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Use of a Human-Derived Liver Cell Line for the Detection of Protective Effect of Dietary Antioxidants Against Food Mutagens

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1. Introduction

Hepatocellular carcinoma is the most frequent form of primary liver cancer, it is one of the most common life threatening solid tumors with global annual diagnosis exceeding one million new cases and remains the third leading cause of cancer death (Ahmedin et al., 2007). Human diet often contains compounds that cause DNA damage. Common dietary mutagens would include N-nitroso compounds (Tricker & Preussmann, 1991), fungal toxins (Gelderblom et al., 2001), or cooked meat carcinogens (Layton et al., 1995). High nitrate levels in processed foods may be a risk factor, possibly through their ability to form N-nitroso compounds in vivo (Ferguson et al., 2004). N-nitroso compounds are known hepatocarcinogenic agents and have been implicated in the etiology of several human cancers (Bansal et al., 2005). N-Nitrosamines are mutagenic and carcinogenic compounds widely present in the human diet and have been detected at ppb levels in a wide variety of matrices such as bacon, ham, frankfurters, sausages, cheese, beer, rubber, ground water, smoked tobacco and cosmetics (Filho et al., 2003). N-nitrosopiperidine (NPIP) is a potent extrahepatic carcinogen inducing tumours mainly in the esophagus and the nasal cavity (Gray et al., 1991). N-nitrosodibutylamine (NDBA) produces tumours in the esophagus and urinary bladder in rat, although the liver is its major target tissue for carcinogenesis (Williams et al., 1993). N-nitrosopyrrolidine (NPYR) induce mainly liver tumors in rats (Gray et al., 1991) and is a weak pulmonary carcinogen in mice (Wong et al., 2003) and N-nitrosodimethylamine (NDMA) is the most commonly encountered volatile N-nitrosamine in food samples and is a potent liver, lung and kidney carcinogen (Preussmann & Stewart, 1984).

Heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs) are formed during the high-temperature cooking of meat and fish. To date, more than 20 different heterocyclic amines (HCAs) have been identified in cooked foods and they can be classified into two main groups called carbolines and aminoimidazoazaarenes (AIAs) (Toribio et al., 2007). AIAs are formed at the normal cooking temperatures of 100–225°C while heating

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foodstuffs in the presence of creatinine, amino-acids and sugars, involving Maillard reaction (Ristic et al., 2004). Among those, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (8-MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-f]-quinoxaline (4,8-diMeIQx) and 2-amino-1-methyl-6-phenyl-imidazo[4,5-b]pyridine (PhIP) are the most abundant (Skog et al., 1998). Benzo(a)pyrene (BaP), an important PAH, is a potent systemic and local carcinogen known to induce skin, lung, and stomach tumours in animal models (Ueng et al., 2001). NPIP, NDBA, NPYR and HCAs were categorized as Group 2B: possible causative agents in human cancer, while NDMA and B(a)P as Group 2A: probable causative agents in human cancer (IARC, 1993).

N-nitrosamines, HCAs and B(a)P are DNA reactive chemicals that require metabolic activation, usually by various cytochrome P450 (CYP) enzymes for interaction with DNA (Ingelman-Sundberg, 2002). It has been suggested that DNA damage and free radical damage are in part involved in the carcinogenic action induced by N-nitrosamines (Bartsch et al., 1989). 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 food mutagens in the cell. In a previous work we showed that N-nitrosamines (Arranz et al., 2007; García et al., 2008a,b), benzo(a)pyrene (Delgado et al., 2009) and heterocyclic amines (Haza et al., 2011) were able to generate DNA strand breaks and oxidized bases. The increasing appreciation of the importance of food mutagens as potential human carcinogens stimulated intense research on protective dietary factors in chemical carcinogenesis.

Current evidence strongly supports a contribution of polyphenols to the prevention of various diseases associated with oxidative stress, such as cancer and cardiovascular, neurodegenerative and age-related diseases (Kanazawa et al., 2006). Phenolics have been reported to have a capacity to scavenge free radicals (Havsteen, 2002). Gallic acid (3,4,5-trihydroxybenzoic acid, GA) is a polyhydroxyphenolic compound, which can be found in various natural products, like gallnuts, tea leaves, bark, green tea, apple-peels, grapes, strawberries, pineapples, bananas, lemons, and in red and white wine (Madlener, et al., 2007). GA is a strong antioxidant that possesses antimutagenic and anticarcinogenic activities (Inoue, et al., 1994; Stich, et al., 1982) and exerts antiproliferative effects on cancer cells by generating hydrogen peroxide (Lapidot, et al., 2002). It inhibits melanogenesis which may be related to GA's antioxidant activity in scavenging reactive oxygen species (Seo et al., 2003).

Piceatannol (3-hydroxyresveratrol or astringinine, PCA) is a phenolic compound that occurs naturally in grapes and red wine (McDonald et al., 1998). The total amount of PCA in red-grape wine has been reported to be up to 15 mg/l (Cantos et al., 2003), however the biotransformation of the abundant red wine component, resveratrol (*trans*-3,5,4-trihydroxystilbene), contributes to increase PCA concentrations at tissue level (Piver et al., 2004). Both substances are synthesized in plants in response to fungal or other environmental stress, classifying them as phytoalexins. Piceatannol has been identified as the active ingredient of *Melaleuca leucadendron* (white tea tree), *Cassia garretiana* (Asian legume) and *Rheum undulatum* (Korean rhubarb), which are used in traditional herbal medicine (Tsuruga, et al., 1991; Matsuda, et al., 2000) and as the antileukemic compound in the seeds of *Euphorbia lagascae*, which is used in folk medicine to treat cancer, tumors and warts (Ferrigni et al., 1984). Teguo et al. 2001 also detected piceatannol in cell suspension cultures of *Vitis vinifera* (wine grapes).

