Effects of Red Wine and Vodka on Collateral-Dependent Perfusion and Cardiovascular Function in Hypercholesterolemic Swine

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Background—Moderate consumption of alcohol, particularly red wine, has been shown to decrease cardiac risk. We used a hypercholesterolemic swine model of chronic ischemia to examine the effects of 2 alcoholic beverages on the heart.

Methods and Results—Yorkshire swine fed a high-cholesterol diet underwent left circumflex ameroid constrictor placement to induce chronic ischemia at 8 weeks of age. One group (HCC, n=9) continued on the diet alone, the second (HCW, n=8) was supplemented with red wine (pinot noir, 12.5% alcohol, 375 mL daily), and the third (HCV, n=9) was supplemented with vodka (40% alcohol, 112 mL daily). After 7 weeks, cardiac function was measured, and ischemic myocardium was harvested for analysis of perfusion, myocardial fibrosis, vessel function, protein expression, oxidative stress, and capillary density. Platelet function was measured by aggregometry. Perfusion to the ischemic territory as measured by microsphere injection was significantly increased in both HCW and HCV compared with HCC at rest, but in only the HCW group under ventricular pacing. Microvessel relaxation response to adenosine 5'-diphosphate was improved in the HCW group alone as was regional contractility in the ischemic territory, although myocardial fibrosis was decreased in both HCW and HCV. Expression of proangiogenic proteins phospho-endothelial nitric oxide synthase and vascular endothelial growth factor was increased in both HCW and HCV, whereas phospho-mammalian target of rapamycin was increased only in the HCV group. Expression of Sirt-1 and downstream antioxidant phospho-FoxO1 was increased only in the HCW group. Protein oxidative stress was decreased in the HCW group alone, whereas capillary density was increased only in the HCV group. There was no significant difference in platelet function between groups.

Conclusion—Moderate consumption of red wine and vodka may reduce cardiovascular risk by improving collateral-dependent perfusion through different mechanisms. Red wine may offer increased cardioprotection related to its antioxidant properties. (Circulation. 2012;126[suppl 1]:S65–S72.)

Key Words: alcohol ■ animal model ■ antioxidants ■ ischemia ■ myocardial perfusion

An intriguing paradox in cardiovascular health is the repeatedly validated finding that moderate alcohol consumption decreases cardiovascular risk.1 Many studies have demonstrated a “J-shaped” dose-dependent relationship between alcohol consumption and cardiovascular mortality, wherein moderate alcohol intake of 20 to 30 g/day, equivalent to that found in 2 to 3 glasses of wine, is associated with maximal cardiovascular protection.2 Numerous mechanisms have been suggested to explain the cardioprotective effect of alcohol, including increased high-density lipoprotein (HDL) cholesterol,2 reduced plasma viscosity and fibrinogen concentration,4 improved endothelial function,5 and decreased inflammation.6 Nonetheless, a definitive explanation for this phenomenon remains elusive.

Beer, wine, and spirits have all been associated with reduced cardiovascular risk, suggesting that ethanol itself likely exerts some protective effect on the cardiovascular system.7 However, some head-to-head comparisons between wine and other alcoholic beverages have shown that wine drinkers have even lower risk of coronary artery disease and
cardiac death compared with beer and spirit drinkers who avoided wine. In particular, several substances unique to red wine have been investigated for their antioxidant, proangiogenic, and anti-inflammatory properties. The most popular of these compounds is resveratrol (3,5,4′-trihydroxystilbene), a plant polyphenol that has been shown to have antioxidant, anticancer, and antiaging effects. Resveratrol has been shown to activate Sirtuins, a family of deacetylases known to act on numerous cellular pathways. Among these is the Forkhead class O (FOXO) pathway, which regulates the cellular response to oxidative stress. Alcendor et al demonstrated that hearts of transgenic mice overexpressing Sirt-1 were protected from oxidative stress through the activation of FOXO1, and resveratrol has been shown to upregulate both Sirt-1 and FOXO1 in rat hearts. Thus, red wine may have additional cardioprotective effects due to the antioxidant properties of resveratrol.

Not surprisingly, interventional studies looking at the cardiovascular effects of alcohol treatment in humans are scarce, and observational studies are limited by patient variation and a high degree of bias. We designed a controlled animal study using a swine model of hypercholesterolemia and chronic ischemia to investigate the effects of resveratrol-containing red wine and resveratrol-free vodka on the heart. We hypothesized that these alcoholic beverages would have beneficial effects on perfusion, cardiovascular function, and oxidative stress in ischemic myocardium.

