Myocardial protection in beating heart cardiac surgery: I: Pre- or postconditioning with inhibition of es-ENT1 nucleoside transporter and adenosine deaminase attenuates post-MI reperfusion-mediated ventricular fibrillation and regional contractile dysfunction

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Objective: To determine the role of the p-nitrobenzylthioinosine-sensitive equilibrative nucleoside transporter 1 (es-ENT1) in postmyocardial infarction reperfusion injury-mediated ventricular fibrillation and regional dysfunction. We used erythro-9 (2-hydroxy-3-nonyl)-adenine and p-nitrobenzylthioinosine to inhibit both adenosine deamination and transport in a canine model of off pump acute myocardial infarction.

Methods: Anesthetized adult dogs (n = 37), instrumented to monitor the percentage of systolic segmental shortening and wall thickening using sonomicrometry, underwent 90 minutes of left anterior descending coronary artery occlusion and 120 minutes of reperfusion. Myocardial coronary blood flow, adenosine triphosphate pool, infarct size, and the incident of ventricular fibrillation and cardioversion were also measured. The dogs received an intravenous infusion of the vehicle (control) or 100 μM of erythro-9 (2-hydroxy-3-nonyl)-adenine and 25 μM p-nitrobenzylthioinosine before ischemia (preconditioning group) or just before reperfusion (postconditioning group).

Results: In the control group, adenosine triphosphate depletion was associated with the accumulation of more inosine than adenosine during ischemia and washed out during reperfusion. Myocardial adenosine and inosine were the major nucleosides in the pre- and postconditioning groups during ischemia and remained detectable during reperfusion. In both groups, recovery of systolic segmental shortening and wall thickening and a reduction in the incidence of ventricular fibrillation (P < .05 vs the control group) coincided with retention of myocardial nucleosides. The infarct size in the 3 groups was not significantly different, independent of myocardial blood flow during ischemia.

Conclusions: Preconditioning or postconditioning with erythro-9 (2-hydroxy-3-nonyl)-adenine/p-nitrobenzylthioinosine significantly reduced the incidence of ventricular fibrillation and cardioversion and attenuated regional contractile dysfunction mediated by postmyocardial infarction reperfusion injury. It is concluded that p-nitrobenzylthioinosine-sensitive equilibrative nucleoside transporter 1 played a major role in these events.

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Supplemental material is available online.

Reperfusion of the ischemic heart is a prerequisite intervention for myocardial salvage and patient survival. In addition to preexisting ischemic injury, reperfusion of an infarcted heart creates more aggressive conditions that trigger a surge of free radicals and promote ventricular arrhythmia and fibrillation. Therefore, targeting postischemic reperfusion injury that occurs with thrombolytic therapy, percutaneous coronary intervention, and coronary artery bypass grafting (CABG) represents a major scientific and clinical challenge and opportunity.

Ischemia depletes adenosine triphosphate (ATP), transiently elevates adenosine, and progressively accumulates inosine (Figure E1). On reperfusion, both adenosine and inosine are released from the ischemic cells by way of the p-nitrobenzylthioinosine (NBMPR)-sensitive equilibrative nucleoside transporter (es-ENT1), where they are converted to hypoxanthine and xanthine. Both metabolites are substrates for xanthine oxidase, catalyzing the formation of...
superoxide radicals on reperfusion. The es-ENT1 transporter plays a major role in on- and off-pump canine models of myocardial stunning. Specific binding sites of [3H]-

METHODS

The biochemical reagents, EHNA and NBMPR, were obtained from Sigma-Aldrich (St. Louis, Mo).

Animal Model

The experiments conformed to the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (publication no. 5377-3; available at http://www.nap.edu/catalog/5140. html).

