Effect of Red Wine Consumption on Low-Density Lipoprotein Oxidation and Atherosclerosis in Aorta and Coronary Artery in Watanabe Heritable Hyperlipidemic Rabbits

Yasuhisa Wakabayashi*

Department of Medicine, University of Kitasato Medical School, 1-15-1 Kitasato, Sagamihara 228-8555, Japan

Red wine was given to Watanabe heritable hyperlipidemic (WHHL) rabbits (7 mL/kg/day, approximately 23 mg total polyphenol/kg/day) from the age of 4 months for 14 months. With WHHL rabbits given a comparable dose of alcohol used as references, the effect of wine phenolics on the morphology of atherosclerotic lesions in the coronary artery and aorta was investigated in light of the susceptibility of plasma intermediate and low-density protein (ILDL) to oxidation. ILDL of animals consuming red wine showed reduced susceptibility to oxidation, but atherosclerotic lesion progression was not prevented. In WHHL rabbits, a dose of wine phenolics sufficient to inhibit ILDL oxidation might not be adequate to prevent the progression of atherosclerosis morphologically.

Keywords: *Red wine; phenolic compounds; alcohol; antioxidant; LDL oxidation*

INTRODUCTION

Moderate consumption of alcoholic beverages has been reported to reduce the risk of coronary heart disease (Klatsky et al., 1974; Kozararevic et al., 1980). In a controlled animal experiment in which rabbits were fed a high cholesterol diet along with water or different beverages containing equal amounts of alcohol for 3 months, every alcoholic beverage except beer was demonstrated to reduce the incidence of coronary arteriosclerosis, the greatest protection being provided by red wine (Klurfeld and Kritchevsky, 1981). Renaud and de Lorgeril (1992) demonstrated that the consumption of red wine was a primary correlate in the French paradox, the unexpected low incidence of coronary heart disease mortality in certain French populations consuming high-fat diets and having a high plasma cholesterol concentration. Subsequently, phenolic compounds in red wine were shown to protect low-density lipoprotein (LDL) from oxidation in vitro (Frankel et al., 1993). This antioxidant effect provided a plausible explanation for the French paradox, since evidence had been accumulated by that time that oxidatively modified LDL played a crucial role in atherogenesis (Steinberg et al., 1989). Since then, the putative protective effect of red wine on atherosclerosis has been widely advocated. However, inhibition of LDL oxidation by wine phenolics has been little documented in conjunction with morphological alteration in atherosclerosis. The present study tested whether red wine phenolics would provide any benefit in the morphology of atherosclerosis in Watanabe heritable hyperlipidemic (WHHL) rabbits. Red wine was given from the age of 4 months, when coronary atherosclerosis develops, until the age of 18 months, when all animals show the disease (Watanabe et al., 1985). WHHL rabbits given a comparable dose of alcohol alone were used as references.

* Telephone: 81-42-778-8022. Fax: 81-42-778-8441. E-mail: yasiwaka@med.kitasato-u.ac.jp.

MATERIALS AND METHODS

Animals. Homozygous WHHL rabbits were kindly supplied from the Institute for Experimental Animals, Kobe University School of Medicine. They were housed individually in metal cages in a room controlled to 22 °C and 40−45% humidity on a 14 h/10 h light/dark cycle. A CR1 rabbit diet containing 3.4% vegetable fat, 0.3% animal fat, 61 mg % cholesterol, and 20.6% protein (Clea Japan Inc, Tokyo) was given (120 g daily). Animals had unrestricted access to water. At 4 months of age, the animals were assigned to either the red wine or the ethanol drinking groups. Because sex-related differences in atherogenesis do not occur in this model (Watanabe et al., 1988), group assignment was random. The wine-drinking group was composed of 4 males and 1 female (mean body weight, 2.8 ± 0.1 kg). The ethanol-drinking group was composed of 4 males and 3 females (mean body weight, 2.7 ± 0.1 kg).

