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Short Communication

In vitro cytotoxicity to human cells in culture of some phenolics from olive oil

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Abstract

The neutral red in vitro cytotoxicity assay was used to evaluate the comparative responses of human cells isolated from tissues of the oral cavity to olive oil phenolics. The cell lines used included normal gingival fibroblasts, immortalized, nontumorigenic gingival epithelial cells, and carcinoma cells from the salivary gland. No differences in the relative sensitivities to the phenolics amongst the three cell types were noted. In general, for all cell types, the sequence of increasing cytotoxicity was: oleuropein aglycone > oleuropein glycoside, caffeic acid > *o*-coumaric acid > cinnamic acid ≫ tyrosol, syringic acid, protocatechuic acid, vanillic acid. Cytotoxicity was noted only at phenolic concentrations far exceeding those attainable after habitual consumption, thus indicating that consumption of phenol-rich olive oil is safe.

Keywords: Olive oil; Cytotoxicity; Phenolic compounds; Oleuropein

1. Introduction

Free radicals and oxidative stress are recognized as important factors in the etiology of many chronic diseases. Dietary components with antioxidant activity therefore have received particular attention because of their potential to modulate oxidative stress associated with chronic disease. Thus, for example, the lower incidence of coronary heart disease [1,2] and some types of cancer [3,4] in people of the Mediterranean area led to the hypothesis that a diet rich in fruits, vegetables, grains, and legumes has a beneficial effect on human health. The major fat component of the so-called "Mediterranean diet" is olive oil.

Phenolic compounds in virgin olive oil, although considered among its several "minor" constituents, may play a major role in preventing chronic human disease. Specific health-promoting effects of olive oil phenolics include inhibition of the oxidation of low-density lipoproteins (LDLs) thought to be involved in the onset of atherosclerosis [5,6], scavenging of free radicals [7,8], reducing inflammation through inhibition of lipooxygenase activity, sparing prostaglandin generation, and lessening of the generation of reactive oxygen species (ROS) by leukocytes [9], and enhancement of the macrophage-mediated response [10].

This study evaluated the in vitro cytotoxicity of nine phenolic compounds in olive oil towards cell lines derived from tissues of the oral cavity. Although there is some information on the response of cultured mammalian cells to constituents of olive oil [11–13], there are no studies on the response of cells derived from the oral cavity, i.e., the initial site of exposure upon ingestion of olive oil. This is important in light of the potential cytotoxicity of phenolic molecules in high concentrations [14] and the putative anticarcinogenic activities of olive oil phenolics [15]. Furthermore, to determine whether olive oil phenolics are preferentially antiproliferative to carcinoma cells, studies were performed with nonmalignant and malignant cells. Because of national and international concerns to develop strategies to prevent cancer, considerable research has focused on the identification of compounds isolated from dietary sources that might prevent or postpone the

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onset of cancer. However, many of the in vitro toxicology studies designed to identify naturally occurring anticarcinogens are performed solely with cells derived from malignant tissues, thereby not allowing to attribute specific tumor-preventive properties to individual compounds. Thus, an important aspect of this study was the comparative responses of normal and malignant cells to the test agents. The cell lines included normal GN61 gingival fibroblasts, immortalized, non-tumorigenic S-G gingival epithelial cells, and malignant HSG_1 cells derived from the salivary gland.

2. Experimental

2.1. Cell lines

The human gingival epithelial S-G cell line was obtained from F.H. Kasten, Quillen College of Medicine, East Tennessee State University, Johnson City, TN, USA [16–18], the human salivary gland carcinoma HSG_1 cell line from H. Sakagami, Department of Dental Pharmacology, Meikai University School of Dentistry, Saitama, Japan, and normal human gingival GN61 fibroblasts from D.A. Tipton, College of Dentistry, University of Tennessee, Memphis, TN, USA. Cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, and 2.5 μg/ml amphotericin B (Fungizone), termed the growth medium, and were maintained in a humidified atmosphere with 5.5% CO_2 at 37 °C. Cultures were dissociated with 0.05% trypsin–0.2% EDTA.

2.2. Test agents

3,4-Dihydroxybenzoic acid (protocatechuic acid), 2-(4-hydroxyphenyl)ethanol (tyrosol), cinnamic acid, syringic acid, vanillic acid (Fluka Chemical Co., Milwaukee, WI), *o*-coumaric acid, caffeic acid (Sigma Chemical Co., St. Louis, MO), oleuropein glycoside (Extrasynthese, Genay, France), and oleuropein aglycone were solubilized in ethanol. Oleuropein aglycone was synthesized by enzymatic digestion of its glycoside with β-glucosidase and its purity was checked by reverse-phase HPLC [19]. Stock solutions of all test agents were made at high enough concentrations of ethanol so that when diluted prior to use, the residual solubilizing ethanol solution ($\leq 1\%$) was not cytotoxic.

