# The HNO donor Angeli's salt offers potential haemodynamic advantages over NO• or dobutamine in ischaemia-reperfusion injury in the rat heart *ex vivo*

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**Running head:** Advantages of HNO over NO• or dobutamine in I-R

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**ABSTRACT**

Available inotropic pharmacotherapy for acute heart failure (HF) remains largely ineffective at ameliorating marked impairments in contractile function. Nitroxyl (HNO), the redox sibling of NO•, has recently attracted interest as a therapeutic approach for acute HF. We now compare the impact of ischaemia-reperfusion (I-R) injury on acute haemodynamic responsiveness of the HNO donor, Angeli's salt (AS), to that of NO• and dobutamine. Dose-response curves to bolus doses of AS, diethylamine NONOate (DEA/NO, both 0.001−1 μmol) and dobutamine (0.1−100 nmol) were performed in rat isolated hearts, following I-R or normoxic perfusion. An additional 10 μmol dose of Angeli's salt was included, to permit roughly equivalent inotropic responses to dobutamine. Changes in cardiac contraction, heart rate and coronary flow (CF) were determined. Although AS and DEA/NO elicited comparable dose-dependent increases in CF in normoxic hearts, only AS vasodilation was preserved after I-R. AS and dobutamine elicited dose-dependent inotropic responses in normoxic hearts and I-R blunted inotropic responses to both. Dobutamine however increased heart rate, which was exacerbated by I-R; this was not evident with AS. Further, AS infusion during reperfusion (1 μM), in a separate cohort of rat hearts, improved recovery of cardiac contractility, with lower incidence of I-R-induced ventricular fibrillation. In conclusion, these observations suggest that HNO offers haemodynamic advantages over NO• following I-R. Although I-R suppresses inotropy to both agents, residual contractile responses to AS following I-R is likely free of concomitant pro-arrhythmic events. HNO donors may thus offer haemodynamic advantages over existing pharmacotherapy in acute HF.

