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ORIGINAL CONTRIBUTION

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Effect of complex polyphenols and tannins from red wine on DNA oxidative damage of rat colon mucosa *in vivo*

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Summary *Background:* Dietary polyphenols have been reported to have a variety of biological actions, including anti-carcinogenic, antioxidant and anti-inflammatory activities.

Aim of the study: In the present study we have evaluated the effect of an oral treatment with complex polyphenols and tannins from red wine and tea on DNA oxidative damage in the rat colon mucosa.

Methods: Isolated colonocytes were prepared from the colon mucosa of rats treated for ten days with either wine complex polyphenols (57.2 mg/kg/d) or thearubigin (40 mg/kg/d) by oral gavage. Colonocyte oxidative DNA damage was analysed at the single cell level using a modification of the comet assay technique.

Results: The results show that wine complex polyphenols and tannins induce a significant decrease (–62 % for pyrimidine and –57 % for purine oxidation) in basal DNA oxidative damage in colon mucosal cells without affecting the basal level of single-strand breaks. On the other hand, tea polyphenols, namely a crude extract of thearubigin, did not affect either strand breaks or pyrimidine oxidation in colon mucosal cells.

Conclusions Our experiments are the first demonstration that dietary polyphenols can modulate *in vivo* oxidative damage in the gastrointestinal tract of rodents. These data support the hypothesis that dietary polyphenols might have both a protective and a therapeutic potential in oxidative damage-related pathologies.

Key words Antioxidant – oxidative stress – flavonoids – comet assay – isolated colonocytes

Introduction

Polyphenolic compounds are a wide class of plant-derived molecules that are found in high concentrations in fruits, vegetables and beverages such as wine and tea (1). Several epidemiological studies have reported a negative correlation between the intake of flavonoid polyphenols and the incidence of cardiovascular diseases and cancer (2, 3). Colon cancer is one of the most prevalent neoplastic diseases in the developed world (4) and animal studies indicate that polyphenolic compounds from wine and tea *may* exert a protective action in experimental colon carcinogenesis (5–8).

Polyphenols possess a broad spectrum of biological activities, including anti-inflammatory (9, 10), anti-oxidative (11), anti-mutagenic (12) and anti-angiogenetic (13) actions that could all contribute to the anti-carcinogenic effect.

The antioxidant activity of monomeric polyphenols has been repeatedly demonstrated. The major monomeric phenols from green tea and wine, i. e. epigallocatechin gallate, epigallocatechin, epicatechin gallate, epicatechin and catechin, exhibit scavenger activity against free radicals generated in the aqueous phase and chain-breaking properties against lipid peroxidation *in vitro* (14, 15). Polymerised polyphenols and crude polyphenol extracts from black or

green tea or wine also protect against oxidative damage to blood cells *in vitro* (16, 17) and oxidation of low density lipoproteins (LDL) (18, 19). Protection against free-radical-induced cytotoxicity and DNA damage by dietary flavonoids *in vitro* has also been demonstrated in Chinese hamster cells (20), cultured human colonocytes (21) and human lymphocytes (22). On the other hand, little evidence of a protective role against oxidative damage of these compounds *in vivo* has been provided. An increase in plasma antioxidant capacity has been reported following catechin absorption in humans (23), and tea consumption has been reported to decrease blood and urine biomarkers of oxidative stress (24). However, no changes have been reported in some markers of oxidative stress in humans drinking green tea (25).

On this basis, the aim of the present study was to verify whether treatment with complex polyphenols and tannins from wine and tea *in vivo* affects basal DNA oxidative damage in rat colon mucosal cells. For this purpose, we have used the comet assay, a technique allowing the detection of single-strand breaks (SSBs) and oxidised DNA bases at the single-cell level (26, 27, 28).

Materials and methods

Preparation of wine and tea polyphenols and tannins

A red wine complex polyphenol and tannin powder (WCPT) free of low molecular weight polyphenols was prepared as previously described (29) with a yield of approximately 0.8 g per liter of red wine. Approximately half of the material consisted of proanthocyanidins with a mean degree of polymerisation of 6.3, the other half consisting of derived tannins of unknown structure, formed from grape phenolic constituents in the course of wine making.