2.3. Neutral red cytotoxicity assay

Individual wells of a tissue culture 96-well microtiter plate were inoculated with 0.2 ml of growth medium containing sufficient cells, approximately 3.4×10^4, to provide 70% confluence after 24 h of incubation. Thereafter, the growth medium was replaced with exposure medium, consisting of DMEM, 10% Serum Plus (JRH Biosciences, Lenexa, KS), 2% FBS and antibiotics, unamended and amended with varied concentrations of the test agents. Serum Plus is a supplement containing a low level of fetal serum proteins and is enhanced with specific growth promoting factors. Six to eight replicate wells were used per concentration of test agent. After 24 h of exposure, cytotoxicity was assessed with the neutral red (NR) cytotoxicity assay, which is based on the uptake and lysosomal accumulation of the supravital dye, NR [20,21].

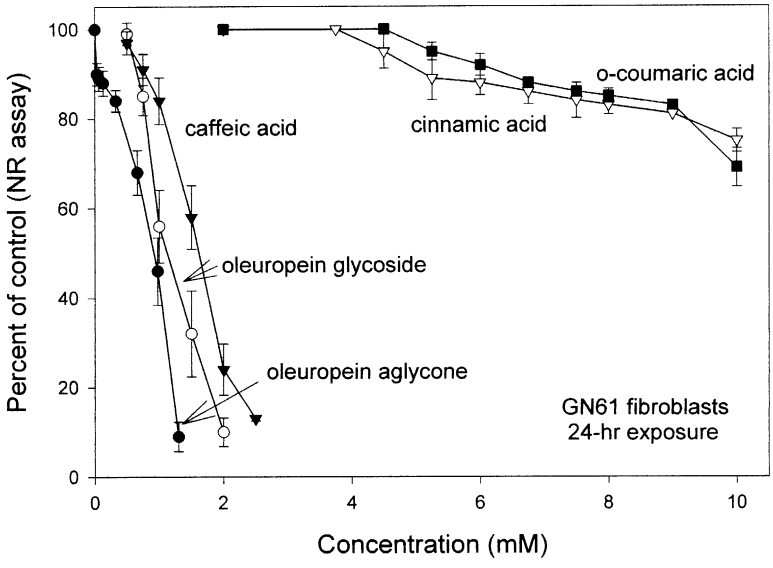

Fig. 1. Viability of human gingival GN61 fibroblasts exposed to oleuropein aglycone, oleuropein glycoside, caffeic acid, *o*-coumaric acid, and cinnamic acid for 24 h. Viability, based on the incorporation of NR into viable cells, was reported as the arithmetic mean percentages of control ± SEM.

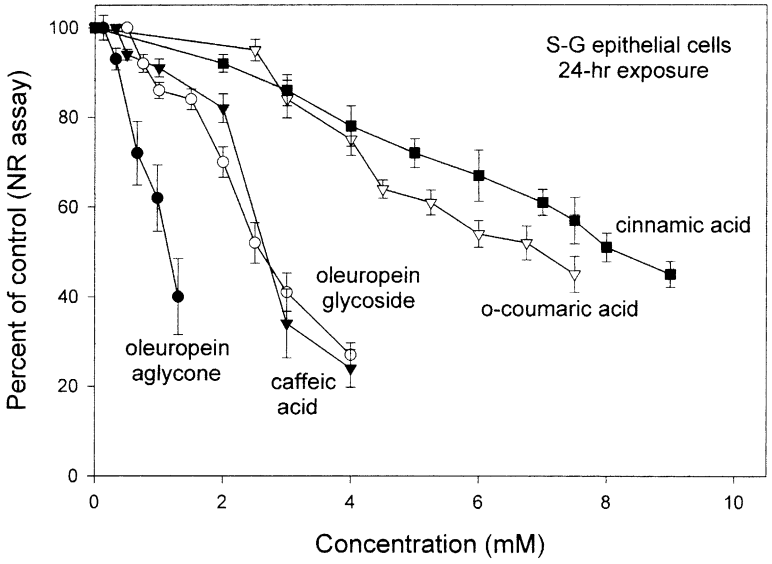

Fig. 2. Viability of human gingival S-G epithelial cells exposed to oleuropein aglycone, oleuropein glycoside, caffeic acid, *o*-coumaric acid, and cinnamic acid for 24 h. Viability, based on the incorporation of NR into viable cells, was reported as the arithmetic mean percentages of control ± SEM.

