ± 0.05 ^{a,c,d}	1.62 ± 0.2 ^{a,b,d,e}	3.62 ± 0.6 ^{a,b,c,e}	5.95 ± 0.2 ^{a,b,c,d}
22:6, n-3 (DHA)	0.97 ± 0.1 ^{b,c,d,e}	1.10 ± 0.1 ^{a,c,d,e}	5.02 ± 0.4 ^{a,b}	5.92 ± 0.8 ^{a,b}	6.75 ± 0.3 ^{a,b}
Total SFA	38.4 ± 3.5	33.8 ± 4.2	35.6 ± 4.0	35.8 ± 3.3	34.5 ± 2.6
Total MUFA	17.5 ± 2.0 ^b	45.6 ± 3.9 ^{a,c,d,e}	27.5 ± 2.9 ^b	22.1 ± 2.3 ^b	24.4 ± 3.0 ^b
Total PUFA	44.1 ± 5.2 ^b	20.6 ± 1.9 ^{a,c,d,e}	36.9 ± 3.1 ^b	42.1 ± 3.7 ^b	41.1 ± 3.7 ^b
Total LCPUFA	16.2 ± 1.5 ^b	3.98 ± 0.5 ^{a,c,d,e}	17.9 ± 1.4 ^b	13.4 ± 1.1 ^b	15.2 ± 1.2 ^b
Total n-6 LCPUFA	14.6 ± 0.7 ^{b,d,e}	1.92 ± 0.05 ^{a,c}	11.2 ± 0.8 ^{b,d,e}	2.97 ± 0.2 ^{a,c}	2.00 ± 0.05 ^{a,c}
Total n-3 LCPUFA	1.58 ± 0.1 ^{c,d,e}	2.06 ± 0.1 ^{c,d,e}	6.70 ± 0.4 ^{a,b,d,e}	10.4 ± 0.9 ^{a,b,c,e}	13.2 ± 0.8 ^{a,b,c,d}
LCPUFA n-6/n-3 ratio	9.24 ± 0.6 ^{b,c,d,e}	0.93 ± 0.04 ^{a,c,d,e}	1.67 ± 0.1 ^{a,b,d,e}	0.29 ± 0.03 ^{a,b,c,e}	0.15 ± 0.02 ^{a,b,c,d}

Values are expressed as g fatty acid/100 g of fatty acid methyl esters (FAME) and represent the mean ± SEM ($n=12$ rats/experimental group). Statistical significances were assessed at $p < 0.05$ by one-way ANOVA and Newman–Keuls' test. Values sharing the same letter in each row are not statistically significant. Saturated fatty acids (SFA) correspond to 14:0, 16:0 and 18:0. Monounsaturated fatty acids (MUFA) correspond to 14:1, n-7, 16:1, n-7 and 18:1, n-9. Polyunsaturated fatty acids (PUFA) correspond to 18:2, n-6, 18:3, n-3, 20:4, n-6, 20:5, n-3, 22:5, n-3, and 22:6, n-3; n-6 long-chain polyunsaturated fatty acids (LCPUFA) are 20:4, n-6; n-3 LCPUFA are 20:5, n-3, 22:5, n-3, and 22:6, n-3; n-6/n-3 ratio: 20:4, n-6/ (20:5, n-3+22:5, n-3+22:6, n-3).

Table 2
Fatty acid composition of triacylglycerols of epididymal adipose tissue obtained from experimental groups SFO, CO, RMO, SIO and ChO.

