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ORIGINAL CONTRIBUTION

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Effect of complex polyphenols and tannins from red wine (WCPT) on chemically induced oxidative DNA damage in the rat

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Summary *Background:* Flavonoids are polyphenolic antioxidants occuring in vegetables and fruits as well as beverages such as tea and wine which have been thought to influence oxidative damage.
Aim of the study: We wanted to verify whether a complex mixture of wine tannins (wine complex polyphenols and tannins, WCPT) prevent chemically-induced oxidative DNA damage *in vivo*.
Methods: Oxidative DNA damage was evaluated by measuring the ratio of 8-hydroxy-2'-deoxyguanosine (8OHdG)/ 2-deoxyguanosine (2dG) x 10^{-6} in hydrolyzed DNA using HPLC coupled with electrochemical and UV detectors.
Results: We treated rats with WCPT (57 mg/kg p.o.) for 14 d, a dose 10-fold higher than what a moderate wine drinker would be exposed to. WCPT administration significantly reduced the ratio of 8OHdG/2dG x 10^{-6} in liver DNA obtained from rats treated with 2-nitropropane (2NP) relative to controls administered 2NP only (33.3 ± 2.5 vs. 44.9 ± 3.2 x 10^{-6} 2dG; μ ± SE; $p<0.05$). On the contrary, pretreatment with WCPT for 10 d did not protect the colon mucosa from oxidative DNA damage induced by 1,2-dimethylhydrazine (DMH). 2NP and DMH are hepatic and colon carcinogens, respectively, capable of inducing oxidative DNA damage.
Conclusions: WCPT have protective action against some types of chemically-induced oxidative DNA damage *in vivo*.

Key words Wine polyphenols – 8-hydroxy-2'-deoxyguanosine – oxidative damage – 2-nitropropane – 1,2-dimethylhydrazine

Introduction

DNA damage, mediated by reactive oxygen species, has recently attracted much attention because of its supposed relationship with basic pathological processes like aging and carcinogenesis (1).

It has been suggested that polyphenols may affect oxidative damage. Green tea polyphenols have been shown to reduce the incidence of lung cancer and the formation of 8-hydroxy-2'-deoxyguanosine (8OHdG), a marker of oxidative DNA damage, in mice administered 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone) (2). Wei and Frenkel (3) also reported that the treatment of mice with (-)epigallocatechin gallate prior to the application of 12-O-tetradecanoyl-phorbol-13-acetate decreased polymorphonuclear leukocyte infiltration, H_2O_2 formation, and the oxidation of normal bases to 5-hydroxymethyl-2'-deoxyuridine and 8OHdG.

Furthermore 2-wk administration of a 2 % green tea infusion or of a crude catechin extract in drinking water had a protective effect against liver oxidative DNA damage and hepatotoxicity in rats treated with 2-nitropropane (2NP) (4). The formation of 8OHdG in liver after 2 NP in DNA has been proposed as one of the mechanisms underlying its carcinogenicity (5, 6).

The lower incidence of coronary diseases in populations where red wine is a traditional drink, referred to as

the "French paradox", is attributed to a high dietary level of phenolics with strong antioxidant and oxygen radical scavenging properties (7, 8). A direct correlation between the phenolic content of wines and their ability to scavenge superoxide radicals has been established (9) but no distinction has been made between the various types of phenolic components.

Phenolic compounds have very diverse structures, ranging from rather simple molecules to polymers. The higher molecular weight compounds among these (Mw >500) are usually designated by the term tannins (10). The major phenolics in green tea are monomeric flavanols (epicatechin, epigallocatechin, and the corresponding gallic esters). In contrast, red wine phenolics are mainly polymeric material, including genuine grape tannins (proanthocyanidins) and complex polyphenols formed by various reactions during wine-making and aging. Health-related properties of all these molecules, such as free-radical scavenging abilities (11, 12) or interactions with proteins and enzyme inhibition (13–16), have been shown to increase with both the chain length (degree of polymerization) and the extent of galloylation, suggesting that complex phenols and tannins may be more active than monomeric phenolics.

However, while monomeric flavonoids (17, 18) and flavonols (19) have been shown to be absorbed into the blood circulation system after an oral administration, there is no evidence on the bioavailability and biological effects of tannins and related complex polyphenols from wine (WCPT).

On this basis we wanted to verify whether complex polyphenols and tannins from red wine (WCPT) have a preventive activity against oxidative DNA damage. As an experimental model we studied DNA damage induced by 2NP in the liver of rats and by 1,2-dimethylhydrazine (DMH), a specific intestinal carcinogen, in rat colonic mucosa.

