Oxidative DNA Damage Is Prevented by Extracts of Olive Oil, Hydroxytyrosol, and Other Olive Phenolic Compounds in Human Blood Mononuclear Cells and HL60 Cells1,2

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Abstract

Our aim in this study was to provide further support to the hypothesis that phenolic compounds may play an important role in the anticarcinogenic properties of olive oil. We measured the effect of olive oil phenols on hydrogen peroxide (H2O2)-induced DNA damage in human peripheral blood mononuclear cells (PBMC) and promyelocytic leukemia cells (HL60) using single-cell gel electrophoresis (comet assay). Hydroxytyrosol [3,4-dihydroxyphenyl-ethanol (3,4-DHPEA)] and a complex mixture of phenols extracted from both virgin olive oil (OO-PE) and olive mill wastewater (WW-PE) reduced the DNA damage at concentrations as low as 1 μmol/L when coincubated in the medium with H2O2 (40 μmol/L). At 10 μmol/L 3,4-DHPEA, the protection was 93% in HL60 and 89% in PBMC. A similar protective activity was also shown by the dialdehydic form of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA) on both kinds of cells. Other purified compounds such as isomer of oleuropein aglycon (3,4-DHPEA-EA), oleuropein, tyrosol, [p-hydroxyphenyl-ethanol (p-HPEA)] the dialdehydic form of elenolic acid linked to tyrosol, caffeic acid, and verbascoside also protected the cells against H2O2-induced DNA damage although with a lower efficacy (range of protection, 25–75%). On the other hand, when tested in a model system in which the oxidative stress was induced by phorbole 12-myristate 13-acetate-activated monocytes, p-HPEA was more effective than 3,4-DHPEA in preventing the oxidative DNA damage. Overall, these results suggest that OO-PE and WW-PE may efficiently prevent the initiation step of carcinogenesis in vivo, because the concentrations effective against the oxidative DNA damage could be easily reached with normal intake of normal olive oil. J. Nutr. 138: 1411–1416, 2008.

Introduction

Epidemiological evidence and numerous animal studies indicate that olive oil may possess anticarcinogenic properties. Case control studies have shown an inverse correlation between olive oil consumption and cancer in different sites (1–8) and animal studies demonstrated a protective activity against chemically induced carcinogenesis, such as Dimethylbenz[a]anthracene-induced mammary tumors (9) and Azoxymethane-induced colon carcinoma (10). Furthermore, olive oil may protect from UV-induced skin cancer (11) and it reduces the incidence of spontaneous appearance of liver tumors in mice (12).

Mutations in somatic cells play a central role both in cancer initiation and in other stages of the carcinogenic process (13). Such genetic alterations are caused by exposure to genotoxic substances of environmental origin and/or are endogenously produced. Among the endogenously produced genotoxic substances, the reactive oxygen species seem to be of particular importance, because they are continuously produced in all aerobic organisms both as by-products of normal oxygen metabolism and as bactericidal agents by activated phagocytic cells (14). Therefore, the oxidative stress has been strongly correlated to the onset of various degenerative diseases, particularly cancer (15). For these reasons, the chemoprevention ability of olive oil has been ascribed to minor phenolic compounds that possess a potent antioxidant activity (16,17). The phenolic composition of olive oil is rather complex and includes the phenolic alcohols hydroxytyrosol [3,4-dihydroxyphenyl-ethanol (3,4-DHPEA)]5 and tyrosol

5 Abbreviations used: 3,4-DHPEA, 3,4-dihydroxyphenyl-ethanol or hydroxytyrosol; 3,4-DHPEA-EA, isomer of oleuropein aglycon; 3,4-DHPEA-EDA, dialdehydic form of elenolic acid linked to hydroxytyrosol; OO-PE, olive oil phenolic extract; p-HPEA, p-hydroxyphenyl-ethanol or tyrosol; p-HPEA-EDA, dialdehydic form of elenolic acid linked to tyrosol; PMA, phorbol 12-myristate 13-acetate; WW-PE, wastewater phenolic extract.
[p-hydroxyphenylethanol (p-HPEA)] and their secoiridoid precursors such as the dialdehydic form of eлиноic acid linked either to hydroxytyrosol (3,4-DHPEA-EDA) or to tyrosol (p-HPEA-EDA), and the isomer of oleuropein aglycon (3,4-DHPEA-EA) (18). Because of their hydrophilic properties, a consistent amount of phenols is lost during olive oil production in the olive mill wastewater (19), which could therefore be a relevant source of such compounds (20–22).

