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Ethanol Promotes New Vessel Growth in Remote Non-Ischemic Myocardium

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Abstract

Background—Epidemiologic studies have demonstrated that daily low to moderate alcohol consumption is cardioprotective as compared to abstainers and high alcohol consumption. Our group reported that alcohol consumption improves angiogenesis in chronically ischemic myocardium. We developed a clinically relevant follow-up study to assess the effect of moderate alcohol consumption on new vessel growth in normal myocardium remote from an ischemic territory in a swine model.

Materials and Methods—Sixteen male Yorkshire swine underwent placement of an ameroid constrictor to induce chronic myocardial ischemia. Post-operatively animals were supplemented with either 90ml of ethanol daily (ETOH) or 80g of sucrose of equal caloric value (SUC). Seven weeks after ameroid placement, myocardial tissue from a territory remote from the ischemia was harvested for analysis.

Results—Both groups had similar microvascular relaxation to endothelial dependent and endothelium independent vasodilators. Also, both groups had similar myocardial perfusion at rest and with demand pacing. The ETOH group had significantly increased arteriolar and capillary density in the non-ischemic myocardium compared to the SUC group. Ethanol supplementation also increased expression of pro-angiogenesis proteins VEGF and VECADHERIN, and decreased expression of anti-angiogenesis proteins angiostatin and endostatin.

Conclusions—Ethanol supplementation increased capillary and arteriolar density, up-regulated pro-angiogenesis and pro-survival proteins, and down-regulated anti-angiogenesis protein expression. These findings suggest that at moderate doses, ethanol directly promotes new vessel growth in the non-ischemic myocardium remote from chronic ischemia.

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Keywords

ischemia; angiogenesis; alcohol; myocardium

Introduction

Despite numerous advancements in the surgical, medical and percutaneous treatments of coronary artery disease, it is still one of the leading causes of death in the United States¹. Given the significant burden of disease, there is growing interest in identifying lifestyle modifications to reduce the development of cardiovascular disease and improve myocardial function after acute myocardial infarction. Epidemiologic studies conducted over the last half century have consistently demonstrated that there is an inverse relationship between low to moderate alcohol consumption and cardiovascular disease²⁻⁵. These studies have shown that people who regularly consume low-moderate doses of alcohol have a lower mortality than abstainers or heavy drinkers. The reduction in mortality among low to moderate drinkers is primarily due to a decrease in the incidence of fatal ischemic heart disease^{4, 6}. Experimental studies have investigated this phenomenon and determined that low to moderate dose of alcohol has many beneficial pleiotropic effects on lipids, platelet activity and fibrinolysis⁷. Conversely, people who consume large quantities of alcohol have a higher morbidity and mortality compared to people who consume low doses of alcohol and abstainers.

In our lab, we previously reported that daily moderate alcohol consumption in the form of vodka and red wine in a swine model of metabolic syndrome and chronic myocardial ischemia improves myocardial endothelial function and perfusion, increases insulin signaling in peripheral tissues, and reduces oxidative stress⁸⁻¹⁰. In another study of moderate alcohol supplementation, we reported that alcohol improves insulin signaling, perfusion, endothelial dysfunction, and angiogenesis in chronically ischemic myocardium^{11, 12}.

Although it is generally accepted that daily low to moderate alcohol consumption is cardioprotective, little is known about the effects of alcohol on normally perfused myocardium remote from an ischemic territory. Therefore, we developed a clinically relevant swine model of daily moderate alcohol consumption and chronic myocardial ischemia to define the effect of alcohol on myocardial perfusion, endothelial function and angiogenic potential in normally perfused myocardium remote from an ischemic territory.

Methods

Animal Model

Fourteen intact male Yorkshire miniswine (Parsons Research, Amherst, MA) were fed 500g/day of regular chow (Sinclair Research, Columbia, MO) daily and underwent ameroid constrictor placement to the left circumflex artery to simulate conditions of chronic myocardial ischemia as described previously¹³. Male swine were selected to eliminate the known effect of estrogens on cardiac pathophysiology. Postoperatively, one group was supplemented with 90 ml of ethanol daily (50%/V, EtOH, n = 7) and the control group was

supplemented with 80g of sucrose of equal caloric value to the alcohol (SUC, n = 7). After 7 weeks of diet supplementation, all animals were anesthetized and the heart was exposed via median sternotomy. The animals were euthanized by exsanguination and samples from the normally perfused myocardium remote from the ischemic territory (in the distribution of the left anterior descending coronary artery) was collected. Tissue samples were placed in krebs solution for microvessel studies or rapidly frozen in liquid nitrogen for histologic and protein expression analysis.