Surgical Procedures and Model of Acute MI

A total of 37 microfilaria-free adult dogs, of either sex and weighing 22.5 ± 2 kg, were used in the present study. The dogs were anesthetized with an initial intravenous injection of 35 mg/kg sodium pentobarbital (Abbott Laboratories, Chicago, Ill) and intubated and ventilated using a Bennett MA1 respirator (Puritan-Bennett, Berkeley, Calif). Next, a median sternotomy was performed, and the heart was supported in a pericardial sling. The anterior and posterior segments of the left ventricle were instrumented with 2 pairs of lead nitrate zirconate piezoelectric crystals to monitor systolic segmental shortening (SS%, 10 mm) and wall thickening (WT%, epicardium to endocardium) using a pulse transit sonomicrometry system (Trition Technology, San Diego, Calif). Millar transducers were placed intravenicularly by way of the apex and into the carotid artery to monitor the left ventricular and systemic pressures, respectively. The analog data were digitized at 200 Hz and stored on a hard disk on a personal computer and subsequently analyzed using interactive Crunch software developed in our laboratory. Regional myocardial contractility and wall thickening were measured at steady state conditions. The results were calculated as the percentage of change in the end-diastolic length in both SS% and WT% for each beat (10–25 beats). The body and heart temperatures were maintained at 37 ± 1°C. The right phrenic nerve was transected, and the sinoatrial node was crushed and then paced at 150 bpm. A silastic snare was placed around the left anterior descending (LAD) artery just before the first diagonal branch. Porcine-based heparin was intravenously injected (400 U/kg) as an initial bolus followed by 200 U/kg/hr (Elkins-Sinn, Cherry Hill, NJ). Arterial blood gases, pH, and hematocrit were routinely determined and maintained (partial oxygen pressure, 100–140 mm Hg; partial carbon dioxide pressure, 30–40 mm Hg; pH, 7.32–7.48; and hematocrit, about 30%).

Assessment of Adenine Nucleotide Pool Metabolism

Transmural Tru-Cut needle biopsy specimens (5–10 mg) were obtained at baseline before and after drug administration, during LAD artery occlusion, and after 120 minutes of reperfusion, as previously described and published on line (see E-Methods).

Regional Myocardial Blood Flow and Infarct Size

Coronary blood flow was measured using nonradioactive color microspheres (NuFlow Hydron-Coat, Irvine, Calif), as previously described and published on line (see E-Methods).

Experimental Protocol

Myocardial blood flow, systolic SS%, WT%, and myocardial biopsy were obtained at baseline, during LAD occlusion, and after reperfusion. Matching measurements and biopsies were obtained from the nonischemic coronary artery territory of the left ventricle. The LAD coronary artery was snared for 90 minutes. A single bolus of the vehicle (saline and 0.05% dimethylsulfoxide) or vehicle solution containing 100 μM EHNA and 25 μM NBMPR (50 mL) was administered intravenously 5 minutes before LAD artery occlusion (preconditioning group) or just before reperfusion (postconditioning group).

Statistical Analysis

The data are presented as the mean ± standard error of the mean. Sequential measurements were compared using analysis of variance for repeated measures using SAS (SAS Institute, Cary, NC). When significant differences were detected between the groups’ pairs of mean values, they were compared using Tukey’s post hoc tests. Differences were considered significant if the probability value for comparison of least squares means was less than 0.05; the F ratios are provided with the figures. The frequency of VFib was analyzed using Fisher’s exact probability test.
during LAD artery occlusion and no or small infarction (0–5%) were rejected from the study (7 of the 37 dogs). All dogs (n = 8 in each group) survived to the end of reperfusion period, despite events of VFib during reperfusion.

Myocardial ATP

At baseline, the myocardial ATP levels were as follows: control group, 30 ± 3 nmol/mg protein; preconditioning group, 28 ± 3 nmol/mg protein; postconditioning group, 29 ± 2 nmol/mg protein; and nonischemic posterior left ventricular segment, 30 ± 2 nmol/mg protein. Ninety minutes of LAD artery occlusion decreased the myocardial ATP levels equally in all groups (>70%). The ATP level in the control, preconditioning, and postconditioning groups was 4.8 ± 2, 7.5 ± 2, and 8.0 ± 2 nmol/mg protein, respectively (P < .01 vs baselines). The circumflex vascular bed had normal ATP levels. Unlike the control and postconditioning groups, the ATP levels partially recovered in the preconditioning group, to 11.3 ± 3 and 13.3 ± 2 nmol/mg protein at the end of 30 and 120 minutes of reperfusion, respectively (P < .05 vs baseline and other groups, analysis of variance, F = 5.87).

