Ethanol Promotes Arteriogenesis and Restores Perfusion to Chronically Ischemic Myocardium

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Background—Moderate alcohol consumption is known to be cardioprotective compared with either heavy drinking or complete abstinence. We assessed the hypothesis that ethanol supplementation would improve myocardial function in the setting of chronic ischemia.

Methods and Results—Sixteen male Yorkshire swine underwent placement of an ameroid constrictor into the left circumflex artery to induce chronic myocardial ischemia. Postoperatively, animals were supplemented with either 90 mL of ethanol (EtOH) daily (50%/V, EtOH) or 80 g of sucrose of equal caloric value (SUC), serving as controls. Seven weeks after ameroid placement, arteriolar density (1.74±0.210% versus 3.11±0.368% area of arterioles per low-powered field in sucrose (SUC) versus EtOH; *P=0.004), myocardial perfusion (ratio of blood flow to the at-risk myocardium compared with the normal ventricle during demand pacing was 0.585±0.107 versus 1.08±0.138 for SUC versus EtOH; *P=0.014), and microvascular reactivity were significantly increased in ethanol-treated animals compared with controls in the at-risk myocardium. Analysis of vascular endothelial growth factor and NOTCH pathway signaling suggested proneovascular and proliferative activity in the ischemic area. The average peak blood alcohol level in the treatment group was 40±4 mg/dL, consistent with levels of moderate drinking in humans.

Conclusions—Ethanol supplementation increased arteriolar density and significantly improved myocardial perfusion and endothelium-dependent vasorelaxation in chronically ischemic myocardium. These findings suggest that, at moderate doses, ethanol directly promotes vasculogenesis and improves microvascular function, resulting in significant improvements in myocardial perfusion in the setting of chronic ischemia. (Circulation. 2013;128[suppl 1]:S136-S143.)

Key Words: alcohol ▼ angiogenesis ▼ coronary disease ▼ endothelium ▼ ischemia ▼ microcirculation ▼ myocardium

Cardiovascular disease remains the leading cause of mortality in the developed world, despite decades of pharmacological, interventional, and surgical advancements. Although both the private and academic sectors have made significant progress in understanding cardiovascular disease processes, as a scientific and medical community we still struggle to restore myocardial perfusion and function in the setting of both chronic ischemia secondary to coronary artery disease and after acute insults from ischemia and reperfusion. However, several clinical retrospective and observational studies in recent years have focused on the cardio-specific effects of one of the most widely consumed pharmacological agents in the Western world, perhaps second only to caffeine—alcohol. In the early 1990s, reports in the medical literature began to validate what society had observed for decades, that despite a diet high in saturated fats, rates of cardiovascular disease in the Mediterranean were lower than in the rest of the Western world. The attribution of this phenomenon to the concomitant consumption of red wine coined the term French paradox. Furthermore, the dose–response relationship of a J- or U-shaped curve of alcohol consumption established that moderate drinkers seemed to afford cardioprotection compared with either heavy drinkers or abstainers.

Despite the generally accepted relationship that moderate drinking conferred cardioprotection, controversy persisted on whether these cardiovascular benefits resulted from the lifestyle of the moderate drinker, the alcohol itself, or other vasoactive compounds in alcoholic beverages, such as the resveratrol found in red wine. Large animal models have allowed prospective studies with minimal confounders to demonstrate not only generalized antioxidative and anti-inflammatory properties of both alcohol and polyphenols, such as resveratrol, but have also suggested proangiogenic and antiangiogenic effects of polyphenols, as well as proangiogenic effects of ethanol.

Our laboratory uses a large animal (porcine) model of chronic myocardial ischemia to study and modulate the neurovascular response to various pharmacological, cell, and diet-modulated therapies. Although we have previously studied the effects of alcohol-free supplementation with purified resveratrol, as well as several alcohol-containing compounds, a

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prospective large-animal study with unaltered, pharmaceutical-grade ethanol is required to delineate and clarify the observed cardioprotective effects of alcohol consumption in both clinical observations and recent animal studies on cardiac and vascular health. The current experiment was designed to assess the hypothesis that ethanol supplementation alone would improve myocardial function in the setting of chronic ischemia.

