Antioxidative Capacity of Wine on Human LDL Oxidation *in Vitro*: Effect of Skin Contact in Winemaking of White Wine

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To assess the antioxidative effect of the non-alcoholic components of wine, human low-density lipoprotein (LDL) was oxidized *in vitro* by copper ions in the presence of polyphenolic extracts from three wines: standard red wine (R), standard white wine (W1), and white wine the must of which had been in contact with grape solids during 8 h before fermentation (W2). Lipoprotein peroxidation was monitored as the formation of conjugated dienes, of thiobarbituric acid reactive substances (TBARS), and fluorescent substances. At equal volume-of-extract additions to LDL, the lag phase of diene production increased proportionally with the polyphenol concentration of each extract. By the addition of equal phenolic substance concentration (8 μmol of gallic acid equiv/L) the timing of lag phase was 410 $\pm$ 8, 442 $\pm$ 11, and 516 $\pm$ 37 min for W1, R, and W2 respectively compared to 78 $\pm$ 6 min for control LDL without added extract. At 9 h of incubation, TBARS and fluorescence production were drastically inhibited by W1 but completely inhibited by R and W2. At 24 h of oxidation only fluorescence was still inhibited. The results indicate that the polyphenols contained in wines could inhibit protein derivatization but only delay lipid peroxidation and that the type, as well as the concentrations, of polyphenols of the different wines have varying protective effects. The wine-making process that includes the pre-incubation of the must with the grape skin prior to and during fermentation (red and certain white wines) was the most effective in preventing LDL oxidation *in vitro*.

Keywords: *Wine extracts; polyphenols; lipoprotein peroxidation; atherosclerosis*

INTRODUCTION

The pathogenesis of atherosclerosis is recognized as being multifactorial (Ross, 1993) and one of the major contributors to the process is peroxidation (Parthasarathy and Rankin, 1992), particularly of low-density lipoprotein (LDL). Oxidation of LDL (oxLDL) *in vivo* can be induced by cells that secrete reactive oxygen species into the medium or by intracellular lipoperoxides that are transferred to LDL. The resultant modified lipoproteins are recognized by cell-surface "scavenger" receptors on monocytes-derived macrophages, are internalized and, when the accumulation of intracellular cholesterol is excessive, leads to foam cell formation (Brown and Goldstein, 1983). The infiltration and deposition of these cell type in the arterial wall are considered the initiating steps in atheromatous plaque development and subsequent cardiovascular disease. The disease is known to be exacerbated by a high consumption of saturated fatty acids (National Research Council Committee on Diet and Health, 1989), but in France, despite a diet which is high in saturated fat, the incidence of coronary artery disease is low. This "French Paradox" has been attributed, in part, to a relatively high wine consumption (Renaud and de Lorgeril, 1992). Similarly, the incidence of cardiovascular disease is low in Mediterranean countries (Keys, 1970; ERICA Research Group, 1988), but, to date, this has been linked to the high percentage of monounsaturated fatty acids of the Mediterranean-type diet (Masana et al., 1991); equally so, however, the low incidence could be due to the regular consumption of wine, which is an integral part of the Mediterranean diet.

Debate continues as to whether the observed inverse relationships between a moderate wine intake and atherosclerosis are casual or causal (Hegsted and Ausman, 1988; Rimm et al., 1991; Jackson and Beaglehole, 1993), and opinions are varied (Camargo et al., 1985; Moore and Pearson, 1986; Meade et al., 1986) as to what may be the mechanisms accounting for the putative anti-atherogenicity; the antioxidative property being the most recent and controversial (De Whalley et al., 1990; Frankel et al., 1993a; Fuhrman et al., 1995; Sharpe et al., 1995; De Rijke et al., 1996; Caldú et al., 1996), particularly with regard to whether it is the alcohol *per se* or other constituents of wine that produce the effect.

The present study was undertaken to assess the effect of the non-alcoholic components of differently-processed white and red wines on the susceptibility of LDL to oxidation. We have studied this effect *in vitro* because *in vitro* oxidation of LDL is considered an approach to what occurs *in vivo* (Esterbauer et al., 1992). Our first objective was to do a preliminary study about the *in vitro* effect of wine phenolics on LDL oxidation and subsequently, to extend the study *in vivo* analyzing the antioxidant effect of polyphenols on LDL of wine consumers.