	Fatty acid composition (g per 100 g FAME)				
	Groups				
	(a) SFO	(b) CO	(c) RMO	(d) SIO	(e) ChO
Fatty acid					
18:2, n-6 (LA)	12.7 ± 1.8 ^{d,e}	11.8 ± 1.2 ^{d,e}	10.5 ± 1.7 ^{d,e}	4.52 ± 0.4 ^{a,b,c,e}	2.01 ± 0.3 ^{a,b,c,d}
18:3, n-3 (ALA)	0.21 ± 0.02 ^{b,c,d,e}	1.70 ± 0.3 ^{a,d,e}	2.32 ± 0.5 ^{a,b,d,e}	8.21 ± 0.8 ^{a,b,c}	10.3 ± 1.2 ^{a,b,c}
20:4, n-6 (AA)	4.42 ± 0.8 ^{b,d,e}	3.50 ± 0.2 ^{a,c,d,e}	4.11 ± 0.4 ^{b,d,e}	1.85 ± 0.4 ^{a,b,c}	1.78 ± 0.2 ^{a,b,c}
20:5, n-3 (EPA)	0.23 ± 0.03 ^{b,c,d,e}	0.45 ± 0.05 ^{a,c,d,e}	0.67 ± 0.05 ^{a,b,d,e}	1.78 ± 0.3 ^{a,b,c}	2.16 ± 0.5 ^{a,b,c}
22:6, n-3 (DHA)	0.43 ± 0.05 ^{b,c,d,e}	0.97 ± 0.05 ^{a,d,e}	0.99 ± 0.1 ^{a,d,e}	4.63 ± 0.8 ^{a,b,c}	4.71 ± 0.7 ^{a,b,c}
Total SFA	61.2 ± 4.7	57.5 ± 4.2	56.9 ± 4.5	56.4 ± 3.9	57.2 ± 4.4
Total MUFA	20.4 ± 1.8	23.7 ± 2.8	24.1 ± 3.4	22.1 ± 2.0	21.4 ± 2.2
Total PUFA	18.4 ± 1.4	18.8 ± 1.5	19.0 ± 1.3	21.5 ± 1.6	21.4 ± 1.7
Total LCPUFA	5.15 ± 0.3 ^{d,e}	5.02 ± 0.3 ^{d,e}	5.81 ± 0.4 ^{d,e}	8.35 ± 0.8 ^{a,b,c}	8.73 ± 0.9 ^{a,b,c}
Total n-6 LCPUFA	4.44 ± 0.2 ^{b,d,e}	3.60 ± 0.3 ^{a,d,e}	4.15 ± 0.3 ^{b,d,e}	1.92 ± 0.1 ^{a,b,c}	1.83 ± 0.1 ^{a,b,c}
Total n-3 LCPUFA	0.71 ± 0.04 ^{b,c,d,e}	1.42 ± 0.05 ^{a,c,d}	1.70 ± 0.05 ^{a,b,d,e}	6.40 ± 0.4 ^{a,b,c}	6.90 ± 0.6 ^{a,b,c}
LCPUFA n-6/n-3 ratio	6.30 ± 0.5 ^{b,c,d,e}	2.50 ± 0.2 ^{a,d,e}	2.40 ± 0.2 ^{a,d,e}	0.30 ± 0.04 ^{a,b,c}	0.30 ± 0.05 ^{a,b,c}

Values are expressed as g fatty acid/100 g of fatty acid methyl esters (FAME) and represent the mean ± SEM ($n=12$ rats/experimental group). Statistical significances were assessed at $p < 0.05$ by one-way ANOVA and Newman–Keuls' test. Values sharing the same letter in each row are not statistically significant. Identification of saturated and unsaturated fatty acids and their relationships are shown in Table 1.

fatty acid composition of this tissue is less remarkable than that produced in hepatic tissue. Only groups that received a higher amount of ALA (SIO and ChO groups) showed a significant modification in the fatty acid composition compared to the control group (SFO). ALA, EPA and DHA levels were increased whereas LA and AA were reduced ($p < 0.05$). The modification of the fatty acid profile was also observed from the individual values for n-6 LCPUFA and n-3 LCPUFA and for the n-6/n-3 LCPUFA ratio.

3.3. Hepatic activity and expression of Δ -5 and Δ -6 desaturase enzymes

Fig. 1 shows the hepatic Δ -5 and Δ -6 desaturase activities (A and B) and expressions (C and D) within the different experimental groups. Both enzymatic activity and expression of Δ -5 and Δ -6 desaturase enzymes were modified by the increased incorporation of dietary ALA. Compared with the control group (SFO), it is observed that supply of low amounts of ALA (i.e. CO and RMO groups) resulted in higher values for the enzymatic activity and expression. However, activity and expression were reduced for