**Methods**

**Study Design**

Sixteen intact male Yorkshire miniswine (Parson’s Research, Amherst, MA) with unlimited access to drinking water were fed an unmodified diet (Teklad Miniswine Diet 8753; Harlan Laboratories Inc, South Easton, MA) at 3% to 5% of their body weight daily until they reached a weight of 20 to 30 kg, at which point all animals underwent ameroid constrictor placement (Research Instruments SW, Escondido, CA) to induce chronic myocardial ischemia. For all surgical procedures, anesthesia was induced with an intramuscular injection of 5 mg/kg tiletamine HCl (Telazol; Fort Dodge Animal Health, New York, NY). Animals were endotracheally intubated and mechanically ventilated at 12 to 20 breaths/min. Anesthesia was maintained with a gas mixture of 1.5 to 2.0 L/min of O₂ and 0.75%–3.0% of isoflurane. For the ameroid placement procedure, peri-operative antibiotic prophylaxis consisted of a single dose of intravenous enrofloxacin (5 mg/kg) given after induction of anesthesia and continued orally (enrofloxacin 68 mg PO daily) for 5 days postoperatively in addition to peri-operative aspirin at 325 mg/d for prophylaxis against thromboembolic events starting 1 day before the operation and continuing for a total of 5 days.

Surgical approach for ameroid constrictor placement was via a mini left thoracotomy through the fourth intercostal space, and a titanium ameroid constrictor (1.75–2.25 mm internal diameter) was placed around the proximal left circumflex coronary artery (LCx) just distal to its takeoff from the left main coronary artery. During a 2-minute temporary occlusion of the LCx, isotope-labeled gold microspheres (nonradioactive) were injected into the left atrium to determine the myocardial territory at risk through shadow labeling after cardiac harvest as described below. The pericardium was reapproximated with 3 interrupted 4-0 Nurolon sutures (Ethicon, Somerville, NJ). Postoperative pain for all survival operations was controlled with buprenorphine HCl intramuscular injection (0.03 mg/kg) at the end of the operation and placement of a transdermal fentanyl patch (4 mg) given after induction of anesthesia and continued orally (fentanyl 50 µg PO daily) for 5 days postoperatively in addition to peri-operative aspirin at 325 mg/d for prophylaxis against thromboembolic events starting 1 day before the operation and continuing for a total of 5 days.

For the final operation, a right external jugular vein cutdown was performed for placement of a 7F triple-lumen central venous catheter. Thirty milliliters of blood was drawn for laboratory testing. The right common carotid artery was then accessed and cannulated with a 5F arterial sheath through the same skin incision. The right iliac artery was surgically accessed and cannulated with a 5F arterial sheath for coronary angiography. Coronary angiography of the left and right coronary circulation was performed using <7 mL of omnipaque contrast through an Amplatz Right 1 catheter. Assessment of thrombolysis in myocardial infarction flow, Rentrop, and Blush scores was performed by an interventional cardiologist (R.J.L.) who was blinded to the study groups.

**Myocardial Blood Flow and Shadow Labeling**

At the time of ameroid placement, during a 2-minute temporary occlusion of the LCx, 1.5×10⁷ (5 mL) isotope-labeled gold microspheres (BioPhysics Assay Laboratory Inc, Worcester, MA) were injected into the left atrium to determine the myocardial territory at risk (AAR) and the remote normal ventricle (NV) through shadow labeling. For perfusion analysis, lutetium and europium microspheres (BioPhysics Assay Laboratory) were injected into the left atrium during the final operation under rest conditions (lutetium) and during demand pacing at 180 bpm (europium) with an external pacing pulse generator (Medtronic, Minneapolis, MN) while simultaneously withdrawing arterial blood from the femoral artery catheter with the Harvard PHD 2000 automated syringe pump (Harvard Apparatus, Holliston MA). Of the 10 transmural myocardial sections collected, the sample with the highest gold microsphere density was determined to be the NV (in the distribution of the left anterior descending coronary artery), whereas the myocardial section with the lowest count was determined to be the AAR (in the distribution of the occluded LCx). Myocardial blood flow to each sample was calculated using the following equation:

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

**Coronary Angiography, Hemodynamic Measurements, and Blood Draws**

One week after ameroid placement and diet modification, all animals in the EtOH group were sedated with 5 mg/kg tiletamine 1 hour and 30 minutes after meal/supplement consumption, and jugular venous blood was sampled for serum ethanol levels. This postprandial time was determined to be the peak serum alcohol measurement as empirically determined by our previous studies. After induction of general anesthesia for the final operation, a right external jugular vein cutdown was performed for placement of a 7F triple-lumen central venous catheter. Thirty milliliters of blood was drawn for laboratory testing. The right common carotid artery was then accessed and cannulated with a 5F arterial sheath through the same skin incision. The right iliac artery was surgically accessed and cannulated with a 5F arterial sheath for coronary angiography. Coronary angiography of the left and right coronary circulation was performed using <7 mL of omnipaque contrast through an Amplatz Right 1 catheter. Assessment of thrombolysis in myocardial infarction flow, Rentrop, and Blush scores was performed by an interventional cardiologist (R.J.L.) who was blinded to the study groups.

**Pressure–Volume Conductance Catheter Measurements**

At the beginning of the harvest operation, a multisite pressure-volume (PV) conductance catheter (Ventri-Cath; Millar Instruments, Inc, Houston, TX) was placed into the LV via the right common carotid arterial sheath under fluoroscopic guidance and was used in conjunction with the Micro-Tip Pressure Volume System-Ultra Foundation System and PowerLab hardware, and LabChart Pro software (ADInstruments, Colorado Springs, CO). PV loops were obtained at baseline and during demand pacing at 180 bpm. Before the cardiac harvest, continuous PV measurements were taken during supradiaphragmatic inferior vena cava occlusion by compressing the inferior vena cava against the thoracic spine with a sponge stick. This conductance method of PV measurement has been successfully validated in animal experiments. The end-systolic PV relationship was measured as the linear slope of the relationship of LV pressure over LV volume at the end of systole. The end-systolic PV relationship value is calculated as the slope of the upper left corner of the PV loops (pressure on y axis and volume on x axis) during temporary occlusion of the inferior vena cava.
Immunohistochemistry

Twelve-micrometer-thick sections of frozen myocardial tissue from each NV and AAR were formalin-fixed and incubated with goat antibody against endothelium-specific CD31 (R&D Systems, Minneapolis, MN) followed by DyLight-conjugated anti-goat secondary antibody (Jackson ImmunoResearch, West Grove, PA) for capillary density and with mouse antibody against smooth muscle actin (Sigma-Aldrich, St. Louis, MO) followed by DyLight-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch) for arteriolar density. Sections were mounted in Vectashield mounting medium with 4’,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Three representative microscopic images of each sections were digitally captured and analyzed for capillary density (CD31-positive structures between 5 and 25 μm² in cross-sectional area) and arteriolar density (smooth muscle actin–positive structures that contained with CD31) and analyzed using Image J software (National Institutes of Health, Bethesda, MD). Arteriolar density was calculated as the percent of each representative low-power field (×10) staining for smooth muscle actin, whereas capillary density was reported as the percent of each representative high-power field (×20) staining for CD31. A negative control using only the secondary antibodies was conducted for each slide. Researchers were blinded to study group at the time of microvessel studies and analysis.

Microvessel Studies

Coronary arterioles (80–180 μm diameter) were isolated and placed in a microvessel chamber as previously described. 14 Briefly, vessels were preconditioned to 30% to 40% of their baseline diameter with thromboxane-A, analog U46619 (0.1–1.0 μmol/L) and then treated with endothelium-dependent vasodilators ADP (10−5−10−4 mol/L) and substance P (10−5−10−4 mol/L) and the endothelium-independent vasodilator sodium nitroprusside (10−5−10−2 mol/L). Responses were defined as percent relaxation to baseline diameter after vasoconstriction. All reagents were obtained from Sigma Aldrich (St. Louis, MO). Researchers were blinded to study group at the time of microvessel studies. For each animal, single measurements were made for microvessel baseline diameter, preconditioned diameter, and microvessel diameter in response to serial concentration of each vasoactive substance.