Myocardial Adenosine and Inosine

Adenosine and inosine were not detectable in the myocardial tissue before LAD artery occlusion in any group (Figure 1). At the end of 90 minutes of occlusion, a low adenosine level was found in the control group, with a marked increase in inosine (5.8 ± 0.40 nmol/mg protein; Figure 1, B). In the preconditioning group, both adenosine and inosine progressively accumulated, reaching peaks at 90 minutes of LAD artery occlusion (7.8 ± 0.7 and 3.3 ± 0.5 nmol/mg protein, respectively). Adenosine and inosine declined but remained significantly detectable during reperfusion. Similar to the control group, adenosine was low in the postconditioning group at the end of 90 minutes of LAD artery occlusion (0.2 ± 0.1 nmol/mg protein); inosine was 7.3 ± 0.6 nmol/mg protein. Unlike the control group, inosine remained detectable during reperfusion in the postconditioning group during reperfusion.

Regional Systolic SS% and WT%

The baseline SS% and WT% in the control, preconditioning, and postconditioning groups were 22% ± 3%, 20% ± 4%, and 19% ± 2% and 24% ± 5%, 19% ± 3%, 21% ± 3%, and 22% ± 3%, respectively. At the onset of LAD artery occlusion, systolic SS% switched from positive shortening to a negative paradoxical bulging (Figure 2, A) and a concomitant profound wall thinning (Figure 2, B) in all groups. In the control group, SS% was −25% ± 3% and WT% was −77% ± 3% at 90 minutes of ischemia and remained depressed during reperfusion (Figure 2, B). The change in WT% was almost threefold greater than the change in SS% at the end of ischemia. In the preconditioning group, the SS% (−18% ± 2%) and WT% (−50.3% ± 2%) were also similar to those of the control group. A marked recovery was observed in SS% in this group, reaching +75% ± 2% and +78.4% ± 4% at 30 and 120 minutes of reperfusion, respectively. The WT% in this group was less negative (−15% ± 2%) at 30 minutes of reperfusion than that at the end of ischemia (−55% ± 4%) and WT% had improved at the end of reperfusion (25% ± 4%). In the postconditioning group, SS% at the end of ischemia was −22% ± 3% but had recovered to +30% ± 3% and +53% ± 3% after 30 and 120 minutes of reperfusion, respectively. The WT% changed from −55% ± 3% at the end of ischemia to a less negative −36% ± 5% and then to positive thickening (12% ± 3%) at 30 and 120 minutes of reperfusion, respectively.

FIGURE 1. Effect of erythro-9 (2-hydroxy-3-nonyl)-adenine/p-nitrobenzylthioinosine (EHNA/NBMPR) on (A) myocardial adenosine and (B) inosine in a canine model of 90 minutes of left anterior descending (LAD) artery occlusion and reperfusion showing statistically significant differences between groups with respect to (A) adenosine (P < .001, analysis of variance, F = 11.81; *P < .05 versus baseline and control group, #P < .05 versus all other groups, analysis of variance; n = 8 dogs/group). EHNA/NBMPR; PreC, preconditioning; PostC, postconditioning; LV, left ventricle.
Ventricular Fibrillation

The incidence of fibrillation was recorded during postischemic reperfusion. VFib was more frequent at 25 to 30 minutes of reperfusion. All hearts that were resuscitated with hand massage and electric cardioversions completed the study (Figure 3, A). One or more episodes of VFib that required cardioversion occurred in 6 of 8 hearts in the control group, 2 of 8 in the preconditioning group, and 4 of 8 in the postconditioning group (P < .05, Fisher’s exact probability test). The number of electric cardioversions needed to achieve successful resuscitation (Figure 3, B) was also lower in the EHNA/NBMPR-treated groups (preconditioning, 1.8 ± 0.8, and postconditioning, 1.5 ± 0.3) than in the control group (8.8 ± 4; P < .05).

Myocardial Blood Flow and Infarct Size

The myocardial blood flow before LAD artery occlusion was 2.2 ± 0.2, 2.1 ± 0.1, and 2.01 ± 0.2 mL/min/g in the control, preconditioning, and postconditioning groups, respectively (n = 8/group). The coronary blood flow during LAD artery occlusion was 0.14 ± 0.05, 0.12 ± 0.03, and 0.15 ± 0.04 mL/min/g in the control, preconditioning, and postconditioning groups, respectively (P = NS). The ischemic area (risk area) relative to the total area of the left ventricle was 39% ± 6%, 37% ± 4%, and 32% ± 5% respectively.
in the control, preconditioning, and postconditioning groups, respectively. The infarct size normalized to the risk area was not significantly different among the 3 groups (35% ± 5%, 24% ± 7%, and 28% ± 6% for the control, preconditioning, and postconditioning groups, respectively; \( P = .08 \)).