Materials and Methods

Animal Model

Twenty-seven intact male Yorkshire swine (Parsons Research, Amherst, MA) were fed a high-cholesterol diet (500 g once daily; Sinclair Research, Columbia, MO) starting at 4 weeks of age and continuing for the duration of the experiment. At 8 weeks age, swine underwent left circumflex (LCx) amiodor placement (Research Instruments SW, Escondido, CA) to induce chronic ischemia and were then divided into 3 groups. One group (HCC, n = 9) continued on the high-cholesterol diet alone, the second (HCW, n = 9) received a high-cholesterol diet supplemented with 375 mL of red wine daily (black Mountain pinot noir, 12.5% alcohol v/v, 0.3–0.5 µg/mL resveratrol; Haro Hills, CA), and the third group (HCV, n = 9) received high-cholesterol diet supplemented with 112 mL of vodka daily (Rubinoff vodka, 40% alcohol v/v, Somerville, MA). Resveratrol content in this particular variety of pinot noir was quantified for in accordance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” (National Institutes of Health publication no. 5377-3 1996).

Measurement of Global and Regional Myocardial Function

Heart rate, mean arterial pressure, developed left ventricular pressure, first derivative of left ventricular pressure, and regional myocardial contractility in the ischemic area at risk (AAR) were recorded before cardiac harvest using intraventricular and intra-aortic single-sensor pressure catheters (Millar Instruments, Houston, TX) and the Sonometrics system (Sonometrics Corp. London, Ontario, Canada) as previously described.

Myocardial Perfusion Analysis

Myocardial perfusion was measured through isotope-labeled microspheres (BioPAL, Worcester, MA). Gold-labeled microspheres that were 1.5×10⁷ were injected during temporary LCx occlusion at to identify the AAR. Lutetium (resting heart rate) and Europium (pacing to 160 beats/min) labeled microspheres were injected at the final procedure at the same time as simultaneously withdrawing arterial blood from the femoral artery catheter. Harvested left ventricular samples were completely dried in a 60°C oven and then exposed to neutron beams and microsphere densities measured (BioPAL). Myocardial blood flow in the AAR was determined using the following equation:

\[ \text{Blood flow} = \frac{\text{withdrawal rate} / \text{tissue weight}}{\text{(tissue microsphere count / blood microsphere count)}} \]

Microvessel Studies

Coronary arterioles (80–180 µm diameter) from the AAR were isolated and placed in a microvessel chamber. Vessels were maximally preconstricted with thromboxane-A₂ analog U46619 (0.1–1.0 µmol/L) and then treated with endothelium-dependent vasodilator adenosine-5′-diphosphate (10⁻⁹ to 10⁻⁵ mol/L) and...
endothelium-independent vasodilator sodium nitroprusside (10⁻⁹ to 10⁻⁴ mol/L). Responses were defined as percent relaxation of the preconstricted diameter. All reagents were obtained from Sigma-Aldrich (St Louis, MO).

Quantification of Fibrosis

Twelve-micron-thick sections from the AAR were fixed and trichrome-stained. Digital images of the stained slices were captured using Aperio slide scanning software (Aperio Technologies, Vista, CA). The amount of blue-stained collagen was quantified in a blinded fashion for 3 randomly selected 10X fields per section using Image J software (National Institutes of Health, Bethesda, MD) and expressed as a percentage of the total section area. Measurements from the 3 fields were averaged to obtain representative percent fibrosis for each section.

Immunoblotting

Sixty micrograms of total protein from AAR homogenates were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Invitrogen, San Diego, CA) and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA). Membranes were incubated with antibodies against endothelial nitric oxide synthase, phospho-endothelial nitric oxide synthase, mammalian target of rapamycin, phospho-mammalian target of rapamycin, FOXO1, phospho-FOXO1 (Cell Signaling Technology, Danvers, MA), Sirt-1 (Santa Cruz Biotechnology, Santa Cruz, CA), and vascular endothelial growth factor (Vascosan, Billerica, MA). Immune complexes were detected with chemiluminescence (Amer sham, Piscataway, NJ) and photographed using GeneSnap software (Kodak, Rochester, NY) and photographed using Image J software. Alpha-tubulin (Cell Signaling Technology, Danvers, MA) was used as a loading control. Expression of phosphorylated proteins was expressed as a ratio of phosphorylated:total protein.

Protein Oxidative Stress

Dinitrophenylhydrazine-derivatized tissue homogenates containing 30 μg of total protein from the AAR were separated as described previously. Membranes were incubated with primary antibody to dinitrophenylhydrazine followed by horseradish peroxidase-linked secondary antibody per manufacturer’s recommendations (Millipore, Billerica, MA). Immune complexes were visualized with chemiluminescence. Densitometric analysis of entire lanes was performed using Image J software.

