Basic nutritional investigation

Monounsaturated and ω-3 but not ω-6 polyunsaturated fatty acids improve hepatic fibrosis in hypercholesterolemic rabbits

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Manuscript received January 27, 2004; accepted June 7, 2004.

Abstract

Objective: Although the influence of saturated fatty acids, monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), lipids, cholesterol levels, and other blood lipids has been established, few studies have examined the influence of these dietary lipids on the composition and histologic damage of organs in situations of hypercholesterolemia. Biliary lipids come from the liver, and this organ is essential in cholesterol homeostasis; thus, it may be helpful to evaluate the inter-relations among biliary, hepatic lipids, and hepatotoxic effects in situations of hypercholesterolemia with different dietary lipids. This study investigated whether administration of diets differing in fatty acid profiles (ω-3 PUFAs, ω-6 PUFAs, or MUFAs) influence the content of biliary lipids, the lithogenic index of gallbladder bile, and the development of hepatic fibrosis in hypercholesterolemic rabbits.

Methods: Thirty rabbits were randomized to one of five groups. A control group received rabbit chow for 80 d. The remaining four groups received a 50-d diet that contained 3% lard and 13% cholesterol to provoke hypercholesterolemia. After this period, three groups were fed for another 30 d on a diet enriched with ω-6 PUFAs, MUFAs, and ω-3 PUFAs, respectively. Liver, bile, and plasma lipid compositions, lipid peroxidation in hepatic mitochondria, and histologic hepatic lesions were analyzed.

Results and conclusions: There was a beneficial effect of MUFAs and ω-3 PUFAs on hepatic fibrosis in hypercholesterolemic rabbits because both dietary fats led to recovery from hepatic lesions. However, because intake of ω-3 PUFA provoked lithogenic bile in rabbits, MUFA intake would be more advisable.

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Keywords: Rabbit; Lipid peroxidation; Liver; Bile; Steatohepatitis; Lipids

Introduction

The administration of diets high in cholesterol and saturated fats to induce hypercholesterolemia and hyperlipemia in laboratory animals is a widely used method to investigate different aspects of atherosclerosis, cholesterol metabolism, and biliary lipid secretion [1,2]. A good model to study metabolism of cholesterol and biliary acid is the rabbit because of its similarity to humans [3] and its rapid development of atheromatotic lesions [4].

Diets with different fatty acid profiles are used in the management of hyperlipemia (hypercholesterolemia and hypertriglyceridemia). In this sense, diets rich in ω-3 and ω-6 polyunsaturated fatty acids (PUFAs) and monounsaturated fatty acids (MUFAs) have been used [5,6]. The degree of dietary fat saturation also affects the metabolism of different biliary lipids by altering bile secretion and content; however, there is no consensus on the occurrence or direction of these changes.

This study was supported by grant ALI96-11901 from the CICYT, Ministerio de Educación y Ciencia. Concepción M. Aguilera received a postdoctoral fellowship from the Spanish Ministry of Education. M. Carmen Ramírez-Tortosa and José L. Quiles received a Ramón y Cajal contract from the Ministry of Science and Technology and the University of Granada.

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The influence of dietary \(-3\) PUFA on biliary lipid composition is even more controversial. Although the addition of \(-3\) fatty acids to the diet of the rat increases the content and secretion of cholesterol in the bile\[7\], fish oil in humans decreases levels of biliary cholesterol and the cholesterol saturation index\[8\] and increases the bile content of phospholipids and biliary acids. In the case of the rabbit, the effects of \(-3\) PUFA on bile acids are unknown.

In relation to the possible influences of dietary MUFA on the composition and excretion of biliary lipids in the bile, available data are scarce. In dogs, dietary olive oil as the MUFA source enhances excretion of bile acids in response to food in comparison with sunflower oil rich in \(-6\) PUFA, whereas no changes reportedly take place in biliary concentrations of phospholipids and cholesterol\[9\].

The influence that saturated fatty acids, MUFA, and PUFA exert on levels of cholesterol and other plasmatic lipids and on lipoprotein content in different species including humans has been established\[10,11\]. Nevertheless, few studies have examined the influence of these dietary lipids on the composition and histologic damage of organs subjected to hypercholesterolemia. Because biliary lipids come from the liver and this organ is essential in cholesterol homeostasis, it maybe helpful to evaluate the inter-relations among biliary, hepatic lipids, and hepatotoxic effects in situations of hypercholesterolemia when the dietary lipid quality is manipulated.