Materials and methods

Chemicals and standards

DMH and 2NP were obtained from Sigma, Milan, Italy; 8OHdG, used as chromatographic standard, was synthesised as reported in a previous paper (20). Quercetin-3-glucoside was purchased from Extrasynthese (Lyon, France). (+)-Catechin and (-)-epicatechin were purchased from Sigma (St. Louis, MO, USA). Epicatechin-3-O-gallate and benzylthioethers of catechin, epicatechin, epicatechin-3-O-gallate and epigallocatechin were obtained by thiolysis of concentrated skin extract and purified by semi-preparative HPLC as described earlier (21, 22). Toluene-α-thiol was purchased from Merck (Darmstadt, Germany).

Complex polyphenols and tannins from wine (WCPT)

To prepare the fraction defined in this paper as "wine complex polyphenols and tannins" or "WCPT", we used a 2-year old red wine, vintage 1994, made from *Vitis vinifera* grapes (variety Cabernet Sauvignon) by standard red wine making procedures at the Arzens Cooperative winery (Arzens, Aude, France).

A WCPT powder free from low molecular weight phenols was obtained by using the following procedure: the wine was first de-alcoholized under vacuum, filtered to remove tartaric precipitates and deposited onto a Relite Diaion Column (Mitsubichi, Japan). After washing with water to remove sugars and organic acids, the wine 'whole phenolic pool' was eluted with 90 % ethanol, concentrated under vacuum to eliminate the ethanolic solvent and atomized. Thus, 380 g of wine phenolic powder was obtained, starting from 140 l of wine. Batches of this powder were then dissolved in water and chromatographed on a Toyopearl TSK HW-50 (F) column (TosoHaas, Stuttgart, Germany) using a procedure upscaled from that described earlier for fractionation of grape skin tannins (22). After washing with water and eluting the low molecular mass phenols with a mixture of ethanol:water:trifluoroacetic acid (55:45:0.005, v:v:v), the polymeric fraction was recovered with acetone:water (60:40, v:v). The acetone fractions were pooled, concentrated under vacuum and atomized, yielding the WCPT powder (0.8 g/l of wine).

Phenolic analysis of the wine was achieved by HPLC after fractionation on Toyopearl TSKgel HW-50 (F) as described earlier (22, 23). Low molecular weight phenols were analyzed by HPLC-DAD. The wine fraction containing polymeric material and the WCPT powder were analyzed by HPLC-DAD to check that they were free of low molecular weight phenolics and were then submitted to thiolysis followed by HPLC analysis. The latter technique provides qualitative (mean degree of polymerization and proportions of each constitutive unit) and quantitative (concentration) information on proanthocyanidin units, which may be either present as native grape tannins or included in derived tannins. All quantitations were based on peak areas. A calibration curve established with quercetin-3-glucoside (at 360 nm) was used to quantify flavonols. The concentrations of monomeric flavanols and of the various thiolysis products were calculated from peak areas at 280 nm, using calibration curves established for each individual compound.

Animals

Male Fisher 344 rats (180–200 g) were purchased from Nossan (Correzzana, Milan, Italy). After their arrival from the supplier animals were quarantined for 1 week, during which they were fed a standard lab chow. The rats were then shifted to a diet with a composition based on the AIN76 diet, modified to contain a high amount of fat

(230 g/kg corn oil w/w), a low level of cellulose (20 g/kg w/w), and a low level of calcium (1.3 g/kg 2/2), similar to the diet of some western human populations. Dietary components were purchased from Piccioni (Gessate, Milan, Italy).

In vivo experiments

Rats were administered 2NP (100 mg/kg) i.p. in saline supplemented with 0.1 % Tween80. Control animals were administered the vehicle alone. All animals were killed with decapitation after ether anesthesia and were treated according to the European Union Regulations on the Care and Use of Laboratory Animals (Law 86/609/EC, 1986).

We studied the effect of WCPT administration on 2NP-induced DNA oxidative damage in the liver and colon mucosa. In these experiments we used a dosage of WCPT (57 mg/kg) which was about ten times higher than the exposure of a man of about 70 kg drinking a moderate amount of red wine (0.5 l/d). The animals were treated for 14 d by gavage with WCPT in the morning; controls received a gavage of water alone. Treatments did not modify body weight. After 14 d one group of rats was given an i.p. injection of 2NP and the controls an injection of vehicle alone. Animals were sacrificed 15 h following 2NP, their livers were excised and frozen at -80°C until analysis.