Myocardial Lysate Preparation

Myocardial lysis buffer was prepared by adding 1 protease inhibitor tablet (Roche Diagnostics, Indianapolis, IN), 250 μL each of phosphatase inhibitor cocktail 2 and 3 (Sigma-Aldrich), and 320 μL of 2 mol/L sodium fluoride to 25 mL of radioimmunoprecipitation assay buffer (RIPA Buffer; Boston BioProducts, Ashland, MA) at 4°C. The RIPA-soluble myocardial lysate was then prepared by homogenizing 150 to 200 mg of frozen (−80°C) myocardial tissue from the AAR in 1.0 mL of the above prepared RIPA buffer at 4°C using 6×2.0 mm diameter zirconium-oxide beads (Next Advance Inc, Averill Park, NY) in the Bullet Blender Blue tissue homogenizer (Next Advance) for two 5-minute cycles. Lysates were then incubated at 4°C for 30 minutes on a shaker and then centrifuged at 14 000g for 10 minutes at 4°C.

The SDS-soluble lysate fraction was prepared by resolubilizing the pellet remaining from the centrifugation of the RIPA-soluble lysate as prepared above (pellet contains zirconium-oxide beads used in previous lysate preparation) in 1.0 mL of 2% SDS, 151 mmol/L Tris-Base, 106 mmol/L Tris-HCl, and 0.51 mmol/L EDTA. Protease inhibitors, phosphatase inhibitors, and sodium fluoride were added at the same concentration as above. The pellet and SDS buffer were then homogenized in the Bullet Blender for two 5-minute cycles, heated for 3 minutes at 100°C, centrifuged at 10 000g for 5 minutes, and the supernatant stored in liquid nitrogen, which is designated as the SDS-soluble lysate fraction. Total protein concentrations for all lysates were determined by Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) and Synergy Mx multimode microplate reader 7191000 (BioTek Instruments, Inc, Winooski, VT).

Western Blot Analysis

For each sample, 60 μg of total protein from the RIPA-soluble or SDS-soluble myocardial lysate was fractionated by SDS-PAGE using the NuPage Novex Bis-Tris Mini Gel system (Invitrogen, Carlsbad, CA) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were incubated at 4°C overnight with antibodies against vascular endothelial growth factor receptor (VEGFR) 2 (Cell Signaling, Danvers, MA; SDS-soluble), VEGFR3 (Millipore; RIPA-soluble), Notch 2 (Cell Signaling; SDS-soluble), Jagged 1 (Cell Signaling; SDS-soluble), Jagged 2 (Cell Signaling; RIPA-soluble), delta-like ligand 4 (Cell Signaling; RIPA-soluble), endostatin (Millipore; RIPA-soluble), and interleukin 8 (IL-8; Santa Cruz Biotechnology, Santa Cruz, CA; RIPA-soluble) followed by the appropriate horseradish peroxidase–linked secondary antibodies (1:3000; Jackson ImmunoResearch). Immunoreactive complexes were visualized using electrochemiluminescence and photographed using GeneSnap software (Syngene, Cambridge, UK). Densitometry of electrochemiluminescence signal was performed using Image J software (National Institutes of Health). To ensure and correct equal protein loading, membranes were probed with GAPDH (Cell Signaling), a constitutively expressed housekeeping protein. Raw data collected as arbitrary light units from electrochemiluminescence fluorescence and Image J densitometry were averaged for each group and expressed as fold change compared with the mean of the control group (SUC) using Microsoft Excel Software (Microsoft, Redmond, WA). Researchers were blinded to study group during analysis of Western blot data.