In the present study, we investigated whether administration of diets with different fatty acid profiles in \(-3\) PUFA, \(-6\) PUFA, and MUFA, which have proven hypolipemic effects, influence the content of biliary lipids, the lithogenic index of gallbladder bile, and the development of hepatic fibrosis in rabbits that have hypercholesterolemia induced by dietary cholesterol and saturated fat.

**Materials and methods**

**Animals and diets**

Thirty male New Zealand rabbits (Laboratory Animal Service, University of Granada, Granada, Spain) weighing 3 to 3.2 kg were randomized to one of five experimental groups and were kept in individual cages under a 12-h light, 12-h dark cycle with free access to food (150 g/d) and water. One group, which served as the control (group C), received a standard chow diet for 80 d. The other groups received a 50-d diet that contained 95.7% standard chow, 3% lard, and 1.3% cholesterol (Abbott Laboratories S.A., Granada, Spain) to provoke hypercholesterolemia\[12\]. After this period, rabbits in the hypercholesterolemic control group (H) were killed, and the remaining three groups received a 30-d, semisynthetic, and isoenergetic diet composed of 98.25% standard chow and 1.75% fat. These three groups differed only in their lipid source (Table 1): \(-3\) polyunsaturated fatty acid; \(-6\) polyunsaturated fatty acid; MUFA, polyunsaturated fatty acid; SFA, saturated fatty acid

\* Grams per 100 g of total fatty acids recovered.

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**Table 1**

<table>
<thead>
<tr>
<th>C diet</th>
<th>H diet</th>
<th>M diet</th>
<th>P6 diet</th>
<th>P3 diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td></td>
<td></td>
<td></td>
<td>6.9</td>
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<tr>
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<td>21.47</td>
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</tr>
<tr>
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<tr>
<td>18:0</td>
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<td>9.23</td>
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<td>4.37</td>
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<td>18:1(-9)</td>
<td>27.52</td>
<td>29.88</td>
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<td>31.19</td>
</tr>
<tr>
<td>18:1(-7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2(-6)</td>
<td>34.05</td>
<td>28.3</td>
<td>6.81</td>
<td>55.92</td>
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<tr>
<td>18:3(-3)</td>
<td>2.64</td>
<td>2.23</td>
<td>0.66</td>
<td>0.07</td>
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<tr>
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<td>1.09</td>
<td>0.79</td>
<td>0.28</td>
<td>0.15</td>
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<tr>
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<td>0.25</td>
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<td>20:4(-6)</td>
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<tr>
<td>22:6(-3)</td>
<td>0.56</td>
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<td>0.05</td>
<td>0.12</td>
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<tr>
<td>SFA</td>
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<td>31.81</td>
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<tr>
<td>MUFA</td>
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<td>30.67</td>
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<tr>
<td>PUFA</td>
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<td>0.74</td>
<td>0.22</td>
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</tbody>
</table>

C, control (standard rabbit chow); H, hypercholesterolemic; M, monounsaturated fatty acid; MUFA, monounsaturated fatty acid; P3, \(-3\) polyunsaturated fatty acid; P6, \(-6\) polyunsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid
Plasma lipid composition

At the end of the experimental period, rabbits were anesthetized with sodium pentothal at doses of 16 mg/kg of body weight and exsanguinated from a cannulated carotid. Blood was collected into tubes coated with ethylene-diaminetetra-acetic acid, and plasma was separated by centrifugation at 1750g for 10 min. Plasma levels of cholesterol, triacylglycerols (TG), and phospholipids (PL) were measured by enzymatic colorimetric methods using commercial kits (Boehringer Mannheim, Mannheim, Germany).

Determination liver lipid composition

An excised liver fragment was standardized for all rabbits and immediately frozen in liquid nitrogen until analyzed. About 200 mg of liver from each rabbit was minced in a tissue homogenizer. Total lipids were extracted by lysis. About 200 mg of liver from each rabbit was minced and immediately frozen in liquid nitrogen until analysis at 1750g for 10 min. Plasma levels of cholesterol, triacylglycerols (TG), and phospholipids (PL) were measured by enzymatic colorimetric methods using commercial kits (Boehringer Mannheim, Mannheim, Germany).