A crude thearubigin fraction (TR) was prepared from Lattakari Assam black tea (Importers Ltd, Guildford, UK) that was ground coarsely using a laboratory scale knife mill (Gryphon 11–30-Brook Crompton Parkinson Motors, Huddersfield, England). Approximately 3 kg of ground material was soaked overnight in chloroform (5 litres) in a large beaker placed in an operating fume cupboard to remove a proportion of the caffeine. The soaked leaf was filtered on a Buchner funnel and the residue washed with chloroform (1 litre) and dichloromethane (1 litre). The extracted leaf was placed between sheets of filter paper and solvent residues allowed to evaporate overnight in an operating fume cupboard.

The partially decaffeinated black tea leaf (80 g) was placed in a pre-warmed vacuum flask (1 litre capacity) and freshly boiled water added gently in convenient aliquots until full while the contents were agitated by swirling. The flask was capped and the tea brewed for 10 min, with inversion at 30-s intervals. The brew was filtered through glass wool into a 5-litre separating funnel and allowed to cool to approx. 65 °C and an equal volume of chloroform was carefully added and the funnel capped. The contents were mixed vigorously by inversion for 1 min and allowed to stand until two layers separated. The organic phase, including any remaining emulsion, was run off to waste and the partition step repeated once.

The decaffeinated brew was similarly partitioned against equal volumes of ethyl acetate to remove orange-red theaflavins, residual flavanols (catechins) and flavonol glycosides. This procedure was continued until the organic phase was colourless (usually 15 stages). The aqueous phase was allowed to stand overnight in the separating funnel and was then run into a pear-shaped flask for evaporation at room temperature (approx. 22 °C) under reduced pressure, aliquoted and freeze-dried to yield the TR. There are appreciable losses during work up and yield is considerably less than theoretical. The procedure was repeated as necessary to obtain the required quantity.

Animals and treatments

Male Fisher 344 rats weighing approximately 100 g were purchased from Nossan (Milan, Italy). One week after arrival the animals were shifted from standard lab chow to a high fat diet containing 230 g/kg corn oil, 20 g/kg cellulose and 1.3 g/kg calcium, thus mimicking the typical diet of human populations at high risk of colon cancer, as previously described (30). Diet components were purchased from Piccioni (Gessate, Milan, Italy).

Rats were treated with WCPT (57.2 mg/kg/d), TR (40 mg/kg/d) or water by oral gavage (0.5 ml) each day for ten days before sacrifice. For WCPT, we assumed a human exposure of about 5.7 mg/kg/d for a moderate wine drinker (a 70 kg man drinking 0.5 l wine per day), considering that the WCPT content of red wine was 0.8 g/l. In the present experiments we used a dose ten times higher relative to the exposure levels calculated above.

For TR, we based our calculations on the average UK consumption (3–4 cups of tea per day) for people over the age of 10 years which equates to approximately 11 g of black tea based upon 3.1 g per typical UK tea bag. This average, of course, includes non-drinkers, so the mean for those drinking tea would be higher. The average TR content of domestic brews varies substantially with the method of brewing. A currently popular method, dipping a teabag in hot water for 75 s, extracts approximately 0.3 mg/ml, equal to an average consumption of about 210 mg TR per day (some 3 mg/kg body weight for a 70 kg adult). Recognising that some methods of brewing achieve a higher extraction rate, and that some people consume considerably more than 3–4 cups per day (30), it was decided to use a baseline of 4 mg/kg body weight/day, increasing this by a factor of ten to a dose of 40 mg/kg/day.

Isolation of colonocytes

At the end of this treatment period rats were killed by decapitation, the colon removed, and colon mucosa cells were isolated using the procedure described by Pool-Zobel et al. (28). After washes in oxygenated (100 % O_2) Hanks' balanced salt solution Ca-Mg-free (HBSS) the colon was filled with a solution (4 mg/ml) of proteinase K in HBSS (Boehringer, Mannheim, Germany). The two extremities of the colon were ligated and the piece incubated at 37°C for 30 min. The cells of the epithelium were subsequently released with slight agitation after cutting open the colon segment and the suspension was centrifuged for 8 min at 1000 x g. The supernatant was discarded and the pellet resuspended in HBSS.

Comet assay

The comet assay procedure was used to measure the basal level of SSBs and the basal level of both pyrimidine and purine oxidation in the DNA of acutely isolated colonocytes.

