Polyphenols synergistically inhibit oxidative stress in subjects given red and white wine

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Abstract

Aim of this study was to analyse the relationship between the plasma levels of polyphenols and the antioxidant activity of red and white wine. Twenty healthy subjects (HS) were randomly allocated to drink 300 ml of red (n = 10) or white (n = 10) wine for 15 days. Ten HS who refrained from any alcohol beverage for 15 days were used as control. Urinary PGF-2α-H2O2-III, a marker of oxidative stress and plasma levels of polyphenols were measured. Urinary PGF-2α-III significantly fell in subjects taking wine with a higher percentage decrease in subjects given red wine (−38.5 ± 6%, p < 0.001) than in those given white wine (−23.1 ± 6%). Subjects taking red wine had higher plasma polyphenols than those taking white wine (1.9 ± 0.6 μM versus 1.5 ± 0.33 μM, p < 0.001). Plasma polyphenols were inversely correlated with urinary PGF2α (r = 0.37, p < 0.001). No changes of urinary isoprostanes were observed in subjects who refrained from wine intake.

In vitro study demonstrated that only a mixture of polyphenols, all in a range corresponding to that found in human circulation, inhibited LDL oxidation and PKC-mediated NADPH oxidase activation. Such inhibitory effects were more marked using the concentrations of polyphenols detected in human circulation after red wine intake.

This study shows that red wine is more antioxidant than white wine in virtue of its higher content of polyphenols, an effect that may be dependent upon a synergism among polyphenols.

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1. Introduction

“French paradox” is a term coined in the last decade to indicate lower incidence of coronary heart disease (CHD) in France compared to other Western countries such as United States, despite similar intake of animal fat [1]. This phenomenon has been attributed to increased intake of wine, particularly red wine [1]. Accordingly, several epidemiological studies demonstrated an inverse relationship between consumption of alcohol beverage and CHD and a recent meta-analysis supported the view that light-to-moderate wine intake is associated with lower incidence of CHD [2–4]. In survivors from myocardial infarction, drinking about 2 drinks/day of ethanol (mostly wine) was associated with decrease in cardiovascular events [5]. The mechanism that underlies this alleged CHD protection of wine, however, remains debatable [6]. The difficulty is in part due to distinguish between the potential benefits of alcoholic versus non-alcoholic components of wine. It is also unclear if in human moderate intake of wine affects mechanisms implicated in the pathogenesis of atherosclerosis and its complications. There are data, for example to indicate that wine has an antiplatelet effect in experimental animals [7–9]; also,
in people who participate in the Lyon Diet Heart Study an inverse relationship between wine ethanol intake and platelet aggregation has been reported [10].

Antioxidant effect is another putative mechanism accounting for the cardioprotective effect of wine [1], but data concerning the antioxidant effect of wine in vivo are divergent [11]. The reason for these equivocal findings is unclear, however the methods to assess oxidative stress in vivo are often unreliable and this may partially explain discrepant data. Urinary excretion of isoprostanes, a class of eicosanoids derived from fatty acids interaction with oxygen free radicals, is considered a reliable method to assess oxidative stress in vivo [12]. So far there is only one study exploring the effect of wine on isoprostanes: the study showed no effect of red and white wine but a significant decrease of isoprostanes after supplementation with dehalcoholised red wine, suggesting that polyphenolic content of wine exerts an antioxidant effect [13]. To further explore the role of polyphenols on the antioxidant effect of wine, we undertook an intervention study that examined if the antioxidant effect of wine is dependent upon its polyphenolic content. As red wine has higher polyphenolic content than white wine [14], we compared the antioxidant effect of red and white wine supplementation to a population of healthy subjects. Also, we examined if the antioxidant effect of wines was dependent upon the plasma concentration of polyphenols. Finally, we performed in vitro study to analyse the mechanism through which polyphenols exert an antioxidant effect.

2. Materials and methods

2.1. Study design

We recruited 20 healthy volunteers who had no evidence of cardiovascular disease and had no risk factors for atherosclerosis such as hypertension, diabetes, dislipidaemia, obesity and smoking habit.

None of the subjects took antioxidant vitamins or other types of antioxidants or antiplatelet drugs in the month before the study.

