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ORIGINAL CONTRIBUTION

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Antioxidant and radical scavenging properties *in vitro* of polyphenolic extracts from red wine

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Summary *Background & Aims* Red wine polyphenols inhibit chemically-induced oxidative DNA damage *in vivo* in experimental animals through a mechanism which is still unclear. On this basis, we tried to clarify the mechanisms of inhibition of DNA oxidation *in vitro* by wine extracts containing monomeric and polymeric phenols (WE) and monomer-free complex polyphenols and tannins (WCPT) from red wine. *Methods* Oxidative DNA damage was induced by incubating DNA with GSH/Fe^{3+} or cumene hydroperoxide (CumOOH) *in vitro* and using 8-OH–2-deoxyguanosine (8-OHdG) levels as a measure of DNA oxidation. Levels of 8-OHdG were determined by HPLC coupled with electrochemical detector (ESA). *Results and conclusions* WCPT and WE, at μM concentrations, reduced concentration-dependently oxidative DNA damage induced by GSH/Fe^{3+}. WCPT and WE also reduced DNA oxidation by CumOOH. In conclusion, complex polyphenols and tannin extracts from red wine, with or without small molecular phenols, prevent oxidative DNA damage through a dual mechanism, iron binding and direct free radical scavenging.

Key words Oxidative DNA damage – Reactive oxygen species – Iron chelation – Polyphenols – Antioxidants

Introduction

Biochemical studies suggest that the oxidation induced by reactive oxygen species may be important in a number of pathological conditions such as cancer and aging (1, 2). Formation of reactive oxygen species is closely related to the redox state of transition metals (3). One-electron reduction of oxygen by ferrous ion generates the superoxide anion, which is converted into hydrogen peroxide by enzymatic and non-enzymatic mechanisms (4). Hydrogen peroxide can produce the hydroxyl radical through the Fenton reaction, which requires ferrous iron or copper; moreover, ferrous iron can react with oxygen, forming perferryl ion (FeO^{2+} and FeO^{3+}) species, which are more reactive than the hydroxyl radical itself (5).

Polyphenols, including tannins, have been shown to have antioxidant and free radical-scavenging effects (6, 7); some phenols are known to have a strong chemical affinity for iron and can bind free ferrous ions.

We previously reported that red wine extracts composed of complex polyphenols and tannins, isolated with a method that eliminates monomeric phenols (WCPT), inhibit chemically induced oxidative DNA damage *in vivo* in the rat liver and intestine (8, 9). We also demonstrated that a phenolic powder from red wine containing both monomeric and complex phenols (WE) could inhibit azoxymethane (AOM)-induced colon carcinogenesis in the rat (10).

In this study we tried to clarify the mechanism of action of WCPT and WE on oxidative DNA damage *in vitro*.

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Materials and methods

Chemicals and standards

Herring sperm DNA, GSH, ferric chloride, ferrous sulphate, cumene hydroperoxide (CumOOH), DMSO and deferoxamine (DFO) were obtained from Sigma (Sigma-Aldrich, Milan, Italy). Complex polyphenols and tannins from red wine (WCPT) and wine phenolic total extract (WE) were prepared as described in detail elsewhere (8–10). WCPT had the following constitutive units: 60.7 % epicatechin, 18.1 % catechins, 17.9 % epigallocatechin and 3.3 % epicatechin 3-O-gallate. The mean molecular weight of this mixture was calculated to be about 1000; therefore, we used 1.8, 3.5 and 18 µg/ml of WCPT and WE in order to reach approximate concentrations of 1.8, 3.5 and 18 µM, in the range of the those used in the literature for other polyphenols in *in vitro* assays (11, 12).

Oxidative DNA damage induced by GSH/Fe^{3+}

Herring sperm DNA (0.7 mg/ml, final concentration) was incubated in air for 2 h at 37 °C in the dark with the test substances in the presence of 3 µM $FeCl_3$, 15 µM GSH and 4 mM HEPES, pH 7, as reported by Park and Floyd (13). We considered the basal oxidative DNA damage in herring sperm as control values.