MATERIALS AND METHODS

Bovine serum albumin (BSA, fraction V), α-tocopherol, and 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline) were from Sigma (St. Louis, MO). Trolox was from Aldrich. Silica

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Table 1. Analysis of Several Indices of the Wines[a]

	W1	W2	R
density kg/L	0.9921	0.9933	0.9967
ethanol content (% vol)	14.10	13.00	11.80
titratable acidity as tartaric acid (g/L)	5.95	5.35	5.05
volatile acidity as acetic acid (g/L)	0.38	0.35	0.49
pH	3.25	3.45	4.00
free sulfur dioxide (mg/L)	15	15	13
total sulfur dioxide (mg/L)	35	33	31
malic acid (g/L)	0.40	0.46	0.07
lactic acid (g/L)	0.01	<0.005	1.34
total phenolics as gallic acid [mg/L (GAE)[b]]	292	620	1941

[a] The elaborated wines were analyzed according to Office International de la Vigne et du Vin methods. The total phenolics values have been previously corrected for the SO_2 concentration. [b] GAE = gallic acid equivalents.

gel 100 C18-reversed phase was from Fluka (Fluka Chemie AG). Thiobarbituric acid (TBA) was from Serva (Heidelberg, NY), malondialdehyde (1,1,3,3-tetraethoxypropane), EDTA, $CuSO_4$, ethanol, methanol, formic acid, heptane, and other chemicals were from Merck (Darmstadt, Germany). Bradford reagent was purchased from Bio-Rad (Hercules, CA), and the cholesterol, triglyceride, and phospholipid enzymatic assay kits from Boehringer Mannheim (Mannheim, Germany).

Winemaking. Wines used in this experience were made in the pilot winery in the Institut Català de la Vinya i el Vi (INCAVI).

White wines were made with the white variety "Macabeu" also known as "Viura", one of the widest planted in Spain. Grapes were divided into two groups in the following way:

W1: crushing followed by pressing in a horizontal press.

W2: crushing, destemming, skin contact (Boulton et al., 1995) during 8 h at 19 °C pressing in a horizontal press.

The juices obtained followed the same process: 24 h settling, racking off the clarified juice, and inoculation with yeast strain P-29 *Saccharomyces cerevisiae* at 1.5×10^6 cells/mL level with a liquid culture at exponential growth. Fermentation was maintained at 19 °C. After fermentation and subsequent racking, fining was made with 50 mg/L gelatin and 400 mg/L bentonite. Filtration was done with diatomaceus earth (DE). Wines were cold stabilized against bitartrate precipitation by storage for 1 week at −4 °C and DE filtered. Bottling was done after sterile filtration with 0.65 μm membrane filters.

Both wines are representatives of the current commercial wine production.

The red wine R was made with "Tempranillo" grapes, one of the most widely planted and used for red wines in Spain. Grapes were crushed, destemmed, and inoculated with yeast strain P-29 *S. cerevisiae* at 1.5×10^6 cells/mL. Fermentation started, and after 4 days of fermentation with the grape solids pressing was done. 4 days later fermentation ended. The obtained wine was fined with egg white 100 mg/L and DE filtered. Cold stabilization was obtained by storage at −4 °C for 1 week and subsequent DE filtration. Bottling was done after sterile filtration with 0.65 μm membrane filters.

Analysis of Finished Wines. After bottling, the finished wines were analyzed (Table 1) according to methods of the Office International de la Vigne et du Vin (OIV) (1990). Total phenolics were determined by the Folin−Ciocalteu method, and values were corrected for the interference caused by the sulfur dioxide in the following way: we added 0, 1, 2, 4, 6, or 8 mL of a sulfur dioxide solution at 1000 mg/L in a 100 mL volumetric flask, filled up with the wine, mixed, and left covered at room temperature. This operation was repeated for the three wines (W1, W2, and R). After 4 days, we analyzed all the solutions according to Folin method and we used the standard addition method for every wine to substract the sulfur dioxide concentration value.

Preparation of Wine Phenolics. The wine samples were diluted 1/5 and filtered in a 0.45 μm filter. Phenolic extracts were obtained by high-pressue liquid chromatography (HPLC) using a C18 semi-preparative column (10 cm length and 7.5 mm diameter) that was filled with 3.3 g of silica gel C18-reversed phase with a particle size of 0.040−0.063 mm. Formic acid (1.91 μmol/L), methanol, de-ionized H_2O, and methanol−formic acid (1:1) were used, consecutively, as mobile phase. The eluent was monitored with a UV detector (Perkin Elmer LC35) set at 280 nm.