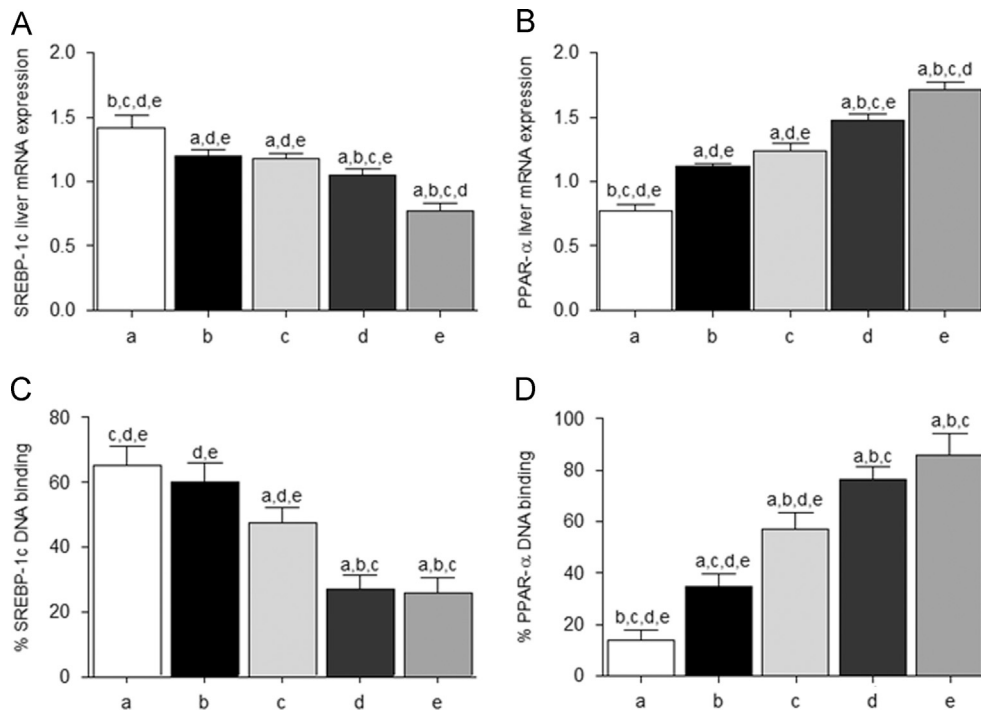
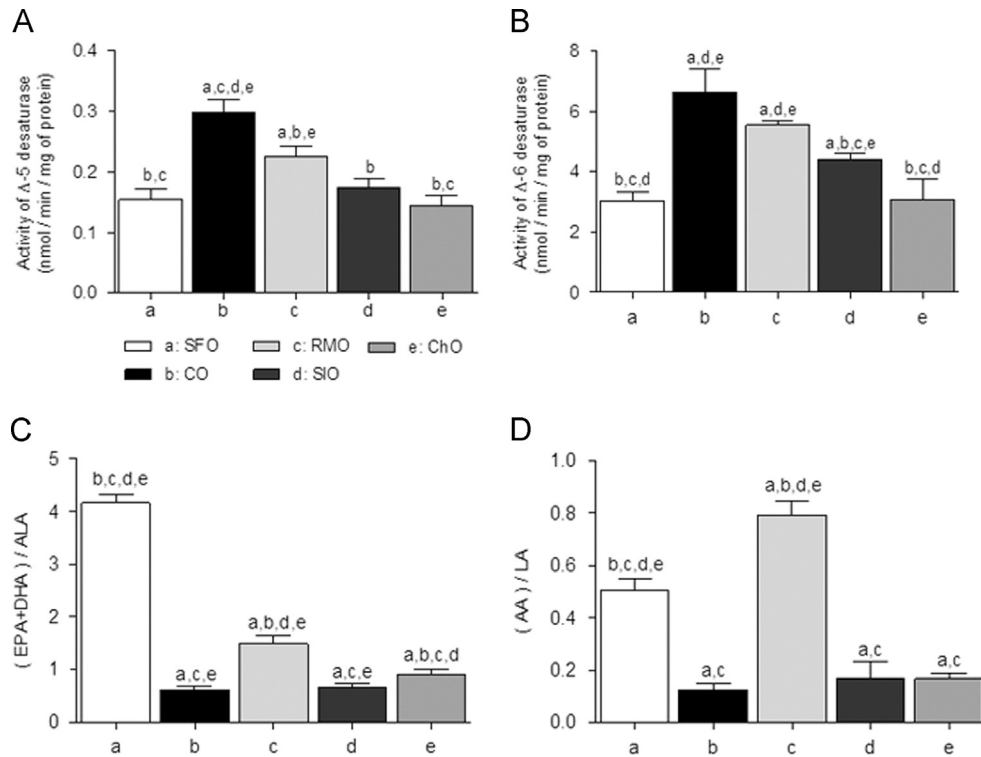
higher ALA amounts (SIO and ChO) in spite of the higher amount of tissue n-3 LCPUFA observed for these groups (Table 1), which reflects the regulatory mechanism of n-3 LCPUFA for the activity and expression of these enzymes.

3.4. Hepatic expression and DNA-binding activity of SREBP-1c and PPAR- α transcription factors

Modification of hepatic expression and DNA-binding activity of SREBP-1c and PPAR- α transcription factors are shown in Fig. 2(A–D). Increasing ALA content of diets inversely reduced both the expression (A) and the DNA-binding activity (C) of SREBP-1c transcription factor, and increases the expression (B) and the DNA-binding activity of PPAR- α transcription factor.

3.5. Hepatic and epididymal lipogenic and lipolytic enzymatic activities

The enzymatic activity of hepatic (ACC and FAS) and epididymal (ACC, G-6-PDH and MA) lipogenic enzymes and of the hepatic



lipolytic enzyme CPT-1 are shown in Fig. 3. Activity of lipogenic enzymes was reduced by the increase of dietary ALA. Hepatic ACC (A) and FAS (B) and epididymal G-6-PHD (E) and MA (F) showed less activity than the respective enzyme activity of the control in

all experimental groups. Epididymal ACC (D) which is substantially less active than the hepatic enzyme, was only reduced in ChO group. Hepatic lipolytic enzyme CPT-1 (C) was significantly increased by the dietary supply of ALA.

