Alcohol-Free Red Wine Enhances Plasma Antioxidant Capacity in Humans[1]

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ABSTRACT Moderate wine consumption is reputed to exert a protective effect against coronary heart disease (CHD). The nature of the protective compounds is unclear and the mechanisms are incompletely understood. We studied whether the nonalcoholic component of wine increases plasma antioxidant capacity measured as total radical-trapping antioxidant parameter (TRAP), and whether such an effect is associated with the presence of phenolic compounds in plasma. The TRAP and plasma levels of phenolic compounds were measured in 10 healthy subjects after the ingestion of 113 mL of tap water (control) and alcohol-free red and white wine at 1-wk intervals. Both alcohol-free wines possessed an in vitro dose-dependent peroxyl-radical activity, but red wine, with a polyphenol concentration of 363 ± 48.0 mg/L quercetin equivalent (QE), was 20 times more active (40.0 ± 0.1 mmol/L) than white wine (1.9 ± 0.1 mmol/L), which has a polyphenol concentration of 31 ± 1 mg QE/L. The ingestion of alcohol-free red wine caused significant increases in plasma TRAP values and polyphenol concentrations 50 min after ingestion. Alcohol-free white wine and water had no effects on either of the plasma values. The parallel and prompt increase of antioxidant status and of circulating levels of polyphenols in fasting subjects after bolus ingestion of a moderate amount of alcohol-free red wine suggests that polyphenols are absorbed in the upper gastrointestinal tract and might be directly involved in the in vivo antioxidant defenses. J. Nutr. 128: 1003–1007, 1998.

KEY WORDS: • *red wine* • *antioxidants* • *total polyphenols* • *total antioxidant potential* • *humans*

Many epidemiological studies have reported an inverse correlation between moderate alcohol consumption and coronary heart disease (CHD)[3] (Friedman and Kimball 1986, Lazarus et al. 1991, Rimm et al. 1991). The relation between alcohol consumption and risk of cardiovascular disease has been described as a J-shaped curve (Gronbaek et al. 1994, Stampfer et al. 1988), with the lowest risk attributed to light drinkers and the highest to nondrinkers and heavy drinkers. These observations have tended to characterize light drinking as protective. The positive effects of moderate quantities of alcohol were attributed in part to an increase in plasma levels of HDL (Gaziano et al. 1993, Sillanaukee et al. 1993, Taskinen et al. 1987) and to an inhibition of platelet aggregation (Demrow et al. 1995, Folts et al. 1994) through a reduction in the thrombotic tendency (Renaud and de Lorgeril 1992). Alcohol intake has also been shown to affect platelet activity indirectly, through an HDL-mediated stimulation of the production of prostacyclin, a potent vasodilator and platelet anti-aggregator (Guivernau et al. 1989). There are, however, also contrary effects of alcohol. Ethanol metabolism is responsible for generation of free radicals (Situnayake et al. 1990); it may enhance lipid peroxidation and may reduce glutathione levels in hepatic cells (Videla and Valuenzela 1982).

There is no clear evidence that one type of alcoholic beverage is more protective than another, although Friedman and Kimball (1986) reported that beer and wine were associated with a reduction in CHD and that this reduction was greater than that for spirits in nonsmokers. Recently Gronbaek et al. (1995) showed that light and moderate wine drinking was strongly associated with lower mortality from CHD and other causes, whereas a similar intake of alcohol from spirits led to increased risk.

Wine is an important component in Mediterranean dietary traditions; it is claimed to contribute to the explanation of the "French paradox" (Frankel et al. 1993, Renaud and de Lorgeril 1992), i.e., the fact that some regions of France have lower mortality rates for CHD than North-European countries although exposed to similar CHD risk factors (dietary fat intake or plasma cholesterol). Red wine has been shown ex vivo to inhibit LDL oxidation (Kondo et al. 1994), increase antioxidant capacity in humans (Whitehead et al. 1995), reduce the susceptibility of human plasma to lipid peroxidation (Furhman et al. 1995) and raise plasma levels of HDL cholesterol and apolipoprotein A-I (Lavy et al. 1994). In all of these studies, the administration of white wine with the same alcohol content (Furhman et al. 1995, Lavy et al. 1994, Whitehead et al. 1995), or pure alcohol (Kondo et al. 1994) failed to show any protective effect.

The question, however, remains unanswered: is the nonalcoholic fraction of wine, represented mainly by phenolic compounds, the primary factor responsible for this protective effect?