All gave informed consent to participate in the study which was approved by the Ethical Committee of our University.

All subjects underwent a run-in period of 1 week during which they refrained from consuming wine or alcohol and NSAID and were asked for their dietary habit.

All subjects consumed a typical Mediterranean diet based on carbohydrates, olive oil, fruit and vegetables with no apparent difference in the amount of flavonoids consumed with the food: all subjects refrained from consuming chocolate, tea or coffee 1 week before and during the whole period of the study. After the run-in phase, the subjects were randomly allocated to consume a total of 300 mL/day of red (total polyphenolic content 1.8 g/L) (n = 10, males 4, females 6, mean age 45 ± 6 years) or white (total polyphenolic content 0.25 g/L) (n = 10, males 5, females 5, mean age 42 ± 3 years) wine during dinner. In order to exclude confounding factors related to the alcohol content of wine, red and white wine had the same percentage of alcohol (12.5%, v/v). Throughout the follow-up any other type of alcoholic drink was forbidden. At baseline and after 15 days of follow-up a blood sample was taken between 8 and 9 a.m. from each subject who had fasted for at least 12 h; therefore, the interval between the last drink and blood collection was 12 h. A bottle of wine (750 ml) was given to each subject every 2 days and the residual amount present in each bottle was measured to check for compliance. Apart from routine analysis, laboratory study consisted of measuring the urinary content of PGF2α-III, and the plasma concentrations of polyphenols and ethanol. The same protocol was applied to 10 healthy subjects (males 5, females 5, mean age 42 ± 3 years) having similar clinical characteristics but refraining from taking any alcohol beverage for 15 days.

2.2. Urinary eicosanoid assays

Urinary PGE2α-III was measured by previously described and validated EIA assay method [15,16]. Ten milliliter urine aliquots were extracted on a C-18 SPE column; the purification was tested for recovery by adding a radioactive tracer (tritiated PGE2α-III) (Cayman chemical). The eluates were dried under nitrogen, recovered with 1 ml of buffer, and assayed in a PGE2α-III specific EIA kit (Cayman chemical). PGE2α-III concentration was corrected for recovery and creatinine excretion and expressed as pg/mg of creatinine.

2.3. Conjugated dienes

The standard oxidation assay was performed using a Perkin-Elmer Lambda 4B UV/vis spectrometer.

The measurement of the 234 nm absorption was started and the absorption was measured at intervals of 10 min for a period of 1 h.

Oxidative modifications of LDL (50 μg protein/ml, equal to 0.1 μM) was performed by 0.1 mM cupric sulfate in presence and absence of a mixture of polyphenols corresponding to the mean values achieved after wine intake. For white wine the mixture was composed by: catechin (0.013 μM), caffeic acid (0.187 μM) and resveratrol (1.33 μM). For red wine the mix was composed by catechin (0.056 μM), caffeic acid (0.192 μM) and resveratrol (1.72 μM).

The absorbance changes with time (A/ΔA/min) were computed and the diene versus time profile was divided into three consecutive time phases: lag phase, propagation phase and decomposition phase.

The length of lag phase was determined as the intercept of the tangent with the extrapolated line for the slow reaction. The maximum rate of oxidation was derived from the slope of the tangent or the peak of ΔA/min curve. With a molar absorbance ε234nm, for conjugated dienes of 29,500 mol−1 cm−1, the rate in micromoles conjugated dienes formed per liter and minute (μM min−1) was given by (ΔA/min) × 33.8. In addition the maximum amount of dienes
performed in platelet homogenates according to Ref. [20].

2.4.4. Platelet NADPH oxidase activity

All non-specified products were provided by Sigma–Aldrich.

2.4.3. Ethanol detection

The assay solution contained 400 µl Tyrode buffer and 0.25 mM lucigenin. After preincubation at 37 °C for 3 min, the reaction was started by adding 100 µl of platelet homogenates in presence or less of AA 0.5 mM.

The chemiluminescent signal was expressed as counts per minute (cpm) for an average of 10 min and corrected by protein concentration (cpm/mg).

2.4.5. Superoxide anion (O2−) production

Superoxide anion (O2−) produced by platelets was measured by using lucigenin chemiluminescence method and dihydroethidium cytofluorimetric analysis. The chemiluminescence of lucigenin was detected with a Bio-Orbit 1251 luminometer.