Oxidative DNA damage induced by CumOOH

Herring sperm DNA (0.7 mg/ml) was incubated in the presence of air at 37 °C for 2 h in the dark with 7.9–16 mM CumOOH in 4 mM HEPES pH 7.0. We considered the basal oxidative DNA damage in herring sperm as control values.

Measurement of 8-OHdG levels in DNA

After incubation with GSH/Fe^{3+} or CumOOH, with or without the addition of WCPT or WE, DNA was precipitated by ethanol in the presence of 20 % 10 M ammonium acetate, washed with 70 % ethanol, dried, dissolved in 20 mM acetate buffer pH 5.2, denatured at 90 °C for 3 min and digested to a nucleoside pool; 8-OHdG levels were measured with HPLC coupled with electrochemical detection as previously reported (8).

Statistics

Data are analyzed with one-way ANOVA using the Statgraphics Statistical Package (Statistical Graphic Corporation, Rockville, MD, USA). Differences were considered statistically significant when the probability levels *P was < 0.05 and **P < 0.01.

Results and discussion

In our experimental conditions, Fe^{3+} was reduced to Fe^{2+} by GSH, starting a free radical generating reaction which induced a 30-fold increase in 8-OHdG levels in co-incubated DNA, when compared to control levels. Incubation with CumOOH generated free radicals and it in turn induced a significant, although less dramatic, elevation of 8-OHdG levels in DNA (about 2.5-fold compared to control levels) (Table 1). When the amount of CumOOH was increased in the incubation mixture, the levels of 8-OHdG in DNA increased in a concentration-dependent manner (Table 1).

As a control of the reliability of the system for measuring oxidation damage and radical scavenging effects, we also tested the effect of DMSO, a solvent endowed with radical scavenging activity. DMSO reduced oxidation damage induced by GSH/Fe^{3+} and CumOOH (Table 1); on the contrary the iron chelator deferoxamine (DFO) inhibited only 8-OHdG formation induced by GSH/Fe^{3+}, but had no effect on CumOOH-induced DNA

Table 1 Effect of DMSO and deferoxamine (DFO) on DNA oxidation in vitro induced by Fe^{3+}/GSH and cumene hydroperoxide (CumOOH)

	Levels of 8-OHdG/dG x 10^{-5}			
	DNA	DNA + GSH 3 µM Fe^{3+}	DNA +	
	–		7.9 mM CumOOH	16 mM CumOOH
Control	5.0 ± 0.28	121.8 ± 11.7	10.0 ± 0.77	21.4 ± 1.5
DMSO (20 mM)	–	60.5 ± 5.2**	7.6 ± 0.58*	–
DMSO (200 mM)	–	35.0 ± 2.53**	6.0 ± 0.62*	12.0 ± 1.2**
DFO (2 mM)	–	5.9 ± 0.35**	9.4 ± 0.8	–

*P < 0.05 relative to control; ** P < 0.01 relative to control. DNA oxidation is expressed as levels of 8-OHdG/dG x 10^{-5}

damage. It appears that oxidative DNA damage catalyzed by iron can be reduced by agents that bind iron itself or scavenge free radicals; on the contrary oxidative DNA damage induced by CumOOH is insensitive to iron chelation and can be reduced only by radical scavengers.

WCPT and WE (3.6 and 18 µg/ml) strongly inhibited oxidative DNA damage induced by GSH/Fe^{3+}, whereas a smaller, although still significant, effect was observed at 1.8 µg/ml (Fig. 1). WCPT and WE (3.6 and 18 µg/ml) also protected against oxidative DNA damage induced by CumOOH (Fig. 2) while at concentration of 1.8 µg/ml no effect was found (data not shown). However, the effect of WCPT and WE on CumOOH-induced DNA damage was not so pronounced (about 20 %) compared with the reduction in iron-induced DNA damage (about 70 %) (Fig. 2).

