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Effect of long-term ingestion of weakly oxidised flaxseed oil on biomarkers of oxidative stress in LDL-receptor knockout mice

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Abstract

The effect of oxidised fatty acids on atherosclerosis progression is controversial. Thus, our objective was to evaluate the effect of long-term consumption of weakly oxidised PUFA from flaxseed oil on oxidative stress biomarkers of LDL-receptor$^{(-/-)}$ mice. To test our hypothesis, mice were separated into three groups. The first group received a high-fat diet containing fresh flaxseed oil (CONT−), the second was fed the same diet prepared using heated flaxseed oil (OXID), and the third group received the same diet containing fresh flaxseed oil and had diabetes induced by streptozotocin (CONT+). Oxidative stress, aortic parameters and non-alcoholic fatty liver disease were assessed. After 3 months, plasma lipid profile, glucose levels, body weight, energy intake and dietary intake did not differ among groups. Likewise, oxidative stress, plasma malondialdehyde (MDA), hepatic MDA expressed as nmol/mg portion (ptn) and antioxidant enzymes did not differ among the groups. Hepatic linoleic acid, α-linolenic acid, arachidonic acid and EPA acid declined in the OXID and CONT+ groups. Aortic wall thickness, lumen and diameter increased only in the OXID group. OXID and CONT+ groups exhibited higher concentrations of MDA, expressed as μmol/mg ptn per %PUFA, when compared with the CONT− group. Our results suggest that ingestion of oxidised flaxseed oil increases hepatic MDA concentration and is potentially pro-atherogenic. In addition, the mean MDA value observed in all groups was similar to those reported in other studies that used xenobiotics as oxidative stress inducers. Thus, the diet applied in this study represents an interesting model for further research involving antioxidants.

Key words: Flaxseeds: Oxidation: Malondialdehyde: Mice: Atherosclerosis

Atherosclerosis is the pathological process that underlies the two major types of CVD: myocardial infarction and stroke[1]. Several factors influence the atherosclerotic process in its different stages, including heredity, age, arterial pressure, smoking status, sedentarism and diet[2]. Given the risks and ethical concerns, animal models have been applied to investigate the effect of these factors on atherosclerosis progression during life[3]. Murine models have been GM to better mimic dyslipidaemia, inflammation and oxidative stress, all conditions present in fatty streaks and atherosclerotic plaque development[4–6]. Although genetic engineering of mice is straightforward, causing the animal to carry several customised mutations, most are not available from centralised repositories, making studies less reproducible[7]. The use of GM mice normally maintained by repositories, such as LDL-receptor (LDLr) or ApoE knockout (C57BL/6 strain) associated with a high-fat diet, has shown good results in terms of hypertriacylglycerolaemia, hypercholesterolaemia and atherosclerotic disease that progress to myocardial infarction and stroke[3,8,9], but a few changes have been observed in inflammation, and almost none in oxidative stress biomarkers such as hepatic or plasma malondialdehyde (MDA) and antioxidant enzyme activities[10,11]. In general, animal protocols involving diabetes induction, exposure to cigarette smoke, administration of xenobiotics, acute exercise, radiation or intermittent hypoxia have caused changes in oxidative biomarkers routinely analysed in clinical studies[6,12–17]. A common aspect of most of these models is the use of a high-fat diet, rich in SFA, consisting primarily of lard[18]. In fact, this type of diet has been efficient in promoting dyslipidaemia and inflammation[8,19,20].

According to Khan-Merchant *et al.*[21], dietary oxidised lipids, if incorporated into LDL, could be pro-atherogenic. Indeed, the effect of oxidised lipids consumption on oxidative stress associated with atherosclerosis is controversial. Although some studies have pointed to anti-atherogenic activity[5,22,23], others have suggested exactly the opposite, reporting oxidised fatty

Abbreviations: ALA, α-linolenic acid; CONT+, high-fat diet prepared with fresh flaxseed oil + streptozotocin; CONT−, high-fat diet containing fresh flaxseed oil; IS, internal standard; LDLr, LDL-receptor; MDA, malondialdehyde; OXID, high-fat diet prepared with heated flaxseed oil; ptn, portion; TBARS, thiobarbituric acid reactive substances.

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acids consumption as a risk factor for atherosclerosis[24,25]. In general, studies that identified anti-atherogenic activity after high doses of oxidised fatty acids and oxidised cholesterol consumption observed impaired cholesterol absorption[5] or activation of PPAR-α in the liver and vasculature, inhibiting monocyte recruitment and smooth vascular cells proliferation and migration[23]. Studies that observed atherogenic effects reported that the amount of oxidised lipids in the diet largely determines the levels of oxidised lipids in circulating lipoproteins, increasing inflammation and oxidative stress, accelerating the onset of atherosclerosis lesions formation, mainly when associated with a diet containing a high amount of fat and cholesterol[21,24–27]. In a very interesting study, Awada *et al.*[24] observed that the consumption of oxidised *n*-3 PUFA triggered oxidative stress and inflammation in the upper intestine of mice. In another study, Staprans *et al.*[25,28] found that an oxidised-cholesterol diet resulted in a 32–38 % increase in fatty streak lesions in LDLr$^{(-/-)}$ and ApoE$^{(-/-)}$ mice, respectively.

The effect of long-term oxidised fatty acids intake on atherosclerosis progression seems to depend on the type of PUFA. *n*-3 PUFA intake has been recommended as a supplement (capsules) or replacement for SFA and *n*-6 PUFA in foods and diet. These PUFA are highly susceptible to oxidation. Large amounts of strongly oxidised PUFA are not considered unsafe, because the oxidation products present an unpleasant odour and are poorly absorbed. However, there is no enough information regarding the toxicity of long-term weakly oxidised PUFA. Thus, our objective was to evaluate the effect of long-term consumption of weakly oxidised PUFA from flaxseed oil on *in vivo* oxidative stress, using an animal model currently applied to investigate atherosclerosis.

Methods and materials

Material

Gold flaxseed oil was obtained by applying cold pressure and by filtration from Pazze Ind. Alim. Ltda. Thiobarbituric acid (TBA), TCA, 14 % BF3-methanol, butylated hydroxytoluene (BHT), cumene hydroperoxide and tetraethoxypropane (TEP) 97 % were purchased from Sigma Chemical Co. HPLC-grade solvents were purchased from Merck SA. Milli-Q water was used to prepare all aqueous solutions (Millipore Corp.).

Study design

First, 50-ml tubes containing 35 ml of flaxseed oil were heated at 100°C for 10 h. Preliminary assays proved that this condition (100°C/10 h) resulted in a weakly oxidised oil as the peroxide value was above the fresh sample (2·67 meq O_2/kg) but below the legislation limit (15 meq O_2/kg). Exposing the oil to direct sunlight or fluorescent light or leaving the oil at room temperature for a longer period of time can also promote its oxidation[29,30]. Next, 200 parts per million of tertiary butylhydroquinone (TBHQ) was added and the samples were kept under refrigeration (4°C) until diet preparation. Oil samples were characterised according to their oxidation level as fresh (0 h) and heated (10 h). The high-fat diets were formulated as previously described[31]. Flaxseed oil (fresh and heated) was used to replace two-thirds of the lard in the high-fat diet. All diets were extrused. The oxidative markers of the diets were evaluated after extrusion. The mineral mixture of the high-fat diet was modified to make it more similar to 'cafeteria diet'[32]. Thus, salt content was doubled (from 74·0 to 148·0 g/kg of mineral mixture), and Se was reduced from 0·01025 mg to 0·00512 g/kg of the mineral mixture. In addition, Fe was increased from 6·06 g to 18·18 g/kg of the mineral mixture. Finally, fibre content was adjusted from 50·0 g to 100·0 g/kg of the diet to enable the addition of polyunsaturated oil as a substitute for lard, maintaining the diet in powder form instead of paste.

