Dietary non-tocopherol antioxidants present in extra virgin olive oil increase the resistance of low density lipoproteins to oxidation in rabbits

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Abstract

Consumption of a range of dietary antioxidants may be beneficial in protecting low density lipoprotein (LDL) against oxidative modification, as studies have demonstrated that antioxidants other than vitamin E may also function against oxidation of LDL in vitro. In the present study, the effect of polyphenol antioxidants on the susceptibility of LDL to copper-mediated oxidation was investigated after feeding semi-purified diets to 3 groups of New Zealand white (NZW) rabbits. All diets comprised 40% energy as fat with 17% energy as oleic acid. Dietary fatty acid compositions were identical. Oils with different polyphenol contents were used to provide the dietary source of oleic acid — refined olive oil, extra virgin olive oil and Trisun high oleic sunflower seed oil. Polyphenolic compounds (hydroxytyrosol and p-tyrosol) could only be detected in the extra virgin olive oil. Vitamin E was equalised in all diets. LDL oxidizability in vitro was determined by continuously monitoring the copper-induced formation of conjugated dienes after 6 weeks of experimental diet feeding. The lag phase before demonstrable oxidation occurred was significantly increased in the high polyphenol, extra virgin olive oil group (P < 0.05) when compared with combined results from the low polyphenol group (refined olive oil and Trisun), even though the LDL vitamin E concentration in the high polyphenol group was significantly lower. The rate of conjugated diene formation was not influenced by the presence of dietary polyphenols. Results demonstrate that antioxidants, possibly phenolic compounds which are present only in extra virgin olive oil, may contribute to the endogenous antioxidant capacity of LDL, resulting in an increased resistance to oxidation as determined in vitro.

Keywords: Antioxidant; Low density lipoprotein; Conjugated diene formation; Olive oils; Polyphenols; Rabbits

1. Introduction

Low density lipoprotein (LDL) that has been modified by oxidation has been implicated in the development of atherosclerosis [1]. Circulating LDL particles in plasma are protected from the
effects of lipid peroxidation by cellular and extra-
cellular antioxidant mechanisms which serve to
trap reactive oxygen species close to their site of
formation and which also function to inhibit the
chain reaction of free radical formation. LDL
particles contain fat-soluble endogenous antioxi-
dants that can also prevent or limit the chain
reaction of lipid peroxyl radical formation. These
natural antioxidants, which include ubiquinol, vi-
tamin E, lycopene and β-carotene, are preferen-
tially oxidised before oxidation of LDL
polyunsaturated fatty acids [2,3]. Vitamin E is
quantitatively the most abundant antioxidant
present in LDL, and in vitro studies suggest that
synergistic interaction between vitamin C and the
tocopheroxyl radical maintains vitamin E in the
functional, reduced state [4,5]. It may be of benefit
to increase both the range and concentration of
endogenous antioxidants contained within LDL
in order to gain optimal protection against oxida-
tive modification. Oral supplementation with vitai-
nin E but not β-carotene has been shown to
independently increase the resistance of LDL to
oxidation as determined by in vitro methods [6–
9], and other dietary antioxidants may also be of
importance [10], although it is not clear to what
extent antioxidant supplementation will prevent
atherosclerosis in the presence of high levels of

Polyphenolic antioxidants are present in un-
refined olive oils, and they have been demon-
strated to contribute considerably to the oxidation
stability of the oil [12]. Consumption of Mediter-
anean diets, with olive oil as the major fat, has
been shown in epidemiological studies to be asso-
ciated with a reduced incidence of coronary heart
disease (CHD) [13]. In terms of the oxidation
modification hypothesis of atherosclerosis, in-
creased olive oil consumption at the expense of
polyunsaturated fats would increase the intake of
monounsaturated acids which are less susceptible
to oxidation, and may also result in increased
consumption of dietary polyphenolic antioxi-
dants. Polyphenolic compounds present in red
wine and green tea (including flavonoids, cate-
chins and flavonols) were recently shown to in-
hbit the oxidation of human LDL in vitro
[14,15], and polyphenols from other dietary
sources, such as olive oil, may also have similar
effects [16]. The concentration of polyphenols is
known to vary among olives of different varieties
and from different locations [17] and is also
known to be influenced by the oil extraction
procedure. Virgin olive oil produced from olives
of good quality is consumed unrefined and there-
fore contains polyphenols which are usually re-
moved from other edible oils as a consequence of
the refining process [18]. To investigate the effects
of different olive oil types on LDL oxidizability,
experimental diets were fed to 3 groups of female
New Zealand white (NZW) rabbits. The diets
were planned to have identical fatty acid composi-
tions and vitamin E contents and differed only in
terms of the dietary oleic acid source. Oleic acid
provided 17% energy of each experimental diet
and was provided by either a refined olive oil, an
extra virgin olive oil or Trisun high oleic
sunflower seed oil. In this way, the effects on LDL
oxidation of components present in the unsa-
ponifiable fractions of the oils could be compared
independent of dietary fatty acid composition or
vitamin E concentration.