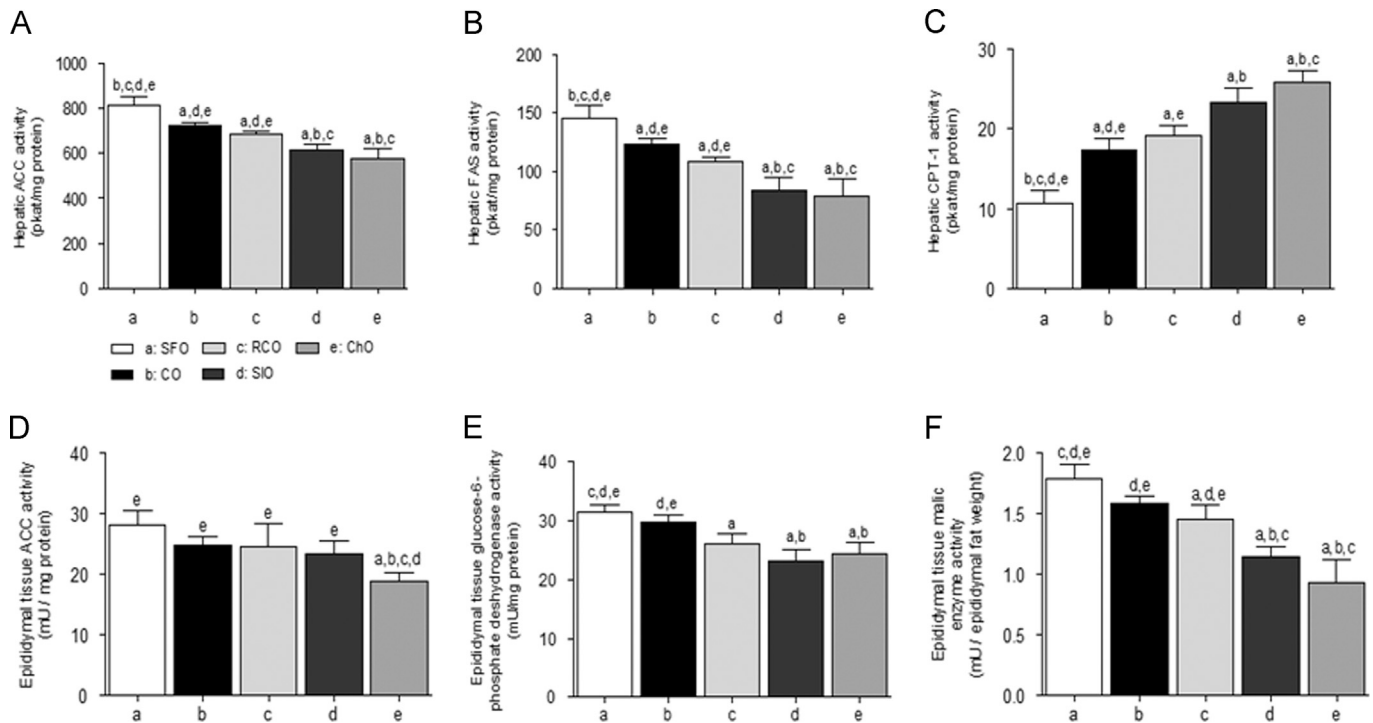


Fig. 3. Lipogenic and lipolytic enzyme activities of hepatic and epididymal tissue of experimental groups SFO, CO, RMO, SIO and ChO. Hepatic ACC activity (A), FAS activity (B), CPT-1 activity (C), epididymal tissue ACC activity (D), G-6-PDH activity (E), and MA activity (F). Values represent means \pm SEM ($n=12$ rats/experimental group). Significant differences between the groups are indicated by the letter identifying each group ($p < 0.05$; one-way ANOVA and the Newman–Keuls' test).

Table 3
Oxidative stress-related parameters (hepatic and plasma) in experimental groups SFO, CO, RMO, SIO and ChO.

	Groups				
	(a) SFO	(b) CO	(c) RMO	(d) SIO	(e) ChO
A. Liver parameters					
GSH ($\mu\text{mol/g}$ liver)	$5.19 \pm 0.4^{d,e}$	$5.36 \pm 0.3^{d,e}$	$5.49 \pm 0.4^{d,e}$	$6.73 \pm 0.4^{a,b,c}$	$6.89 \pm 0.5^{a,b,c}$
GSSG ($\mu\text{mol/g}$ liver)	0.41 ± 0.04	0.43 ± 0.05	0.42 ± 0.05	0.39 ± 0.05	0.44 ± 0.04
Total GSH equivalents ($\mu\text{mol/g}$ liver)	$5.60 \pm 0.5^{d,e}$	$5.80 \pm 0.4^{d,e}$	$5.90 \pm 0.5^{d,e}$	$7.10 \pm 0.4^{a,b,c}$	$7.30 \pm 0.5^{a,b,c}$
GSH/GSSG ratio	$12.7 \pm 1.5^{d,e}$	$12.5 \pm 1.3^{d,e}$	$13.1 \pm 1.6^{d,e}$	$17.3 \pm 1.4^{a,b,c}$	$15.7 \pm 1.5^{a,b,c}$
Protein carbonyls (nmol/mg protein)	2.38 ± 0.2	2.29 ± 0.3	2.41 ± 0.3	2.47 ± 0.2	2.50 ± 0.3
F2-isoprostanes (pg/mg protein)	8.96 ± 1.1	9.40 ± 1.2	8.67 ± 1.2	9.05 ± 1.5	10.1 ± 1.1
TBARs (nmol/mg protein)	0.11 ± 0.04	0.13 ± 0.05	0.14 ± 0.04	0.10 ± 0.05	0.12 ± 0.04
B. Plasma parameters					
TBARs (nmol/mg protein)	0.06 ± 0.02	0.05 ± 0.01	0.05 ± 0.02	0.04 ± 0.02	0.05 ± 0.02
Antioxidant capacity (mM Trolox equivalents)	1.29 ± 0.03	1.30 ± 0.1	1.25 ± 0.05	1.32 ± 0.05	1.28 ± 0.05