Animals

In total, twenty eight male, homozygous, LDLr knockout mice (3 months old) with a C57BL/6 background, weighing 24·61 (SE 0·29) g, were purchased from the Faculty of Pharmaceutical Sciences, University of São Paulo. The mice were housed in plastic cages (five animals per cage) at constant room temperature (22 ± 2°C) and relative humidity (55 ± 10 %), under a 12 h light–12 h dark cycle. Food and water were available *ad libitum*. Animals were divided into three groups and fed a high-fat diet, where two-third of the lard used to prepare the diet was substituted by fresh flaxseed oil in the 'negative control' group (CONT−) or by heated flaxseed oil in the OXID group. Another group was fed a high-fat diet prepared with fresh flaxseed oil, but had type 1 diabetes induced by streptozotocin (180 mg/kg) intraperitoneally without fasting, which was applied at the beginning of the trial, characterising the 'positive control' group (CONT+). The diabetic rat appears to be the most appropriate systemic oxidative stress model[16]. Dietary intake was recorded daily, and the animals were weighed individually twice a week. After 3 months, the mice were deprived of food for 8 h and anaesthetised with isoflurane. Blood samples were collected by heart puncture, immediately centrifuged (1600 ***g*** for 15 min at 4°C), frozen in liquid N_2 and stored (−80°C) for future analysis. Serum lipoprotein concentrations (total cholesterol – MS 10009010068; LDL-cholesterol – MS 10009010136; HDL-cholesterol – MS 10009010026; TAG – MS 10009010070) and glucose levels (MS 10009010236) were quantified using Labtest Diagnóstica SA. commercial kits for enzymatic colorimetric tests. The liver was excised and weighed. Small pieces of the larger lobe were frozen for analyses. The heart and aorta were collected and fixed in 10 % formol for 24 h. Subsequently, the samples were stored in 70 % ethanol, until stereological analysis. The animal protocol was conducted in accordance with 'National guidelines for the care and use of animals', and was approved by the Ethic Committee for Animal Studies of the Faculty of Pharmaceutical Sciences (Protocol CEUA/FCF 429).

Methods

Oxidative markers of the flaxseed oil. Lipid hydroperoxide concentrations were determined as previously described[33]. The absorbance readings were measured at 510 nm using a UV–Vis mini 1240 spectrophotometer (Shimadzu Scientific Instrument). The hydroperoxide content was determined using

a standard curve prepared with known concentrations of cumene hydroperoxide. Concentrations were expressed as meq O_2/kg of oil. The amount of thiobarbituric acid reactive substances (TBARS) was determined according to previously described procedures[34]. Measurements were taken in duplicate and values are expressed as mg/kg of oil.

Hexanal by headspace solid-phase microextraction coupled with GC-MS. The hexanal content of the samples was determined according to the procedures previously described[35] with some modifications. An emulsion was prepared with 10 % of the oil, and 990 µl of this emulsion was added to 10 µl of internal standard (1 µl of MBIK/ml in methanol) and hermetically sealed in a 20-ml headspace glass vial with a polypropylene hole cap and PTFE/silicone septa (Supelco). Analysis was carried out in an Agilent 7890A GC-MS (Agilent Technologies). The stationary phase was a ZB-5 MS capillary column (5 % polysilarylene/95 % polydimethylsiloxane; 30 m × 0·32 mm; 1 µm film thickness; Phenomenex®; Phenomenex Inc.). The ion source and quadrupole temperatures were set at 230 and 150°C, respectively. Ultra-pure He was the carrier gas, operated at a constant flow of 1·0 ml/min. The oven temperature was maintained at 40°C for 5 min, increased to 100°C at 4°C/min and then to 220°C at 17°C/min; the final temperature was maintained for 10 min. All mass spectra were acquired in electron-impact (EI) mode with an ionisation voltage of 70 eV and a mass range of 35–300 *m/z*. Total ion content (TIC) and selected-ion monitoring chromatogram were employed as data acquisition mode using the National Institute of Standards and Technology (NIST) library. The following retention times and quantification ions were used: internal standard (IS) 8·7 min (43, 58 and 100 *m/z*) and hexanal 11·7 min (44, 56 and 72 *m/z*). All quantifications were based on the peak area ratio of the signal of the analyte and the IS signal. A standard curve (µg/ml hexanal = 39·112 hexanal:IS area ratio; r 0·995) was prepared with five concentrations of hexanal (0–0·08 µg/ml of fresh emulsion) and the same amount of IS. Results were expressed as ρg hexanal/ml of emulsion; two independent replicates were run per sample.

Fatty acids composition

Samples were esterified according to the previously described procedure[36]. Oil samples (1·5 mg) and tissue homogenates (10 mg) were transferred to tubes containing 1 mg of IS (tricosanoic acid methyl ester (C23 : 0)), 50 µl 0·5 % BHT and 1 ml 0·5 M-methanolic NaOH. Fatty acids quantification was carried out using a GC equipped with a G3243A MS detector (Agilent 7890A GC System; Agilent Technologies Inc.). A fused silica capillary column (J&W DB-23 Agilent 122–236; 60 m × 250 mm inner diameter) was used to inject 1 µl of the sample. High-purity He was used as the carrier gas at a flow rate of 1·3 ml/min with a split injection of 50:1. The oven temperature was programmed from 80 to 175°C at a rate of 5°C/min, followed by another gradient of 3°C/min to 230°C, which was maintained for 5 min. The GC inlet and transfer line temperatures were 250 and 280°C, respectively. GC-MS was performed using 70 eV EI in scan acquisition and quantified by TIC. The fatty acids were identified by NIST and by comparing the retention time with those of four purified standard mixtures of fatty acid methyl esters (4-7801; 47085-U; 49453-U and 47885-U; Sigma Chemical Co.). All mass spectra were acquired over an *m/z* range of 40–500. Samples were analysed in triplicate and results are expressed as percentage of fatty acids in oil or mg/100 mg of hepatic tissue.

Oxidative stress biomarkers

The assessment of aortic wall thickness and lumen. The stereological parameters were estimated using Visiopharm (version 4.6.3.857) stereologic NEWCAST™ software. The ascending aorta was isolated from the heart at the height of the aortic sinus and at the beginning of the aortic arch. Each ascending aorta was weighed and measured by a digital caliper. Subsequently, samples were placed in sucrose (7 %) overnight, frozen and sectioned in a cryostat Leica (Leica Imaging Systems) at a thickness of 10 µm. The Cavalieri's principle was used to estimate the volume of the aortic lumen and wall compartments[37,38].

Determination of malondialdehyde concentration in liver homogenates. MDA concentration was determined by reverse-phase HPLC, following the protocol previously described[39], with modifications. Liver homogenates (0·05 ml) were mixed with 12·5 µl of 0·2 % BHT and 6·25 µl of 10 N NaOH. About 20 µl of the TBA–MDA conjugate derivative was injected for HPLC (Agilent Technologies 1200 Series) in a Phenomenex reverse-phase C18 analytical column (250 mm × 4·6 mm; 5 mm; Phenomenex) with an LC8-D8 pre-column (Phenomenex AJ0-1287) and was fluorometrically quantified at an excitation of 515 nm and emission of 553 nm. The HPLC pump delivered the isocratic mobile phase: 60 % PBS (10 mmol, pH 7·1) + 40 % methanol at a flow rate of 1·0 ml/min. A standard curve was prepared using TEP. The results are expressed as ηmol MDA/mg protein. According to Frankel[30], precursors of MDA are endoperoxides produced as secondary products of PUFA containing three or more double bonds. Thus, considering that SFA and MUFA are not precursors of MDA, and that the amount of PUFA observed in the liver was different among the groups, the results were also expressed as µmol/mg protein per %PUFA.

Antioxidant enzyme activities in liver homogenates. Superoxide dismutase (SOD) activity was determined according to the previously described procedure[40]. Liver homogenates containing 0·024 µg/µl of protein (25 µl) were placed into a microplate with 200 µl of freshly prepared 0·1 mM-EDTA, 62 µM-Nitrotetrazolium blue chloride (NBT) and 98 µM-NADH in 50 mM-PBS (pH 7·4). The reaction was initiated with the addition of 25 µl of freshly prepared 33 µM-phenazine methosulphate in 50 mM-PBS (pH 7·4) containing 0·1 mM-EDTA. Absorbance at 560 nm was continuously monitored over 5 min as an index of NBT reduction. A standard curve was prepared using SOD (Sigma Chemical Co.) (0·173–2·77 U/mg portion (ptn)). Glutathione peroxidase (GPx) activity was determined according to a previously described procedure[41], with modifications. In brief, 30 µl of the homogenate (with 25 µg/µl ptn) was incubated at 37°C for 5 min with 125 µl of 0·1 M-PBS and 1 mM-EDTA (pH 7·4), 5 µl of freshly prepared 0·08 M-GSH and 5 µl of freshly prepared glutathione reductase (GR) (9·6 U). Next, 30 µl

of 4 mg/ml NADPH and 5 µl of 0·46 % TBHQ were added to the reaction. Absorbance at 340 nm was continuously monitored over 4 min at 37°C. A standard curve was prepared using GPx enzyme (Sigma Chemical Co.) (2·08–25 U/mg ptn). GR activity was determined as previously described[42], with modifications. Liver homogenate containing 4·0 µg/µl of protein (20 µl) was incubated for 5 min at 37°C with 180 µl of reaction medium containing 2 ml of 0·1 M PBS with 1 mM-EDTA (pH 7), 1·5 ml of 0·005 M EDTA, 1·5 ml of milli-Q water, 10 mg of glutathione disulphide and 2 mg of NADPH. Absorbance at 340 nm was continuously monitored. A standard curve was prepared using GR enzyme (Sigma Chemical Co.) (0·003–0·25 U/mg ptn). All enzymatic assays were performed using a plate reader (Multi-Detection microplate reader; Synergy – BioTek) integrated with Gen 5 software. Samples were analysed in triplicate.