Several studies established the single cell gel electrophoresis (SCGE) or Comet assay as a suitable method for assessing the ability of phytochemicals to protect cells against genotoxic effect of several xenobiotics (Collins, 2005). 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 (Figure 1).

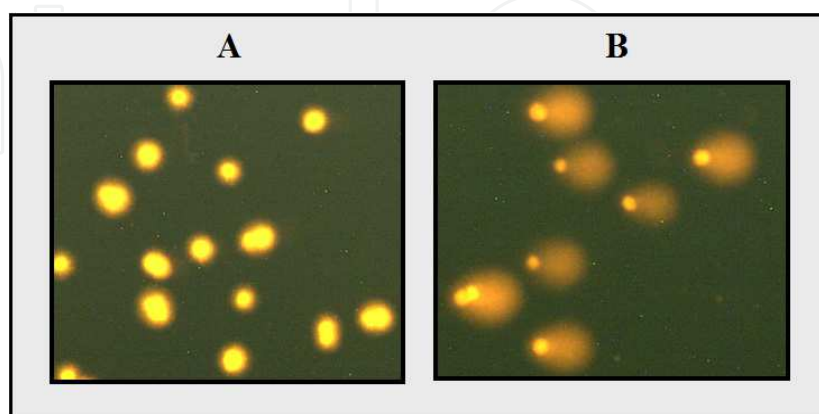


Fig. 1. HepG2 cells untreated (A) and treated (B) with N-nitrosopyrrolidine (NPYR) and incubated with Fpg enzyme, visualized under fluorescence microscopy and using comet assay.

As part of our program to evaluate the protective effects of dietary polyphenols with different chemical structure (previously we have evaluated, flavonols and flavanols) we sought to determine whether gallic acid (as representative of phenolic acids) or piceatannol (stilbenes) could protect human hepatoma cells (HepG2) from oxidative DNA damage induced by food mutagens.

2. Material and methods

2.1 Chemicals

Gallic acid (GA), piceatannol (PCA) and food mutagens used in this study are shown in Figures 2 and 3. N-nitrosodimethylamine (NDMA), N-nitrosodibutylamine (NDBA), N-nitrosopyrrolidine (NPYR), N-nitrosopiperidine (NPIP), benzo(a)pyrene (BaP), dimethyl sulfoxide (DMSO) and low melting point agarose (LMP) were purchased from Sigma-Aldrich (St. Louis, MO). 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (8-MeIQx), 2-amino-3,4,8-trimethyl-3H-imidazo[4,5-f]quinoxaline (4,8-diMeIQx) and 2-amino-1-methyl-6-phenyl- imidazo[4,5-b]pyridine (PhIP), were purchased from Toronto Research Chemicals Inc. (North York, On. Canada). 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. Food carcinogens and polyphenols were dissolved in sterile DMSO. The stock solutions were stored deep frozen (-80°C).

2.2 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% CO₂ atmosphere.

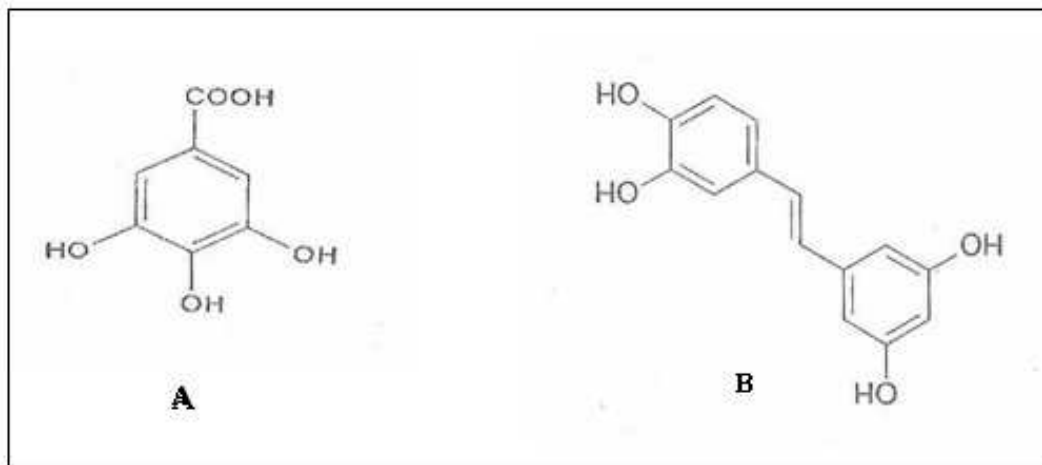


Fig. 2. Chemical structures of gallic acid (A) and piceatannol (B).

2.3 Analysis of DNA damage (strand breaks and oxidized purines/pyrimidines) induced by galic acid or piceatannol 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 galic acid and piceatannol. The SCGE assay was carried out according to the protocol of Olive et al. (1992) with minor modifications.

