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Antigenotoxic Effect of Grape Seed Procyanidin Extract in Fao Cells Submitted to Oxidative Stress§

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The protective effects of grape seed procyanidin extract on the repair of H_2O_2-induced DNA lesions were tested using Fao cells. Cells were exposed to 600 μM H_2O_2 for 3 or 21 h. A procyanidin extract from grape seed (PE) was incubated or preincubated (1 h) during the exposure to H_2O_2. The ability of procyanidins to protect against the genotoxicity of H_2O_2 was compared with those of the monomeric flavanols (+)-catechin and (−)-epicatechin and the flavonol quercetin. After treatment, DNA damage was monitored using alkaline single-cell gel electrophoresis (the comet assay) (Aherne, S. A.; O'Brien, N. M. *Nutr. Cancer* **1999**, *34*, 160−166). At the end of the experiment, PE significantly decreased the damage caused by H_2O_2. The results also showed that quercetin was the most effective of the flavonoids tested, which is consistent with its powerful antioxidant character. The results indicate that procyanidins are more effective than the corresponding individual monomers, catechin and epicatechin, at preventing DNA lesions in hepatocytes and that this protection is higher after preincubation than after co-incubation.

KEYWORDS: Procyanidins; quercetin; comet assay; DNA damage; oxidative stress; H_2O_2; hepatocytes

INTRODUCTION

Reactive oxygen species (ROS) are produced in cells by cellular metabolism and by exogenous agents. These species react with biomolecules in cells, including DNA. ROS induce numerous lesions in DNA, which cause deletions, mutations, and other lethal genetic effects. If left unrepaired, this damage may contribute to a number of degenerative processes, including cancer and aging (*2*, *3*). Characterization of this damage to DNA has indicated that both the sugar and the bases are susceptible to oxidation. About 100 different modifications have been identified, including the appearance of AP sites, cross-linking to protein, and single- and double-strand breakage (*4*). This last type of breakage is highly toxic and mutagenic and can cause chromosome aberrations, whereas single-strand breaks are repaired. Living organisms have evolved several systems that recognize and repair the various forms of DNA damage induced by oxidation. These DNA repair systems are key processes in the secondary defense system, which copes with damage caused by ROS and which is not destroyed by the primary defense mechanisms. Primary defense mechanisms include antioxidant enzymes, nonenzymatic endogenous antioxidants, and exogenous antioxidant molecules, which together are usually able to maintain ROS at nonharmful levels. Polyphenols are exogenous antioxidants, and this is the most studied property of these compounds. Experimental and epidemiological data have revealed that moderate red wine consumption prevents various types of cancer. This is largely due to the phenolic compounds in wine. The flavonoids present in red wine comprise, among others, flavonols and procyanidins (derivatives of flavan-3-ols), the distinct antioxidative potentials of which are of great importance for explaining their beneficial effects. The antioxidative effects of flavonoids involve mechanisms such as metal-chelating, free-radical scavenging with the formation of less reactive flavonoid aroxyl radicals (*5*), inhibiting certain oxidative enzymes (*6*), and activating detoxifying/defensive proteins (*7*). The standard antioxidant capacity of flavonoids can be determined from their reduction potentials (700−450 mV), which are lower than those of alkylperoxyl and superoxide radicals (2300−1000 mV). Therefore, flavonoids may inactivate these damaging oxyl species and prevent their deleterious consequences.

In this study, we examined the antigenotoxic and protective effects of a procyanidin extract from grape seed, of their stable monomers the flavan-3-ols (+)-catechin and (−)-epicatechin, and of the flavonol quercetin on H_2O_2-induced genotoxicity in rat Fao cells. The DNA cleavage/repair was identified by alkaline single-cell electrophoresis, a very sensitive method for detecting strand breaks and measuring repair kinetics at the level of single cells.

§ This research was done at the Rovira i Virgili University in Tarragona, Spain. Dr. Llópiz and Dr. Céspedes were visiting scientists from the University of Cuba and did not carry out any of the experiments reported in the paper at that institution.

