Red Wine Inhibits Monocyte Chemotactic Protein-1 Expression and Modestly Reduces Neointimal Hyperplasia After Balloon Injury in Cholesterol-Fed Rabbits

An-Ning Feng, MD; Yuh-Lien Chen, PhD; Ya-Ting Chen, BS; Yaw-Zon Ding, MD; Shing-Jong Lin, MD, PhD

Background—Wine consumption decreases the risk of myocardial infarction. Intimal hyperplasia contributes to restenosis after angioplasty. Local ethanol delivery inhibits intimal hyperplasia after balloon injury in rabbit iliac and pig coronary arteries. The effects of wine consumption on intimal response and monocyte chemotactic protein-1 (MCP-1) expression were studied in cholesterol-fed rabbits.

Methods and Results—Male rabbits were fed a 2% cholesterol diet together with red wine (12.5% vol, 5 mL/kg body wt per day; n=7), white wine (13.3% vol, 5 mL/kg body wt per day; n=7), or no wine as a control (n=8) for 6 weeks. A balloon injury of the abdominal aorta was performed at the end of the third week. Abdominal aortas were harvested at the end of 6 weeks. Neointimal hyperplasia was measured morphometrically. MCP-1 expression was determined by Northern blot, in situ hybridization, and immunohistochemistry. Rabbits fed red wine had significantly less neointimal hyperplasia than did control rabbits (intima/media area ratio 0.59 \pm 0.05 [red wine group] versus 0.79 \pm 0.07 [control group], P<0.05). However, rabbits fed white wine showed a trend (but not significant) toward less intimal response compared with control rabbits (intima/media area ratio 0.65 \pm 0.04 [white wine group] versus 0.79 \pm 0.07 [control group], P=0.165). Both red wine and white wine significantly reduced MCP-1 mRNA and protein expression in the aorta.

Conclusions—Long-term consumption of red wine and white wine inhibits MCP-1 expression, and in the small number of animals studied, red wine modestly reduces neointimal hyperplasia. Since red wine exhibits higher antioxidant capacity than does white wine, the decreased intimal response might be partly attributed to its antioxidant effects. (Circulation. 1999;100:2254-2259.)

Key Words: alcohol \(\text{vessels} \text{restenosis} \text{antioxidants} \text{proteins}

An inverse relation between moderate alcohol consumption and the risk of coronary heart disease has been demonstrated in several types of studies.\(^1\)\(^-\)\(^9\) A number of mechanisms have been suggested, including increased levels of HDL,\(^3\) inhibition of platelet aggregation,\(^2,10\) antioxidant capacity,\(^11,12\) antithrombogenesis,\(^10\) and reduction of psychosocial stress. In a large prospective population study,\(^13\) compared with either nondrinkers or liquor drinkers, wine drinkers are at lower risk of cardiovascular disease or death from coronary artery disease. The antioxidant contents in red wine, especially flavonoids, which increase the antioxidant capacity of serum in vivo, may play an important role.\(^11,12,14,15\)

Coronary angioplasty is a routine interventional procedure for the treatment of coronary artery disease. Restenosis, however, remains a major problem.\(^16\) Complex mechanisms including vessel wall remodeling, smooth muscle cell proliferation, monocyte recruitment, foam cell accumulation, and neointimal hyperplasia have been suggested to be responsible for the development of restenotic lesions after angioplasty.\(^17\)\(^-\)\(^19\) Several antioxidants, such as probucol,\(^20\) butylated hydroxytoluene,\(^21\) and vitamin E,\(^22\) have been shown to prevent the progression of atherosclerosis and to inhibit neointimal thickening and macrophage accumulation after balloon injury of the artery in cholesterol-fed animals. Local alcohol delivery has been demonstrated to inhibit neointimal hyperplasia after balloon injury in rabbit iliac arteries\(^23\) and pig coronary arteries.\(^24\) Moderate alcohol feeding has also been found to attenuate postinjury vascular cell proliferation in a rabbit angioplasty model.\(^25\) In the present investigation, the effects of wine consumption on monocyte chemotactic protein-1 (MCP-1) expression, a potent chemoattractant for circulating monocytes, and on neointimal thickening after balloon injury of the aorta in cholesterol-fed rabbits were studied.
Methods

