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Kazuhiro Kaneda  
*Osaka Dental University*

Masami Miyamae  
*Osaka Dental University*

Shingo Sugioka  
*Osaka Dental University*

Chika Okusa  
*Osaka Dental University*

Yoshitaka Inamura  
*Osaka Dental University*

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Sevoflurane Enhances Ethanol-Induced Cardiac Preconditioning Through Modulation of Protein Kinase C, Mitochondrial $K_{\text{ATP}}$ Channels, and Nitric Oxide Synthase, in Guinea Pig Hearts

Kazuhiro Kaneda, DDS*, Masami Miyamae, MD, PhD†, Shingo Sugioka, DDS, PhD‡,
Chika Okusa, DDS*, Yoshitaka Inamura, DDS*, Naochika Domae, MD, PhD§,
Junichiro Kotani, DDS, PhD¶, Vincent M. Figueredo, MD‖

*Postdoctoral Researcher, #Assistant Professor, §Professor, Department of Anesthesiology, Osaka Dental University, Osaka, Japan
†Assistant Professor, &Professor, Department of Internal Medicine, Osaka Dental University, Osaka, Japan
¶Institute for Heart and Vascular Health, Albert Einstein Medical Center, Philadelphia, USA

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Conflict of Interest: The authors have no conflicts of interest to report.

Key Words: sevoflurane, preconditioning, ethanol, mitochondrial $K_{\text{ATP}}$ channel, protein kinase C, nitric oxide synthase

Corresponding Author & Reprints: Masami Miyamae, MD, PhD
Department of Internal Medicine,
Osaka Dental University
8-1 Kuzuha hanazono-cho Hirakata,
Osaka 573-1121, Japan
TEL : 81-72-864-3079, FAX : 81-72-864-3179
E-mail: kyotomiya@hotmail.com

Short Title: Sevoflurane and ethanol preconditioning
Implication Statement

Sevoflurane enhances cardiac preconditioning induced by regular ethanol consumption, an effect, mediated in part by PKC and mitochondrial $K_{\text{ATP}}$ channels. Modulation of NOS expression by regular ethanol consumption may also play a role in this enhanced cardioprotection.
Abstract

**Background:** Volatile anesthetics and regular ethanol consumption induce cardioprotection mimicking ischemic preconditioning. We investigated whether sevoflurane enhances ethanol preconditioning and whether inhibition of protein kinase C (PKC) and mitochondrial $K_{\text{ATP}}$ channels attenuated this enhanced cardioprotection. The effects of regular ethanol consumption on expression of inducible (iNOS) and endothelial (eNOS) nitric oxide synthase were determined.

**Methods:** Isolated perfused guinea pig hearts underwent 30 min global ischemia and 120 min reperfusion (Control:CTL). The ethanol group (EtOH) received 2.5% ethanol in their drinking water for 6 weeks. Anesthetic preconditioning was elicited by 10 min exposure to sevoflurane (1 MAC; 2%) in ethanol (EtOH+SEVO) or non-ethanol (SEVO) hearts. PKC and mitochondrial $K_{\text{ATP}}$ channels were inhibited with chelerythrine (CHE) and 5-hydroxydecanoate (5-HD) pretreatment, respectively. Contractile recovery was assessed by monitoring of left ventricular developed (LVDP) and end-diastolic (LVEDP) pressures. Infarct size was determined by triphenyltetrazolium chloride staining. Expression of iNOS and eNOS were determined by Western blot analysis.

**Results:** After ischemia-reperfusion, hearts from the EtOH, sevoflurane (SEVO), and EtOH+SEVO groups had higher LVDP and lower LVEDP compared with CTL. Infarct size was reduced in EtOH and SEVO hearts compared with CTL (27% and 23% vs. 45%, respectively, p<0.001). Sevoflurane further reduced infarct size in EtOH hearts (27% vs. 15%, p<0.001). CHE and 5-HD abolished cardioprotection in both SEVO and EtOH cardioprotected hearts. iNOS expression was reduced and eNOS expression was increased in EtOH hearts.