We investigated the hypothesis that the ingestion of alcohol-free red wine increases antioxidant potential in humans,

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[3] Abbreviations used: ABAP, 2,2′-diazobis (2-amidinopropane) dihydrochloride; CHD, coronary heart disease; MPA, metaphosphoric acid; QE, quercitin equivalent; TRAP, total radical-trapping antioxidant parameter; Trolox, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid.

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and that this effect is due to the prompt absorption of the wine's polyphenol fraction. The purpose of the study was to evaluate the effect of the nonalcoholic fraction of red and white wines on the total radical-trapping antioxidant parameter (TRAP) and on the total plasma levels of polyphenols in vivo in humans.

SUBJECTS AND METHODS

Subjects. Ten healthy volunteers (six women and four men), aged 25–50 y, nonsmokers, were selected after ascertaining that they had not been taking vitamin supplements and/or drugs known to interfere with intestinal absorption and/or the P450 enzymatic system. Each subject fasted beginning 12 h before reporting to the laboratory in the morning, and drank 113 mL of dealcoholated red wine. This amount corresponds to 300 mL of non-dealcoholated wine. The experiment was repeated after 1 wk on the same subjects using dealcoholated white wine, and 1 wk later using tap water (control).

The subjects maintained their usual diet throughout the 2 wk of the study. Venous blood samples were obtained by antecubital venipuncture, by vacutainer EDTA Na^+ at base line and at 30, 50 and 120 min after wine ingestion. The blood samples were immediately centrifuged at 12,000 × *g* for 3 min, avoiding unnecessary exposure to light. An aliquot of the plasma was immediately stabilized with metaphosphoric acid (MPA) 0.75 mol/L (BDH Laboratory Supplies Poole BH15 1 TD, UK) for vitamin C analysis (Deutsch and Weeks 1965). Plasma samples were stored at −80°C until analysis.

The study protocol was approved by the Human Ethics Committee of the National Institute of Nutrition and written informed consent was obtained from each volunteer.

Wine samples. One red wine ("Chianti Classico Rocca Castagnoli" Farm, Siena, Italy), and one white wine ("Valdadige" bottled by Terre Fredde, Trento, Italy) were used in the study. Red wine was chosen because it is richer than white wine in polyphenols, which have strong in vitro antioxidant activity (Frankel et al. 1995, Macheix et al. 1990). To separate the antioxidant effect of pure alcohol from that of the nonalcoholic fraction, wines were dealcoholized before the tests in a rotary evaporator at 25°C for 4 h. To avoid mechanical stress, the vacuum was applied progressively and gradually up to −3 MPa. Each of the two wines was processed in a large single batch sufficient for all of the in vitro and in vivo experiments.

Chemicals and reagents. The HPLC-grade methanol and acetone were produced by Carlo Erba, Milan, Italy. All other chemicals came from Sigma Chemical, St Louis, MO, unless otherwise stated. 6-Hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) came from Aldrich Chemical (Milwaukee, WI). 2,2′-Diazobis (2-amidinopropane) dihydrochloride (ABAP) came from Wako Pure Chemical Industries (Osaka, Japan).

Solutions and reagents for TRAP assays were made with the use of Milli-Q (Millipore, Bedford, MA) double-distilled water (resistance > 18 mW/cm^2) and passed through a Chelex 100 resin Na^+ form.

Total phenolic compound concentrations in plasma and wine. Total phenolic compound concentrations in plasma and wine were measured by the Folin-Ciocalteau method (Swain and Hills 1959), modified to remove protein interferences. Total phenolic compounds were determined after a procedure of extraction/hydrolysis, precipitating protein with 0.75 mol/L MPA. For hydrolyzing the conjugated forms of polyphenols, 1 mL of 1.0 mol/L Hcl was added to 500 μL of the sample (plasma or diluted wine), vigorously vortexed for 60 s and incubated at 37°C for 30 min. Later, 1.0 mL of 2.0 mol/L NaOH in 75% methanol was added, and the resulting mixture vortexed for 3 min and incubated at 37°C for 30 min. This step breaks the links of polyphenols with lipids and provides a first extraction of polyphenols. Then, 1.0 mL of 0.75 mol/L MPA was added after mixing for 3 min to remove plasma proteins; the sample was centrifuged at 1500 × *g* for 10 min. The supernatant was removed and kept on ice in the dark, while polyphenols were extracted again by adding 1.0 mL of a 1:1 (v/v) solution of acetone:water and centrifuged for 10 min at 2700 × *g*. The two supernatants were combined and filtered through a HV 0.45-μm filter (Millipore SJHV004NS). Samples (200 μL) were assayed for total polyphenols with the Folin-Ciocalteau reagent. The results were expressed as milligrams quercetin equivalent (QE) per liter.

