Moderate Daily Intake of Red Wine Inhibits Mural Thrombosis and Monocyte Tissue Factor Expression in an Experimental Porcine Model

L. Casani, MS; E. Segales, MS; G. Vilahur, MS; A. Bayes de Luna, MD, PhD, FESC; L. Badimon, PhD, FESC

Background—Moderate consumption of red wine has been epidemiologically associated with a reduction in cardiovascular disease, but its mechanism of action is not fully understood. The objective was to study whether the protective effects of a daily intake of red wine (Tempranillo, 12.8% alcohol vol/vol) could be related to inhibition of thrombosis in an experimental model of diet-induced hyperlipemia.

Methods and Results—For 100 days, animals were fed a western-type proatherogenic diet containing 2% cholesterol and 20% saturated fat. Three doses of red wine were studied (20, 30, and 40 g wine-ethanol/d) and compared with placebo-control animals not taking any wine. Thrombosis under flow conditions was evaluated by radioisotopic quantification of deposited platelets on damaged arteries. Changes in RhoA translocation in platelets and monocyte tissue factor expression were also analyzed. Mural platelet deposition was significantly reduced in animals ingesting red wine with their food. Expression of RhoA in the platelet cytoplasm (inactive form) was increased in wine-fed animals. Tissue factor mRNA expression in lipopolysaccharide-stimulated monocytes was reduced in wine-fed animals. Total cholesterol levels were not significantly different among groups.

Conclusions—Moderate red wine intake significantly reduces platelet deposition triggered by damaged vessel wall, partially explained by inhibition of RhoA translocation to the platelet membrane. Hence, a daily moderate intake of wine seems to inhibit different pathways that converge in a reduced thrombotic risk on vessel wall injury. (Circulation. 2004;110:460-465.)

Key Words: wine ■ thrombosis ■ cardiovascular disease ■ tissue factor
TABLE 1. Analysis of Red Wine Composition According to Manufacturers

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol degree</td>
<td>12°67</td>
</tr>
<tr>
<td>Density</td>
<td>994</td>
</tr>
<tr>
<td>Tannins (including catechines), g/L</td>
<td>3.36</td>
</tr>
<tr>
<td>Anthocyanins, ppm</td>
<td>448</td>
</tr>
<tr>
<td>Resveratrol, ppm</td>
<td>1.75</td>
</tr>
</tbody>
</table>

bacteria (Bio-Rad). The membranes were incubated with anti-RhoA monoclonal antibody (1:200) (Santa Cruz Biotechnology), then incubated with anti-mouse immunoglobulin (1:10 000). Antibody visualization was performed by a chemiluminescent method (Super-Signal, Pierce).

Blood and Plasma Determinations

Blood was withdrawn by venipuncture from the ear marginal vein in fasting animals at baseline and at days 15, 30, 50, 75, and 100 of the study.

Blood cell counting (System-9000, Serono-Baker Diagnostics) and coagulation parameters (ST4 coagulometer, Diagnostica-Stago) were followed up. Blood alcohol concentration was determined by use of an alcohol dehydrogenase assay kit (332-UV) according to the manufacturer’s instructions (Sigma Chemical). Liver enzymes, total cholesterol, HDL cholesterol, and triglycerides were determined enzymatically (Kodak Ektachem DT, Eastman Kodak Co).

Figure 1. Total platelet deposition (PLT × 10⁶/cm²) of nonheparinized blood over severely damaged vessel wall at 2 different shear rates, 212 s⁻¹ (n=9, group A; n=9, group B) and 1696 s⁻¹ (n=9 per group). Black bars, hyperlipemic control animals; hatched bars, wine-treated animals. *Significantly different (P<0.05).