Animal Experiments
Twenty-two male New Zealand White rabbits, 3 months of age and weighing 2.79±0.19 kg (2.5 to 2.8 kg), were housed in the animal center of Taipei Veterans General Hospital, Taipei, Taiwan. After 1 week on a commercial rabbit chow diet (Scientific Diet Services), the animals were then fed a 2% high-cholesterol diet (Purina Mills Inel) and randomly allocated to 1 of 3 groups: 1, red wine group (1989 Chateau La Vieille Cure, Pessac, Bordeaux, France, 12.5% vol; n=7); 2, white wine group (1994 Bouchard Pere & Fils, Pouilly-Fuisse, Beune, France, 13.3% vol; n=7); and 3, control group (n=8). Wine was fed at a dose of 5 mL/kg body wt via nasogastric tube every day for 6 weeks. Body weight was recorded before and at the end of the third and sixth week of the experiment.

At the end of the third week of high-cholesterol feeding and wine consumption, the animals in each group were fasted for 12 hours and anesthetized with an intramuscular injection of xylazine (5 mg/kg) and ketamine hydrochloride (35 mg/kg). The surgery was performed under sterile conditions. The right femoral artery was exposed through an incision line 1.5 to 2.0 cm below the inguinal ligament, and an arteriotomy was performed. A 3F arterial embolectomy balloon catheter (Baxter Healthcare) was introduced retrogradely into the lower abdominal aorta for 16 cm; measurement was from the tip of the catheter. Denudation was then performed by inflating the balloon with normal saline and slowly pulling it back with the feeling of resistance. This procedure was repeated 3 times. The surgical wound was closed, and the animals were continued on a high-cholesterol diet and wine consumption.

At the end of 6 weeks of study, the rabbits were killed. The abdominal aorta and iliac artery were harvested for 16 cm by measuring from the previous arteriotomy site. The arterial specimens were dissected gently free of adhering tissues and then rinsed with ice-cold PBS. The abdominal aorta was cut into 6 segments. A small part of each arterial segment was taken, immersion-fixed with 4% buffered paraformaldehyde, paraffin-embedded, and then cross-sectioned for morphometry, in situ hybridization, and immunohistochemistry. The remaining larger portion of each arterial segment was immediately frozen in liquid nitrogen for RNA isolation.

Biochemical Measurement
Blood samples were collected before and 2, 4, and 6 weeks after the high-cholesterol diet feeding for measurement of plasma cholesterol and triglyceride; Kodak Ektachem DT 60 and DTSC analyzers were used.

Morphometric Measurement
One cross section (thickness 5 μm) was obtained from each segment of the abdominal aorta. The specimens were stained with hematoxylin and eosin. Morphometric analysis of these arterial sections was performed by the use of an LV-2 Image Analyzer (Winhow Instruments). For each cross section of arterial specimen, the intimal and medial areas were measured, and the intima/media area ratio was determined.

RNA Isolation and Northern Blot Analysis
The expression of MCP-1 in the arterial specimen was examined at the RNA level by Northern blot analysis. Total RNA was isolated from aortic lysate by the guanidine thiocyanate/phenol chloroform method, which was followed by ethanol precipitation. mRNA samples (20 to 25 μg per lane) were electrophoresed on 1.2% agarose paraformaldehyde gel, transferred onto a nylon membrane (Nyttran, 0.45 μm, Schleicher & Schuell Inc), and fixed by ultraviolet irradiation. After hybridization with 32P-labeled MCP-1 probes, the membrane was washed at room temperature twice with 4X standard saline citrate (SSC; 1× SSC contains 0.15 mol/L NaCl and 0.015 mol/L sodium citrate, pH 7.0) containing 1% SDS, twice with 1× SSC containing 1% SDS (at 37°C), and twice with 0.2× SSC containing 0.1% SDS for a 15-minute period per wash. Blots were exposed to x-ray films (X-OMAT, Kodak) at −70°C. Autoradiographic results were scanned and analyzed by optical densitometry (Molecular Dynamics). The GAPDH mRNA levels served as the internal standard to normalize the MCP-1 signals.