All animals were observed to ensure complete consumption of food and supplement, had unlimited access to water, and were housed in a warm, non-stressful environment for the duration of the experiment.

The Institutional Animal Care and Use Committee of the Rhode Island Hospital approved all experiments. Animals were cared for in compliance 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” (NIH publication no. 5377-3 1996).

Surgical Interventions

Anesthesia—Anesthesia was induced with an intramuscular injection of telazol (4.4 mg/kg). Animals were endotracheally intubated, mechanically ventilated at 12 – 20 breaths per minute, and general anesthesia was maintained with a gas mixture of oxygen at 1.5 – 2 liters/min and isoflurane at 0.75 – 3.0% concentration.

Ameroid Constrictor Placement—Animals were given a single dose of intravenous enrofloxacin 5mg/kg for antibiotic prophylaxis and general anesthesia was induced and maintained. Animals were prepped and draped in the usual sterile fashion. The heart was exposed through a left mini-thoracotomy and pericardiotomy. The left atrial appendage was retracted, and the left circumflex artery was dissected at the take off of the left main coronary artery. The ameroid constrictor was placed around the left circumflex artery (Research Instruments SW, Escondido, CA). The pericardium was loosely re-approximated followed by a layered closure of the surgical incision. Post-operative pain was controlled with a single dose of intramuscular Buprenorphine (0.03 mg/kg) and a 72 hour Fentanyl patch (4µg/kg). All animals received 325mg of aspirin daily starting 1 day pre-operatively and continuing for a total of 5 days for prophylaxis against thrombo-embolic events. All animals continued perioperative antibiotics: enrofloxacin 68mg orally daily for 5 days.

Cardiac Harvest—Under general anesthesia, the heart was exposed via median sternotomy. Animals were euthanized by exsanguination and the normally perfused, non-ischemic myocardial samples from the left anterior descending coronary artery territory was collected for further analysis.

Protein Expression

Frozen tissue samples were homogenized and in a radio-immunoprecipitation assay solution (Boston BioProducts, Ashland, MA) supplemented with protease and phosphatase inhibitors. Forty micrograms of whole-tissue lysates were fractionated by SDS-PAGE

4-12% Bis-Tris gels (NuPage Novex Mini Gel, Invitrogen, Carlsbad, CA) and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were incubated overnight at 4°C with primary antibodies at dilutions recommended by the manufacturer against phosphorylated-endothelial nitric oxide synthase (p-ENOS S1177), ENOS, vascular endothelial growth factor (VEGF), VEGF-receptor 1 (VEGFR1), VEGFR2, vascular endothelial cadherin (VECADHERIN), heat shock protein 27 (HSP27), HSP90, phosphorylated p38 (PP38 T180/Y182), p38, phosphorylated nuclear factor kappa-light-chain-enhancer of activated B cells (pNFκB S536), NFκB, (all from Cell Signaling, Danvers, MA), NADPH oxidase2 (NOX2 Sigma Aldrich, St Louis, MO), endostatin (Millipore), angiostatin (Calbiochem, Billerica, MA), and VEGFR3 (Abcam, Cambridge, MA). Membranes were incubated with the appropriate horseradish peroxidase-linked secondary antibody for one hour at room temperature (Jackson ImmunoResearch, West Grove, PA). Immune complexes were visualized with enhanced chemiluminescence and images were captured with a digital camera system (G-Box, Syngene, Cambridge, England). Band densitometry was quantified as arbitrary light units using Image-J software (National Institutes of Health, Bethesda, MD). All membranes were probed with GAPDH to correct for loading error.

Myocardial Perfusion

Myocardial perfusion was measured by injecting gold isotope-labeled microspheres (Biophysics Assay Laboratory) into the left atrium at the time of ameroid placement during a brief left circumflex artery occlusion. At the final operation, Lutetium microspheres were injected into the left atrium at rest and Europium microspheres were injected while pacing at 160 beats/minute. While injecting the microspheres, blood was simultaneously withdrawing from a femoral artery catheter. Samples of the ischemic myocardium in the circumflex artery distribution and blood were dried at 60° for >48h and microsphere density was quantified with a gamma counter after exposure to neutron beam radiation (Biophysics Assay Laboratory). Myocardial blood flow to each sample was calculated using the following equation:

$$\text{Blood Flow} = \frac{\text{Withdrawal Rate}}{\text{Tissue Weight}} \times \frac{\text{Tissue Microsphere Count}}{\text{Blood Microsphere Count}}$$