The method was validated in plasma by performing a recovery test, adding increasing amounts of quercetin to a plasma sample. The recovery percentage was of 92 ± 7 (n = 12). Quercetin response was linear in the range from 0.72 to 9.0 mg/L (r = 0.999) (data not shown). The estimated limits of detection were 0.72 mg/L of quercetin.

The reproducibility of the method was assessed by measuring three plasma samples at different concentrations of quercetin (0.72, 1.43 and 2.86 mg/L). The average CV percentages were 2.6, 1.0 and 2.0% for the within-day variation (triplicate analysis) and 11.2, 10.2 and 8.5% for between-day variation (9 d in duplicate analysis), respectively.

The effect of time of storage on total polyphenol content of plasma was assessed by measuring total polyphenols immediately after blood withdrawal and at different times of storage at −80°C. We did not observe any significant variation in total plasma levels of polyphenols stored at −80°, confirming previous results (de Rijke et al. 1996).

Samples were assayed in duplicate and protein content was determined in each sample (Layne 1957). The amount of protein in each sample was negligible (data not shown).

Total radical-trapping antioxidant potential: TRAP assay. The method employed to assess the in vitro and in vivo TRAP of wine was developed in our laboratory (Ghiselli et al. 1995). It is based on the protection afforded by plasma, or any other substance, against the decay of a fluorescent target, R-phycoerythrin (R-PE), during a controlled peroxidation reaction. The reaction mixture is 1.5×10^{-8} mol/L R-PE in 75 mmol/L phosphate buffer, pH 7.0. In this study, alcohol-free wines (neutralized at pH 7.4 with NaOH) or human plasma was added to the reaction mixture to obtain a final volume of 2.0 mL and pre-incubated at 37° C for 5 min in 10-mm quartz fluorometer cells. The oxidation reaction was started by adding ABAP to a final concentration of 4.0 mmol/L. The decay of R-PE fluorescence was monitored every 5 min for 90 min on a Perkin-Elmer (Norwalk, CT) LS-5 Luminescence Spectrometer equipped with a thermostatically controlled cell-holder. The monochromators operated at an excitation wavelength of 495 nm/5 nm slit width and at an emission wavelength 575 nm/5 nm slit width. All TRAP assays were performed immediately after plasma collection. The results were standardized using Trolox, a water-soluble analog of α-tocopherol and, according to Wainer et al. (1985), expressed as micromoles of peroxyl radicals trapped per liter of plasma.

Plasma antioxidants. Plasma carotenoids (lutein + zeaxanthin, lycopene, β-cryptoxanthin, α- and β-carotene), and α-tocopherol were simultaneously determined by HPLC (Maiani et al. 1989). Plasma samples (200 μL) were deproteinized by the addition of ethanol, containing ascorbic acid (5 g/L) and *d*-α-tocopherol acetate (<1 mg/L) as an internal standard. The mixture was vortexed and then extracted twice with hexane containing butylated hydroxytoluene (0.1 g/L). The combined hexane layers were evaporated completely. The residues were reconstituted in 50 μL diethyl ether and 150 μL of mobile phase (methanol/acetonitrile/tetrahydrofuran, 50:45:5, v/v/v) to bring the samples up to their original volume; 50 μL were injected by an autosampler into a reversed-phase C_{18} column and eluted isocratically at a flow rate of 1 mL/min. The peaks were detected with a variable spectrophotometer detector (Perkin-Elmer L. C. 95) connected to a personal computer (PE Nelson 1020, Perkin-Elmer).

Ascorbic acid was determined by the microfluorimetric method (Deutsch and Weeks 1965). The uric acid assay was performed by the enzymatic colorimetric test, Peridochrom (Boehringer, Mannheim GmbH, Germany). Plasma sulphydryl groups were measured immediately after plasma collection by the method described by Ellman (1959).

Plasma antioxidants and total polyphenols were assayed within 3 months of blood sampling.