**Conclusions:** Sevoflurane enhances cardiac preconditioning induced by regular EtOH consumption. This effect is mediated in part by modulation of PKC and mitochondrial $K_{\text{ATP}}$ channels, and possibly by altered modulation of NOS expression.
The number of words is 261.
Introduction

Volatile anesthetics induce cardiac preconditioning in a manner similar to ischemic preconditioning.\textsuperscript{1} Since Kersten et al.\textsuperscript{2}, first documented anesthetic preconditioning with isoflurane, investigators have sought to elucidate the involved mechanisms mediating this cardioprotection. Recently, sevoflurane was shown to confer additional cardioprotection to late ischemic preconditioning, mediated via mitochondrial $K_{\text{ATP}}$ channels.\textsuperscript{3} These data suggests that sevoflurane may enhance cardioprotection in myocardium, which is already preconditioned.

Epidemiological studies have shown that mortality rates for people who regularly drink alcohol in moderation are lower than in abstainers, primarily due to decreased fatal ischemic heart disease.\textsuperscript{4-7} Further, moderate alcohol consumers have lower mortality from myocardial infarction compared with abstainers.\textsuperscript{8} These beneficial cardiac effects may be due to the pleiotropic effects of ethanol on lipids\textsuperscript{9}, platelets, and fibrinolytic activity.\textsuperscript{10,11} We have demonstrated that regular ethanol consumption renders hearts more tolerant to ischemia-reperfusion injury, to a degree similar to acute ischemic preconditioning.\textsuperscript{12-14} We found this ethanol-induced preconditioning is mediated through myocyte adenosine A\textsubscript{1} receptor\textsuperscript{12,13} and protein kinase C (PKC) activation.\textsuperscript{14} Ethanol and volatile anesthetics-induced cardiac preconditioning, thus, appear to share common signaling pathways. Guiraud et al.\textsuperscript{15}, confirmed that the combination of ethanol and ischemic preconditioning were synergistic in reducing infarct size.

We hypothesized that anesthetic preconditioning with sevoflurane would enhance ethanol-induced myocardial preconditioning, and that this effect is potentially mediated by PKC activation and modulation of mitochondrial $K_{\text{ATP}}$ channels.\textsuperscript{16} Both sevoflurane and
acute ethanol exposure increase nitric oxide release.\textsuperscript{17,18} Thus, we examined the effect of chronic ethanol exposure on the expression of inducible and endothelial nitric oxide synthases (iNOS and eNOS).
Methods

This study was conducted in accordance with the Guidelines for Animals Research at Osaka Dental University (ODU), and with the approval of the Animal Experiment Committee of ODU, Osaka, Japan. Male Hartley guinea pigs were fed Lab Diet (RC4™, Oriental Yeast, Tokyo, Japan) and given water ad libitum.

Isolated Heart Perfusion and Measurement of Function

Animals weighing 650-700g were given heparin (1000 units i.p.), then anesthetized with pentobarbital (60 mg/kg i.p). The hearts were excised and immediately arrested in cold isosmotic saline containing 20 mmol/L KCl. The aorta was cannulated and the isolated hearts perfused at 70 mmHg on a nonrecirculating isovolumic perfused heart apparatus, using a Krebs-Henseleit (KH) perfusate (mmol/L): 118 NaCl, 4.0 KCl, 2.52 CaCl2, 24.8 NaHCO3, 1.7 MgSO4, 1.2 KH2PO4, 11.0 glucose, 0.5 EDTA and 8 units/L insulin. The perfusate was insufflated continuously with 95%O2/5%CO2. The hearts were paced at 240 beats/min using platinum-tipped electrodes connected to a Grass Instruments (Quincy, MA, USA) SD-5 stimulus generator. Left ventricular developed pressure (LVDP) was measured from a 2.5 French, high-fidelity micromanometer (Nihon-Kohden, Tokyo, Japan) passed into a compliant latex balloon inserted into the LV, and recorded on a PowerLab 2/20 Data Recording System (ADInstruments, Hayward, Australia). The balloon was connected to a Y-adapter with one end used to advance the micromanometer and the other used to fill the LV balloon with bubble-free water to an end-diastolic pressure (LVEDP) of 10 mmHg. The maximum rate of increase of LV pressure (+dP/dtmax) and the minimum rate of decrease of LV pressure (-dP/dtmin) were calculated using Chart™5 (ADInstruments). Coronary flow
(CF) was measured by collecting effluent. Global ischemia was achieved by clamping the aortic inflow line. Temperature of the heart was continuously monitored by a digital thermometer (PTW-100A, Unique Medical, Japan). During ischemia, hearts were maintained at 37 °C by enclosure in a water-jacketed air chamber. Warmed perfusate kept in the lower part of the chamber saturated the air with humidity and prevented cooling by evaporation.

