

# Consumption of red wine polyphenols reduces the susceptibility of low-density lipoproteins to oxidation in vivo<sup>1-4</sup>

Shailja V Nigdikar, Norman R Williams, Bruce A Griffin, and Alan N Howard

See corresponding editorial on page 220.

**ABSTRACT** Red wine polyphenols (RWPPs) were obtained from red wine by absorption and elution from a resin column. Red wine (375 mL/d), white wine (375 mL/d), RWPPs (1 g/d, equivalent to 375 mL red wine/d) in capsules, RWPPs (1 g/d) dissolved in white wine, or a control alcoholic drink (40 g ethanol/d) was given to groups of 6–9 healthy men for 2 wk. Plasma LDL was separated by ultracentrifugation and desalted by dialyzing against a phosphate buffer without EDTA. In the copper-catalyzed peroxidation of LDL (copper-diene assay), the mean lag time increased by 17.8 min after red wine, 14.2 min after RWPP capsules, and 11.7 min after RWPPs in white wine. These groups also showed decreases in thiobarbituric acid-reactive substances, lipid peroxides, and conjugated dienes and increases in plasma and LDL polyphenols. The only change with white wine was an increase in thiobarbituric acid-reactive substances; there were no changes after the control drink. In a second study, RWPPs (1 and 2 g/d) and vitamin E [1000 IU (671 mg)/d] were given for 2 wk. In the copper-diene assay the addition of 10  $\mu$ mol EDTA/L abolished the increased lag time of 17.7 min seen with 1 g RWPP/d and changed the increased lag time from 13.2 to 4.5 min seen with 2 g RWPP/d. Vitamin E increased lag time by 67.6 min with dialysis without EDTA and by 50.5 min with EDTA. When the column method was used for desalting LDL, all 3 treatments produced an increase in lag time. The failure of some authors to obtain antioxidant effects with the consumption of red wine may be due to the differing techniques. *Am J Clin Nutr* 1998;68:258–65.

**KEY WORDS** Lipid peroxidation, red wine, white wine, polyphenols, LDL, men, antioxidants

## INTRODUCTION

A moderate consumption of alcohol-containing beverages decreases mortality (1–4) from coronary artery disease (CAD) and one mechanism of protection may be the increased plasma concentration of HDL cholesterol (5). It was proposed that the so-called French paradox, the low CAD mortality in France despite a diet high in saturated fat, can be explained by the population's high consumption of wine (6). A moderate intake of wine is associated with lower mortality from CAD compared with spirits or beer (7–9). A major question is whether red wine is superior to other alcoholic beverages in its putative cardioprotective effect.

Red wine but not white wine contains abundant polyphenols, which inhibit the oxidation of human LDL in vitro (10). Oxidation of the polyunsaturated lipid components of LDL with reactive oxygen species may have a role in atherosclerosis (11). Whether wine polyphenols can reduce the susceptibility of LDL to oxidation in vivo is controversial. An Israeli group (12) claimed that the consumption of red wine but not white wine by volunteers reduced the propensity of LDL to undergo lipid peroxidation (in response to copper ions), determined by decreases in the content of thiobarbituric acid-reactive substances (TBARS), lipid peroxides, and conjugated dienes in LDL as well as a substantial prolongation of the lag phase before oxidation. The antioxidant effects were not secondary to changes in plasma vitamin E or  $\beta$ -carotene content but were related to the elevation in polyphenol concentrations in plasma and LDL. A Dutch group (13) examined the effect of the nonalcoholic components of red wine by reducing its alcohol content. The dealcoholized red wine did not affect the susceptibility of LDL to copper-mediated oxidative modification as measured by lag time before and after red or white wine consumption. The results of the Dutch study did not show a beneficial effect of red wine consumption on LDL oxidation. The design of the 2 studies differed considerably, the most important difference being the method used for the copper-catalyzed peroxidation.

The present study was designed to investigate the discrepancies in results reported by the 2 groups. The study protocol used by the Israeli group was followed as much as possible.

<sup>1</sup>From the COAG Laboratory, Papworth Hospital, Papworth Everard, Cambridge, United Kingdom; The Center for Nutrition and Food Safety, School of Biological Sciences, University of Surrey, Guildford, United Kingdom; and the Howard Foundation, Whitehill House, Great Shelford, Cambridge, United Kingdom.

<sup>2</sup>Presented in part previously in abstract form in *Proc Nutr Soc* 1997;56:288A.

<sup>3</sup>Supported by a grant from the Howard Foundation, Cambridge, United Kingdom. Nutriproducts Ltd (Grandview, Leeds, United Kingdom) donated the red wine polyphenols.

<sup>4</sup>Address reprint requests to AN Howard, Whitehill House, Granhams Road, Great Shelford, Cambridge CB2 5JY, United Kingdom. E-mail: Howard\_Foundation@whcambdemon.co.uk.

Received September 30, 1997.

Accepted for publication March 2, 1998.

Different methods for removing EDTA from LDL preparations before oxidation were also compared. In addition, total polyphenols were extracted as a powder from the same red wine used in our study and were then given to volunteers to investigate the importance of wine polyphenols as antioxidants in the absence of alcohol.

## SUBJECTS AND METHODS

### Subjects

The study protocol was approved by the local research ethics committee of the Papworth Hospital NHS Trust. All subjects signed informed consent documents. Thirty healthy men aged 35–65 y who were nonsmokers, normolipemic, nonobese, and consuming a standard UK diet participated in the study. The volunteers discontinued wine consumption 2 wk before each study. All volunteers were asked to maintain their usual diet and lifestyle.

### Study 1: comparison of red and white wines, red wine polyphenols, and the control alcoholic drink

The volunteers were divided into groups of 6–9 subjects and given daily for 2 wk the following: 1) French *Cabernet sauvignon* red wine (1/2 bottle, 375 mL); 2) French vin ordinaire white wine (1/2 bottle, 375 mL); 3) French vin ordinaire white wine (1/2 bottle, 375 mL) plus 1 g red wine polyphenols obtained as described below; 4) red wine polyphenols as a powder (*see below*), 1 g in 2 gelatin capsules; and 5) a control drink (400 mL/d) containing 10% (by vol) ethanol as vodka and lemonade (containing no polyphenols) obtained from a supermarket. For practical reasons only 6 subjects were studied per week. They were allocated randomly to the various groups with a period of  $\geq 4$  wk between each treatment period.

