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 knockout (C57BL/6 strain) 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 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\(^{25}\) or activation of PPAR-\(\alpha\) in the liver and vasculature, inhibiting monocyte recruitment and smooth vascular cells proliferation and migration\(^{25}\). 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.\(^{25,26}\) 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 \textit{in vivo} oxidative stress, using an animal model currently applied to investigate atherosclerosis.

\section*{Methods and materials}

\textbf{Material}

Gold flaxseed oil was obtained by applying cold pressure and by filtration from Pazzio Ind. Alim. Ltda. Thiobarbituric acid (TBA), TCA, 14% BF3-methanol, butylated hydroxytoluene (BHT), cumene hydroperoxide and tetraethoxypropylene (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.).

\textbf{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 value (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 extruded. 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 'catereria 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.

\textbf{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 \textit{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% formalin 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).

\textbf{Methods}

\textbf{Oxidative markers of the flaxseed oil.} Lipid hydroperoxide concentrations were determined as previously described\(^{35}\). 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 O₂/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\(^{(33)}\) 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 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 chromatograms 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 = 59·112 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 pg hexanal/ml of emulsion; two independent replicates were run per sample.

**Fatty acids composition**

Samples were esterified according to the previously described procedure\(^{(33)}\). 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 1·5 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 μmol 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\(^{(38)}\). 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 μM-PBS (pH 7·4). The reaction was initiated with the addition of 25 μl of freshly prepared 33 μM-phenazine methosulphate in 50 μM-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.) (0·173–2·777 U/mg portion (ptn)). Glutathione peroxidase (GPs) activity was determined according to a previously described procedure\(^{(11)}\), 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 mM-PBS and 1 mM-EDTA (pH 7·4), 5 μl of freshly prepared 0·08 μM-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 40 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

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**Fig. 1.** Oxidative markers evaluated in fresh and heated (100°C/10 h) flaxseed oil samples: (a) hydroperoxides (meq O₂/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 χ² 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.).

### 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)

<table>
<thead>
<tr>
<th>Nutrients (g/100 g)</th>
<th>Mean SE</th>
<th>Mean SE</th>
<th>Pt‡</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CONT†</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moisture</td>
<td>6.52 0.11</td>
<td>6.71 0.03</td>
<td>0.169</td>
</tr>
<tr>
<td>Ashes</td>
<td>3.43 0.11</td>
<td>3.20 0.02</td>
<td>0.105</td>
</tr>
<tr>
<td>Lipids</td>
<td>30.32 0.20</td>
<td>30.87 0.40</td>
<td>0.287</td>
</tr>
<tr>
<td>Proteins</td>
<td>16.79 0.30</td>
<td>16.62 0.03</td>
<td>0.596</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>42.94 0.57</td>
<td>42.60 0.45</td>
<td>0.668</td>
</tr>
<tr>
<td>Energy (kJ/100 g)</td>
<td>2141.41 1.59</td>
<td>2153.71 8.16</td>
<td>0.214</td>
</tr>
<tr>
<td>Energy (kcal/100 g)</td>
<td>511.81 0.38</td>
<td>514.75 1.95</td>
<td>0.214</td>
</tr>
<tr>
<td><strong>OXID</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14 : 0</td>
<td>0.49 0.01</td>
<td>0.50 0.01</td>
<td>0.969</td>
</tr>
<tr>
<td>C16 : 0</td>
<td>18.67 0.01</td>
<td>18.85 0.01</td>
<td>0.156</td>
</tr>
<tr>
<td>C18 : 0</td>
<td>0.60 0.02</td>
<td>0.59 0.03</td>
<td>0.821</td>
</tr>
<tr>
<td>C18 : 1 n-9 cis</td>
<td>11.70 0.09</td>
<td>11.04 0.45</td>
<td>0.152</td>
</tr>
<tr>
<td>C18 : 1 n-9 trans</td>
<td>24.71 0.02</td>
<td>25.79 0.37</td>
<td>0.018</td>
</tr>
<tr>
<td>C18 : 2 n-6</td>
<td>1.21 0.06</td>
<td>1.11 0.02</td>
<td>0.239</td>
</tr>
<tr>
<td>C18 : 3 n-3</td>
<td>13.17 0.03</td>
<td>14.86 0.14</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Oxidative markers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LOOH (meq O₂/kg oil)§</td>
<td>2.67 0.24</td>
<td>4.70 0.32</td>
<td>0.037</td>
</tr>
<tr>
<td>TBARS (mg/l oil)§</td>
<td>8.85 0.06</td>
<td>14.42 0.06</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

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 g), casein (151 g), dextrin (100 g), sucrose (100 g), lard (100 g), flaxseed oil (200 g), fibre (100 g), mineral mixture (AIN-93-MX: 37.8 g), vitamin mixture (AIN-93-M-VX: 10.8 g), L-cysteine (1.94 g), choline bitartrate (2.7 g) and tertiary butylhydroquinone (0.06 g). In the mineral mix, salt content was doubled (from 74 g/kg to 148 g/kg of mineral mixture). Se was reduced from 0.0125 g to 0.00512 g/kg and Fe was increased from 0.06 g to 181.8 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.