Liver steatosis analysis. Liver tissue samples were fixed in 10 % formalin for 24 h and then embedded in paraffin wax. The samples were cut into 5-µm sections and stained with haematoxylin–eosin to evaluate steatosis and inflammation. Steatosis, hepatocellular ballooning and lobular inflammation were determined histopathologically and graded as described elsewhere[43]. The degree of steatosis was graded using the following four-point scale: grade 0, steatosis involving <5 % of

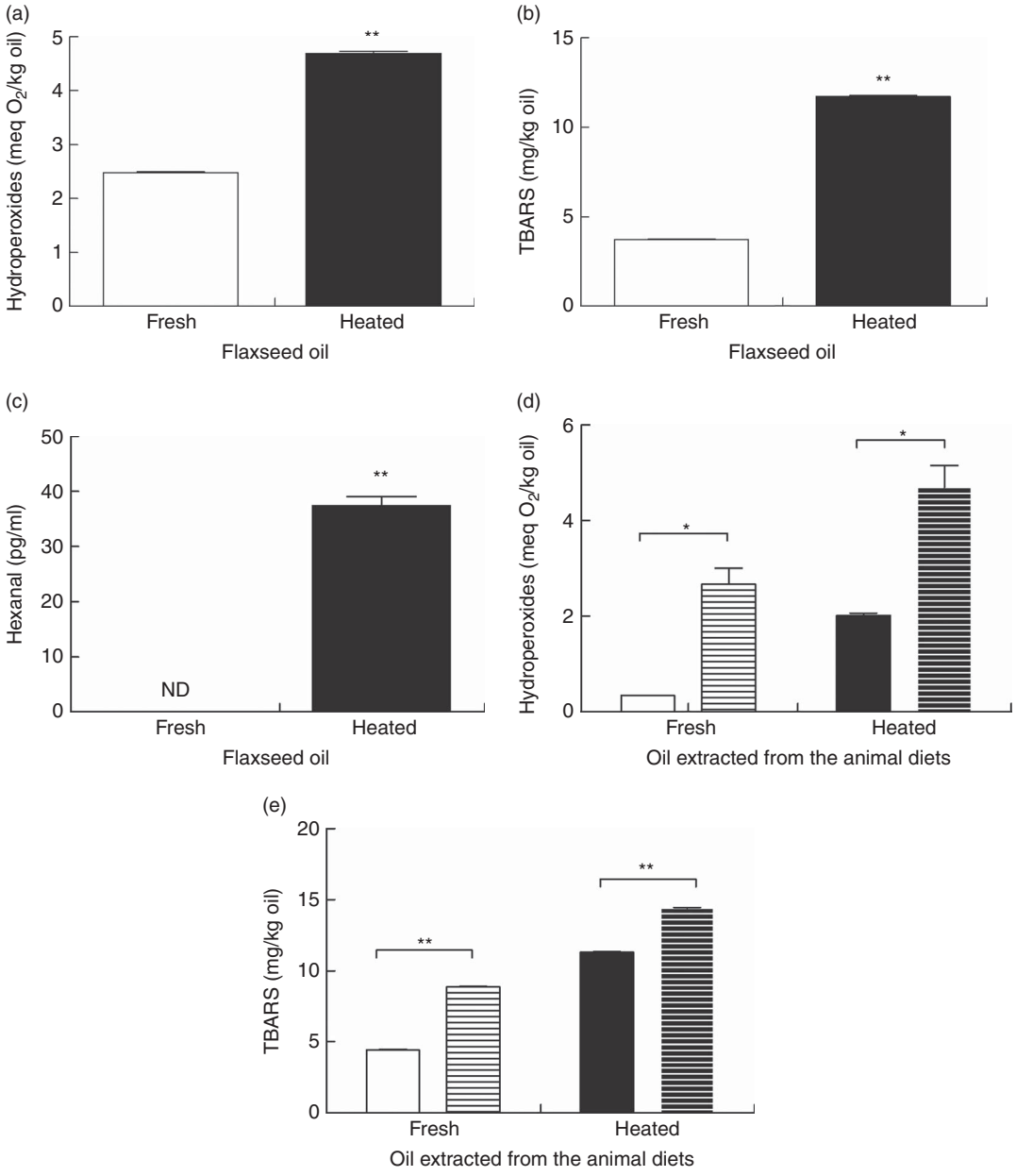

Fig. 1. Oxidative markers evaluated in fresh and heated (100°C/10 h) flaxseed oil samples: (a) hydroperoxides (meq O_2/kg oil); (b) thiobarbituric acid reactive substances (TBARS) (mg/kg oil) and (c) hexanal (pg/ml) concentrations, and in the oil extracted from the diets before and after extrusion: hydroperoxides (d) and TBARS (e) concentrations. Values are means (*n* 2), with standard errors represented by vertical bars. * $P<0·05$; ** $P<0·01$. ■, Before; ▤, after.

hepatocytes; grade 1, steatosis involving up to 33 % of hepatocytes; grade 2, steatosis involving 33–66 % of hepatocytes; and grade 3, steatosis involving >66 % of hepatocytes. Lobular inflammation was also graded on a four-point scale: grade 0, no foci; grade 1, fewer than two foci per 20× field; grade 2, two to four foci per 20× field; and grade 3, more than four foci per 20× field. Hepatocyte ballooning was graded on a three-point scale: 0, none; 1, a few balloon cells; and 2, any/prominent balloon cells. For non-alcoholic fatty liver disease activity score (NAS), features of steatosis, lobular inflammation and hepatocyte ballooning were combined, and the range of values were from 0 to 8.

Statistical analysis. Values are expressed as mean values with their standard errors. Variance homogeneity was previously evaluated for all variables by Hartley's test, and data were submitted to a Box–Cox transformation when necessary. Oxidative markers of the fresh and heated flaxseed oils were compared by *t* test for independent samples, whereas the diet extrusion effect was treated by *t* test for dependent samples. One-way ANOVA or Kruskal–Wallis ANOVA followed by the *post hoc* Tukey's test or multiple comparisons of mean ranks was used to evaluate the differences among the three experimental groups. Non-parametric χ^2 test was applied to compare steatosis levels and NAS index. Significance was set at *P* values <0·05. All the analyses were performed using STATISTICA version 9.0 (StatSoft Inc.).

Results

The oxidative markers of the flaxseed oil samples used to prepare the animals' diet are shown in Fig. 1. Flaxseed oil heated at 100°C for 10 h exhibited a higher concentration of hydroperoxides (Fig. 1(a)), TBARS (Fig. 1(b)) and hexanal (Fig. 1(c)) than fresh flaxseed oil. After mixture, all animal diets were extrused. Given that the extrusion process involves additional heat treatment, the oxidative markers were evaluated in the oils extracted from the diets before and after extrusion. Concentrations of hydroperoxides (Fig. 1(d)) and TBARS (Fig. 1(e)) increased after extrusion. However, this increase was proportional to the oils extracted from both diets. Chemical composition, fatty acids content and oxidative markers in the two diets applied in this study are shown in Table 1. No alterations were observed in nutrients and just a few changes were found in the fatty acids profile. As expected, the OXID diet showed higher hydroperoxides and TBARS values than the CONT diet.