### Study 2: comparison of red wine polyphenols and vitamin E

Twenty of the above mentioned volunteers from the previous study were divided into groups of 6–9 subjects and were given daily for 2 wk the following: 1) a black currant-flavored drink containing 1 g red wine polyphenols [The drink was a commercially available drink powder containing sugar, citric acid, sodium citrate aspartame, and synthetic flavor (Cambridge Manufacturing Co Ltd, Corby, United Kingdom) to which 330 mL water was added immediately before consumption.]; 2) capsules containing 2 g red wine polyphenols/d as a powder, prepared as described below; and 3) 1000 IU (671 mg) *RRR*- $\alpha$ -tocopherol/d (Lane Health Products Ltd, Gloucester, United Kingdom).

Six subjects were studied weekly and were allocated randomly to either receive the black currant-flavored drink or the capsules containing 2 g red wine polyphenols/d with a period of 4 wk between each treatment period. Subjects given 1000 IU *RRR*- $\alpha$ -tocopherol/d were treated at the end of the study because vitamin E can be retained in LDL for many weeks after a dose is administered. The 2 studies took place in sequence over 9 mo and the products were divided equally and consumed after lunch and dinner.

### Preparation of red wine polyphenols

The red wine polyphenols were manufactured by Nutriproducts Ltd (Grandview, Leeds, United Kingdom). Their method of manufacture involves the absorption and removal of the polyphenols by an absorbent resin column from the same *Cabernet sauvignon* red wine described above. Red wine was filtered to remove sedi-

**TABLE 1**

Intake of phenolic compounds from red wine (375 mL) and red wine polyphenols

Peak number and wine phenolic compounds <sup>1</sup>	Red wine <sup>2</sup>	Polyphenol powder <sup>3</sup>
	mg/d	mg · g <sup>-1</sup> · d <sup>-1</sup>
1. Catechin	8	11
2. Epicatechin	6	7
3. Procyanidin dimers	18	12
4. Procyanidin polymers	170	190
5. Quercetin glycosides	3	1.7
6. Quercetin	0.4	1.8
7. Myricetin glycosides	0.8	1.1
8. Myricetin	0.4	0.4
9. Anthocyanin glycosides <sup>4</sup>	4.5	56
10. Polymeric anthocyanidins	8	9
11. Gallic acid	13	0.8
12. Other phenolic acids <sup>5</sup>	13	2
13. <i>trans</i> Resveratrol	3	3
14. <i>cis</i> Resveratrol	0.8	0.3
Total	248	296

<sup>1</sup>Peaks 1–4 are quantified as catechin, peaks 6 and 8 as quercetin, peaks 5 and 7 as quercetin, peaks 9 and 10 as malvidin, peaks 11 and 12 as gallic acid, and peaks 13 and 14 as *trans* resveratrol.

<sup>2</sup>Vintage 1993 kept for 2 y.

<sup>3</sup>Prepared from the vintage 1993 red wine before bottling and kept at 5°C.

<sup>4</sup>Delphinidin, cyanidin, peonidin, petunidin, and malvidin glucosides.

<sup>5</sup>Syringic, caftaric, caffeic, *para*-coumaric, and coumaric acids.

ment, distilled under vacuum at a pressure of  $30 \times 10^3$  Pa at 75–80°C for 1 min, cooled and concentrated under vacuum at 55°C, and then cooled quickly to 25°C by refrigeration. The concentrated wine was passed through an absorbent polystyrene resin column Diaion HP-20 Relite (Supelco Ltd, Poole, United Kingdom), which was then washed with distilled water. The polyphenols were eluted with 45–50% ethanol. The eluate was then concentrated to 35% dry matter under vacuum distillation at 55°C and spray-dried under nitrogen to produce a powder with a moisture content of 3–4%. The polyphenol powder had a dark red color, was soluble in water or aqueous alcohol, and was quite palatable but with an astringency similar to that of red wine.

### Polyphenol contents and intake

The Folin-Ciocalteu method (14) was used to determine total polyphenols (in gallic acid equivalents): red wine, 1.6 g/L; white wine, 0.2 g/L; red wine polyphenol powder, 450 mg/g; white wine containing red wine polyphenol powder, 1.8 g/L; and alcoholic drink, 0 g/L.

The red wine and red wine polyphenol powder from which it was obtained were analyzed by ETS Laboratories (St Helena, CA). One gram of the powder was dissolved in 25 mL methanol, diluted 1:1 with deionized water, centrifuged at  $10000 \times g$  for 3 min at room temperature, and injected into an HPLC apparatus according to a method that quantifies some of the major constituents in wine (15). Undiluted red wine was also injected for similar analysis. The intake (mg/d) of phenolic compounds from the 375 mL red wine and the 1 g red wine phenols used in study 1 are presented in **Table 1**.

The chief differences between the wine and powder were that the powder contained virtually no phenolic acids because they

were not extracted by the resin column used for its preparation and the powder contained  $\approx 12$  times more anthocyanin glycosides (chiefly malvidin glycoside) than the wine. Presumably this was because of the precipitation of these compounds, which occurs when red wine is aged.

### Separation of plasma

Blood samples were drawn into  $K_3EDTA$  (1 mmol/L) 12 h after dinner, before and at the end of the 2-wk period of wine consumption. Samples were centrifuged at  $2000 \times g$  for 15 min at  $4^\circ C$  to obtain plasma. Aliquots of plasma (for lipid and polyphenols) were stored at  $-70^\circ C$  before being analyzed. All other analyses were started on the same day that blood was drawn and samples were stored at  $4^\circ C$  until processed.

### Plasma antioxidant indexes

Plasma lipid peroxides (16) were estimated by lipid absorption with PHM-L liposorb gel (Calbiochem-Novabiochem, Nottingham, United Kingdom) and suspension of the gel in CHOD-iodide reagent (BDH-Merck, Lutterworth, United Kingdom). The mixture was then shaken for 60 min at room temperature and centrifuged at  $10000 \times g$  for 3 min at room temperature, and the optical density of the supernate was measured at 405 nm. Plasma total antioxidant status (17) was measured by using 2,2'-azino-di-[3 ethylbenzthiazoline sulfonate], peroxidase, and hydrogen peroxide obtained as a kit (Randox Laboratories, Crumlin, United Kingdom).