Platelets (2 × 10⁹/ml final concentration) were incubated with or without the two polyphenols mixtures (30 min, 37 °C) and then stimulated with collagen 6 µg/ml. Each sample was added with 5 µM lucigenin, the chemiluminescence obtained at the third minute was measured and O2− production was expressed stimulation index (S.I. = mean level of stimulated platelet luminescence/average level of luminescence in unstimulated platelets) [20].

2.4.6. Phosphorylation of platelet proteins

The platelet suspensions (2 × 10⁹/ml) were incubated for 1 h at 37 °C with 32P (2 µCi/ml of cell suspension), separated from plasma proteins and from excess of 32Pi by centrifugation and suspended in Tyrode’s buffer containing 0.2% bovine serum albumin, 5 µM glucose and 10 mM Hepes, pH 7.35, adjusted to a final concentration of 2 × 10⁸ cells/ml.

32P-labelled platelets were preincubated with or without red or white polyphenols mixtures (30 min, 37 °C) and then stimulated with collagen (6 µg/ml); the reaction was stopped by addition of an equal volume of twice concentrate Laemmli’s buffer, followed by incubation at 95 °C for 5 min.

Protein samples were analysed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), for Western blotting, proteins were electrotransferred to nitrocellulose membranes.

The rate of protein kinase C (PKC) activation (expressed as phosphorylation of 47-kDa PKC-specific substrate) was analysed by autoradiography. The developed spots were calculated by densitometric analysis on a NIH Image 1.62f analyser, the amount of phosphorylation was determined by dividing the areas of the phosphorylated spots of stimulated platelets by the area of control unstimulated platelets; the value was expressed as decrement percentage of phosphorylation [20].

(µM) before onset of decomposition was calculated by the maximum increase of the absorbance according to A × 33.8 [17].

2.4. Plasma polyphenol measurement

The source of chemicals are included in each paragraph; all non-specified products were provided by Sigma–Aldrich.

2.4.1. Enzymatic treatment of plasma

Plasma samples were incubated with a hydrolysing solution to obtain free phenolic compounds by using the technique previously reported [18]. Briefly, 1.5 ml of plasma were mixed with 2 ml of 100 mM acetic acid buffer (pH 5.0) containing 4000 U glucuronidase plus 200 U sulfatase (Carlo Erba, Milan, Italy). The mixture was incubated for 1 h at 37 °C and then extracted three times with ethyl acetate (Carlo Erba). 3.0 ml ethyl acetate was added each time and the mixture was vortexed for 4 min. After centrifuging the mixture for 5 min at 3500 × g, the top layers were removed. The combined extracts were passed through anhydrous sodium sulfate and dried under nitrogen. Samples were stored at −70 °C until use.

2.4.2. HPLC configuration and analysis

The separation of free phenolic compounds was carried out as previously described [18]. Briefly, HPLC system consisted of a Perkin-Elmer series 410 LC Pump with a SEC-4 controller for gradient elution. Mobile phase consisted of two solutions: solution A was 0.22 M acetic acid (Carlo Erba), solution B was methanol (Carlo Erba). A binary gradient (ranging from 7 to 24%) was applied, at a flow-rate of 1 ml/min, to a Wakosil II 5C18 RS analytical column (5 µm, 150 mm × 4.6 mm i.d., SGE), endowed with a SGE 10 mm guard column and maintained at 30 °C.

The plasma extracts were re-dissolved just before the analysis in methanol, and 20 µl were injected in the system. The eluate was monitored with an electrochemical detector, Coulochem II (ESA, Bedford, MA, USA) equipped with an analytical cell model 5011. Settings were as follows: the first electrode (the analytical one) at +600 mV and the second electrode (the reference one) at +400 mV. The output of the detector was connected either to a fluorometer or to a SGE 10 mm guard column and maintained at 30 °C.