Table 2 presents the metabolic parameters observed in the animals according to the experimental groups. No differences were observed between the three groups, except for the lower body weight gain/diet intake found in the CONT+ group. In relation to tissue weight, CONT+ exhibited the highest value for hepatic tissue and lowest for adipose tissue, compared with the other two groups. However, CONT+ did not show higher glucose concentration (13·61 mmol/l) compared with the others

Table 1. Chemical composition, fatty acid content and oxidative markers of the diets containing fresh and oxidised flaxseed oils (Mean values with their standard errors)

	High-fat diets*				
	CONT†		OXID		
	Mean	SE	Mean	SE	*P*‡
Nutrients (g/100 g)					
Moisture	6·52	0·11	6·71	0·03	0·169
Ashes	3·43	0·11	3·20	0·02	0·105
Lipids	30·32	0·20	30·87	0·40	0·287
Proteins	16·79	0·30	16·62	0·03	0·596
Carbohydrates	42·94	0·57	42·60	0·45	0·668
Energy (kJ/100 g)	2141·41	1·59	2153·71	8·16	0·214
Energy (kcal/100 g)	511·81	0·38	514·75	1·95	0·214
Major fatty acids (%)§					
C14 : 0	0·49	0·01	0·50	0·01	0·969
C16 : 0	18·67	0·01	18·85	0·01	0·156
C16 : 1	0·60	0·02	0·59	0·03	0·821
C18 : 0	11·70	0·09	11·04	0·45	0·152
C18 : 1*n*-9 *cis*	24·71	0·02	25·79	0·37	0·018
C18 : 1*n*-9 *trans*	1·21	0·06	1·11	0·02	0·239
C18 : 2*n*-6	13·17	0·03	14·86	0·14	<0·001
C18 : 3*n*-3	29·94	0·14	28·76	0·55	0·061
Oxidative markers					
LOOH (meq O_2/kg oil)§	2·67	0·24	4·70	0·32	0·037
TBARS (mg/l oil)§	8·85	0·06	14·42	0·06	<0·001

CONT, control; OXID, high-fat diet prepared with heated flaxseed oil; LOOH, lipid hydroperoxide; TBARS, thiobarbituric acid reactive substances.
* High-fat diet (g/kg) was composed of starch (195·5), casein (151·20), dextrin (100·0), sucrose (100·0), lard (100·0), flaxseed oil (200·0), fibre (100·0), mineral mixture (AIN-93M-MX; 37·8), vitamin mixture (AIN-93M-VX; 10·8), L-cystine (1·94), choline bitartarate (2·7) and tertiary butylhydroquinone (0·06). In the mineral mix, salt content was doubled (from 74·0 to 148·0 g/kg of mineral mixture), Se was reduced from 0·0125 g to 0·00512 g/kg and Fe was increased from 6·06 g to 18·18 g/kg.
† CONT diet was consumed by high-fat diet prepared with fresh flaxseed oil + streptozotocin and high-fat diet prepared with fresh flaxseed oil groups.
‡ Probability values obtained by the *t* test.
§ Values obtained in the oils extracted from diets.

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Table 2. Diet intake, body weight, blood lipid profile, tissue weight and hepatic fatty acids measured in the animals (Mean values with their standard errors)

	Experimental groups						
	CONT−		OXID		CONT+		
	Mean	SE	Mean	SE	Mean	SE	*P**
Diet intake (g/d)	0·86	0·00	0·90	0·09	0·88	0·07	0·924
Energy intake (kJ/d)	18·37	0·04	19·29	2·05	18·87	1·51	0·907
Energy intake (kcal/d)	4·39	0·01	4·61	0·49	4·51	0·36	0·907
Initial body weight (g)	24·20	0·54	24·50	0·44	25·27	0·51	0·439
Final body weight (g)	28·68	1·25	28·55	0·52	25·73	0·79	0·063
Body weight gain/diet intake	5·21	1·32[a]	4·51	0·64[a]	0·52	0·60[b]	0·006
Blood lipid profile							
Total cholesterol (mmol/l)	7·79	0·26	7·55	0·59	9·10	1·60	0·608
HDL-cholesterol (mmol/l)	1·88	0·05	1·87	0·15	2·05	0·15	0·620
LDL-cholesterol (mmol/l)	2·66	0·11	2·46	0·22	2·90	0·46	0·661
VLDL-cholesterol (mmol/l)	3·24	0·14	3·29	0·29	4·16	1·07	0·545
TAG (mmol/l)	0·84	0·04	0·90	0·10	0·75	0·16	0·271
Glucose (mmol/l)	10·90	0·57	11·03	0·86	13·61	1·95	0·706
Relative tissue weight							
Liver (%)	3·22	0·13[a]	3·08	0·13[a]	3·90	0·26[b]	0·007
Abdominal adipose (%)	1·96	0·26[a]	1·84	0·13[a]	1·21	0·17[b]	0·022
Retro-epididymal adipose (%)	2·91	0·46[a]	2·44	0·25[a]	1·44	0·17[b]	0·015
Retro-peritoneal adipose (%)	0·88	0·15[a]	0·86	0·11[a]	0·36	0·05[b]	0·037
Hepatic fatty acids (mg/g tissue)							
C16 : 0	16·28	0·80[a]	13·79	0·48[b]	13·40	0·63[b]	0·006
C16 : 1	9·66	0·36	9·00	0·27	8·96	0·29	0·208
C18 : 0	0·76	0·05	0·66	0·05	0·48	0·03	0·075
C18 : 1*n*-9 *cis*	12·97	1·32[a]	8·51	0·64[b]	8·60	0·86[b]	0·007
C18 : 1*n*-9 *trans*	0·75	0·05	–		–		–
C18 : 2*n*-6 *cis*	9·72	0·76[a]	7·43	0·58[b]	6·23	0·58[b]	0·002
C18 : 3*n*-3	7·24	0·93[a]	4·45	0·53[b]	4·50	0·58[b]	0·041
C20 : 4*n*-6	1·83	0·10[a]	1·53	0·09[b]	1·34	0·06[b]	0·002
C20 : 5*n*-3	1·52	0·09[a]	1·16	0·13[b]	1·03	0·04[b]	0·001
C22 : 6*n*-3	2·60	0·26	2·19	0·12	2·04	0·11	0·076

CONT−, high-fat diet containing fresh flaxseed oil; OXID, high-fat diet prepared with heated flaxseed oil; CONT+, high-fat diet prepared with fresh flaxseed oil + streptozotocin.[a,b] Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$).
* Probability values obtained by ANOVA or Kruskal–Wallis ANOVA.

(10·96 mmol/l). OXID and CONT+ groups displayed a reduced amount of all PUFA except DHA, when compared with the CONT− group.

Fig. 2 presents histopatological microphotographs of the hepatic tissue. All three groups received a high-fat diet, and no intergroup differences were recorded in steatosis or the NAS index, as observed in Fig. 2(a–c).

Oxidative biomarkers measured in liver and plasma are shown in Fig. 3. No differences were observed in MDA concentration, expressed as ηmol/mg ptn (Fig. 3(a)) in the liver homogenate. However, when concentration was adjusted for the amount of PUFA and expressed as μmol/mg ptn per %PUFA (Fig. 3(b)), the OXID and CONT+ groups showed a higher concentration than the CONT− group. No changes were observed in plasma MDA concentration (Fig. 3(c)) or in antioxidant enzyme activities such as SOD (Fig. 3(d)), GPx (Fig. 3(e)) and GR (Fig. 3(f)). Fig. 4(a–c) shows a representative image of the aorta obtained from the three experimental groups (Fig. 4(a): CONT−; Fig. 4(b): OXID and Fig. 4(c): CONT+), whereas aorta thickness, lumen and total diameter are shown in Fig. 4(d–f), respectively. Samples of the aorta from the OXID group exhibited higher thickness, lumen and total diameter compared with the samples obtained from the other two groups, characterising an isolated effect of PUFA oxidation products on these parameters.

Discussion

On the basis of the hypothesis that PUFA contribute to oxidative stress, whereas SFA are protective[44,45], our results confirmed that a long-term intake of weakly oxidised flaxseed oil containing about 44 % α-linolenic acid (ALA) and 15 % linoleic acid (LNA), as part of a high-fat diet, could increase oxidative stress in the liver of LDLr$^{(-/-)}$ mice. The group fed oxidised flaxseed oil (OXID) showed a similar MDA concentration in the liver (0·25 (SE 0·05) mmol/ mg ptn per % PUFA) to that observed in diabetic mice (CONT+) (0·27 (SE 0·05) mmol/mg ptn per % PUFA), both higher than the value found in the group that received the high-fat diet prepared with fresh flaxseed oil (CONT−) (0·13 (SE 0·01) mmol/mg ptn per % PUFA). In studies carried out with rodents and humans, it was found that oxidised lipids in the diet, including fatty acids and cholesterol, are absorbed and packed into chylomicrons[27,46]. In rodents, oxidised lipids are delivered to the liver, incorporated into serum lipoproteins and transported into VLDL, which is secreted into the circulation[25]. In our study, some of the fatty acids

