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ORIGINAL CONTRIBUTION

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Dietary polyphenols protect against *N*-nitrosamines and benzo(a)pyrene-induced DNA damage (strand breaks and oxidized purines/pyrimidines) in HepG2 human hepatoma cells

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Abstract *Background* Dietary polyphenols have been reported to have a variety of biological actions, including anticarcinogenic and antioxidant activities. *Aim of the study* In the present study we investigated the protective effect of dietary polyphenols against *N*-nitrosodimethylamine (NDMA), *N*-nitrosopyrrolidine (NPYR) and benzo(a)pyrene (BaP)-induced DNA damage (strand breaks and oxidized purines/pyrimidines) in HepG2 cells. *Methods* Human hepatocellular carcinoma (HepG2) cells, which retain many specialized liver functions and drug metabolizing enzyme activities, were used as in vitro model for human hepatocytes. NDMA, NPYR and BaP were employed to induce DNA damage. DNA damage (strand breaks, oxidized pyrimidines and oxidized purines) was evaluated by the alkaline single cell gel electrophoresis or comet assay. *Results* None of the polyphenols concentrations tested in presence or absence of Fpg (formamidopyrimidine-DNA glycosylase), or Endo III (Endonuclease III) caused DNA damage per se. Increasing concentrations of BaP (25–100 μM) induced a significant increase of DNA strand breaks, Fpg and Endo III sensitive sites in a dose dependent manner. Myricetin and quercetin decreased DNA strand breaks and oxidized pyrimidines induced by NDMA, but not oxidized purines. However, both flavonoids reduced oxidized pyrimidines and purines induced by NPYR. DNA strand breaks induced by NPYR were prevented by quercetin, but not by myricetin. BaP-induced DNA strand breaks and oxidized pyrimidines were strongly reduced by myricetin and quercetin, respectively. While oxidized purines induced by BaP were reduced by quercetin, myricetin had no protective effect. (+)-Catechin and (−)-epicatechin reduced DNA strand breaks, oxidized pyrimidines and oxidized purines induced by NDMA. DNA strand breaks, and oxidized purines induced by NPYR were also prevented by (+)-catechin and (−)-epicatechin, while the maximum reduction of oxidized pyrimidines was found by (+)-catechin and (−)-epicatechin at 10 μM. (+)-Catechin and (−)-epicatechin de-

creased also DNA strand breaks and oxidized pyrimidines but not oxidized purines induced by BaP. *Conclusions* Our results clearly indicate that polyphenols protect human derived cells against DNA strand breaks and oxidative DNA damage effects of NDMA, NPYR or BaP, three carcinogenic compounds which occur in the environment.

Key words dietary polyphenols – *N*-nitrosamines – benzo(a)pyrene – DNA damage – comet assay

Introduction

Polycyclic aromatic hydrocarbons (PAH) and *N*-nitrosamines are mutagenic and carcinogenic compounds widely present in the environment. PAH are exogenous factors exposed to humans via inhalation of industrial and automobile emissions, cigarette smoke, and ingesting pyrolysed food products. Benzo(a)pyrene (BaP), an important PAH, is a carcinogen that undergoes metabolic activation through CYP1A1, CYP1A2, and CYP1B1. Thus, BaP is a potent systemic and local carcinogen known to induce skin, lung, and stomach tumours in animal models [36]. Humans are exposed to *N*-nitrosamines from occupational and environmental sources and through in vivo formation of ingested precursor amines and nitrosating agents. These compounds have been detected in a wide variety of matrices such as bacon, ham, frankfurters, sausages, cheese, beer, rubber, ground water, smoked tobacco, cosmetics, etc [9]. *N*-Nitrosodimethylamine (NDMA) is the most commonly encountered volatile *N*-nitrosamine in food samples and it is a potent liver, lung and kidney carcinogen [33]. *N*-Nitrosopyrrolidine (NPYR) induced mainly liver tumours in rats and is a weak pulmonary carcinogen in mice [10]. *N*-nitrosamines require metabolic activation to exert their carcinogenic effects through cytochrome P450 (CYP2E1) [39]. The reactive intermediates of nitrosamine metabolism also have the ability to alkylate nucleophilic sites of DNA [34] resulting in alkali labile adducts, which can lead to the formation of abasic sites and DNA strand breaks. *N*-Nitrosamines and PAH are focus of special interest due to their increasing appreciation as potential human carcinogens. However, in balance, there are also many other dietary agents which have been found to be beneficial in promoting good health and reducing the risks of some of these agents [31].

There was convincing evidence to suggest that fruit and vegetable intake was inversely associated with cancers of the lung, stomach, mouth, pharynx, esophagus and colon [38]. Over the past two decades, plant polyphenols increasingly attracted researchers, food manufacturers, as well as consumers due to their antioxidant properties. Phenolic compounds, abundantly present in fruits, vegetables, and beverages such as tea and red wine, are a large group of compounds with a similar chemical structure [40]. They could influence several important biological functions by their free-radical scavenging ability, signal transduction pathway, stimulating apoptosis, inhibiting inflammation and inhibiting proliferation in human cancer cell lines [30]. Since in the Mediterranean basin, fresh fruit and vegetables as well as wine figure prominently in the general diet, in this study has been evaluated the protective effect of some dietary polyphenols, such as, (+)-catechin; (+)-*trans*-3,3′,4′,5,7-pentahydroxyflavane, (-)-epicatechin; (−)-*cis*-3,3′, 4′,5,7-pentahydroxy- flavane, quercetin; 3,3′,4′,5,7-pentahydroxyflavone and myricetin; 3,3′,4′,5,5′,7-hexahydroxyflavone. These compounds are suggested to act as chemopreventive compounds against lung, stomach, colorectal, epithelial, kidney, uterus, and ovary cancer [25].

The single cell gel electrophoresis (SCGE) or Comet assay has been considered as a useful tool for investigating DNA damage and DNA repair in mammalian cells due to oxidative damage [5]. In this study, the Comet assay was modified to permit the detection of oxidized bases by including a step in which DNA is digested with formamidopyrimidine-DNA glycosylase (Fpg) or endonuclease III (Endo III) to uncover oxidized purines and pyrimidines, respectively. The effects of polyphenols compounds on the formation of Fpg sites or Endo III sites were examined.

The study of the reactions of polyphenolic constituents of the diet with mutagenic nitrosating species or benzo(a)pyrene is an area of great promise in cancer preventive strategies. Thus, the aim of this study was to evaluate the protective effect by dietary polyphenols against *N*-nitrosodimethylamine (NDMA), *N*-nitrosopyrrolidine (NPYR) and benzo(a)pyrene (BaP)-induced DNA damage (strand breaks and oxidized purines/pyrimidines) in human hepatoma cells (HepG2), using the single-cell gel electrophoresis (SCGE) assay. These cells retain many specialized liver functions and drug metabolizing enzyme activities, comparable with human hepatocytes, such as P450-mixed function oxidases, epoxide hydrolase, glucuronlytransferase, and glutathione transferase [7].