2. Animals and methods

2.1. Study design

Twenty four female NZW rabbits (8–10 weeks
old) were obtained from Harlan CPB, Zeist, The
Netherlands. The animals were individually
housed under standard conditions (temperature
18 ± 1°C, humidity 65% ± 10% and day/night
cycles of 12 h). Food and drinking water were
available ad libitum throughout the study and all
animals were clinically observed and weighed
weekly. In the first prestudy week, the animals
acclimatised and received 100% standard rabbit
diet (Stanrab SQC, SDS, England). In the second
prestudy week, the animals were randomised into
3 dietary groups (n = 8) on the basis of body-
weight, and began accustomisation to the experi-
mental diets, receiving 50% standard rabbit diet
and 50% semi-purified diet. In the third (and final)
prestudy week, the animals received 25% standard
rabbit diet and 75% semi-purified diet. From the
first study week up to the end of the study period
(6 weeks), the animals received 100% semi-
purified diets which contained 40% energy as fat. The fat blends of each diet were designed to have virtually identical fatty acid compositions, with 17% energy provided by oleic acid. Only the fat sources of the diets differed — either refined olive oil (Chempri BV, Raamsdonksveer, The Netherlands), extra virgin olive oil (Dante, Milan, Italy), or Trisun high oleic sunflower seed oil (Contined BV, Bennekom, The Netherlands) were used as sources of oleic acid. The composition of the semi-purified diets (expressed in g/1000 kJ) was: calcium caseinate (14.6), experimental fat (10.8), maize starch (27.1), mineral mixture (5.5), vitamin mixture without vitamin E (0.5) and fibre (15.9). Vitamin E was equalised in all diets by supplementing the 2 olive oil group diets with D-\text{\textalpha}-tocopherol (Sigma, St. Louis, MO, USA) to the level found in the Trisun group diet. Determination of fatty acid composition and vitamin E concentration was carried out on all fat blends as described in experimental methods (Table 1). After 6 weeks on the full experimental diets, blood samples were collected from the ear vein of each animal into Na\text{\textsubscript{2}}-ethylene diamine tetraacetic acid (EDTA, final concentration 1 mg/ml). Plasma was prepared immediately by centrifugation for 10 min at 2000 \times g and either used freshly for LDL isolation and vitamin C and uric acid analysis or stored at –70°C under argon.

2.2. Laboratory analyses

All chemicals were of analytical grade and were obtained from Merck, Darmstadt, Germany, unless otherwise stated. The concentration of vitamin E in representative samples of the fat blends of the experimental diets was determined by high performance liquid chromatography (HPLC). Following liquefaction and homogenisation at 60°C, approximately 200 mg of each fat blend was accurately weighed and dissolved in chloroform/isopropanol (1:2, v/v). Samples were injected onto a reversed phase C18 column, with methanol/isopropanol/water (50:50:6, v/v) as the mobile phase, with detection at 292 nm. Vitamin E was quantified using \text{\textalpha}, \text{\textgamma} and \text{\textdelta} tocopherol as the external standards; results were expressed as tocopherol equivalents (TE) (1 mg D-\text{\textalpha}-tocopherol = 1 TE, 1 mg D-\text{\textgamma} tocopherol = 0.1 TE).