Statistical Analysis

All results were reported as means±SEM. All data between 2 group comparisons was analyzed by Student t test, with significant differences between groups defined as P≤0.05. Microvessel relaxation data were analyzed by 2-way ANOVA with Bonferroni post-test, comparing differences between groups at each dose. All statistical analysis was performed

Table 1. Metabolic Profile at Time of Cardiac Harvest

<table>
<thead>
<tr>
<th></th>
<th>SUC</th>
<th>EtOH</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST, IU/L</td>
<td>20.7±1.6</td>
<td>23.7±1.4</td>
<td>0.18</td>
</tr>
<tr>
<td>ALT, IU/L</td>
<td>61.1±3.4</td>
<td>73.0±7.0</td>
<td>0.15</td>
</tr>
<tr>
<td>TRG, mg/dL</td>
<td>14.6±2.6</td>
<td>14.0±1.7</td>
<td>0.86</td>
</tr>
<tr>
<td>TC, mg/dL</td>
<td>68.3±3.5</td>
<td>66.9±2.4</td>
<td>0.75</td>
</tr>
<tr>
<td>HDL, mg/dL</td>
<td>30.3±1.8</td>
<td>29.0±1.0</td>
<td>0.56</td>
</tr>
<tr>
<td>LDL, mg/dL</td>
<td>35.1±2.2</td>
<td>35.0±1.5</td>
<td>0.98</td>
</tr>
</tbody>
</table>

Calculated LDL, mg/dL=TC−(TRG/5)−HDL. ALT indicates alanine transaminase; AST, aspartate transaminase; EtOH, ethanol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SUC, sucrose; TC, total cholesterol; and TRG, triglycerides.

Table 2. Cardiac Catheterization Results

<table>
<thead>
<tr>
<th></th>
<th>SUC</th>
<th>EtOH</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ameroid occlusion, %</td>
<td>99.9±0.1</td>
<td>100.0±0.0</td>
<td>0.34</td>
</tr>
<tr>
<td>TIMI score</td>
<td>0.57±0.30</td>
<td>0.14±0.14</td>
<td>0.22</td>
</tr>
<tr>
<td>Rentrop score, left</td>
<td>1.57±0.30</td>
<td>1.00±0.38</td>
<td>0.26</td>
</tr>
<tr>
<td>Rentrop score, right</td>
<td>1.14±0.46</td>
<td>0.67±0.33</td>
<td>0.43</td>
</tr>
<tr>
<td>Blush score</td>
<td>0.71±0.29</td>
<td>0.14±0.14</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Left and right coronary angiographic analysis for the thrombolysis in myocardial infarction flow grade (TIMI, 0=no contrast flow through stenosed segment, 1=a portion flows through the stenosed segment, 2=contrast opacifies the distal segment, but perceptively slower more proximally, 3=contrast flows briskly and clears rapidly from the distal segment), Rentrop (0=no visible collateralization, 1=faint filling of only side branches of coronary artery of interest, 2=partial filling of artery of interest, and 3=complete filling of artery of interest), and Blush (0=no blush or contrast density, 1=minimal blush, 2=moderate blush, 3=normal blush compared with nonaffected coronary artery) scores between groups. 9 EtOH indicates ethanol; SUC, sucrose; and TIMI, Thrombolysis in Myocardial Infarction grade flow.
and statistical images generated using GraphPad Prism 5.0 Software (GraphPad Software Inc, San Diego, CA). The animals were received in 2 deliveries of 8 animals and were assigned deidentifying numbers on arrival. The first 4 animals were assigned to the EtOH group, and each subsequent 4 animals were assigned to alternating study arms. Researchers were blinded to the study group during all animal interactions.

Results

Animal Model

One animal in the EtOH group died 6 days after ameroid placement after a 3-day course of metronidazole for gastrointestinal distress; necropsy revealed a perforated gastric ulcer. One animal in the SUC group died immediately after ameroid placement secondary to persistent LCx occlusion, leading to ventricular fibrillation arrest. Both animals were excluded from analysis, leaving a total of 14 animals with even study arms of 7 animals in each of the 2 groups. During the ameroid placement procedure, 3 animals from the EtOH group and 4 animals from the SUC group required 75 mg of intravenous amiodarone for atrial fibrillation with resolution of arrhythmia. There were no obvious behavioral changes in any of the remaining animals or any obvious behavioral differences between groups. All animals regularly consumed all of their chow, including diet supplementation of ethanol or sucrose.