DISCUSSION

The hypothesis underlying the present study was that ATP catabolites (adenosine and inosine), accumulating during ischemia and released on reperfusion by way of the ENT1, contribute to post-MI reperfusion injury-mediated VFib and contractile dysfunction. A combination of EHNA and NBMPR was used to modulate the adenosine and inosine metabolism and compartmentalization during ischemia and reperfusion. The unique feature of pre- and postconditioning with EHNA/NBMPR is that their actions are confined within the ischemic myocardium without affecting nonischemic tissues. Because acute MI is irreversible, new modalities that target post-MI reperfusion injury and improve functional recovery despite MI represent a clinical challenge and opportunity.

ATP depletion during LAD artery occlusion was similar in all groups, suggesting that EHNA/NBMPR intervention does not prevent ischemic injury, per se, but instead targets reperfusion injury by modulating adenosine and inosine metabolism and transport during ischemia and reperfusion. Numerous investigators have characterized the phenomena of classic ischemic preconditioning and postischemic preconditioning in various models, demonstrating that endogenous adenosine formation and release from ischemic cells is required for activation of the adenosine receptor subtypes that mediate the anti-infarction signaling mechanisms. Because acute MI is irreversible, new modalities that target post-MI reperfusion injury and improve functional recovery despite MI represent a clinical challenge and opportunity.

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In clinical settings, emergent thrombolytic therapy, percutaneous coronary intervention, and/or CABG are performed to restore blood perfusion to the ischemic myocardium as early as possible. In these cases, we believe that pre- and postconditioning with EHNA/NBMPR might not reduce preexisting MI but targets post-MI-mediated complications associated with reperfusion injury. These drugs could be infused intravenously or by intracoronary administration using the transcatheaters before coronary intervention. Previous clinical trials have demonstrated the safety and efficacy of exogenous adenosine as an adjunct to cold cardioplegia during CABG and as an adjuvant to thrombolytic therapy in the Acute Myocardial Infarction Study of Adenosine (AMISTAD) trials. In the latter trial, a reduction in the infarct size was observed in the early, but not late, MI and only at a high dose of adenosine (70 μg/mL/min for 3 hours). However, the over all mortality was not affected by adenosine therapy. Because adenosine has a short half life in humans and animals (seconds), higher doses of adenosine were used to reach pharmacologic efficacy.
Adenosine deamination also increases the levels of unwanted free radical substrates for the vascular xanthine oxidase. Therefore, EHNA would prolong the half life of exogenous adenosine and reduce the dose of adenosine in future clinical trials. Our intervention introduces the concept of “global myocardial protection” during off-pump beating heart CABG surgery without hypothermia, cardioplegic arrest on pump. The infusion of EHNA/NBMPR in a model of the off-pump beating heart provides site-specific protection at the ischemic vascular bed and wherever oxidative stress might occur during surgery. Targeting post-MI reperfusion injury with EHNA/NBMPR has important clinical potential in cases of post-MI thrombolytic therapy, stenting, and CABG.

**Study Limitations**
The present study had the obvious experimental model limitations that must be considered, including species-, age-, and model-related differences. The issue of the species-dependent presence of xanthine oxidase remains debatable. However, it is believed that the porcine heart resembles the human anatomy and coronary distribution. Also, some concerns have been recognized regarding the utility of color microspheres to measure myocardial blood flow because of the loss of microspheres and the yield of color recovery during extraction of microspheres and colors and analysis. Another limitation was the use of load-dependent estimates of regional function. Echocardiography would be more appropriate to monitor global function in the presence of a significant MI. Finally, the doses of EHNA and NBMPR were greater than the Ki values (0.05 nM). EHNA and NBMPR were used according to our previous in the presence of a significant MI. Finally, the doses of phy would be more appropriate to monitor global function and xanthine in free radical-mediated reperfusion injury. *Circulation*. 1988;78: 1336-45.