### LDL antioxidant indexes

#### Separation of LDL

LDL was separated by density gradient ultracentrifugation with a Beckman bench top model Optima TLX with a TLA 100.4 rotor (Beckman, Palo Alto, CA). The density of 24 mL plasma was adjusted to 1.3 kg/L by adding solid sodium bromide. Aliquots of 1 mL were layered under 2 mL of a 1.006-kg/L density solution. Spinning took place at  $540000 \times g$  for 20 min at  $4^\circ C$ . The orange-yellow LDL band ( $\approx 0.4$  mL from each tube) was removed and pooled. Aliquots of 1 mL were added to 1 mL of a 1.154-kg/L density solution layered under 1 mL of a 1.063-kg/L density solution and spun for 30 min at  $540000 \times g$  at  $4^\circ C$ . The visible LDL layer (containing 0.7–1.0 g LDL protein/L) was removed and processed immediately. All density solutions contained EDTA (2  $\mu\text{mol/L}$ ) as an antioxidant.

#### Subsequent treatment of LDL

All reagents were prepared by using reverse osmosis water treated with an ion-exchange resin column to remove trace elements (Elga, High Wycombe, United Kingdom). Three methods of treating the LDL were used. Method A was used exclusively in study 1 and all 3 methods were used in study 2.

**Method A (final dialysis without EDTA).** LDL (2.5 mL) was dialyzed against 5 L of 10 mmol sodium phosphate-buffered saline (PBS)/L containing 2  $\mu\text{mol}$  EDTA/L with a dialysis cassette (Slide-A-Lyzer Perstorp; Pierce Chemical Co, Rockford, IL) at  $4^\circ C$  for 1 h, then with 5 L of 10 mmol PBS/L without EDTA at  $4^\circ C$  overnight.

**Method B (continuous dialysis with EDTA).** LDL was removed and dialyzed at  $4^\circ C$  as above except that dialysis was with 10  $\mu\text{mol}$  EDTA/L in 10 mmol PBS/L.

**Method C (column treatment).** LDL was passed through an EcNo-pac 10DG desalting column (Bio-Rad, Hemel Hempstead,

United Kingdom) (18). The column was washed twice with 10 mmol PBS/L and treated by mixing with 5 g chelex-100 resin/L (Bio-Rad) and decanting the liquid. LDL (600  $\mu\text{L}$ ) was then loaded on the column and eluted with 3 mL PBS buffer at a flow rate of 0.6 mL/min with a peristaltic pump (Ismatec, Weston Super Mare, United Kingdom).

### LDL oxidation

Lipoprotein (diluted with 10 mmol PBS/L to give a final concentration of 50 mg LDL protein/L) was incubated in the presence of copper sulfate (5  $\mu\text{mol/L}$ ) at  $37^\circ C$  for 5 h (19) and oxidation was terminated by refrigeration and addition of 0.1 mmol EDTA/L. LDL oxidation was determined as follows. Lag time was determined by continuously measuring (20) the increase in absorbance at 234 nm according to the method developed by Esterbauer et al (21). At the end of the incubation, conjugated diene formation (21) was determined by measuring the difference of absorbance at the end of the reaction and at baseline and by using the extinction coefficient of  $2.95 \times 10^4$  (mol/L) $^{-1} \cdot \text{cm}^{-1}$ . TBARS (22) were determined by adding 1 mL TCA:TBA:HCL reagent (15% trichloroacetic acid:0.375% thiobarbituric acid:0.25 mol HCl) to a 0.5-mL aliquot of the LDL solution (oxidized as above), mixing the sample, and heating it in boiling water at  $100^\circ C$  for 10 min. The absorbance of the supernate was measured against a blank at 535 nm and the concentration of TBARS was calculated by using an extinction coefficient of  $1.56 \times 10^5$  (mol/L) $^{-1} \cdot \text{cm}^{-1}$ . Lipid peroxides (23) were determined by using a commercially available reagent (cholesterol color reagent, CHOD-iodide method; Diagnostic Merck, Darmstadt, Germany).

### Other assays

Plasma and LDL polyphenols were measured by the Folin-Ciocalteu reaction (14) with a gallic acid standard. The results were expressed as mg gallic acid/g protein. Plasma and LDL protein were measured by the Bradford method (24) by using the Bioquant reagent (Merck, Darmstadt, Germany). Plasma lipids were measured by conventional methods with kits (Instrumentation Laboratories Ltd, Milan, Italy).

### Statistics

All results are given as means  $\pm$  SD. For each index, differences between the before and after values were compared by the paired *t* test (two-tailed). Analysis of covariance with baseline values as covariants was performed to compare different treatments. *P* values were calculated by using the adjusted means. The computer software programs EXCEL (version 5; Microsoft, Redmond, WA) and ASTUTE (DDU Software, Leeds, United Kingdom) were used for the computations.

## RESULTS

In both studies 1 and 2, there were no significant baseline differences in any index between treatments.

### Study 1: comparison of red and white wine, red wine polyphenols, and the alcoholic drink

As shown in **Table 2**, after 2 wk, plasma polyphenols increased 38% with red wine, 27% with white wine plus 1 g red wine polyphenols, and 28% with red wine polyphenol capsules, but not with white wine. Plasma lipid peroxides decreased by 32% with red wine, 29% with white wine plus 1 g red wine polyphenols, and

**TABLE 2**Concentration of polyphenols and lipid peroxides in plasma of volunteers consuming red wine, white wine, white wine with added polyphenols, polyphenol powder, or alcohol (control) for 2 wk<sup>1</sup>