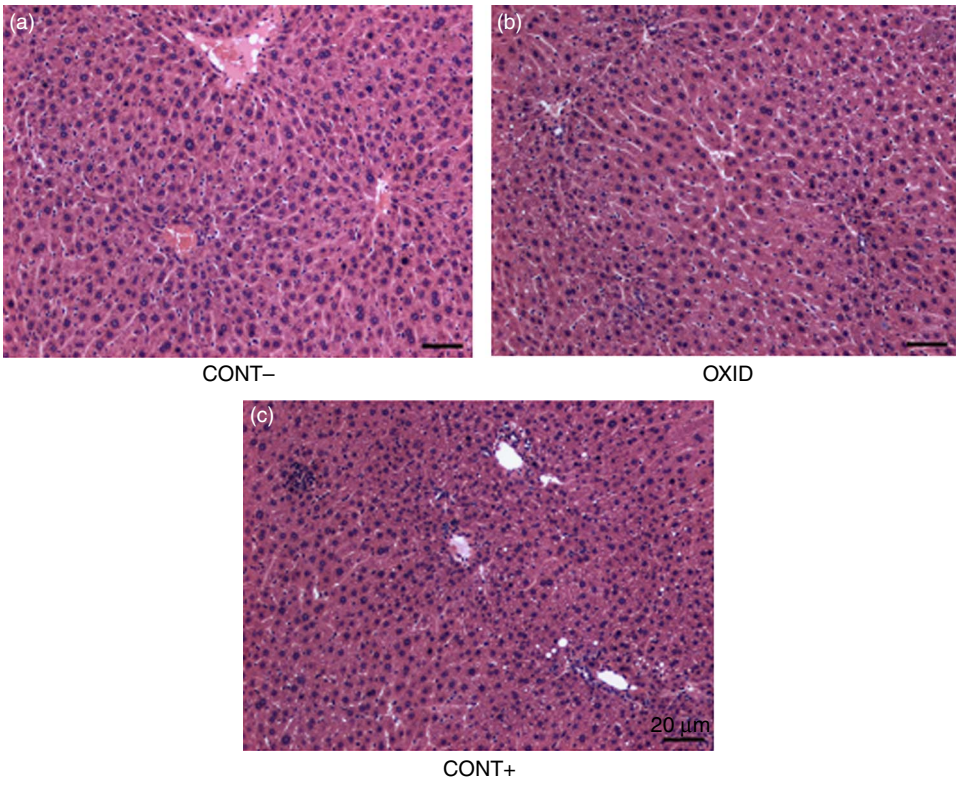

Fig. 2. Liver samples were stained with haematoxylin–eosin, the magnification is 100× and the scale bars represent 20 µm. The liver of mice fed a high-fat diet prepared with fresh flaxseed oil (CONT–) (a), a high-fat diet prepared with heated flaxseed oil (OXID) (b) and fed a high-fat diet prepared with fresh flaxseed oil + streptozotocin (CONT+) (c) showed similar liver steatosis.

present in the blood lipid profile of the OXID animals were already oxidised, increasing the concentration of hepatic MDA and likely other *n*-alkanals. These compounds form adducts with lysine *ε*-amino residues in the apo B, modifying the LDL molecules and increasing the expression of CD36 scavenger receptors, which favours modified LDL uptake by macrophages, leading to an increase in aortic lesions[47–50]. It has been reported that the intake of oxidised PUFA affects the antioxidant defence system[24]. Compounds produced from thermally induced autoxidation of PUFA are metabolised via addition of GSH in the liver by gluthatione-*S*-transferase and excreted as mercapturate conjugates in the urine[14,47,51]. Thus, depletion of intracellular GSH via 'Michael addition' can also contribute to raising oxidative stress. In addition, minor components in the flaxseed oil and their decomposition during heating could be contributing to the effects observed in the animals. However, more studies must be carried out aiming to evaluate the specific contribution of each minor component to oxidative stress.

On the other hand, the results observed in the CONT+ group can be explained by the fact that hyperglycaemia contributes to oxidative stress through different mechanisms, including increased polyol pathway flux, increased intracellular formation of advanced end products, activation of protein kinase C or over-production of the superoxide anion by the mitochondrial electron transport chain[52].

Except for MDA concentration measured in the liver, no changes were observed in the other biomarkers evaluated in our model. Some hypotheses can be raised to explain this result. First, the flaxseed oil was submitted to low oxidation (4·69 meq O_2/kg), considering 15·00 meq O_2/kg as the legal limit for commercial food-grade oils[53] Staprans *et al.*[26] supplemented female Sprague–Dawley rats by gastric intubation with oxidised maize oil, containing about three times more peroxides than the flaxseed oil used in our study. The authors reported an increase in serum peroxides and TBARS compared with a group fed a lipid-free sucrose diet. Khan-Merchant *et al.*[21] observed an increase in aortic lesion areas of more than 100 % in LDLr$^{(-/-)}$ mice fed 5·6 mg of oxidised LNA by gavage associated with a high-fat diet, whereas the amount estimated in our study was only 2·9 mg of oxidised LNA + ALA. We chose a low oxidation level in order to mimic the realistic values found in foods considered safe for human consumption and also to avoid diet rejection by the animals, owing to the strong odour characteristic of the secondary products of PUFA oxidation[54], as oral supplementation was used in our study instead of gastric intubation. It has been reported that lipid hydroperoxides are acutely toxic to rodents, but their effect tends to be less severe after oral administration, because of their reduced absorption across the enterocytes[47]. Thus, the low level of oil oxidation associated with reduced absorption of its oxidation products could have contributed to the lack of significant alterations in the biomarkers, except for hepatic MDA. Our second hypothesis is based on the type of oxidative marker selected in different studies. Short-chain aldehydes generated from lipid peroxidation are usually classified into 2-alkenals

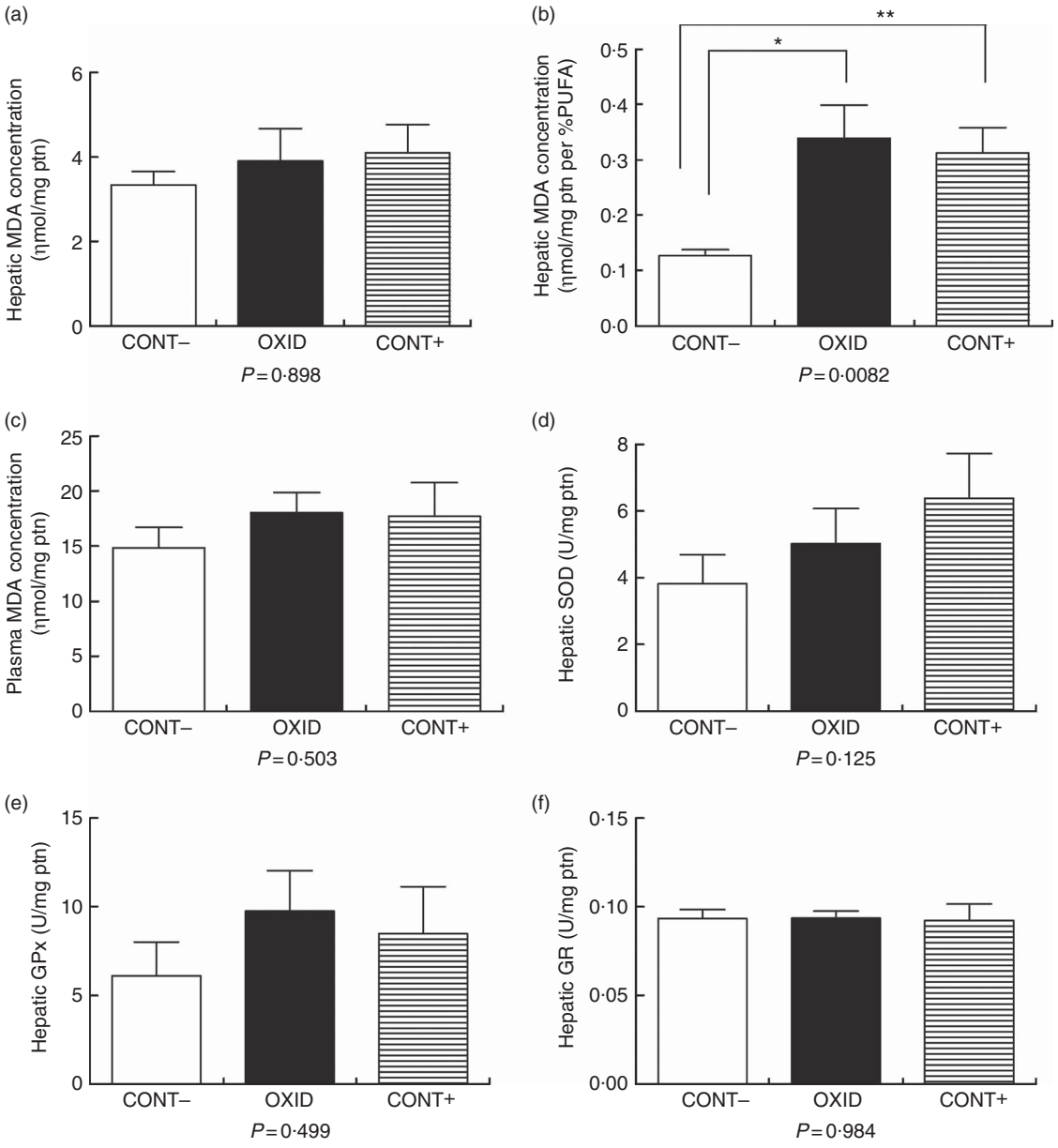

Fig. 3. Hepatic malondialdehyde (MDA) content expressed as ηmol/mg portion (ptn) (a) and μmol/mg ptn per %PUFA (b), plasma MDA content expressed as ηmol/mg ptn (c) and enzymatic activity expressed as U/mg ptn for superoxide dismutase (SOD) (d), glutathione peroxidase (GPx) (e) and glutathione reductase (GR) (f). Values are means (n 10), with standard errors represented by vertical bars. * $P<0·05$; ** $P<0·01$. CONT–, high-fat diet prepared with fresh flaxseed oil; OXID, high-fat diet prepared with heated flaxseed oil; CONT+, high-fat diet prepared with fresh flaxseed oil + streptozotocin.