Cells were counted and the vitality assessed by means of the Trypan Blue exclusion test. The final concentration was adjusted to about 10^7 cells/ml and the suspension was kept on ice until use. Twenty µl of cell suspension were then mixed with 75 µl of 0.7 % low-melting point agarose (maintained at 37 °C) and kept for 10 min on ice in order to solidify the agarose. After this time, a further layer of agarose was added on each slide and left again to solidify for 10 min on ice. The slides were subsequently transferred to a lysis solution [1 % N-lauroyl-sarcosine, 2.5 mM NaCl, 100 mM Na_2EDTA, 1 % TritonX–100, 10 % dimethylsulfoxide (DMSO)] and incubated 1 h at 4°C in order to obtain lysis of the cytoplasms and deproteinisation of the nuclei.

In order to test the capability of the cells to withstand oxidation damage, we submitted some of the slides to *in vitro* oxidative stress before the lysis step. This was accomplished by incubating the slides with different concentrations of H_2O_2 in phosphate-buffered saline for 5 min at 4°C (to minimise repair). The number of H_2O_2-induced SSBs in each condition was then measured.

For analysing oxidised DNA bases, two bacterial repair enzymes were used to introduce breaks at pyrimidine or purine oxidation sites respectively. After completion of the lysis step the slides were washed three times in endonuclease buffer (40 mM HEPES-KOH, 100 mM KCl, 0.5 mM EDTA, 0.2 mg/ml bovine serum albumin (BSA), pH 8.0) and each slide was then incubated at 37°C for 45 min with 50 µl of either endonuclease III (endo-III) for pyrimidine, or formamidopyrimidine glycosilase (fpg) for purine oxidation detection (both kindly provided by Dr. A. R. Collins, Rowett Res. Inst., Aberdeen, Scotland, UK, upon isolation from *E. Coli*). The enzymes were dissolved in the same buffer at the concentration of 1 µg protein/ml and sealed with a coverslip. The specific pyrimidine or purine oxidative damage was calculated as the difference between the damage detected in the respective enzyme-treated slides and their corresponding controls (buffer-incubated slides).

Enzyme incubation was not performed for SSB measurement, and the slides were subjected to electrophoresis following the lysis step.

All the slides of the experiment were placed in an ice-cold electrophoresis chamber (model GNA–200, Pharmacia, Milan, Italy) in an alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA) for 20 min to allow DNA unwinding. At the end of this time, current was switched on and the electrophoresis was conducted for 20 min at 25 V and 300 mA.

At the end of this time the slides were removed from the electrophoresis chamber and washed three times, 5 min each, with neutralisation buffer (40 mM tris-HCl, pH 7.4). Finally, the slides were stained with ethidium bromide (20 µg/ml) overnight and analysed on the following day.

Microscopical analysis was carried out by means of a Labophot–2 microscope (Nikon, Japan) provided with epifluorescence and equipped with a rhodamine filter (excitation wavelength 546 nm; barrier 580 nm).

Each experimental point was run in duplicate and the images of 50 randomly chosen nuclei per slide were captured and analysed using custom-made imaging software coupled with a CCD camera (model C5985, Hamamatsu, Japan). The amount of DNA fluorescence migrating in the tail was measured in each nucleus and the nuclei classified into five categories from 0 to 4 with increasing tail migration (class 0: 0–6 %, class 1: 6.1–17 %; class 2: 17.1–35 %; class 3: 35.1–60 %; class 4: 60.1–100 % DNA migrated in the tail). Data were expressed in arbitrary units (AU), obtained by multiplying the percentage of each comet type in a slide by its category number as described by Wollowski et al. (32).

Statistical analysis

Differences between groups were analysed by means of Student's *t* test.

Results

A high fat diet as employed in the present experiments did not alter *per se* basal DNA oxidative damage in the rat colon mucosal cells. In fact, with the comet assay technique we did not observe any statistically significant difference among the level of oxidised pyrimidines in isolated colonocytes between standard lab chow-fed and high fat diet-fed rats (69.4 ± 14.72 and 40.6 ± 15.71 AU respectively). Similarly, no significant differences were detected in the basal level of SSBs between the two groups of ani-

mals (71.4 ± 6.30 and 82.0 ± 14.94 AU in the standard lab chow- and high fat diet-fed rats respectively).

Treatment with WCPT and TR did not produce any detectable toxic effect on the rats, whose weight and general condition remained normal during the whole experiment.