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(µM) before onset of decomposition was calculated by the maximum increase of the absorbance according to A × 33.8 [17].
2.4.7. Statistical analysis
Comparisons among groups taking red or white wine, before and after supplementation, and comparison with the control group, were carried out by one-way and repeated measures ANOVA and were replicated as appropriate with non-parametric tests (Wilcoxon and Kolmogorov–Smirnov (z) tests) in case of non-homogeneous variances as verified by Levene’s test. MANOVA with a Bonferroni test for multiple comparisons was applied in in vitro experiments. The correlation analysis was carried out by Pearson’s test. Data are presented as mean ± S.D. [21]. All calculations were made using personal computer software (Stat View II, Abacus Concepts, Berkley, California).

3. Results
At baseline urinary excretion of PGF2α-III was 340.2 ± 61 pg/mg creatinine in subjects allocated to red wine and 336.7 ± 58 pg/mg of creatinine in those allocated to white wine; there was no difference in PGF2α-III between the two groups. At the end of follow-up, in subjects given red wine and white wine urinary excretion of PGF2α-III decreased from 340.2 ± 61 to 216.5 ± 54 (p < 0.001) and from 336.7 ± 58 to 258.8 ± 62 (p < 0.001), respectively. The percentage decrease of PGF2α-III was significantly higher in subjects given red wine compared to those given white wine (−38.5 ± 6% versus −23.1 ± 6%, p < 0.001) (Fig. 1). Ethanol was not detected in the blood 12 h after the last drink (not shown). In subjects who refrained from taking any alcohol beverages, urinary excretion of PGF2α-III was 338.0 ± 54 pg/mg creatinine at baseline and 335.3 ± 54 pg/mg creatinine after 15 days of follow-up (p > 0.05).

At baseline, analysis of plasma polyphenols revealed the presence of three polyphenols, namely resveratrol, caffeic acid and catechin; we were unable to find detectable amount of quercetin or other polyphenols in human circulation. Resveratrol, caffeic acid and catechin significantly increased after intake of both white and red wine; however, subjects given red wine had higher plasma concentration of polyphenols compared to those given white wine (Table 1). At the end of wine intake, an inverse correlation between plasma concentration of polyphenols and urinary excretion of PGF2α-III was found (Fig. 2). No changes of lipid profile or other metabolic variables were observed in the groups at the end of follow-up (not shown).

In order to assess if polyphenols, that were detected in human blood, influenced LDL oxidation in vitro, native LDL were incubated with 1 μM of each poliphenol or with a mixture of polyphenols, all in a range <1 μM. Two mixtures were chosen on the basis of plasma polyphenol concentrations achieved after red or white wine intake. “Red wine mixture” and “white wine mixture” were the sum of the mean concentration of resveratrol, catechin and caffeic acid observed after red and white wine intake, respectively. While LDL oxidation was not influenced by up to 1 μM single polyphenol

### Table 1

<table>
<thead>
<tr>
<th>Flavonoids (μM)</th>
<th>White wine</th>
<th>Red wine</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Catechin</td>
<td>ND</td>
<td>0.013 ± 0.002</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>0.073 ± 0.01</td>
<td>0.187 ± 0.03</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>0.716 ± 0.03</td>
<td>1.33 ± 0.300</td>
</tr>
<tr>
<td>Total flavonoid content</td>
<td>0.791 ± 0.04</td>
<td>1.501 ± 0.337</td>
</tr>
</tbody>
</table>

* p < 0.02 ANOVA post hoc test.
§ p < 0.007 between the two groups after 15 days of wine consumption.
Fig. 3. Effect of catechin (0.013 μM), caffeic acid (0.187 μM) and resveratrol (1.33 μM) for “white wine mix” and catechin (0.056 μM), caffeic acid (0.192 μM) and resveratrol (1.72 μM) for “red wine mix” on copper-induced LDL oxidation (panel A), platelet $O_2^-$ production (panel B), platelet NADPH oxidase (RCU: relative chemiluminescence units) (panel C), and PKC activation (panel D). Data are expressed as mean ± S.D. of five experiments ( * $p < 0.01$, ** $p < 0.001$).

(data not shown), the mixtures of polyphenols significantly inhibited the formation of conjugated dienes, the extent of which was dependent on the concentration of the polyphenols in the mixture; thus “red mix” had more inhibitory effect than “white mix” (Fig. 3, panel A).