Briefly, HepG2 cells were plated on to multiwell systems at a density of 1.5×10^5 cells/ml culture medium. 24 hr after seeding, cells were exposed to galic acid (0.1-5 µM), or piceatannol (0.1-5 µM) or solvent, for another 24 h at 37 °C and 5% CO₂. The solvent concentration in the incubation medium never exceeds 0.1%. After treatments, 10µl of a suspension of 1×10^5 cells were mixed with 70µl 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 other slides to be treated with Fpg or Endo III. After solidification, the cells were lysed in darkness for 1 hour in a high salt alkaline buffer (2.5M NaCl, 0.1M EDTA, 0.01M Tris, 1% Triton X-100, pH 10). The slides were then equilibrated 3x5 minutes in enzyme buffer (0.04M HEPES, 0.1M of Fpg or Endo III at 1µg/ml in enzyme buffer for 30 min at 37°C in a humid dark chamber. Control slides were incubated with 30µl of enzyme buffer only. Following enzyme treatment, the slides were placed in electrophoresis buffer (0.3M NaOH, 1mM 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 25V. After electrophoresis, the slides were neutralized using 0.4M Tris pH 7.5 and KCl, 0.5mM EDTA, 0.2 mg/ml BSA, pH 8). After this time, slides were incubated with 30µl fixed in methanol. Subsequently, the DNA was stained

with ethidium bromide (10 µg/ml) in Tris acetate EDTA (TAE 1X) during 5 minutes and examined in a fluorescence microscope (OLYMPUS BH-2) connected to a computerized image analysis system (Comet Score 5.5). Olive tail moment (OTM) as defined by Olive et al. (1992) 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.

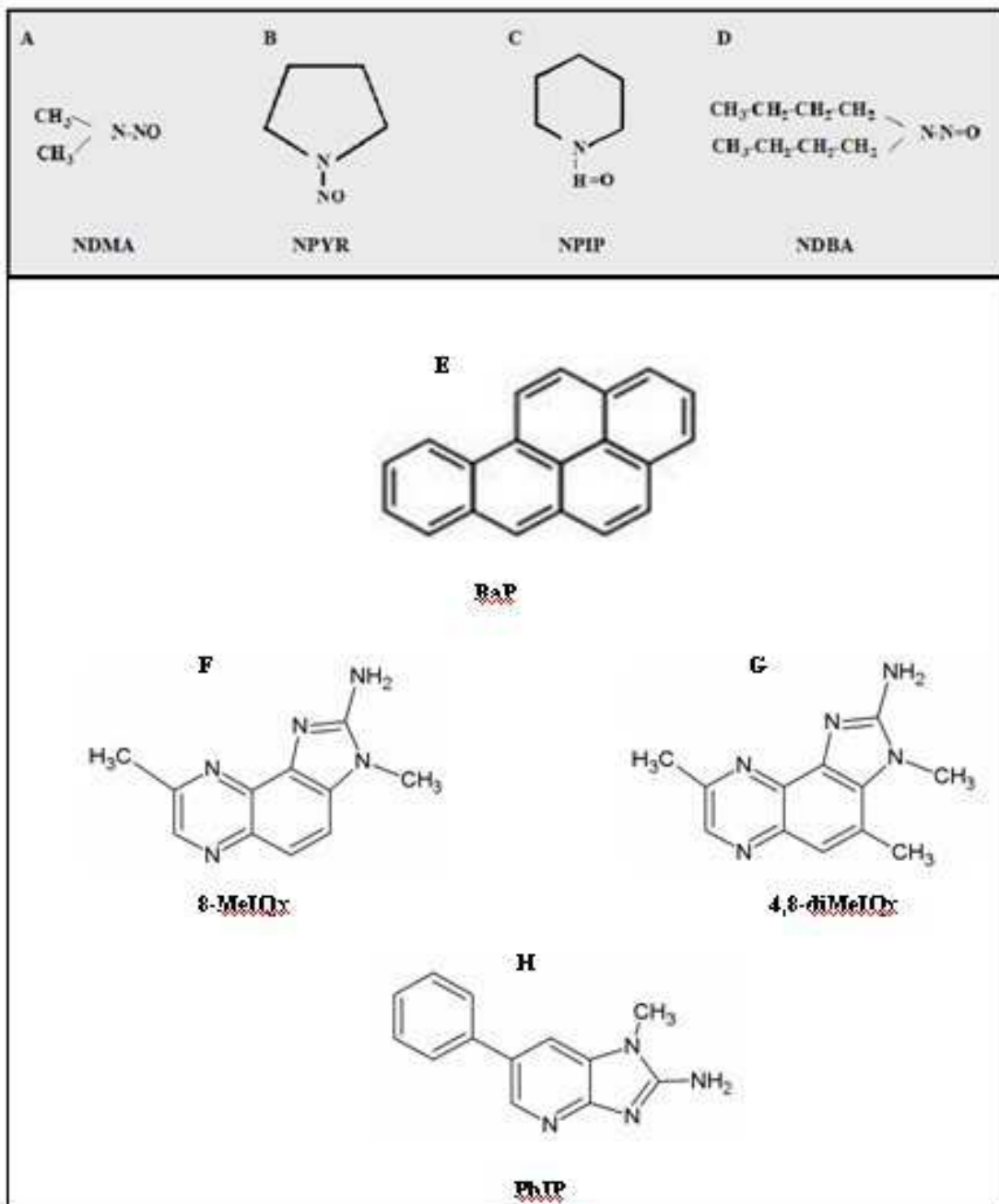


Fig. 3. Chemical structures of food mutagens used in this study: (A) N-nitrosodimethylamine (NDMA), (B) N-nitrosopyrrolidine (NPYR), (C) N-nitrosopiperidine (NPIP), (D) N-nitrosodibutylamine (NDBA), (E) benzo(a)pyrene (BaP), (F) 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (8-MeIQx), (G) 2-amino-3,4,8-trimethyl-3H-imidazo[4,5-f]quinoxaline (4,8-diMeIQx) and (H) 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP).