**Keywords:** Cardioprotection; Nitric oxide; Nitroxyl; Vasodilation; Ventricular function

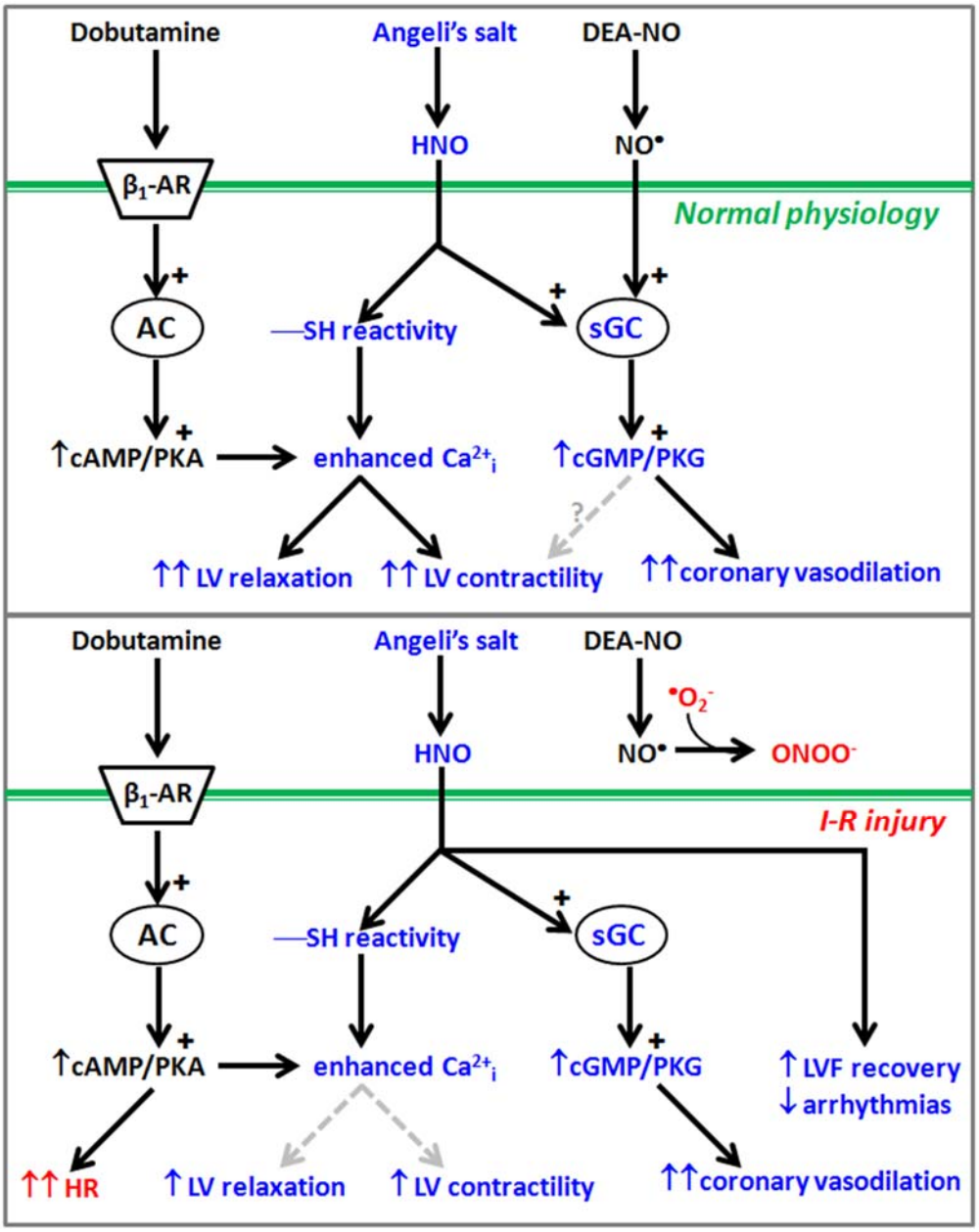
**Chemical compounds studied in this article:** sodium trioxodinitrate (Angeli's salt, PubChem CID: CID 10129945); diethylamine NONOate (DEA/NO, PubChem CID: 9571404); dobutamine (PubChem CID: 36811); Sodium nitroferricyanide (III) dihydate/Sodium nitroprusside dihydrate sodium nitroprusside (SNP, PubChem CID: 11963579)

**Abbreviations:** AC, adenylyl cyclase; AS, Angeli's salt; $\beta_1$-AR, $\beta_1$-adrenoceptor; CGRP, calcitonin gene-related peptide; CK, creatine kinase; DEA/NO, diethylamine NONOate; DOB, dobutamine; DRC, dose-response curve; HF, heart failure; HNO, nitroxyl; I-R, ischaemia-reperfusion; LV, left ventricle; LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; LV±dP/dt, first derivative of LV pressure; MI, myocardial infarction; NO•, nitric oxide; $\bullet O_2^-$, superoxide;

$ONOO^-$, peroxynitrite; PKA, protein kinase A; PKG, protein kinase G; ROS, reactive oxygen species; RyR2, ryanodine receptors; SERCA, sarco/endoplasmic reticulum $Ca^{2+}$-ATPase; sGC, soluble guanylyl cyclase, SH, thiol; SNP, sodium nitroprusside; U46619, 9,11-dideoxy-9α,11α-methanoepoxy-prostaglandin F2α.

**GRAPHICAL ABSTRACT**

In contrast to the $\beta_1$-adreneoceptor ($\beta_1$-AR) agonist dobutamine and the NO• donor diethylamine NONOate (DEA/NO), the vasodilator properties of the nitroxyl (HNO) donor Angeli's salt were preserved after ischaemia-reperfusion (I-R) injury. Although I-R injury blunted the inotropic effects of both Angeli's salt and dobutamine, the HNO donor did not increase heart rate (HR). Angeli's salt, continuously perfused during reperfusion, also improved post-ischaemic recovery of left ventricular function (LVF) and reduced the incidence of arrhythmias. HNO donors may thus offer haemodynamic advantages over existing pharmacotherapy in acute heart failure. AC, adenylyl cyclase; $\bullet O_2^-$ , superoxide; $ONOO^-$ , peroxynitrite; PKA, protein kinase A, PKG, protein kinase G; sGC, soluble guanylyl cyclase, SH, thiol. On the figure, blue and red text represent beneficial and detrimental effects, respectively; grey arrows indicate more modest effects than black arrows.