Supplement	Plasma polyphenols	Plasma lipid peroxides
	mg/g protein	μmol/L
Red wine (375 mL/d) (n = 9)		
Baseline	16.2 ± 5.7 <sup>2</sup>	2.2 ± 0.6
After 2 wk	22.6 ± 2.7 (23.2) <sup>a,B</sup>	1.5 ± 0.3 (1.3) <sup>A</sup>
Change	6.4 ± 3.0	-0.75 ± 0.3
P <sup>3</sup>	0.002	0.04
White wine (375 mL/d) (n = 9)		
Baseline	18.9 ± 5.1	1.7 ± 0.6
After 2 wk	20.3 ± 1.5 (20.4) <sup>a,c,D,E</sup>	2.1 ± 0.6 (2.2) <sup>A,B,C,D</sup>
Change	1.4 ± 3.6	0.4 ± 0.3
P	0.45	0.02
White wine (375 mL/d) + polyphenol powder (1 g/d) (n = 6)		
Baseline	17.6 ± 4.2	1.7 ± 0.5
After 2 wk	22.6 ± 1.7 (22.9) <sup>e,F</sup>	1.2 ± 0.2 (1.2) <sup>B</sup>
Change	5.0 ± 2.4	-0.5 ± 0.2
P	0.02	0.01
Polyphenol powder (1 g/d) (n = 9)		
Baseline	21.0 ± 3.0	1.9 ± 0.6
After 2 wk	26.9 ± 5.4 (26.6) <sup>g,B,D,F</sup>	1.4 ± 0.3 (1.3) <sup>C</sup>
Change	5.8 ± 2.4	-0.5 ± 0.3
P	0.02	0.02
Alcoholic drink (400 mL 10% ethanol/d) (n = 6)		
Baseline	23.9 ± 1.2	1.6 ± 0.2
After 2 wk	24.0 ± 1.5 (23.2) <sup>e,E</sup>	1.5 ± 0.2 (1.6) <sup>D</sup>
Change	0.1 ± 0.2	-0.1 ± 0.2
P	0.86	0.46
Experimental SD (ANCOVA)	3.04	0.41

<sup>1</sup> Adjusted means (in parentheses) within a column with the same superscript are significantly different:  $P < 0.05$  (lowercase),  $P < 0.01$  (uppercase).<sup>2</sup>  $\bar{x} \pm SD$ .<sup>3</sup> Paired *t* test.

28% with red wine polyphenol capsules. With white wine consumption there was an increase of 23% in lipid peroxides.

The LDL-associated antioxidant effects of the consumption of red wine, white wine plus 1 g red wine polyphenols, and red wine polyphenol capsules were all in the same direction and of similar magnitude (Table 3). LDL polyphenols increased 26%, 62%, and 29%, respectively. In the copper-catalyzed peroxidation assay, lag time increased 31%, 21%, and 27%, respectively; the formation of TBARS decreased by 22%, 24%, and 25%, respectively; conjugated dienes decreased by 15%, 11%, and 12%, respectively; and lipid peroxides decreased by 22%, 23%, and 25%, respectively. After white wine there was a 21% increase in TBARS and a 14% increase in conjugated dienes. No oxidative or antioxidative effects were observed after the control drink. In the copper-catalyzed peroxidation assay the lag times and shape of the curves for baseline samples before treatment were comparable with those published by the Israeli group (12).

Analysis of covariance showed that for almost all comparisons there was no significant difference between any of the groups in which red wine polyphenols were administered (red wine, white wine plus 1 g red wine polyphenols, and red wine polyphenol capsules). In every comparison there was a significant difference between groups administered white wine or the control drink and those given red wine polyphenols (Tables 1 and 2). There was a trend for all of the alcoholic drinks to produce an increase in plasma HDL cholesterol, which was only significant with white wine plus red wine polyphenols and alcohol. No

significant changes in plasma lipids were observed with the red wine polyphenol capsules (Table 4).

### Study 2: comparison of red wine polyphenols and vitamin E

The objectives of this study were to determine the concentration of total antioxidants in plasma and to compare different methods of preparation of LDL before the copper-diene assay (Table 5). Total antioxidants in the plasma were increased by 11% and 15% by red wine polyphenols (1 and 2 g/d, respectively) and by 7% by vitamin E. When EDTA was omitted from the final dialysis buffer, red wine polyphenols consumption either as a drink (1 g/d) or capsules (2 g/d) produced an increase in lag time of 30% and 21%, respectively, and of 12% and 22%, respectively, by the column method. The addition of EDTA to the dialysis buffer abolished the effect with 1 g red wine polyphenols/d and resulted in only a small increase in lag time of 7% with 2 g/d. There were no significant differences between the lag times obtained by the dialysis without EDTA and the column method.

Analysis of covariance showed no significant differences in the increase in lag time between the drink and capsules. Vitamin E produced a large increase in lag time with the dialysis without EDTA and with the column method of 107% and 110%, respectively. Inclusion of EDTA in the dialysis buffer resulted in an increase of 66%, which was not significantly different from the increase after the other 2 methods. The increase in lag times with vitamin E was significant (analysis of covariance), being 4–5-fold greater than with red wine polyphenols.

**TABLE 3**

Concentration of LDL polyphenols and copper-catalyzed peroxidation assays in volunteers consuming red wine, white wine, white wine with added polyphenols, polyphenol powder, or alcohol (control) for 2 wk<sup>1</sup>