Materials and methods

Chemicals

N-nitrosodimethylamine (NDMA), *N*-nitrosopyrrolidine (NPYR), benzo(a)pyrene (BaP), quercetin, myricetin, (+)-catechin, and (−)-epicatechin, dimethyl sulfoxide (DMSO) and low melting point agarose (LMP) were purchased from Sigma–Aldrich (St Louis, MO). Formamidopyrimidine-DNA glycosylase (Fpg) and Endonuclease III (Endo III) were obtained from Trevigen Inc. (Gaithersburg, MD). All other chemicals and solvents were of the highest grade commercially available. *N*-nitrosamines, benzo(a)pyrene, and polyphenols were dissolved in sterile DMSO. The stock solutions were stored deep frozen (−80°C).

HepG2 cells

Human hepatocellular carcinoma (HepG2) cells were purchased from Biology Investigation Center Collection (BIC, Madrid, Spain). Only cells of passage 10–17 were used in the experiments. The cells were cultured as monolayer in Dulbecco's Modified Eagle Medium supplemented with 10% v/v heat inactivated foetal calf serum, 50 U/ml penicillin and 50 μg/ml streptomycin and 1% v/v L-glutamine. Culture medium and supplements required for the growth of the cells were purchased from Gibco Laboratories (Life Technologies, Inc., Gaithersburg, MD 20884-9980). Cell cultures were incubated at 37°C and 100% humidity in a 5% CO_2 atmosphere.

Analysis of DNA damage induced by benzo(a)pyrene or polyphenols in the Alkaline Comet assay

Cell viability was routinely determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay in order to select non-toxic concentrations of chemicals. The SCGE assay was carried out according to the protocol of Olive et al. [32] with minor modifications. Induction of DNA damage (strand breaks and oxidative DNA damage) by NPYR and NDMA has been previously evaluated [1]. Results obtained indicated that cells treated with NPYR and NDMA, after conversion to nucleoids, and incubated with Fpg had significant increases in DNA damage, the lowest effective concentrations being 5 and 27 mM, respectively. However, when HepG2 cells were treated with NPYR and NDMA and without enzymes the concentration of NPYR and NDMA required to cause a significant increase in DNA damage was 50 and 135 mM, respectively. For this reason this concentration range was used in subsequent studies.

Briefly, HepG2 cells were plated on to multiwell systems at a density of 1.5 x 10^5 cells/ml culture medium. 24 h after seeding, cells were exposed to BaP, (25–100 μM), or to quercetin (1–10 μM), or to myricetin, (1–10 μM), or to (+)-catechin (10–50 μM) or to (−)-epicatechin (10–50 μM) or to the solvent for another 24h at 37°C and 5% CO_2. The solvent concentration in the incubation medium never exceeded 0.1%. After incubation, 10 μl of a suspension 1 x 10^5 cells were mixed with 70 μl of LMP agarose type VII (0.75% concentration in PBS), distributed on slides that had been pre-coated with LMP agarose type VII (0.30% concentration in PBS), and left to set on an ice tray. Three slides were prepared for each concentration of the compound tested, one slide for control and the others slides to be treated with Fpg or Endo III. After solidification, the cells were lysed in darkness for 1 h in a high salt alkaline buffer (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris, 1% Triton X-100, pH 10). The slides were then equilibrated 3 × 5 min in enzyme buffer (0.04 M HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, pH 8). After this time, slides were incubated with 30 μl of Fpg or Endo III at 1 μg/ml in enzyme buffer for 30 min at 37°C in a humid dark chamber. Control slides were incubated with 30 μl enzyme buffer only. Following enzyme treatment, the slides were placed in electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH 13, cooled in a refrigerator) in darkness for 40 min. Electrophoresis was performed in a cold-storage room, in darkness, in a Bio-Rad subcell GT unit containing the same buffer, for 30 min at 25 V. After electrophoresis, the slides were neutralized using 0.4 M Tris pH 7.5 and fixed in methanol. Subsequently, the DNA was stained with ethidium bromide (10 μg/ml) in Tris acetate EDTA (TAE 1X) during 5 min and examined in a fluorescence microscope (OLYMPUS BH-2) connected to a computerized image analysis system (Comet Score 1.0). Olive tail moment (OTM) as defined by Olive et al. [32] was determined and expressed as arbitrary units (AU). OTM $= I \times L$, where I is the fractional amount of DNA in the comet tail (% DNA in the tail) and L is the distance from the centre of the comet head to the centre of tail distribution.

Analysis of DNA damage induced by a simultaneous treatment of *N*-nitrosamines or benzo(a)pyrene and polyphenols in the Alkaline Comet assay

HepG2 cells were plated on to multiwell systems at a density of 1.5 x 10^5 cells/ml culture medium. 24 h after seeding, the corresponding polyphenols concentrations were added to the wells and plates were

incubated for 24 h at 37°C and 5% CO_2. After incubation, cells were simultaneously treated with: (1) NPYR (50 mM without enzymes and 5 mM with Endo III or FPg) [1] and quercetin (0.1–5 μM) or myricetin (0.1–5 μM) or (+)-catechin (10–50 μM) or (−)-epicatechin (10–50 μM). Or (2) NDMA (135 mM without enzymes and 27 mM with Endo III or Fpg) [1] and quercetin (0.1–5 μM) or myricetin (0.1–5 μM) or (+)-catechin (10–50 μM) or (−)-epicatechin (10–50 μM). Or (3) BaP (50 μM with and without enzymes) and quercetin (1–10 μM) or myricetin (1–10 μM) or (+)-catechin (10–50 μM) or (−)-epicatechin (10–50 μM). Then plates were incubated for another 24 h at 37°C and 5% CO_2. After the treatments, the cells were processed as described above.

Statistical analysis

Images of 50 randomly selected cells per concentration were evaluated and the test was carried out three times. The reported OTM is the mean ± standard deviation (SD) of three independent experiments. Thus, we compare three means of OTM from three different experiments. Cultures without *N*-nitrosamines or BaP or polyphenols were considered as negative controls. In all experiments the following negative controls have been included: C_0, cells treated with solvents; C_1, cells treated without enzymes; C_2, cells incubated with Endo III; C_3, cells incubated with Fpg. Induction of DNA damage by N-nitrosamines or BaP was defined as 100% of genotoxicity. The Student's *t* test was used for statistical comparison between simultaneous treatments and controls, and differences were considered significant at $P \leq 0.05$.

Results

DNA damage induction by benzo(a)pyrene and polyphenols

No cytotoxicity has been previously found at the concentrations of polyphenols tested (data not shown). Cell viability was always above 80% of control viability. None of the polyphenols (quercetin 0.1–10 μM, myricetin 0.1–10 μM, (+)-catechin 10–50 μM and (−)-epicatechin 10–50 μM) concentrations tested in presence or absence of Fpg or Endo III, caused DNA damage per se (Fig. 1). For this reason this concentration range was used in subsequent studies.