The protocol for the NR assay was as follows. A foil-wrapped aqueous stock suspension of NR (4 mg/ml) stored at room temperature was diluted to a working concentration of 40 μg/ml NR in exposure medium and incubated overnight at 37 °C. Prior to use, this solution was centrifuged to remove fine dye crystals. After a 24-h exposure of the cells to the test agents, the medium was removed, 0.2 ml of the NR-containing medium was added per well, and incubation was continued for 1 h at 37 °C. Cells were then rapidly washed and fixed with 0.2 ml of 0.5% formalin–1% $CaCl_2$ (v/v) and the NR incorporated into viable cells was released into the supernatant with 0.2 ml of 1% glacial acetic acid–50% ethanol. Absorbency was recorded at 540 nm with a microtiter plate spectrophotometer. Experiments were performed, at least, three times. Cytotoxicity graphic data were constructed as the arithmetic mean percentages of control ± standard errors of the mean (SEM). Linear regression analysis was used to compute the concentration of test agent needed to reduce absorbency of NR by 50%, termed the midpoint cytotoxicity, or NR_{50}, value. To determine the concentration of test agent at which cytotoxicity was noted initially, the experimental data were analyzed with a one-way analysis of variance (ANOVA) followed by Tukey's multiple range test for significant difference. To be considered

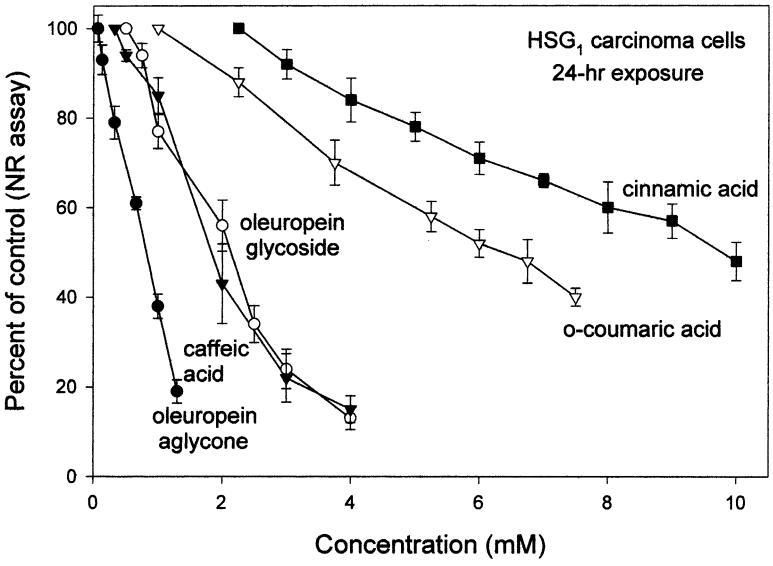

Fig. 3. Viability of human carcinoma HSG_1 cells exposed to oleuropein aglycone, oleuropein glycoside, caffeic acid, *o*-coumaric acid, and cinnamic acid for 24 h. Viability, based on the incorporation of NR into viable cells, was reported as the arithmetic mean percentages of control ± SEM.

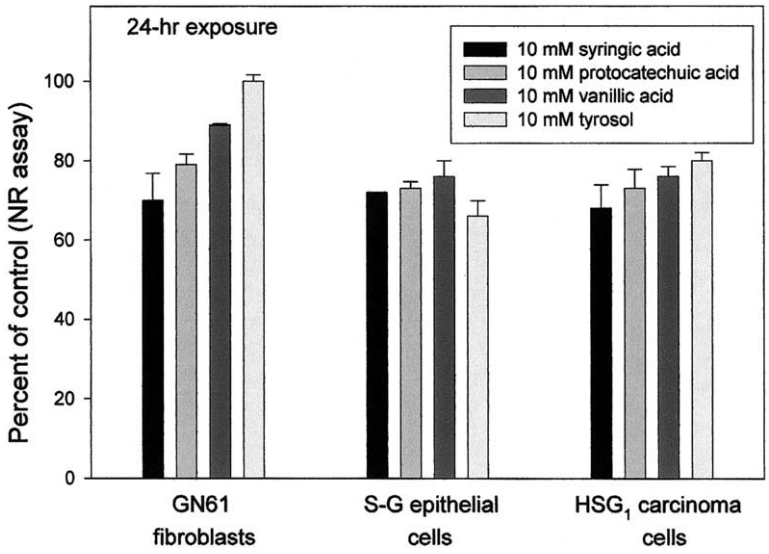

Fig. 4. Viability of human gingival GN61 fibroblasts, gingival S-G epithelial cells, and HSG_1 carcinoma cells exposed for 24 h to a 10-mM concentration of syringic acid, protocatechuic acid, vanillic acid, and tyrosol. Viability, based on the incorporation of NR into viable cells, was reported as the arithmetic mean percentages of control ± SEM.