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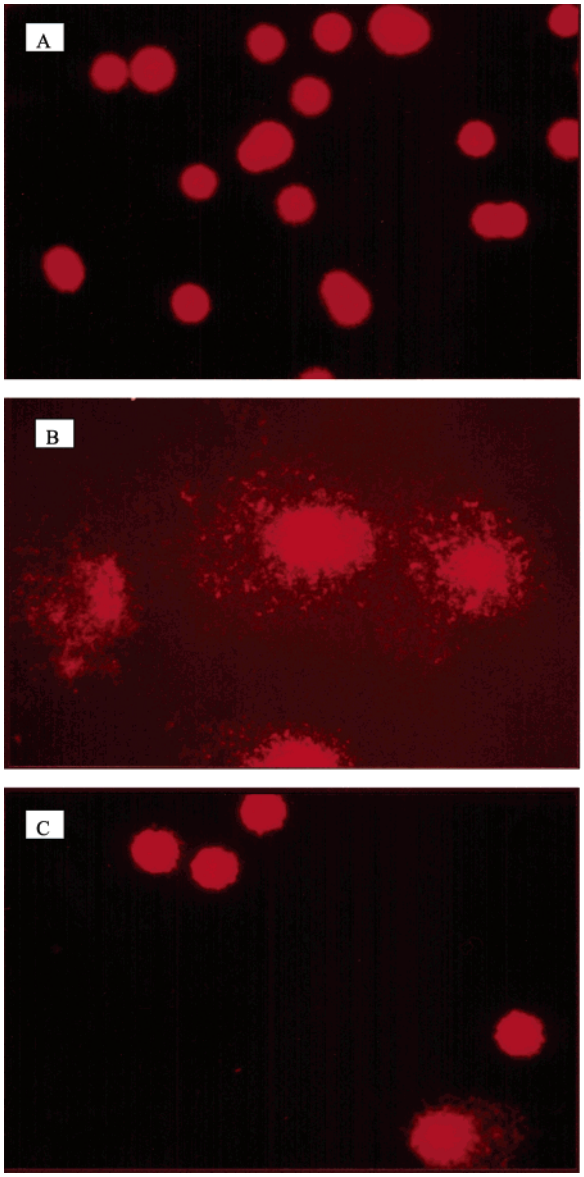

Figure 1. Representative comet images of Fao cells after treatment with flavonoids and/or H_2O_2 for 3 h: (**A**) control (level 0); (**B**) 600 μM H_2O_2 (levels 3 and 4); (**C**) 25 μM quercetin and 600 μM H_2O_2 (levels 1 and 2). Comet assay was carried out as described under Materials and Methods.

MATERIALS AND METHODS

Fao cells were routinely cultured in F-12 Coon's Modification medium (Sigma), supplemented with 5% fetal bovine serum (BioWhittakker), 0.1% fungizone (BioWhittakker), and 1% penicillin/streptomycin (BioWhittakker). Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2. They were seeded 4−5 days before use in six-well plates (Corning) at a density of (3−4) × 10^5 cells/well. Cells were incubated with 50−600 μM H_2O_2 (Sigma) so that the H_2O_2 concentration which induced considerable DNA damage could be found. Cells were incubated with 600 μM H_2O_2 and at the same time or 1 h previously exposed to the flavonoids at the concentrations described below. Stock solutions were prepared by dissolving flavonoids in ethanol (Merck). The cultures were supplemented with (+)-catechin (Fluka) and (−)-epicatechin (Fluka) to a final concentration of 150 μM and then incubated for 3 or 21 h. In the case of quercetin (Fluka), cells were supplemented with a concentration of 150 μM for 3 h and a concentration of 25 μM for 21 h. For the procyanidin extract (Les Dérivés Résiniques et Terpéniques, Dax, France) obtained from grape seed and consisting of 21.3% monomers, 17.4% dimers, 16.3% trimers, 13.3% tetramers, and 31.7% higher polymers, cells were supplemented with a concentration of 75 μM for 3 h and a concentration of 25 μM for 21 h. The concentration of the procyanidin extract was calculated by taking a mean MW = 1399. The concentrations used were previously determined to be nontoxic (*8*). Controls with the equivalent ethanol content of flavonoid solutions (0.3% final concentration in the culture medium) were incubated without H_2O_2 under the same conditions.

At the end of each incubation period, the hepatocytes were washed once with phosphate buffer (pH 7) and then scraped in 1 mL of buffer containing 0.1% (v/v) Triton X-100 (Panreac). The comet assay was performed according to the method of Piperakis (*9*). Briefly, trypsinized cells were suspended in 80 μL of 0.5% low-melting-point agarose in PBS and placed on a micro slide glass covered with 0.5% agarose in PBS. The slide was immersed in a lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM TRIS, 1% Triton X-100, 10% DMSO, pH 10) at 4 °C for 1 h. After lysis, the slide was placed in the electrophoresis buffer (0.3 M NaOH, 1 mM Na_2EDTA, pH 12) and electrophoresed for 20 min at 25 V, 250 mA, and 4 °C. After electrophoresis, the slide was neutralized (0.4 M Tris, pH 7.5), stained with ethidium bromide (20 μg/mL), and covered with coverslips. The "comet tail" was observed using a fluorescence microscope (Olympus) equipped with a 515−560-nm excitation filter and a 590-nm barrier filter. Fifty nuclei on each slide were visually scored from 0 (undamaged nucleus) to 4 (severely damaged nucleus) (see **Figure 1**). Samples were scored blindly, and results were expressed as a percentage of arbitrary units.