The antioxidative activity of olive oil phenols has been demonstrated in several in vitro systems; for example, it was shown that hydroxytyrosol: 1) prevents the tert-butylhydroperoxide-induced death of HepG2 cells (23); 2) counteracts the low-density lipoprotein oxidation both chemically produced (24) and cell mediated (25); and 3) protects different cell types such as CaCo-2 (26), erythrocytes (27), and PC12 (28) from hydrogen peroxide (H2O2)-induced cytotoxicity as evidenced by several methods like the leakage of lactate dehydrogenase and the 3-[4,5-dimethyl(thiazol-2-yl)]-3,5-diphenyltetrazolium bromide assay. More recently, some intervention studies have investigated the DNA protective potential of olive oil phenols with conflicting results. Weinbrenner et al. (29) found a decreased amount of 8-oxo-7,8-dihydro-2’-deoxyguanosine in mitochondrial DNA of mononuclear cells and in urine after short-term consumption of olive oil with a linear trend significantly correlated to the content of phenols. Similarly, Salvini et al. (30) showed a 30% reduction of oxidative DNA damage in peripheral blood lymphocytes during intervention on postmenopausal women with virgin olive oil containing high amounts of phenols. On the other hand, no significant effect was detected on urinary excretion of etheno-DNA adducts after consumption of phenol-rich olive oil (31). Furthermore, it was shown that the urinary excretion of oxidation products of guanine was not modified after assumption of olive oil with low, medium, and high phenolic content (32).

In this study, we have investigated the potential protective activity of different olive oil phenols on oxidative DNA damage induced either by H2O2 or by phorbol-myristate-acetate (PMA) activated monocytes. This last ex vivo model, recently developed in our laboratory (33), has the advantage that, instead of treating the cells with extreme nonphysiological levels of a single oxidant, it exposes the cells to several oxidative species, which are continuously produced at low concentrations and represent the natural oxidative stress in more realistic conditions. The oxidative DNA damage was detected by the highly sensitive comet assay (34).

Materials and Methods

Materials and cell line. Human promyelocytic leukemia cells (HL60), obtained from the American Type Culture Collection, were cultured in RPMI 1640 medium as previously described (33). 3,4-DHPEA was obtained from Cayman Chemicals, oleuropein glucoside was purchased from Extrasynthése, p-HPEA from Janssen Chemical, and caffeic acid from Fluka.

Preparation of phenol extracts from olive oil and olive mill wastewater. The mechanical oil extraction process was performed at industrial plant as follows. Green olives (Olea europaea L.) from cultivar Coratina at the ripening stage of 0.90, evaluated as the pigmentation index, were crushed using a hammer crusher; the malaxation was carried out for 40 min at 25°C and the oil was extracted by centrifugation (9600 × g; 1 min) using a decanter (Rapanelli Mod. 400 ECO/G) at a low level of water addition. A phenol methanolic extract was obtained from the virgin olive oil (OO-PE), which contained 650 mg/kg of total phenols, as water addition. A phenol methanolic extract was obtained from the virgin olive oil (OO-PE) to obtain a total concentration of 3,4-DHPEA–containing compounds (3,4-DHPEA-EDA, 3,4-DHPEA-EA, and verbascoside) corresponding to 10 mmol/L. Similarly, the purified phenolic compounds were dissolved in the same solution at the concentration of 10 mmol/L. All samples were then divided into aliquots and stored at −20°C in the dark. The compounds were thawed just before use and diluted in RPMI 1640 medium (Bio-Whittaker, Boehringer Ingelheim) to the desired concentrations. All the solutions were sterilized by filtration on 0.22-μm filters (Celbio).

We investigated the antioxidant potential of olive oil phenols in 2 different conditions as follows: 1) the cell suspensions (HL60 or PBMC) were enriched with different compounds in RPMI 1640 complete medium and then immediately treated with 40 μmol/L H2O2 for 30 min at 37°C; 2) the cells (lymphocytes) were exposed to the reactive oxygen species produced by activated monocytes. For this purpose, cell suspensions were enriched with different compounds in RPMI 1640 complete medium and then coincubated for 1 h at 37°C and 5% CO2.
with the adherent monocytes (prepared as reported above) either activated or not with PMA (2 μmol/L). In both cases, after incubation, the cells were recovered and we evaluated the viability and the DNA damage using the trypan blue exclusion technique and the comet assay, respectively (33).