Supplement	LDL		LDL copper-catalyzed peroxidation		
	Polyphenols	Lag time	TBARS <sup>2</sup>	Conjugated dienes	Lipid peroxides
	mg/g protein	min	μmol/g protein	μmol/g protein	μmol/g protein
Red wine (375 mL/d) (n = 9)					
Baseline	33.6 ± 6.6 <sup>3</sup>	51.6 ± 7.5	34.7 ± 5.7	685 ± 75	236 ± 84
After 2 wk	42.3 ± 8.1 (44.1) <sup>A</sup>	69.3 ± 18.3 (71.6) <sup>A,B</sup>	26.9 ± 4.8 (27.5) <sup>A</sup>	579 ± 84 (624) <sup>b,c,A,D</sup>	155 ± 57 (131) <sup>A,B</sup>
Change	8.7 ± 5.1	17.8 ± 10.8	-7.8 ± 0.9	-106 ± 57	-81 ± 24
P <sup>4</sup>	0.001	0.008	0.0004	0.0008	0.008
White wine (375 mL/d) (n = 9)					
Baseline	39.3 ± 6.0	63.8 ± 18.6	30.1 ± 6.0	732 ± 126	170 ± 48
After 2 wk	38.5 ± 9.9 (36.4) <sup>A,B,C</sup>	63.6 ± 9.1 (58.2) <sup>c,A</sup>	36.5 ± 6.9 (39.4) <sup>A,B,C,D</sup>	833 ± 138 (848) <sup>A,E,F,G</sup>	188 ± 57 (203) <sup>A,C,D</sup>
Change	-0.7 ± 9.3	-0.2 ± 5.1	6.5 ± 0.9	101 ± 90	18 ± 9
P	0.82	0.95	0.0001	0.012	0.22
White wine (375 mL/d) + polyphenol powder (1 g/d) (n = 6)					
Baseline	31.1 ± 2.7	54.8 ± 2.5	39.9 ± 3.9	809 ± 83	217 ± 73
After 2 wk	45.8 ± 8.8 (49.2) <sup>d,B</sup>	66.5 ± 5.1 (66.7) <sup>d</sup>	30.5 ± 2.7 (28.5) <sup>B</sup>	719 ± 49 (685) <sup>b,E,H</sup>	128 ± 56 (115) <sup>C,E</sup>
Change	14.7 ± 6.1	11.7 ± 2.7	-9.3 ± 1.2	-90 ± 49	-89 ± 17
P	0.0001	0.007	0.0003	0.007	0.005
Polyphenol powder (1 g/d) (n = 9)					
Baseline	37.0 ± 4.5	51.7 ± 5.7	37.9 ± 13.5	803 ± 71	216 ± 39
After 2 wk	47.6 ± 6.3 (47.0) <sup>c,C</sup>	65.9 ± 12.9 (68.1) <sup>c</sup>	28.3 ± 6.9 (27.3) <sup>C</sup>	709 ± 45 (680) <sup>c,F,I</sup>	149 ± 45 (136) <sup>D,F</sup>
Change	10.6 ± 6.6	14.2 ± 7.2	-9.6 ± 6.0	-93 ± 66	-67 ± 6
P	0.002	0.006	0.02	0.004	0.004
Alcoholic drink (400 mL 10% ethanol/d) (n = 6)					
Baseline	39.4 ± 4.9	54.0 ± 5.1	36.5 ± 5.6	750 ± 127	137 ± 49
After 2 wk	43.7 ± 1.7 (41.4) <sup>d,c</sup>	56.6 ± 4.7 (57.3) <sup>d,B</sup>	29.3 ± 2.0 (29.0) <sup>D</sup>	770 ± 76 (767) <sup>D,G,H,I</sup>	174 ± 47 (208) <sup>B,E,F</sup>
Change	4.3 ± 4.4	2.6 ± 3.4	-7.2 ± 6.1	20 ± 76	36 ± 3
P	0.08	0.14	0.07	0.6	0.18
Experimental SD (ANCOVA)	6.46	10.66	4.02	56.7	40.3

<sup>1</sup> Adjusted means (in parentheses) within a column with the same superscript are significantly different:  $P < 0.05$  (lowercase),  $P < 0.01$  (uppercase).

<sup>2</sup> Thiobarbituric acid-reactive substances.

<sup>3</sup>  $\bar{x} \pm$  SD.

<sup>4</sup> Paired  $t$  test.

## DISCUSSION

The ability of red wine consumption to increase antioxidant activity in vivo has been highly controversial. The findings of another study published in this Journal by an Israeli group (12) were disputed 1 y later by the findings of a Dutch group, also published in this Journal (13). There were some major differences in the protocols used by these 2 groups, primarily in the product studied and in the techniques for analysis of LDL oxidation (Table 6). The findings of our study largely agreed with the findings of the Israeli group (12). In our study, red wine consumption increased plasma and LDL polyphenols and enhanced antioxidant activity as judged by decreased plasma total peroxides, increased lag time, and decreased LDL lipid peroxides and TBARS in the copper-catalyzed peroxidation of LDL-conjugated dienes.

However, our group (UK) observed much less antioxidant activity with red wine consumption than did the Israeli group (IS) (lag time: UK, 31%; IS, 380%; TBARS: UK, 22%; IS, 44%; conjugated dienes: UK, 15%; IS, 48%; lipid peroxides: UK, 30%; IS, 40%). This could be explained by the much greater increase in total polyphenol concentration in LDLs observed by the Israeli group (UK: 26%; IS: 400%). A 16-fold difference in effect cannot be explained easily. The Folin-Ciocalteu method

for determination of polyphenols is unreliable because any substance containing a phenolic group could interfere. Future research should use HPLC analysis as currently being developed by those working in this field.

The Israeli group (12) found that white wine consumption had a prooxidant effect, which was partially confirmed by us (lag time: UK, 0%; IS, 33%; TBARS: UK, 21%; IS, 28%). The reason for this effect is unclear. Thus, red wine but not white wine had antioxidant activity when given to volunteers, and this difference was most likely due to the content of wine polyphenols, which are abundant in red wine but not in white wine. A polyphenol extract of red wine obtained as an alcohol-free powder gave the same results as did red wine and had equivalent antioxidant activity both in plasma and in LDL. The difference observed between the results of the Israeli (12) and Dutch (13) groups could not be explained by the use of dealcoholized wine by the Dutch. The Israeli group suggested that the effect of white wine may have been due to the prooxidative properties of alcohol (25), but we found no changes with a control solution of alcohol.

EDTA is often added to plasma and LDL to chelate trace quantities of copper or iron, which could catalyze the oxidation of lipids (26). Others (19) prefer to desalt LDL and remove

TABLE 4

Concentration of total cholesterol, HDL cholesterol, LDL cholesterol, and triacylglycerol in plasma