We also treated rats with a specific intestinal carcinogen, 2-dimethylhydrazine (DMH). The animals were treated for 10 d with WCPT (57 mg/kg/d) by gavage. Controls received water alone. After 10 d DMH (20 mg/kg) or saline was administered by gavage. Rats were sacrificed 24 h after DMH administration, their colon was excised, rinsed with saline, and frozen at -80 °C until analysis. The colons were then thawed, the mucosa layer removed by scraping with a scalpel and processed for DNA analysis.

Isolation and hydrolysis of DNA

Isolation and hydrolysis of liver or colon mucosa DNA was performed using a published method with a few modifications (24). Briefly, livers (1 g w.w.) were homogenized in 10 ml of ice-cold 0.15 M NaCl 0.015 M sodium citrate buffer pH 7 and centrifuged at 600 x g x 15 min. The pellet thus obtained was first solubilized in 6 ml of a pH 8 buffer containing 0.01 M Tris-HCl, 0.01 M EDTA, 0.01 M NaCl, sodium dodecyl sulfate 0.5 %, and RNAse 20 μg/ml, and incubated at 37 °C for 1 h in the dark. Proteinase K (final concentration 100 μg/ml) was then added and incubated at 37 °C overnight in the dark. The lysed mixture was extracted with chloroform:isoamyl alcohol (10:2, v:v) and DNA was precipitated from the aqueous phase as reported by Fiala et al. (6). The DNA was then dried and stored under argon gas at -20 °C until further processing.

DNA was finally solubilized in 300 μl of 20 mM acetate buffer pH 5.2 and denatured at 90 °C for 3 min. P1 nuclease (50 UI/mg of DNA) was added and incubated at 37 °C for 1 h in the dark. The incubation mixture was then digested for 1 h at 37 °C with alkaline phosphatase (10 IU/mg of DNA) after the addition of 20 μl of 0.4 M TRIS-HCl buffer pH 8.8. All samples were protected from light to avoid artefactual light-induced oxidation. The hydrolyzed mixture was filtered by Micropure-EZ Enzyme Remover (Amicon Inc., MA, USA) and 90 μl were injected into the HPLC apparatus.

HPLC analysis

The separation of 8OHdG and 2-deoxyguanosine (2dG) was performed with an LC/9A Shimadzu HPLC pump using two detectors: UV (Biorad) and Coulochem (ESA model 5100 with a 5010 analytical cell). For chromatographic separation we used a C18 reverse-phase column (Supelco, 5 mm, i.d. 0.46 x 25 cm); the eluting solution was H_2O:CH_3OH (92:8 v/v) with 50 mM KH_2PO_2 pH 5.5 at a flow rate of 1 ml/min. The 8OHdG and 2dG were detected using an ESA Coulochem II electrochemical detector in line with an UV detector as reported previously (24). The detectors were connected to a Shimadzu integrator for the determination of peak areas. The retention times for 2dG and 8OHdG were about 9 min and 13 min, respectively. The detection limit for 8OHdG was 20 pg. The 8OHdG levels were expressed as molar ratio 8OHdG/2dG x 10^{-6}.

Statistics

Data were analyzed with one-way ANOVA by calculating the contrasts between means with the Least Significant Difference method (LSD) and multifactorial analysis of variance using the Statgraphics Statistical Package (Statistical Graphic Corporation, Rockville, MD, USA). Differences were considered statistically significant when the probability level P was <0.05.

Results

The WCPT powder contained trace amounts of flavonol aglycones (6 mg/g, i.e., 15 % of the wine flavonol initial content) but no anthocyanins, flavonol glycosides, flavanol monomers nor phenolic acids. Sugar analysis also confirmed that this powder contained no polysaccharides. Although some losses occurred in the last purification step, proanthocyanidin composition of the tannin powder, as determined by thiolysis, was qualitatively similar to that of the wine, with the following constitutive units: 18.1 % catechin, 60.7 % epicatechin, 3.3 % epicatechin 3-O-gallate, and 17.9 % epigallocatechin, and a mean degree of polymerization of 6.3. Genuine proanthocyanidin units represented approximately half of the