The polyphenol concentration in the experimental diet oils was determined. Representative samples of the oils were taken, and after homogenisation, approximately 20 g of oil (weighed to the nearest 0.1 mg) was diluted with 10 ml hexane. One ml syringic acid (Sigma, St. Louis, MO, USA) was added as internal standard, together with 10 ml extraction eluent (methanol/water, 40:60, v/v). The tube was then shaken for 2 min and, after centrifugation, the lower, water/methanol phase was transferred to a clean tube. The extraction phase was repeated, then 10 ml hexane was added to the combined methanol/water extracts. The tube was shaken for one minute and then centrifuged to separate the two layers. The water/methanol phase was then transferred to a 100 ml, round bottomed flask and evaporated to 1–2 ml on a rotary evaporator (vacuum, 65°C). The remaining sample solution was then passed through a 45 μM filter. A standard curve was prepared at the same time: approximately 20 g Ceres MCT dietary oil (Van den Bergh Foods, Rotterdam) was weighed to the nearest 0.1 mg into each of 6 centrifuge tubes, and 10 ml hexane was added. A known volume of a solution of tyrosol isomers in water (200 μg/ml of d-\text{\textomega}, m-\text{\textomega}, and p-tyrosol) was added to each tube (range 0–200 μg/ml). Syringic acid (1 ml) was added and the standards extracted as described above. 15 μl of each standard and sample solution was then injected onto a reversed phase HPLC column (ODS Hypersil 5 μM, 10 cm \times 4.6 mm). Gradient

Table 1

<table>
<thead>
<tr>
<th>Diet group</th>
<th>Vit E</th>
<th>Fatty acid composition (energy percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>12:0</td>
</tr>
<tr>
<td>Extra virgin olive oil</td>
<td>23.2</td>
<td>9.5</td>
</tr>
<tr>
<td>Trisun oil</td>
<td>28.8</td>
<td>10.0</td>
</tr>
<tr>
<td>Refined olive oil</td>
<td>25.7</td>
<td>10.2</td>
</tr>
</tbody>
</table>

Vitamin E is expressed as mg \text{\textalpha}-tocopherol equivalents/100 g fat blend.
elution was used to separate the polyphenolic components, using water/acetic acid (980:20, v/v) and methanol. The polyphenol peaks were detected at a wavelength of 274 nm and quantified by comparison with the peak areas of the standards.

2.3. Plasma and LDL analyses

Vitamin C was determined spectrophotometrically in fresh plasma following deproteination with 150 mM trichloroacetic acid (TCA). Uric acid was determined in plasma by an enzymatic, colorimetric method. Both determinations were carried out using a Cobas Mira S discrete analyzer and kits supplied by Boehringer Mannheim, Germany. α-Tocopherol was determined in plasma and LDL samples as previously described [19]. Plasma total cholesterol, LDL cholesterol, LDL triacylglycerol and LDL phospholipid were determined using enzymatic, colorimetric methods (Boehringer Mannheim, Germany). Determination of malondialdehyde (MDA) in fresh plasma was used as a possible index of lipid peroxidation in vivo. Malondialdehyde determination was carried out essentially as described by Wong et al. [20], except that the HPLC eluent was monitored using fluorescence detection. The excitation wavelength was 537 nm and the emission wavelength was 554 nm.

2.4. Lipoprotein isolation and in vitro oxidation studies

Plasma lipoproteins were isolated by discontinuous density gradient ultracentrifugation in a SW 41Ti rotor (Beckman Instruments, Palo Alto, USA) for 24 h at 4°C [21]. LDL was isolated in a density range of 1.019–1.063 g/ml. Density gradient solutions contained 0.1 mM Na₂EDTA to inhibit LDL oxidation during the isolation procedure. The susceptibility of LDL to copper-mediated oxidation was determined by monitoring the formation of conjugated dienes, essentially as described by Princen et al. [6]. Following LDL isolation, the LDL protein content was determined using bovine serum albumin (BSA) (Fraction V, Sigma, St. Louis, MO, USA) as the standard [22]. LDL was then diluted to 40 μg LDL protein per ml, using 1.18 M NaCl, 0.1 mM Na₂EDTA, to equalise EDTA concentrations in all samples. For conjugated diene assay, 40 μg LDL protein was incubated with phosphate buffered saline (PBS), pH 7.4, in a Varian Carey 3 UV–VIS spectrophotometer equipped with an automatic cell changer. The temperature of the assay mixture was brought to 30 ± 1°C, then 25 μl freshly prepared 2 mM CuCl₂·2H₂O was added to initiate oxidation of LDL lipids. The assay volume was 1000 μl and the final concentration of EDTA was 25 μM together with a copper ion concentration of 50 μM. The absorbance at 234 nm was monitored at two minute intervals until oxidation was complete. The oxidation profile of each LDL sample was plotted as absolute absorbance against time and the lag phase and maximum rate of each LDL oxidation reaction were determined as previously described [23].