Values represent means \pm SEM ($n=12$ rats/experimental group). Statistical significances were assessed at $p < 0.05$ by one-way ANOVA and Newman–Keuls' test. Values sharing the same letter in each row are not statistically significant. Total GSH equivalents = GSH + 2GSSG.

3.6. Hepatic antioxidative status and hepatic and plasma oxidative stress parameters

Hepatic antioxidative status and oxidative stress parameters (A) and plasma oxidative stress parameters (B) are shown in Table 3. Hepatic antioxidative status was modified only in SIO and ChO groups which showed increased GSH content and a higher GSH/GSSG ratio compared to control. Parameters of oxidative stress (protein carbonyls, F2-isoprostanes and TBARS) were not modified by the ALA dietary intervention. Plasma parameters of

oxidative stress (TBARS and antioxidant capacity) were also not modified by dietary ALA.

3.7. Hepatic SOD, CAT, GPX and GR activities

Activity of hepatic antioxidative enzymes SOD (A), CAT (B), GPX (C), and GR (D) are shown in Fig. 4. The activity of these antioxidative enzymes was not modified in CO and RMO groups, compared to SFO group. However, larger ALA supply such as

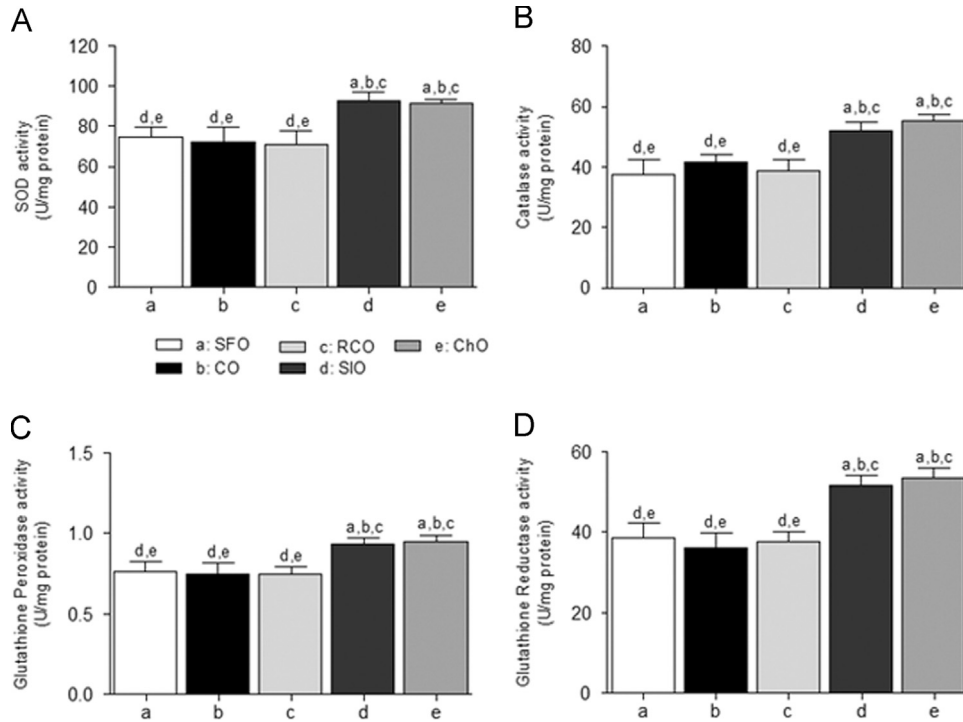


Fig. 4. Hepatic superoxide dismutase (A), catalase (B), glutathione peroxidase (C) and glutathione reductase (D) activity. Values represent means \pm SEM ($n=12$ rats/experimental group SFO, CO, RMO, SIO and ChO). Significant differences between the groups are indicated by the letter identifying each group ($p < 0.05$; one-way ANOVA and the Newman–Keuls' test).