Supplement	Cholesterol	HDL cholesterol	LDL cholesterol	Triacylglycerol
	<i>mmol/L</i>			
Red wine (375 mL/d) ( <i>n</i> = 9)				
Baseline	5.69 ± 1.41 <sup>1</sup>	0.94 ± 0.27	3.82 ± 1.20	2.03 ± 0.75
After 2 wk	5.76 ± 0.93	0.96 ± 0.15	3.84 ± 0.99	2.06 ± 0.90
Change	0.07 ± 0.84	0.01 ± 0.18	0.02 ± 0.63	0.02 ± 1.20
<i>P</i> <sup>2</sup>	0.82	0.87	0.92	0.96
White wine (375 mL/d) ( <i>n</i> = 9)				
Baseline	5.76 ± 1.20	0.99 ± 0.21	3.76 ± 0.99	2.29 ± 0.75
After 2 wk	5.70 ± 1.35	1.02 ± 0.21	3.71 ± 1.14	2.09 ± 0.72
Change	-0.06 ± 0.57	0.03 ± 0.06	-0.04 ± 0.45	-0.20 ± 0.33
<i>P</i>	0.78	0.081	0.78	0.11
White wine (375 mL/d) + polyphenol powder (1 g/d) ( <i>n</i> = 6)				
Baseline	6.03 ± 1.37	1.18 ± 0.12	4.13 ± 1.05	1.63 ± 1.20
After 2 wk	6.08 ± 1.13	1.25 ± 0.15	4.22 ± 0.96	1.35 ± 0.76
Change	0.05 ± 0.49	0.07 ± 0.05	0.08 ± 0.25	-0.28 ± 0.49
<i>P</i>	0.82	0.025 <sup>3</sup>	0.46	0.21
Polyphenol powder (1 g/d) ( <i>n</i> = 9)				
Baseline	5.79 ± 1.05	0.97 ± 0.21	3.85 ± 0.90	2.38 ± 2.40
After 2 wk	5.71 ± 0.99	1.00 ± 0.30	3.78 ± 0.66	2.08 ± 1.77
Change	-0.08 ± 0.36	0.03 ± 0.12	-0.07 ± 0.33	-0.30 ± 0.69
<i>P</i>	0.54	0.40	0.56	0.23
Alcoholic drink (400 mL 10% ethanol/d) ( <i>n</i> = 6)				
Baseline	4.14 ± 0.71	0.82 ± 0.25	3.00 ± 0.56	0.70 ± 0.12
After 2 wk	4.56 ± 0.83	0.98 ± 0.29	3.18 ± 0.69	0.88 ± 0.29
Change	0.42 ± 0.71	0.16 ± 0.12	0.18 ± 0.59	0.18 ± 0.22
<i>P</i>	0.22	0.035 <sup>3</sup>	0.50	0.10

<sup>1</sup> $\bar{x} \pm \text{SD}$ .<sup>2</sup>Paired *t* test.<sup>3</sup>*P* < 0.05.

EDTA after its separation and use either dialysis or an absorption column. In the method used by the Dutch group (13), EDTA is not removed, but excess copper is added to compensate for the chelating effect of EDTA. To determine the importance of the preparation of LDL before oxidation, we compared dialysis with and without EDTA and a column method. When red wine polyphenols were given at a dose of 1 and 2 g/d, results from dialysis without EDTA and the column method were comparable and resulted in increased lag times of >6 min. Adding EDTA to the dialysis buffer had a pronounced effect. With the 1-g/d dose there was no increase in lag time and with the 2-g/d dose the increase was only 4.5 min compared with 13.2 min for dialysis without EDTA and 11.8 min for the column method. Thus, there was an inhibiting effect of EDTA in the copper-diene assay when polyphenols were examined. The failure of the Dutch group (13) to obtain an increase in lag time and hence enhanced antioxidant activity could be explained by the relatively high concentration of EDTA in their LDL samples.

In copper-catalyzed peroxidation of LDL, vitamin E is a sacrificial antioxidant and conjugated diene formation commences when all of the vitamin E present has been oxidized (27). During dialysis without EDTA it is possible for antioxidants such as vitamin E in LDL to be oxidized (28) by trace amounts of metals present in the buffer; this would lead to faster peroxidation of LDL and reduced lag time. The mechanism by which polyphenols protect LDL from oxidation is unknown. There are several possibilities. It is known that red wine polyphenols function as metal chelators similar to EDTA in the *ex vivo* copper-catalyzed peroxidation of LDL (29–31). First, polyphenols could form a complex with some

of the copper added during the catalyzed peroxidation of LDL. Second, they could protect vitamin E from oxidation because of trace elements in the dialyzing medium or by radical trapping.

We prefer the first possibility because it fits with the data we obtained with the column method, ie, the possibility of oxidation of vitamin E was limited and the lag time was prolonged. It is well established that dietary supplements of vitamin E given in large quantities (1000 IU/d) increase the vitamin E content of LDL and increase the lag time in copper-catalyzed peroxidation (32). To study the importance of vitamin E, the same 3 methods for desalting LDL before oxidation were compared in volunteers given a large dose of vitamin E. For dialysis without EDTA, dialysis with EDTA, and the column method the lag times were 67.6, 50.5, and 64.6 min, respectively. Thus, EDTA had no inhibiting effect on the copper-diene test at the low concentrations of EDTA (10  $\mu\text{mol/L}$ ) used in the dialysis buffer. This finding also suggests that there was no loss of vitamin E during dialysis without EDTA.

Further work is needed to test the hypothesis that polyphenols act by chelating trace elements without EDTA and to determine the mechanism by which this occurs. Such a property could be important *in vivo* because polyphenols could provide LDL with a component *in situ* for chelating trace elements and inhibiting catalytic peroxidation.

The Israeli group found an increase in HDL cholesterol with red wine but not with white wine. We found no significant changes on HDL cholesterol with red wine or red wine polyphenols. It is well known that alcohol intake increases HDL cholesterol (33) and we found that alcohol alone and white wine with added polyphenols raised HDL cholesterol. We conclude

**TABLE 5**

Plasma total antioxidant status and lag times in copper-catalyzed peroxidation with different methods for desalting LDL in volunteers consuming red wine polyphenols or vitamin E for 2 wk<sup>1</sup>