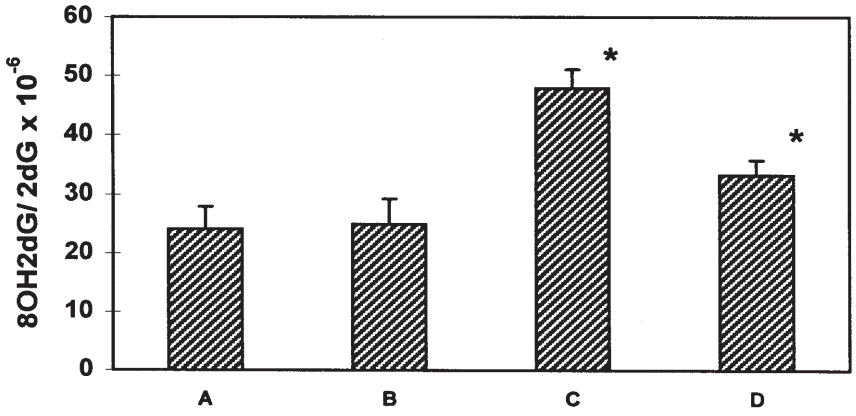

Fig. 1 Levels of 8OHdG in liver DNA in control rats and rats administered with WCPT and 2NP. A: controls (n = 5); B: animals administered WCPT (57 mg/kg/d) for 14 d (n = 5); C: controls treated with 2NP (100 mg/kg i.p.) 15 h before sacrifice (n = 5); D: animals administered WCPT (57 mg/kg/d) for 14 d and treated with 2NP (100 mg/kg, i.p.) 15 h before sacrifice (n = 5). * $p < 0.05$ A vs C and C vs D. Data are expressed as ratio 8OHdG/2dG x 10^{-6} and are $\mu \pm$ SE.

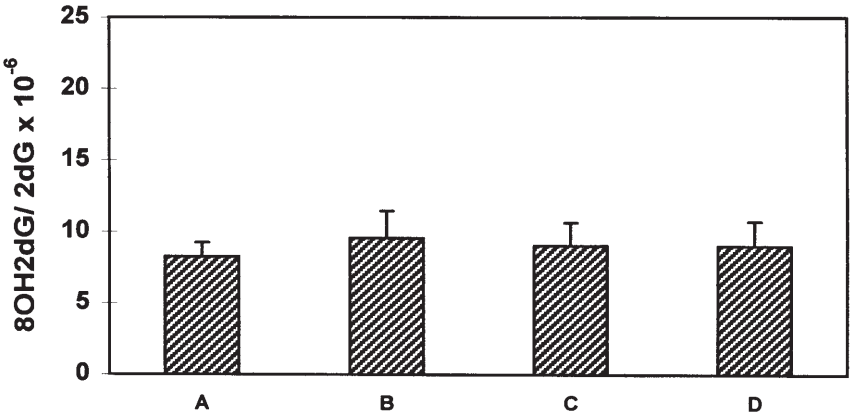

Fig. 2 Levels of 8OHdG in colon mucosa DNA in control rats and rats administered with WCPT and 2NP. A: controls (n = 5); B: animals administered WCPT (57 mg/kg/d) for 14 d (n = 5); C: controls treated with 2NP (100 mg/kg i.p.) 15 h before sacrifice (n = 5); D: animals administered WCPT (57 mg/kg/d) for 14 d and treated with 2NP (100 mg/kg, i.p.) 15 h before sacrifice (n = 5). Data are expressed as in Fig. 1.

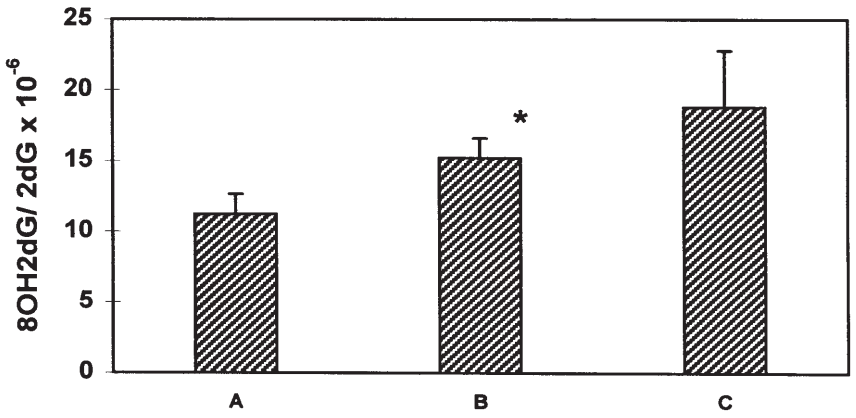

Fig. 3 Levels of 8OHdG in colon mucosa DNA in control rats and rats administered with WCPT and DMH. A: controls (n = 11); B: rats sacrificed 24 h after the treatment with DMH (20 mg/kg) by gavage (n = 26); C: animals administered WCPT (57 mg/kg/d) for 10 d and then sacrificed 24 h after the treatment with DMH (20 mg/kg n = 5). * $p < 0.05$, A vs B, with multifactorial analysis of variance. Data are expressed as in Fig. 1.

material present in the tannin powder (498 mg/g), the other half presumably consisting of 'derived polyphenols', as expected in a 2-year old red wine.