After 14 months of treatment, the 18-month-old rabbits were anesthetized with 25 mg/kg pentobarbital sodium intravenously. Following local anesthesia with 2% xylocaine hydrochloride, the carotid artery was isolated to insert a cannula for exsanguination. The body was perfused with 500 mL of Ringer's solution, given via ear vein, over approximately 30 min. When the heart beat had stopped, the heart and the aorta from the ascending aorta to just distal to the left renal artery were removed. The heart was immediately immersion-fixed in 4% buffered paraformaldehyde. Following fixation, the ventricle was cut horizontally into eight equal pieces from the coronary sulcus to the apex, and each cross section was embedded in paraffin (Watanabe et al., 1988). The 3 μm sections were then prepared and stained with hematoxylin-eosin, Masson's trichrome stain, and van Gieson's elastica stain. Aorta were cleaned of loose adventitial tissue, opened longitudinally to expose the intimal surface, and pinned on a corkboard. The intima was digitally recorded by a Panasonic video camera (model NV-DJ1, Osaka). To evaluate plaque morphology microscopically, segments of descending thoracic aorta were cut transversely, fixed, embedded, and stained in the same manner as described above.

Luminal Narrowing Ratio of Coronary Arteries and Extent of Aortic Atherosclerotic Lesion. Shiomi et al. (1990) and Kroon et al. (1993) assessed coronary atherosclerosis by determining degrees of diameter narrowing in the most severely affected segment in three or four major coronary

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arteries, namely, the left anterior descending artery, left circumflex artery, left septal artery, and right coronary artery. In the present study, it was assessed by determining the luminal narrowing ratio in all of the segments of coronary arteries (four major coronary arteries and their branches) in the eight cross section specimens, on the condition that each arterial segment was transversely dissected. Usually, six to ten arterial segments were observed in each cross section specimen. Therefore, approximately 50−80 segments were observed per heart. The image of each segment was observed with a model BX 50 microscope (Olympus Optical Co. Ltd., Tokyo) and was recorded in a Macintosh computer using an image analyzing system (model DXC-108, Olympus Optical Co. Ltd., Tokyo). The luminal narrowing ratio of each segment was determined by calculating the area of plaque and lumen (area of plaque divided by that of lumen, expressed in %) with a MacScope package and a National Institute of Health image processing program. The area of lumen was termed as that bounded by the internal elastic lamina. The video-recorded image of the aortic intima was processed in a similar manner, and the extent of atherosclerotic lesion (area of visible atheromatous lesion divided by area of surface, expressed in %) was determined. All animal experiments were conducted according to the University guidelines for animal experiments.

Red Wine and Alcohol. Japanese pinot noir (Fujimien 1989 and 1993, Suntory Yamanashi Winery) was used in the study. Total phenolic content was determined according to the Folin−Ciocalteu method (Singleton and Rossi, 1965) to be 3430 mg/L (1989 vintage) and 3160 mg/L (1993 vintage). Alcohol content by volume was 11.9% (1989) and 11.8% (1993). A dose of 7 mL/kg body weight of red wine (approximately 23 mg total polyphenol/kg/day) was diluted with water, and 50 mL was prepared in a plastic bottle hung in front of individual cages, subject to spontaneous drinking. This dose was estimated from the studies performed by Fuhrman et al. (1995) and Nigdikar et al. (1998). For animals consuming alcohol alone, 1 mL/kg body weight of industrial ethanol (99.5%) was prepared in the same manner. Final concentration of alcohol in the diluted beverages was determined by the enzymatic method using aca analytical test packs (Dade Behring, Wilmington, DE). It was approximately 38−39 g/L (825−847 mmol/L) in bottles containing red wine and 36−40 g/L (782−868 mmol/L) in bottles containing ethanol when beverages were prepared for 3.0 kg animals. Those values were comparable but lower than predicted. Whether and how the animals consumed alcoholic beverages was tested via a pilot study with four rabbits, $n = 2$ per group. The animals did not finish drinking 50 mL of alcoholic beverage at a time, but the bottles were usually empty by the next morning. Little liquid was found spilt over the floor, and collected urine gave an alcoholic scent. Red wine or ethanol was freshly prepared daily in the morning and given for 5 days a week for 14 months without a break. The dose was adjusted weekly following measurements of body weights.

General Condition and Biochemical Determinations. General appearance and intake of food and alcoholic beverages were observed daily. At the study period of 0 (4 months of age), 1, 3, 4, 6, 10, and 14 months, blood was collected from the external jugular vein after an overnight fast. Packed cell volume was determined, and plasma was separated and analyzed for levels of total cholesterol, HDL cholesterol, triglyceride, total protein, total bilirubin, albumin, iron, sugar, urea nitrogen, uric acid, creatinine, and electrolytes, as well as activities of aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transferase, alkaline phosphatase, cholinesterase, lactate dehydrogenase, amylase, and creatine kinase. The analysis was performed with a model AU 600 autoanalyzer (Olympus Optical Co. Ltd., Tokyo) at Sumitomo Metal Bio-Science Inc. (Tokyo). To assess any possible relationship between progression of atherosclerosis and the serum level of glycated protein products, serum fructosamine was measured by a colorimetric procedure according to the method described by Johnson et al. (1982).