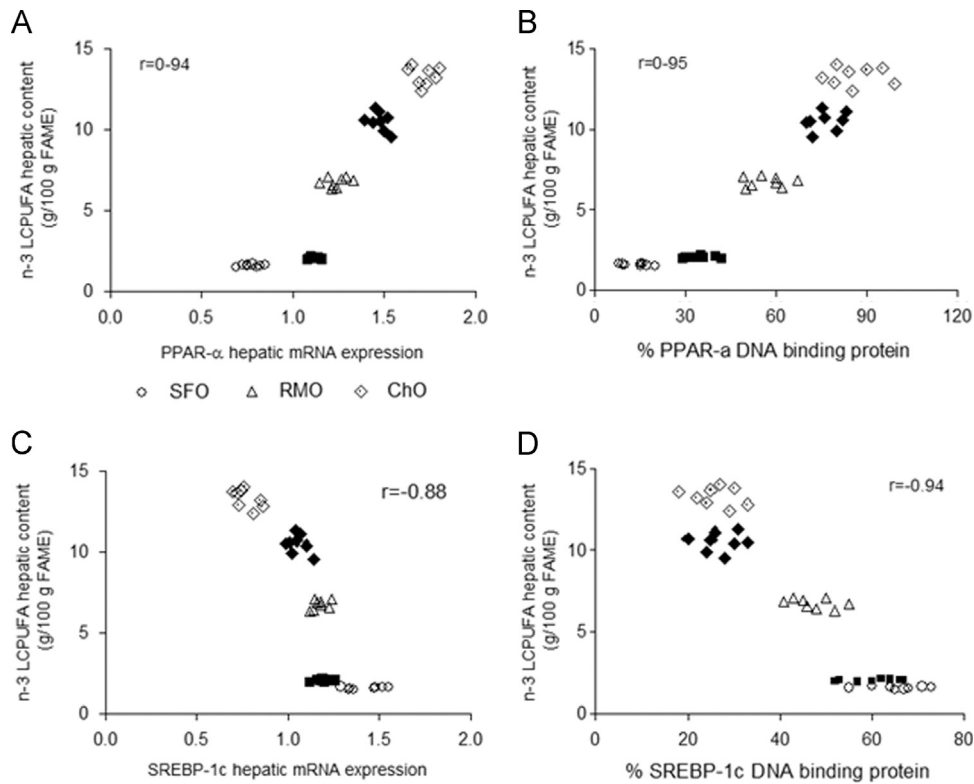


Fig. 5. Correlation between hepatic n-3 LCPUFA content with: hepatic PPAR- α mRNA expression (A), % PPAR- α DNA-binding protein (B), hepatic SREBP-1c mRNA expression and (C) % SREBP-1c DNA-binding protein of the different experimental groups SFO, CO, RMO, SIO and ChO.

obtained from SIO and ChO groups, resulted in higher activity of antioxidative enzymes.

3.8. Correlation between hepatic content of n-3 LCPUFA with the expression and DNA-binding activity of PPAR- α and SREBP-1c transcription factors

Fig. 5 shows the correlation between hepatic content of n-3 LCPUFA and the expression (A) and DNA-binding activity (B) of PPAR- α transcription factor and the expression (C) and DNA-binding activity (D) of SREBP-1c transcription factor. It was observed that the hepatic accretion of n-3 LCPUFA produced in the different experimental groups showed a high positive correlation between tissue expression ($r=0.94$) and DNA-binding activity ($r=0.95$) of PPAR- α and a negative correlation for the expression ($r=-0.88$) and DNA-binding activity ($r=-0.94$) of SREBP-1c.

3.9. General physiological parameters

Supplementary Table S3 shows physiological parameters related to the different experimental groups (initial and final body weight, hepatic and epididymal adipose tissue weight). No significant differences were observed for these parameters during the experimental period of ALA intervention. Food intake and the weight of other organs (heart, brain, kidney, etc.) were also not modified during the intervention (data not shown).

4. Discussion

The different oils used as fat energy sources in the isocaloric diets did not affect the weight of the animals or the weight of the liver and epididymal adipose tissue, which indicates that the diets produced a similar physiological effect in animals, independent of the source of the provided lipids. The fatty acid composition of hepatic triacylglycerides (Table 1) showed a remarkable difference between the diets formulated with the different oils. The increase in the ALA content of the oils is reflected in a decrease in the levels of hepatic LA and AA (with the exception of the RMO group) and in an increase of hepatic EPA and DHA, thus indicating that the greater contribution of ALA from the diets allows a higher conversion in EPA and DHA, as can be observed in the SIO and ChO groups. This effect was also observed for the decrease of the n-6/n-3 LCPUFA ratio. The behavior of the epididymal tissue was similar to that observed in hepatic tissue, although the increase of EPA and DHA in epididymal triacylglycerides was lower than that observed in hepatic tissue. Adipose tissue, in general, is not a good reservoir of LCPUFA (as observed from values obtained in SFO group), compared to the hepatic tissue but the biggest contribution of ALA (SIO and ChO groups) would allow a greater deposit of n-3 LCPUFA in adipose tissue. The significant decrease of the n-6/n-3 LCPUFA ratio observed for SIO and ChO groups accounts for the greater capacity of the epididymal tissue for n-3 LCPUFA deposition. Preliminary studies have shown that in rats fed with sacha inchi oil or chia oil, it is observed a significant increase of n-3 LCPUFA content of other tissues, such as the brain [18,23]. In this regard, when there is no contribution of dietary DHA, the administration of ALA significantly increases DHA synthesis in rat brain [24], which clearly demonstrates the importance of ALA for the conservation of a minimum of DHA accretion in the brain. For this reason there is some consensus that although the conversion of ALA to DHA is low, it is very important for mammals and particularly to humans [9,11]. Conversion from ALA to DHA in humans is less than 1% [40]. However, recent studies have demonstrated that the administration of either chia oil or sacha inchi oil produced a significant increase of DHA in different tissues [41,42]. Young subjects who consumed sacha inchi