Blood Alcohol Levels, Glucose, and Metabolic Profile

Peak blood alcohol content in the ethanol-supplemented animals was 39.6±3.93 mg/dL. At the time of the final operation, there was no statistical difference in baseline blood glucose level between groups (44.7±7.69 versus 63.3±8.73 mg/dL, P=0.136 for SUC and EtOH, respectively) or weight at the time of cardiac harvest (41.8±1.06 versus 40.0±0.97 kg, P=0.232 for SUC and EtOH, respectively). There were no differences in hepatic function parameters or lipid profile between groups (Table 1).

Coronary Angiography

Closure of the ameroid constrictor was angiographically verified to be >99% for each animal before cardiac harvest. Although <1 in both groups, the average thrombolysis in myocardial infarction perfusion score in the ethanol group was 4× lower than in controls and, a 5-fold reduction in Blush score was observed in the ethanol group as well (also <1 in both groups), although neither of these trends reached statistical significance. Rentrop scores were not significantly different between the 2 groups (Table 2).

LV Hemodynamic Measurements

Hemodynamic parameters measured with PV transduction of the LV before cardiac harvest demonstrated no significant differences in several functional measurements, including cardiac output, contractility, and compliance (Table 3). The slope of the end-systolic PV relationship during inferior vena caval occlusion, a preload-independent measure of LV contractility, was not statistically different between the SUC and EtOH groups (2.51±0.247 versus 2.04±0.180 mmHg/mL in...
the SUC and EtOH groups, respectively; \( P = 0.186 \), with the \( r^2 \) values for the linear regression of end-systolic PV relationship measuring 0.971±0.014 in the SUC group and 0.985±0.003 in the EtOH group.

**Myocardial Perfusion to the At-Risk Myocardium Preserved With Alcohol Supplementation**

Myocardial perfusion was measured by neutron activation of isotope-labeled microspheres injected into the left atrium at rest and during demand pacing at 180 bpm. There was a significant increase in myocardial perfusion in the ethanol-supplemented group during demand pacing as assessed by the ratio of blood flow between the AAR and NV (Figure 1). Perfusion to the NV was not statistically different between the SUC and EtOH groups at rest (1.02±0.188 versus 0.877±0.115 mL/min per gram, respectively; \( P = 0.552 \)) or during demand pacing (0.572±0.127 versus 0.560±0.142 mL/min per gram, respectively; \( P = 0.952 \)).

**Arteriolar Density Was Significantly Higher With Alcohol Supplementation**

There was no difference in capillary density in the AAR between the 2 groups. Arteriolar density, however, was significantly higher in the AAR of the ethanol-supplemented animals compared with the SUC controls (Figure 2).

**Microvascular Function/Reactivity Significantly Improved With Alcohol Supplementation**

In the AAR, we observed significant improvements in endothelium-independent vasorelaxation in response to sodium nitroprusside with ethanol supplementation. Similarly, we observed significantly improved endothelium-dependent vasorelaxation in response to both ADP and substance-P with ethanol supplementation (Figure 3). Baseline characteristics were similar between groups (Table 4).

**Proneovascular/Angiogenic Expression in Both VEGF and NOTCH Pathways With Alcohol Supplementation**

Expression of VEGF receptors 2 and 3 was significantly decreased in the ethanol-supplemented group (Figure 4). Expression of the Notch 2 receptor and Notch ligands Jagged 1 and 2 was significantly higher in the ethanol-supplemented group, whereas the expression of the Notch ligand delta-like ligand 4 was significantly decreased (Figure 5). Significant decreases in the expression of endostatin and the cytokine IL-8 were observed in the ethanol-supplemented group (Figure 6).

**Discussion**

In this model of chronic myocardial ischemia, animals were supplemented with either moderate doses of ethanol or sucrose of equal caloric value, serving as controls. The mean peak blood alcohol level measured 1.5 hours after meal/ethanol consumption was 40 mg/dL in the treatment group, which correlates with a standard breathalyzer test of 0.04 or half of the 0.08 limit of legal intoxication for driving a motor vehicle in most US states. There were no significant increases in liver transaminases, cholesterol profile, or fasting glucose level between groups or observable behavioral changes to suggest any negative systemic effects of the dosing of ethanol used for the duration of the study. Although various studies on alcohol intake in humans have demonstrated moderate consumption to be cardioprotective compared with abstinence or heavy drinking,\(^3,4\) there is no standard as to how moderate