Supplement	Plasma total antioxidant status <i>mmol/L</i>	Lag time		
		Dialysis without EDTA (A)	Dialysis with EDTA (B)	Column (C)
Wine polyphenols				
Drink (1 g/d) ( <i>n</i> = 6)				
Baseline	1.8 ± 0.25 <sup>2</sup>	60.0 ± 9.1	64.3 ± 9.3	54.2 ± 11.3
After 2 wk	1.9 ± 0.25 (1.7) <sup>a,b</sup>	77.7 ± 20.6 (80.3) <sup>A</sup>	64.5 ± 9.6 (68.1) <sup>A</sup>	60.6 ± 11.5 (61.4) <sup>A</sup>
Change	0.2 ± 0.25	17.7 ± 7.6	0.3 ± 0.2	6.4 ± 0.25
<i>P</i> <sup>3</sup>	0.0008	0.02	0.9 <sup>d</sup>	0.005
Capsules (2 g/d) ( <i>n</i> = 6)				
Baseline	1.3 ± 0.25	62.7 ± 6.1	67.7 ± 8.8	54.0 ± 5.4
After 2 wk	1.5 ± 0.25 (1.6) <sup>a</sup>	75.8 ± 6.9 (74.8) <sup>B</sup>	72.2 ± 7.6 (73.4) <sup>B</sup>	65.8 ± 5.4 (66.7) <sup>B</sup>
Change	0.2 ± 0.25	13.2 ± 0.7	4.5 ± 1.2	11.8 ± 0.25
<i>P</i>	0.03	0.004	0.02 <sup>d</sup>	0.003
Vitamin E (1000 IU/d) ( <i>n</i> = 8)				
Baseline	1.5 ± 0.28	63.1 ± 5.1	76.6 ± 9.9	58.5 ± 6.2
After 2 wk	1.6 ± 0.09 (1.6) <sup>B</sup>	130.8 ± 10.8 (129.1) <sup>A,B</sup>	127.1 ± 23.5 (122.2) <sup>A,B</sup>	123.1 ± 12.7 (121.5) <sup>A,B</sup>
Change	0.1 ± 0.28	67.6 ± 5.7	50.5 ± 13.5	64.6 ± 6.5
<i>P</i>	0.0002	<0.0001	0.0005 <sup>d</sup>	<0.0001
Experimental SD (ANOVA)	0.082	10.1	16.3	9.84

<sup>1</sup> Adjusted means (in parentheses) within a column with the same superscript are significantly different: *P* < 0.05 (lowercase), *P* < 0.01 (uppercase). There were no significant differences between the results with the column method and those with dialysis with and without EDTA.

<sup>2</sup>  $\bar{x} \pm SD$ .

<sup>3</sup> Paired *t* test.

<sup>4</sup> Significantly different from dialysis without EDTA, *P* < 0.01.

that 2 wk was not long enough to observe a significant effect in all groups receiving alcohol. No changes in lipids were observed by the Dutch group, as was expected because their wine was dealcoholized.

St Leger et al (34) were the first to draw attention to the inverse association between wine consumption and mortality from CAD in different countries and they expressed the hope that the factor in wine would never be isolated because it was

already present in a palatable form. Not everyone shares their view. If the increased antioxidant capacity produced by red wine polyphenols has any clinical benefit, especially in the prevention of CAD, the use of wine polyphenols as a food ingredient could be advantageous in populations for whom alcohol is prohibited because of social, health, or religious reasons.


Although some authors do not believe that wine consumption prevents CAD (35), the intake of flavonols, which are abundant in

**TABLE 6**

Differences in protocols between the Israeli and Dutch groups

	Israeli group (12)	Dutch group (13)	Present study
Product	Red and white Israeli wines	Dealcoholized Italian wines	French wines
Amount given (mL/d)	400	550	375
Study duration (wk)	1–2	4	2
Prediet treatment	No change	Low flavonoid diet, 2 wk white wine	Without wine 2 wk
Subjects	17 men (25–45 y)	19 men, 5 women (23–63 y)	26 men (35–65 y)
Temperature of plasma storage (°C)	4	–80, with saccharose	4, analysis same day
Plasma estimations			
Polyphenols	Done	Not done	Done
TBARS assay with AAPH	Done	Not done	Not done
Lipids	Done	Done	Done
Lipid peroxides	Not done	Not done	Done
Subsequent preparation of LDL after UC	Dialysis against PBS, no EDTA in final dialyzing buffer	EDTA added and not removed	Dialysis against PBS, no EDTA in final dialyzing buffer
LDL estimations	Polyphenols	Not done	Polyphenols
Amount of copper used for peroxidation (μmol/L)	10	38	5
Estimations with copper peroxidation	Lag time, conjugated dienes, TBARS, lipid peroxides	Lag time, conjugated dienes, oxidation rate	Lag time, conjugated dienes, TBARS, lipid peroxides

<sup>1</sup> TBARS, thiobarbituric acid–reactive substances; PBS, phosphate-buffered saline; UC, ultracentrifugation; AAPH, azobis(2-amidinopropane).

wine, has been shown to be inversely correlated with mortality from CAD (36, 37). Red wine significantly reduced aortic atherosclerosis in cholesterol-fed rabbits compared with white wine or spirits (38). It is not clear what amounts of polyphenols are absorbed from red wine or whether their putative benefit is due to an antioxidant effect. The value of wine polyphenols can only be established by primary or secondary prevention trials in relatively large numbers of the population or patients with CAD. In view of the potential benefit, such trials seem appropriate. 

We thank David Frappe for statistical advice and assistance, Judy West for technical assistance, and Wolfgang Leonhardt (University of Dresden) and Richard Bruckdorfer (Royal Free Hospital, London) for advice on the methodology used.