Microvessel Studies

Epicardial arterioles ranging in size from 80-120µm diameter, 1-2mm length from the chronically-ischemic left circumflex territory were isolated and cannulated and pressurized (40 mmHg) with dual micropipettes in a microvessel chamber as previously described¹⁴. The vessels were pre-constricted by 20-50% of their baseline diameter with the thromboxane-A₂ analog U46619 (0.1-1.0µM). Microvessels were exposed to an endothelium-dependent vasodilator adenosine-5'-diphosphate (ADP 10⁻⁹ to 10⁻⁴ mol/L), which is a receptor mediated vasodilator that acts mainly through nitric oxide. Microvessels were also treated with an endothelium-independent vasodilator sodium nitroprusside (SNP 10⁻⁹ to 10⁻⁴ mol/L), which is a cyclic guanosine monophosphate-mediated vasodilator

(Sigma Aldrich) as described previously.¹⁴ The percent relaxation from the initial pre-constricted diameter was recorded.

Immunohistochemical Staining for Angiogenesis

Frozen myocardium was sectioned (12- μ m-thickness) and fixed in 10% formalin for 10 minutes. Sections were blocked with 1% bovine serum albumin in phosphate buffered saline for 1 hour at room temperature and incubated with antibodies against porcine endothelial marker CD-31 (R&D Systems, Minneapolis, MN) and smooth muscle actin (SMA, Sigma Aldrich), followed by the appropriate alexa-fluor conjugated antibody (Jackson ImmunoResearch) for 45 minutes. Slides were then mounted with Vectashield with 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Images were captured at X20 magnification with a Nikon E800 Eclipse microscope (Nikon, Tokyo, Japan) at the same exposure in three random fields. Capillaries were defined as structures between 5-25 μ m² in cross-sectional area and arterioles were defined by co-localization of SMA and CD-31 staining. Arteriolar density and capillary density was measured using Image J software in a blinded fashion. The percent capillary and arteriolar density for each animal was averaged from the three randomly selected myocardial tissue sections.

Data Analysis

All results are reported as mean $\pm$ standard error of the mean. Microvessel responses were analyzed with a two-way ANOVA followed by a post-hoc Bonferroni test. A Student's T-test was used to compare the means of all other studies using GraphPad Prism 5.0 Software (GraphPad Software Inc., San Diego, CA).

Results

Animal Model

All animals included in the analysis survived the entire experiment. None of the animals developed wound infection or dehiscence of the mini-thoracotomy site. One animal in the SUC group died shortly after placement of the ameroid constrictor due to persistent ST elevations and ventricular fibrillation arrest. One animal from the ETOH group died on post-operative day 6 and necropsy revealed a perforated gastric ulcer. The two animals that did not survive were excluded from the analysis. We previously reported that there was no difference in cardiac output, contractility or compliance in the ETOH group compared to SUC.¹¹

Angiogenesis Protein Expression—In the normally perfused myocardium remote from the ischemic territory, there was a significant up-regulation of pro-angiogenesis proteins VEGF, VECADHERIN, HSP27, HSP90, P38, pNF κ B, NF κ B in the ETOH group compared to the SUC group. There was also a significant down regulation of anti-angiogenesis proteins angiostatin and endostatin in the ETOH group compared to the SUC group (Table 1).

Myocardial Perfusion—Myocardial perfusion to the non-ischemic normally perfused myocardium in the left anterior descending coronary artery territory was measured at the

time of the final operation, and there was no difference in flow at rest between the SUC and ETOH groups ($1.02 \pm 0.19 \text{ ml/min/g}$, $0.88 \pm 0.12 \text{ ml/min/g}$ $p=0.55$). Also there was no difference in myocardial perfusion during demand pacing at 160 beats/min in the SUC and ETOH groups ($0.57 \pm 0.13 \text{ ml/min/g}$, $0.56 \pm 0.14 \text{ ml/min/g}$ $p=0.95$ respectively) (Figure 1).

Microvessel Reactivity—Microvessel relaxation in response to ADP, an endothelium-dependent vasodilator, was not significantly different in the ETOH group compared to the SUC group (2-way ANOVA $p=0.75$ Figure 2A). There was a significant increase in the microvessel relaxation in the ETOH group compared to SUC in response to SNP, an endothelium-independent vasodilator (2-way ANOVA $p=0.03$ Figure 2B). The baseline diameter and percent precontraction of the ADP-treated microvessels was not statistically significant (Figure 2C). Of note, the baseline diameter and percent preconstruction of the SNP-treated microvessels was approaching significance (Figure 2C).