DNA Probes
The following DNA fragments were used as probes: a 0.7-kb EcoRI and BamHI fragment containing full-length MCP-1 cDNA and a 1.3-kb PstI fragment containing GAPDH cDNA (received from American Type Culture Collection).

In Situ Hybridization and Immunohistochemical Analysis
To examine the cellular expression and localization of the MCP-1 gene and protein, in situ hybridization and immunohistochemistry were performed on serial sections of the aorta. The first tissue section was hybridized with digoxigenin (DIG)-labeled MCP-1 cDNA. The second and third sections were incubated with smooth muscle cell–specific and macrophage-specific antibodies to identify smooth muscle cells and macrophages, respectively. The last section was used to detect MCP-1 protein expression.

In Situ Hybridization
MCP-1 cDNA was labeled with DIG-dUTP according to the manufacturer’s instructions (Boehringer-Mannheim Biochemica) and used as a probe for in situ hybridization, which was performed according to a previously published method. Paraffin-embedded arterial sections (5-μm thickness) were placed onto poly-L-lysine–coated slides, deparaffinized, treated with proteinase K (1 μg/mL) for 15 minutes at 37°C, and acetylated (0.25% acetic anhydride in 0.1 mol/L triethanolamine and 0.9% NaCl) for 10 minutes. Sections were then washed with 2× SSC and prehybridized with 100 μL prehybridization solution (5× SSC, 5× Denhardt’s solution, 50% deionized formamide, 250 μg/mL yeast RNA, 250 μg denatured salmon sperm DNA, and 4 mmol/L EDTA for 3 hours). Hybridization was performed at 50°C for 16 to 24 hours in a humid chamber with prehybridization solution (25 μL per section) containing 10 ng/μL DNA probe. After hybridization, sections were washed at 42°C twice in 2× SSC, once in 0.2× SSC, and twice in 0.1× SSC for 15 minutes per wash. Sections were then blocked for 30 minutes, incubated with alkaline phosphatase–conjugated anti-DIG antibody for 30 minutes, and detected with a color solution containing 337.5 μg/mL nitro blue tetrazolium salt and 175 μg/mL 5-bromo-4-chloro-3-indolyl phosphate for 10 to 30 minutes according to the manufacturer’s instructions. In some experiments, tissue sections were hybridized with DIG-labeled probes plus 50-fold unlabeled cDNA, which abolished the signals as controls.

Immunohistochemistry
For immunohistochemistry, 3 serial paraffin-embedded sections were used for each arterial specimen. The arterial sections were deparaffinized, rehydrated, and washed with PBS. Nonspecific binding was blocked by preincubation with PBS containing either 1% normal serum or 5 mg/mL bovine serum albumin for 1 hour at room temperature. Sequentially, the second serial section was incubated with mouse anti-smooth muscle actin (1:400 dilution, I4A, Sigma Chemical Co) for 1 hour at 37°C, which identified vascular smooth muscle cells. The third serial section was incubated with mouse anti-rabbit macrophage (1:50 dilution, Ram II, Dako Corp) for 1 hour at 37°C, which reacted with the rabbit monocyte/macrophage cell population. These 2 sections were then incubated with FITC–conjugated goat anti-mouse secondary antibody (1:400 dilution, Sigma) at room temperature for 1.5 hours. Each incubation was followed by three 5-minute washes in PBS. The last serial section was incubated with goat anti-human MCP-1 primary antibody (1:15 dilution, R&D Systems) for 1 hour at 37°C. The sections were then incubated with biotinylated conjugated horse anti-goat IgG for 1 hour at room temperature. Antigen-antibody complexes were localized by incubation with avidin/biotin/horseradish peroxidase complex for 1.5 hours at room temperature and by subsequently using 0.5 mg/mL 3,3′-diaminobenzidine/0.01% hydrogen peroxide.
in 0.1 mol/L Tris-HCl buffer, pH 7.2, as a chromogen (Vector Labs). Negative control was performed by omitting primary antibody during the incubation of arterial sections.