E-METHODS

Surgical Procedures and Model of Acute MI
A total of 37 microfilaria-free adult dogs, of either sex and weighing 22.3 ± 2 kg, were used in the present study. The dogs were anesthetized with an intravenous injection of 35 mg/kg sodium pentobarbital (Abbot Laboratories, Chicago, Illinois), intubated, and ventilated using a Bennett MA1 respirator (Puritan-Bennett, Berkeley, Calif). Additional doses of 10 mg/kg sodium pentobarbital were injected as needed to maintain anesthesia. A median sternotomy was performed, and the heart was supported in a pericardial sling. The anterior (ischemic) and posterior (nonischemic) segments of the left ventricle were instrumented with 2 pairs of lead titanate zirconate piezoelectric crystals to monitor systolic segmental shortening and systolic wall thickening using a pulse transit sonomicrometry system (Triton Technology, San Diego, Calif). Micromanometer balloon-tipped catheters (Millar Instruments, Houston, Tex) were placed intraventricularly by way of the apex and into the carotid artery to monitor the left anterior descending artery just before the first diagonal branch. The right phrenic nerve was transected to eliminate diaphragmatic contractions. The sinoatrial node was crushed, and the heart atrially paced at 150 bpm using a Medtronic 5880A pacemaker (Medtronic, Minneapolis, Minn). The rationale for electrical pacing of hearts is to minimize variations in the heart rates among dogs of the same group and between groups. Porcine-based heparin was injected intravenously (400 U/kg) as an initial bolus followed by 200 U/kg/hr (Elkins-Sinn, Cherry Hill, NJ). Arterial blood gases, pH, and hematocrit were routinely determined and maintained (partial oxygen pressure, 100–140 mm Hg; partial carbon dioxide pressure, 30–40 mm Hg; pH, 7.32–7.48; and hematocrit, about 30%).

Assessment of Adenine Nucleotide Pool Metabolism
Myocardial adenine nucleotides (adenosine triphosphate [ATP], adenosine diphosphate, and adenosine monophosphate), adenine nucleosides (adenosine and inosine), purine bases (hypoxanthine and xanthine), and nicotinamide adenine dinucleotide (NAD<sup>+</sup>) were determined at baseline, during 90 minutes of left anterior descending artery occlusion and 120 minutes of reperfusion. Transmural Tru-Cut needle (Travenol Laboratories, Deerfield, Ill) biopsy specimens (5–10 mg) were obtained at baseline and after drug administration. Serial biopsies were obtained during 90 minutes of normothermic ischemia and 120 minutes of reperfusion. Each biopsy was immediately frozen and stored in liquid nitrogen (−196°C). Each biopsy specimen was extracted, neutralized, and analyzed using high-performance liquid chromatography, as previously described, and the values were normalized per milligram protein.

Measuring Coronary Flow With Colored Microspheres
Measurements of regional coronary flow were performed as previously described before and during ischemia. Fluorescent-labeled microspheres (15 μm in diameter) were injected into the left atrium of the anesthetized dogs, and reference blood samples were simultaneously withdrawn from a femoral artery. At the end of each study, each heart was weighed and transmurally cubed and divided into the epicardium and endocardium and accurately weighed. The tissue samples and reference blood samples were digested with potassium hydroxide and filtered, and the fluorescent dye was extracted with dimethylformamide as a solvent. The fluorescence of each sample was determined for each color using an automated spectrofluorometer. The coronary blood flow was expressed as the mean milliliters per minute per gram of tissue weight of the transmural sections of the myocardium.

Tissue Histologic Examination
Tissue samples (200–400 mg) were obtained at the end of each study from the ischemic and nonischemic segments of the left ventricle and fixed in glutaraldehyde and prepared for thin sectioning and electron microscope analysis, as previously described by Hayte. The samples were analyzed by a histologist (H.A.) who was unaware of the groups.

Myocardial NAD<sup>+</sup>
Figure 4 shows the effects of acute myocardial ischemia and reperfusion on tissue NAD<sup>+</sup> in all groups. At baseline, all groups had similar NAD<sup>+</sup> levels. The decline in myocardial NAD<sup>+</sup> was more pronounced (50%) in the untreated control group (from 3.7 ± 0.3 to 1.8 ± 0.3 nmol/mg protein, about a 50% reduction from baseline) during ischemia and continued to decline to 1.4 ± 0.2 and 1.2 ± 0.2 nmol/mg protein (50% and 63%) during 30 and 120 minutes of reperfusion. In the preconditioning and postconditioning groups, the NAD<sup>+</sup> levels were maintained during ischemia and during reperfusion (Figure E2).