**Experimental Protocol**

Animals were assigned to one of 8 groups (n=10 each; Figure 1). Animals were initially given 1.25 % ethanol in their drinking water for 1 week and then 2.5 % ethanol for 6 weeks. Serum ethanol levels at time of sacrifice were measured by gas chromatography (Shimadzu, Kyoto, Japan). Liver enzymes were measured to rule out the possible liver tissue damage.

After a 20 min equilibration, baseline LVDP, LVEDP and CF were recorded. All hearts were subjected to 30 min of ischemia followed by 120 min of reperfusion. Anesthetic preconditioning was elicited by administration of sevoflurane (2%) for 10 min followed by 10 min washout before ischemia. Sevoflurane was insufflated by passing the 95%O₂/5%CO₂ gas mixture through a calibrated vaporizer (ACOMA, Tokyo, Japan). Samples of coronary perfusate were collected anaerobically from the aortic cannula for measurement of sevoflurane concentration by an organic vapor sensor (OSP, Saitama, Japan). To investigate the role of PKC and mitochondrial Kᵦₐₜₚ channels activation, their inhibitors chelerythrine (CHE) and 5-hydroxydecanoate (5-HD) were administered for 20 min, starting 10 min before sevoflurane (EtOH+SEVO+CHE, EtOH+SEVO+5HD) or saline
vehicle (CTL+CHE, CTL+5HD) administration. Chelerythrine (Alexis-Calbiochemicals, Tokyo, Japan) and 5-HD (Sigma Chemicals, St. Louis, MO, USA) were dissolved in distilled water, and added to the KH perfusate to a final concentration of 10 µM and 200 µM, respectively. We performed experiments in the following order: first a CTL, followed by ETOH, SEVO, ETOH+SEVO, CTL+CHE, and so forth until the first series of experiments were completed. This sequence was repeated a total of ten times.

**Determination of Myocardial Infarct Size**

At the end of experiments, the hearts were quickly frozen at –80 °C for 15 min, then sliced into 2 mm thick transverse sections from apex to base (6 slices/heart). After removing the right ventricle and defrosting, each slice was weighed and incubated at 37 °C with 1% triphenyltetrazolium chloride (Sigma Chemicals) in phosphate buffer (pH 7.4) for 10 min and then fixed in 10% formalin for at least 5 h to distinguish red stained viable tissue from pale unstained necrotic tissue. Each slice was photographed and the necrotic area was determined using Adobe Photoshop® CS (Adobe, CA, USA) and multiplied by the weight of the slice, then expressed as a fraction of left ventricle.

**Western Blot Analysis**

Separate experiments were performed (n=4 in each group) to examine iNOS and eNOS expression. Myocardial tissue samples were collected before ischemia, and homogenized in ice-cold homogenizing buffer containing in mM: 250 sucrose, 20 HEPES (pH 7.5), 10 KCl, 2 EGTA, 2 MgCl$_2$, 25 NaF, 50 β-glycerophosphate, 1 Na$_3$VO$_4$, 1 PMSF, 1% Triton X and protease inhibitor leupeptin (10 µg/ml). The homogenate was centrifuged at 1000g and 4 °C
for 5 min. The supernatant was re-centrifuged at 10000g and 4 °C for 15 min. The protein concentration was estimated with a Bradford assay. Equivalent amounts (50 µg) of protein samples were loaded and separated on a 5-10% SDS-PAGE gradient gel, then electrically transferred overnight to a PVDF membrane (Millipore Co., Billerica, USA). After blocking with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T), the membranes were incubated for 2 hr at 4 °C in TBS-T containing 5% milk and 1:200 dilution of rabbit primary antibody for iNOS and eNOS (H-174 and H-159, Santa Cruz Biotechnology, USA). Membranes were incubated with a 1:1000 dilution of horseradish peroxidase–labeled anti-rabbit immunoglobulin G (NA 934V, GE Healthcare, UK). The same blot was stripped and re-blotted with antibodies to α-tubulin to confirm equal protein loading. Bound antibody signals were detected with enhanced chemiluminescence (Pierce Biotechnology, IL, USA) and visualized using VersaDoc 5000® Imaging System (Bio-Rad). Quantitative analysis of the band densities was performed by Quantity One® software (Bio-Rad).