These results demonstrate that the presence of monomeric phenols is not essential for protection against free radical damage. In fact, the fraction containing monomers (WE) had about the same activity compared to the polymeric fraction obtained from red wine (WCPT), demonstrating that antioxidant activity is not restricted to monomeric polyphenols such as (-)epigallocatechin gallate (14) or other flavanols (15).

Our results also indicate that the antioxidant effects are very striking in the presence of an iron-catalyzed oxidation system, indicating iron chelation as the main explanation for this effect. In the past some authors have indicated that the metal-chelating activity of polyphenols was a minor mechanism explaining their antioxidant action (11), a conclusion not shared by more recent studies (12, 16) and contradicted by the present results.

However, since a reduction in DNA damage induced by hydroxyl radicals generated by CumOOH was also observed, WE and WCPT have direct radical-scavenging activity too.

In conclusion, the *in vitro* activity of red wine polyphenols against DNA oxidation may explain their protective effect against induced-oxidative damage *in vivo* (8, 9) and, at least in part, the inhibitory activity against experimental colon azoxymethane (AOM) carcinogenesis (10), since 1,2-dimethylhydrazine, a precursor of AOM, a specific colon carcinogen with DNA-methylating activity, is also an inducer of DNA oxidative damage (7).

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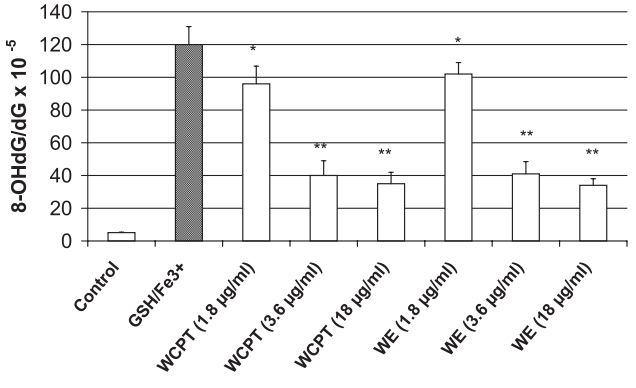

Fig. 1 Effect of WCPT (1.8, 3.6 and 18 µg/ml) and WE (1.8, 3.6 and 18 µg/ml) on 8-OHdG formation induced by GSH/ 3 µM Fe^{3+} in herring sperm DNA. Control values were obtained in the absence of GSH/3 µM Fe^{3+}. Each value is the mean of 8 individual determinations ± SEM. * $P < 0.05$ relative to controls; ** $P < 0.01$ relative to controls.

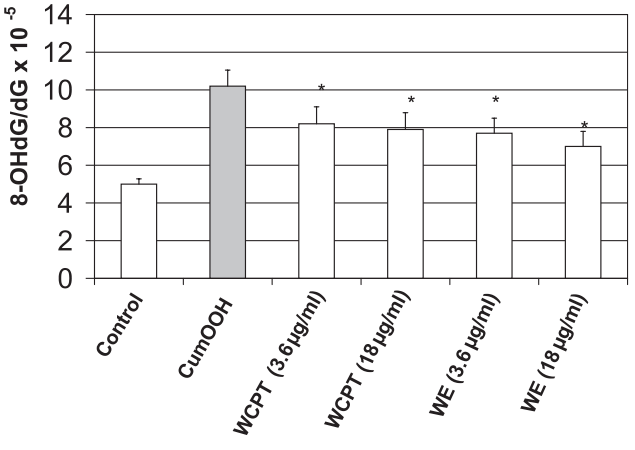

Fig. 2 Effect of WCPT and WE (3.6 and 18 µg/ml) on 8-OHdG formation induced by 7.9 mM CumOOH in herring sperm DNA. Control values were obtained in the absence of CumOOH. Each value is the mean of 8 individual determinations ± SEM. * $P < 0.05$ relative to controls.

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