The precision and reproducibility of the specific methods were monitored by using reference material (SRM 968) from the National Institute of Standards and Technology (Gaithersburg, MD). SRM 968 includes a known concentration of plasma β-carotene, retinol and α-tocopherol. The precision, expressed as a CV was 7.0 and 3.0 for β-carotene and α-tocopherol, respectively. A pool of human sera was utilized for the quality control of the ascorbic acid, and a commercial control was used for uric acid. The precision was 4.1 for ascorbic

TABLE 1

Total phenolic compound concentrations and total radical-trapping antioxidant parameter (TRAP) of alcohol-free red and white wines[1]

	Phenolic compound	TRAP[2]	Regression[3]
	mg QE/L[4]	*mmol/L*	*r*
Alcohol-free red wine	3636 ± 48	40.0 ± 0.1	0.999
Alcohol-free white wine	31 ± 1	1.9 ± 0.1	0.998

[1] Values are means ± SEM of three separate experiments.
[2] TRAP value is expressed as millimoles of peroxyl radicals trapped per liter of wine.
[3] Regression coefficient (*r*) of linear regression of lag-phase vs. 2, 4 and 8 μL of diluted alcohol-free red 1:200 (v/v) and white 1:2 (v/v) wine.
[4] QE, quercetin equivalents.

acid and 1.4 for uric acid. The reproducibility was 1.7 and 12.4 for uric acid and β-carotene, respectively.

Statistics. Statistical analyses (paired *t* test and linear regression) were performed using Statview II (Abacus Concepts, Berkeley, CA) software for Macintosh. The data are expressed as means ± SEM. A *P*-value < 0.05 was considered significant.

RESULTS

In vitro study. **Table 1** shows the phenolic compound concentrations and the in vitro TRAP of the two alcohol-free wines. The total phenolic concentration of red wine was, as expected, much higher than that of white wine. The total radical-trapping antioxidant capacity of red wine was at least 20 times stronger than that of the white wine. The regression between increasing amounts of wines and the length of lag-phase was significant for both wines, with correlation coefficients close to 1 ($r = 0.999$ and $r = 0.998$) (Table 1).

In vivo study. **Table 2** describes the time trend of plasma TRAP levels after the ingestion of alcohol-free wine. Base-line TRAP values gradually rose in subjects who drank red wine, reaching a peak 50 min after ingestion ($P < 0.004$) and falling back to base line after 2 h. The plasma TRAP did not change when subjects drank white wine or water (Table 2).

TABLE 2

Plasma total radical-trapping antioxidant parameter (TRAP) levels before and after drinking 113 mL of water and alcohol-free red or white wine in human subjects[1,2]

	TRAP		
Time, *min*	Water	Alcohol-free red wine	Alcohol-free white wine
		μmol/L	
0	1130 ± 46	1108 ± 48	1241 ± 107
30	1142 ± 25	1157 ± 57	1166 ± 83
50	1145 ± 41	1267 ± 56**	1164 ± 79
120	1145 ± 47	1114 ± 51	1226 ± 103

[1] Values are means ± SEM, $n = 10$ for alcohol-free red and white wine and $n = 9$ for water.
[2] TRAP value is expressed as micromoles of peroxyl radicals trapped per liter of plasma.
** Significantly different ($P < 0.004$) from time 0.

TABLE 3

Plasma total polyphenols concentrations before and after drinking 113 mL of water or alcohol-free red or white wine in human subjects[1]

	Total polyphenols		
Time, *min*	Water	Alcohol-free red wine	Alcohol-free white wine
		mg QE/L plasma[2]	
0	6.99 ± 0.74	7.17 ± 0.41	7.06 ± 0.54
30	7.07 ± 0.57	8.52 ± 0.80	7.43 ± 0.46
50	7.01 ± 0.54	10.15 ± 1.21*	7.51 ± 0.55
120	7.01 ± 0.58	8.48 ± 0.93	7.01 ± 0.39

[1] Values are means ± SEM, $n = 10$ for alcohol-free red and white wine and $n = 9$ for water.
[2] QE, quercetin equivalents.
* Significantly different ($P < 0.05$) from time 0.

To evaluate whether the ingestion of alcohol-free wines affected the antioxidant capacity of plasma via changes in other plasma antioxidants, we measured the circulating levels of vitamin C, vitamin E, thiol groups, uric acid and five carotenoids. Antioxidant concentrations were unaffected by the ingestion of water, alcohol-free red wine or alcohol-free white wine (data not shown).

Base-line plasma polyphenol levels increased after ingestion of alcohol-free red wine, reaching their highest value after 50 min ($P < 0.05$) and decreasing after 2 h (**Table 3**). Plasma levels of polyphenols did not change when subjects drank white wine or water.

DISCUSSION

For more than 20 years, evidence has accumulated that drinkers of alcohol in moderate quantities have lower CHD mortality rates than nondrinkers (Friedman and Kimball 1986, Lazarus et al. 1991, Rimm et al. 1991). The epidemiologic data are confusing as to which alcoholic beverage is more protective, but evidence suggests that it may be wine (Gronbaek et al. 1995). It has been claimed that wine might indeed be one of the most active ingredients involved in the protective effect of the Mediterranean diet and might contribute to an explanation for the "French Paradox," the apparent compatibility of a diet rich in saturated fats with a low incidence of mortality for CHD in some regions of France. It is not clear which component of wine is responsible for these protective effects, although recent evidence has proposed that the active compound may not be alcohol itself.