Statistical Analysis

All data are expressed as mean±SD. Two-factor repeated-measures analysis of variance was used to evaluate differences over time between groups. If differences were observed, a Tukey post-hoc test was used to confirm the significance of differences between groups. The differences in expressions of iNOS and eNOS were determined by unpaired Student’s t test. Power analysis demonstrated a sample size of n=10 hearts per group was sufficient. A value of p<0.05 was considered statistically significant.
**Results**

Of a total of 92, 10 hearts were not used secondary to intractable ventricular fibrillation after reperfusion (four in CTL, three in CTL+CHE, two in CTL+5HD and one EtOH+SEVO+CHE) and 2 hearts were not used due to aortic rupture. Additional hearts were studied until each group had n=10 successful experiments. There was no significant difference in body weight among groups. Serum ethanol levels at time of sacrifice was 2.7±0.5 mmol/L. Ethanol was not detected in the effluent of the 10 EtOH hearts sampled after 50 min washout but before ischemia or sevoflurane exposure. There was no difference in liver enzymes levels between EtOH and CTL animals. The concentration of sevoflurane in the coronary perfusate after 10 min of exposure was 0.36±0.09 mM. Sevoflurane was not detected in the effluent during the baseline, ischemic, and reperfusion periods.

**Hemodynamics**

Baseline LVDP, +dP/dt_{\text{max}}, -dP/dt_{\text{min}} and CF were similar among the 8 groups. Administration of sevoflurane or treatment with CHE and 5-HD did not significantly affect LVDP or CF. Recovery of LVDP was greater in EtOH, SEVO, and EtOH+SEVO compared with CTL throughout the reperfusion period. Recovery of LVDP in EtOH+SEVO hearts was abolished by administration of CHE and 5-HD. Treatment with CHE and 5-HD alone did not affect the recovery of LVDP. LVEDP increased to 600% of baseline in CTL after ischemia-reperfusion. The increase in LVEDP was significantly less in EtOH, SEVO, and EtOH+SEVO compared with CTL hearts during reperfusion period. LVEDP in CHE and 5-HD groups was similar to that of CTL hearts. Treatment with CHE and 5-HD alone did not affect the increased LVEDP.
All groups had reduced $+\frac{dP}{dt_{\text{max}}}$ after reperfusion compared with baseline. Recovery of $+\frac{dP}{dt_{\text{max}}}$ was significantly greater in SEVO, EtOH and SEVO+EtOH compared with CTL hearts, but not in hearts pretreated with CHE and 5-HD. Changes of $-\frac{dP}{dt_{\text{min}}}$ during the reperfusion period were similar to those of $+\frac{dP}{dt_{\text{max}}}$. There was no significant difference in CF among all groups throughout the experiment. This suggests that changes in CF could not account for the improved contractile recovery of SEVO or ETOH hearts (Table 1).

*Myocardial Infarct Size*

Myocardial infarct size in EtOH and SEVO groups was significantly reduced by approximately 50% compared with control hearts (EtOH:27±6%, SEVO:23±7% vs. CTL:45±11%, p<0.001). The administration of sevoflurane in EtOH hearts decreased infarct size compared with EtOH alone (EtOH+SEVO:15±4%, vs. EtOH: 27±6%, p<0.001). Myocardial infarct size in EtOH and SEVO hearts pretreatment with CHE and 5-HD was not different compared with CTL hearts (EtOH+SEVO+CHE:44±12%, p=0.777, EtOH+SEVO+5-HD:45±9%, p=0.852 vs. CTL). Treatment with CHE and 5-HD alone did not affect the infarct size compared with the CTL group (p=0.884; 0.355, respectively, vs. CTL) (Figure 2).