LDL Separation. At the completion of the study (14-month study period), blood was drawn and collected into tubes containing EDTA (final concentration, 1 mg/mL). LDL was separated from plasma by nonsequential density gradient ultracentrifugation. A density solution of 1.006 kg/L (0.9 mL) was layered over 1.8 mL of plasma in a polycarbonate tube. This was placed in an RP100AT4 rotor (Hitachi Koki Co, Katsuta, Japan) and centrifuged at 411000*g* (av) at 4 °C for 3.5 h with a CS-120 ultracentrifuge (Hitachi Koki Co, Hitachinaka, Japan). The top layer containing very low-density lipoprotein (0.9 mL) was removed. The rest was mixed, covered with 0.9 mL of a density solution of 1.182 kg/L, vortexed for a few seconds, and spun again in the same manner. The visible LDL layer was removed by aspiration. This LDL-containing fraction (d 1.006−1.063) was applied to an agarose gel electrophoresis system to discern its purity, using a Helena REP Lipo-30 Electrophoresis System (Helena Laboratories, Beaumont, TX). This fraction yielded a single broad band on an agarose gel. This fraction, however, contained both intermediate-density lipoprotein (IDL) and LDL, hence termed ILDL hereafter. ILDL was diluted with 10 mM phosphate buffer containing 150 mM NaCl, pH 7.2 (PBS), and EDTA was removed by ultrafiltration using a Centriprep-30 tube (molecular weight cutoffs, 30 000 daltons, Amicon, Beverly, MA). The protein content in ILDL (free of EDTA) was determined using a protein assay kit (Bio-Rad, Hercules, CA).

ILDL Oxidation ex Vivo. *(1) Measurement of the Formation of Conjugated Dienes following Copper Ion Induced Oxidation (Esterbauer et al., 1989).* ILDL was diluted with PBS to a concentration of 50 μg protein/mL. Oxidation was initiated by the addition of freshly prepared $CuSO_4$ solution (final concentration, 2 μM), and incubation was performed at 37 °C. Changes in absorbance at 234 nm were continuously monitored using a thermostat-controlled U-3220 spectrophotometer (Hitachi, Tokyo, Japan), and the lag time and oxidation rate were determined. The lag time was defined as the intercept between the tangent of the absorbance curve during the propagation phase with the baseline and was expressed in minutes. The oxidation rate was calculated from the slope of the absorbance curve during the propagation phase and was expressed in Δ absorbance units/min.

(2) Thiobarbituric Acid Reactive Substances (TBARS) Assay. ILDL was diluted with PBS to a concentration of 150 μg protein/mL. This native ILDL was incubated with $CuSO_4$ (final concentration, 2 μM) at 37 °C for 3 h. The reaction was terminated by the addition of 20 μM of EDTA and refrigeration. Then, 1 mL of native ILDL or oxidized ILDL was mixed with 1 mL of 20% trichloroacetic acid and 1 mL of the reagent containing 8.8 M acetic acid and 2.3 M 2-thiobarbituric acid, and TBARS were assayed according to the method described by Buege and Aust (1978). TBARS were expressed as lipid peroxide content in nmol/malondialdehyde equiv/mg ILDL protein, with tetramethoxypropane used as reference. The ratio of lipid peroxide content in the oxidized ILDL to the native ILDL was calculated and was defined as an increase in TBARS formation following oxidation, expressed in %.

Electrophoretic Migration Distance on Agarose Gel Electrophoresis. ILDL was adjusted to 50 μg protein/mL with PBS and applied to an agarose gel electrophoresis system as described above. Following electrophoresis and staining, the distance from the sample well (cathode side) to the middle of the (single) band was measured on a gel. This was termed the migration distance and was expressed in mm.