As shown in Fig. 2, increasing concentrations of BaP (25–100 μM) induced a significant increase of DNA strand breaks, Fpg sensitive sites and Endo III sensitive sites in a dose dependent manner. Results revealed that HepG2 cells treated with a concentration of 25 μM BaP slightly increased the DNA strand breaks (Fig. 2a1, b1), whereas the Fpg sensitive sites (Fig. 2a3, b3) and the Endo III sensitive sites were markedly increased (Fig. 2a2, b2) in comparison with their respective controls. The maximum increase of DNA strand breaks, Fpg sensitive sites and Endo III sensitive sites were exhibited by 100 μM BaP. In subsequent simultaneous treatments with BaP and

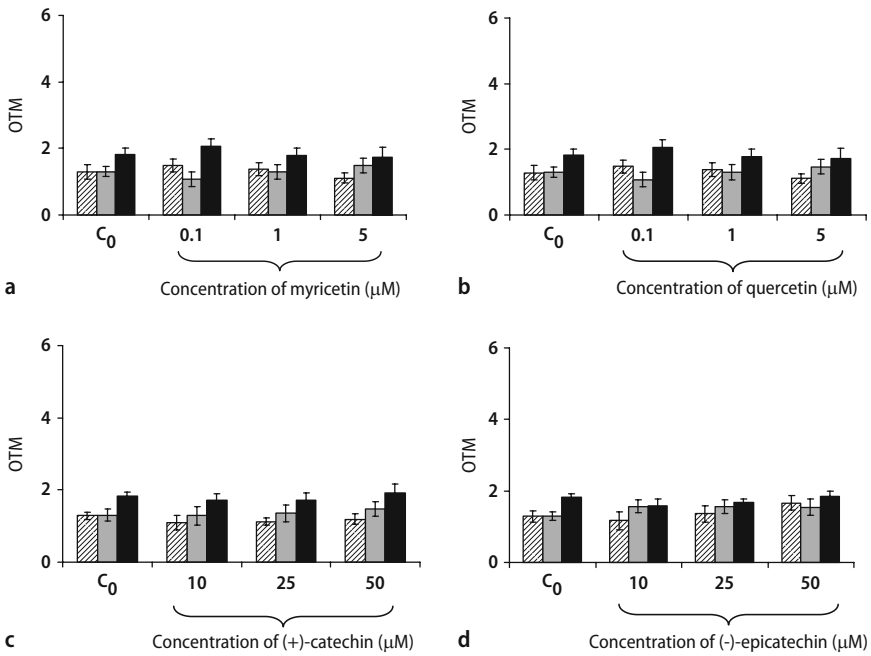

Fig. 1 Induction of DNA strand breaks and oxidized purines/pirimidines by **a** myricetin, **b** quercetin, **c** (+)-catechin and **d** (−)-epicatechin on human HepG2 cells incubated without enzymes (*diagonally shaded bar*), with Endo III (*lightly shaded bar*) or Fpg (*darkly shaded bar*). C_0 HepG2 cells without (+)-catechin, (−)-epicatechin, myricetin or quercetin and incubated without enzymes (*diagonally shaded bar*), with Endo III (*lightly shaded bar*) or Fpg (*darkly shaded bar*)

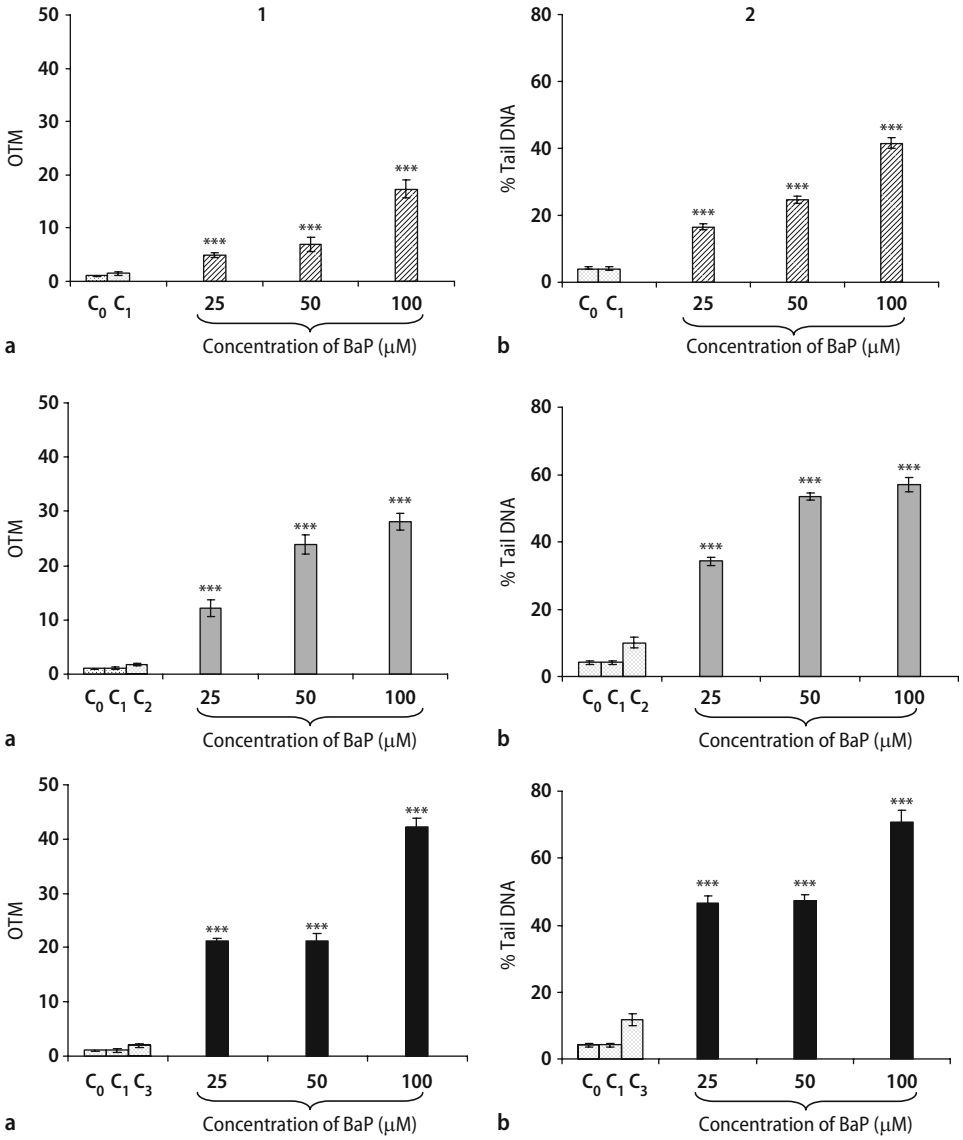

Fig. 2 Effect of BaP-induced DNA strand breaks and oxidative damage in human HepG2 cells expressed as **a** OTM in AU, and **b** % Tail DNA. (1) (C_0) control cells treated with solvent. (C_1) control cells treated without enzymes. HepG2 cells treated with BaP and incubated without enzymes (*shaded with dots*). (2) (C_2) control cells treated with Endo III. HepG2 cells treated with BaP and incubated with Endo III (*shaded with dots*). 3. (C_3) control cells treated with Fpg. HepG2 cells treated with BaP and incubated with Fpg (*diagonally shaded bar*)

polyphenols, the HepG2 cells were incubated with the concentration of 50 μM BaP for 24 h.