2.4 Analysis of DNA damage (strand breaks and oxidized purines/pyrimidines) induced by a simultaneous treatment of food mutagens and galic acid or piceatannol in the Alkaline Comet assay

Induction of DNA damage (strand breaks and oxidative DNA damage) by NDMA, NPYR (Arranz et al., 2007) NPIP, NDBA (García et al., 2008), B(a)P (Delgado et al., 2008), 8-MeIQx, 4,8-diMeIQx and PhIP (Haza and Morales, 2010) have been previously evaluated by our laboratory. HepG2 cells were plated on to multiwell systems at a density of 1.5×10^5 cells/ml culture medium. 24 h after seeding, the corresponding galic acid or piceatannol concentrations were added to the wells and plates were incubated for 24hr at 37°C and 5% CO₂. After incubation, cells were simultaneously treated with the concentrations of food mutagens that caused a significant increased on DNA damage and previously evaluated by our laboratory. NPYR (50mM without enzymes and 5mM with EndoIII or Fpg enzymes), NDMA (135mM without enzymes and 27mM with EndoIII or Fpg enzymes), NDBA (3 mM), NPIP (44 mM), BaP (50 µM), Me IQx (500 µM), 4,8-diMeIQx (200 µM) or PhIP (300 µM), and different concentrations of galic acid (0.1-5µM) or piceatannol (0.1-5µM) for another 24 hours at 37°C and 5% CO₂. After the treatments, the cells were processed as described above (Figure 4.)

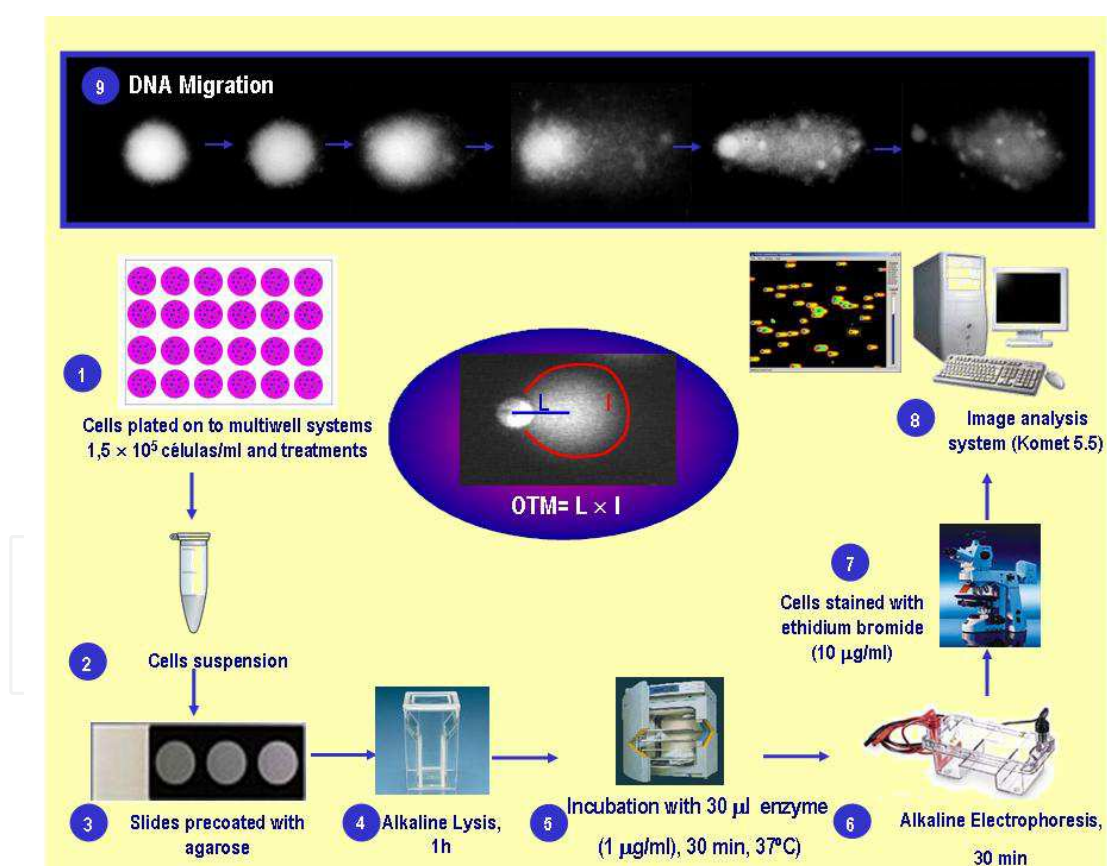


Fig. 4. Comet assay procedure.

2.5 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 \pm standard deviation (S.D.) of three

independent experiments. Thus, we compare three means of OTM from 3 different experiments. Cultures without N-nitrosamines or polyphenols were considered as negative controls. In all experiments the following negative controls have been included: cells treated with solvents and treated without enzymes, cells incubated with Endo III and cells incubated with Fpg. Induction of DNA damage by N-nitrosamines 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 $p \leq 0.05$.

3. Results

3.1 DNA damage (strand breaks and oxidized purines/pyrimidines) induced by galic acid or piceatannol in the Alkaline Comet assay

No cytotoxicity has been previously found at the concentrations of galic acid or piceatannol tested (data not shown). Cell viability was always above 80% of control viability. At non-cytotoxic concentrations (0.1-5 μ M) piceatannol and galic acid did not induce DNA strand breaks and oxidative DNA damage (Table 1). For this reason this concentration range was used in subsequent studies. DNA damage was not measured at cytotoxic concentrations (> 5 μ M) because under these conditions DNA damage is caused as a consequence of necrosis or apoptosis (Henderson et al., 1998).