Fatty Acid Content. ILDL was adjusted to 150 μg protein/mL with PBS, and its fatty acid content was analyzed by high performance liquid chromatography using the internal standard method. The system was composed of LC-6A (Shimadzu Co., Kyoto, Japan) equipped with a fluorescence spectrophotometer monitoring at 412 nm with excitation at 255 nm. A Lichrospher RP8 column (25 mm × 0.46 mm, Kanto Chemical Co, Tokyo) was used, and column temperature was maintained at 40 °C in a column oven. Fatty acid derivatives with 9-anthryldiazomethane reagent were eluted by a programmed linear gradient with A ($CH_3CN/H_2O/CH_3OH = 90/60/1$) and B ($CH_3CN$) within 128 min. The flow rate was 2.1 mL/min. The retention times, peak areas, and percentage distribution of the compounds were computed with an integrator (model CR-5A,

Table 1. Plasma Biochemical Variables at 14 and 18 Months of Age[a]

	ethanol ($n = 7$)		red wine ($n = 5$)	
	4 months	18 months	4 months	18 months
total protein (g/L)	64 ± 2	67 ± 2	62 ± 1	70 ± 1
iron (mmol/L)	32 ± 3	34 ± 4	39 ± 4	40 ± 5
total bilirubin (mmol/L)	10 ± 2	6 ± 1	12 ± 2	5 ± 1
aspartate amino-transferase (U/L)	32 ± 6	26 ± 4	44 ± 9	30 ±4
alanine amino-transferase (U/L)	96 ± 30	72 ± 13	110 ± 27	52 ± 8
amylase (U/L)	348 ± 26	336 ± 22	316 ± 15	298 ± 9
γ-glutamyl transferase (U/L)	4 ± 1	5 ± 1	4 ± 1	5 ± 1
alkaline phosphatase (U/L)	303 ± 33	48 ± 4	257 ± 11	51 ± 6
urea nitrogen (mmol/L)	7.6 ± 0.6	5.4 ± 0.6	7.7 ± 0.2	4.3 ± 0.5
creatinine (mmol/L)	70 ± 10	90 ± 10	60 ± 10	80 ± 10

[a] Plasma biochemical variables were measured at the ages of 4, 5, 7, 8, 10, 14, and 18 months. Changes in these variables in relation to treatment and time trend were analyzed by repeated measures of ANOVA. The overall changes were not significantly different over the experimental period in both groups. No overall differences were seen between the groups. Only alkaline phosphatase activity revealed a significant decrease over time in both groups ($p < 0.05$), but overall the differences between the groups were not significant.

Shimadzu Co.). An aliquot of 5 μL of the sample was injected using an autosampler. Results were expressed in μM/mg ILDL protein.

Statistical Analysis. All data were expressed as mean ± SEM. Assessment of any interaction between treatment and time trend was performed by a repeated measures of analysis of variance (ANOVA). Each index of ILDL oxidation or morphology obtained at the end of the study was compared and assessed by Mann−Whitney's U test between the two groups. A SAS program was used for each analysis.

RESULTS

General Condition and Plasma Biochemical Determination. Animals consumed the full amount of food and alcoholic beverages given daily. The mean body weight at the end of the experiment was 3.5 ± 0.1 kg in animals consuming ethanol and 3.4 ± 0.2 kg in animals consuming red wine. At the start of the experiment, values of packed cell volume and biochemical parameters were comparable between the two groups. These variables did not show any significant change over the treatment time in animals of both groups (by ANOVA) (Table 1). Only the levels of total cholesterol and alkaline phosphatase showed a decrease toward the end of the experiment. The decline over the treatment period was statistically significant in both groups (Figure 1). However, the overall differences between the two groups were not significant. The levels of HDL cholesterol or triglyceride did not show any significant change during the study period (Figures 2 and 3). Also, the overall differences were not significantly different between the groups. The HDL cholesterol constantly stayed at extremely low levels. The serum fructosamine was 431 ± 36 μmol/L in animals consuming ethanol and 430 ± 51 μmolL in animals consuming red wine at the start of the experiment. It showed a trend to decrease rather than increase over the treatment period, but overall changes were not statistically significant in both groups.