(e.g. acrolein), 4-hydroxy-2-alkenals (e.g. 4-hydroxy-2-nonenal (HNE), 4-hydroxy-2-hexenal (HHE)) and ketoaldehydes (e.g. MDA). Although HNE is the major aldehyde produced during n-6 fatty acids oxidation, and HHE is characteristic of n-3 fatty acids oxidation, MDA is the most abundant specific lipid-peroxidation aldehyde capable of forming adducts with lysine residues[49,51]. For this reason, MDA was the biomarker chosen in our study. However, 4-HHE, propanol, F3-isoprostanes, F4-neuroprostanes and their isomers are more specific products from n-3 fatty acid oxidation[30,55,56]. The major PUFA present in the flaxseed oil is n-3 ALA, accounting for about 44 % of the total. Therefore, 4-HHE, propanol and F3-isoprotanes quantification could be applied to better complement MDA analysis in future studies. The third hypothesis is that in the diet offered to all three groups, Fe and salt contents were increased while Se was decreased, in order to promote a higher response in terms of oxidative stress and also mimic Western diets. Several experiments support the idea that oxidised Hb and Fe overload enhance lipid peroxidation in the liver[45,49,57]. In addition to Fe, Se is a cofactor for GPx activity[58], and the diet of all groups exhibited reduced Se content, contributing to increased oxidative stress, irrespective of the presence of oxidised lipids. Finally, Yin *et al.*[59] reported that the same oxidation products generated *in vitro* by free-radical mechanisms can be

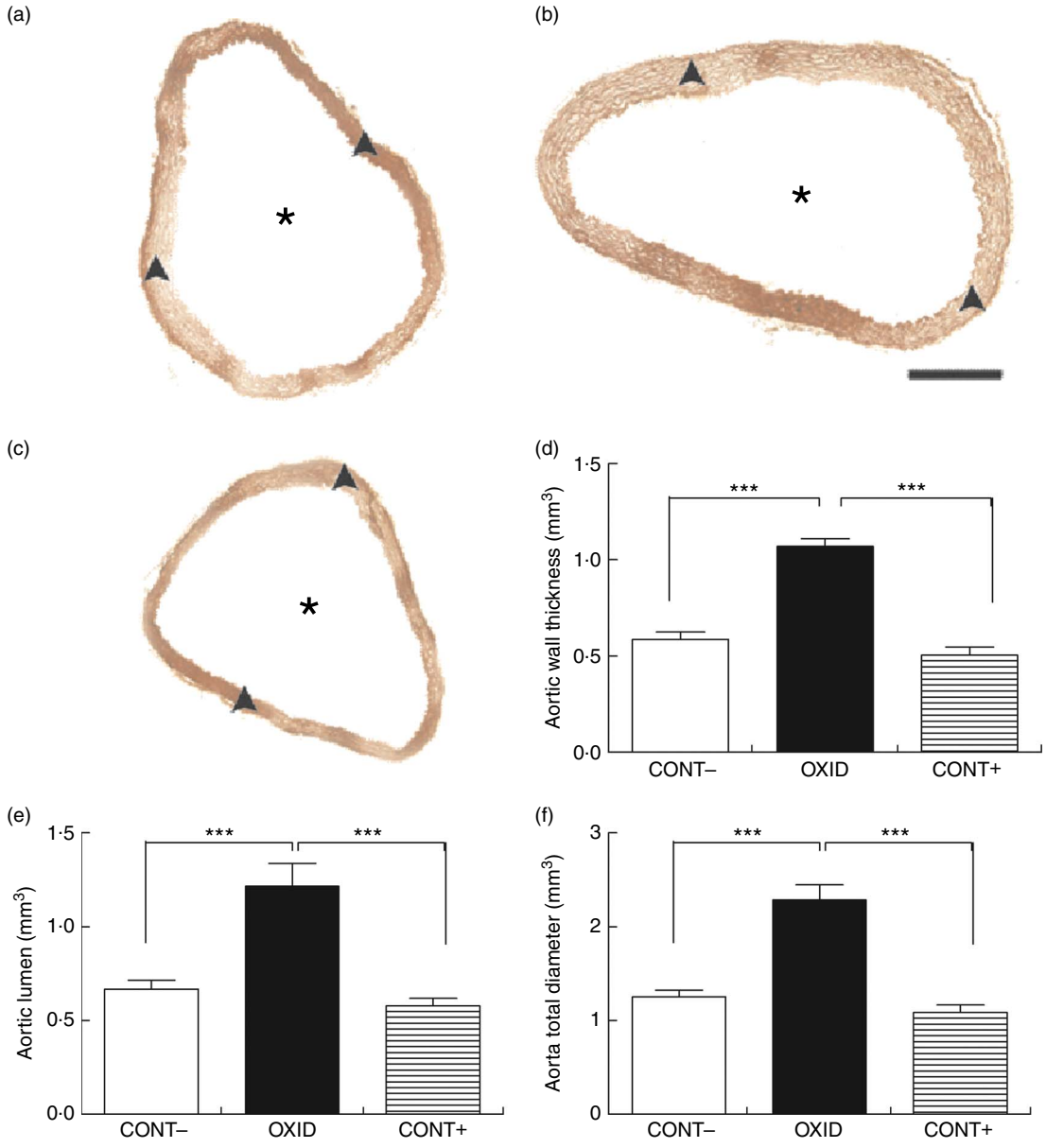

Fig. 4. Representative images of orcein-stained aortas of a high-fat diet prepared with fresh flaxseed oil (CONT–) (a), a high-fat diet prepared with heated flaxseed oil (OXID) (b) and a high-fat diet prepared with fresh flaxseed oil + streptozotocin (CONT+) (c). Values of aortic wall area (d), aortic lumen area (e) and total aorta area (f) are means (n 5), with standard errors represented by vertical bars. *** $P < 0{\cdot}001$. *, Lumen; ▲, vessel wall in the range of 50 µm.

detected *in vivo*. Although the rate is low, part of the ALA is converted into EPA and DHA during fatty acids metabolism. The same authors reported that EPA-derived J3-IsoPs activates the transcription factor Nrf2, which leads to antioxidant cytoprotective gene expressions, regulating detoxification of reactive O_2 species[10,17,49,60]. However, the pro- or anti-atherogenic activity of n-3 fatty acids oxidation products, both from the diet or endogenously produced by non-enzymatic reactions, is controversial. The results of more recent investigations have proposed that some products formed by n-3 fatty acids peroxidation show biological properties, including anti-arrhythmic, anti-inflammatory and antioxidant effects[61]. Thus, as our fourth hypothesis, we suggest that a more severe oxidative stress condition could be obtained by replacing n-3 with n-6 fatty acids in our model.

Even after assuming an increase in the other oxidative stress biomarkers after oxidised fatty acids intake, regardless of whether they come from an n-3 or n-6 source, this does not necessarily represent an atherogenic risk. This question was raised in our previous study involving fish oil and oxidative stress[62]. For example, Penumetcha *et al.*[63] fed LDLr$^{(-/-)}$ mice a diet rich in n-6 fatty acids and observed an increase in oxidative stress, as measured by 8-iso-PG $F_{2\alpha}$, but negatively associated with aortic lesion, suggesting an adaptive response by increasing antioxidant defence.