2.5. Analysis of fatty acid compositions

Total lipids from the fat blends or LDL samples were first extracted into chloroform [24], then transmethylated using methanolic hydrochloric acid. The fatty acid methyl esters (FAME) were then purified by column chromatography prior to GC analysis. FAME analysis was carried out on a Carlo Erba Mega 2 series gas chromatograph (Interscience, Breda, The Netherlands) with flame ionisation detection, using a WCOT fused silica capillary column coated with 0.2 μM CP Sil 88 (Fused silica, Chrompack International, Middelburg, The Netherlands).

2.6. Data analysis

Data was analysed by one way analysis of variance (ANOVA). For LDL oxidizability (defined by the lag phase and the maximum rate), the pre-planned comparison of the extra virgin olive oil group versus Trisun and refined olive oil groups (i.e., high polyphenol versus low polyphenol) was also examined. Data of plasma vitamin C, lag phase and LDL triacylglycerol was log-transformed before ANOVA in order to obtain more equal variances between groups. All data is presented as mean (± S.E.M.) of 8 animals.
Table 2
Polyphenol concentrations (mg/kg) in the dietary oils

<table>
<thead>
<tr>
<th></th>
<th>$p$-Tyrosol</th>
<th>Hydroxytyrosol</th>
<th>Total polyphenol index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Refined olive oil</td>
<td>&lt;</td>
<td>&lt;</td>
<td>2</td>
</tr>
<tr>
<td>Trisun</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>Sunflower seed oil</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>Hardened coconut oil</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>Extra virgin olive oil</td>
<td>26</td>
<td>9</td>
<td>233</td>
</tr>
</tbody>
</table>

The detection limit of each polyphenol is 1 mg/kg. The total polyphenol index includes all unidentified polyphenol peaks. $p$-Tyrosol and $m$-tyrosol were not detected in any samples. < denotes that the concentration of polyphenols was below the detection limit of the assay.

3. Results

3.1. Animals

No significant differences were observed between the experimental dietary groups in terms of bodyweight gain or food consumption.

3.2. Composition of the experimental fat blends

The total fatty acid composition of the diet fat blends is detailed in Table 1. The concentration of alpha tocopherol equivalents in the fat blend mixture of the extra virgin olive oil diet was lower than the concentration in both the refined olive oil and Trisun diets. The results of the polyphenol determinations in the olive oils (extra virgin and refined), Trisun high oleic sunflower seed oil, sunflower seed oil and hardened coconut oil which were used to prepare the fat blends of the semi-purified diets are given in Table 2. Only the extra virgin olive oil demonstrated significant amounts of polyphenols, including the potent antioxidant hydroxytyrosol (3,4-dihydroxyphenylethanol).

3.3. Plasma and LDL lipids

Plasma and LDL lipids were determined after 6 weeks of experimental diet feeding. Mean plasma total cholesterol levels tended to be highest in the extra virgin group and lowest in the Trisun group, but within-group variability was quite large and these differences were not significant (Table 3). LDL triacylglycerol concentrations were highest in the extra virgin olive oil group, but the range was large (0.04–0.9 mmol/l) compared with the other groups. Levels of other LDL constituents and high density lipoprotein (HDL) cholesterol did not differ significantly. The LDL total fatty acid compositions were found to be similar as planned in all groups (Table 4).