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

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>CONT−</th>
<th>OXID</th>
<th>CONT+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
</tr>
<tr>
<td><strong>Diet intake (g/d)</strong></td>
<td>0.86</td>
<td>0.00</td>
<td>0.90</td>
</tr>
<tr>
<td>Energy intake (kJ/d)</td>
<td>18.37</td>
<td>0.04</td>
<td>19.29</td>
</tr>
<tr>
<td>Energy intake (kcal/d)</td>
<td>4.39</td>
<td>0.01</td>
<td>4.61</td>
</tr>
<tr>
<td>Initial body weight (g)</td>
<td>24.20</td>
<td>0.54</td>
<td>24.50</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>28.68</td>
<td>1.25</td>
<td>28.55</td>
</tr>
<tr>
<td>Body weight gain/diet intake</td>
<td>5.21</td>
<td>1.32a</td>
<td>4.51</td>
</tr>
<tr>
<td><strong>Blood lipid profile</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>7.79</td>
<td>0.26</td>
<td>7.55</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.88</td>
<td>0.05</td>
<td>1.87</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>2.66</td>
<td>0.11</td>
<td>2.46</td>
</tr>
<tr>
<td>VLDL-cholesterol (mmol/l)</td>
<td>3.24</td>
<td>0.14</td>
<td>3.29</td>
</tr>
<tr>
<td>TAG (mmol/l)</td>
<td>0.84</td>
<td>0.04</td>
<td>0.90</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>10.90</td>
<td>0.57</td>
<td>11.03</td>
</tr>
<tr>
<td><strong>Relative tissue weight</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver (%)</td>
<td>3.22</td>
<td>0.13a</td>
<td>3.08</td>
</tr>
<tr>
<td>Abdominal adipose (%)</td>
<td>1.96</td>
<td>0.26a</td>
<td>1.84</td>
</tr>
<tr>
<td>Retro-epidymal adipose (%)</td>
<td>2.91</td>
<td>0.46a</td>
<td>2.44</td>
</tr>
<tr>
<td>Retro-peritoneal adipose (%)</td>
<td>0.88</td>
<td>0.15a</td>
<td>0.86</td>
</tr>
<tr>
<td><strong>Hepatic fatty acids (mg/g tissue)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>16.28</td>
<td>0.80a</td>
<td>13.79</td>
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<tr>
<td>C16:1</td>
<td>9.66</td>
<td>0.36</td>
<td>9.00</td>
</tr>
<tr>
<td>C18:0</td>
<td>0.76</td>
<td>0.05</td>
<td>0.66</td>
</tr>
<tr>
<td>C18:1n-9 cis</td>
<td>12.97</td>
<td>1.32a</td>
<td>8.51</td>
</tr>
<tr>
<td>C18:1n-9 trans</td>
<td>0.75</td>
<td>0.05</td>
<td>–</td>
</tr>
<tr>
<td>C18:2n-6 cis</td>
<td>9.72</td>
<td>0.76a</td>
<td>7.43</td>
</tr>
<tr>
<td>C18:3n-3</td>
<td>7.24</td>
<td>0.93a</td>
<td>4.45</td>
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<td>C20:4n-6</td>
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<td>0.10a</td>
<td>1.53</td>
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<tr>
<td>C20:5n-3</td>
<td>1.52</td>
<td>0.09a</td>
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<tr>
<td>C22:6n-3</td>
<td>2.60</td>
<td>0.26</td>
<td>2.19</td>
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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 histopathological 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 mol/mg ptn (Fig. 3(a)) in the liver homogenate. However, when concentration was adjusted for the amount of PUFA and expressed as mol/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, 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−) (0.15 (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. In rodents, oxidised lipids are delivered to the liver, incorporated into serum lipoproteins and transported into VLDL, which is secreted into the circulation. In our study, some of the fatty acids...
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 \( \varepsilon \)-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 glutathione-\( S \)-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 \textit{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 \textit{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. 2.
Liver samples were stained with haematoxylin–eosin, the magnification is 100 \( \times \) and the scale bars represent 20 \( \mu \)m. 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.
(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, 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-isoprostanes 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 \(^{(48)}\), 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

![Graphs showing hepatic MDA concentration, plasma MDA concentration, hepatic GPx activity, and hepatic GR activity](https://www.cambridge.org/core/core_media/coverimage.png)

**Fig. 3.** Hepatic malondialdehyde (MDA) content expressed as \(\eta \text{mol/mg portion (ptn)}\) (a) and \(\mu \text{mol/mg ptn per %PUFA}\) (b), plasma MDA content expressed as \(\eta \text{mol/mg ptn}\) (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.
detected \textit{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 \(\Delta^3\)-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 \textit{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\(_2\), 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. Our results showed that consumption of oil containing a higher amount 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. 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 inducers, such as Yang et al. using CCL (4-5 nmol/mg ptn), Zeng et al. applying ethanol (3-5 nmol/mg ptn), Ibrahim et al. with a high-fat diet containing more Fe and without vitamin E (2-60 nmol/mg ptn), Botelho et al. using a high-fat diet with 30%lard (2-50 nmol/mg ptn), Rosa et al. submitting CF1 mice to sham intermittent hypoxia for 35 d (1-2 nmol/mg ptn) and Lin & Ying 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. This enlargement can be attributed to fracture of load-bearing elastin fibres caused by pulsatile tensile stress. 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 fatty acids, 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 stereological 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


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