Immunostaining for Capillary and Arteriolar Density

Twelve-micron-thick frozen sections of myocardium from the AAR were formalin-fixed and then incubated with antibodies against porcine endothelium marker CD-31 (R&D Systems, Minneapolis, MN) and smooth muscle actin (Sigma Aldrich) followed by the appropriate Alexa-fluor conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Sections were mounted in Vectorshied (Vector Laboratories, Burlingame, CA). Photomicrographs of 3 random 20X fields per section were taken with a Zeiss Axiolab microscope (Carl Zeiss Inc, Thornwood, NY). Capillaries, defined as CD-31-positive structures between 5 and 25 μm² in cross-sectional area, and arterioles, defined as structures containing for both smooth muscle actin and CD-31, were counted using Image J software. Results were averaged for 3 slices per section and are presented as vessels/mm².

Platelet Activity

Whole blood samples drawn 1 hour postprandially were collected directly into tubes containing 3.2% trisodium citrate. Platelet function studies were performed using a Platelet Lumi-Aggregometer (Chronolog Corporation, Havertown, PA). Aggregation response to the agonists adenosine-5’-diphosphate (10 μmol/L) and arachidonic acid (0.5 mmol/L) was measured by impedance aggregation and adenosine 5’-triphosphate secretion using the firefly luciferin-luciferase system as previously described.

Statistical Analysis

All results are presented as mean±SEM. Microvessel responses were analyzed using 2-way, repeated-measures analysis of variance using the Bonferroni method to compare all pairwise contrasts between treatment group means (K=3). Student t test was used to compare blood alcohol content between the HCW and HCV groups. All other comparisons were carried out using one-way analysis of variance with a Neuman-Keuls posthoc test to compare between groups using GraphPad Prism 5.0 Software (GraphPad Software Inc, San Diego, CA). Differences with a probability value <0.05 were considered statistically significant.

Results

Serum Cholesterol and Alcohol

All groups had similarly elevated levels of total serum cholesterol at the end of the experiment (Figure 1A). HDL cholesterol, however, was significantly increased in the 2 alcohol-treated groups compared with controls (Figure 1B). There was no difference in the calculated low-density lipoprotein:HDL ratio between groups (Figure 1C).

Alcohol was detected in the blood of HCW and HCV animals 1 hour after eating but not of HCC animals. There was no significant difference in blood alcohol content between the HCW and HCV groups (Figure 1D).

Coronary Angiography

All animals demonstrated complete occlusion of the LCx at the terminal procedure. There was no significant difference in angiographic collateralization as assessed by the Rentrop score between groups. Radiographic perfusion as assessed by blush scoring tended to be higher in the alcohol-treated groups compared with the control group, but this difference did not reach statistical significance (0.11±0.11 in HCC, 0.50±0.19 in HCW, and 0.44±0.24 in HCV; P=0.31).

Myocardial Function

Heart rate at the time of harvest was significantly lower in the HCW group compared with both the HCC and HCV groups (69.0±2.2 mm Hg in HCW, 5.4 mm Hg in HCC, 69.2±2.2 mm Hg in HCV, and 77.9±1.9 beats/min in HCW, P=0.08). Developed pressure and left ventricular contractility as measured by first derivative of left ventricular pressure were similar between groups (Figure 2A-B). Regional contractility in the AAR was measured as myocardial shortening in 2 axes. In the vertical axis, contractility was decreased in the HCV group compared with both other groups, whereas contractility in the HCC group was marginally improved compared with the HCV group (Figure 2C). On the horizontal axis, regional contractility was significantly improved in the HCV group compared with both HCC and HCV, whereas the contractility in the HCV group was similar to the HCC group (Figure 2D).

Myocardial Perfusion

Under resting conditions, perfusion to the AAR was significantly increased approximately 1.5-fold in both the HCW and...
HCV groups compared with the HCC group (Figure 3A). There was no significant difference between the HCW and HCV groups at rest. However, under ventricular pacing, perfusion in the HCW group was significantly greater than in both HCC and HCV, and there was no longer a significant difference between the HCC and HCV groups (Figure 3B).

**Microvessel Function**

Microvessel relaxation response to adenosine-5′-diphosphate, an endothelium-dependent vasodilator, was significantly improved in the HCW group compared with both HCC and HCV (Figure 4A). There was no significant difference in the relaxation response to endothelium-independent sodium nitroprusside between groups (Figure 4B).

**Myocardial Fibrosis**

Fibrosis was significantly higher in the HCC group compared with HCW and HCV. There was no significant difference in fibrosis between HCW and HCV animals (Figure 4C).