*Western Blot analysis*

The densities of iNOS and eNOS were normalized against that of CTL. Chronic EtOH consumption increased expression of eNOS whereas it reduced expression of iNOS compared with the CTL groups (Figure 3). Ethanol-treated and CTL hearts showed similar
iNOS and eNOS expression in the presence of CHE and 5-HD with and without sevoflurane exposure (data not shown).
**Discussion**

We found that pretreatment with sevoflurane and regular ethanol consumption equally preconditioned hearts against ischemia-reperfusion injury. Furthermore, sevoflurane enhanced the cardioprotective effect of regular ethanol consumption resulting in smaller infarct size. This protection was abolished by inhibiting PKC or mitochondrial K\textsubscript{ATP} channels, suggesting their role in mediating the observed cardiac preconditioning. Finally, we found that regular ethanol consumption increased eNOS expression and reduced iNOS expression. Our finding that hearts treated with sevoflurane or ethanol showed similar improved functional recovery, demonstrated by increased LVDP and lower LVEDP, as well as a nearly 50% decrease in infarct size, following ischemia-reperfusion is consistent with previous reports.\textsuperscript{12,20,21} We observed that despite a significant reduction of myocardial infarct size induced by the combination of ethanol and sevoflurane, compared with either preconditioning therapy alone, recovery of LVDP during reperfusion was comparable among SEVO, EtOH and SEVO+EtOH hearts. This disparity between the functional recovery and infarct size might be explained by myocardial stunning from global ischemia that affected a greater area of the LV than that susceptible to infarction.\textsuperscript{22,23}

A new finding of this study was that ethanol-induced preconditioning is enhanced by anesthetic-induced preconditioning. A possible mechanism for this effect may be that ethanol does not completely activate the signal transduction pathways eliciting cardioprotection. We previously demonstrated that exposure to 2.5 % ethanol for 6 weeks reduces ischemia-reperfusion injury to the same degree as 5%, 10%, and 20% ethanol for 6 to 12 weeks.\textsuperscript{12} Thus, the ethanol dose used in the present study should induce the maximum ethanol-induced cardiac protection. Similarly, the sevoflurane dose (2%) used in the present
study has been previously shown to produce the maximum cardiac protection. The additive preconditioning effect by the combination of sevoflurane and ethanol suggests that there are some differences in either their mechanisms of action or degree of signaling activation.

Opening of $K_{ATP}$ channels is an important step in the signal transduction cascade of cardiac protection from both volatile anesthetics\(^{21}\) and chronic ethanol exposure.\(^ {25}\) For example, Zaugg et al.\(^ {26}\), demonstrated that sevoflurane increased mitochondrial flavoprotein oxidation, an index of mitochondrial $K_{ATP}$ channel activity, and that this effect was completely abolished by chelerythrine (2 µM) in rat cardiomyocytes. Mitochondrial $K_{ATP}$ channels are activated by NO\(^ {27}\), a key mediator in late ischemic preconditioning.\(^ {28}\) Others have shown that preconditioning with sevoflurane enhances the increased NO effluent induced by bradykinin and nitroprusside in isolated guinea pig hearts.\(^ {17}\) Moreover, ethanol consumption increases serum NO production 30 min after drinking in humans.\(^ {29}\) Our data and prior reports suggest that increased eNOS expression due to chronic ethanol consumption could result in increased NO production. This is consistent with a previous study demonstrating that ethanol increases NO production through modulation of eNOS expression.\(^ {18}\) Recently, the importance of eNOS in myocardial ischemic protection was demonstrated in an eNOS knockout mice model.\(^ {30}\) Thus, augmentation of mitochondrial $K_{ATP}$ channel activity by increased NO with the combination of sevoflurane and chronic ethanol consumption might explain the additive cardioprotection in SEVO+EtOH hearts.

Previous experiments suggest that inflammation plays a role ischemia-reperfusion injury.\(^ {31}\) NO derived from iNOS mediates cardiac dysfunction by inducing production of pro-inflammatory cytokines.\(^ {32}\) Cytokines such as interleukin-1β, interleukin-6, and tumor necrosis factor-alpha (TNF-α) are up-regulated rapidly in response to myocardial ischemia.\(^ {33}\)
Sevoflurane has been shown to reduce the production of TNF-α in patients undergoing coronary bypass surgery.\textsuperscript{34} Interestingly, a recent study found that the late phase of ethanol’s myocardial preconditioning correlated with reduced leukocyte-endothelial cell adhesive interactions, suggesting an anti-inflammatory effect.\textsuperscript{35} In the present study, with a crystalloid-perfused heart, leukocyte-endothelial cell adhesive interactions, though, is not a factor. Nevertheless, EtOH hearts demonstrated reduced iNOS expression before ischemia compared with CTL. Thus, regular ethanol consumption could attenuate the inflammatory response to myocardial ischemia resulting from increased NO production via iNOS. Although iNOS is thought to be absent in myocytes under physiologic conditions, there is evidence that iNOS is present in small vessel endothelium, vascular smooth muscle cells, and immune cells that infiltrate the heart.\textsuperscript{36}