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*Figure 2. Microvessel density. Capillary (left) and arteriolar (right) densities in the area at risk, chronically ischemic myocardium in the territory of the occluded left circumflex coronary artery as measured by immunohistochemical staining at the time of cardiac harvest. CD31 indicates cluster of differentiation 31; HPF, ×20 high-powered field; LPF, ×10 low-powered field; and SMA, smooth muscle actin.*

*Figure 3. Microvessel reactivity. Microvascular function of arterioles in the area at risk at the time of cardiac harvest. Endothelium-independent vasorelaxation measured in response to sodium nitroprusside (SNP). Endothelium-dependent vasorelaxation measured in response to ADP and substance-P. ANOVA \( P \) values reported in graphic. Bonferroni post-test \( P \) values depicted as *\( P < 0.05 \), ‡\( P < 0.001 \).*
Table 4. Baseline Microvessel Characteristics

<table>
<thead>
<tr>
<th></th>
<th>SUC</th>
<th>EtOH</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline diameter, µm</td>
<td>137.3±5.5</td>
<td>137.0±6.9</td>
<td>0.98</td>
</tr>
<tr>
<td>Preconstriction diameter, µm</td>
<td>93.1±6.3</td>
<td>94.0±8.7</td>
<td>0.94</td>
</tr>
<tr>
<td>% Preconstriction</td>
<td>−31.9±4.7</td>
<td>−31.9±4.1</td>
<td>1.00</td>
</tr>
<tr>
<td>ADP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline diameter, µm</td>
<td>136.3±5.3</td>
<td>137.3±7.3</td>
<td>0.78</td>
</tr>
<tr>
<td>Preconstriction diameter, µm</td>
<td>91.4±5.6</td>
<td>92.6±8.4</td>
<td>0.91</td>
</tr>
<tr>
<td>% Preconstriction</td>
<td>−32.9±3.3</td>
<td>−31.2±3.9</td>
<td>0.74</td>
</tr>
<tr>
<td>Substance-P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline diameter, µm</td>
<td>140.8±5.8</td>
<td>134.3±7.3</td>
<td>0.51</td>
</tr>
<tr>
<td>Preconstriction diameter, µm</td>
<td>93.5±5.4</td>
<td>90.1±8.0</td>
<td>0.74</td>
</tr>
<tr>
<td>% Preconstriction</td>
<td>−33.8±1.5</td>
<td>−33.3±3.2</td>
<td>0.89</td>
</tr>
</tbody>
</table>

Microvessels sampled from the AAR at the time of cardiac harvest showing baseline diameter, preconstricted diameter with thromboxane-A₂ analog U46619, and percent of preconstruction from baseline. AAR indicates area at risk; EtOH, ethanol; SNP, sodium nitroprusside; and SUC, sucrose.

drinking is defined. The US National Institute on Alcohol Abuse and Alcoholism (a division of the National Institutes of Health) defines low-risk or moderate alcohol consumption as no more than 4 drinks on any day and no more than 14 drinks per week in men or no more than 3 drinks on any day and 7 drinks per week in women, whereas binge drinking is defined as a blood alcohol content reaching 80 mg/dL. Thus, in accordance with the US National Institutes of Health, the peak blood alcohol levels achieved in the current study are 50% of that to be considered binge drinking, and we consider this study to correlate with a level of moderate alcohol consumption in humans.

After 7 weeks of diet supplementation, animals receiving ethanol had significantly improved myocardial perfusion to the ischemic territory. Although an increase in perfusion may be attributable to an increase in vessel density (capillary, arteriolar, or macro-collateral flow), improvements in microvascular reactivity in the at-risk myocardium, or hemodynamic improvements in contractility, the current study suggests that the observed gains in myocardial perfusion are likely secondary to both an increase in arteriolar density and improved microvascular function. Interestingly, a recent study done by our group in high-cholesterol animals supplemented with either vodka or red wine demonstrated a significant increase in capillary density with vodka supplementation alone and improvements in microvascular function with red wine supplementation alone. The current study suggests that ethanol was the acting agent in the later study accountable for improvements in both vascular ingrowth and microvascular function through improved smooth muscle reactivity, as both endothelial-dependent and endothelial-independent microvascular reactivity were improved in the current study with ethanol in the absence of concomitant polyphenols, such as resveratrol, found in red wine.