Isolation and Oxidation of LDL. Sera from healthy volunteers were pooled and 10 μL/mL of a preservative solution containing 0.1 mmol/L chloramphenicol, 0.1 mmol/L gentamicin, 100 mmol/L EDTA, and 0.2 mol/L NaCl was added. LDL (d = 1.019−1.063 g/mL) isolation was by sequential preparative ultracentrifugation essentially as described by Havel et al. (1959). The integrity and the purity of the lipoprotein preparations were checked by agarose gel electrophoresis (Hatch and Lees, 1968). LDL, dialyzed overnight at 4 °C against phosphate-buffered saline (PBS, 10 mmol/L sodium phosphate buffer, pH 7.4, 150 mmol/L NaCl), was oxidized in the absence/presence of polyphenolic extracts from the wines. In one experiment, LDL was oxidized by adding α-tocopherol dissolved in ethanol (final concentration of 1%), and we included a blank that included only pure ethanol. We also compared polyphenols with the hydrophilic vitamin E analogue Trolox (dissolved in ethanol at final concentration of 1%). Oxidative modification was promoted at 37 °C by the addition of freshly prepared $CuSO_4$ (final concentration of 40 μmol/L per 1 mg/mL of total mass lipoprotein). Oxidation was terminated at the times indicated by the addition of EDTA at a final concentration 5-fold excess to that of copper.

Determination of Oxidation Products. The degree of LDL oxidation was determined at the times indicated by monitoring the formation of conjugated dienes (CD), thiobarbituric acid reactive substances (TBARS), and fluorescence.

Conjugated diene production was measured essentially as described by Esterbauer et al. (1989). Absorbance at 234 nm (a distinct peak of the spectrum) was continuously monitored and the amount of dienes present in the lipoprotein calculated from the molar absorption of conjugated diene (E_{234nm} = 27 000 L mol^{-1} cm^{-1}). Results were expressed as μmol of CD/g of lipoprotein. From the kinetic profile several indices of oxidation were calculated as described Kleinveld et al. (1992): initial CD (CD_o) as being the initial amount of dienes present in lipoprotein; lag phase the interval of time between the intercept of the least-squares slope of the curve with the initial absorbance axis and interpreted as LDL resistance to oxidation; maximal CD (CD_{max}) the maximal amount of dienes formed; maximal rate of oxidation calculated from the slope of the curve during the propagation phase (R_{max}); and maximal time (t_{max}) as the time needed to produce the maximal amount of CD.

TBARS were determined as described by Yagi (1976). Briefly, 2-thiobarbituric acid (335 mg) was dissolved in 100 mL of acetic acid (50%). The pH was adjusted to 7.4. 1.5 mL of water and 1 mL of reagent were added to 0.5 mL of LDL (2.5 mg/mL). After 30 min in a bath at 95−100 °C, the tubes were cooled to room temperature and the color was extracted with 2 mL of *n*-butanol. After centrifugation, the absorption of the supernatant was read at excitation 515 nm and emission 548 nm. A calibration curve (0.038−5 μM) was obtained using malondialdehyde (MDA), and the points of this curve were treated in the same way as the LDL samples. Results were expressed as μmol of MDA/g of lipoprotein.

Total fluorescence was measured in lipoproteins by fluorometry (excitation at 360 nm and emission at 430 nm) (Tsuchida et al., 1985). To distinguish between liposoluble and hydrosoluble fluorescence, 1 mL of LDL was extracted with 1 mL of ethanol and 2 mL of heptane, and the fluorescence associated with the heptane phase was measured as above. Hydrosoluble fluorescence was the difference between the total and the liposoluble fraction. Values obtained were expressed as relative fluorescence units (RFU)/g of lipoprotein.

Table 2. Oxidizability Indices of Low-Density Lipoprotein (LDL) in the Absence and Presence of Phenolic Extracts[a]

	LDL control ($n = 2$)	W1 ($n = 2$)	W2 ($n = 2$)	R ($n = 2$)
lag phase (min)	101	152	304	714
CD_0 (μmol/g of LDL)	45	45	45	45
CD_{max} (μmol/g of LDL)	176	181	177	181
CD production (μmol/g of LDL)	132	136	132	136
t_{max} (min)	155	208	360	788
R_{max} (μmol of CD/min/g of LDL)	3.3	3.3	3.3	2.5

[a] LDL (0.25 mg/mL) was oxidized by incubation with $CuSO_4$ (10 μmol/L) in the absence (LDL control) and the presence of equal volume of phenolic extracts which on dilution 1/1500 resulted in final concentrations of 1.14, 2.43, and 7.60 μmol/L gallic acid equivalents for standard white wine (W1), white wine made in contact with grape solids (W2), and standard red wine (R), respectively. Indices were calculated from the conjugated diene (CD) production curves. Results are the mean of two independent experiments.