All results are expressed as means ±SEM (n = 4). The statistical analysis consisted of the General Linear Model procedure of SPSS (2002) combined with Bonferroni's multiple-range test. The level of statistical significance was taken as $p < 0.05$.

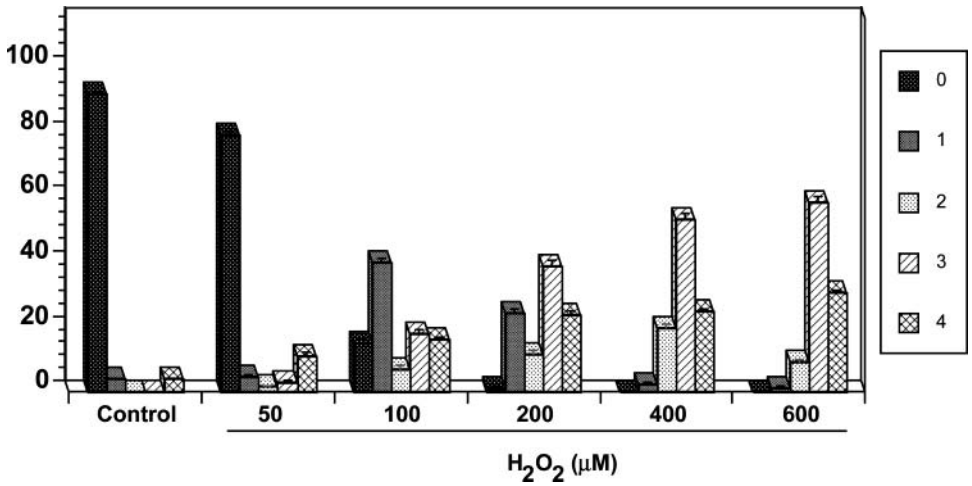

Figure 2. Effects of H_2O_2 concentration on DNA of Fao cells. DNA damage was scored from 0 (undamaged nucleus) to 4 (severely damaged nucleus), and results are expressed as a percentage of arbitrary units. Values are mean ± SEM of four independent experiments.

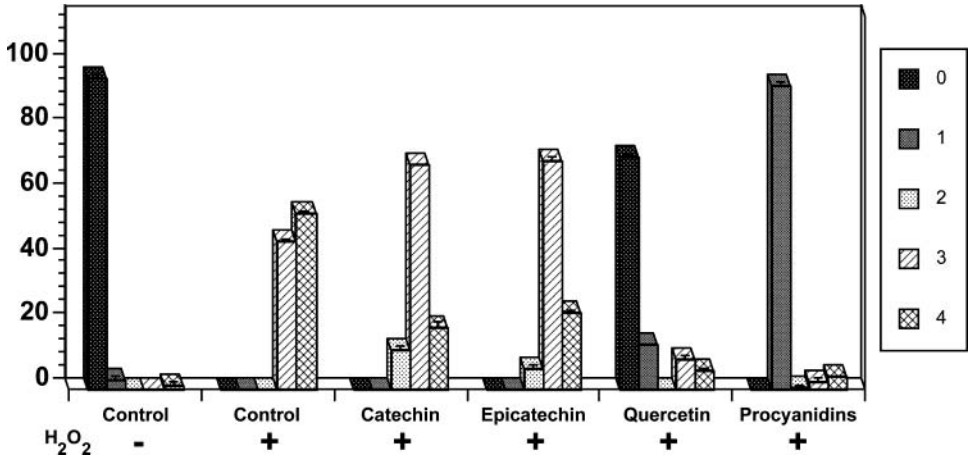

Figure 3. Effects of preincubation (1 h) with flavonoids on DNA damage in Fao cells induced by 600 μM H_2O_2 for 3 h. DNA damage was scored from 0 (undamaged nucleus) to 4 (severely damaged nucleus), and results are expressed as a percentage of arbitrary units. Values are mean ± SEM of four independent experiments.