Then we investigated if polyphenols exerted an antioxidant effect also at cellular levels. We found that the two mixtures influenced collagen-induced platelet production of $O_2^-$, that, in fact, was inhibited in a dose-dependent manner, “red mix” having more antioxidant effect than “white mix” (Fig. 3, panel B). As we have previously demonstrated that platelet production of $O_2^-$ is generated via arachidonic acid-induced NADPH oxidase activation, we tested in vitro if polyphenols affected this platelet pathway [19]. Accordingly, with our study incubation of platelet with NADPH enhanced platelet production of $O_2^-$, that was inhibited dose-dependently by polyphenols (Fig. 3, panel C).

In order to investigate the mechanism through which polyphenols inhibited the activation of platelet NADPH oxidase, we tested in vitro if they influenced the activation of PKC. This study showed that polyphenols inhibited the activation of PKC in a dose-dependent manner (Fig. 3, panel D), so indicating that polyphenols inhibit platelet production of $O_2^-$ via PKC-dependent NADPH oxidase activation.

4. Discussion

In the present study we tested the hypothesis that wine exerts an antioxidant effect via its polyphenolic content. Comparison of the antioxidant effect of two types of wine, namely red and white wine, may be useful to explore this issue because the concentration of polyphenols is about 10-fold higher in red wine.

The result of the interventional study was that both wines exerted an antioxidant effect but the percentage inhibition of isoprostanes was higher in subjects given red wine. Also, plasma concentration of polyphenols increased after supplementation with both types of wines but the increase was significantly higher in subjects given red wine. Finally, we observed a significant inverse correlation between the plasma concentration of polyphenols and the urinary excretion of PGF2α, indicating that the higher is the plasma concentration of polyphenols the lower is the oxidative stress in human. Taken together these findings show that after wine intake the plasma concentration of polyphenols is related to its polyphenolic content and this results in a different inhibition rate of oxidative stress.

These data are apparently in contrast with the study performed by Abu-Amjha Caccetta et al. [13], who performed
Polyphenols may also behave as antioxidant agents by acting at cellular level. For instance, some polyphenols may inhibit the activation of xanthine oxidase, that is a producer of superoxide anion [31]. Other studies have focused the attention on NADPH oxidase, that is one of most important producer of superoxide anion in phagocytic and non-phagocytic cells [32] and demonstrated that also the expression of this enzyme may be down-regulated by polyphenols [33–35]. To further explore the interaction between NADPH oxidase and polyphenols we performed in vitro experiments with human platelets that possess the enzyme and produce ROS upon appropriate stimulus [36]. We observed that single polyphenol did not influence the production of superoxide anion, while the mixture of polyphenols dose-dependently inhibited oxidative stress with a mechanism involving the activation of NADPH oxidase. Then we investigated the mechanism through which polyphenols might down-regulate NADPH oxidase activity and focused our attention on PKC, an enzyme that activates NADPH oxidase via phosphorylation of p47 phox [37]. Actually, the effect of polyphenols such as quercetin, on PKC has been already studied but the concentration used was several orders of magnitude higher than that found in human circulation [9]. Using very low concentrations of polyphenols we observed that polyphenols dose-dependently inhibited PKC activation so suggesting that the inhibition of NADPH oxidase was PKC-mediated.

Epidemiologic studies have consistently shown that moderate alcohol consumption is associated with reduced risk of CHD but the mechanism is still not fully defined. The fact that different types of alcohol beverage are protective against CHD [38] suggests the existence of multiple mechanisms potentially accounting for its cardioprotective effect. The alcoholic component of alcohol beverage could, for instance, be cardioprotective by increasing HDL, or via its preconditioning-like protection [39,40]. As shown by the present study, the non-alcoholic component of alcohol beverage such as polyphenols, could limit LDL accumulation within atherosclerotic plaque via its antioxidant effect. Further study is necessary to assess if the different content of polyphenols in the alcohol beverage may be responsible for a different cardioprotective effect.

In conclusion, our study suggests that the wine exerts in human an antioxidant effect via its polyphenolic content. Polyphenols act via inhibition of LDL oxidation and, at cellular levels, down regulating PKC-mediated NADPH oxidase activation. This property, that is likely attributable to a synergism among the polyphenols contained in the wine, may be useful to develop novel antioxidant treatment to prevent CHD.

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References