Vitamin E Determination. Vitamin E was measured with the method described by Tsen (1961). Briefly, tocopherols from lipoproteins were extracted with heptane and quantified by the addition of a reagent solution that contained bathophenanthroline (4,7-diphenyl-1,10-phenanthroline) and $FeCl_3$. The colored complex resulting from the conversion of Fe^{3+} into Fe^{2+} was measured spectrophotometrically at 534 nm using α-tocopherol as standard. Results were expressed as mg of α-tocopherol equiv/g of lipoprotein.

Other Assays. Cholesterol, triglycerides, and phospholipids for the characterization of lipoproteins were measured enzymatically using commercial kits (Boehringer Mannheim, Germany). Proteins were measured by the method of Bradford (1976) using BSA as standard.

Statistical Analyses. All values are reported as mean ± SEM. Normality of distributions were confirmed prior to statistical comparisons. Differences in the means between two groups were assessed with paired *t*-test and, for three or more groups, by ANOVA. The level of significance was set at $P < 0.05$.

RESULTS

The phenolic component in red wine was substantially higher compared with white wines: 1941 mg of gallic acid equivalents (GAE)/L in conventional red wine versus 620 mg of GAE/L in white wine in contact with grape solids and 292 mg of GAE/L in conventional white wine. Other characteristic indices of the finished wines are also shown in Table 1.

The indices of conjugated diene production resulting from the copper-mediated oxidation of LDL carried out in the presence of identical volumes of the polyphenolic extracts from each wine are summarized in Table 2. Lag phase was increased relative to LDL oxidized without extract. It is of note that, whereas an increase in lag phase corresponded with the phenolic component concentration of each extract, the diene production and maximal rate were similar in all cases; only for red wine was a lower value of the maximal rate of diene production observed. To exclude the possibility that this antioxidant effect was due to a type of metal-chelating activity of the vinous phenols, a 5-fold molar excess of copper was used concurrently in the incubation and similar lag phases were observed: 93 vs 91, 137 vs 143, 320 vs 281, and 774 vs 670 min for LDL oxidized without extract (control LDL), with W1, with W2, and with R extracts, respectively.

The indices of oxidation conducted in the presence of identical concentrations (8 μmol of GAE/L) are presented in Table 3. Although each extract was added at the same phenolic component concentration, the increase in lag phase was different among wine extracts and was observed to be highest for W2 extract. Maximal rate and maximal diene production were inhibited in all cases, and the inhibition was inversely proportional to the increase in lag phase induced by the extracts. The effects on TBARS and fluorescence production are presented in Figures 1 and 2. Relative to the control LDL (without extract additions) incubation, TBARS formation (Figure 1, upper panel) was drastically inhibited by the addition of W1 but completely inhibited by W2 and R extracts for up to the 9 h of the experimental incubation ($P = 0.0001$). However, when oxidation was prolonged for 24 h, this inhibitory effect was largely lost (Figure 2, upper panel). Fluorescence production was also significantly inhibited ($P < 0.0001$) at 9 h of incubation by the addition of each extract (Figure 1, lower panel). In contrast to TBARS, at 24 h all of the extracts were still exerting an inhibitory effect on total fluorescence production (Figure 2, lower panel). The inhibitory effect of R and W2 was significantly higher than that of W1 ($P < 0.0001$). The analysis of liposoluble fluorescence, at 24 h in the presence of the extracts, did not show significant differences relative to LDL incubated without the extract. However, hydrosoluble fluorescence production at 24 h was significantly lower in the presence of the extracts ($P < 0.0001$). R and W2 contained lower concentrations of hydrosoluble components than W1 but without being statistically significant. The vitamin E content of LDL in the course of the experimental incubation is presented in Figure 3. At the start of the incubation the tocopherol equivalent concentrations (a measure of the "potential" antioxidative capacity) were higher in the presence of the extract additions than in their absence (LDL control), and the characteristic progressive loss of vitamin E during oxidation was delayed by the presence of the extracts. Although vitamin E was measurable over a longer time in the presence of those extracts that showed a higher initial antioxidant capacity, after 4 h of oxidation vitamin E was completely consumed in all cases.

Table 3. Oxidizability Indices of Low-Density Lipoprotein (LDL) in the Absence and Presence of 8 μmol/L Gallic Acid Equivalents of Phenolic Extracts[a]

	LDL control ($n = 2$)	W1 ($n = 2$)	W2 ($n = 2$)	R ($n = 2$)
lag phase (min)	78	410	516	442
CD_0 (μmol/g of LDL)	53	53	53	53
CD_{max} (μmol/g of LDL)	169	152	133	140
CD production (μmol/g of LDL)	116	99	80	87
t_{max} (min)	128	470	570	510
R_{max} (μmol of CD/min/g of LDL)	3.4	2.6	2.2	2.4

[a] LDL (0.25 mg/mL) was oxidized by incubation with 10 μmol/L $CuSO_4$ in the absence (LDL control) or presence of extracts of standard white wine (W1), white wine made in contact with grape solids (W2), and standard red wine (R), all of which were diluted to contain 8 μmol/L of gallic acid equivalents. Indices were calculated from the conjugated diene (CD) production curves. Results are the mean of two independent experiments.