The viable cells isolated from the colon mucosa in the controls, in the WCPT- and in the TR-treated group were about 90 % of total cells and cell viability was not affected by the treatments (Table 1).

The results of our experiments showed that WCPT treatment did not modify the basal level of SSBs in colon mucosal cells after ten days of treatment (Fig. 1). Also, Fig. 1 shows that cellular susceptibility to *in vitro* oxidative stress induced by different concentrations of H_2O_2 was not modified by WCPT treatment.

However, we found that WCPT treatment induced a statistically significant decrease (–62 %) in the basal level of pyrimidine oxidation ($p < 0.05$) as detected by using the endo III enzyme modification of the assay (Table 2). A 57 % ($p < 0.05$) decrease in purine oxidation level was also found with the addition of fpg enzyme in the WCPT-treated animals as compared with the controls (Table 2).

Table 3 shows that TR also did not modify the basal level of SSBs in colon mucosal cells after ten days of treatment. Similarly, TR did not reduce significantly the basal level of pyrimidine oxidation detected using the endo III enzyme modification of the assay. A tendency towards decrease was observed that did not reach significance even though the number of animals per group was increased from 9 to 14–15.

Table 1 Effect of polyphenol treatment on cell viability of acutely isolated rat colon mucosal cells

Treatment	n	% Cell Viability
Water	9	92.5 ± 1.59
WCPT 57.2 mg/kg/d	9	90.0 ± 3.89
Water	15	90.6 ± 1.87
TR 40 mg/kg/d	14	87.8 ± 2.51

Animals were treated by gavage (0.5 ml per day of WCPT or TR in water or water alone) for ten days. Values are means ± SEM, n = number of animals, WCPT = wine complex polyphenols and tannins, TR = thearubigin. Cell viability is expressed as the percentage of living cells as evaluated by Trypan Blue exclusion test

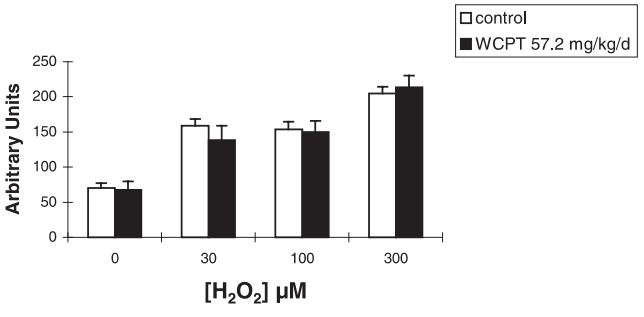

Fig. 1 *Ex vivo* test for antioxidant capability on colon mucosal cells. Acutely isolated colonocytes prepared from rats treated with WCPT or vehicle alone by gavage for ten days were challenged *in vitro* with H_2O_2 and DNA strand breaks were measured by the comet assay. Data are expressed as means ± SEM, n = 4 (WCPT), n = 5 (control).

Table 2 Effect of wine complex polyphenols and tannins treatment on DNA base oxidation in acutely isolated rat colon mucosal cells

Treatment	Pyrimidine oxidation	Purine oxidation
H_2O	53.4 ± 12.18	48.7 ± 9.85
WCPT 57.2 mg/kg/d	20.1 ± 7.62*	21.0 ± 7.89*

Animals were treated by oral gavage (0.5 ml per day of WCPT in water or water alone) for ten days.
Values are means ± SEM of nine animals per group, pyrimidine and purine oxidation are expressed in arbitrary units (AU) and were estimated as the difference between DNA damage detected with endonuclease III (endo III) or formamidopyrimidine-glycosylase *(fpg)* digestion and damage without digestion.
* $p < 0.05$ statistically significant difference from the control group (treated with water) evaluated by the unpaired Student's *t* test

Table 3 Effect of thearubigin treatment on basal SSBs and oxidative DNA damage measured by the comet assay in acutely isolated rat colon mucosal cells

Animal treatment	n	Single-strand breaks	Pyrimidine oxidation
Water	15	83.3 ± 11.11	58.6 ± 13.88
TR	14	78.7 ± 11.44	41.6 ± 10.85
40 mg/kg/d		(–5 %)	(–29 %)

Animals were treated by oral gavage (0.5 ml per day of thearubigin-TR- in water or water alone) for ten days. Values are means ± SEM, n = number of animals, the percent changes are shown in parentheses. DNA damage is expressed in arbitrary units (AU). Oxidative DNA damage on pyrimidine bases was measured by means of the enzyme endo III, and was estimated as the difference between DNA damage detected with enzyme digestion and damage without digestion

Discussion

Our results show that oral treatment with WCPT induced a decrease in basal DNA oxidative damage of acutely isolated rat colon mucosal cells without affecting the DNA basal level of SSBs. Analysis of endogenous levels of DNA strand breaks and oxidised bases in colon cells *in vivo* by means of the comet assay has been reported previously only in human biopsies (33).