**Single cell gel electrophoresis (comet assay).** The single cell gel electrophoresis assay was performed essentially as described by Singh et al. (40). Briefly, cells were included in the low-melting agarose (0.7% in PBS) and placed in a lysis solution (2.5 mol/L NaCl, 100 mmol/L Na₂EDTA, 10 mmol/L Tris-HCl, pH 10, containing freshly added 1% Triton x100 and 10% dimethylsulfoxide) for 1 h at 4°C. Electrophoresis was carried out in freshly made buffer (1 mmol/L Na₂EDTA, 300 mmol/L NaOH, pH 13.0) for 20 min at a fixed voltage of 25V (300 mA). After electrophoresis, the slides were neutralized (0.4 mol/L Tris-HCl, pH 7.5) and stained with ethidium bromide (20 mg/L).

**Comet detection and statistical analysis.** The comets (n = 100) were analyzed 24 h after staining at 400× magnification using a fluorescence microscope (Zeiss, R.G.) equipped with a 50-W mercury lamp. The damage for each slide was expressed in arbitrary units, which is a parameter derived from the “tail moment” as previously described (41).

The results of each experiment, repeated 5 times (n = 5) using PBMC preparations obtained from different donors, were compared using a 1-way ANOVA. When a significant (P < 0.05) treatment effect was detected, the means were compared using Tukey’s post hoc comparison test.

**Results**

HPLC analysis of the phenolic composition of OO-PE and WW-PE showed more 3,4-DHPEA and 3,4-DHPEA-EDA in WW-PE than in OO-PE (Table 1). In addition, verbascoside was not found in the OO-PE and 3,4-DHPEA-EA, p-HPEA-EDA, and (+)-1-acetoxypinoresinol were not present in the WW-PE (Table 1). The extracts were dissolved at concentrations of 9.532 g/L (OO-PE) and 5.266 g/L (WW-PE) to have the same molar concentration of 3,4-DHPEA + 3,4-DHPEA-containing compounds in the culture medium used for cell exposure (Table 1).

3,4-DHPEA was used as reference compound, because several studies have demonstrated its potent antioxidant activity.

We performed preliminary experiments to determine both the concentration of H₂O₂ and the time of exposure of the cells, which gave an appreciable genotoxic effect without cytotoxicity. We found that the optimal conditions for treating cells were 40 μmol/L H₂O₂ and 30 min of incubation at 37°C. Enrichment of the exposure medium during the H₂O₂ treatment with purified 3,4-DHPEA, OO-PE, and WW-PE reduced the DNA damage to both PBMC (Fig. 1) and HL60 cells (Fig. 2). Both 3,4-DHPEA and phenol mixtures significantly decreased the H₂O₂ genotoxicity at concentrations as low as 1 μmol/L. In addition, WW-PE slightly but significantly affected DNA damage in the absence of H₂O₂ (Fig. 2C).

Different phenolic compounds, structurally related to 3,4-DHPEA and present in OO-PE and WW-PE, reduced the DNA damage caused by H₂O₂ (Table 2). In both cell types, the protective activity of 3,4-DHPEA-EDA was similar to that of 3,4-DHPEA (considered the reference compound), whereas the protective activities of 3,4-DHPEA-EA, oleuropein, p-HPEA, and p-HPEA-EDA were significantly lower compared with that of 3,4-DHPEA (Table 2). In addition, caffeic acid and verbascoside had less effect than 3,4-DHPEA in HL60 cells (Table 2).

The ability of the phenols to prevent DNA damage also was investigated in a model system in which the oxidative stress was induced by PMA-activated monocytes. Freshly isolated lymphocytes were coincubated with monocytes (attached to the bottom of the 96-well plate) either stimulated or not with 2 μmol/L of PMA, a protein kinase C activator. After 1 h of incubation at

**TABLE 1** Concentrations of different compounds present in OO-PE and from WW-PE

<table>
<thead>
<tr>
<th>Extract Culture medium</th>
<th>Extract from Culture medium</th>
<th>Concentration mg/g</th>
<th>μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,4-DHPEA</td>
<td>8.0</td>
<td>0.50</td>
<td>1.65</td>
</tr>
<tr>
<td>3,4-DHPEA-EDA</td>
<td>205.7</td>
<td>6.13</td>
<td>8.20</td>
</tr>
<tr>
<td>3,4-DHPEA-EA</td>
<td>133.6</td>
<td>3.37</td>
<td>n.d.</td>
</tr>
<tr>
<td>Verbascoside</td>
<td>n.d.</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>Total 3,4-DHPEA-containing compounds</td>
<td>347.3</td>
<td>10</td>
<td>37</td>
</tr>
<tr>
<td>p-HPEA</td>
<td>7.1</td>
<td>0.49</td>
<td>0.16</td>
</tr>
<tr>
<td>p-HPEA-EDA</td>
<td>238.3</td>
<td>7.47</td>
<td>n.d.</td>
</tr>
<tr>
<td>(+)-1-Acetoxypinoresinol</td>
<td>86.5</td>
<td>n.d.</td>
<td>1.98</td>
</tr>
<tr>
<td>Total phenols</td>
<td>679.2</td>
<td>19.94</td>
<td>10.16</td>
</tr>
</tbody>
</table>

1 n.d., Not detected.