To evaluate, for comparison purposes, the antioxidant capacity of the phenolic extracts, LDL was oxidized in the presence of different concentrations of α-tocopherol (a well-established antioxidant). The effect of the same concentration of α-tocopherol that this used for the

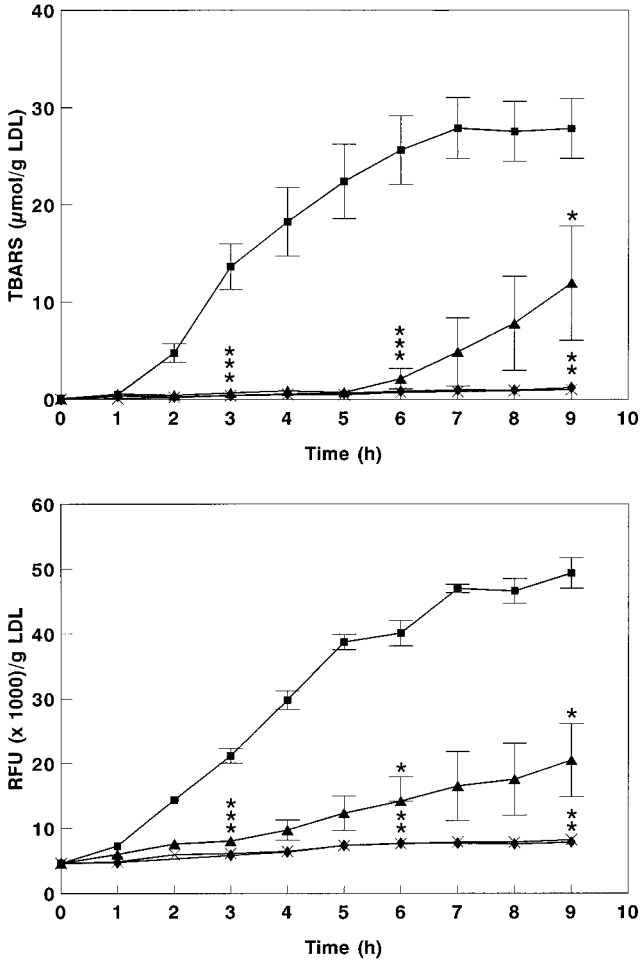

Figure 1. Line plots of thiobarbituric acid reactive substances (TBARS; upper panel) and relative fluorescence units (RFU; lower panel) formation during copper-induced oxidation of 2.5 mg/mL of LDL in the absence (■) or presence of polyphenolic extracts (all at the concentration of 8 μmol of GAE/L) of conventional red wine (♦), conventional white wine (▲), or white wine made in contact with grape solids (×). Each point is the mean and SEM of four independent assays for TBARS and three for fluorescence. Statistical comparisons were made at 3, 6, and 9 h of oxidation. Asterisks refer to $P \leq 0.0001$ compared to LDL control.