New Vessel Growth—The percent capillary density/HPF in the ETOH group was significantly higher compared to SUC (18.75 ± 0.43 , 11.67 ± 0.88 $p<0.001$). The arteriolar count/HPF was also significantly increased in the ETOH group compared to the SUC group (33.67 ± 2.45 , 23.89 ± 1.71 count/HPF $p=0.01$) (Figure 3).

Discussion

In this swine model of chronic myocardial ischemia, we studied the effects of moderate daily alcohol consumption on the non-ischemic myocardium remote from an ischemic territory. In the animals supplemented with alcohol, there was a significant up-regulation of pro-angiogenesis proteins, decrease in anti-angiogenesis proteins, and increase in new vessel growth. Alcohol supplementation also increased microvessel reactivity but did not affect myocardial perfusion. These results suggest that moderate alcohol supplementation has beneficial effects in myocardium remote from an ischemic territory and may help the heart adapt to ischemic insult.

In this study, we examine the effects of moderate doses of alcohol on the heart. In a metaanalysis of 51 epidemiologic studies, low doses of alcohol defined as 25g of alcohol/day or approximately 2 drinks/day reduced the risk of coronary artery disease by 25% compared to abstainers¹⁵. However, high doses of alcohol defined as >89g of alcohol/day or >7 drinks/day is detrimental and increases the risk of coronary artery disease. There is also a difference in the relative risk reduction between genders. In women, lower doses of alcohol (10g of alcohol/day or >1 drink/day) is needed to reduce the risk of heart disease, whereas >52g alcohol/day is harmful in women. In the current study of male swine, we chose a moderate dose of alcohol (45g alcohol/day), and we previously reported that the peak blood alcohol level measured 1.5 hours after alcohol consumption was 40mg/dL, which is considered half the standard legal limit of 0.08 for operating a motor vehicle in the United States¹¹. This study was designed as a follow up to a previous study in which animals were supplemented with 45g of alcohol with and without the cardioprotective polyphenol resveratrol in the form of red wine and vodka respectively⁸⁻¹⁰. Given the evidence from epidemiologic and experimental studies, 45g of ethanol/day is considered a moderate dose of alcohol.

To date, there are few if any studies demonstrating the effects of alcohol in non-ischemic myocardium remote from an ischemic territory. We previously reported that there was no angiographic evidence of improved macrovascular collateral flow¹¹. In the current study, we found that alcohol supplementation up-regulated pro-angiogenesis proteins VEGF and VECADHERIN. There was also a significant down-regulation of anti-angiogenesis proteins angiostatin and endostatin in the alcohol treated group. These changes in the protein expression in the angiogenesis pathway were correlated with histologic evidence of increased alcohol-mediated new vessel growth in the normally perfused myocardium remote from an ischemic territory.

The cardioprotective effect of alcohol in the heart has been observed in many animal models simulating chronic alcohol consumption followed by an ischemic insult. These studies have consistently reported that alcohol elicits protection against subsequent ischemic injury similar to ischemic preconditioning, so-called ethanol-induced preconditioning^{2, 16-18}. Following a pre-conditioning stimulus, there is an increase in nitric oxide, protein kinases such as AKT and P38, and transcription factors such as NFκB, leading to the transcription and synthesis of proteins such as HSP27 and HSP90, which protect the heart against ischemia and subsequent cell death^{17, 18}. Interestingly, we found that there was an increase in P38, NFκB, pNFκB, HSP27 and HSP90 in the ETOH group despite the absence of a direct ischemic injury to the normally perfused myocardium. These results suggest that perhaps the ethanol-induced preconditioning is not limited to only ischemic areas. Ethanol may exert its preconditioning cardioprotection globally in the myocardium, which allows the heart to compensate for future ischemic insults.

In examining the microvessel reactivity, there was no difference in the endothelium dependent vasodilator ADP. Activated ENOS produces nitric oxide, which is a potent vasodilator. This result is not surprising given the fact that there was no difference in ENOS protein expression in the alcohol treated group. However, alcohol supplementation did result in an increase in microvessel relaxation in response to the endothelial independent vasodilator SNP. Although this result is statistically significant, it is not likely to be physiologically significant because the baseline characteristics of the harvested microvessels from the ETOH and SUC groups were approaching significance. Therefore, we conclude that alcohol does not affect microvessel reactivity in the normally perfused myocardium remote from an ischemic territory.

Surprisingly, despite the increase in pro-angiogenesis signaling and new vessel growth, there was no difference in myocardial perfusion either at rest or with demand pacing in the ETOH group. This may be due to the fact that hearts were harvested only 7 weeks after ameroid placement and there was not sufficient time to develop fully mature and functional vessels that are capable of delivering blood and producing a measurable increase in myocardial perfusion. Perhaps the capillaries and arterioles identified histologically are not quite functional and do not significantly contribute to myocardial perfusion at the time of harvest.