## REFERENCES

- Kozararevic D, McGee D, Vojvodic N, Racic Z, Dawber T, Gordon T. Frequency of alcohol consumption and morbidity and mortality. *Lancet* 1980;1:613-6.
- Marmot MG, Rose G, Shipley MJ, Thomas BJ. Alcohol and mortality: a U-shaped curve. *Lancet* 1981;1:580-3.
- Shaper AG, Wannamethee G, Walker M. Alcohol and mortality in British men: explaining the U-shaped curve. *Lancet* 1988;1:1267-73.
- Boffetta P, Garfinkel L. Alcohol drinking and mortality among men enrolled in an American Cancer Society prospective study. *Epidemiology* 1990;1:337-9.
- Gaziano YM, Buring JE, Breslow JL, et al. Moderate alcohol intake increased levels of high density lipoprotein and its subfractions and decreased risk of myocardial infarction. *N Engl J Med* 1993;329:1829-33.
- Renaud S, de Lorgeril M. Wine alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* 1992;339:1523-6.
- Klatsky AL, Armstrong MA, Freedman GD. Relation of alcoholic beverage use to subsequent coronary heart disease hospitalization. *Am J Cardiol* 1986;58:710-4.
- Klatsky AL, Armstrong MA. Alcoholic beverage choice and risk of coronary heart disease mortality: do red wine drinkers fare best? *Am J Cardiol* 1993;71:467-9.
- Grønback M, Deis A, Sørensen TJA, Becker U, Schnohr P, Jensen G. Mortality associated with moderate intakes of wine, beer, or spirits. *Br Med J* 1995;310:1165-9.
- Frankel EN, Waterhouse AL, Teissedre PL. Principal phenolic phytochemicals in selected California wines and their antioxidant activity in inhibiting oxidation of human low-density lipoproteins. *J Agric Food Chem* 1995;43:890-4.
- Witztum JL. The oxidation hypothesis of atherosclerosis. *Lancet* 1994;344:793-8.
- Fuhrman B, Lavy A, Aviram M. Consumption of red wine with meals reduces the susceptibility of human plasma and low-density lipoprotein to lipid peroxidation. *Am J Clin Nutr* 1995;61:549-54.
- de Rijke YB, Demacker PNM, Assen NA, Sloots LM, Katan MB, Stalenhoef AFH. Red wine consumption does not affect oxidizability of low-density lipoproteins in volunteers. *Am J Clin Nutr* 1996;63:329-34.
- Singleton SL, Rossi JA. Colorimetry of total phenols with phosphomolybdic phosphotungstic acid reagents. *Am J Enol Viticulture* 1965;16:144-58.
- Price SF, Breen PJ, Valladao M, Watson BT. Cluster sun exposure and quercetin in Pinot noir grapes and wine. *Am J Enol Viticulture* 1995;46:187-94.
- Gorog P, Semeria FJ, Gorog DA. Activation of the phagocytic system increases initial proliferation in hypercholesterolaemic rabbits. *Atherosclerosis* 1993;111:47-53.
- Miller NJ, Rice-Evans C, Davies MJ, Gopinathan V, Milner A. A novel method for measuring antioxidant capacity and the application to monitoring the antioxidant status in premature neonates. *Clin Sci* 1993;84:407-12.
- Leonhardt W, Hanefeld M, Müller G, et al. Impact of concentrations of glycated hemoglobin,  $\alpha$ -tocopherol, copper and manganese on oxidation of low-density lipoproteins in patients with type I diabetes, type II diabetes and control subjects. *Clin Chim Acta* 1996;254:173-86.
- Kleinvelde HA, Hak-Lemmers LM, Stalenhoef AF, Demacker PN. Improved measurement of LDL susceptibility to copper-induced oxidation. Application of a short procedure for isolating LDL. *Clin Chem* 1992;38:2066-72.
- McDowell IFW, McEneny J, Trimble ER. A rapid method for measurement of the susceptibility to oxidation of low-density lipoprotein. *Ann Clin Biochem* 1995;32:167-74.
- Esterbauer H, Striegl G, Puhl H, Rotheneder M. Continuous monitoring of in vitro oxidation of human low density lipoprotein. *Free Radic Res Commun* 1989;6:67-75.
- Buege JA, Aust SD. Microsomal lipid peroxidation. *Methods Enzymol* 1978;52:302-10.
- El-Saadani M, Esterbauer M, El-Sayed M, Goher M, Massar AY, Jurgens G. A spectrophotometric assay of lipid peroxides in serum lipoproteins using a commercially available reagent. *J Lipid Res* 1989;30:627-30.
- Bradford MM. Analysis of protein. *Anal Biochem* 1976;72:248-54.
- Fernandez-Checa JC, Garcia-Ruiz C, Ookkens M, Kapiowitz N. Impaired uptake of glutathione by hepatic mitochondria from chronic ethanol fed rats. *J Clin Invest* 1991;87:397-405.
- Lamb DJ, Leake DS. The effect of EDTA on the oxidation of low density lipoprotein. *Atherosclerosis* 1992;94:35-42.
- Esterbauer H, Jurgens G, Quenenberger O, Koller E. Autoxidation of human low density lipoprotein: loss of polyunsaturated fatty acids and vitamin E and generation of aldehydes. *J Lipid Res* 1987;28:495-509.
- Scheek LM, Wiseman SA, Tyburg LBM, van Tol A. Dialysis of isolated low density lipoprotein induces a loss of lipophilic antioxidants and increases the susceptibility to oxidation in vitro. *Atherosclerosis* 1995;117:139-44.
- Afansiev IB, Dorozhko AI, Brodskii AV, Kostyuk AV, Potapovitch AL. Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. *Biochem Pharmacol* 1989;38:1763-9.
- Halliwell B, Gutteridge JMC. Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol* 1990;186:1-85.
- Morel I, Lescoat G, Cogrel P, et al. Antioxidant and iron chelating activities of the flavonoids catechins, quercetin and diosmetin on iron loaded rat hepatocyte cultures. *Biochem Pharmacol* 1993;45:13-9.
- Simons LA, Von Konigsmark M, Balasubramanian S. What dose of vitamin E is required to reduce susceptibility of LDL to oxidation. *Aust N Z J Med* 1996;26:496-503.
- Hartung GH, Foreyt JL, Reers RS, et al. Effect of alcohol dose on plasma lipoprotein subfractions and lipolytic enzyme activity in active and inactive men. *Metabolism* 1990;39:81-6.
- St Leger AS, Moore F, Cochrane AL. Factors associated with cardio-mortality in developed countries with particular reference to consumption of wine. *Lancet* 1979;1:1017-20.
- Hennekens CH, Willett W, Rosner B, Cole DS, Mayrent SL. Effects of beer, wine, and liquor in coronary deaths. *JAMA* 1979;242:1973-4.
- Hertog MGL, Feskens EJM, Hollman PCH, Katan MB, Kromhout D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet* 1993;342:1007-11.
- Hertog MGL, Kromhout D, Aravanis C, et al. Flavonoid intake and long-term risk of coronary heart disease and cancer in the Seven Countries Study. *Arch Intern Med* 1995;155:381-6.
- Klurfield DM, Kritchevsky D. Differential effects of alcoholic beverages on experimental atherosclerosis in rabbits. *Exp Mol Pathol* 1981;34:62-71.