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![Figure 1](image-url) **FIGURE 1** Effect of increasing concentrations of 3,4-DHPEA (A), OO-PE (B), and WW-PE (C) on DNA damage of HL60 cells that were untreated or treated with H₂O₂ 40 μmol/L for 30 min. Values are means ± SD, n = 5. Means without a common letter differ, P < 0.05.
37°C, lymphocytes were removed and assayed for DNA damage. The presence of PMA increased DNA damage in lymphocytes (Fig. 3). When lymphocytes were exposed to PMA-activated monocytes in the presence of the different phenolic compounds, the DNA damage was significantly reduced (Fig. 3). Surprisingly, in this experimental system, p-HPEA was more effective than 3,4-DHPEA in preventing oxidative DNA damage. 3,4-DHPEA reduced DNA damage 45% and p-HPEA, 69% (Fig. 3).

### Discussion

Although different in vitro systems have shown that olive oil phenols possess a potent antioxidant activity (16,17) and prevent the reactive oxygen species-mediated cell injury (26–30), there is limited and contradictory evidence for such a protective role on DNA damage. For instance, 3,4-DHPEA promotes both the bleomycin-Fe(III)-dependent DNA damage (42) and the copper-dependent chemical modification of DNA bases (24). In addition, olive oil extracts and purified oleuropein were found to exert a genotoxic effect on Jurkat cells when tested at concentrations of 100 μg/L and 100 μmol/L, respectively (43). Nevertheless, oleuropein and p-HPEA, in concentrations ranging from 10 to 1000 μmol/L, did not prevent the DNA damage induced by exposure of the Jurkat cells to H₂O₂ continuously produced by the enzyme glucose oxidase (43). Furthermore, p-HPEA demonstrated a genotoxic effect on whole blood cells at 50 μmol/L when assayed by the atypical comet assay (44). Recently, oleuropein and olive mill wastewater were demonstrated to be genotoxic by the micronucleus test (45). On the other hand, 3,4-DHPEA showed a protective effect toward the oxidative DNA damage induced by peroxynitrite (ONOO⁻) on neuronal hybridoma cells at high concentrations (0.25–1.0 mmol/L) (46). Instead, we demonstrated that phenols, when used both as purified compounds and in a complex crude extract and regardless of the source (i.e. olive oil or olive mill wastewater) may prevent the H₂O₂-induced DNA damage in a very low concentration range (1–10 μmol/L). These concentrations could be easily reached in the tissues with an ordinary intake of 50 g/d of olive oil, because phenols are effectively absorbed in humans (47). Indeed, a recent intervention study has shown that an intake of 40 mL of olive oil containing a considerable amount of phenols (366 mg/kg) can result in a plasma concentration of 3,4-DHPEA and p-HPEA above 10 μmol/L during the first 4 h after ingestion (48).

Our results agree partially with those published by Quiles et al. (49), who found a slight protective effect (25%) on human prostate cancer cells by 10 μmol/L of 3,4-DHPEA. However, in

### Table 2

<table>
<thead>
<tr>
<th>Compounds, 10 μmol/L</th>
<th>DNA damage reduction</th>
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<tbody>
<tr>
<td></td>
<td>PBMC</td>
</tr>
<tr>
<td>3,4-DHPEA</td>
<td>93 ± 4ᵃ</td>
</tr>
<tr>
<td>3,4-DHPEA-EDA</td>
<td>93 ± 4ᵃ</td>
</tr>
<tr>
<td>3,4-DHPEA-EA</td>
<td>62 ± 7ᵇ</td>
</tr>
<tr>
<td>Oleuropein</td>
<td>60 ± 4ᵃ</td>
</tr>
<tr>
<td>p-HPEA</td>
<td>67 ± 15ᵇ</td>
</tr>
<tr>
<td>p-HPEA-EDA</td>
<td>65 ± 14ᵇ</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>75 ± 8ᵇᶜ</td>
</tr>
<tr>
<td>Verbascoside</td>
<td>78 ± 12ᵇᶜ</td>
</tr>
</tbody>
</table>

ᵃ Values are means ± SD, n = 5. Means in a column without a common letter differ, P < 0.05.