It has been suggested that moderate consumption of oxidised fats is safe, but some lipid oxidation compounds might be

harmful in the long term[54]. Our results showed that consumption of oil containing a higher amounts of oxidised PUFA (OXID), associated with a diet containing a larger amount of salt and Fe and a lower amount of Se, was sufficient to promote an increase in liver MDA equivalent to the concentration observed in the CONT+. Clinically relevant animal models of antioxidant function are essential for improving our understanding of the role of antioxidants in the pathogenesis of complex diseases[6]. Considering that MDA can form adducts with lysine residues in apo B, contributing to atherosclerosis progression, the model developed in our study provided an alternative to promote an increase in oxidative stress, without applying severe forms of induction. The concentration of liver MDA expressed as nmol/mg ptn observed in all groups (3·8 nmol/mg ptn) was similar to or higher than the values found in other studies that applied xenobiotic agents as inductors, such as Yang *et al.*[64] using CCl_4 (4·5 nmol/mg ptn), Zeng *et al.*[65] applying ethanol (3·5 nmol/mg ptn), Ibrahim *et al.*[45] with a high-fat diet containing more Fe and without vitamin E (2·60 nmol/mg ptn), Botelho *et al.*[66] using a high-fat diet with 30 % lard (2·50 nmol/mg ptn), Rosa *et al.*[15] submitting CF1 mice to sham intermittent hypoxia for 35 d (1·2 nmol/mg ptn) and Lin & Ying[67] using a high-fat diet containing 70 % fat (1·4 nmol/mg ptn). Thus, our model could be further used to evaluate antioxidants and atherosclerosis.

In addition to the rise in oxidative stress in the liver caused by consumption of oxidised flaxseed oil, the OXID group showed an increase in aorta wall thickness, aortic lumen and total aortic diameter, when compared with CONT– and CONT+ groups. As all three groups were fed the same high-fat diet, the difference observed in the OXID group was an isolated consequence of oxidised fatty acids intake. This outward hypertrophy without a reduction in lumen size represents adaptive remodelling in response to a rise in pressure[68]. This enlargement can be attributed to fracture of load-bearing elastin fibres caused by pulsatile tensile stress[69]. Therefore, this effect on the aorta was an isolated consequence of oxidised PUFA consumption, and could be an adaptive response to an increase in arterial pressure.

In conclusion, our data showed that the long-term consumption of flaxseed oil containing weakly oxidised ALA and LNA can promote oxidative stress in $LDLr^{(-/-)}$ mice, measured as liver MDA concentration. Taking into account the new trends to replace pro-inflammatory SFA or *n*-6 fatty acids with anti-inflammatory *n*-3[70], our study highlights that oils rich in PUFA must be strongly protected from oxidation during their processing and storage. In addition, the diet used in this study represents an improvement in the current model systems and can be applied in future investigations involving antioxidants and atherosclerosis.

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I. A. C. designed the research protocol; M. S. N. and M. C. K. conducted the research analysis; A. A. B. L. L. and F. V. L. L. carried out the aorta sterological analysis; B. C. performed steatosis analysis; I. A. C. and M. S. N. analysed the data and wrote the manuscript. All the authors read and approved the final version of the manuscript.

There are no conflicts of interest.

References

1. Libby P, Ridker PM & Hansson GK (2011) Progress and challenges in translating the biology of atherosclerosis. *Nature* **473**, 317–325.
2. Zaina S & Lund G (2011) Epigenetics: a tool to understand diet-related cardiovascular risk? *J Nutrigenet Nutrigenomics* **4**, 261–274.
3. Rezvan A, Ni CW, Alberts-Grill N, *et al.* (2011) Animal, *in vitro*, and *ex vivo* models of flow-dependent atherosclerosis: role of oxidative stress. *Antioxid Redox Signal* **15**, 1433–1448.
4. Ishibashi S, Herz J, Maeda N, *et al.* (1994) The two-receptor model of lipoprotein clearance: tests of the hypothesis in 'knockout' mice lacking the low density lipoprotein receptor, apolipoprotein E, or both proteins. *Proc Natl Acad Sci U S A* **91**, 4431–4435.
5. Higley NA, Beery JT, Taylor SL, *et al.* (1986) Comparative atherogenic effects of cholesterol and cholesterol oxides. *Atherosclerosis* **62**, 91–104.
6. Yu R & Schellhorn HE (2013) Recent applications of engineered animal antioxidant deficiency models in human nutrition and chronic disease. *J Nutr* **143**, 1–11.
7. Lloyd K, Franklin C, Lutz C, *et al.* (2015) Reproducibility: use mouse biobanks or lose them. *Nature* **522**, 151–153.
8. Russell JC & Proctor SD (2006) Small animal models of cardiovascular disease: tools for the study of the roles of metabolic syndrome, dyslipidemia, and atherosclerosis. *Cardiovasc Pathol* **15**, 318–330.
9. Hansson GK & Libby P (2006) The immune response in atherosclerosis: a double-edged sword. *Nat Rev Immunol* **6**, 508–519.
10. Oikawa D, Akai R, Tokuda M, *et al.* (2012) A transgenic mouse model for monitoring oxidative stress. *Sci Rep* **2**, 229.
11. Ding T, Yao Y & Praticò D (2005) Increase in peripheral oxidative stress during hypercholesterolemia is not reflected in the central nervous system: evidence from two mouse models. *Neurochem Int* **46**, 435–439.
12. Sakata N, Yoshimatsu G, Tsuchiya H, *et al.* (2012) Animal models of diabetes mellitus for islet transplantation. *Exp Diabetes Res* **2012**, 256707.
13. Kunitomo M, Yamaguchi Y, Kagota S, *et al.* (2009) Biochemical evidence of atherosclerosis progression mediated by increased oxidative stress in apolipoprotein E-deficient spontaneously hyperlipidemic mice exposed to chronic cigarette smoke. *J Pharmacol Sci* **110**, 354–361.
14. Kuiper HC, Miranda CL, Sowell JD, *et al.* (2008) Mercapturic acid conjugates of 4-hydroxy-2-nonenal and 4-oxo-2-nonenal metabolites are *in vivo* markers of oxidative stress. *J Biol Chem* **283**, 17131–17138.
15. Rosa DP, Martinez D, Picada JN, *et al.* (2011) Hepatic oxidative stress in an animal model of sleep apnoea: effects of different duration of exposure. *Comp Hepatol* **10**, 1.
16. Hermans N, Cos P, De Meyer GR, *et al.* (2007) Study of potential systemic oxidative stress animal models for the evaluation of antioxidant activity: status of lipid peroxidation and fat-soluble antioxidants. *J Pharm Pharmacol* **59**, 131–136.
17. Flora SJS (2011) Arsenic-induced oxidative stress and its reversibility. *Free Radic Biol Med* **51**, 257–281.