Compound	Concentration (μ M)	DNA damage (OTM) ^b		
		DNA strand breaks	Endo III	Fpg
Control ^a	0	1.3 \pm 0.3	1.4 \pm 0.2	1.9 \pm 0.5
GA	0.1	1.1 \pm 0.4	1.2 \pm 0.8	2.1 \pm 0.6
	1.0	1.3 \pm 0.6	1.2 \pm 0.4	1.8 \pm 0.3
	5.0	1.4 \pm 0.3	1.4 \pm 0.7	1.6 \pm 0.5
PCA	0.1	1.3 \pm 0.6	1.2 \pm 0.6	2.1 \pm 0.7
	1.0	1.4 \pm 0.4	1.3 \pm 0.8	1.8 \pm 0.8
	5.0	1.5 \pm 0.3	1.5 \pm 0.7	1.8 \pm 0.7

^a OTM median values in control cells without enzymes or incubated with Endo III or Fpg
^b OTM expressed as arbitrary units. The mean DNA damage were calculated from the respective values of three independent experiments.

Table 1. Effect of different concentrations of GA and PCA on DNA strand breaks and on the formation of Endo III and Fpg sensitive sites of human hepatoma cells.

3.2 DNA damage induction by simultaneous treatment of food carcinogens and galic acid or piceatannol in the Alkaline Comet assay

Protection afforded by piceatannol and gallic acid towards NDBA and NPIP-induced oxidative DNA damage was shown in Table 2. No protective effect was shown by piceatannol and gallic acid against NDBA or NPIP-induced DNA strand breaks in HepG2 cells. Gallic acid, but not piceatannol, weakly reduced the Endo III sensitive sites induced by NDBA (28.5%, 0.1 μ M). However, piceatannol reduced the NPIP-induced Endo III sensitive sites at all concentrations tested (28-36%, 0.1-5 μ M) and no effect was shown by

gallic acid. The maximum reduction of Fpg sensitive sites induced by NDBA was found at the highest concentration of piceatannol (5 μ M, 56%). However, the maximum reduction of Fpg sensitive sites induced by NPIP was at the lowest concentration (0.1 μ M, 34.2%). Gallic acid only exerted its protective effect against NPIP-induced Fpg sensitive sites (42.1-23.6%, 0.1-5 μ M).

Compound	Concentration (μ M)	DNA damage (OTM) ^a		
		DNA strand breaks	Endo III	Fpg
NDBA		1.8 \pm 0.4	2.1 \pm 0.3	2.5 \pm 0.2
GA	0.1	1.5 \pm 0.6	1.5 \pm 0.4* (28.5)	2.4 \pm 0.6
	1.0	1.5 \pm 0.4	1.8 \pm 0.4	3.0 \pm 0.3
	5.0	1.6 \pm 0.3	1.9 \pm 0.5	3.0 \pm 0.5
PCA	0.1	1.7 \pm 0.6	1.9 \pm 0.6	3.0 \pm 0.4
	1.0	1.6 \pm 0.5	1.8 \pm 0.5	2.0 \pm 0.3* (20.0)
	5.0	1.8 \pm 0.3	1.9 \pm 0.7	1.1 \pm 0.5** (56.0)
NPIP		1.7 \pm 0.4	2.5 \pm 0.6	3.8 \pm 0.4
GA	0.1	1.7 \pm 0.3	2.3 \pm 0.5	2.2 \pm 0.4** (42.1)
	1.0	1.7 \pm 0.4	2.3 \pm 0.3	2.5 \pm 0.2** (34.1)
	5.0	1.9 \pm 0.4	2.3 \pm 0.5	2.9 \pm 0.4** (23.6)
PCA	0.1	1.7 \pm 0.6	1.8 \pm 0.5** (28.0)	2.5 \pm 0.3** (34.2)
	1.0	1.6 \pm 0.5	1.8 \pm 0.5** (28.0)	2.9 \pm 0.5** (23.6)
	5.0	1.8 \pm 0.3	1.6 \pm 0.6** (36.0)	3.0 \pm 0.6* (21.0)

^a OTM expressed as arbitrary units. The mean DNA damage were calculated from the respective values of three independent experiments. The values in parentheses (%) were defined as the percentage of protection of NDBA or NPIP-induced DNA damage by tested compounds. Asterisks indicate significant difference from control ** P \leq 0.01, * P \leq 0.05.

Table 2. Protective effect of GA and PCA on DNA strand breaks, the formation of Endo III and Fpg sensitive sites of human hepatoma cells induced by 3 mM of NDBA and 44 mM of NPIP.

Table 3 shows the effect of piceatannol and gallic acid against NPYR and NDMA-induced oxidative DNA damage. Results revealed that piceatannol at the lowest concentration reduced the DNA strand breaks induced by NPYR and NDMA (0.1 μ M, 32.2% and 47.6%, respectively). On the contrary, gallic acid did not shown any protective effect against NPYR or NDMA-induced DNA strand breaks. The formation of Endo III sensitive sites induced by NPYR was prevented only by piceatannol at all the concentrations (0.1-5 μ M, 12.5-25%), whereas, both compounds, PCA (0.1-5 μ M, 30.7-19.2%) and GA (0.1-1 μ M, 23%) protected against the formation of Endo III sensitive sites induce by NDMA, respectively. On the other hand, the formation of Fpg sensitive sites induced by NPYR and NDMA were reduced by PCA and GA. At a dose of 0.1 μ M, PCA exhibited the maximum reduction (38.8%) on Fpg sensitive sites induced by NPYR, whereas GA exhibited it at 5.0 μ M (18.5%). PCA and GA also reduced the formation of Fpg sensitive sites induced by NDMA at concentrations of 0.1-1 μ M, respectively (30.9% and 23.8-14.2%).