Bile lipid composition and biliary bile acid determination

Bile was collected from the gallbladder by means of a sterile syringe. Commercial kits (Boehringer Mannheim) were used to analyze cholesterol and PL in the bile. Before biliary acid determination, protein and pigment were removed from the samples according to the method of Levin et al. [14]. Bile acids from gallbladder bile were analyzed as described by Talalay [15] using 3α-hydroxysteroid dehydrogenase and glycolic acid as a standard.

The lithogenic index was calculated according to the method of Metzger et al. [16] and the experimental data of Admirand and Small [17] for the curve of maximum cholesterol solubility.

Lipid peroxidation assay in hepatic mitochondria

Lipid peroxidation was measured as the hydroperoxide content [18] in isolated liver mitochondria [19].

Histologic analysis of hepatic lesions

Whole livers were rapidly dissected out, and the same fraction for all rabbits was taken. For histologic examinations, buffered 4% formaldehyde-fixed, paraffin-embedded tissue sections were stained with hematoxylin and eosin, Gomori’s trichromic stain, periodic acid-Schiff stain, and Gomori’s reticulin silver impregnation stain.

The presentation of steatosis and its distribution in the hepatic parenchyma were evaluated in all rabbit livers according to the effect in zone 3 (pericentral vein), zones 3 and 2 (pericentral vein + central vein), and zones 3, 2, and 1 (pericentral vein + central vein + periportal tract) according to classic models of the hepatic lobule. The inflammatory infiltrate and its localization in the portal tract, parenchyma (lobule; activity, and fibrotic evolution (staging) in the livers were studied according to the terminology of Batts and Ludwig for chronic hepatitis [20]. Moreover, parenchymal iron deposits were analyzed.

Because steatohepatitis may cause central vein sclerosis and pericentral “spidery” fibrosis that isolates individual hepatocytes or clusters of hepatocytes (pericellular fibrosis) with an alteration in reticulin pattern, we evaluated these lesions on a four-point intensity semiquantitative scale (pericellular fibrosis: 0 = no fibrosis, 1 = mild fibrosis, 2 = moderate fibrosis, 3 = severe fibrosis; central vein sclerosis: 0 = no fibrosis, 1 = increase in collagen deposits, 2 = beginning of bridges between central veins, 3 = bridges between two central veins; reticulin pattern: 0 = normal, 1 = mild alteration, 2 = moderate alteration, 3 = severe alteration).

All chemicals and solvents used were of maximal quality and acquired from Merck (Darmstadt, Germany) and Sigma (St. Louis, MO, USA).

Statistical analysis

Before any statistical analysis, all variables were checked for normality and homogeneity of variance by using Kolmogorov-Smirnoff and Levene tests, respectively. When a variable was found not to follow normality, it was log-transformed and reanalyzed. Comparison of results for lipid composition in groups C and H were analyzed by unpaired Student’s t tests. All parameters for groups H, P6, M, and P3 were analyzed by a one-way analysis of variance; to evaluate mean differences between groups, multiple comparison tests were adjusted by Bonferroni’s corrections. Changes in hepatic lesions were analyzed by the Kruskal-Wallis test, and Mann-Whitney U test was performed a posteriori to evaluate mean differences between groups. P < 0.05 was considered statistically significant. Data were analyzed with SPSS 11.0.1 for Windows (SPSS, Inc., Chicago, IL, USA).

Results

Intake of cholesterol and lard significantly boosted the liver weight of rabbits compared with group C. However, after the intake of the different dietary fats, liver weights did not differ with respect to group H (Table 2). The ratio of liver weight to body weight was significantly lower only for group P3 versus group H.

Plasma levels of TG, PL, and TC increased significantly in rabbits fed a diet rich in cholesterol and saturated fat for 50 d. Rabbits in groups P6, M, and P3 had significantly
lower levels of TG, TC, and PL after 30 d of the experimental diets, but only groups P3 and P6 exhibited values TG similar to those in group C (Table 3).

The lipid composition in rabbit livers is listed in Table 3. As has been described for plasma, after cholesterol intake, concentrations of TG, TC, and PL increased significantly compared with group C. Liver TG content did not change after intakes of ω-3 PUFA, ω-6 PUFA, or MUFA. However, liver TC content decreased significantly in groups P6 and P3 versus group H, although group C values were not reached.