## INTRODUCTION

Heart failure (HF) is a major cause of hospitalisation in the ageing population of developed countries. Further, patients admitted for HF exhibit higher rates of in-hospital and post-discharge mortality[1-3]. First-line treatment may currently include diuretic agents (to limit concomitant pulmonary oedema), nitrosovasodilators (to unload the heart), and/or a positive inotrope (in an effort to enhance cardiac contractility)[3-5]. Available inotropic pharmacotherapy (e.g. dobutamine, levosimendan, digoxin, etc) is often ineffective at ameliorating the marked impairments in left ventricular (LV) contractile function. In addition, these interventions can potentially initiate adverse cardiac events (e.g. tachyarrhythmias, further impairments in coronary perfusion, and increased mortality)[5-9]. Development of safer positive inotropes for use in HF scenarios is thus urgently required.

HNO has attracted interest as a new approach for managing acute HF[10,11]. HNO shares the vasodilator properties of its well-known redox sibling NO• (and to hence potentially unload the heart), combined with the additional unique ability to enhance LV contraction. We have recently reported that the HNO donor, Angeli's salt, elicits concomitant increases in LV contractility and coronary flow. This is observed in the absence of cardiac arrhythmias, under normal conditions[12]. In the chronically-failing heart, Angeli's salt retains its positive inotropic effects, again without evidence of pro-arrhythmic tendencies[13]. Both LV function and coronary flow often remain impaired for some time (and perhaps indefinitely) following reperfusion[14,15] and hence is a common trigger of acute HF. Immediately following I-R injury, LV dysfunction is still evolving and is hence a more acute myocardial insult. Whether the inotropic and vasodilator responsiveness of an HNO donor are preserved in the face of such an acute myocardial insult remains to be resolved. In the rat isolated heart, pretreatment with the HNO donor Angeli's salt prior to the ischaemic insult (analogous to a pharmacological preconditioning) has been observed in previous studies to confer cardioprotection[16]. This cardioprotection was however absent when the HNO donor was only administered just prior to reperfusion *in vivo*[17]. Thus, the potential for HNO to confer cardioprotection when administered later in the I-R injury response is not well understood.

The objectives of the current study were (i) to determine whether inotropic and vasodilator responsiveness of the HNO donor Angeli's salt are preserved in the acute phase of I-R injury, and how this compares to more conventional NO• vasodilator and dobutamine inotropic effects, and (ii) to investigate whether Angeli's salt limits I-R injury when infused during reperfusion. Our results suggest

that Angeli's salt (but not a NO• donor) elicited comparable dose-dependent vasodilator responses in normoxic and I-R hearts. Although inotropic responses to both Angeli's salt and dobutamine were blunted by I-R injury, dobutamine-induced tachycardia was exacerbated by I-R (which was not evident with Angeli's salt). Further, Angeli's salt infusion during reperfusion improved recovery of cardiac contractility; the incidence of I-R-induced ventricular fibrillation was also reduced. Hence HNO may offer superior vasodilator responsiveness to its redox sibling NO• with concomitant inotropic responsiveness that is comparable to dobutamine (yet with less pro-arrhythmic potential).

## MATERIALS AND METHODS

This investigation conforms to both the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publications No. 85-23, revised 1996) and the National Health and Medical Research Council (NHMRC) of Australia code of practice for the care and use of animals for scientific purposes. All the procedures involved in this project were approved by RMIT University and Alfred Medical Research Educational Precinct (AMREP) Animal Ethics Committees. All studies were performed in hearts isolated from anaesthetised adult male Sprague-Dawley rats (body weight 280-360 g), purchased from the Animal Resources Centre (Murdoch WA, Australia, for dose-response curve studies, as illustrated in Figure 1A) or bred in-house at AMREP Animal Services (cardioprotection studies, as illustrated in Figure 1B and Supplementary Figure 1).