### Figures

**Figure 2** Effect of increasing concentrations of 3,4-DHPEA (A), OO-PE (B), and WW-PE (C) on DNA damage of PBMC either untreated or treated with H₂O₂ 40 μmol/L for 30 min. Values are means ± SD, n = 5. Means without a common letter differ, P < 0.05.

**Figure 3** Effect of 3,4-DHPEA, p-HPEA, and oleuropein (10 μmol/L) on DNA damage of lymphocytes coincubated with monocytes either untreated or stimulated with PMA (2 μmol/L) for 1 h. Values are means ± SD, n = 5. Means without a common letter differ, P < 0.05.
this study, the experimental conditions were different from our conditions, because the cells were preincubated with the phenols for 24 h and then stressed by H$_2$O$_2$ 60 µmol/L for 5 min in incomplete medium. In addition, we obtained further evidence supporting the hypothesis that the phenolic compounds may efficiently prevent the oxidative DNA damage at low concentrations by means of the ex vivo system of PMA-activated monocytes. This model simulates more closely the oxidative stress in vivo, because the treatment of monocytes with PMA activates both the NADPH oxidase, which catalyses the 1-electron reduction of oxygen to form O$_2^-$, and the NO synthase, which produces NO. These highly reactive compounds may be the precursors of a wide spectra of reactive species that are able to induce DNA damage on lymphocytes (33). It is important to emphasize that in the PMA-activated monocyte system, p-HPEA was more efficient than 3,4-DHPEA in preventing DNA damage. These results were unexpected, because the ortho-diphenols, i.e. 3,4-DHPEA-EA, are more potent radical scavengers and effective antioxidants than simple phenols, i.e. p-HPEA (50). However, the reactive species produced by activated monocytes are different and more complex than the simple H$_2$O$_2$. In addition, a definitive answer to the ability of 3,4-DHPEA and p-HPEA to scavenger different reactive oxygen species is not available. For instance, some studies have shown a potent scavenger activity of 3,4-DHPEA against superoxide anions (51,52), whereas others have found a scavenger activity against H$_2$O$_2$ but not against superoxide anions (53). The latter result is supported by the finding that 3,4-DHPEA protected the cells from H$_2$O$_2$-induced damage but did not affect superoxide anion-induced cell death (28). Data on the effects exerted by p-HPEA on the different reactive oxygen species are not available; therefore, the possibility remains that this compound could efficiently scavenge O$_2^-$, NO, and ONOO$.^{-1}$ On the other hand, it may be possible that p-HPEA acts on other cell functions, such as the endogenous antioxidant systems and DNA damage repair activity, which are independent from the antioxidant characteristic. It was recently found, for instance, that p-HPEA restored intracellular antioxidant defense in J774 A.1 cells (25). Furthermore, an inhibitory effect of p-HPEA on NADPH oxidase of the monocyte with the consequent reduction of superoxide production cannot be excluded, whereas 3,4-DHPEA does not affect this enzyme activity (53).

When the damage was produced by H$_2$O$_2$, the preventive activities of different phenols, in particular 3,4-DHPEA, caffeic acid, and p-HPEA, reflected their antioxidant potential as determined by the 2,2-diphenyl-l-picrylhydrazil radical scavenging test (54,55). Indeed, H$_2$O$_2$ causes DNA strand breaks by generation of the hydroxyl radical (OH$^-$) via the Fenton reaction in the presence of a Fe$^{2+}$. Therefore, olive oil phenols may act, in addition to their interference as free radical scavengers, as metal ion chelators (56). This latter property depends, at least in part, upon the presence in the phenol molecule of catechol moieties; in fact, catechol was reported to form a complex with Fe$^{2+}$ at pH 7.4 hindering the reaction with H$_2$O$_2$ to generate OH$^-$ (28).

Regarding the other secoiridoid compounds, the addition of the dialdehydic form of ellenoic acid to both 3,4-DHPEA and p-HPEA does not modify the DNA damage preventive activity (a similar protective activity was found for 3,4-DHPEA and 3,4-DHPEA-EDA and for p-HPEA and p-HPEA-EDA), whereas the addition of the ellenoic acid to 3,4-DHPEA reduced the effect (3,4-DHPEA-EDA was less active than 3,4-DHPEA). These observations are in agreement with those of Visioli et al. (57), who showed a similar inhibitory potential of these compounds toward oxidation of LDL.

In conclusion, this study showed a potent DNA damage preventive activity of olive oil phenols, providing new evidence to support a possible role of these compounds in the prevention of cancer. In addition, the results obtained with WW-PE suggest that this industrial by-product could be an abundant and inexpensive material to obtain bioactive phenolic compounds.

**Literature Cited**


DNA damage prevention by olive oil phenols 1415