Livers adapted their fatty acid profiles to the different diets (Table 4). Hypercholesterolemic rabbits showed a significantly higher percentage of MUFA due to 18:1ω-9 (oleic acid) compared with group C, but ω-6 PUFA, ω-3 PUFA, and saturation indexes were significantly lower. Intake of sunflower oil provoked a significant increase in ω-6 PUFA, the main group of fatty acids present in this oil. Further, the MUFA percentage in group M increased significantly due to the sharp increase in oleic acid (18:1ω-9). Group P3 showed the highest level of ω-3 PUFA compared with the other groups (C, H, P6, and M).

The molar percentage of biliary bile acids was significantly lower in group H than in group C (Figure 1A). However, rabbits fed MUFA and ω-6 PUFA (groups M and P6, respectively) recovered normal values similar to those in group C. The molar percentage of bile cholesterol in rabbits from group H was significantly higher than that in group C. Thus, as occurred with the percentage of bile acids, groups P6 and M approached the value of group C, while only group M showed no significant differences from group C (Figure 1B). The molar percentage of biliary PL was significantly higher in group H than in group C, but, after intakes of different oils, this parameter in groups M, P6, and P3 reached values similar to those in group C (Figure 1C).

The lithogenic index was significantly higher in group H than in group C. This index decreased significantly in groups P6 and M, with values only in group M reaching a value lower than 1 (Figure 1D). Histologic examination showed a normal liver condition in group C, although with a mild to moderate inflammatory infiltrate (Figure 2A) without steatosis and without an increase in intrahepatic collagen deposits. All livers from group H rabbits developed steatohepatitis characterized by a steatosis with mild liver cell injury. Typical macro- and microvesicular fats had a panlobular distribution (Figure 2B,C), although these were occasionally limited to zone 3 plus 2. Neutrophilic and lymphocytic inflammations were mild to moderate and associated with intracytoplasmic lipid deposits. Central vein sclerosis and pericentral spidery fibrosis were moderate to severe, with an altered reticulin pattern (Figure 2D,E), that
was significantly different from those of group C (Table 5). Parenchymal iron deposition and fibrosis in the portal tract were not found. Liver histology for groups fed different edible oils (M, P6, and P3) showed moderate to severe steatosis (Figure 2F) with mild inflammation, but no significant differences were found with respect to group H. Comparisons of central vein sclerosis, pericellular fibrosis, and reticulin pattern across groups P6, M, P3, and H showed that the fibrosis in group P6 was as severe as that in group H. However, the degree of fibrosis was significantly lower in groups M and P3 than in group H, with only group M showing significant differences with regard to group P6 (Figure 3 and Table 5). Parenchymal iron deposition and fibrosis in the portal tract were not found in these rabbits.

Mitochondrial lipid peroxidation status in group H (8.4 ± 1.2 μM hydroperoxide per milligram of protein) was significantly higher than in group C (2.5 ± 0.5 μM). After intakes of different edible oils, only group M (6.0 ± 0.6 μM) and group P3 (4.3 ± 0.6 μM) showed a significantly lower lipid peroxidation compared with group H. However, group P6 (10.9 ± 1.7 μM) showed mitochondrial lipid peroxidation similar to that of group H.

**Discussion**

Differences in liver fatty acid profiles confirmed that all the rabbits adapted to changes induced by experimental dietary lipids. Histopathologic examination of livers showed that differences in liver weight across groups H, P6, P3, and M compared with group C were due to hepatic steatosis induced by intakes of cholesterol and lard. The hypercholesterolemic effect found in the rabbits is consistent with other studies performed in this animal model [21]. Poorman et al. [22] and Podrez et al. [23] reported that the sharp plasma cholesterol increase after cholesterol intake is due to lower synthesis and secretion of biliary bile acids that provoke an accumulation of these compounds in the liver. Our results support this hypothesis because we found a significant decrease in the molar percentage of bile acids. Lipoproteins are important in elimination of cholesterol from plasma, and some studies have suggested that cholesterol or fish oil ingestion interrupts the intracellular transfer of apolipoprotein B and, hence, assembly of very low-density lipoprotein. This enriches rough endoplasmic reticulum membranes with cholesterol, thereby downregulating the expression of the low-density lipoprotein receptor [24]. Alternatively, the high level of biliary cholesterol could be due to a stronger cholesterol flow from high-density lipoprotein to bile that is mediated by a hepatic high-density lipoprotein receptor [2,25]. Our results confirm that alterations in bile composition are involved in the development of hypercholesterolemia in rabbits fed a diet rich in cholesterol and fat.