### *Materials*

All materials were purchased from Sigma-Aldrich (St. Louis, MO) except where indicated and were of analytical grade or higher and dissolved in distilled water unless otherwise stated. The source and catalogue number of all reagents is detailed in Supplementary Table 1. KCl, $CaCl_2$, and $MgSO_4 \cdot 7H_2O$ were from Merck (Darmstadt, Germany). Sodium trioxodinitrate (Angeli's salt), DEA/NO and U46619 were obtained from Cayman Chemical Company (Ann Arbor, USA). Angeli's salt and DEA/NO were dissolved and diluted in 10 mM NaOH, both prepared daily and kept on ice until the time of use. Dobutamine was dissolved in distilled water with gentle heating, with subsequent dilutions in Kreb's buffer. U46619 was dissolved in a stock solution of 1 mM in 100% ethanol; aliquots stored at -20ºC were further diluted on the day of use in Kreb's buffer. L-LDH standard from hog muscle was from Boehringer Ingelheim (Ingelheim, Germany).

### *Rat isolated heart perfusion*

Hearts were isolated from anaesthetised rats (325 mg/kg sodium pentobarbitone i.p. (Lethabarb; Virbac Animal Heath, Sydney, Australia), cannulated via the aorta and Langendorff-perfused with Kreb's buffer (composition in mM: NaCl 118, KCl 4.7, $MgSO_4 \cdot 7H_2O$1.18, $KH_2PO_4$ 1.2, EDTA 0.5, $CaCl_2$ 1.75, $NaHCO_3$ 25.0 and D-glucose 11) bubbled with 95% $O_2$ /5% $CO_2$ at pH 7.4 and 37ºC under constant pressure perfusion using the ADInstruments Langendorff System® (ADInstruments Pty Ltd, Bella Vista, Australia), as previously described[12,18]. See Supplementary Methods for a more detailed description of the isolation and cannulation of rat hearts for Langendorff-perfusion. A fluid-filled balloon was inserted into the LV to measure LV pressure. Perfusion pressure,

coronary flow, heart rate, LV systolic pressure (LVSP), its derivative LV±dP/dt, LV end-diastolic pressure (LVEDP) and LV developed pressure (LVDP) were continuously recorded throughout the protocol, using the AD Instruments PowerLab data acquisition system. Rat isolated hearts were then allowed to equilibrate for at least 20 min prior to subsequent sham or I-R injury intervention. Hearts exhibiting poor function (e.g. LV+dP/dt <1500 mmHg/sec, heart rate <100 beats/min, or sustained arrhythmias) during the equilibration period were excluded from the study.

***Impact of I-R injury on haemodynamic responses to Angeli's salt***

After equilibration, rat isolated hearts were assigned to either sham normoxic perfusion for a further 55 min, or to I-R injury. During 30 min global ischaemia, hearts were submerged in 37°C Kreb's buffer, followed by 25 min reperfusion. As illustrated in the protocol schema in Figure 1A, both normoxic and I-R-injured hearts were then infused with the thromboxane $A_2$ mimetic U46619 (3 μM, 0.1-1.5 ml/min) via a port just above the aortic cannula, to preconstrict the coronary vasculature sufficiently to cause a reduction in coronary flow by ~50%. A single bolus dose of vehicle was then administered to the heart via a second injection port, prior to construction of a serial dose-response curve, to the HNO donor Angeli's salt, the NO• donor DEA/NO (both 0.001−1 μmol, to compare vasodilator responses) or to the clinically-used inotrope dobutamine (0.1−100 nmol). An additional 10 μmol dose of Angeli's salt was included, to permit roughly equivalent inotropic responses to dobutamine. Respective vehicles comprised 10 mM NaOH (for Angeli's salt and DEA/NO) and Kreb's buffer (for dobutamine). Dose-response curves were performed by administering bolus doses in increasing magnitude 1 min apart. Each heart was subjected to all 3 dose-response curves at the indicated timepoints, in randomised order (as shown in Figure 1A). Between each curve, U46619 infusion was stopped, allowing 5 min washout. The various haemodynamic responses to Angeli's salt, DEA/NO and dobutamine obtained during each dose-response curve were expressed as percentage change from baseline.