The effect of piceatannol and gallic acid against BaP and PhIP-induced oxidative DNA damage was shown in **Table 4**.

Compound	Concentration (μM)	DNA damage (OTM) ^a		
		DNA strand breaks	Endo III	Fpg
NDMA		2.1 \pm 0.6	2.6 \pm 0.2	4.2 \pm 0.5
GA	0.1	2.5 \pm 0.5	2.0 \pm 0.3** (23.0)	3.2 \pm 0.2** (23.8)
	1.0	2.2 \pm 0.6	2.0 \pm 0.4** (23.0)	3.6 \pm 0.4* (14.2)
	5.0	2.1 \pm 0.3	2.5 \pm 0.5	4.9 \pm 0.7
PCA	0.1	1.1 \pm 0.6** (47.6)	1.8 \pm 0.4** (30.7)	2.9 \pm 0.4** (30.9)
	1.0	2.0 \pm 0.4	2.0 \pm 0.4** (23.0)	2.9 \pm 0.3** (30.9)
	5.0	2.1 \pm 0.5	2.1 \pm 0.6** (19.2)	4.3 \pm 0.6
NPYR		3.1 \pm 0.5	4.0 \pm 0.5	5.4 \pm 0.4
GA	0.1	3.2 \pm 0.3	4.0 \pm 0.3	4.8 \pm 0.4* (11.1)
	1.0	3.4 \pm 0.5	4.8 \pm 0.4	4.5 \pm 0.2** (16.6)
	5.0	3.4 \pm 0.3	5.6 \pm 0.5	4.4 \pm 0.4** (18.5)
PCA	0.1	2.1 \pm 0.3** (32.2)	3.5 \pm 0.1** (12.5)	3.3 \pm 0.3** (38.8)
	1.0	3.3 \pm 0.3	3.2 \pm 0.3** (20.0)	4.0 \pm 0.4** (25.9)
	5.0	4.1 \pm 0.5	3.0 \pm 0.3** (25.0)	6.1 \pm 0.7

^a OTM expressed as arbitrary units. The mean DNA damage were calculated from the respective values of three independent experiments. The values in parentheses (%) were defined as the percentage of protection of NDMA or NPYR-induced DNA damage by tested compounds. Asterisks indicate significant difference from control ** $P \leq 0.01$, * $P \leq 0.05$.

Table 3. Protective effect of GA and PCA on DNA strand breaks, the formation of Endo III and Fpg sensitive sites of human hepatoma cells induced by NDMA (135 mM without enzymes and 27 mM with Endo III or Fpg enzymes) and NPYR (50 mM without enzymes and 5 mM with Endo III or Fpg enzymes).

Piceatannol protected against DNA strand breaks induced by BaP and PhIP at all concentrations tested (0.1-5 μM , 60.0-65.3% and 34.7-12.5%, respectively). However, gallic acid only exerted protection against BaP-induced DNA strand breaks at the highest concentrations (1-5 μM , 18.-36%). An important decrease of the formation of BaP-induced Endo III sensitive sites was also shown by piceatannol at the lowest concentration (0.1 μM , 60.5%), whereas gallic acid drastically reduced the formation of Endo III sensitive sites at all the concentrations tested (0.1-5 μM , 79.6-63.9%). On the other hand, only gallic acid showed a weakly protective effect against the PhIP-induced Fpg sensitive sites (0.1-1 μM , 17.7%).

The protective effect of gallic acid and piceatannol against MeIQx and diMeIQx-induced oxidative DNA damage was shown in **Table 5**. No effect was shown by piceatannol and gallic acid against DNA strand breaks and the formation of Endo III sensitive sites induced by MeIQx and diMeIQx. Fpg sensitive sites induced by MeIQx (41.1-31.3%) and diMeIQx (41.1-23.5%) were prevented by gallic acid at all the concentrations tested (0.1-5 μM). However, piceatannol only reduced the formation of Fpg sensitive sites at the lowest concentrations (MeIQx, 25.4% and diMeIQx, 27.4%).

Compound	Concentration (μ M)	DNA damage (OTM) ^a		
		DNA strand breaks	Endo III	Fpg
BaP	50	15.0 \pm 0.5	26.1 \pm 0.4	21.2 \pm 0.7
GA	0.1	15.2 \pm 0.7	5.3 \pm 0.3*** (79.6)	32.1 \pm 0.2
	1.0	12.3 \pm 0.6** (18.0)	5.2 \pm 0.4*** (80.0)	34.2 \pm 0.7
	5.0	9.6 \pm 0.6** (36.0)	9.4 \pm 0.5*** (63.9)	34.2 \pm 0.7
PCA	0.1	6.0 \pm 0.6*** (60.0)	10.3 \pm 0.4** (60.5)	32.3 \pm 0.7
	1.0	6.1 \pm 0.5*** (59.3)	18.6 \pm 0.4** (28.7)	34.2 \pm 0.6
	5.0	5.2 \pm 0.5*** (65.3)	19.1 \pm 0.6** (26.8)	39.3 \pm 0.6
PhIP	300	3.2 \pm 0.5	3.9 \pm 0.7	4.5 \pm 0.7
GA	0.1	3.3 \pm 0.6	3.9 \pm 0.3	3.7 \pm 0.3* (17.7)
	1.0	3.0 \pm 0.5	4.3 \pm 0.7	3.7 \pm 0.4* (17.7)
	5.0	3.0 \pm 0.6	4.5 \pm 0.7	4.2 \pm 0.4
PCA	0.1	2.1 \pm 0.4* (34.7)	3.0 \pm 0.2* (23.0)	4.0 \pm 0.5
	1.0	2.3 \pm 0.3* (28.1)	2.8 \pm 0.4** (28.2)	4.2 \pm 0.5
	5.0	2.8 \pm 0.5* (12.5)	2.6 \pm 0.4** (33.3)	5.8 \pm 0.4

^a OTM expressed as arbitrary units. The mean DNA damage were calculated from the respective values of three independent experiments. The values in parentheses (%) were defined as the percentage of protection of BaP or PhIP-induced DNA damage by tested compounds. Asterisks indicate significant difference from control *** P \leq 0.001, ** P \leq 0.01, * P \leq 0.05.