To explain the mechanism by which wine could exert a beneficial effect, attention has recently been directed to the nonalcoholic fraction of wine, represented mainly by phenolic compounds. The role that phenolic compounds are thought to have in the prevention of oxidative stress–linked diseases is associated mainly with their antioxidant capability. Earlier epidemiologic evidence forthcoming from Hertog et al. (1993), showed that those subjects in a group of adults who were in the highest tertile for flavonoid intake had about three times less risk of dying from cardiovascular diseases than those with the lowest intake. A number of flavonoids have been found to possess antioxidant and free radical–scavenging properties (Hanasaky et al. 1994, Morel et al. 1994) and to be able to inhibit LDL oxidation (Negré-Salvayre and Salvayre 1992,

Mangiapane et al. 1992). They can also inhibit several key enzymes in cellular systems involved in the generation of reactive oxygen species, including prostaglandin cyclooxygenase and lipoxygenase (Laughton et al. 1991, Takahama et al. 1985).

We have shown that alcohol-free red wine displays a stronger in vitro antioxidant activity than alcohol-free white wine. The only consistent difference between red and white wines is the phenolic content, which is 20 times higher in red wine (Frankel et al. 1995, Macheix et al. 1990). Our findings suggest that the in vitro peroxyl-radical–scavenging activity of alcohol-free wines is closely related to their phenol content. Therefore the stronger antioxidant activity of alcohol-free red wine is proportional to its higher phenolic compound concentration.

In vivo studies showed that the antioxidant potential in humans responds to the oral ingestion of phenol-rich beverages such as red wine (Whitehead et al. 1995) and tea (Serafini et al. 1996). These responses were believed to be elicited by the rapid absorption into the circulation of polyphenols (Serafini et al. 1996). Furhman et al. (1995) expanded these findings, showing that the reduced susceptibility of LDL to oxidation after dietary consumption of red wine was associated with an increase in total polyphenol levels in plasma LDL.

We have demonstrated for the first time that the nonalcoholic fraction of red wine does significantly raise total plasma antioxidant capacity and that this increase is paralleled by a concomitant increase in plasma concentrations of phenolic compounds. On the basis of in vitro measure of TRAP, we calculated that an increase of 2.5 mg/L of quercetin raises TRAP by ~140 μmol/L. We found that 50 min after red wine ingestion, total plasma polyphenols increase by 2.98 mg QE/L. At that time, TRAP was ~159 μmol/L greater than at time zero, which is in line with the in vitro calculation. Thus we can reasonably speculate that the changes in plasma antioxidant potential may be entirely attributable to the phenolic fraction of red wine. The lack of change in plasma phenolic compounds and antioxidant potential after the ingestion of alcohol-free white wine can be explained by the very low concentration of phenolic compounds in white wine.

Information on the metabolic fate of ingested polyphenols is very limited and contradictory (Das 1971, Gugler et al. 1975, Hackett and Griffiths 1983, Hollman et al. 1995, Unno and Takeo 1995). Direct evidence that flavonoids are absorbed by humans is provided by Maiani et al. (1997), who showed the appearance of epigallocatechingallate in plasma 30 min after the ingestion of 300 mL of green tea. Our results show that plasma polyphenols peak 50 min after wine drinking, suggesting that the absorption of polyphenols might occur, under the fasting conditions of our experiment, mainly in the gastroduodenal region and, in lesser amounts, in the jejunum. What happens to the dietary phenols that escape absorption in these regions and progress toward the colon is still unclear, but their possible intraluminal antioxidative effect should not be neglected.

In another paper, we reported that the ethanol moiety of wine is likely to improve the absorption of phenolic compounds by reducing their interaction with salivary proteins (Serafini et al. 1997), suggesting a specific and separate role for the alcoholic fraction of wine. Thus it can be argued that the in vivo response to wine drinking under everyday conditions might be even greater than that observed in this study with alcohol-free wine.

Our findings shed further light on the nature of the beneficial effects of moderate wine consumption. This is in line with epidemiologic evidence and gives supporting evidence for the recommendation that wine, in moderate quantities, should be consumed with meals on the basis of its protective effects (Willet et al. 1991).

In conclusion, although more information on the bioavailability of phenolic compounds is necessary, our results suggest that the ingestion of plant foods and products, such as red wine, may modulate redox status by providing a vast array of bioactive compounds.

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