18. Breslow JL (1996) Mouse models of atherosclerosis. *Science* **272**, 685–688.
19. Jawien J, Nastalek P & Korbut R (2004) Mouse models of experimental atherosclerosis. *J Physiol Pharmacol* **55**, 503–517.
20. Jové M, Pamplona R, Prat J, *et al.* (2013) Atherosclerosis prevention by nutritional factors: a meta-analysis in small animal models. *Nutr Metab Cardiovasc Dis* **23**, 84–93.
21. Khan-Merchant N, Penumetcha M, Meilhac O, *et al.* (2002) Oxidized fatty acids promote atherosclerosis only in the presence of dietary cholesterol in low-density lipoprotein receptor knockout mice. *J Nutr* **132**, 3256–3262.
22. Eder K (1999) The effects of a dietary oxidized oil on lipid metabolism in rats. *Lipids* **34**, 717–725.
23. Kämmerer I, Ringseis R & Eder K (2011) Feeding a thermally oxidised fat inhibits atherosclerotic plaque formation in the aortic root of LDL receptor-deficient mice. *Br J Nutr* **105**, 190.
24. Awada M, Soulage CO, Meynier A, *et al.* (2012) Dietary oxidized *n*-3 PUFA induce oxidative stress and inflammation: role of intestinal absorption of 4-HHE and reactivity in intestinal cells. *J Lipid Res* **53**, 2069–2080.
25. Staprans I, Pan X-M, Rapp JH, *et al.* (1998) Oxidized cholesterol in the diet accelerates the development of aortic atherosclerosis in cholesterol-fed rabbits. *Arterioscler Thromb Vasc Biol* **18**, 977–983.
26. Staprans I, Rapp JH, Pan X-M, *et al.* (1993) The effect of oxidized lipids in the diet on serum lipoprotein peroxides in control and diabetic rats. *J Clin Invest* **92**, 638–643.
27. Staprans I, Rapp JH, Pan X-M, *et al.* (1996) Oxidized lipids in the diet are incorporated by the liver into very low density lipoprotein in rats. *J Lipid Res* **37**, 420–430.
28. Staprans I, Pan X-M, Rapp JH, *et al.* (2000) Oxidized cholesterol in the diet accelerates the development of atherosclerosis in LDL receptor- and apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* **20**, 708–714.
29. Cao J, Zou X-G, Deng L, *et al.* (2014) Analysis of nonpolar lipophilic aldehydes/ketones in oxidized edible oils using HPLC-QqQ-MS for the evaluation of their parent fatty acids. *Food Res Int* **64**, 901–907.
30. Frankel EN (2005) *Lipid Oxidation*. Bridgwater: The Oily Press.
31. Tallman DL & Taylor CG (2003) Effects of dietary fat and zinc on adiposity, serum leptin and adipose fatty acid composition in C57BL/6J mice. *J Nutr Biochem* **14**, 17–23.
32. Sampey BP, Vanhoose AM, Winfield HM, *et al.* (2011) Cafeteria diet is a robust model of human metabolic syndrome with liver and adipose inflammation: comparison to high-fat diet. *Obesity (Silver Spring)* **19**, 1109–1117.
33. Shantha NC & Decker EA (1994) Rapid, sensitive, iron-based spectrophotometric methods for determination of peroxide values of food lipids. *J AOAC Int* **77**, 421–424.
34. McDonald RE & Hultin HO (1987) Some characteristics of the enzymic lipid peroxidation system in the microsomal fraction of flounder skeletal muscle. *J Food Sci* **52**, 15–21.
35. Garcia-Llatas G, Lagarda M, Romero F, *et al.* (2007) A headspace solid-phase microextraction method of use in monitoring hexanal and pentane during storage: application to liquid infant foods and powdered infant formulas. *Food Chem* **101**, 1078–1086.
36. Shirai N, Suzuki H & Wada S (2005) Direct methylation from mouse plasma and from liver and brain homogenates. *Anal Biochem* **343**, 48–53.
37. Van Vré EA, van Beusekom HM, Vrints CJ, *et al.* (2007) Stereology: a simplified and more time-efficient method than planimetry for the quantitative analysis of vascular structures in different models of intimal thickening. *Cardiovasc Pathol* **16**, 43–50.
38. Jordão MT, Ladd FVL, Coppi AA, *et al.* (2011) Exercise training restores hypertension-induced changes in the elastic tissue of the thoracic aorta. *J Vasc Res* **48**, 513–524.
39. Hong Y-L, Yeh S-L, Chang C-Y, *et al.* (2000) Total plasma malondialdehyde levels in 16 Taiwanese college students determined by various thiobarbituric acid tests and an improved high-performance liquid chromatography-based method. *Clin Biochem* **33**, 619–625.
40. Ewing JF & Janero DR (1995) Microplate superoxide dismutase assay employing a nonenzymatic superoxide generator. *Anal Biochem* **232**, 243–248.
41. Flohe L & Gunzler WA (1984) Assays of glutathione peroxidase. *Methods Enzymol* **105**, 114–121.
42. Torres LL, Quaglio NB, de Souza GT, *et al.* (2011) Peripheral oxidative stress biomarkers in mild cognitive impairment and Alzheimer's disease. *J Alzheimers Dis* **26**, 59–68.
43. Kleiner DE, Brunt EM, Van Natta M, *et al.* (2005) Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* **41**, 1313–1321.
44. Zou Y, Li J, Lu C, *et al.* (2006) High-fat emulsion-induced rat model of nonalcoholic steatohepatitis. *Life Sci* **79**, 1100–1107.
45. Ibrahim W, Lee U-S, Yeh C-C, *et al.* (1997) Oxidative stress and antioxidant status in mouse liver: effects of dietary lipid, vitamin E and iron. *J Nutr* **127**, 1401–1406.
46. Staprans I, Rapp JH, Pan X-M, *et al.* (1994) Oxidized lipids in the diet are a source of oxidized lipid in chylomicrons of human serum. *Arterioscler Thromb* **14**, 1900–1905.
47. Grootveld M, Atherton MD, Sheerin AN, *et al.* (1998) *In vivo* absorption, metabolism, and urinary excretion of alpha, beta-unsaturated aldehydes in experimental animals. Relevance to the development of cardiovascular diseases by the dietary ingestion of thermally stressed polyunsaturate-rich culinary oils. *J Clin Invest* **101**, 1210–1218.
48. Uchida K (2000) Role of reactive aldehyde in cardiovascular diseases. *Free Radic Biol Med* **28**, 1685–1696.
49. Adibhatla RM & Hatcher JF (2010) Lipid oxidation and peroxidation in CNS health and disease: from molecular mechanisms to therapeutic opportunities. *Antioxid Redox Signal* **12**, 125–169.
50. Viana M, Villacorta L, Bonet B, *et al.* (2005) Effects of aldehydes on CD36 expression. *Free Radic Res* **39**, 973–977.
51. Uchida K (2003) 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress. *Prog Lipid Res* **42**, 318–343.
52. Rains JL & Jain SK (2011) Oxidative stress, insulin signaling, and diabetes. *Free Radic Biol Med* **50**, 567–575.
53. Agência Nacional de Vigilância Sanitária (Anvisa) (2005) Resolução RDC n° 270, de 22 de setembro de 2005. In *Regulamento técnico para óleos vegetais, gorduras vegetais e creme vegetal (Technical Regulations for Vegetable Oils, Vegetable Fats and Vegetable Cream)*, Vol. RDC 270, [A-ANdV Sanitária, editor]. Brasilia: Diário Oficial da União; Poder Executivo.
54. Dobarganes C & Márquez-Ruiz G (2003) Oxidized fats in foods. *Curr Opin Clin Nutr Metab Care* **6**, 157–163.
55. Negre-Salvayre A, Coatrieux C, Ingueneau C, *et al.* (2008) Advanced lipid peroxidation end products in oxidative damage to proteins. Potential role in diseases and therapeutic prospects for the inhibitors. *Br J Pharmacol* **153**, 6–20.
56. Esterbauer H, Gebicki J, Puhl H, *et al.* (1992) The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radic Biol Med* **13**, 341–390.
57. Vinchi F, Muckenthaler MU, Da Silva MC, *et al.* (2014) Atherogenesis and iron: from epidemiology to cellular level. *Front Pharmacol* **5**, 328–347.
58. Fairweather-Tait SJ, Bao Y, Broadley MR, *et al.* (2011) Selenium in human health and disease. *Antioxid Redox Signal* **14**, 1337–1383.

British Journal of Nutrition

59. Yin H, Brooks JD, Gao L, *et al.* (2007) Identification of novel autoxidation products of the ω-3 fatty acid eicosapentaenoic acid *in vitro* and *in vivo*. *J Biol Chem* **282**, 29890–29901.
60. Gao L, Wang J, Sekhar KR, *et al.* (2007) Novel *n*-3 fatty acid oxidation products activate Nrf2 by destabilizing the association between Keap1 and Cullin3. *J Biol Chem* **282**, 2529–2537.
61. Roy J, Le Guennec J-Y, Galano J-M, *et al.* (2015) Non-enzymatic cyclic oxygenated metabolites of omega-3 polyunsaturated fatty acid: bioactive drugs? *Biochimie* **120**, 56–61.
62. Carrepeiro MM, Rogero MM, Bertolami MC, *et al.* (2011) Effect of *n*-3 fatty acids and statins on oxidative stress in statin-treated hypercholestorelemic and normocholesterolemic women. *Atherosclerosis* **217**, 171–178.
63. Penumetcha M, Song M, Merchant N, *et al.* (2012) Pretreatment with *n*-6 PUFA protects against subsequent high fat diet induced atherosclerosis – potential role of oxidative stress-induced antioxidant defense. *Atherosclerosis* **220**, 53–58.
64. Yang J, Li Y, Wang F, *et al.* (2010) Hepatoprotective effects of apple polyphenols on CCl4-induced acute liver damage in mice. *J Agric Food Chem* **58**, 6525–6531.
65. Zeng T, Guo F-F, Zhang C-L, *et al.* (2008) The anti-fatty liver effects of garlic oil on acute ethanol-exposed mice. *Chem Biol Interact* **176**, 234–242.
66. Botelho PB, Guimarães JP, Mariano KR, *et al.* (2015) Effect of echium oil combined with phytosterols on biomarkers of atherosclerosis in LDLr-knockout mice: echium oil is a potential alternative to marine oils for use in functional foods. *Eur J Lipid Sci Tech* **117**, 1561–1567.
67. Lin C-c & Yin M-c (2008) Effects of cysteine-containing compounds on biosynthesis of triacylglycerol and cholesterol and anti-oxidative protection in liver from mice consuming a high-fat diet. *Br J Nutr* **99**, 37–43.
68. Prewitt RL, Rice DC & Dobrian AD (2002) Adaptation of resistance arteries to increases in pressure. *Microcirculation* **9**, 295–304.
69. Laurent S & Boutouyrie P (2015) The structural factor of hypertension large and small artery alterations. *Circ Res* **116**, 1007–1021.
70. van Diepen JA, Berbée JFP, Havekes LM, *et al.* (2013) Interactions between inflammation and lipid metabolism: relevance for efficacy of anti-inflammatory drugs in the treatment of atherosclerosis. *Atherosclerosis* **228**, 306–315.

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