3.4. Dietary effects on antioxidant and MDA concentrations

Plasma vitamin C level was significantly lower in the extra virgin olive oil group (Table 5). No significant differences between the groups were observed in the plasma levels of vitamin E or uric acid, but the LDL vitamin E concentration was significantly lower in the extra virgin olive oil group. The MDA concentration in the refined olive oil group was significantly lower than in the Trisun group (Table 5), but was not different from the mean level observed in the extra virgin olive oil group.

3.5. Dietary effects on in vitro LDL oxidizability

No significant differences were observed in the length of the lag phase (mean $\pm$ S.E.M.) for LDL samples from the Trisun (219 $\pm$ 12 min) and the refined olive oil (216 $\pm$ 16 min) groups. The lag phase in the extra virgin olive oil group (283 $\pm$ 38 min) was not significantly different from that observed in the other experimental groups. Pre-planned comparison of results from the extra virgin olive oil group (high polyphenol) with the low polyphenol group (Trisun and refined olive oil) demonstrated a significantly longer (30%) lag phase for the extra virgin olive oil LDL samples (283 $\pm$ 38 min) than found for the combined low polyphenol groups (218 $\pm$ 9, $P < 0.05$). The conjugated diene assay absorbance curves for all groups are presented in Fig. 1. No
differences in the maximum rate of LDL oxidation (expressed as nmol dienes/min/mg LDL protein) were observed among the groups (6.1 ± 0.84 (extra virgin olive oil), 6.64 ± 0.47 (Trisun) and 6.1 ± 0.37 (refined olive oil)).

4. Discussion

Atherosclerosis is promoted by genetic and environmental factors which elevate levels of LDL, the major carrier of cholesterol in human blood. However, it appears that LDL may only be atherogenic after it has undergone chemical or oxidative modification [25,26]. In terms of this mechanism, antioxidants that can prevent lipid peroxidation may be critically important in preventing the oxidative modification of LDL particles. Human LDL contains a variety of antioxidants that can inhibit lipid peroxidation, including \( \alpha \)-tocopherol (biologically the most active form of vitamin E), ubiquinol-10, \( \beta \)-carotene, lycopene and other (oxy)carotenoids. \( \alpha \)-Tocopherol is the most abundant antioxidant in LDL and many clinical studies aimed at increasing the resistance of LDL to oxidation have used supplementation with vitamin E, either singly [27,28], or in combination with \( \beta \)-carotene and/or vitamin C [6–8]. Vitamin E supplementation has been shown to increase the resistance of LDL to oxidative modification, but several studies have also shown that the oxidative susceptibility of LDL from non-vitamin E supplemented donors is not related to the LDL \( \alpha \)-tocopherol content [29–31]. These results suggest that vitamin E does contribute to the protection of LDL against oxidation, but other antioxidant factors may also be of importance.

Potent natural antioxidants, although present in small amounts in dietary sources, may also contribute to the oxidative resistance of LDL. Olive oil contains a relatively low concentration of \( \alpha \)-tocopherol but is known to be highly resistant to oxidative degradation. This is due, in part, to its low content of polynsaturated fatty acids, but may also be due to a high concentration of polyphenolic antioxidants, especially in high quality extra virgin oils. The quality grading of an olive oil is based on the acidity level, which is related to the free fatty acid content. Extra virgin olive oil is the highest quality oil and may have a maximum 1% acidity. If the acidity level is greater than 3.3%, the oil is then termed ‘lampante’ and must be refined before it is suitable for human consumption. Only unrefined oils have a significant content of polyphenols, which are responsible for the organoleptic properties of high quality olive oils. The polyphenol fraction of extra virgin olive oil is very complex, but compounds which have been identified in this fraction include 3,4-dihydroxyphenylethanol (hydroxytyrosol), 4-hy-

**Table 3**

<table>
<thead>
<tr>
<th></th>
<th>Extra virgin oil</th>
<th>Trisun</th>
<th>Refined oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma total cholesterol</td>
<td>7.0 ± 0.95</td>
<td>5.7 ± 0.88</td>
<td>6.2 ± 0.92</td>
</tr>
<tr>
<td>LDL total cholesterol</td>
<td>2.4 ± 0.40</td>
<td>2.3 ± 0.35</td>
<td>2.5 ± 0.44</td>
</tr>
<tr>
<td>LDL triacylglycerol</td>
<td>0.24 ± 0.12</td>
<td>0.14 ± 0.06</td>
<td>0.16 ± 0.07</td>
</tr>
<tr>
<td>LDL phospholipid</td>
<td>0.62 ± 0.11</td>
<td>0.58 ± 0.07</td>
<td>0.63 ± 0.12</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>1.38 ± 0.23</td>
<td>1.20 ± 0.16</td>
<td>1.35 ± 0.12</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M, n = 8 per group. All results are expressed as mmol/l. *LDL triacylglycerol was significantly higher in the extra virgin olive oil group, P < 0.05.