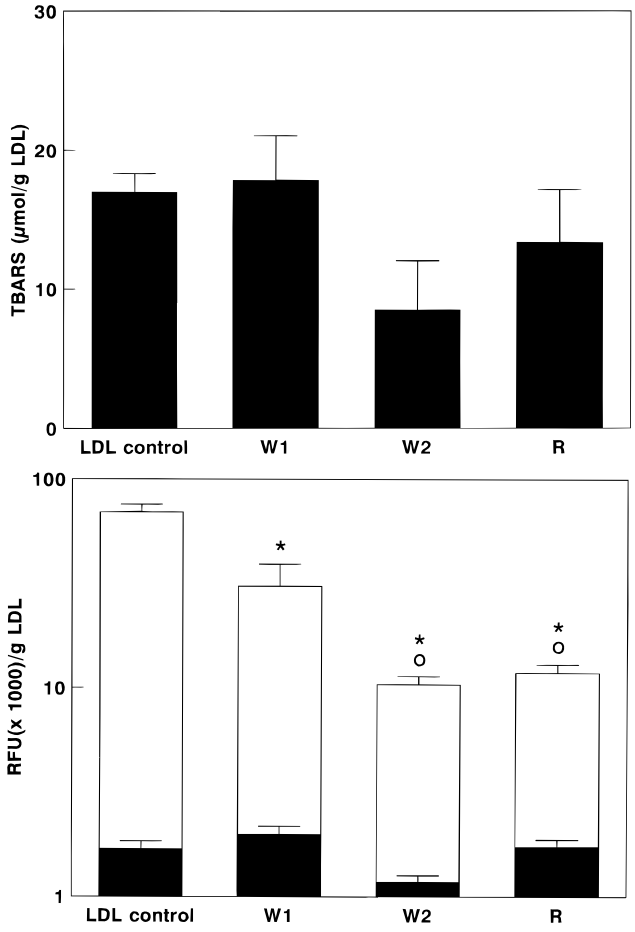

Figure 2. Histograms of thiobarbituric acid reactive substances (TBARS; upper panel) and relative fluorescence units (RFU; lower panel) production at 24 h of copper-induced oxidation of 2.5 mg/mL LDL without (LDL control) or in the presence of polyphenolic extracts (all added at 8 μmol of GAE/L) from conventional red wine (R), conventional white wine (W1), or white wine made in contact with grape solids (W2). Total fluorescence is presented as hydrosoluble (open block) and liposoluble fluorescence (shaded block). Results are the mean and SEM of nine independent assays for TBARS and five for fluorescence. Asterisks refer to $P < 0.0001$ vs control; the open circles refer to $P < 0.0001$ R and W2 vs W1.

extracts (8 μmol/L) resulted in a lower increase of the lag phase. To obtain comparable increases in lag phase that had been observed for the extracts at the concentration of 8 μmol/L, 5−8-fold higher concentrations (40−60 μmol/L) of α-tocopherol were needed (Table 4). In order to compare polyphenols with a hydrophilic antioxidant, we repeated the experiment oxidizing LDL with Trolox and we also included α-tocopherol. In this last experiment lag phases obtained with 8 μmol/L of the extracts were 303 min for W1, 471 min for W2, and 399 min for R vs 58 min for LDL control. When we added the two antioxidants above mentioned we obtained the following lag phases for Trolox: 262 min at 8 μmol/L, 308 min at 30 μmol/L, 429 min at 40 μmol/L, 446 min at 50 μmol/L, and 501 min at 60 μmol/L; for α-tocopherol, 147 min at 8 μmol/L, 220 min at 30 μmol/L, 296 min at 40 μmol/L, 375 min at 50 μmol/L, and 432 at 60 μmol/L vs 58 min for LDL control. In order to analyze the possible antioxidant effect of alcohol, we also oxidized LDL in the presence of 1% or 10% of pure ethanol. When 1% ethanol was added we did not find differences in lag phase, but at 10% ethanol a certain antioxidant effect was observed (results shown in Table 4).

DISCUSSION

The relative contributions to a low incidence of cardiovascular disease of wine and other constituents such as mono-unsaturated fatty acids (mainly oleic acid from olive oil) in the Mediterranean-type diet are the subject of considerable investigation, but the observation that light-to-moderate alcohol consumption confers a lower atherosclerosis risk-factor status appears to have considerable support. However, the mechanism for such anti-atherogenicity is far from clear. To date, the best-supported hypothesis is based on the observation that, with moderate consumption of alcohol, high-density lipoprotein (HDL) components (cholesterol and apolipoproteins AI and AII) are increased (Camargo et al., 1985; Moore and Pearson, 1986). These plasma constituents have been well-established, in epidemiological studies, as being inversely correlated with the incidence of cardiovascular disease (CVD), and the proposed mechanism is that the HDL lipoprotein acts as a captor of the excess of cholesterol and its subsequent transport to the liver for irreversible catabolism. The components increased in HDL could be explained by the cholesteryl ester transfer protein (CETP). Some authors have described a reduction of the CETP activity after a

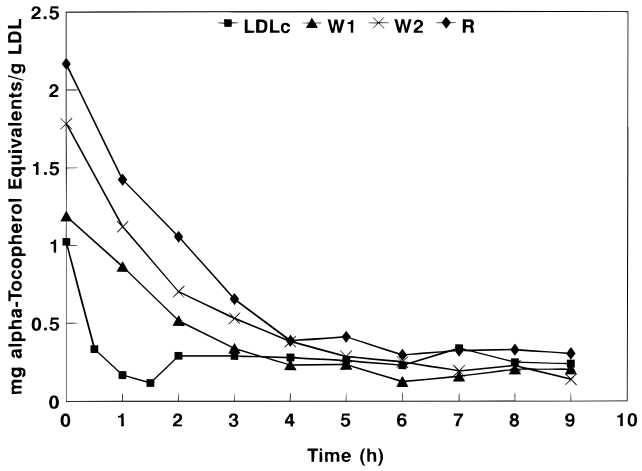

Figure 3. Vitamin E concentration during the course of copper-induced oxidation of 2.5 mg/mL LDL in the absence (LDL control) or presence of polyphenolic extracts (all added at 8 μmol of GAE/L) from conventional red wine (R), conventional white wine (W1), or white wine made in contact with grape solids (W2). Vitamin E concentration is expressed as mg of α-tocopherol equiv/g of LDL. Each point is the mean of four independent LDL oxidation experiments.