Table 4. Protective effect of GA and PCA on DNA strand breaks, the formation of Endo III and Fpg sensitive sites of human hepatoma cells induced by BaP and PhIP.

4. Discussion

The aim of the present study was to evaluate the protective effect of gallic acid and piceatannol towards food mutagens-induced oxidative DNA damage (strand breaks and oxidized purines/pyrimidines) in human hepatoma cells (HepG2), using the single-cell gel electrophoresis (SCGE) assay. There is speculation that oxidative DNA damaged is involved in cancer development (Mastaloudis et al., 2004). Otherwise, metabolism of food mutagens in cells could generate reactive oxygen species that arise in DNA strand breaks or alkali labile sites, including abasic sites. Previous studies in our research group showed that NDBA, NPIP (García et al., 2008), NDMA and NPYR (Arranz et al., 2007) were able to generate oxidized bases. In addition, during the metabolic process, BaP also produces reactive oxygen species (ROS) via cytochrome P4501A1 (Burczynski and Penning 2000). In a previous investigation we also showed that HepG2 cells treated with BaP or HCAs induced Fpg and Endo III sensitive sites, indicating the presence of oxidized purines and pyrimidines, respectively (Delgado et al., 2009, Haza et al., 2010).

Polyphenols have an important activity as antioxidants and also a remarkable role on carcinogen activation in vivo and on carcinogenesis (Dolara et al., 2005; Lambert et al., 2005). However, some flavonoids such as gallic acid, piceatannol, resveratrol, quercetin and myricetin showed cytotoxicity for a number of cell lines or even induced oxidative DNA strand breakage in human lymphocytes or in HepG2 cells at concentrations higher than 100

μM (Hadi et al., 2007; Johnson and Loo, 2000). The mechanism by which these flavonoids induce DNA damage at higher concentrations might be due to the pro-oxidant properties of these compounds (Wu et al., 2004). Thus, it is important to evaluate whether the adverse effect of GA and PCA on DNA in human hepatoma cells, as shown in Table 1. Our results indicate that none of the dietary polyphenols (GA, PCA) concentrations tested (0.1-5 μM) caused DNA strand breaks, or oxidized purine or pyrimidine bases per se in HepG2 cells (Table 1), although at concentrations higher than 5 μM induced DNA strand breaks and oxidative DNA damage in HepG2 cells (data not shown). Approximately, people in the United States ingest each day 1g of tannic acid (TA) (Sanyal et al., 1997). As one of the food additives, TA is probably hydrolyzed in the acidic pH in the stomach, releasing the 10 potentially reactive GA residues (Brune et al., 1989). GA concentration in the stomach could achieve a maximum of 1.5mmol/L. GA from tablets and tea was rapidly absorbed, but the highest GA concentration observed in plasma was only 1.83 $\mu\text{mol/L}$ and 2.09 $\mu\text{mol/L}$, respectively (Shahzad et al., 2001). Thus, considering the uptake of hydrolysable TA, the concentrations of GA used in this study would not be absurd.

In the present study, we observed that, GA was less efficient than piceatannol to reduce DNA damage induced by food mutagens tested. The presence of three or four hydroxyl groups present in GA and PCA respectively, results in differing protective effects against food mutagens.

Compound	Concentration (μM)	DNA damage (OTM) ^a		
		DNA strand breaks	Endo III	Fpg
MeIQx	500	3.9 ± 0.6	5.0 ± 0.2	5.1 ± 0.5
GA	0.1	3.8 ± 0.3	5.0 ± 0.4	3.0 ± 0.2** (41.1)
	1.0	3.8 ± 0.4	5.4 ± 0.4	3.2 ± 0.3** (37.2)
	5.0	3.7 ± 0.4	6.0 ± 0.5	3.5 ± 0.6** (31.3)
PCA	0.1	3.8 ± 0.4	5.9 ± 0.4	3.8 ± 0.1** (25.4)
	1.0	4.0 ± 0.3	5.1 ± 0.5	4.2 ± 0.4** (17.6)
	5.0	4.2 ± 0.5	5.0 ± 0.6	6.1 ± 0.6
diMeIQx	200	3.9 ± 0.2	4.8 ± 0.5	5.1 ± 0.5
GA	0.1	3.9 ± 0.4	4.6 ± 0.5	3.0 ± 0.3** (41.1)
	1.0	3.8 ± 0.6	5.4 ± 0.6	3.5 ± 0.3** (31.3)
	5.0	3.9 ± 0.5	6.2 ± 0.5	3.9 ± 0.5** (23.5)
PCA	0.1	3.8 ± 0.4	5.8 ± 0.3	3.7 ± 0.4** (27.4)
	1.0	4.5 ± 0.3	5.4 ± 0.3	4.8 ± 0.3
	5.0	5.1 ± 0.6	5.0 ± 0.5	6.3 ± 0.5

^a OTM expressed as arbitrary units. The mean DNA damage were derived from three independent experiments. The values in parentheses (%) were defined as the percentage of protection of MeIQx or diMeIQx-induced DNA damage by tested compounds. Asterisks indicate significant difference from control, ** P ≤ 0.01.