statistically significant, the P-value of the effect being considered must be ≤ 0.05.

3. Results and discussion

The cells were exposed to the test agents at concentrations up to 10 mM, as for some test agents (e.g., syringic acid), and this appeared to be their limit of solubility. Based on 24-h cytotoxicity data, the test agents were grouped into two categories, those for which complete cytotoxicity curves were generated (Figs. 1–3) and NR_{50} values calculated; this group included oleuropein aglycone, oleuropein glycoside, caffeic acid, *o*-coumaric acid, and cinnamic acid. The second group, for which midpoint cytotoxicity values could not be determined (i.e., >10 mM), included syringic acid, protocatechuic acid, vanillic acid, and tyrosol (Fig. 4).

Based on the concentration of test agent evoking initial cytotoxicity, the responses of the immortalized S-G and HSG_1 cells were very similar and demonstrated greater sensitivity than the normal GN61 fibroblasts. In particular, as compared with the S-G and HSG_1 cells, the GN61 cells better tolerated *o*-coumaric acid, cinnamic acid, tyrosol, syringic acid, protocatechuic acid, and vanillic acid (Table 1). However, based on the concentrations of test agent causing midpoint cytotoxicity, the sensitivities to a 24-h exposure to olive oil phenolics for the normal (i.e., GN61) and transformed (i.e., the S-G and HSG_1) cell lines were approximately equivalent and followed the sequence oleuropein aglycone > oleuropein glycoside, caffeic acid > *o*-coumaric acid > cinnamic acid ≫ tyrosol, syringic acid, protocatechuic acid, vanillic acid. Cytotoxicity was observed at concentrations that by far exceed those attained after consumption of olive oils, even if highly

Table 1
Concentrations of olive oil test agents causing initial cytotoxicity towards human cells in culture

Test agent	Concentration (mM)		
	GN61	S-G	HSG_1
Oleuropein aglycone	0.66	0.66	0.33
Oleuropein glycoside	1.0	1.0	1.5
Caffeic acid	1.5	3.0	2.0
o-Coumaric acid	6.0	3.0	3.0
Cinnamic acid	6.75	4.0	5.0
Tyrosol	>10.0	3.0	5.0
Syringic acid	9.0	6.0	5.0
Protocatechuic acid	10.0	5.0	7.5
Vanillic acid	10.0	10.0	10.0

Table 2
Midpoint cytotoxicity (NR_{50}) values of olive oil test agents towards human cells in culture

Test agent	NR_{50} values (mM)		
	GN61	S-G	HSG_1
Oleuropein aglycone	0.9	1.1	0.8
Oleuropein glycoside	1.2	2.8	2.3
Caffeic acid	1.6	2.9	2.1
o-Coumaric acid	>10	6.7	6.4
Cinnamic acid	>10	9.1	9.8

NR_{50} values for tyrosol, syringic acid, vanillic acid, and protocatechuic acid were >10 mM.

rich in phenolic compounds. Such concentrations have been reported to be associated with formation of hydrogen peroxide in cell culture media, which may explain the observed cytotoxicity [22]. The relatively high concentrations of olive oil phenolics, needed to induce cytotoxicity, may be relevant to predicting the potential consequences of overexposure to polyphenolics in general. This is of particular concern because of the availability, and hence safety to human health, of dietary supplements (particularly, antioxidants) administered excessively as pills and tablets and containing polyphenolics derived from fruits and vegetables [23,24].

In conclusion, olive oil phenolics, even at high concentrations, appeared not to exert cytotoxic effects on cells derived from the buccal mucosa. Moreover, given that for any specific phenolic test agent, cytotoxicity, as noted by the NR_{50} value, was observed at comparable concentrations towards both the malignant (HSG_1) and the nonmalignant (S-G; GN61) cells (Table 2); tumor-preventing activities of olive oil phenolics are, at present, yet to be fully demonstrated.

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