Previous data on wine polyphenols showed a protective effect of these compounds against experimentally-induced oxidative stress (18, 19). Duthie and Dobson (21) have

shown that the polyphenols quercetin and myricetin did not induce changes in the basal level of DNA strand breaks or oxidised DNA bases upon *in vitro* treatment of cultured human colon mucosal cells, while they decreased hydrogen peroxide-induced strand breaks. Furthermore, it has been shown that anthocyanins did not reduce endogenous oxidative damage in human colon cells in culture (34).

In our experimental conditions, we used a different class of polyphenols, mainly consisting of polymeric compounds. Both wine and tea have a particular phenolic composition compared to other food sources, due to the conversion of genuine plant components to other (presumably polymeric) molecular species during processing. In particular, thearubigins arise from enzymatic oxidation of green tea catechins during black tea fermentation. Similarly, grape polyphenols have been reported to proceed to various types of derived tannins during wine making and ageing (35, 36).

While the intestinal absorption and metabolic fate of polyphenols *in vivo* are not well known, they are at least partly absorbed from the gastrointestinal tract (37). Epithelial cells of the small and large intestines are obviously exposed to the highest concentrations of dietary polyphenols. These compounds show affinity for cellular membranes and it has been reported that this feature is essential for their protective action against hydrogen peroxide cytotoxicity (38). Thus, it is possible that upon oral treatment WCPT and/or their metabolites accumulate in colonocytes and exert some biological actions in various intracellular compartments.

The biological mechanism by which WCPT decrease basal oxidative damage in colon mucosal cells can only be hypothesised. A free-radical scavenging action (14, 15) and an iron-chelating activity (39) of polyphenols have been described. In our experiments, we have not found any effect of WCPT treatment on hydrogen peroxide-induced increase in DNA strand breaks in isolated colonocytes *in vitro*. This result seems to indicate that the repeated oral treatment with WCPT does not increase the scavenging capacity of colonocytes towards the hydroxyl radical. Thus, the antioxidant action of WCPT that we have observed might rather be due to different and possibly manifold mechanisms: among the possibilities are the inhibition of enzymes involved in free radical production (9, 40), the induction of xenobiotic metabolism, possibly leading to detoxification of intracellular reactive chemicals (41), the inhibition of intestinal iron absorption, as demonstrated for tea polyphenols (42) or the potentiation of cellular repair systems.

At variance with WCPT, black tea polyphenols (TR) did not modify the basal levels of DNA oxidative damage in colon mucosal cells using the same schedule used for WCPT, although a tendency towards decrease was observed. The basal levels of DNA SSBs were also not modified by TR treatment.

An antioxidative action has been demonstrated *in vitro* for whole tea extracts (17) and tea polyphenols (16) and there is evidence that tea consumption decreases oxidative damage in humans (23). TR are a class of condensed polyphenols present in black tea and represent 10–20 % of the total dry weight of tea (43). An inhibitory effect of TR on lipid peroxidation and on macrophage nitrite production has been shown *in vitro* (44, 40). It was calculated that 8–10 cups of tea daily would be required to ensure a significant increase of the antioxidant capacity *in vivo* in humans (45) and, accordingly, the dose of TR that we have employed was chosen on the basis of high human tea consumption. Thus, it is possible that other polyphenolic molecules such as theaflavins, even though less represented as percentage of dry weight, are an important contribution to the antioxidant activity of black tea.

In conclusion, our data show for the first time that dietary polyphenols can modulate *in vivo* oxidative damage in the gastrointestinal tract of rodents. In order to define the protective and therapeutic potential of these compounds in oxidative damage-related pathologies, further research will have to clarify whether these effects can be observed in other organs which are subjected to oxidation damage (such as brain and heart), the role of the single components of the polyphenol crude extracts and the relative antioxidant potency of each.

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