Recent studies demonstrate that acute (as opposed to chronic) ethanol exposure fails to exert a cardioprotection when ethanol is present during ischemia-reperfusion.\textsuperscript{37} Although not withdrawn from the drinking water before sacrifice in this study, serum ethanol levels were low (2.7±0.5 mmol/L) at the time of sacrifice. Ethanol was washed out prior to ischemia using a crystalloid ethanol-free buffer. Ethanol was not detected after 50 min perfusion in the effluent. Ethanol freely diffuses in myocytes. Thus, tissue levels of ethanol were likely negligible at the time of study.

A limitation of the present study is that we did not directly measure PKC levels and mitochondrial $K_{\text{ATP}}$ channel activity before and during ischemia-reperfusion. The doses of CHE and 5-HD used in the present study have been previously found to reduce PKC levels and inhibit mitochondrial $K_{\text{ATP}}$ channel activity, respectively.\textsuperscript{14,38} Furthermore, we and other investigators have previously shown that the same concentrations of CHE and 5-HD inhibit
the preconditioning-like effects of ethanol or sevoflurane alone.\textsuperscript{14,25,38} Prolonged ingestion of ethanol may lead to changes in heart rate and blood pressure. Although we did not measure heart rate or blood pressure prior to sacrifice in this study, a previous study demonstrated that feeding 10 % ethanol (a much higher dose) for 40 weeks to guinea pigs did not alter hemodynamics compared with control\textsuperscript{39}. Finally, animals were not randomized to each study group although the order of study was standardized.

In conclusion, sevoflurane enhances cardiac preconditioning induced by regular ethanol consumption. Our findings suggest that PKC and mitochondrial $K_{\text{ATP}}$ channels play a role in mediating this cardioprotection. NOS expression is altered by regular ethanol consumption.
References


Table Legend

Table 1: Data are presented as mean ± SD. LVDP=left ventricular developed pressure; LVEDP=left ventricular end-diastolic pressure; CF=coronary flow; +dP/dt_{max}=maximum rate of increase of LV pressure; -dP/dt_{min} = maximum rate of increase of LV pressure; CTL=control; EtOH=ethanol; SEVO=sevoflurane CHE=chelerythrine; 5-HD=5-hydroxydecanoate. *p<0.05 vs. CTL , n=10 for each group
**Figure Legends**

Figure 1: Schematic illustration of the experimental protocol of this study. All hearts were subjected to 30 min global ischemia followed by 120 min reperfusion. Anesthetic preconditioning (APC) was elicited by administration of sevoflurane (2% or 1 MAC) for 10 min with a 10 min washout period. Ethanol-treated animals received 2.5% ethanol in their drinking water for 6 weeks. CTL=control; EtOH=ethanol; SEVO=sevoflurane; CHE=chelerythrine, PKC inhibitor; 5-HD=5-hydroxydecanoate, mitochondrial $K_{ATP}$ channel inhibitor

Figure 2: Infarct size as a percentage of LV in eight groups. Treatment with sevoflurane (1 MAC) and 2.5 % ethanol for 6 weeks equally reduced infarct size compared with control. The combination of sevoflurane and 2.5 % ethanol conferred a further reduction of infarct size. This additional cardioprotective effect was abolished by CHE and 5-HD in SEVO+EtOH. 5-HD and CHE treatment alone did not affect infarct size. Data are presented as mean ± SD. *p<0.001 vs. CTL, #p<0.001 vs. EtOH and SEVO

Figure 3: Western blot analysis of iNOS and eNOS from left ventricular samples before ischemia in control and ethanol-treated hearts (n=4 for each group). In ethanol-treated hearts, iNOS expression was significantly reduced and eNOS expression was significantly increased compared with control. *p<0.05 vs. CTL
### Table 1. Hemodynamic Variables

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