***Impact of Angeli's salt on I-R injury***

Hearts were isolated from male Sprague-Dawley rats (300-360 g) under ketamine-xylazine anaesthesia (100 and 12 mg/kg i.p., respectively), Langendorff-perfused and equilibrated as described above. After equilibration, hearts were assigned to either sham normoxic perfusion for a further 60 min, or to I-R injury (30 min global ischaemia with 30 min reperfusion, as illustrated in Figure 1B). The Kreb's perfusion buffer during reperfusion was randomly allocated to supplementation

with Angeli's salt (1 μM), or vehicle control. The impact of Angeli's salt on recovery of LV function post I-R injury was assessed, expressing each parameter as the percentage change from pre-ischaemic values. Rate-pressure product (RPP) was calculated as the product of heart rate and LVDP. The area under the curve (AUC) for the time-course of post-ischaemic recovery of LV function in the presence and absence of Angeli's salt during reperfusion was calculated using Graphpad Prism® (version 6.0, San Diego, CA).

***Impact of I-R on myocardial injury and incidence of arrhythmias***

Levels of lactate dehydrogenase (LDH) in coronary effluent were determined in sham and I-R hearts allocated to the dose-response curve protocol, to ensure significant I-R injury had occurred. LDH was measured from the rate of reduction in absorbance during conversion of NADH to its oxidised form $NAD^+$ at 340 nm, in the presence of sodium pyruvate, using a UV/VIS spectrophotometer (Lambda 25; PerkinElmer, Waltham, MA, USA). A standard curve was constructed using hog muscle L-LDH, from which coronary effluent LDH concentrations were then derived. The incidence of arrhythmias (specifically of ventricular fibrillation) during the first 10 min of reperfusion was also examined in I-R hearts allocated to both the dose-response curve protocol, and the cardioprotection protocol, during the first 10 min of reperfusion. Occurrence of ventricular fibrillation was defined from LVP recordings according to Lambeth Conventions[19] where beats were no longer distinguishable from one another and LVDP was <5 mmHg. Total duration of ventricular fibrillation during the first 10 min of reperfusion was calculated.

***Statistical analysis***

All results were expressed as mean ± standard error of the mean (SEM), with the number of independent experiments denoted as 'n'. All statistical comparisons were performed using Graphpad Prism® (version 6.0, San Diego, CA). $P<0.05$ was considered statistically significant.

Dose-response curve studies: Evaluation of the vasodilator and cardiac contractile responses to each dose-response curve in sham versus I-R-injured hearts were compared using two-way ANOVA with Sidak *post hoc* analysis for multiple comparisons. LDH results, and the incidence of arrhythmias, in normoxic versus I-R hearts were also compared using Student's unpaired *t*-test.

Cardioprotection studies: The time-course of post-ischaemic recovery of LV function in the presence and absence of Angeli's salt during reperfusion was also compared using two-way ANOVA with Sidak *post hoc* analysis for multiple comparisons. The Area-Under-the-Curve (AUC) figures derived for each

of sham normoxic hearts, and I-R hearts in the presence of vehicle or Angeli's salt in the perfusion buffer were derived using Graphpad Prism, to calculate the area represented by the change in each parameter with time, over the 30 min of reperfusion, for each individual heart. The pooled data for the derived AUC for the recovery of each parameter of LV function during reperfusion in each experimental group AUC was then compared using one-way ANOVA with Dunnett's *post hoc* analysis for multiple comparisons.