Figure 2. Perfusions with nonheparinized blood. Perfusions were run for 3 minutes over mildly and severely damaged vessel wall, at 212 s⁻¹ (n=16 per group) and 1696 s⁻¹ (n=16 per group). Black bars, hyperlipemic control animals; hatched bars, hyperlipemic with 40 g wine-ethanol/d animals; open bars, hyperlipemic with 20 g wine-ethanol/d animals. *Significantly different (P<0.05), HD group vs HL group, and †significantly different (P<0.05), LD group vs HL group.
LDL Oxidation
Lipoprotein oxidation capacity was analyzed in EDTA-collected blood. Samples were obtained at the end of the perfusion experiment and in the control study at baseline and 30, 50, and 120 minutes after red wine administration. LDLs were isolated by sequential ultracentrifugation, and oxidation was induced by copper as described. Lipoprotein oxidation was also analyzed by thiobarbituric acid–reactive substances.

Tissue Factor Expression in Induced Monocytes
Blood collected in sodium citrate from normolipemic animals fed with and without 40 g wine-ethanol was used to obtain monocytes by an elutriation centrifuge. Elutriated monocytes were harvested on RPMI 1640 medium with Glutamax-1 (Gibco) overnight and then adhered on 100-mm plastic dishes (Falcon) and induced with lipopolysaccharide (LPS) (10 μg/10^6 cells). Four hours later, supernatants were aspirated and centrifuged; dishes were scraped with Tripure (Boehringer Mannheim Corp) and processed to obtain RNA by conventional techniques. The centrifuged supernatants (700 g, 5 minutes) containing nonadherent cells were also dissolved with Tripure.

Tissue factor (TF) mRNA and a control GAPDH mRNA were amplified in semiquantitative reverse transcription–PCR analysis. RNA (0.5 μg) was reverse-transcribed in a final 15-μL reaction mixture, performed at 42°C for 1 hour. cDNA (1 μL) was amplified in a total 25-μL reaction. The specific oligonucleotides selected for TF amplification were as follows: sense, 5′-AGAGTTTCAACACCTATTACCTGGA-3′ and antisense, 5′-AGTTTTCTCCTTTATTACCAT-3′; for GAPDH, sense, 5′-GTCACCAAGGGCTGCTTTAAA 3′ and antisense, 5′-ACGGAAGGCGCATGCAGTGA 3′. Amplification of TF was performed by 35 cycles of 95°C for 2 minutes, 55°C for 40 seconds, and 72°C for 1 minute, followed by a final extension of 72°C for 10 minutes. A ratio of TF/GAPDH was used to normalize TF values. PCR products were resolved by electrophoresis in 1.5% agarose gels stained with ethidium bromide.

Data Analysis
Multiple group means were compared by single-factor or multiple-factor ANOVA, followed by Fisher’s protected least significant difference and Scheffé’s F test. Results are expressed as mean±SEM, and a value of P<0.05 was considered significant.

Results
Thrombotic Response and Ex Vivo Platelet Aggregation
The thrombotic response to severely damaged vessel wall was significantly decreased in the group ingesting 30 g wine-ethanol/d of red wine at both high and low shear rates (Figure 1).

Platelet deposition also showed a statistically significant reduction with 20 and 40 g wine-ethanol/d in native blood perfusions over mildly and severely damaged vessel wall and at both shear rate conditions (Figure 2)

When heparinized blood was used, the inhibitory effects of red wine on platelet deposition were clearly significant in severely damaged substrates (P<0.05) at high and low shear rates in perfusions of 3 and 5 minutes (Figure 2B). In eroded vessel wall, because of the low levels of platelet deposition (platelet adhesion), the reduction was significant (P<0.05) only at a low shear rate (Figure 3A), whereas results did not reach significance at high shear rate.
Red wine intake did not produce any significant difference in Blood and Plasma Determinations when compared with the hyperlipemic placebo group (data not shown). Ingestion of these light to moderate doses of red wine did not cause any significant reduction in either whole blood or platelet deposition over severely damaged vessel wall at low shear rate (Figure 3D) compared with normolipemic animals not taking any wine. Ingestion of these light to moderate doses of red wine did not cause any significant reduction in either whole blood or platelet-rich plasma aggregation ex vivo induced by collagen or ADP (data not shown).

**Rho-A Protein Translocation**

The expression of RhoA protein in the platelet membrane (active form) showed a reduction in groups ingesting wine (Figure 4). RhoA protein in platelet cytoplasm (inactive form) was increased in groups ingesting the high dose of red wine (40 g wine-ethanol/d) compared with the hyperlipemic placebo group (data not shown).