ILDL Oxidation (Table 2). As compared to the ILDL of ethanol-drinking animals, the ILDL of animals consuming red wine demonstrated more resistance to oxidation, as indicated by extension of the lag time and

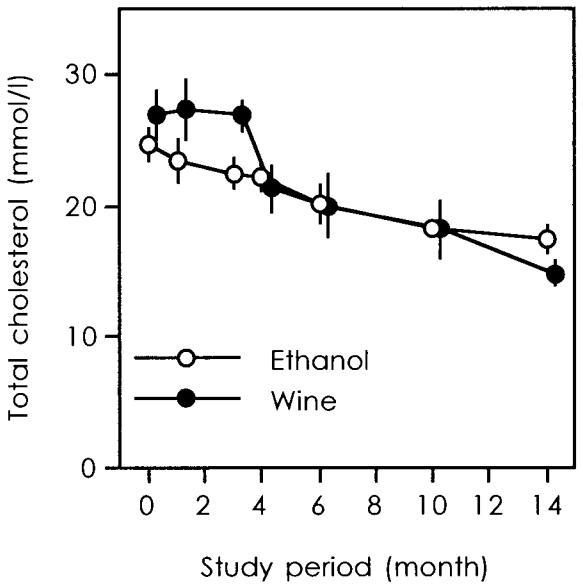

Figure 1. Levels of total cholesterol. The decrease over the treatment time was significant in both groups ($p < 0.05$), but overall the differences between the two groups were not significant (analyzed by ANOVA).

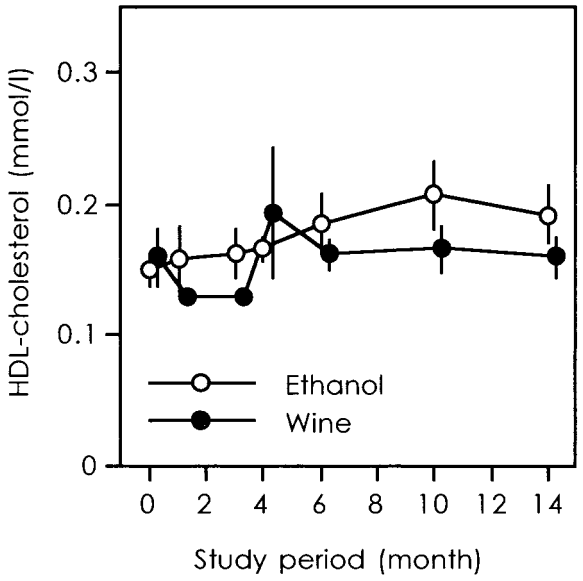

Figure 2. Levels of HDL cholesterol in animals treated with ethanol or red wine.

reduction in oxidation rate and TBARS formation. Each index was significantly different between the two treatments.

Electrophoretic Migration Distance on Agarose Gel Electrophoresis. The migration distance of native ILDL from animals consuming ethanol was 9.8 ± 0.2 mm, and that from animals consuming red wine was 9.2 ± 0.2 mm. The difference was statistically significant ($p < 0.05$). This indicated that ILDL from animals consuming red wine was less negatively charged and hence less oxidized than that from ethanol-consuming animals.

Fatty Acid Content. There was no significant difference in any fatty acid content in ILDL between the groups, except in the α-linolenic acid content. Linoleic acid was the richest fatty acid in ILDL. Its content was 4.65 ± 0.24 μmol/mg ILDL in the animals consuming ethanol and 4.28 ± 0.33 μmol/mg ILDL in the animals

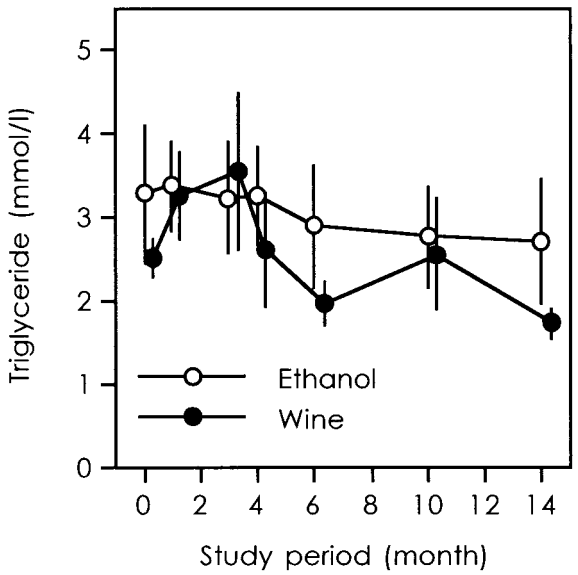

Figure 3. Levels of triglyceride in animals treated with ethanol or red wine.