oil show a significant increase of EPA and DHA in erythrocyte phospholipids [41,42], while in pregnant women who have a very low intake of ALA [22] the daily consumption of chia oil (10.1 g ALA per day) starting from the sixth month of pregnancy and until the sixth month post-partum, produced a significant increase in the DHA levels of breast milk [43]. However, this increment was only observed until the third month of nursing, in spite of the continuous supply of ALA, which is attributed to a physiological self-regulatory process [43]. It is noteworthy to mention that in these women EPA significantly increased in the fatty acid composition of erythrocyte phospholipids, but DHA and AA content did not change in these cells, which reflects the complex regulatory metabolism of n-6 and n-3 LCPUFA [43].

The highest contribution of ALA, which produced a higher content of EPA and DHA in hepatic triacylglycerides, also produced a consistent reduction in the activities of Δ -5 and Δ -6 desaturase enzymes (Fig. 1A and B). This effect would result from a negative feedback of the products of these enzymes (EPA and DHA), effect which has been described in literature [44]. A similar effect was observed in the expression of such enzymes (Fig. 1C and D). Since both enzymes perform the conversion of LA and ALA to the respective n-6 and n-3 LCPUFA, this regulatory effect also explains the decrease of AA that was observed for the SIO and ChO groups in hepatic (Table 1) and epididymal (Table 2) triacylglycerides. Diets that provide preformed EPA and/or DHA produced a significant decrease in the expression of Δ -5 and Δ -6 desaturase enzymes both in hepatic and brain tissues, while this decreasing effect is lower when ALA is the fatty acid supplied [45]. Both EPA and DHA can inhibit the activity and the expression of these enzymes [46] which implies a decrease in the synthesis and the ability to bind to DNA of SREBP-1 c (Fig. 2A and C). Conversely, the increase of ALA in diets produced an increase both in the expression and in the ability to bind to DNA of PPAR- α (Fig. 2B and D). This effect can be explained, in part, because the increase of dietary ALA resulted in a significant increase in the levels of n-3 LCPUFA, which in turn may increase the activity and the expression of PPAR- α [47].

Activation of PPAR- α by n-3 fatty acids and the subsequent cyto-protective effect produced is directly linked with the different routes of cyto-protection ascribed to this nuclear transcription factor [48,49], notably the inactivation of the nuclear- κ B (NF- κ B) transcription factor through the formation of non-functional complexes between PPAR- α and the sub-unit p65 of NF- κ B [50]. The inactivation produces a blockage of the inflammatory response of NF- κ B decreasing, for example, the expression of pro-inflammatory genes among which is the gene that encodes for TNF- α [17]. In this context, in non-alcoholic fatty acid liver disease (NAFLD), the main hepatic disease that currently affects the population, the reduction of hepatic n-3 LCPUFA levels [21]. This situation may result from a low intake of these fatty acids and/or an alteration in their synthesis or an increase in their oxidation or lipo-peroxidation, produces a drastic reduction in the hepatic activity of PPAR- α along with an increase in the activity and expression of NF- κ B [21,51,52]. However, when the reduction of hepatic levels of n-3 LCPUFA is prevented, the pro-inflammatory effect which characterized NALFD via activation of PPAR- α is reduced [17,53]. Another cyto-protective action described for n-3 LCPUFA in particular at the hepatic level, is the insulin-sensitizing potential that these fatty acids have on the hormone insulin-like growth factor-1 (IGF-1), a key hormone involved in the extra-pancreatic regulation of secretion and tissue sensitivity to insulin [54]. ALA, via activation of PPAR- α , may sensitize the action of IGF-1, improving the action of insulin in adipose tissue and skeletal muscle [48,55]. In this regard ALA is associated with a lower prevalence of insulin resistance in normo-weight subjects [56].