**Blood and Plasma Determinations**

Red wine intake did not produce any significant difference in biochemical parameters, blood cell counts, or platelet counts.

**TABLE 2. Plasma Lipid Composition**

<table>
<thead>
<tr>
<th></th>
<th>Total Cholesterol, mg/dL</th>
<th>HDL Cholesterol, mg/dL</th>
<th>Non-HDL Cholesterol, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A (n=3)</td>
<td>515.07±52.27</td>
<td>69.79±5.63</td>
<td>439.64±51.7</td>
</tr>
<tr>
<td>Group B (n=3)</td>
<td>407.43±36.56</td>
<td>59.57±4.99</td>
<td>323.03±45.50</td>
</tr>
<tr>
<td>Group HD (n=4)</td>
<td>333.5±45.11</td>
<td>55±8.51</td>
<td>273.1±44.23</td>
</tr>
<tr>
<td>Group LD (n=4)</td>
<td>538±47.14*</td>
<td>86.5±6.55*</td>
<td>434.7±40.11</td>
</tr>
<tr>
<td>Group HL (n=4)</td>
<td>415±47.22</td>
<td>55.5±4.13</td>
<td>355.2±51.90</td>
</tr>
<tr>
<td>Normolipemic (n=4)</td>
<td>64.5±5.78</td>
<td>60.75±1.31</td>
<td>29.42±5.46</td>
</tr>
</tbody>
</table>

Values were obtained at the end of the experimental period. Group A, 30 g wine-ethanol/d; group B, control group; group HD, 40 g wine-ethanol/d; group LD, 20 g wine-ethanol/d; group HL, hyperlipemic placebo group.

Liver enzymes were increased by the hypercholesterolemic diet (baseline AST, 32±2 U/L, and ALT, 41±3 U/L; hyperlipemic AST, 64±9 U/L, and ALT, 68±9 U/L, P<0.05). Interestingly, wine-fed hyperlipidemic animals did not show any impairment in liver enzymes (AST, 51±2 U/L [HD], and 58±9 U/L [LD]; ALT, 34.25±6 U/L [HD], and 65±13 U/L [LD]). No detectable levels of ethanol were found in blood as expected, because the last red wine ingestion was the day before the experimental procedure.

Plasma levels of total cholesterol and HDL cholesterol were significantly increased in the LD group (Table 2).

**LDL Oxidation**

The LDL oxidation, measured as maximal conjugated dienes, was significantly lower (P<0.0015) in the LD group than in the HL group (Table 3). Lag time was not significantly increased by red wine ingestion. The maximal velocity of conjugated diene formation was significantly reduced for the LD group but was not affected in the HD group with respect to the control group. The thiobarbituric acid-reactive substances test confirmed the lower oxidative capability of LDL from the group LD respect to the HD group (P<0.001). No differences in these parameters were found for normolipemic animals fed or not fed red wine.

**Tissue Factor Expression in Induced Monocytes**

Quantification of DNA resulting from the reverse transcription–PCR for TF showed that isolated peripheral blood monocytes, differentiated by adhesion, from wine-fed animals have both a lower TF mRNA content and a lower capacity to induce TF mRNA expression in the presence of LPS (Figure 5).

**Discussion**

Our results demonstrate that chronic intake of red wine with meals induces a significant inhibitory effect of platelet deposition on damaged vessel wall and that this inhibitory effect is more evident with the high dose of wine than with the lower doses. One single dose of red wine in association with the meal in normolipemic animals showed a reduction in platelet deposition, although it did not reach significance in...
all conditions tested. These results indicate that moderate intake of red wine could exert a beneficial antithrombotic effect.

Thrombotic risk in the control hyperlipemic animals increased with perfused lesion severity, perfusion time, and shear rate conditions, as described previously. Groups ingesting red wine showed the same platelet deposition pattern but with lower growth, suggesting that the reduction in platelet deposition will probably be the result of a passivation effect of red wine in platelets. There is evidence that flavonoids present in red wine inhibit cAMP and cGMP phosphodiesterases, increasing platelet levels of cAMP and cGMP. As a result, platelet cytosolic calcium ([Ca^2+]_i) levels would decrease, reducing the level of in vivo platelet activity. Activation of platelets implies different responses, including shape change, adhesion, aggregation, and secretion. Shape change of platelets could be regulated by both calcium-dependent and -independent pathways. We have found that red wine ingestion reduced the translocation of RhoA into membranes (active site), accompanied by an increase in the levels of cytoplasmic RhoA (inactive), corresponding to a reduction in platelet deposition.