Table 2. Oxidative Susceptibility of ILDL of Animals Consuming Ethanol and Red Wine[a]

	ethanol ($n = 7$)	red wine ($n = 5$)	p
lag time (min)	23.2 ± 2.7	53.7 ± 1.5	<0.01
oxidation rate during propagation phase (Δ absorbance/min × 10^{-3})	0.34 ± 0.08	0.13 ± 0.03	<0.01
TBARS in native ILDL[b] (nmol/mg ILDL)	1.9 ± 0.4	2.1 ± 0.4	NS
TBARS in oxidized ILDL[c] (nmol/mg ILDL)	3.2 ± 1.0	2.5 ± 0.5	NS
increase in TBARS formation following oxidation (%)	159 ± 17	123 ± 6	<0.05

[a] Paired data were compared and analyzed by Mann−Whitney's U test. NS: not significant. [b] TBARS measured before oxidation. [c] TBARS measured following oxidation by the addition of $CuSO_4$ and incubation for 3 h at 37 °C. TBARS was expressed as lipid peroxide content in nmol/malondialdehyde equiv/mg ILDL protein, with tetramethoxypropane used as reference.

consuming red wine. The content of α-linolenic acid was 0.29 ± 0.05 μmol/mg ILDL in the ethanol-consuming animals and 0.13 ± 0.04 μmol/mg ILDL in the animals consuming wine ($p < 0.05$). However, the amount itself was small, and no significant correlation was found between α-linolenic acid content in ILDL and such oxidation parameters as lag time, oxidation rate, or TBARS formation rate. Therefore, the different α-linolenic acid contents in ILDL would not fully explain the different susceptibility of ILDL to oxidation in the present study.

Morphological Analysis (Table 3). The numbers of arterial segments observed in the eight cross section specimens were 71 ± 5 in the heart of the animals consuming ethanol and 80 ± 6 in the heart of the animals consuming red wine. These values were comparable. Plaque lesion was found in specimens from cross sections 1−6 and in approximately 10% of arterial segments in the hearts of both groups. Limited to arteries with plaque lesion, the mean luminal narrowing ratio was 79.3 ± 3.2% in the arteries of animals consuming ethanol (range 28.6−99.8%) and 75.2 ± 4.8% in the arteries of animals consuming red wine (range 20.4−98.8%). When the luminal narrowing ratios were calculated in all of the arterial segments, they were approximately 8% in both groups. When taking one of the most severely affected arterial segment, the average luminal narrowing ratios in animals consuming ethanol and red wine were 91.6 ± 3.0% and 96.7 ± 0.9%, respectively. All of these indices were not significantly different between the groups. The extent of atheromatous lesion on the aortic intimal surface was nearly 100% in both groups.

Table 3. Index of Atherosclerosis in Aorta and Coronary Artery in Animals Consuming Ethanol and Red Wine for 14 Months

	ethanol ($n = 7$)	red wine ($n = 5$)	p
Aorta			
extent of atheromatous change on the intimal surface (%)	98.0 ± 1.3	97.6 ± 1.1	NS
Coronary Artery			
total segments of coronary arteries observed in eight cross-sections (per heart)	71 ± 5	80 ± 6	NS
number of arterial segments with plaque (per heart)	7 ± 1	8 ± 1	NS
incidence of plaque lesion in the total segments (%)	10 ± 2	10 ± 1	NS
luminal narrowing ratio in the segments with plaque (%)	79.3 ± 3.2	75.2 ± 4.8	NS
luminal narrowing ratio in the total segments (%)	8.0 ± 1.2	7.5 ± 1.0	NS
luminal narrowing ratio in the most severely affected segment (%)	91.6 ± 3.0	96.7 ± 0.9	NS

Plaque of coronary arteries and aorta was evaluated in light of degrees of fibrosis, accumulation of foamy cells, cellular infiltration, fatty degeneration, or intimal thickening, by grading from (−) to (3+). Each index was graded as (2+) to (3+) in both groups, and there was no significant difference in any index between the two treatments.

DISCUSSION

The epidemiological study dubbed "French paradox" (Renaud and de Lorgeril, 1992) was hypothesized to be partially explainable on the basis of the antioxidant actions of wine phenolics and their ability to inhibit LDL oxidation in vitro (Frankel et al., 1993). This hypothesis assumed that oxidized LDL plays a causative role in atherosclerosis and more generally that deleterious oxidation of various sorts contributed to disease development (Kinsella et al., 1993). The present study tested whether a fixed dose of wine phenolics in ethanol was more efficacious than a comparable dose of alcohol alone in altering lesion progression in the WHHL rabbit. Further, this study sought to determine whether the changes in the resistance of plasma LDL to oxidation ex vivo could be associated with observed changes in either the extent or morphology of arterial lesions.