DNA damage induction by a simultaneous treatment of *N*-nitrosamines or benzo(a)pyrene and polyphenols in the Alkaline Comet assay

The effect of myricetin on NDMA-induced DNA damage in HepG2 cells is shown in Fig. 3.1 Myricetin decreased DNA strand breaks (Fig. 3, a1) and the formation of Endo III sensitive sites (Fig. 3, a2) induced by NDMA, but not the formation of Fpg sensitive sites (Fig. 3, a3). The maximum reduction of Endo III sensitive sites (32% inhibition compared with the control) was observed at the lowest concentration (0.1 μM). Myricetin reduced the formation of Endo III sensitive sites (1–5 μM, 16–19%) and the formation of Fpg sensitive sites (0.1–5 μM, 30–43%) induced by NPYR (Fig. 3, b2, b3), but not DNA strand breaks (all the concentrations increased the genotoxic effect of NPYR) (Fig. 3, b1). Protective effect of myricetin on BaP-induced DNA damage is shown in Fig. 3, c1, c2 and c3. Myricetin (5–10 μM) weakly reduced DNA strand breaks (20–5%) and the formation of Endo III sensitive sites induced by BaP, but not the formation of Fpg sensitive sites (concentrations of 5–10 μM increased the oxidative DNA damage induced by BaP). The maximum inhibition of Endo III sensitive sites (75%) induced by BaP was found at the lowest concentration (1 μM).

The effect of quercetin on NDMA-induced DNA damage in HepG2 cells is shown in Fig. 3.2. None of the quercetin concentrations tested reduced the for-

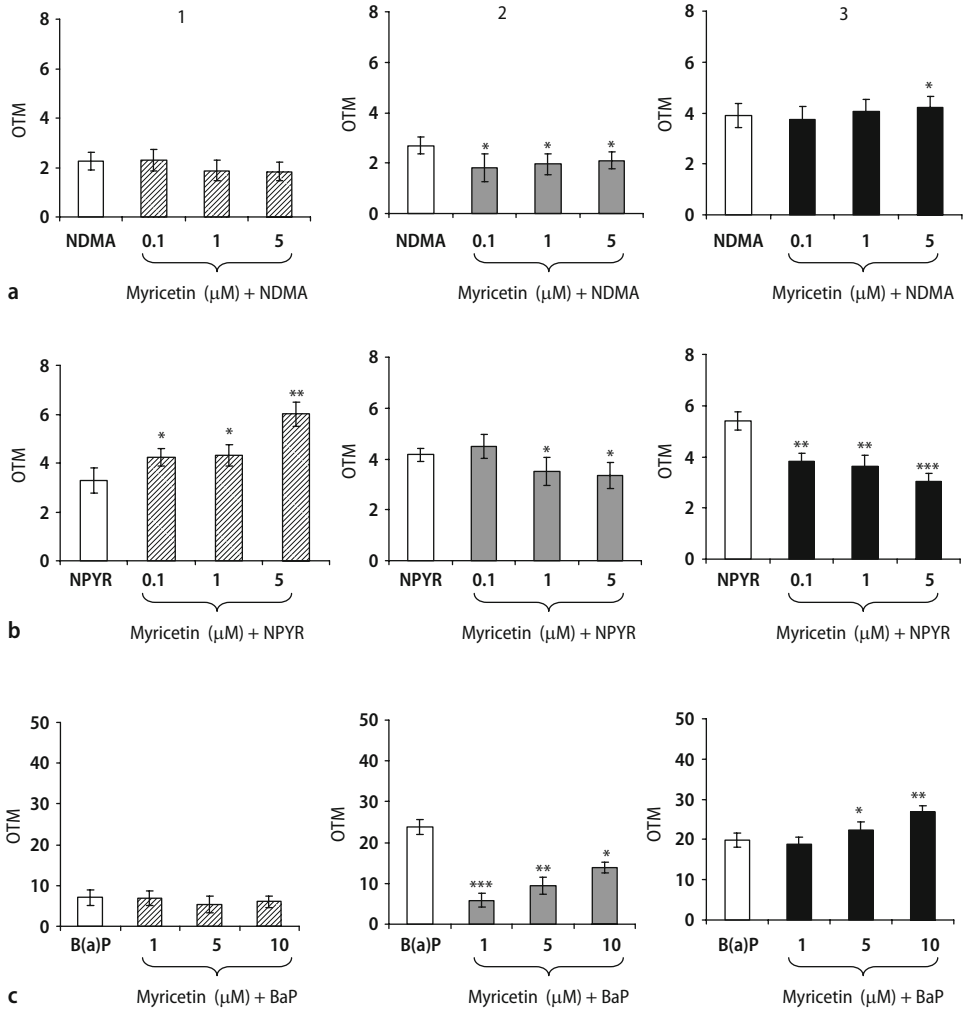

Fig. 3.1 Effect of 3.1 Myricetin, 3.2 Quercetin, 3.3 (+)-Catechin and 3.4 (−)-Epicatechin on **a** NDMA, **b** NPYR and **c** BaP-induced DNA damage in human HepG2 cells. OTM median values in control cells without enzymes and incubated with Endo III or Fpg were 1.47 ± 0.37, 1.85 ± 0.43, 2.11 ± 0.40 AU, respectively. (1) Cells treated with NDMA (135 mM) or NPYR (50 mM) or BaP (50 μM) and incubated without enzymes (*lightly shaded bar*). Cells treated with NDMA (135 mM) or NPYR (50 mM) or BaP (50 μM) and polyphenol and incubated without enzymes (*shaded with dots*). (2) Cells treated with NDMA (27 mM) or NPYR (5 mM) or BaP (50 μM) and incubated with Endo III (*darkly shaded bar*). Cells treated with NDMA (27 mM) or NPYR (5 mM) or BaP (50 μM) and polyphenol and incubated with Endo III (*unfilled bar*). (3) Cells treated with NDMA (27 mM) or NPYR (5 mM) or BaP (50 μM) and incubated with Fpg (*diagonally filled bar*). Cells treated with NDMA (27 mM) or NPYR (5 mM) or BaP (50 μM) and polyphenol and incubated with Fpg (*unfilled bar*). Asterisks indicate significant difference from control $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$

mation of Fpg sensitive sites induced by NDMA (Fig. 3, a3). On the contrary, concentrations of 1–5 μM increased the oxidative DNA damage induced by NDMA. Quercetin inhibited the formation of Endo III sensitive sites induced by NDMA (Fig. 3, a2) at all the concentrations tested (0.1–5 μM, 26%). However, quercetin (0.1–5 μM) reduced the formation of Fpg sensitive sites (18–27%) induced by NPYR (Fig. 3, b3). DNA strand breaks (0.1–1 μM, 2–10%) and the formation of Endo III sensitive sites (1–5 μM, 18%) induced by NPYR were also prevented by quercetin (Fig. 3, b1, b2). Protection afforded by quercetin towards BaP-induced DNA damage occurred in a dose-dependent manner. Quercetin induced a dose-dependent protective effect towards DNA strand breaks (Fig. 3, c1). At 1 μM, BaP genotoxicity was reduced by 6%, and the reduction reached 27% at 10 μM. The formation of Fpg sensitive sites (Fig. 3, c3) was reduced by 8–14% (5–10 μM) and the maximum inhibition of the formation of Endo III sensitive sites (Fig. 3, c2) was observed at 1 μM (82%).