Table 4. Comparison of the Antioxidant Effect of Phenolic Extracts with α-Tocopherol[a]

	lag phase (min)
LDL control	78
W1 (8 μmol GAE/L)	410
W2 (8 μmol GAE/L)	516
R (8 μmol GAE/L)	442
α-tocopherol	
(8 μmol/L)	199
(40 μmol/L)	398
(50 μmol/L)	500
(60 μmol/L)	583
ethanol	
1%	82
10%	99

[a] Lag phase of conjugated diene (CD) production was measured when low-density lipoprotein (LDL) (0.25 mg/mL) was oxidized with $CuSO_4$ (10 μL/L) without (LDL control) and in the presence of phenolic extracts from red wine (R), standard white wine (W1), and white wine made in contact with grape solids (W2). All the extracts were added at 8 μmol/L gallic acid equivalents (GAE), while incubations with α-tocopherol (dissolved in ethanol at 1% final concentration) were varied to achieve a similar lag phase as that of the wine extracts. LDL was also oxidized in the presence of 1% or 10% ethanol. Results are the mean of two independent experiments.

moderate alcohol consumption (Fumeron et al., 1995; Hannuksela et al., 1996). Another credible mechanism is the influence of alcohol on hemostasis (Renaud and de Lorgeril, 1992). Alcohol reduces platelet aggregation and also influences the coagulation system by reducing the concentrations of fibrinogen in the circulation (Meade et al., 1986) and, as such, would play a major role in atherogenesis.

Recently, accelerated atherosclerosis has been linked to the modifications in lipoproteins (particularly LDL) induced by oxidative processes and alcohol itself may be acting as a protective factor by reducing the susceptibility of lipoproteins to oxidative modification because it has been described as a potent *in vitro* antioxidant (Serafini et al., 1994), or by facilitating the absorption of other antioxidants via the intestinal lumen (Muller and Fugelsang, 1994). However, beverages with a high alcohol content, as vodka, do not confer this protection *in vitro* (Kondo et al., 1994) and in relation to wine, the type/class of wine consumed appears to be critical. For example, red wine inhibits the oxidation of LDL *in vitro* (De Whalley et al. 1990; Frankel et al., 1993a) but its effect *in vivo* is controversial (Fuhrman et al., 1995; Sharpe et al., 1995; De Rijke et al., 1996). Studies about white wine consumption do not show antioxidant properties and may, indeed, attribute a pro-oxidant effect to white wine (Fuhrman et al., 1995; Sharpe et al., 1995). Although these latter findings are contrary to our previous report (Caldú et al., 1996), the suggestion that the alcohol content of the beverage is not the pertinent factor is valid.

On the other hand, the anti-atherogenic effect of red wine may be due, in part, to the anti-oxidative properties of the polyphenolic content of the wine (De Whalley et al., 1990; Frankel et al., 1993b). Grape solids are rich in polyphenols and fermentation of must with skin and seeds results in a polyphenolic content of red wine which is particularly high (Sims and Bates, 1994; Kanner et al., 1994).

The present study was designed to confirm and to extend our previous findings (Caldú et al., 1996), to which end the phenolic components of the same wines described in this paper were investigated: R, W1, and W2. As expected, R had the highest phenolic concentration, followed by W2 because of the maceration of these two wines with grape solids. Finally, W1 showed the lowest amount of polyphenols.

The standard white wine polyphenolic content, although in lower concentrations than the other two, had an antioxidative effect since, as our results demonstrated, the polyphenolic extract from any of the three wines studied increased the lag phase of the conjugated diene production resulting from the copper-induced oxidation of LDL.