**Table 4**

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Extra virgin oil</th>
<th>Trisun</th>
<th>Refined oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>2.7 ± 0.2</td>
<td>3.0 ± 0.4</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>16:0</td>
<td>15.3 ± 0.9</td>
<td>14.5 ± 1.0</td>
<td>14.6 ± 0.4</td>
</tr>
<tr>
<td>18:0</td>
<td>10.7 ± 0.3</td>
<td>12.0 ± 0.3</td>
<td>10.7 ± 0.3</td>
</tr>
<tr>
<td>18:1 (n-9)</td>
<td>44.1 ± 1.5</td>
<td>44.1 ± 1.5</td>
<td>41.8 ± 2.0</td>
</tr>
<tr>
<td>18:2 (n-6)</td>
<td>16.9 ± 0.4</td>
<td>18.5 ± 0.5</td>
<td>18.5 ± 1.5</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M., n = 8 per group. No significant differences were observed.
Table 5
Antioxidant and malondialdehyde concentrations

<table>
<thead>
<tr>
<th></th>
<th>Extra virgin oil</th>
<th>Trisun</th>
<th>Refined oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL vitamin E (µg)</td>
<td>7.1 ± 0.7</td>
<td>10.0 ± 1.0</td>
<td>9.0 ± 0.4</td>
</tr>
<tr>
<td>Plasma vitamin E (µg/l)</td>
<td>11.7 ± 2.5</td>
<td>13.4 ± 2.5</td>
<td>14.4 ± 2.7</td>
</tr>
<tr>
<td>Plasma vitamin C (µg/l)</td>
<td>2.4 ± 0.29</td>
<td>3.4 ± 0.34</td>
<td>3.3 ± 0.61</td>
</tr>
<tr>
<td>Plasma uric acid (µmol/l)</td>
<td>192 ± 37</td>
<td>152 ± 38</td>
<td>146 ± 51</td>
</tr>
<tr>
<td>Plasma malondialdehyde (nmol/l)</td>
<td>610 ± 33</td>
<td>628 ± 47</td>
<td>491 ± 22</td>
</tr>
</tbody>
</table>

aLDL vitamin E expressed as µg vitamin E/mg LDL protein. All values are mean ± S.E.M.; n = 8 per group.
bLDL vitamin E and plasma vitamin C significantly lower in the extra virgin oil group. P < 0.05.
cPlasma malondialdehyde significantly lower in the refined oil group than in the Trisun group. P < 0.05.

dihydroxyphenylethanol (tyrosol), 4-hydroxyphenylacetic acid, protocatechuic acid, syringic acid, vanillic acid, caffeic acid and p-coumaric acid [32]. The antioxidant activity of these polyphenolic compounds has recently been compared in terms of their ability to inhibit the formation of peroxides [32]. In this system, hydroxytyrosol, caffeic acid and protocatechuic acid were the most protective against peroxide formation (having protection factors of 15.2, 5.7 and 2.7, respectively, compared with a protection factor of 1.0 for vanillic acid). The synthetic antioxidant, butylated hydroxytoluene (BHT), has a protection factor of 4.4, demonstrating the particularly potent antioxidant activity of hydroxytyrosol; a separate study has confirmed that hydroxytyrosol is a more powerful antioxidant than BHT [33].