Table 5. Protective effect of GA and PCA on DNA strand breaks, the formation of Endo III and Fpg sensitive sites of human hepatoma cells induced by MeIQx and diMeIQx.

Our results revealed that GA only prevented the DNA strand breaks induced by BaP. However, it protected cells against oxidative DNA damage-induced by food mutagens. GA

removed oxidized pyrimidines induced by NDBA and BaP and oxidized purines induced by NDMA, NPYR, NPIP, MeIQx, diMeIQx and PhIP. This may be due to the antioxidant property of GA (Gali et al., 1992). Phenolic hydroxyl groups are known to be potent in scavenging free radicals and the OH group at the para position to the carboxylic group is especially effectual for the antioxidant activity (Son and Lewis, 2002). Thus the three hydroxyl groups present in GA may be responsible for its antioxidant activity. Although, Lu et al (2006) showed that the protective effect of GA derivatives seemed to depend more on their molecular polarities rather than antioxidant activities.

Our results also showed that DNA strand breaks induced by NDMA, NPYR, BaP and PhIP were reduced by PCA. In addition, PCA also removed the oxidized pyrimidines induced by NDMA, NPYR, NPIP, BaP and PhIP and the oxidized purines induced by NDMA, NPYR, NDBA, NPIP, MeIQx and diMeIQx. Supporting that, an additional hydroxyl group in the chemical structure of the PCA would significantly affect the biological activity against the mutagen. Shahidi and Wanasundara (1992) and Makena and Chung (2007) reported that the position and number of hydroxyl groups are crucial in the inhibitory effects of polyphenols. Moreover, it has been found that the biological activity of trans-Resveratrol (t-RES) and its analogues (PCA) significantly depends on the structural determinants, which are i) the number and position of hydroxyl groups (Wolter et al., 2002) ii) intramolecular hydrogen bonding (Fang et al., 2002), iii) stereoisomery and iv) double bond (Wright et al., 2001). Thus, the protective effect of GA and PCA against food mutagens may vary with the structure and dose of the individual compounds and the mutagenic compound.

Taking together our results PCA was more efficient against DNA strand breaks induced by NDMA, NPYR, BaP and PhIP than GA. GA only prevent DNA strand breaks induced by BaP. Comparing the protective effect of both compounds against BaP, PCA showed higher protective effect (60-65%) than GA (18-36%). In addition PCA was also the most active against oxidized pyrimidines induced by NDMA, NPYR, NPIP, BaP and PhIP. GA only showed reduction of the oxidized pyrimidines induced by NDMA and BaP. This reduction was higher (23%, NDMA and 80-63%, BaP) than the observed by PCA against these two compounds. On the contrary, we also showed that GA and PCA at all concentrations increased oxidized purines 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 (Johnson and Loo, 2000). In addition, phenolic compounds have both antioxidant and prooxidant effects depending on the experimental conditions [28]. However, GA and PCA were very efficient to remove oxidized purines induced by all the mutagens tested with the exception of NDBA (GA) and PhIP (PCA). Therefore, although there is a general structure-activity relationship (López-Lázaro, 2002) that shows that some subclasses of polyphenols 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.

5. Conclusion

Our results indicate that, PCA was more efficient than GA to reduce DNA damage induced by food mutagens tested. PCA at the concentrations tested protect human hepatoma derived cells against DNA strand breaks induced by NDMA, NPYR, BaP and PhIP, oxidized pyrimidines induced by NDMA, NPYR, NPIP, BaP, PhIP and oxidized purines induced by

NDBA, NDMA, NPYR, NPIP, MeIQx and diMeIQx. However, GA at the concentrations tested only protects human hepatoma cells against DNA strand breaks induced by BaP, oxidized pyrimidines induced by NDMA and BaP and oxidized purines induced by NDMA, NPYR, NPIP, PhIP, MeIQx and diMeIQx.

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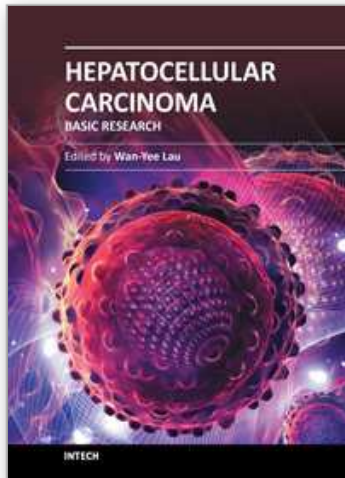
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Hepatocellular Carcinoma represents a leading cause of cancer death and a major health problem in developing countries where hepatitis B infection is prevalent. It has also become increasingly important with the increase in hepatitis C infection in developed countries. Knowledge of hepatocellular carcinoma has progressed rapidly. This book is a compendium of papers written by experts to present the most up-to-date knowledge on hepatocellular carcinoma. This book deals mainly with the basic research aspect of hepatocellular carcinoma. The book is divided into three sections: (I) Biomarkers / Therapeutic Target; (II) Carcinogenesis / Invasion / Metastasis; and (III) Detection / Prevention / Prevalence. There are 18 chapters in this book. This book is an important contribution to the basic research of hepatocellular carcinoma. The intended readers of this book are scientists and clinicians who are interested in research on hepatocellular carcinoma. Epidemiologists, pathologists, hospital administrators and drug manufacturers will also find this book useful.

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