**Statistical Analysis**
Values were expressed as mean±SEM. Data were analyzed by ANOVA followed by the Dunnett test.

**Results**
There was no significant difference in body weight among groups at baseline and at 3 and 6 weeks after feeding with 2% high-cholesterol diet. Plasma lipid levels, especially plasma cholesterol levels, showed rapid increase after high-cholesterol feeding. But there was no significant difference in cholesterol and triglyceride levels among 3 groups at baseline and at 2, 4, and 6 weeks of feeding, as shown in the Table.

<table>
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<th>Red Wine</th>
<th>White Wine</th>
<th>Control</th>
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<tr>
<td></td>
<td>TG, mg%</td>
<td>CHOL, mg%</td>
<td>TG, mg%</td>
</tr>
<tr>
<td>Baseline</td>
<td>110±22</td>
<td>49±14</td>
<td>64±10</td>
</tr>
<tr>
<td>2 weeks</td>
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<td>4 weeks</td>
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<td>6 weeks</td>
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Values are mean±SEM. TG indicates triglycerides; CHOL, cholesterol.

The rabbits that consumed red wine had significantly and modestly less neointimal hyperplasia than did the control group in terms of intima/media area ratio (0.59±0.05 [red wine group] versus 0.79±0.07 [control group], \( P<0.05 \); Figure 1). However, compared with the control group, rabbits that consumed white wine showed a trend (but not significant) toward less intimal response (intima/media area ratio 0.65±0.04 [white wine group] versus 0.79±0.07 [control group], \( P=0.165 \), Figure 1).

The MCP-1 mRNA in the aortic tissue was shown by Northern blot analysis to be significantly reduced after either red or white wine consumption compared with the absence of wine (Figure 2).

**In Situ Hybridization Combined With Immunohistochemistry for MCP-1 Detection**
The control group showed markedly thickened neointima, which exhibited strong MCP-1 mRNA and protein expression (Figure 3, CA and CD). MCP-1 mRNA and protein were also detected in the medial layer. MCP-1–positive cells exhibited immunoreactivity toward smooth muscle cell–specific (Figure 3, CB) and macrophage-specific (Figure 3, CC) antibodies. On the contrary, the red wine group showed less intimal thickening with faint expression of MCP-1 mRNA (Figure 3, RA) and protein (Figure 3, RD). Also, the MCP-1 mRNA and protein could not be clearly detected in the underlying media. Many smooth muscle cells (Figure 3, RB) but few macrophages (Figure 3, RC) were present in the thickened neointima. The distribution and expression of MCP-1 mRNA, smooth muscle cells, macrophages, and MCP-1 protein in the aorta of the white wine group were similar to those in the red wine group, as shown in Figure 3, WA to WD.

**Table**
Plasma Lipid Levels at Baseline and at 2, 4, and 6 Weeks After High-Cholesterol Feeding in Different Groups of Rabbits

<table>
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Values are mean±SEM. TG indicates triglycerides; CHOL, cholesterol.
Figure 3. Detection of MCP-1 mRNA and protein expression in serial sections of abdominal aortas from control (C) group, red wine (R) group, and white wine (W) group. Lumen (L) is uppermost in all sections. Internal elastic lamina is indicated by arrows. A, In situ hybridization to MCP-1 mRNA. Strong staining was observed in thickened neointima and media of control (CA) group. Only weak staining was observed in parts of the less thickened neointima of red wine (RA) and white wine (WA) groups. B, Staining for smooth muscle–specific antibody. Staining was obvious in thickened intima and throughout the media of control (CB), red wine (RB), and white wine (WB) groups. C, Staining for macrophage-specific antibody. Staining was observed in a few cells in the thickened intima of control (CC), red wine (RC), and white wine (WC) groups. D, Staining for MCP-1–specific antibody. Strong staining was observed in the markedly thickened intima and media of control (CD) group. However, only weak staining was observed in parts of less thickened neointima of red wine (RD) and white wine (WD) groups. Magnification ×130.
Discussion