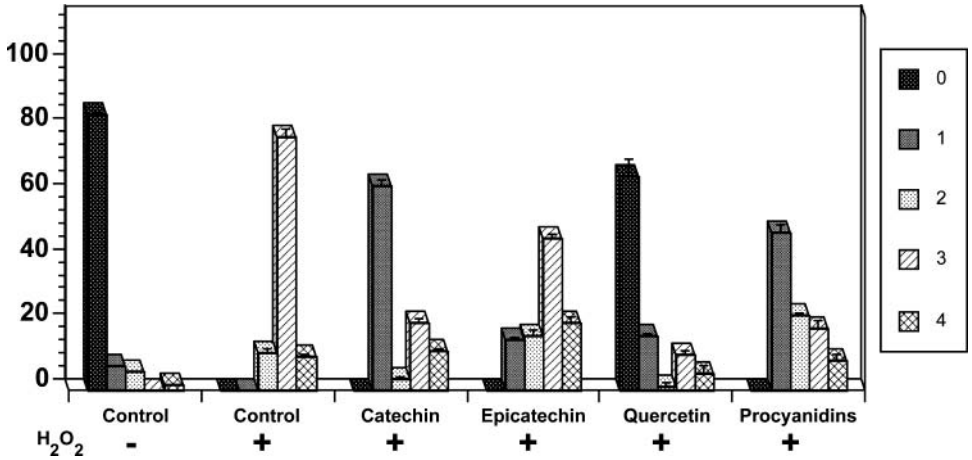

Figure 4. Effects of preincubation (1 h) with flavonoids on DNA damage in Fao cells induced by 600 μM H_2O_2 for 21 h. DNA damage was scored from 0 (undamaged nucleus) to 4 (severely damaged nucleus), and results are expressed as a percentage of arbitrary units. Values are mean ± SEM of four independent experiments.

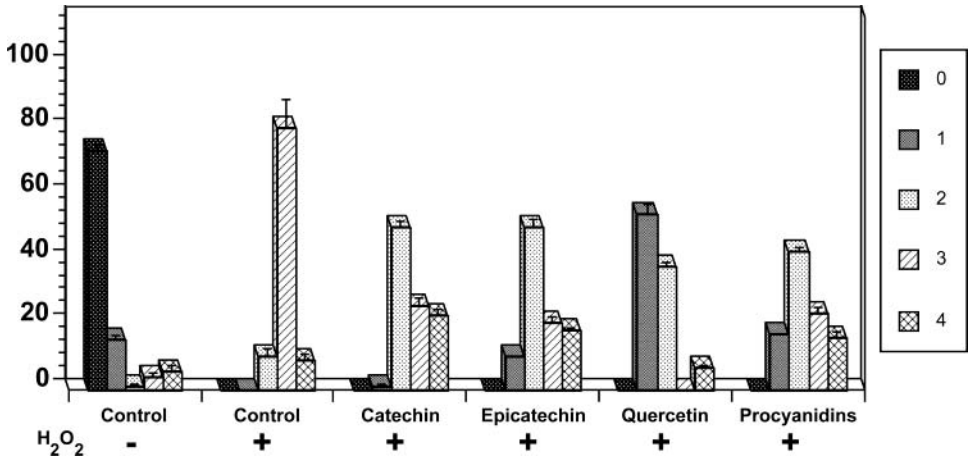

Figure 5. Effects of simultaneous incubation with flavonoids and 600 μM H_2O_2 for 21 h on DNA damage in Fao cells. DNA damage was scored from 0 (undamaged nucleus) to 4 (severely damaged nucleus), and results are expressed as a percentage of arbitrary units. Values are mean ± SEM of four independent experiments.

RESULTS

The images of DNA damage were classified into five grades (0−4), as is shown in **Figure 1**. **Figure 1A** shows level 0 (undamaged nucleus), **Figure 1B** shows levels 3 and 4 (very and severely damaged nucleus), and **Figure 1C** shows levels 1 and 2 (slightly and moderately damaged nucleus).

Incubating Fao cells with increasing quantities of H_2O_2 (50, 100, 200, 400, and 600 μM) for 3 h led to increasingly greater damage (**Figure 2**). At the lowest concentration, 50 μM, the results were similar to control cells (not treated with flavonoids or H_2O_2): 78.99 and 91.97%, respectively, of cells had a damage level of 0. Above 200 μM, there were no cells with a damage

level of 0, and the percentage of cells at levels 3 and 4 increased to 90% at 600 μM of H_2O_2.