The effect of dietary lipids on plasma has been well studied, but few studies have addressed the influence of different fatty acids on the synthesis and secretion of biliary lipids and their relation to liver damage. Only hypercholesterolemic rabbits fed a diet rich in MUFA normalized molar percentages of all biliary lipids, leading to a lithogenic index lower than 1. In contrast, molar percentages of bile cholesterol increased significantly in rabbits fed a diet rich in ω-3 PUFA, whereas the molar percentage of biliary acids sig-
nificantly decreased, inducing a lithogenic bile. The high cholesterol content found in bile of rabbits fed a diet rich in ω-3 PUFA could be due to an increase in cholesterol flow from the plasma to the bile by high-density lipoprotein scavenger receptor class B type I receptor. Our results show that dietary ω-3 PUFA decreases plasma and hepatic levels of cholesterol, thus increasing cholesterol and biliary acid excretion by the bile.

Liver histology showed the hypercholesterolemic diet (group H) provoked steatohepatitis with a thickening of the central vein wall that involved adjacent parenchyma in a spider-like pattern that isolated individual or clusters of hepatocytes. These histologic results are characteristic of human non-alcoholic steatohepatitis as described Burt et al. [26] and Sheth et al. [27]. These studies concluded that the histologic diagnostic of non-alcoholic steatohepatitis requires the presence of macrovesicular steatosis with parenchymal inflammation. Moreover, according to the diagnostic criteria proposed by an international group of pathologists [28], it also must include hepatocytic damage, fibrosis, and neutrophilic inflammation, with or without Mallory’s hyaline bodies. Our rabbit model meets the requirements to be considered a human-like non-alcoholic steatohepatitis model, except that microvesicular steatosis is found in rabbits and macrovesicular steatosis is found in humans. Other investigators [29] have described the presence of lipid droplets in the cytoplasm of rabbits (microvesicular steatosis), which may suggest a species-specific feature. No significant differences in the activity parameter were found between groups H and C due to the mild to moderate inflammatory infiltrate in the liver from group C. This inflammatory infiltrate is difficult to explain, but it was not due to the intracytoplasmic lipid deposits that provoke steatohepatitis, as in other studies. Parameter staging, an indicator of chronic hepatitis evolution as described by Batts and Ludwig [20], did not differ significantly among rabbits in groups C and H. This signifies that in this type of lesion, the fibrosis in the portal tract with or without a bridge formation between them, is irrelevant.

When the hypercholesterolemic diet was changed by supplementation with edible oils, there was a regression in the hepatic collagen deposits, leading to a significant decrease in pericellular fibrosis, reticulin pattern, and central vein sclerosis in groups M and P3. Rabbits fed a ω-6 PUFA diet showed no significant differences from group H. The intake of experimental diets may activate a mechanism to remove hepatic cholesterol and lipids, thereby inhibiting synthesis and/or degradation of collagen. Fatty acids are thought to be responsible for hepatocyte damage, and genetic disorders of fatty acid metabolism have been associated with steatosis [30]. Fatty acid β-oxidation occurs in mitochondria and peroxisomes. Mitochondria catalyze the β-oxidation of the bulk of short-, medium-, and long-chain fatty acids derived from the diet, and this pathway constitutes the major process by which fatty acids are oxidized to

![Fig. 1. Molar percentages of bile acids (A), bile cholesterol (B), phospholipids (C), and lithogenic index (D) from hypercholesterolemic rabbits after 30 d of feeding with different dietary fats. †Groups H, P6, M, and P3 versus group C; *groups P6, M, and P3 versus group H (P < 0.05). For each variable, values with different superscript letters are significantly different for groups P6, M, and P3 (P < 0.05). C, control group (n = 6); H, hypercholesterolemic group (n = 6); M, group fed monounsaturated fatty acids (n = 6); P3, group fed ω-3 polyunsaturated fatty acids (n = 6); P6, group fed ω-6 polyunsaturated fatty acids (n = 6).](image-url)
generate energy. Peroxisomes are involved preferentially in the β-oxidation chain shortening of very long-chain fatty acids with the production of H₂O₂ during the process [31]. Some key enzymes of these fatty acid oxidation systems are transcriptionally regulated by peroxisome proliferator-activated receptor-α, a member of the nuclear hormone receptor superfamily [32]. In this sense, Clarke [33] reported that PUFAs, particularly those from the ω-3 family, increase the fatty acid oxidative capacity of tissues through their ability to function as ligand activators of peroxisome proliferator-activated receptor-α and thereby induce transcription of several gene-encoding proteins affiliated with fatty acid oxidation. Our results agree with those of Clarke because rabbits fed a diet rich in ω-3 PUFAs showed a mild hepatic fibrosis, whereas those fed a diet rich in ω-6 PUFAs showed advanced hepatic fibrosis. However, these biochemical pro-

Fig. 2. (A) Liver from a rabbit in the control group demonstrates no steatosis and a mild-to-moderate inflammatory infiltrate (arrows; hematoxylin and eosin, original magnification 100×). (B) Liver from a hypercholesterolemic rabbit shows hepatic steatosis in the three zones of the classic lobule (hematoxylin and eosin, original magnification 100×). (C) Liver from another hypercholesterolemic rabbit displays macro- and microvesicular fat associated with inflammatory infiltrate (arrows) characteristic of steatohepatitis (hematoxylin and eosin, original magnification 600×). (D) Central vein sclerosis and pericentral spidery fibrosis with pericellular fibrosis (Gomori’s trichromic stain, original magnification 200×). (E) Detail shown at 600×. (F) Liver from a rabbit fed a diet rich in monounsaturated fatty acids discloses moderate steatosis in zone 3 plus zone 2 (hematoxylin and eosin, original magnification 100×). CV, central vein; PT, portal tract.
cesses increase mitochondrial free radical generation and subsequent lipid peroxidation, thereby provoking liver damage [34]. Our results concerning mitochondrial lipid peroxidation status showed that rabbits fed diets with ω-3 PUFA and MUFA had less production of reactive oxygen species than did rabbits fed a diet with ω-6 PUFA. Both mechanisms could explain the greater hepatic damage in rabbits fed ω-6 PUFA.

In summary, our results show a beneficial effect of MUFA and ω-3 PUFA on hepatic fibrosis induced by a hypercholesterolemic diet because both dietary fats resulted in recovery from hepatic lesions. However, because intake of ω-3 PUFA provoked lithogenic bile in the rabbits, MUFA intake would be more advisable. Moreover, lipid metabolism disorders have been associated with hepatic steatosis and hepatic fibrosis, so that consumption of MUFA could be recommended to manage these liver pathologies, but further investigation is needed in humans.

Table 5
Fibrosis in hypercholesterolemic rabbits after 30 d of feeding with different dietary fats*

<table>
<thead>
<tr>
<th>Groups</th>
<th>Central vein sclerosis</th>
<th>Pericellular fibrosis</th>
<th>Reticul pattern</th>
<th>Overall score</th>
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<tr>
<td>C</td>
<td>0.0 ± 0.0</td>
<td>0.29 ± 0.18</td>
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<td>H</td>
<td>2.0 ± 0.41</td>
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<td>P6</td>
<td>2.17 ± 0.17</td>
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<td>6.33 ± 0.55</td>
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<td>M</td>
<td>1.67 ± 0.33</td>
<td>0.83 ± 0.31</td>
<td>0.83 ± 0.31</td>
<td>3.33 ± 0.76</td>
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<tr>
<td>P3</td>
<td>1.50 ± 0.22</td>
<td>1.33 ± 0.21</td>
<td>1.33 ± 0.21</td>
<td>4.16 ± 0.60</td>
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</table>

C, rabbits fed a control diet (n = 6); H, hypercholesterolemic rabbits (n = 6); M, rabbits fed a diet rich in monounsaturated fatty acid (n = 6); P6, rabbits fed a diet rich in ω-3 polyunsaturated fatty acid (n = 6); P3, rabbits fed a diet rich in ω-6 polyunsaturated fatty acid (n = 6).

* Results are expressed as mean ± standard error of the mean. Values with different superscript letters are significantly different for groups P6, M, and P3 at P < 0.05.

† P < 0.05, groups P6, M, and P3 versus group H.
‡ P < 0.05, groups H, P6, M, and P3 versus group C.
References