E-References
FIGURE E1. Role of adenine nucleosides metabolism and transport by way of es-NT1 (compartmentalization) in purine-mediated post-myocardial infarction reperfusion injury. The figure illustrates purine metabolism and compartmentalization in relation to ischemic and reperfusion injury in the untreated control group (A). Ischemia is associated with intracellular adenosine triphosphate (ATP) depletion. Sympathetic stimulation at the onset of ischemia results in neurotransmitter and ATP co-release, elevating extracellular ATP. Ecto- and endo-5'-nucleotidase (endot) and adenosine deaminase continue breaking down ATP and adenosine monophosphate (AMP) during ischemia to adenosine in the intracellular and extracellular compartments. Adenosine is rapidly deaminated to inosine by adenosine deaminase. Extracellular inosine is converted by inosine phosphorylase (IPLase) to hypoxanthine, and the latter is oxidized to xanthine and superoxide radicals during cardiac ischemia and reperfusion. Intracellular inosine and the remaining adenosine are rapidly released on reperfusion by way of the p-nitrobenzylthioinosine (NBMPR)-sensitive adenine nucleoside transporter-1 (es-ENT1), allowing abrupt formation of hypoxanthine, xanthine, and free radicals. The effect of preischemic treatment with EHNA/NBMPR of purine metabolism and compartmentalization is depicted in part B. Adenosine is maintained inside and outside cells. C. Effect of MI postconditioning with EHNA/NBMPR. Intracellular inosine is the major end product of ATP depletion during ischemia. Similar to the control group, noncardiac ATP is broken down to xanthine, producing free radicals in the circulation. Infusion of EHNA/NBMPR after MI but before releasing the left anterior descending coronary artery occlusion allowed entrapment of intracellular inosine, limiting the reperfusion injury mediated by free radicals. ADP, Adenosine diphosphate; AMP, adenosine monophosphate; ecto-5'-NTDase, 5'-nucleotidase; es-ENT1, equilibrative p-nitro-benzylthioinosine (NBMPR)-sensitive adenine nucleoside transport 1; A1R, adenosine receptor 1; IPLase, inosine phosphorylase; XO, xanthine oxidase; O2-, molecular oxygen; O2-, superoxide radical. The font size reflects the amount of purine at ischemia or reperfusion.
FIGURE E2. Effect of erythro-9 (2-hydroxy-3-nonyl)-adenine/p-nitrobenzylthioinosine (EHNA/NBMPR) on myocardial nicotinamide adenine dinucleotide (NAD+) in a canine model of 90 minutes left anterior descending (LAD) artery occlusion and reperfusion. There was statistical significance between groups ($P < .05$, 2-way analysis of variance). *$P < .05$ vs baseline and control group. #$P < .05$ vs post-ischemic treatment group, analysis of variance, $n = 8$ dogs/each group. LV, Left ventricle.

TABLE E1. Reasons for excluding dogs from the study

<table>
<thead>
<tr>
<th>Reason</th>
<th>Control group (n)</th>
<th>EHNA/NBMPR group (n)</th>
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<tbody>
<tr>
<td></td>
<td>Before ischemia</td>
<td>After ischemia</td>
</tr>
<tr>
<td>Fatal anesthesia</td>
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<td>0</td>
</tr>
<tr>
<td>Baseline hypotension or tachycardia (&gt;200 bpm)</td>
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<td>2</td>
</tr>
<tr>
<td>Significant collateral flow (&gt;20% during LAD occlusion)</td>
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<td>2</td>
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<tr>
<td>Total excluded dogs (n)</td>
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<td>4 (36.4%)</td>
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<tr>
<td>Total dogs possible (n)</td>
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<td>11</td>
</tr>
<tr>
<td>Dogs used (n)</td>
<td>8 (61.5%)</td>
<td>8 (66.7%)</td>
</tr>
</tbody>
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EHNA/NBMPR, Erythro-9 (2-hydroxy-3-nonyl)-adenine; NBMPR, p-nitrobenzylthioinosine; LAD, left anterior descending (artery).