Conclusion

This study demonstrates that moderate doses of alcohol have global effects on the heart in non-ischemic myocardium remote from an ischemic territory. Despite the absence of a direct

ischemic insult, there was still up-regulation of pro-angiogenesis proteins and an increase in new vessel growth in the normally perfused myocardium remote from an ischemic territory. These changes may contribute the overall cardioprotective effect of alcohol and allow the myocardium to compensate for ischemic stress. Furthermore, by increasing new vessel growth in the normally perfused myocardium, it may also protect the heart from future ischemic insult by increasing collateral blood flow in both the ischemic and non-ischemic myocardium. These findings shed light on another possible mechanism by which alcohol in low to moderate doses is cardioprotective.

Although this data supports the growing evidence that low to moderate doses of alcohol consumption are cardioprotective, clinicians must weigh these benefits cautiously when advising patients at risk for developing cardiovascular disease. Even low-moderate doses of alcohol carry risk for certain malignancies, accidental injury, fetal alcohol syndrome, etc². For some patients, it may be difficult to limit their consumption to drink only in moderation. From a public health perspective, there is little data that compels abstainers to start consuming alcohol, however alcohol can be part of a healthy lifestyle if consumed responsibly and in moderation.

Limitations

While this is an established and validated large animal model of chronic myocardial ischemia, there are several limitations to this study. First, because this is a costly and technically difficult study, the sample size in each group was relatively small. Second, all animals were harvested 7 weeks after ameroid placement and alcohol supplementation. Perhaps if the study duration was extended, further changes in myocardial remodeling and myocardial perfusion may have taken place. Finally, animals were supplemented with one dose of alcohol, 45g/day. Given the known J-shaped curve of alcohol supplementation and cardiovascular risk, it would have been beneficial to also study the effect of low and high doses of alcohol in the myocardium to establish a dose-response curve.

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Figure 1.

Myocardial perfusion in the normally perfused myocardium remote from an ischemic territory at rest and with demand pacing at 160 beats per minute. SUC sucrose controls. ETOH ethanol treated animals.

Figure 2.

Microvessel relaxation in normally perfused myocardium remote from ischemic territory. A. Microvessel relaxation in response to endothelium-dependent vasodilator adenosine-5'-diphosphate (ADP). B. Microvessel relaxation in response to endothelium-independent vasodilator sodium nitroprusside. C. Baseline characteristics of microvessels. SUC sucrose control. ETOH ethanol treated animals.

Figure 3.

New vessel growth in normally perfused myocardium remote from ischemic territory. SUC sucrose control. ETOH ethanol treated animals. ** $p < 0.01$ *** $p < 0.001$

Table 1

Angiogenesis protein expression in the normally perfused myocardium remote from an ischemic territory.
Fold change $\pm$ SEM compared to SUC. SUC sucrose control. ETOH ethanol treated animals.

	SUC	ETOH	p Value
pENOS (S1177)	1.00 $\pm$ 0.051	0.96 $\pm$ 0.061	0.6
ENOS	1.00 $\pm$ 0.022	1.01 $\pm$ 0.023	0.72
VEGF	1.00 $\pm$ 0.066	1.80 $\pm$ 0.044	<0.01
VEGFR1	1.00 $\pm$ 0.016	1.01 $\pm$ 0.027	0.65
VEGFR2	1.00 $\pm$ 0.056	1.03 $\pm$ 0.030	0.63
VEGFR3	1.00 $\pm$ 0.065	0.96 $\pm$ 0.040	0.63
VECADHERIN	1.00 $\pm$ 0.029	1.13 $\pm$ 0.028	0.01
ANGIOSTATIN	1.00 $\pm$ 0.039	0.79 $\pm$ 0.043	0.004
ENDOSTATIN	1.00 $\pm$ 0.036	0.89 $\pm$ 0.025	0.03
HSP27	1.00 $\pm$ 0.135	1.66 $\pm$ 0.028	<0.01
HSP90	1.00 $\pm$ 0.039	1.36 $\pm$ 0.059	<0.01
pP38 (T180/Y182)	1.00 $\pm$ 0.035	1.02 $\pm$ 0.036	0.73
P38	1.00 $\pm$ 0.02	1.14 $\pm$ 0.060	0.05
pNFKB (S536)	1.00 $\pm$ 0.049	1.21 $\pm$ 0.024	0.002
NFKB	1.00 $\pm$ 0.106	1.37 $\pm$ 0.062	0.01
NOX2	1.00 $\pm$ 0.040	0.99 $\pm$ 0.031	0.85