## RESULTS

### *Baseline characteristics and impact of I-R on rat isolated hearts*

Baseline haemodynamic characteristics of all buffer-perfused rat hearts used in this study, at the end of 20 min equilibration, prior to any intervention are shown in Table 1. There were no differences between hearts allocated to either subsequent sham normoxic perfusion, or I-R, at the end of the equilibration period. To determine whether hearts were haemodynamically-stable for the full duration of the dose-response curve protocol (after either normoxic persion or I-R injury), their haemodynamic characteristics just prior to each of the 3 dose-response curves (before U46619 preconstriction) are shown in Table 2. Recovery of LVDP, LV±dP/dt and coronary flow were significantly impaired in hearts subjected to I-R, with concomitant marked elevation in LVEDP, indicative of injury. Importantly, all haemodynamic characteristics, whether subjected to normoxic perfusion or I-R, were maintained thereafter at a comparable level for the construction of all 3 dose-response curves. Supplementary Figure 2 shows the time course of coronary flow in representative rat isolated hearts subjected to dose-response curves constructed after normoxic sham perfusion or I-R injury. The parallel changes in contractile parameters LVDP and LV+dP/dt are shown in Supplementary Figure 3. Both supplementary figures illustrate the haemodynamic stability of the hearts over the full timecourse of the study. Myocardial cell death, assessed by LDH release into the coronary effluent, was also significantly elevated compared to sham, from 11 ± 5 to 329 ± 63 U/L ($P<0.001$). Reperfusion-induced ventricular fibrillation occurred in four of the seven hearts subjected to I-R in the dose-response curve protocol (mean duration of ventricular fibrillation 271 ± 99 sec), but in none of the sham hearts.

### *Impact of I-R injury on vasodilator responses to Angeli's salt*

In hearts subjected to normoxic perfusion, both the HNO donor Angeli's salt (0.001−10 μmol) and the NO• donor DEA/NO (0.001−1 μmol) elicited dose-dependent vasodilation in the coronary vasculature (under U46619 preconstriction, Figures 2A and 2B). Each induced a comparable increase in coronary flow at the highest dose studied (by ~70−80%). The inotrope dobutamine tended to reduce coronary flow at lower doses (0.1−1 nmol) in sham hearts. A modest vasodilator response to dobutamine was observed at higher doses (10−100 nmol, $p<0.005$ for dose on two-way ANOVA, Figure 2C), but this was considerably less (by ~50%) than that induced by Angeli's salt or DEA/NO. Following I-R injury, the robust vasodilator response to Angeli's salt was preserved, as shown in

Figure 2A. The mild dobutamine-induced vasodilation was similarly intact following I-R injury (Figure 2C). In contrast, the vasodilator response to DEA/NO was significantly (and markedly) impaired following I-R injury (to <50% of that evident in sham hearts, Figure 2B).

***Impact of I-R injury on LV contractile responses to Angeli's salt***

In normoxic sham hearts, Angeli's salt elicited a dose-dependent positive inotropic effect, on both LVSP and LVDP (Figures 3A and 3B, both $p<0.0001$ for dose on two-way ANOVA). Both parameters were increased ~80% above baseline at the highest dose of Angeli's salt used in this study (10 μmol). DEA/NO by comparison did not elicit a statistically significant effect on either LVSP or LVDP with increasing doses under normoxic conditions (Figure 3C-3D, both p=NS for dose on two-way ANOVA). Dobutamine also exerted a positive inotropic response to Angeli's salt in sham hearts, on both LVSP and LVDP with increasing dose under normoxic conditions (Figure 3E-3F, both $p<0.0001$ for dose on two-way ANOVA). Similar enhancement of LV+dP/dt was evident with both Angeli's salt (which again was increased ~80% above baseline with 10 μmol) and dobutamine, but not in response to DEA/NO (Figure 4).

Following I-R injury, inotropic responsiveness to both Angeli's salt and dobutamine were significantly blunted (Figure 3). At the highest dose studied, Angeli's salt increased LVDP by 19.1 ± 5.4% above baseline (Figure 3B), comparable to the residual inotropic response to dobutamine after I-R injury (Figure 3F). The residual inotropic responses to both Angeli's salt (Figure 4A) and dobutamine (Figure 4C) on LV+dP/dt were similar to that observed on LVDP following I-R injury. By comparison, DEA/NO remained without significant impact on each of LVSP, LVDP and LV+dP/dt post I-R (Figures 3C, 3D and 4C, respectively).