Figure 3.3 shows the protective effect of (+)-catechin on NDMA-induced DNA damage in HepG2 cells. (+)-Catechin at the lowest concentration (10 μM) showed the maximum reduction of DNA strand breaks (38%), the formation of Endo III (62%) and Fpg sensitive sites (48%) induced by NDMA (Fig. 3, a1–a3). DNA strand breaks (12–24%) induced by

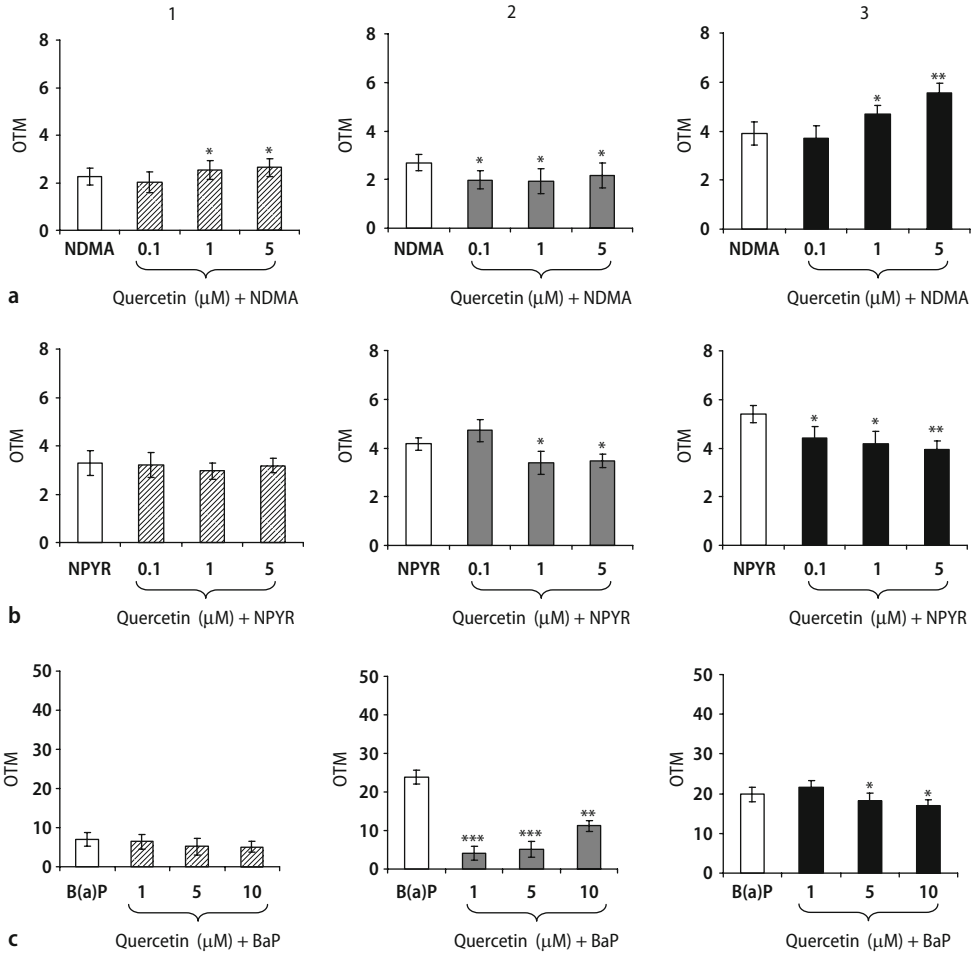

Fig. 3.2 continued

NPYR (Fig. 3, b1) and the formation of Fpg sensitive sites (24–48%) was also prevented by (+)-catechin (10–50 μM, Fig. 3, b3). However, the maximum inhibition of formation of Endo III sensitive sites (47%) was observed at the lowest concentration (10 μM). Figure 3, c1, c2 and c3 show the effect of (+)-catechin on BaP-induced DNA damage. (+)-Catechin decreased DNA strand breaks (25–50 μM, 49%, Fig. 3, c1) and the formation of Endo III sensitive sites, but not the formation of Fpg sensitive sites (all the concentrations increased oxidative DNA damage induced by BaP, Fig. 3, c3). The maximum inhibition of Endo III sensitive sites (82%) was found at 10 μM (Fig. 3, c2).

Figure 3.4 shows the protective effect of (−)-epicatechin on NDMA-induced DNA damage in HepG2 cells. (−)-Epicatechin at the lowest concentration (10 μM) showed the maximum reduction of DNA strand breaks (34%), the formation of Endo III (55%) and Fpg sensitive sites (40%) induced by NDMA (Fig. 3, a1–a3). Protection afforded by (−)-epicatechin towards NPYR-induced DNA damage is showed on Fig. 3, b1–b3.

(−)-Epicatechin at the lowest concentration (10 μM) showed the maximum reduction of DNA strand breaks (34%), the formation of Endo III (34%) and Fpg sensitive sites (50%) induced by NPYR. However, (-)-epicatechin (10–50 μM) reduced DNA strand breaks induced by BaP (Fig. 3, c1) in a dose dependent manner (27–39%). The maximum inhibition of the formation of Endo III sensitive sites (63%, Fig. 3, c2) was found at the lowest concentration (10 μM). The formation of Fpg sensitive sites (Fig. 3, c3) was weakly reduced (5–10%) at 25–50 μM.

Discussion

In the present study we evaluate the protective effect of dietary polyphenols against N-nitrosodimethylamine (NDMA), *N*-nitrosopyrrolidine (NPYR) or benzo(a)-pyrene (BaP)-induced DNA damage (strand breaks and