The present study investigated the effects of two types of olive oils (refined and extra virgin) on the resistance of LDL to in vitro oxidation, compared with a diet of identical fatty acid composition but with Trisun high oleic sunflower seed oil as the oleic acid source. The fat blends of all diets were shown to have similar total fatty acid compositions and the ω-tocopherol content of all diets was planned to be similar. Plasma vitamin C levels were found to be lowest in the group receiving the extra virgin olive oil. No vitamin C was added to the diets and a lower concentration of ascorbate in plasma could possibly reflect either increased utilisation (perhaps due to the lower LDL vitamin E concentration) or decreased production due to its functional replacement by dietary antioxidant components (e.g., polyphenols). LDL cholesterol and phospholipid concentrations were similar in all groups, but LDL triacylglycerol (TAG) concentrations were significantly higher in the extra virgin olive oil group. The variability in LDL TAG concentrations in the extra virgin group was twice that observed in the Trisun and refined oil groups, due to the presence of two animals in this group with high LDL TAG levels (0.90 mmol/l and 0.56 mmol/l). There was no general trend for higher LDL TAG concentrations on the extra virgin olive oil diet. Plasma MDA concentrations were decreased in the refined oil group when compared only with values obtained in the Trisun group. MDA is a decomposition product of polyunsaturated fatty acid peroxides and a decreased plasma level may reflect a reduced degree of lipid peroxidation in vivo. However, because of the similarity between the Trisun and the refined oil groups in terms of LDL fatty acid composition and in vitro LDL oxidizability, it is not clear from

![Fig. 1. The effect of extra virgin olive oil (EVOV), Trisun and refined olive oil (ROV) on copper-mediated LDL conjugated diene formation in vitro, n = 8 per group.](image-url)
this study what factors in the refined olive oil diet may be functioning in vivo and not in vitro.

Significant amounts of polyphenol compounds (in particular tyrosol and hydroxytyrosol) were detected solely in the fat blend mixture of the extra virgin olive oil diet. LDL oxidation resistance was determined by the measurement of copper-induced conjugated diene formation, and no differences in either the lag phase or the maximum rate of diene formation were observed between the refined olive oil group and the Trisun group. The oxidizability of LDL from rabbits receiving the high polyphenol diet was shown to be significantly reduced when compared with LDL from animals receiving low polyphenol diets, even though the LDL vitamin E content on the polyphenol-rich diet was significantly lower. Only the length of the lag phase was increased on the high polyphenol diet, the maximum rate of conjugated diene formation was similar in all groups. This result would suggest that additional antioxidants were present in the high polyphenol group which functioned to delay the propagation of peroxidation of LDL lipids, but once these antioxidants had been depleted, the rate of the oxidation reaction proceeded at a similar rate in all groups due to the highly similar LDL fatty acid compositions.

It can not be confirmed that the antioxidant responsible for the increased LDL oxidation resistance observed in the extra virgin olive oil group was hydroxytyrosol or another polyphenolic compound such as caffeic or syringic acid, but vitamin E, which may be an important antioxidant component in olive oil, was controlled for by equalising the level in all diets. This measure was not totally successful, however, as the dietary vitamin E level in the extra virgin olive oil group was 19% and 10% lower than in the Trisun and refined olive oil groups, respectively. This resulted in the lower LDL vitamin E concentration in the extra virgin olive oil group, a result which could be expected to increase the oxidizability of LDL, and which emphasises the potency of additional active antioxidant components present in this diet. Polyphenols tend to be water-soluble in character and, although they may not be expected to become incorporated into the lipid core of the lipo-protein, they may bind to surface components. A recent report has demonstrated binding of wine polyphenolics to LDL in vivo [34]. The presence of hydroxytyrosol was certainly demonstrated in the extra virgin olive oil and excluded in the other oils used in the study, and hydroxytyrosol has recently been demonstrated to possess free radical scavenging properties and to be able to quench both the hydroxyl radical and the peroxyl radical [35], antioxidant properties which would increase resistance to oxidation as determined by the in vitro conjugated diene assay.

In conclusion, this study has demonstrated that dietary antioxidants present in unrefined, extra virgin olive oils may significantly increase the oxidation resistance of LDL particles to an in vitro oxidative stress. In addition to red wine and tea, which have recently been shown to contain polyphenolic antioxidants with positive effects on LDL oxidizibility both in vitro and in vivo [14,15,34], unrefined olive oils may also possess compounds with similar antioxidant properties.

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