The polyphenolic extracts acted as strong antioxidants. We compared the effect of polyphenolic extracts with α-tocopherol, a lipophilic natural antioxidant contained in LDL and considered efficient in coronary disease treatment (Stephens et al., 1996). But as polyphenols are water-soluble, we also compared the polyphenolic extracts with Trolox, the hydrophilic analogue of α-tocopherol. We used either the natural antioxidant (α-tocopherol) or the water-soluble antioxidant (Trolox), and the polyphenolic extracts showed a higher capacity to inhibit LDL oxidation: to achieve increments in the lag phase similar to those obtained with 8 μmol/L of the polyphenolic extracts, additions of 5−8-fold concentrations of α-tocopherol and 4−8-fold concentrations of Trolox were required. The effect of the polyphenols in increasing the lag phase could not be considered to result from a metal-chelating capability (i.e., the removal of the oxidation-promoting copper ions from the incubation system) since addition of a 5-fold excess of copper ions to the system did not overcome the antioxidative effect of the polyphenols.

The extracts used in our study were free from alcohol, and therefore the effects were attributable to the nonalcoholic components of the wine that were present in the red and white wines. When the LDL was oxidized with equal volumes of each extract, the increases observed in the lag phases were in direct relation to the concentrations of the polyphenols of the different extracts. This indicates that the differences in the antioxidant capacity of these wines resulted from the relative amounts of the polyphenols contained in the extracts and, as such, R would be the most protective followed, in decreasing capacity, by W2 and W1.

Interestingly, when the extracts were added to the incubation system in equal polyphenolic concentrations (8 μmol of GAE/L), the inhibitory effect of the extract from W2 was higher than that from W1 and even from R. These results suggest that the types of polyphenols contained in the extracts, as well as the relative concentrations, are critical in the putative antioxidative function of the wines. Recently, while we prepared this manuscript, Frankel et al. (1995) published a paper about the different antioxidant properties of red and white wines with different phenolic composition.

Other measures of the inhibition of oxidation by the extracts support the above findings. The TBARS and fluorescent compound production were also inhibited by all of the three polyphenolic extracts, the effect being, in descending order, R, W2, and W1. TBARS production is a measure of the aldehydes (mainly malondialdehyde, hexanal, and 4-hydroxinonenal) produced from a progressive oxidative decomposition of fatty acids (mainly arachidonic and docosahexanoic) of the lipid moiety of the LDL (Gebicki et al., 1991). Fluorescence, on the other hand, is almost entirely associated with derivatization of apoprotein (apo B) moiety of the LDL and results from the binding of the protein's free amino groups with the aldehyde products from the lipid peroxidation (Esterbauer et al., 1990). The inhibition of total fluorescence production was greater by R and W2 than by W1 and the effect appeared to be greater in the hydrosoluble than the liposoluble fraction. These findings together with the observation that the addition of the extracts reduces the total fluorescence but not the TBARS production over the 24 h of oxidation, suggest that the polyphenols of wine may delay but not inhibit lipid oxidation while continuing to inhibit the derivatization of apo B. As such, there could be further enhancement of the anti-atherogenic effect since changes in the apo B structure diminishes its normal recognition by the LDL receptor (Witztum and Steinberg, 1991) and, if drastically abnormal, causes the apo B to be taken up by the macrophage scavenger receptor (Haberland et al., 1982) with an increased likelihood of accumulation of cholesterol in the cells leading to foam cell formation.

The natural resistance of LDL to oxidation reflects the endogenous antioxidant (e.g., vitamin E) content of the lipoprotein (Bowry et al., 1992). Our analysis showed that the initial antioxidant capacity of the incubation system was greater in the presence of the extracts. However, it seems that the antioxidant effect of the extracts could not be explained through their capacity to delay the vitamin E consumed during LDL oxidation. In fact, the production of conjugated diene, TBARS, or fluorescence was inhibited even at 9 h of oxidation, but the vitamin E content of the lipoprotein was already exhausted at 4 h of oxidation.

In conclusion, it is clear from the present data that the polyphenols of wine have an *in vitro* antioxidative capacity. How this ability functions *in vivo* or, indeed, which of the polyphenols is the active ingredient is, for the moment, unclear since there is a paucity of information regarding the absorption, metabolism, and excretion (Das, 1971; Gugler et al., 1975; Griffith, 1982; Hackett, 1983) of wine polyphenols. Further, the results demonstrate that white wine, although with a polyphenolic concentration considerably lower than that of red wine, could have a greater antioxidative capacity when both wines are at the same phenolic concentration. We also can conclude that winemaking that includes maceration of the must with grape solids improves the antiatherogenic potential of wine.

ABBREVIATIONS

CD, conjugated dienes; CETP, cholesteryl ester transfer protein; CVS, cardiovascular disease; GAE, gallic acid equivalents; HDL, high-density lipoproteins; HPLC, high-performance liquid chromatography; LDL, low-density lipoproteins; TBARS, thiobarbituric acid reactive substances.

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