Preincubating cells with catechin, epicatechin, quercetin, or PE for 1 h before exposure to 600 μM H_2O_2 for 3 h (**Figure 3**) showed that quercetin, with 71% of cells at level 0, and PE, with 93.6% of cells at level 1, were the compounds that best protected cells against DNA cleavage. Catechin and epicatechin, on the other hand, only slightly improved the DNA damage caused by H_2O_2 (the percentage of cells at level 4 was lower). Although 50% of the cells incubated with H_2O_2 for 3 h were at level 3 and 50% at level 4 (**Figure 3**), after 21 h most (80%) were at level 3 (**Figure 4**). When cells pretreated with flavonoids were exposed to H_2O_2 for 21 h (**Figure 4**), quercetin again showed the best protective effects (66% of undamaged cells), followed by PE (71% at levels 1 and 2), catechin (67% at levels 1 and 2), and, finally, epicatechin (45% at level 3). If flavonoids and H_2O_2 were simultaneously added and incubated for 21 h (**Figure 5**), the protective effect was lower than when cells were preincubated. However, quercetin was yet again the best protector against DNA damage caused by H_2O_2.

The results of using flavonoids to treat Fao cells incubated with H_2O_2 showed that quercetin provided greatest protection against DNA damage, followed by procyanidin extract, catechin, and epicatechin, in this order.

DISCUSSION

Excessive ROS generation in vivo may result in DNA damage. At least two major human problems, aging and carcinogenesis, involve DNA damage (*2*, *3*). Quercetin (*1*, *10*−*18*) and tea catechins (*16*, *19*−*23*) have been at the center of several genetic toxicity and carcinogenicity investigations. Larger molecules, such as procyanidins, which are present in significant amounts in red wine, have only recently attracted attention (*24*). A diet enriched with red wine solids delayed the onset of tumors in transgenic mice that spontaneously develop externally visible tumors (*25*), and a polyphenolic fraction from grape seeds inhibited skin tumorigenesis in mice (*26*). A complex mixture of wine polyphenols protected against some types of chemically induced oxidative DNA damage in the rat (*27*). In a previous experiment, we exposed Fao cells to H_2O_2 under the same conditions as in the current study: PE protected the cell membrane and was the most powerful at protecting against lipid peroxidation and activating the glutathione cycle (*8*). Our present aim was to determine whether grape seed procyanidin extract had this powerful antigenotoxic effect on H_2O_2-induced DNA damage.

The protective effects of flavonoids against oxidative DNA damage shown here are in accordance with the structure−activity relationships of these compounds. All flavonoids have at least two phenyl rings (A and B rings) in their chemical structure separated by a pyran ring (C ring). Quercetin is a flavonol that satisfies all of the determinants of antioxidant flavonoids given by Bors et al. for maximal radical scavenging potential (*28*), whereas catechin and epicatechin have only a catechol structure in the B ring. As well as having the best free radical scavenging properties, quercetin has three potential metal-binding sites (*29*), which could have chelated the iron ions present in the cells and, hence, depressed the Fenton reaction. Catechins, on the other hand, have only one. Therefore, the fact that quercetin has the highest protective effect against oxidative DNA damage agrees with its structural characteristics: it is a more effective radical scavenger and metal chelator than flavan-3-ols, catechin, and epicatechin.

On the other hand, the fact that oligomer procyanidins have a greater protective effect than the monomer components catechin and epicatechin agrees with the experiments of Bors and co-workers, who used pulse radiolysis to show that an increase in the reactivity of procyanidins correlates linearly with the number of reactive sites, defined as the catechol groups in the individual molecules (*30*). This correlation was also shown when the Trolox-equivalent antioxidant capacity (TEAC) assay (*31*, *32*) or the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) assay (*33*) was used to determine the antioxidant potential. Ursini and co-workers also concluded that polymeric procyanidins are better antioxidants than the corresponding monomers, catechin, and epicatechin, because if the catechol moieties are very close, their oxidation potentials are much lower than those of catechins (*34*). It is more difficult to find an explanation based on molecular structure for the different protective effects of catechin and epicatechin on damaged DNA, after incubations for 21 h. The only distinction between these two stereoisomers is the different spatial distributions of the OH substituent in the C3: the 3-hydroxy group is closer to the 2′-hydrogen atom in catechin than in epicatechin, which seems to cause repulsion between the 2′-H and 3-OH groups (*35*).

The recovery of the damage was more marked in the cells treated with the flavonoids tested before the H_2O_2 incubation, which suggests that simple scavenging of ROS by flavonoids was not the only cause of DNA damage protection, although the scavenger activity of these compounds has been shown elsewhere. There is no clear evidence of the intracellular location of polyphenols and even less evidence in the case of procyanidins, although some studies suggest that they interact with cell membranes and intracellular proteins. In vivo and in vitro we have shown that grape seed procyanidin extract activates antioxidative enzymes and the glutathione cycle (*8*, *36*), so these compounds enhance cellular antioxidant potential during preincubation.

The protective action of the flavonoids studied here on DNA damage may be a contribution to the putative antitumorigenic potential of red wine.

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