**Protein Expression**

Expression of proangiogenic proteins phospho-endothelial nitric oxide synthase and vascular endothelial growth factor was significantly increased in both HCW and HCV compared with HCC (Figure 5A–B). Expression of Sirt-1 was significantly increased only in the HCW group, although it tended to be higher than control in the HCV group as well (Figure 5C). However, expression of antioxidant transcription factor phospho-FOXO1 was higher in the HCW group than both other...
groups (Figure 5D), whereas expression of prosangiogenic phospho-mammalian target of rapamycin was highest in the HCV group (Figure 5E).

**Protein Oxidative Stress**
Total oxidative stress in the AAR as measured by the OxyBlot assay was significantly lower in the HCW group than both other groups (Figure 5F).

**Vessel Density**
Capillary density in the AAR was higher in the HCV group than in both HCC and HCW (608 ± 30 vessels/mm² in HCC, 600 ± 39 vessels/mm² in HCW, and 786 ± 73 vessels/mm² in HCV; P = 0.02). There was no significant difference in arteriolar density between the groups (36.1 ± 5.1 vessels/mm² in HCC, 39.6 ± 6.0 vessels/mm² in HCW, and 37.5 ± 5.4 vessels/mm² in HCV; P = 0.90).

**Platelet Function**
When stimulated with adenosine-5'-diphosphate, there was no significant difference in platelet aggregation as measured by impedance (9.67 ± 1.33 ohm in HCC, 10.33 ± 0.56 in HCW, and 12.17 ± 1.40 in HCV; P = 0.52) or adenosine 5'-triphosphate secretion (0.85 ± 0.18 Lum in HCC, 0.93 ± 0.56 in HCW, and 0.67 ± 0.10 in HCV; P = 0.26). Similarly, when stimulated with arachidonic acid, there was no significant difference in aggregation as measured by impedance (6.67 ± 3.38 ohm in HCC, 6.83 ± 1.89 in HCW, and 11.83 ± 1.19 in HCV; P = 0.14) or adenosine 5'-triphosphate secretion (1.31 ± 0.96 Lum in HCC, 1.39 ± 0.46 in HCW, and 1.73 ± 0.39 in HCV; P = 0.47).

**Discussion**
In this study, we demonstrated that both red wine and vodka improve perfusion to ischemic myocardium, but they do so by different mechanisms. Both beverages also decrease myocardial fibrosis in ischemic myocardium, but only red wine increases perfusion during ventricular pacing, improves segmental shortening and microvessel function, and reduces oxidative stress.

Serum alcohol levels in the swine were somewhat lower than we expected considering the body weight of the animals at the time of euthanasia was only 30 to 40 kg. It may simply be that swine metabolize alcohol differently than humans or that swine serum alcohol levels peak at a different time than that which we chose to draw their blood. Fortunately, the serum alcohol levels achieved in the swine in this experiment approximate that of an adult human after one to two alcoholic drinks and were sufficient to produce significant effects.

We specifically chose to use a model of hypercholesterolemia and chronic myocardial ischemia in this study because these comorbidities are very likely to be present in the at-risk patient population that might benefit from the cardioprotective effects of alcohol. Like other studies, we found that...
alcohol supplementation was associated with increased serum HDL cholesterol, whereas total cholesterol levels were unaffected. Although some groups, including our own, have shown that high doses of resveratrol decrease total cholesterol and the low-density lipoprotein:HDL ratio, we did not see these effects with the low doses of resveratrol present in our red wine. HDL cholesterol transports low-density lipoprotein from the periphery to the liver where it is metabolized; thus, the favorable lipid profile created by alcohol supplementation may play a role in cardioprotection and the prevention of atherosclerosis.

A key finding in this study is that both red wine and vodka significantly increased perfusion in the ischemic territory at rest. Two mechanisms can lead to increased blood flow in the setting of chronic ischemia: neogenesis of vessels or dilation of resistance arterioles. Hypercholesterolemia has been shown to cause endothelial dysfunction, reducing the ability of coronary arterioles to dilate. In a previous study, we found that high-dose purified resveratrol reversed this endothelial dysfunction in the ischemic territory of hypercholesterolemic swine related to its antioxidant effects. Similarly, despite a manifold reduction in resveratrol content, red wine in this study significantly decreased oxidative stress and improved endothelium-dependent microvessel relaxation, phenomena that were not seen in vodka-treated swine. The finding that Sirt-1 and the antioxidant transcription factor phospho-FOXO1 were also upregulated only in the wine group suggests that these effects were in fact mediated by resveratrol.