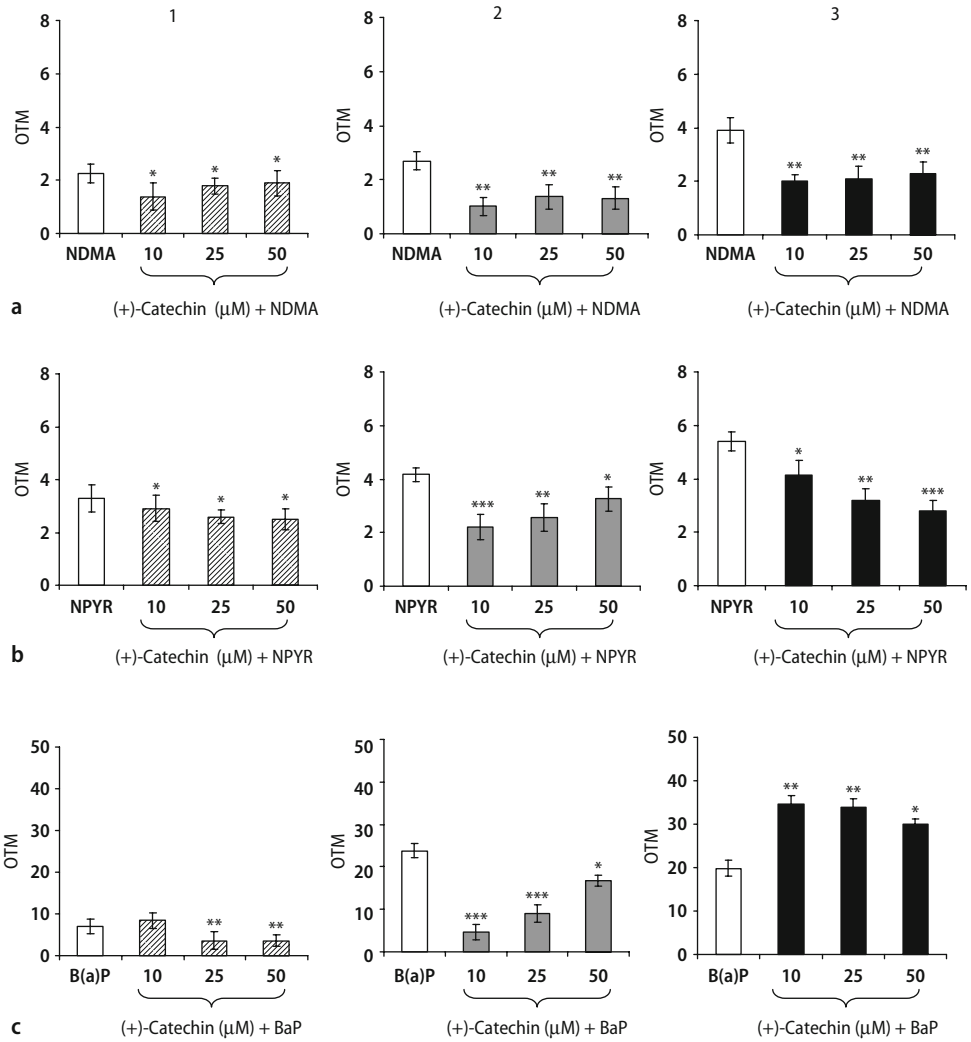

Fig. 3.3 continued

oxidized purines/pyrimidines) in HepG2 cells, using the repair enzymes Fpg and Endo III and the Comet assay. The carcinogenic and mutagenic effects of BaP have been extensively investigated in mammalian and other animal cell systems [14]. Our results showed that BaP (25–100 μM) induced a significant increase of DNA strand breaks in a dose dependent manner. During the metabolic process, BaP produces reactive oxygen species (ROS) via cytochrome P4501A1 (CYP1A1) [3]. These ROS and metabolites can cause oxidative DNA damage and form adducts with DNA. To determine the role of oxidative DNA damage in the observed effects of BaP, we tested whether BaP was able to generate oxidized bases. In the present study we showed that HepG2 cells treated with BaP induced Fpg sensitive sites and Endo III sensitive sites, indicating the presence of oxidized purines and pyrimidines, respectively. This can be further evidence for oxidative DNA damage caused by BaP.

It has been suggested that DNA alkylation and free radical damage are in part involved in the carcinogenic action induced by N-nitrosamines [4]. Strand breaks or alkali labile sites, including abasic sites, may be results of the action of reactive oxygen species that arise during the metabolism of *N*-nitrosamines in the cell. In a previous work we showed that NPYR and NDMA were able to generate oxidized bases [1, 2].

There are a considerable number of reports of anticarcinogenic and antimutagenic effects of polyphenols [15]. However, a significant increase in the consumption of polyphenols may not be without risks [26]. Certain flavonoids, most notably quercetin, are genotoxic in vitro, increasing the frequency of revertants in mutagenicity assays, inducing strand breakage in isolated rat nuclei and increasing chromosomal aberrations in mammalian cells [35]. On the contrary, our results indicate that none of the dietary polyphenols concentrations tested caused DNA strand

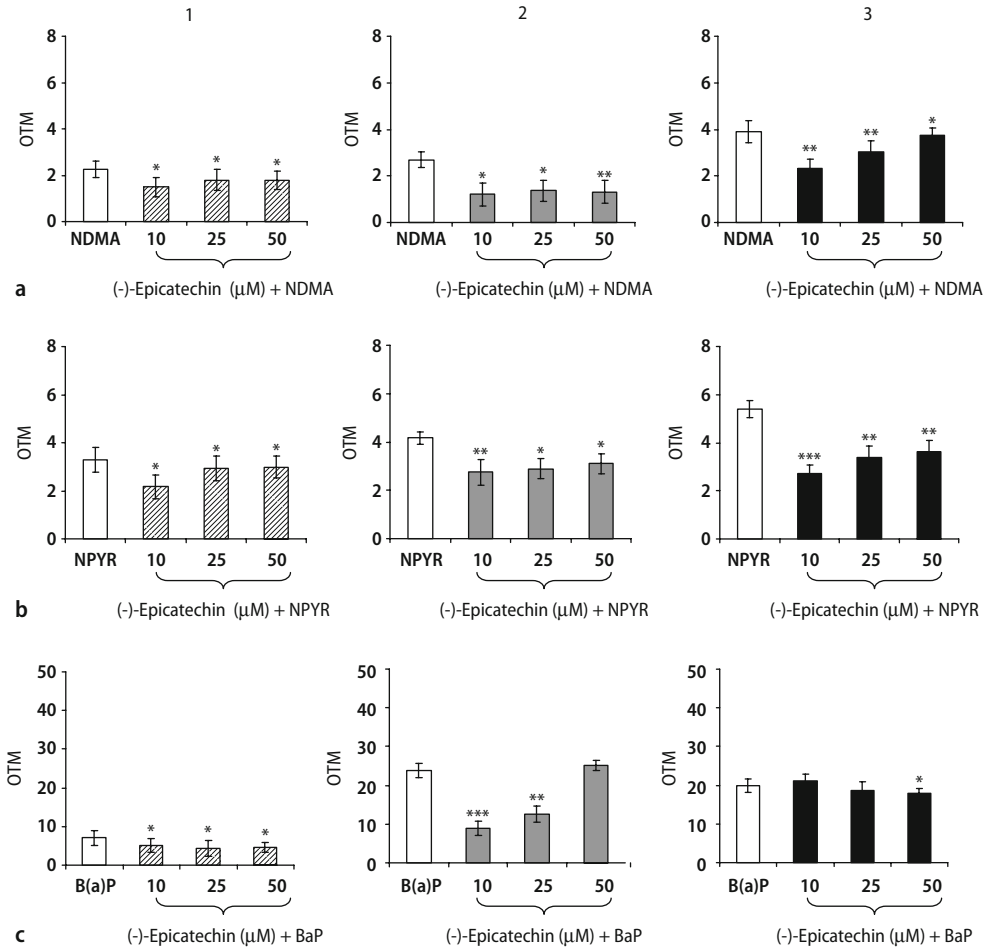

Fig. 3.4 continued

breaks, or oxidized purine or pyrimidine bases per se. These results are in part in agreement with previous reports, which have revealed that some polyphenols such as (+)-catechin, or (−)-epicatechin, were not mutagenic in the Salmonella/microsomal mutagenicity assay using tester strain TA102, while quercetin was found mutagenic [27].

Myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone) and quercetin (3,3′,4′,5,7-pentahydroxyflavone) are two of the most frequently studied flavonoids in the class of flavonols [16]. In the present study, quercetin protected against DNA strand breaks and oxidized pyrimidines induced by BaP, NDMA, and NPYR, while myricetin protected against DNA strand breaks and oxidized pyrimidines induced by BaP and NDMA and towards oxidized pyrimidines and purines induced by NPYR. This variable effect against NPYR is surprising given the similar chemical structure of quercetin and myricetin. This indicates that only an additional hydroxyl group at position 5′ in the chemical structure of myricetin would significantly affect the biological activity against NPYR. In addition, the protective effect of quercetin and myricetin was higher towards BaP than against NDMA and NPYR. Thus, the presence and number of polyhydroxyl groups in the chemical structure and/or the kind of mutagen used to induce DNA damage has an important role in the protective effect. Makena and Chung [27] also reported that the position and number of hydroxyl groups are crucial in the inhibitory effects of polyphenols.

Catechins are also found in a variety of plants and are present in particularly high amounts in tea leaves, such as (+)-catechin and (−)-epicatechin. In the present study (+)-catechin; (+)-*trans*-3,3′,4′,5,7-pentahydroxyflavane, and (−)-epicatechin; (−)-*cis*-3,3′,4′,5,7-pentahydroxy- flavane, reduced DNA strand breaks induced by BaP, NDMA and NPYR. (+)-Catechin and (−)-epicatechin also protected against oxidized purines and pyrimidines induced by NDMA and NPYR, however they only protected against oxidized pyrimidines induced by BaP. Yen et al. [41] reported that (−)-epicatechin had protective effect against BaP-induced DNA damage in Chang

liver cells at concentrations of 10–100 μM. However, Dhawan et al. [8] showed that (+)-catechin and (−)-epicatechin and their gallate esters, cannot afford protection against DNA damage induced in human lymphocytes by heterocyclic amine (Trp-P-2). Our results also indicate that (+)-catechin, with similar chemical structure to (−)-epicatechin, showed a higher protective effect than (−)-epicatechin against DNA damage induced by BaP, or NDMA, while these compounds exhibited a similar level of protection against NPYR. The spatial arrangement of substituents on (+)-catechin and (−)-epicatechin could be more important than the backbone alone in the protective effect and/or the mutagen tested.

Taken together our results, (+)-catechin and (−)-epicatechin showed higher protection against DNA strand breaks induced by BaP, NDMA and NPYR than myricetin or quercetin. In addition, they also protected against oxidized purines and pyrimidines induced by NDMA, NPYR and BaP (with the exception of (+)-catechin that increased oxidative DNA damage induced by BaP). Quercetin and myricetin were the most active flavonoids against oxidized pyrimidines induced by BaP, but not against oxidized purines. On the contrary, they increased oxidative DNA damage induced by BaP. This effect could be attributed to the excess of reactive oxygen species (ROS) produced by BaP. They might cause irreparable oxidative DNA damage [20]. In addition, phenolic compounds have both antioxidant and prooxidant effects depending on the experimental conditions [17]. Therefore, although there is a general structure-activity relationship [21] that shows that some subclasses of flavonoid can be more potent antimutagens, these structural considerations can change depending on the substitution pattern of the molecule and/or the kind of compound used to induce DNA damage.

The protective effect of dietary polyphenols tested in this study, in part, could be due to the free radical scavenging efficiency of these compounds. Flavonoids can inhibit oxidative damage to DNA and therefore, prevent or reduce cellular oxidative stress [22]. According to Cotelle et al. [6], the protective effect of quercetin and myricetin may be associated with the presence of two or three hydroxyl groups in the B ring of its molecule. Ricardo da Silva et al. [34] also reported that (+)-catechin and (−)-epicatechin scavenge superoxide and hydroxyl radicals. Another possibility is that flavonoids, as metal-ion-chelating agents [37], could have chelated iron ions present in the cells and hence depressed the Fenton reaction. Morel et al. [28] investigated the antioxidant and iron chelating activities of (+)-catechin, and quercetin using cell cultures. They showed that both compounds could chelate iron in vitro, and that (+)-catechin was more potent antioxidant than quercetin, which can explain, in part, the protective effect of these flavonoids in the oxidative DNA damage-induced by BaP, NDMA or NPYR.

Other mechanisms proposed to explain this protective effect might also include inhibition of phase I metabolizing enzymes, such as cytochrome P450 (CYP), which metabolically activates procarcinogens to reactive intermediates that trigger carcinogenesis [11]. Flavonoid interactions with CYPs have been reviewed recently [18]. Hatch et al. [19] reported that polyphenols could diminish the production of the active metabolites of the xenobiotic through downregulation of the relevant enzymes, and/or directly interfere with DNA adduct formation. Finally, another mechanism claimed to be responsible for the protective effect of flavonoids is the induction of phase II metabolizing enzymes such as glutathione S-transferase (GST), NAD(P)H:quinine oxidoreductase (NQO) and UDP-glucuronyltransferase (UGT) by which carcinogens are detoxified and therefore more readily eliminated from the body [12].

All the polyphenol used in our work were in μM concentrations and several studies on bioavailability of flavonoids, indicate that they are poorly bioavailable and reach only nanomolar to low micromolar concentrations in plasma [23]. Moreover, since conjugation is a biologic defense mechanism serving the objective of inactivating xenobiotics as well as endogenous bioactive compounds and rendering water-soluble to facilitate their excretion, the presence of free polyphenols as unconjugated compounds from the circulation or the urine following absorption is almost irrelevant [13]. This does not preclude the possibility that flavonoids may accumulate in tissues where they might exert local antioxidant effects or that very low concentrations of flavonoids may modulate cell signalling, gene regulation, angiogenesis, and other biological processes by non-antioxidant mechanisms, which may explain the purported health benefits of flavonoids [29].

In conclusion, our results clearly indicate that polyphenols protect human derived cells against DNA strand breaks and oxidative DNA damage effects of BaP, NDMA and NPYR, three carcinogenic compounds which occur in the environment. Further investigation is in progress to evaluate and better understand the mechanisms by which polyphenols exert their protective effects.