In the present study, we were able to demonstrate that long-term consumption of a moderate amount of wine, especially red wine, could suppress the expression of MCP-1 and modestly reduce the intimal response to balloon deendothelialization in cholesterol-fed rabbits. A number of studies have shown an inverse correlation between coronary heart disease risk and moderate alcohol intake. However, the mechanisms by which moderate alcohol consumption protects the heart remain unclear. Administration of wine has been shown to inhibit in vivo platelet activity and thrombosis in stenosed canine coronary arteries. Inhibition of platelet reactivity by wine has been suggested to be one explanation for protection from coronary heart disease in the French paradox. Besides, increased levels of HDL and reduction in lipoprotein(a) might be responsible for the decreased risk of myocardial infarction.

Monocyte recruitment and macrophage-derived foam cell accumulation, as well as smooth muscle cell migration and proliferation, which lead to intimal hyperplasia, are some major mechanisms responsible for the development of postangioplasty restenosis. Lipid peroxidation plays an important role in these processes. A number of antioxidants, including probucol, butylated hydroxytoluene, and vitamin E, have been shown to inhibit neointimal thickening and macrophage accumulation after balloon injury of the artery in hypercholesterolemic animals. Consumption of red wine with meals reduced the susceptibility of human plasma and LDL to lipid peroxidation. Inhibition of oxidation of LDL with red wine has been demonstrated in healthy volunteers. An in vitro comparison of red wine, white wine, and various fruit juices showed the high antioxidant capacity of red wine in addition to its ability to increase the antioxidant capacity of serum in vivo. Flavonoids are scavengers of superoxide anions and have been shown to inhibit the oxidative modification of LDLs by macrophages. Dietary antioxidant flavonoids have been suggested to reduce the risk of coronary heart disease. The polyphenolic substances (flavonoids) in red wine, which have potent antioxidant properties that could inhibit the oxidation of human LDL, have been thought to be responsible for protection against cardiovascular morbidity and mortality. The antioxidant flavonoids in red wine might also play an important role in the reduction of intimal response and inhibition of MCP-1 expression after balloon injury observed in the present study. Since red wine exhibited much greater antioxidant capacity than did white wine, this fact might explain why red wine but not white wine significantly attenuates the intimal response in our restenosis animal model.

In previous studies, we demonstrated that local delivery of 15% ethanol may reduce phenotype conversion of smooth muscle cells, decrease smooth muscle cell proliferation, and inhibit intimal hyperplasia after balloon injury in rabbit iliac arteries and pig coronary arteries. Moderate alcohol feeding (an average of 2.5 mL alcohol per 500 mL water daily for 10 weeks) has also been shown to reduce neointimal formation, the extent of lipid oxidation, and the number of foam cells in the neointimal area and may decrease the expression of MCP-1 and PDGF by reducing LDL oxidation in a rabbit model of postangioplasty restenosis. Therefore, in addition to antioxidant polyphenolic compounds, the alcohol content in red wine could also make significant contributions to the attenuation of intimal response.

In conclusion, long-term consumption of red and white wine could suppress MCP-1 expression, and red wine modestly decreases intimal thickening after balloon injury in cholesterol-fed rabbits. The ethanol content as well as the phenolic antioxidants in red wine might be responsible for these favorable effects. Since antioxidant protocol has been shown to effectively inhibit neointimal thickening and macrophage accumulation in animals and to reduce the rate of restenosis after balloon angioplasty in humans, it would be worthwhile to determine whether red wine might alter restenosis after coronary angioplasty.

Acknowledgments

This study was partially supported by research grants NSC-85-2331-B-010-046 and NSC-86-2314-B-010-029 from the National Science Council, CI-84-20 and CI-85-17 from the Yen Tjing-Ling Medical Foundation, and V-308 and V-330 from Taipei Veterans General Hospital, Taipei, Taiwan.

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Circulation. 1999;100:2254-2259
doi: 10.1161/01.CIR.100.22.2254

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