On the other hand, the increased perfusion in the vodka-treated group appears to be related to increased capillary density, mediated by increases in phospho-endothelial nitric oxide synthase, vascular endothelial growth factor, and phospho-mammalian target of rapamycin, all potent mediators of angiogenesis in ischemic myocardium. Alcohol has actually been shown to inhibit mammalian target of rapamycin activity in cultured myocytes, but it may act differently in vivo or in the setting of ischemia.

The different mechanisms by which red wine and vodka improve perfusion may also explain why perfusion was only increased in the wine group under ventricular pacing. In the

Figure 5. Immunoblotting. Expression of proangiogenic proteins phospho-eNOS, VEGF, and phospho-mTOR, antioxidant proteins Sirt-1 and phospho-FOXO1, and total protein oxidation in the AAR were measured by immunoblotting. Both alcoholic beverages increased the expression of phospho-eNOS and VEGF (A–B), whereas only red wine significantly increased the expression of Sirt-1 and phospho-FOXO1 (C–D), although Sirt-1 tended to be elevated in the HCV group as well. Phospho-mTOR was only upregulated in the HCV group (E). Protein oxidative stress was significantly reduced in the HCW group compared with both other groups (F). *P<0.05, †P<0.01, ‡P<0.001. eNOS indicates endothelial nitric oxide synthase; VEGF, vascular endothelial growth factor; mTOR, mammalian target of rapamycin; FOXO, Forkhead class O; AAR, area at risk.
setting of greater oxygen demand, capillaries in the ischemic territory of vodka-treated swine would provide minimal benefit with regard to perfusion because of their small size and fixed number. On the other hand, the improved ability of resistance arterioles to relax in the wine-treated animals likely allows them to adjust for the increased oxygen demand of ventricular pacing and increase blood flow accordingly. Thus, the antioxidant properties of resveratrol-containing red wine provide additional benefit over alcohol alone with regard to myocardial perfusion.

Although wine and vodka supplementation had no effect on global left ventricular function as measured by developed left ventricular pressure and first derivative of left ventricular pressure, there were significant differences in regional function in the ischemic territory. Red wine supplementation improved contractility in the ischemic territory, likely related to the improvements in blood flow discussed previously. Vodka supplementation, however, actually decreased regional contractility in the vertical axis, although myocardial fibrosis in the AAR was decreased compared with controls. Chronic ethanol ingestion has been associated with increased myocardial fibrosis and global contractile dysfunction, but the amounts of alcohol given in this study were insufficient to cause cardiomyopathy. It may be that at lower doses, alcohol actually decreases fibrosis in the ischemic territory. Although we did not specifically examine the molecular basis for decreased fibrosis in these animals, this finding likely relates to the increased myocardial perfusion in the ischemic myocardium in these groups. Antioxidants have also been shown to decrease myocardial remodeling and fibrosis after ischemic injury, so this may play a role in the wine-treated animals as well, although they did not demonstrate any less fibrosis than the vodka-treated animals. The decreased contractility in the vodka group could be related to some other mechanism that we did not examine such as increased apoptosis.

A number of studies have shown that the cardioprotective effects of alcohol may actually be related to an inhibitory effect on platelet aggregation. Our results showed no significant difference in platelet aggregation with either red wine or vodka supplementation, although the small numbers of animals assayed led to substantial margins of error. Nonetheless, both red wine and vodka clearly had beneficial effects on myocardial perfusion, fibrosis, and serum lipid profiles with red wine leading to additional improvements in perfusion and regional function due to its antioxidant properties. These findings shed new light on the mechanisms by which moderate alcohol intake might reduce cardiovascular risk. Whether these beneficial effects are also seen in patients remains to be seen.

Limitations

Although the beneficial effects of red wine are frequently ascribed to resveratrol, red wine is a complex substance containing many other compounds, about some of which very little is known. Furthermore, the activation of Sirt-1 may not be specific to resveratrol as once thought. A recent study showed that white wine, which contains little resveratrol, and tyrosol, a phenolic compound found in white wines and olive oil, also increased expression of Sirt-1 and FOXO in rat hearts, although cardioprotection as measured by reduction in infarct size was greatest with resveratrol and red wine. Thus, the antioxidant effect we saw in this study may not be specific to red wine, and future studies investigating other beverages or compounds present in red wine would be useful. In addition, even among red wines, there is a large variation in actual resveratrol content. Although Californian pinot noir is reported to have one of the highest resveratrol contents, the amount of resveratrol in the wine we chose for this study was lower than that reported for other red wines. Whether or not the effects we saw are dose-dependent remains to be seen.

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Disclosures

None.

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