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References

1. Arranz N, Haza AI, García A, Möller L, Rafter J, Morales P (2007) Protective effects of organosulfur compounds towards *N*-nitrosamine-induced DNA damage in the single-cell gel electrophoresis (SCGE)/HepG2 assay. Food Chem Toxicol 45:1662–1669
2. Arranz N, Haza AI, García A, Rafter J, Morales P (2007) Protective effect of-vitamin C towards *N*-nitrosamine-induced DNA damage in the single-cell gel electrophoresis (SCGE)/HepG2 assay. Toxicol in vitro 21:1311–1317
3. Burczynski ME, Penning TM (2000) Genotoxic polycyclic aromatic hydrocarbon *ortho*-quinones generated by aldo-keto reductases induce CYP1A1 via nuclear translocation of the aryl hydrocarbon receptor. Cancer Res 60: 908–915
4. Bartsch H, Hietanen E, Malaveille C (1989) Carcinogenic nitrosamines: free radical aspects of their action. Free Radical Bio Med 7:637–644
5. Collins AR, Duthie SJ, Dobson VL (1993) Direct enzymic detection of endogenous oxidative base damage in human lymphocyte DNA. Carcinogenesis 14:1733–1735
6. Cotelle N, Bernier J, Catteau J, Pommery J, Wallet J, Gaydou EM (1996) Antioxidant properties of hydroxy-flavones. Free Radical Bio Med 20:35–43
7. Doostdar H, Demoz A, Burke MD, Melvin WT, Grant MH (1990) Variation in drug-metabolizing enzyme activities during the growth of human HepG2 hepatoma cells. Xenobiotica 20:435–441
8. Dhawan A, Anderson D, de Pascual-Teresa S, Santos-Buelga C, Clifford MN, Ioannides C (2002) Evaluation of the antigenotoxic potential of monomeric and dimeric flavanols, and black tea polyphenols against heterocyclic amine-induced DNA damage in human lymphocytes using the Comet assay. Mutat Res 515:39–56
9. Filho PJS, Rios A, Valcárcel M, Caramao EB (2003) Development of a new method for the determination of nitrosamines by micellar electrokinetic capillary chromatography. Water Res 37:3837–3842
10. Gray R, Peto R, Branton P, Grasso P (1991) Chronic nitrosamine ingestion in 1,040 rodents—the effect of the choice of Nitrosamine, the species studied, and the age of starting exposure. Cancer Res 51:6470–6491
11. Galati G, O'Brien PJ (2004) Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. Free Radical Bio Med 37:287–303
12. Galati G, Teng S, Moridani MY, Chan TS, O'Brien PJ (2000) Cancer chemoprevention and apoptosis mechanisms induced by dietary polyphenolics. Drug Metabol Drug Interact 17:311–349
13. Goldberg DM, Yan J, Soleas GJ (2003) Absorption of three wine-related polyphenols in three different matrices by healthy subjects. Clin Biochem 36: 79–87
14. Harvey RG (1991) Polycyclic aromatic hydrocarbons: chemistry and carcinogenicity. In: Coombs M (ed) Cambridge University Press, Cambridge
15. Heo MY, Yu KS, Kim KH, Kim HP, Au WW (1992) Anticlastogenic effect of flavonoids against mutagen-induced micronuclei in mice. Mutat Res 284: 243–249
16. Hertog MGL, Feskens EJM, Kromhout D, Hertog MGL, Hollman PCH, Hertog MGL, Katan MB (1993) Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. Lancet 342:1007–1011
17. Hanasaki Y, Ogawa S, Fukui S (1994) The correlation between active oxygen scavenging and antioxidative effects of flavonoids. Free Radical Bio Med 16:845–850
18. Hodek P, Trefil P, Stiborová M (2002) Flavonoids-potent and versatile biologically active compounds interacting with cytochromes P450. Chem Biol Interact 139:1–21
19. Hatch FT, Lightstone FC, Colvin ME (2000) Quantitative structure-activity relationship of flavonoids for inhibition of heterocyclic amine mutagenicity. Environ Mol Mutagen 35:279–299
20. Johnson MK, Loo G (2000) Effects of epigallocatechin gallate and quercetin on oxidative damage to cellular DNA. Mutat Res 459:211–218
21. López-Lazaro M (2002) Flavonoids as anticancer agents: structure–activity relationship Study. Curr Med Chem 2:691–714
22. Lipkin M, Reddy B, Newmark H, Lamprecht SA (1999) Dietary factors in human colorectal cancer. Annu Rev Nutr 19:545–586
23. Lotito SB, Frei B (2006) Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: cause, consequence, or epiphenomenon? Free radical Bio Med 41: 1727–1746
24. Murdok DJL, Barnett YA, Barnett CR (2004) DNA damage and cytotoxicity in pancreatic b-cells expressing human CYP2E. Biochem Pharmacol 68:523–530
25. Maggiolini M, Recchia AG, Bonofiglio D, Catalano S, Vivacqua A, Carpino A, Rago V, Rossi R, Ando S (2005) The red wine phenolics piceatannol and myricetin act as agonists for estrogen receptor alpha in human breast cancer cells. J Mol Endocrinol 35:269–281
26. Mennen LI, Walker R, Bennetau-Pelissero C, Scalbert A (2005) Risks and safety of polyphenol consumption. Am J Clin Nutr 81:326S–329
27. Makena P, Chung K (2007) Effects of various plant polyphenols on bladder carcinogen benzidine-induced mutagenicity. Food Chem Toxicol 45:1899–1909
28. Morel I, Lescoat G, Cogrel P, Sergent O, Pasdeloup N, Brissot P, Cillard P, Cillard J (1993) Antioxidant and iron-chelating activities of the flavonoids catechin, quercetin and diosmetin on iron-loaded rat hepatocyte cultures. Biochem Pharmacol 45:13–19
29. Manach C, Williamson G, Morand C, Scalbert A, Remesy C (2005) Bioavailability and bioefficiency of polyphenols in humans. I. Review of 97 bioavailability studies. Am J Clin Nutr 81: 230S–242S
30. Neuhouser ML (2004) Review: dietary flavonoids and cancer risk: evidence from human population studies. Nutr Cancer 50:1–7
31. Ow YY, Stupans I (2003) Gallic acid and gallic acid derivatives: effects on drug metabolizing enzymes. Curr Drug Metab 4:241–248
32. Olive PL, Wlodek D, Durand RE, Banáth JP (1992) Factors influencing DNA migration from individual cells subjected to gel electrophoresis. Exp Cell Res 198:259–267
33. Preussmann R, Stewart BW (1984) *N*-Nitroso carcinogens. In: Searle CE (ed) Chemical carcinogenesis. ACS Monograph, Washington DC, pp 643–828
34. Ricardo da Silva JM, Rigaud J, Cheynier V, Cheminat A, Moutounet M (1991) Procyanidin dimers and trimers from grape seeds. Phytochemistry 30: 1259–1264
35. Sahu SC, Washington MC (1991) Effects of antioxidants on quercetin-induced nuclear DNA damage and lipid peroxidation. Cancer Lett 60:259–264

36. Ueng Y, Shyu C, Liu T, Oda Y, Liao J, Chen C (2001) Protective effects of baicalein and wogonin against benzo[a]pyrene- and aflatoxin B1-induced genotoxicities. Biochem Pharmacol 62: 1653–1660
37. Williams RJ, Spencer JPE, Rice-Evans C (2004) Flavonoids: antioxidants or signalling molecules? Free Radical Bio Med 36:838–849
38. World Cancer Research Fund (1997) Food, nutrition and the prevention of cancer: a global perspective. Nutrition 15:523–526
39. Yang CS, Smith T, Ishizaki H, Hong JY (1991) Enzyme mechanism in the metabolism of nitrosamines. In: O'Neil IK, Chen J, Bartsh H (eds) Relevance to human cancer of nitroso compounds, tobacco smoke and mycotoxins. IARC, Lyon, pp 567–573
40. Yao LH, Jiang YM, Shi J, Tomas-Barberan FA, Datta N, Singanusong R, Chen SS (2004) Flavonoids in food and their health benefits. Plant Foods Hum Nutr 59:113–122
41. Yen GC, Ju JW, Wu CH (2004) Modulation of tea and tea polyphenols on benzo(a)pyrene-induced DNA damage in Chang liver cells. Free Radical Res 38:193–200