Pre-treatment of rats with WCPT (57 mg/kg) for 14 d did not modify the 8OHdG levels in liver DNA in controls. In preliminary time-course experiments, we treated rats i.p. with 2NP (100 mg/kg) and determined the liver levels of 8OHdG. The maximum effect of 2NP liver was observed 15 h after administration. In animals treated with 2NP, the administration of WCPT had a protective effect against induced oxidative damage. In fact, the levels of 8OHdG after 2NP administration were significantly decreased by WCPT pre-treatment (Fig. 1). On the contrary, we did not find any variation in 8OHdG levels in the colon mucosa, either in controls or in animals treated with 2NP (Fig. 2).

We observed a significant increase of 8OHdG levels in the colon mucosa of rats administered DMH compared to controls. However, pre-treatment of rats with WCPT (57 mg/kg/d) for 10 d was not protective against DMH-induced DNA oxidative damage of the colon mucosa (Fig. 3). We report data derived from three different experiments, including the inter-experiment variation in the statistical analysis.

Discussion

Dietary antioxidants, such as vitamin C, E, and β-carotene, are supposed to protect against cancer and aging by limiting damage to DNA by free radicals, including reactive oxygen species released during cellular respiration. We planned this study to verify whether other putative dietary antioxidants, such as WCPT, can protect against chemically-induced oxidative damage.

Flavonoids are regular components of the human diet. They are polyphenolic antioxidants occurring in vegetables, fruits and in beverages such as tea and wine. Red wine in particular has become a source of interest after the discovery of the so-called "French Paradox" (i.e., the relatively low incidence of cardiovascular diseases in some Mediterranean countries despite the high intake of saturated fat) (25). Flavonoids have been suggested to reduce the incidence of myocardial infarction and the risk of death from coronary heart disease (26). The fact that red wine consumption can prevent the oxidation of low density lipoproteins suggests a possible mechanism for these effects (27).

It is also established that many flavonoids are scavengers of free radicals, antioxidants, chelating agents and modifiers of various enzymatic and other biological functions (28). A recent study reports that green tea protects against liver oxidative DNA damage and hepatotoxicity in rats treated with 2NP (4). The formation of 8OHdG in the liver induced by 2NP is one of the major mechanisms underlying its carcinogenicity (5, 6).

Notwithstanding the information on green tea polyphenols, data on the effect of wine polyphenols on oxidative DNA damage are lacking in the literature. We directed our attention on a polyphenol extract from red wine consisting in a complex mixture of genuine grape tannins (i.e., proanthocyanidins) and "derived-tannins", formed from grape phenolic compounds during vinification.

Our results show that pre-treatment with WCPT for 2 weeks reduces the oxidant action of 2NP on liver DNA in rats. In fact 8OHdG levels are significantly decreased by WCPT after administration of 2NP. On the contrary, the levels of 8OHdG do not increase in colon mucosa after 2NP exposure. These data are coherent with the lack of induction of intestinal tumors after chronic 2NP administration. This compound induces oxidative damage intracellularly through the formation of reactive metabolites in various pathways which are accountable for the toxicity of 2NP in mammalian cells (29). Hepatocytes express most classes of enzymes implicated in the formation of DNA damaging metabolites from 2NP, i.e., specific cytochrome P450-dependent monooxygenases and sulfotransferases. The enzymes are less expressed in the colon mucosa, explaining the lack of effect of 2NP at that level.

We also evaluated the effect of WCPT on DMH-induced colon damage. DMH is a colon-specific a methylating carcinogen, but the involvement of anion superoxide in DMH carcinogenesis is also suspected (30). We report in this paper that DMH increases the level of oxidative damage in colon mucosal DNA, in accordance with previous observations (31). However, pre-treatment with WCPT for 10 d does not protect colon mucosa from oxidative DNA damage induced by DMH.

In conclusion, WCPT at doses comparable to human exposure have protective actions against some, but not all, types of chemically induced oxidative damage.

It is not clear which of the individual compounds of the WCPT mixture used in our experiments is responsible for the observed effects. Data on the metabolism and pharmacokinetics of these compounds are still lacking.

As a possible mechanism of action we can suggest is that protection against oxidative DNA may be due to the iron chelating effect of some proanthocyanidins. Iron, in fact, plays an important role in the formation of hydroxyl radicals through a Fenton reaction (32, 33). However such a mechanism of action, although interesting, is still a matter of speculation.

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