At the start of the experiment, body weight, packed cell volume, and some of biochemical parameters were comparable to those of 3-month-old WHHL rabbits in the study performed by Norido et al. (1993). Significant changes in biochemical parameters were not found over the treatment time except for total cholesterol and alkaline phosphatase activity, but the decline in total cholesterol and alkaline phosphatase activity over age was also observed in normal WHHL rabbits (Norido et al., 1993). The mechanism was unknown but could be ascribed to aging (Watanabe et al., 1988). The body weight at the end of the study was comparable to the

value of 3.6 ± 0.1 kg for untreated WHHL rabbits at a comparable age (19 months old) in the study performed by Hansen et al. (1994). Water-drinking controls were not used in the present study. However, if findings of Norido et al. (1993) and Hansen et al. (1994) were taken into comparison, it was assumed that rabbits tolerated treatments of red wine or ethanol without apparent adverse effects.

Red wine used in the present study contained comparable amounts of phenolic compounds as the various red wines shown in the literature (Kanner et al., 1994; Frankel et al., 1995). The daily amount would be approximately 400 mL and 1300 mg phenolics for 60 kg body weight human adults. In the study in which healthy human subjects consumed 400 mL of Israeli red wine, divided between lunch and supper time, Fuhrman et al. (1995) demonstrated that the total phenolic content in LDL increased by 41% in 14 days and LDL gained resistance against peroxidation. In the recent study in which healthy human subjects consumed 375 mL of French cabernet sauvignon (containing 1.6 g/L polyphenol) or 450 mg/day polyphenol, Nigdikar et al. (1998) showed that plasma polyphenol increased by 38% and 28%, respectively, after treatment for 14 days and LDL showed reduced susceptibility to oxidation. Assuming that the bioavailability of red wine and polyphenol in rabbits was not so different from that in humans, a comparable dose of red wine or wine phenolics used in the present study would be expected to inhibit LDL oxidation in WHHL rabbits. Indeed, LDL (ILDL) of animals consuming red wine (over 14 months) demonstrated resistance against oxidation as compared to ILDL of animals consuming ethanol alone, even in the presence of high levels of total cholesterol and low levels of HDL cholesterol. Despite these changes, there was no morphological difference in aorta or coronary artery between the two treatments.

The extent of aortic atherosclerosis was almost 100% in animals consuming either red wine or ethanol. The lesions observed in the present study were more severe than those of 18-month-old WHHL rabbits (66 ± 7%) used as controls in the study performed by Shiomi et al. (1990). The luminal narrowing ratios in coronary arteries with plaque lesion were also comparable between the two treatments (75−80%). That in the most severely affected segment was 91−96% in animals of both treatments. Because the hearts were not subject to perfusion-fixation in the present study, luminal narrowing ratios would not represent an exact value. However, relative comparison between the two treatments was considered possible as all hearts were treated in the same manner. Shiomi et al. (1990) reported that the mean luminal narrowing ratio in the maximum lesion of three major coronary arteries was 46 ± 7% in WHHL rabbits used for controls. Hansen et al. (1994) studied normal 19-month-old WHHL rabbits and showed that coronary arteriosclerosis at proximal portions was observed in all 11 animals and the degree of stenosis averaged 65%. Although direct comparison would not be simply made because of the reason described above, it could be estimated that the degree of coronary arteriosclerosis in the present study was comparable to or even worse than that in normal WHHL rabbits in the studies performed by Shiomi et al. (1990) and Hansen et al. (1994). Therefore, in light of the benefit in morphology, it would be stated that neither red wine nor ethanol was superior to water alone.