Alcohol and polyphenols have been shown to inhibit in vitro platelet aggregation when added to platelets obtained from healthy volunteers and challenged with thrombin and ADP. However, the high concentrations required (>130 μmol/L) are not reached by feasible moderate intake of wine. Alcohol intake has also been shown to affect platelet activity indirectly, through an HDL-mediated stimulation of the production of prostacyclin, and inhibit the production of thromboxane A_2, perhaps by inhibiting platelet cyclooxygenase activity, or may be promoting an increase in platelet membrane fluidity. Nevertheless, a diet rich in saturated fat, like that ingested by our animals, reduces platelet surface area and membrane fluidity compared with platelets from normolipemic animals. This could be the reason why the ingestion of red wine cannot significantly reduce platelet aggregation in our study, results that are in concordance with those found by Rand et al using thrombin as aggregating agent.

This study also shows for the first time that ingestion of red wine reduces TF expression in LPS-stimulated monocytes from normolipemic animals. Resveratrol, a wine polyphenol component, showed inhibitory effects on TF expression in human endothelial cells and monocytes in vitro studies using high doses of the isolated compound. In the pathogenesis of atherosclerosis, modified LDL particles may become oxidized, then promoting foam-cell formation and enhancing the atherosclerotic lesion. Antioxidant therapies had been proposed to stop or delay this process. Phenolic compounds of the red wine had been shown to exert these activities in vitro and in vivo but in vivo studies are performed with healthy volunteers ingesting about 400 mL of red wine for only 2 or 4 weeks. In our hyperlipemic experimental model, the group ingesting a low dose of wine (LD group) showed better changes in oxidative parameters than the groups ingesting the higher amount of wine. In vivo, the antioxidant protective effect of red wine can be overshadowed by the pro-oxidant effect of the ethanol present in wine, and in groups ingesting 30 and 40 g of alcohol, the antioxidant protective effect of polyphenols may be overshadowed by the pro-oxidant effect of ethanol. The balance between alcohol and polyphenols of a wine may be critical for its in vivo effect on LDL. The wine used in the study contains high amounts of resveratrol (76 μmol/L) and catechins (3.36 g/L), phenolic compounds with demonstrated cardioprotective effects. A moderate and regular con-
sumption of red wine could lead to a modification of cell function that is evidenced in the beneficial effect of red wine on the thrombotic response.

Considering that thrombosis is an integral part of atherosclerosis and coronary artery disease, it is possible that the decreased risk of CHD associated with moderate intake of red wine could be caused by a combination of vascular and antithrombotic effects. The ability of red wine to reduce thrombotic risk, the reduction in monocyte TF expression, the reduction in membrane Rho-A protein translocation, and the global antioxidant effect are major steps to confirm that a moderate intake of red wine can be included as a real cardioprotective factor. Results obtained in oxidative modification of LDL indicated that a low ingestion of red wine, preferably a wine rich in polyphenols, is better than a higher amount of wine, whereby the pro-oxidant effects of ethanol are overshadowing the beneficial effects. Interestingly, the platelet aggregation response to wine-ethanol has been shown to differ according to the type of diet.29,30 We have demonstrated that a moderate dose of red wine taken with a diet rich in saturated fats is able to produce beneficial antiatherothrombotic effects. Therefore, patients at risk, especially those with an exclusive western diet, will receive the most benefit from the moderate red wine intake.

Limitations of the Study
With the experimental design used in this study, we can only draw conclusions about the antithrombotic effects of the chronic intake of a moderate amount of red wine. We cannot identify whether alcohol or nonalcoholic components of red wine are the protective components or whether ingestion of white wine or hard alcoholic beverages (spirits) will produce effects similar to those of red wine.

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