Hepatic and epididymal capacity for biosynthesis and degradation of fatty acids was modified by the dietary intervention with ALA. Hepatic ACC and FAS activities were inhibited mainly in SIO and ChO groups (Fig. 3A and B). The same action occurred for ACC, G-6-PDH and MA activities of epididymal tissue (Fig. 3D–F), which can be interpreted as a lower lipogenic activity of these tissues in response to the ALA content of diets. In addition, it was observed an increase of the hepatic fatty acid oxidative capacity which is reflected in the substantial increase of the CTP-I activity in all experimental groups, being remarkable for SIO and ChO groups, aspect also previously described for n-3 LCPUFA [18]. In this regard, ALA and its n-3 LCPUFA derivatives through the activation of PPAR- α together with the inactivation of SREBP-1 exert one of the most important mechanisms proposed for the regulation of energy metabolism by n-3 PUFA [57]. A study with Zucker fatty rats fed a diacylglycerol rich in ALA, demonstrated that ALA increased the beta-oxidation of fatty acids, thus inhibiting the development of fatty liver [58]. The activation of PPAR- α , the subsequent increase in the action of IGF-1 and the improvement in the action of insulin would be the mechanism that may explain the cyto-protective effect of ALA [48]. In mice fed a diet high in fat, the supplementation of the diet with *rosa mosqueta* oil prevents hepatic steatosis by improving insulin sensitivity and the hepatic oxidative status [59]. In addition, in rats in which insulin resistance is induced by a diet high in sucrose, the consumption of chia seeds (17% ALA) improves the dysfunction of adipose tissue induced by sucrose and also produces a better response of the metabolism of glucose in skeletal muscle, improving dyslipidemia [60]. It is also observed a positive modification in the activity of hepatic transcription factors linked to lipogenesis and an improvement in the activity of hepatic antioxidant enzymes [61]. Another interesting aspect is that diets with high content of sucrose increases oxidative stress (both systemic and tissue), but it is also observed an increase in the activity of antioxidant enzymes in the epididymal adipose tissue as a protective response [62]. It was recently reported that in knockout rats for Δ -5 and Δ -6 desaturase enzymes, a high intake of ALA prevents the development of a pro-lipogenic state and the development of fatty liver [63], indicating an anti-lipogenic effect of ALA, which would be independent of its conversion to n-3 LCPUFA.

The largest contribution of dietary ALA did not mean an increase in hepatic and plasma oxidative stress parameters (measured as protein carbonyls, F2-isoprostanes, TBARs and antioxidant capacity) (Table 3). However, the largest ALA supply improved the antioxidant status of hepatic tissue with the increase of GSH content and of the GSH/GSSG ratio without modification of the levels of GSSG. GSH is regarded as one of the most important non-enzymatic cellular antioxidants, both for its antioxidant role as such and as a cofactor for the GPX enzyme [64]. In relation to the enzymes involved in the protection against oxidative stress (SOD, CAT, GPX and GR), it was observed that a higher dietary contribution of ALA (SIO and ChO groups) increases the activity of these important enzymes (Fig. 4), aspect that together with the increase of GSH (Table 3) reinforces the concept that ALA improves the status of hepatic oxidative stress protection. It has been described that tissue non enzymatic oxidation of n-3 LCPUFA may increase the nuclear erythroid 2-related factor 2 (Nrf2) transcription factor which may positively regulate the expression of antioxidant enzymes [65]. Results regarding the increase of the antioxidant capacity in liver and plasma (Table 3) are interesting as they point out that ALA supply in large amounts could regulate the activity of antioxidant enzymes (Fig. 4). It has been reported that a large intake of n-3 LCPUFA (EPA+DHA) leads to an increase of the oxidative stress [66,67], although this fact was not observed in the current study. In conclusion, ALA may have a fundamental role in rodents, and possible in other mammals, along to be the substrate

for n-3 LCPUFA synthesis [9,11]. Although this conversion is low, it is accepted to be sufficient for supply the physiological requirements of n-3 LCPUFA. It is generally concluded that ALA may possess also important healthy properties for human being [68,69].

5. Conclusion

Vegetable oils having different content of LA and increasing high content of ALA generated important hepatic and epididymal modifications in the rat: (i) increasing the accretion of ALA and its transformation to EPA and DHA; (ii) reducing the desaturation activity and expression of Δ -5 and Δ -6 desaturases as a regulatory action due to the tissue increasing of n-3 LCPUFA, (iii) increasing the expression and DNA-binding activity of the transcription factor PPAR- α ; (iv) decreasing the expression and DNA-binding activity of the transcription factor SREBP-1c; (v) inhibiting the activity of hepatic lipogenic enzymes ACC, FAS and increasing the activity of the fat pro-oxidizing enzyme CPT-I; (vi) decreasing the activity of epididymal lipogenic enzymes ACC, G-6-PDH and MA; and (vii) increasing the hepatic antioxidant status (high GSH and GSH/GSSG ratio and the activity of antioxidant enzymes SOD, CAT, GPX y GR). Supply of diets with high ALA content, such as those received by the SIO and ChO groups, improve tissue n-3 LCPUFA content, the antioxidant status, the capacity of fat oxidation and reduce the activity of fat synthesis. All these metabolic effects may have healthy effects in those animals having diets high in ALA. Vegetable oils rich in ALA, which are now commercially available in many countries, may efficiently supply the chronic dietary deficit of n-3 LCPUFA as we have demonstrated in an animal model.

Author contributions

Rodrigo Valenzuela, María Catalina Hernandez-Rodas, Cynthia Barrera, Alejandra Espinosa and Macarena Marambio performed all experimental protocols. Rodrigo Valenzuela, Miguel Ángel Rincón-Cervera and Alfonso Valenzuela conducted the analysis of result. Rodrigo Valenzuela and Alfonso Valenzuela wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.plefa.2016.02.002>.

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