***Impact of I-R injury on LV relaxation responses to Angeli's salt***

In hearts subjected to normoxic perfusion, Angeli's salt elicited dose-dependent enhancement of LV-dP/dt ($p<0.01$ for dose on two-way ANOVA), enhancing LV-dP/dt by ~50% at the highest dose studied (Figure 4B). This was accompanied by a non-significant tendency for a dose-dependent reduction in LVEDP with Angeli's salt ($p=0.1$, Figure 5A). Similarly, dobutamine enhanced both parameters of cardiac relaxation in sham hearts ($p<0.05$ and $p<0.0001$ for dose on two-way ANOVA, Figures 4E and 5E, respectively). The tendency for both Angeli's salt and dobutamine to enhance cardiac relaxation was also blunted by I-R injury, particularly on LV-dP/dt (Figure 4B and 4F). In

contrast, DEA/NO was without impact on either LV-dP/dt or LVEDP, in either the absence or presence of I-R injury (Figures 4D and 5C).

***Impact of I-R injury on chronotropic responses***

In normoxic sham hearts, Angeli's salt elicited a modest yet significant dose-dependent increase in heart rate (Figure 5B, $p<0.0001$ for dose on two-way ANOVA), which was increased ~10% above baseline at the highest dose studied. This heart rate-response was unaffected by prior I-R injury. Although DEA/NO was without impact on heart rate in sham hearts, a reduction in heart rate was observed at the highest dose in hearts subjected to I-R injury ($p<0.001$, Figure 5D). Dobutamine exerted a similar, modest positive chronotropic action to Angeli's salt in sham hearts ($p<0.0001$ for dose on two-way ANOVA), but a more marked dobutamine-induced tachycardia was clearly evident following I-R injury ($p<0.01$, Figure 5F)

***Impact of Angeli's salt on I-R injury***

As shown in Figure 6, LV contractile function remained markedly blunted for up to 30 min reperfusion following 30 min global ischaemia in rat isolated hearts, on each of LVSP, LVDP, LV+dP/dt and RPP. Continuous infusion with the HNO donor Angeli's salt (1 μM, n=6) during reperfusion however significantly improved recovery of LV function. LVSP was significantly increased by administration of Angeli's salt during reperfusion ($p<0.05$, Figures 6A and 6B), while LVDP was almost completely restored by Angeli's salt, particularly on AUC analysis ($p<0.05$, Figure 6D). By the end of 30 min reperfusion, Angeli's salt improved recovery of LVDP by ~65% (compared to only ~20% for vehicle, $p<0.05$, Figures 6C and 6D), with recovery of RPP improved by Angeli's salt by ~40% (compared to ~15% recovery for vehicle, $p<0.05$, Figures 6G and 6H). Lastly, the duration of ventricular fibrillation in the first 10 min of reperfusion was significantly lower in Angeli's salt-treated hearts compared to its NaOH vehicle control ($p<0.05$, Figure 7). These favourable properties of Angeli's salt during reperfusion were again superior to the effects of an NO• donor, as shown in Supplementary Figures 4 and 5.

## DISCUSSION

This study demonstrates for the first time that the coronary vasodilator responsiveness to the HNO donor, Angeli's salt, is completely preserved in the face of the acute myocardial insult, immediately following I-R injury *in vitro*. Further, administration of Angeli's salt for the full duration of reperfusion improved post-ischaemic recovery of LV contractile function, with reduced duration of ventricular fibrillation. In contrast, coronary vasodilator responsiveness to an NO• donor was markedly blunted following I-R injury, and there was no evidence of NO• donor-induced benefit on the recovery of LV function. Although LV inotropic responsiveness to both Angeli's salt and dobutamine were markedly impaired in the acute phase of I-R-induced LV dysfunction, residual contractile responses to dobutamine (but not Angeli's salt) were accompanied by an exacerbated tachycardia. These findings suggest that HNO donors may offer haemodynamic advantages over existing pharmacotherapy in acute HF.

In the current study, both Angeli's salt and DEA/NO were effective dilators of the coronary vasculature in the normoxic rat isolated heart. