Lipid peroxidation and glutathione system in hyperlipemic rabbits: influence of olive oil administration

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Abstract

We studied the effect of supplementation (10% w/w) of a hyperlipemic diet (1% cholesterol) with olive oil (OLIV) for 6 weeks in four groups of 10 rabbits each. At the end of this period, we determined lipid peroxidation, glutathione content, and glutathione peroxidase, reductase and transferase activities in liver, brain, heart, aorta and platelets. The atherogenic diet increased tissue lipid peroxidation and decreased the protective antioxidant effect of glutathione. Dietary supplementation with olive oil reduced tissue lipid peroxidation by 71.6% in liver, 20.3% in brain, 84.5% in heart, 63.6% in aorta, 72% in platelets. The ratios total/oxidized glutathione were increased in all tissues (49% in liver, 48% in brain, 45% in heart, 83% in aorta, 70% in platelets). Olive oil increased glutathione peroxidase and transferase activities in all tissues. We conclude that in rabbits made hyperlipemic with a diet rich in saturated fatty acids, olive oil decreased tissue oxidative stress. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Olive oil; Lipid peroxidation; Glutathione; Atherosclerosis; Oxidative stress

1. Introduction

Oxygen-derived free radicals play an important role in the pathogenesis of atherosclerosis, mainly by oxidizing low-density lipoproteins (LDL) and generating species that can react with oxygen in the vascular wall [1]. In addition, the production of free radicals is increased in the leukocytes of patients with hypercholesterolemia [2].

One of the treatments indicated for atherosclerosis is a diet poor in saturated fatty acids and rich in poly- and monounsaturated fatty acids [3]. In several experimental models, this dietary measure has been shown to reduce the thrombogenic capacity of the arterial wall and of the platelets, and to lead to reductions in atheromatous lesions [4,5].

In the Mediterranean region, olive oil is one of the main sources of dietary fatty acids. Olives and their oil contain oleic acid and a series of polyphenols [6] which have been shown in studies in vitro to inhibit platelet function and thromboxane synthesis [7], and to stimulate the uptake of free radicals by leukocytes [7,8].

The present study in an experimental model of atherogenesis in rabbits was designed to evaluate the effects of dietary supplementation with olive oil...
on the main indicators of tissue oxidative status as an expression of the cellular damage caused by free radicals, and on antioxidant defence mechanisms, particularly glutathione.

2. Materials and methods

2.1. Animals

A total of 40 male white New Zealand rabbits were used. The animals were 2 months old at the start of the experiment, and had a mean body weight of 2376 ± 31 g. The experimental protocol was approved by the Ethical Committee for Animal Care of the University of Málaga; at all times the animals received care in compliance with the criteria in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences.

2.2. Experimental groups

The rabbits were randomly divided into four groups of 10 animals each. Group 1 animals (controls) were fed a commercial standard diet (Lapin Entretien Ref/112, Laboratorios Panlab, Barcelona). Group 2 animals (atherogenic diet) received a commercial atherogenic diet consisting of a standard diet supplemented with 1% cholesterol, and a high saturated, monounsaturated and polyunsaturated fatty acid content (Laboratorios Panlab). Group 3 animals (OLIV) were given the standard diet enriched with 10% olive oil (Aceites Minerva, Málaga, Spain). Group 4 animals (atherogenic diet+OLIV) were fed with the atherogenic diet enriched with 10% olive oil.

Olive oil was used because of its high content in monounsaturated fatty acids. Table 1 shows the fatty acid composition of each of the four diets tested, and of olive oil alone. Each group was kept on the diet for 6 weeks. Food intake was recorded periodically to avoid differences between groups in the amount of feed consumed. The OLIV-enriched diet was prepared fresh daily.

2.3. Sample collection

The animals were anesthetized with a subcutaneous injection of thalamonal (0.5 ml/kg b.wt.), ketamine (50 mg/kg b.wt.) and midazolam (2.5 mg/kg b.wt.). A medial laparotomy was done to obtain blood from the inferior vein cava. Blood was kept at 37°C for 45 min, then centrifuged at 4500 × g for 15 min at 4°C to obtain serum. Part of the blood was anticoagulated with 3.8% trisodium citrate (1:10 v/v) and was then centrifuged at 800 × g for 10 min at 18°C to obtain platelet-rich plasma (PRP). Platelet count was recorded, then PRP was centrifuged at 2500 × g for 25 min at 18°C. The platelet pellet was washed twice with phosphate-buffered saline (pH 7.4). Tissue samples from the abdominal aorta, liver, heart and brain were obtained.

2.4. Analytical techniques

To determine the basic serum lipid profile we used standard spectrophotometric techniques to measure the concentration of total cholesterol, high density lipoprotein (HDL) cholesterol and triglycerides. Fatty acids were quantified by gas chromatography. Samples were previously dried, methylated and extracted with hexane, then injected into a Hewlett-Packard Model 5890 (Avondale, PA, USA) gas chromatograph.

2.5. Determination of lipid peroxidation

Enriched membrane fractions were used to measure lipid peroxidation. This kind of preparation allows a better coefficient of variation (4.8 ± 0.2%), than in other cell fractions (in our laboratory: 34.9 ± 6.5% in whole cells or 15.8 ± 2.1% in mitochondrial fraction). All samples were diluted 1:10 v/v in buffer containing 50 mM Tris, 100 mM NaCl, 0.5 mM KCl, 3.1 mM CaCl₂, 1 mM MgSO₄, 0.55 mM KH₂PO₄, and 3.20 M sucrose (pH 7.4). The samples were minced and homogenized in a Braun potter at 600 bats, then centrifuged at 1000 × g for 10 min at 4°C. The pellet was discarded and the supernatant was centrifuged at 20,000 × g for 20 min at 4°C. The supernatant was removed and the pellet diluted (1:10 v/v) in the buffer mentioned above without sucrose. All samples were set in crushed ice throughout the procedure.

The products resulting from the reaction with thiobarbituric acid (TBARS), of which the most significant is malondialdehyde (MDA), were used as indi-
cators of lipid peroxidation in membrane fractions. Briefly [9], membrane concentrates were diluted (1:4, v/v) in the buffer solution described above with 20 mM Tris, and were divided into 1.8-ml aliquots. Then 0.1 ml ferrous sulfate and 0.1 ml of ascorbic acid (FeAs) in increasing equimolar concentrations were added. FeAs was used to induce lipid peroxidation via the formation of hydroxyl anions. Test tubes were incubated at 37°C for 45 min with continuous shaking. Blanks which contained only tissue were incubated at 4°C. The reaction was stopped and thiobarbituric acid reactive substances (TBARS) were analyzed using 1 ml 0.5% thiobarbituric acid in 20% trichloroacetic acid. The products used in the samples were added to the blanks. After agitation, the samples were incubated at 100°C for 15 min and then centrifuged at 1000 g for 15 min at 4°C. The amount of TBARS produced was measured as the spectrophotometric absorbance of the supernatant at 532 nm.

The absorbances were compared to that of a standard curve obtained using malondialdehyde-bis-diethyl-acetal. The protein content of the samples was determined using the Bradford method [10]; the results were expressed as nmol of TBARS/mg of protein.

2.6. Determination of glutathione and related enzymes

2.6.1. Total glutathione levels

Total glutathione levels (TG) were determined by spectrofluorometry according to the technique described by Hissin and Hill [11]. Briefly, 200 mg of tissue was homogenized in 0.1 M sodium phosphate buffer (pH 8.0) with 25% phosphoric acid at a proportion of 1:20. The mixture was centrifuged at 13000×g for 15 min at 4°C, and the supernatant was collected. Two spectrophotometry cuvettes per sample were prepared with 1.8 ml sodium phosphate buffer, 100 µl supernatant of the sample, and 100 µl o-phthalaldehyde. The preparations were shaken and incubated for 15 min at 4°C, and a spectrophotometric reading was then obtained at an excitation wavelength of 350 nm and an emission wavelength of 440 nm. The results were compared against a standard curve for commercial glutathione processed as described above, and were expressed as µmol TG/g tissue.

To find the percentages of total glutathione corresponding to oxidized and reduced forms, 200 µl of the sample supernatant was incubated with 8 µl 4-vinylpyridine for 1 h at room temperature; the sample was then processed as described above to determine oxidized glutathione (GSSG). Results are done as the ratios total/oxidized glutathione.

2.6.2. Enzyme activities related with glutathione

Enzyme activities related with glutathione (glutathione peroxidase, glutathione reductase and glutathione transferase) were determined by spectrophotometric kinetics. Tissue samples weighing approximately 300–400 g were diluted in 4 ml 0.1 M potassium phosphate buffer (pH 7.0) with 1 ml 25% phosphoric acid. The mixture was homogenized and centrifuged at 13000×g for 15 min at 4°C. The supernatant was used to determine protein concentrations according to the Bradford method [10].

2.6.3. Glutathione peroxidase

Glutathione peroxidase (GSHpx) was measured according to the method of Flohé and Gunzler [12]. Briefly, a volume equivalent to 25 µg protein was taken from each supernatant and 0.1 M potassium phosphate buffer was added to a volume of 880 µl, together with 53 µl glutathione reductase, 133 µl GSH, and 100 µl nicotinamide-adenine dinucleotide phosphate (NADPH). The microcuvette was shaken by inversion and incubated at 37°C for 3 min. Then 100 µl terbutyl-hydroperoxide was added, and the preparation was read at 340 nm; the decrease in absorbance was recorded every 30 s for 5 min.

2.6.4. Glutathione reductase activity

Glutathione reductase activity (GSSGrd) was determined according to the technique described by Flohé and Gunzler [12]. The amounts of sample and buffer were the same as in the GSHpx analyses. One hundred microliters of NADPH was added, and the microcuvettes were shaken by inversion and incubated as described above for GSHpx. Then 100 µl GSSG was added, the cuvettes were again shaken by inversion, the preparation was read at 340 nm, and the decrease in absorbance was recorded every 30 s for 5 min.
2.6.5. Glutathione transferase activity

Glutathione transferase activity (GSHtf) was determined with the method described by Warholm et al. [13]. One hundred microliters of GSH was added to the volumes of sample and buffer indicated for the other enzyme determinations. The mixture was shaken by inversion and incubated at 37°C for 3 min. Then 50 μl 1-chloro 2,4-dinitrobenzene was added, the mixture was shaken by inversion, and the preparation was read at 340 nm as for the other enzyme activities.

The results for GSHpx and GSSGrd were expressed as units/min, taking into account a molar extinction coefficient for NADPH of 6.22 cm⁻² mol⁻¹. The results for GSHtf were calculated with a correction coefficient of 0.1042.

2.7. Statistical analysis

All results were expressed as the mean ± standard error of the mean (S.E.M.). The groups were compared with Student’s t-test and analysis of variance, and significance of the differences was determined with the Newmann–Keuls test. All analyses were done with the Statistical Package for Social Sciences (SPSSx, version 3.0).

3. Results

3.1. Effect of the atherogenic diet

Feeding with a diet rich in SFA led to mixed hy-
perlipemia:hypercholesterolemia (85.9 ± 14.5 mg/dl in the control group, 1759 ± 166 mg/dl in hyperlipemic animals) and hypertriglyceridemia (117 ± 18 mg/dl in the control group, 571 ± 64 mg/kg in hyperlipemic rabbits). The levels of HDL-cholesterol decreased (31.2 ± 4.8 mg/dl in controls, 17.9 ± 1.5 mg/dl in hyperlipemic animals).

The production of TBARS increased significantly in all tissues in animals given the hyperlipemic diet (Table 2). By way of example, Fig. 1 shows the curves for mean FeAs-induced TBARS in aortic tissue from all four experimental groups.

The ratios total/oxidized glutathione were significantly lower in animals that consumed the hyperlipemic diet (Table 3). There was a proportional increase in the oxidized fraction of glutathione in this group.

Enzymatic activities related with glutathione showed some modifications in rabbits fed with the hyperlipemic diet (Table 4). Glutathione peroxidase activity increased, although not significantly, in brain and platelets; GSSGrd showed a significant decrease in aortic tissue only; and GSHTf was lower in all tissues.

3.2 Effect of olive oil on experimental atherogenesis

The administration of dietary olive oil to hyperlipemic rabbits reduced serum cholesterol to 1342 ± 149, decreased triglycerides to 364 ± 43.2, and increased HDL-cholesterol to 29.5 ± 4.7.

In normolipemic animals, the production of FeAs-induced TBARS was significantly reduced in the liver and heart, whereas in hyperlipemic animals olive oil significantly reduced these values in all tissues (Table 1, Fig. 1).

In normolipemic controls, dietary olive oil significantly increased glutathione ratio in the heart and platelets (Table 4). In animals given the hyperlipemic diet, olive oil increased glutathione system in all tissues.

Enzymatic activities related with glutathione were not modified by olive oil in normolipemic animals. However, in hyperlipemic rabbits, GSHpx was higher in all tissues, GSSGrd was significantly increased in the aorta and platelets, and GSHTf activity was higher in all tissues after treatment with olive oil (Table 4).

Table 3
Ratios total/oxidized glutathione in tissues from different groups of rabbits

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Normolipemic diet (NLD)</th>
<th>NLD+OLIV</th>
<th>Atherogenic diet (ATD)</th>
<th>ATD+OLIV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>17.5 ± 1.8</td>
<td>16.2 ± 1.8</td>
<td>11.1 ± 1.0b</td>
<td>18.3 ± 1.7c</td>
</tr>
<tr>
<td>Brain</td>
<td>16.4 ± 1.4</td>
<td>16.6 ± 1.9</td>
<td>11.5 ± 0.9d</td>
<td>17.6 ± 1.8c</td>
</tr>
<tr>
<td>Heart</td>
<td>18.2 ± 2.0</td>
<td>28.7 ± 3.1a</td>
<td>12.0 ± 1.5a</td>
<td>17.6 ± 1.9c</td>
</tr>
<tr>
<td>Aorta</td>
<td>17.2 ± 1.9</td>
<td>17.9 ± 2.0</td>
<td>11.0 ± 1.3d</td>
<td>20.5 ± 2.5d</td>
</tr>
<tr>
<td>Platelets</td>
<td>17.5 ± 1.8</td>
<td>24.4 ± 2.6o</td>
<td>11.4 ± 1.3d</td>
<td>20.0 ± 2.5b</td>
</tr>
</tbody>
</table>

OLIV, olive oil.

aP < 0.05, versus NLD.
bP < 0.05, versus ATD.
cP < 0.1, versus ATD.
dP < 0.05, versus NLD.
eP < 0.05, versus ATD.
fP < 0.001, versus NLD.
4. Discussion

Our results show that a diet rich in SFA can lead to changes in the oxidative stress system in many different tissues in the rabbit. These changes were manifested mainly as an increase in lipid peroxidation capacity and a decrease in the antioxidant defence capacity of the glutathione system. Dietary supplementation with olive oil reduced lipid peroxidation and favored tissue antioxidant defence mediated by the glutathione system.

As shown by changes in the serum lipid profile, olive oil reduced hyperlipemia in our experimental model, and we found a significant decrease in the atherogenic index (total/HDL-cholesterol index).

Previous studies have reported contradictory findings with regard to the effect of olive oil on the plasma lipid profile. Schick et al. [14] and Navarro et al. [15]; in experimental animals, and Sirtori et al. [16]; in humans, found that a diet rich in olive oil led to no significant modifications in plasma lipids. In contrast, Wahrburg et al. [17] and Grundy et al. [18] showed that in humans, olive oil reduced levels of LDL cholesterol and increased HDL cholesterol; these results are similar to the findings in the present experimental study.

Our finding that tissue peroxidative capacity was enhanced in animals fed with a hyperlipemic diet is compatible with results reported by others [19,20]. These earlier studies postulated that an increase in the production of free radicals might have consequences in hyperlipemic patients. The results reported here are also compatible with the oxidative hypothesis of atherosclerosis [1], according to which superoxide anions lead to the oxidation of LDL. Our findings document increases in the peroxidative capacity of these tissues (TBARS) (Table 2, Fig. 1).

The decrease in total glutathione and proportional increase in its oxidized fraction, together with the increase in GSHpx activity and the decrease in GSHtf activity, indicate that oxidative tissue stress
occurred in hyperlipemic animals. This oxidative stress may account for the earlier finding in our laboratory [4,5] of a reduction in vascular prostacyclin production and an increase in platelet thromboxane synthesis, in relation with increased endothelial lipid peroxide production in the same experimental model as that used in the present study.

Increased tissue oxidative stress can lead to cell damage. Lipid peroxides may increase the production of platelet thromboxane and reduce that of vascular prostacyclin [21]; this may favor thrombotic processes in hyperlipemia, which would then coincide with the well-known thrombogenic potential of the atheromatous plaque itself. In our experimental model, we observed such an effect in platelets and in the arterial wall, and also noted an increase in oxidative stress in other tissues, suggestive of systemic cellular stress caused by lipid peroxides. This oxidative stress may be exacerbated in vivo by the ischemia (reduced arterial blood flow) resulting from atherosclerosis.

The administration of diets enriched in oils rich in n-3 or n-6 fatty acids was shown to reduce the capacity of brain and liver tissues to produce lipid peroxides [15,22–24]. Moreover, research with the same experimental model as that used in the present study found that diets rich in n-6 fatty acids modified oxidative stress in several tissues [25].

Clement and Bourre [26] postulated that this effect of n-6 fatty acid derivatives may result from enhanced antioxidative defence mechanisms in the brain. Our findings in animals given olive oil, which also contains n-6 polyunsaturated fatty acids, are compatible with these results: when tissue oxidative stress increased, olive oil decreased lipid peroxidation and augmented the glutathione-based defence mechanism in the brain and in other tissues. In two earlier studies with the same experimental model as that reported here, we previously found that γ-linoleic acid and olive oil curtailed the increase in platelet thrombotic activity and reduced arterial atheromatous lesions [5] (De La Cruz et al., unpublished data). This antioxidative effect may play an important role in the antithrombotic effect of these fatty acid that occurred in our experimental model.

With regard to the effects of olive oil on free radicals, Scaccini et al. [27] and Aviram et al. [28] showed that a diet rich in olive oil decreased the capacity of the VLDL and HDL fractions of cholesterol to oxidize free radicals, a process that occurs in human hyperlipemia and in models of atherogenesis in experimental animals [1]. The present findings show that a diet rich in olive oil reduces tissue oxidative stress not only by decreasing lipid peroxidation, but also by enhancing the glutathione antioxidative defence system.

Several mechanisms have been proposed to explain the antioxidant capacity of olive oil. One possible mechanism is the increase in tissue sensitivity to the lipid antioxidant effects of vitamin E [27]. Another possibility, suggested by Reaven et al. [28], is the antioxidative ability of oleic acid itself, the major component of olive oil. Recent work by De La Puerta et al. [8] showed that the polyphenols in olive oil (oleuropein, tyrosol, hydroxytyrosol and caffeic acid) inhibit the in vitro activity of leukocyte 5-lipoxygenase, and also inhibit the formation of oxygen-reactive species in these cells in a concentration-dependent way.

We found no reports in earlier studies that olive oil affects the tissue glutathione system. Joulain et al. [29] described an inhibitory effect on GSHpx activity in leukocytes incubated with n-6 polyunsaturated fatty acids, but found no effect after incubation with monounsaturated fatty acids. In our study, no such effect was found in normolipemic animals, whereas GSHpx activity was inhibited in hyperlipemic rabbits. This finding may reflect an active defence system that attempts to counteract the increase in lipid peroxides in hyperlipemic animals by increasing the activity of the glutathione system. In an early study [25], we demonstrated that hyperlipemic rabbits treated with evening primrose oil showed higher antioxidant capacity than normolipemic animals, as well as in the present study with olive oil. Probably, these antioxidant substances increase its effects in high oxidative stress situations, such as occurs with other antioxidant drugs [30].

In summary, we found that in our model of experimental atherogenesis, a diet rich in olive oil reduced the increase in tissue oxidative stress and enhanced the antioxidative defence system. These mechanisms may avoid the morphological and functional damage caused by free radicals in a variety of tissues. The effects may also constitute an additional explanation of the ability of the Mediterranean diet — in which
olive oil is a major component – to prevent cardiovascular disease.

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References

[26] M. Clement, J.M. Bourre, Alteration of alfa-tocopherol content in the developing and peripheral nervous system: per-
sistance of high correlation with total and specific (n-6) poly-
Felice, G. Tomasi, Effect of dietary oils on lipid peroxidation
and on antioxidant parameters of rat plasma and lipoprotein
[28] P. Reaven, S. Parthasarthy, B. Grasse, E. Miller, D. Stein-
berg, J.L. Witztum, Effects of oleate-rich and linoleate-rich
diets on the susceptibility of low density lipoprotein to oxy-
ative modification in mildly hypercholesterolemic subjects,
glutathione peroxidase activity in human blood mononuclear
cells upon in vitro incubation with n-3 fatty acids, Biochem.
[30] J.A. González-Correa, J.P. De La Cruz, E. Martín-Aurioles,
M.A. López-Egea, P. Ortiz, F. Sánchez de la Cuesta, Effects
of S-adenosyl-l-methionine on hepatic and renal oxidative
stress in an experimental model of acute biliary obstruction