Although coronary angiography verified ≥99% closure of the ameroid constrictor in all animals, it failed to demonstrate any robust improvements in macrovascular collateral flow at our 7-week harvest point. This finding is not unexpected as <2 months is a relatively short time frame to expect angiographically observable improvements in coronary circulation. It is reasonable to postulate, however, that with the significant increases in arteriolar ingrowth observed in the ethanol-supplemented animals at 7 weeks, at a later time point these anatomic changes may have developed into improved collateral flow observable in macrocirculation.

Supporting our findings of the direct arteriogenic effects of ethanol in the ischemic territory were proneovascular changes in protein expression in the VEGF and NOTCH pathways. VEGF receptors 2 and 3 were both decreased in the ischemic territory, which is consistent with the internalization and degradation of activated VEGF receptors seen during vascular proliferation. Proproneovascular Notch signaling was also increased in the ethanol-supplemented group through an increase in the Notch 2 receptor and proangiogenic Notch ligands Jagged 1 and 2. Notch signaling has been shown to regulate endothelial progenitor cell activity during recovery from vascular injury. In our study, the Notch ligand delta-like ligand 4 was decreased in the ethanol-treated group, consistent with the well-known antiangiogenic effects of this particular Notch ligand, which plays a greater role in tumor angiogenesis rather than vascular repair after injury. The data presented do suggest that the improvements seen in vessel density, vascular function, and ultimately myocardial perfusion are direct effects of ethanol supplementation through the VEGF and NOTCH pathways. A recent murine study investigating the effects of ethanol supplementation during ischemia and reperfusion injury also demonstrated that ethanol protected at-risk myocardium through rapid increases in VEGF production, resulting in a significant increase in myocardial vascularity.

Significantly attenuated expression of both endostatin and IL-8 was observed in the EtOH group, which supports the antiangiogenic role of these proteins. In fact, antiangiogenic
sequences of IL-8 have been incorporated into artificial cytokine-like peptides developed as a novel antitumor agent. However, IL-8 has also been used to promote lymphatic vessel regeneration for postsurgical lymphedema and has been shown to play an important role in tumor growth, tumor angiogenesis, and metastasis.

In light of the observed preservation of myocardial perfusion, increase in arteriolar ingrowth, and improvement in microvascular function in the at-risk myocardium with ethanol supplementation, one would expect to see concomitant improvements in hemodynamic measurements. Although there were measurable improvements in stroke work, ejection fraction, and contractility (as measured by LV dP/dt max), these improvements did not reach statistical significance. This is likely because of the markedly low developed pressures in the LV (69 and 67 mm Hg at rest in the SUC and EtOH groups, respectively; Table 3), which likely places the animals below pressures of autoregulation, thus masking hemodynamic improvements that would otherwise have manifested. The observed hypotension is likely secondary to the general anesthetic and might have been overcome with the use of a vasopressor after the induction of anesthesia.

Although this study corroborates the growing body of data supporting the cardioprotective effects of moderate ethanol consumption, the ultimate question at hand remains as to whether physicians should encourage moderate alcohol consumption to their patients. Heavy ethanol consumption has many well-established detrimental health effects to several organ systems, including hepatic cirrhosis and negative cardiovascular effects, in addition to being an extremely difficult supplement for many people to consume only in moderation. Thus, although it may be reasonable not to discourage current moderate drinkers from abstaining and to encourage current heavy drinking to moderate their consumption, it is likely unwise to encourage current abstainers to drink in moderation without psychosocial, behavioral, and overall health considerations.

Limitations
Although this is a well-established and validated model of chronic myocardial ischemia, the small sample size does limit the interpretation of seemingly different means, not reaching statistical significance by parametric measures. Due to the small sample size in this large-animal study, it is possible that in several instances differences that did not reach statistical significance, such as in baseline glucose levels, liver transaminases, and cholesterol profiles at the time of the harvest operation, would have done so had the sample size been larger.
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Disclosures
None.

References
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