In the study in which low doses of the antioxidants probucol or vitamin E (both 0.025% of diet by weight) were given to WHHL rabbits from the age of 5−7 months, LDL showed reduced susceptibility to oxidation over the 6-month treatment period, but attenuation of the progression of aortic atherosclerosis was not associated, as compared to nontreatment animals (Kleinveld et al., 1994). The dose of probucol (25 mg/day for rabbits) was equivalent to its therapeutic dose (usually 1000 mg/day for human adults). In contrast, the extent of aortic lesion was demonstrated to be attenuated following treatment with a large dose of probucol (1% of the diet by weight) in WHHL rabbits in the studies performed by Carew et al. (1987) (from the age of 6−8 weeks over 20 months) and Kita et al. (1987) (from the age of 2 months over 6 months), irrespective of hypocholesterolemic effect. These results indicated that from the morphological point of view, the disease process appeared to be dependent upon LDL oxidation and that attenuation in the progression of atherosclerosis appeared to be dependent upon the dose of this particular antioxidant in the WHHL rabbit.

Development of coronary arteriosclerosis was protected by red wine given to Dutch belted rabbits fed with a high cholesterol diet for 3 months (Klurfeld and Kritchevsky, 1981). The animals (2.2 kg body weight) daily consumed 33 mL of red wine, diluted to adjust the alcohol content to 9.5%. The dose was larger by 1.7 times (by alcohol content) than that in the present study. Plasma level of total cholesterol, initially 17.48 ± 3.33 mmol/L, was 22.83 ± 1.55 mmol/L in the animals consuming red wine and 16.42 ± 2.41 mmol/L in the animals consuming ethanol. These values were not extremely different from those of the present study (Figure 1). However, the plasma levels of HDL cholesterol at both initial and end points were in contrast to those of the present study (Figure 2). The initial value of 0.80 ± 0.21 mmol/L increased to 1.81 ± 0.26 mmol/L and 2.02 ± 0.05 mmol/L following consumption of red wine and ethanol, respectively. In the present study, HDL cholesterol was determined enzymatically after serum was precipitated by dextran sulfate-magnesium (by autoanalyzer). Shiomi et al. (1990) measured HDL cholesterol enzymatically following lipoprotein fractionation in 18-month-old WHHL rabbits used as controls and reported it to be 0.36 ± 0.05 mmol/L. Thus, HDL cholesterol metabolism appeared to be different between the two species of rabbits. This possibility, as well as a different dose or treatment time, might account for different lesion progression in the coronary artery following consumption of red wine between the two species of rabbits and/or between hereditary and acquired hypercholesterolemia. Therefore, one possibility that might explain the failure to show any morphological attenuation in the present study would be that the dose of antioxidant provided as nonalcoholic components of red wine had not been adequate to this particular genetic model of rabbit.

A possible benefit would have been afforded by wine phenolics if the morphological evaluation had been performed at earlier ages, because the defense mechanism against oxidation might be reduced over age. However, there was evidence against this possibility. In the study performed by Kleinveld et al. (1994), the lag time showed a trend to increase rather than decrease and the rate of conjugated diene production showed a trend to decrease rather than increase over 6

months in LDL of nontreated WHHL rabbits. Although these trends were not statistically significant and the observed period was from the age of 5−7 months to 11−13 months, the evidence might indicate that WHHL rabbits maintained or gained little, if any, antioxidant capacity or that the dose of oxidized LDL was not increased over age. The decline in blood cholesterol (and presumably LDL) over age (Figure 1) would support the latter possibility.

Coronary arteriosclerosis was assessed by the morphological extent and nature of plaque lesion in the present study. However, the reduction of coronary events was demonstrated to be achieved with minimal angiographic improvement in the clinical trials of cholesterol lowering (Brown et al., 1990; Watts et al., 1992; Waters et al., 1994). The finding indicated that, for atherosclerotic clinical events (indeed, the key parameter in the French paradox) to take place, more factors in addition to morphological severity would be required. Such factors as instability of plaque (Brown et al., 1993), dysfunction of endothelium adjoining to the lesion (Leung et al., 1993), and increased thrombogenicity (Lacoste et al., 1995) were proposed. If the hypothesis of the French paradox was correct, wine phenolics with or without alcohol could modify these factors via an antioxidant mechanism or in combination with an antioxidant mechanism and other mechanisms.

ABBREVIATIONS USED

ANOVA, analysis of variance; EDTA, ethylenediaminetetraacetic acid; HDL, high-density lipoprotein; IDL, intermediate density lipoprotein; ILDL, intermediate-density lipoprotein plus low-density lipoprotein; LDL, low-density lipoprotein; PBS, phosphate-buffered saline; TBARS, thiobarbituric acid reacting substances; WHHL, Watanabe heritable hyperlipidemic.

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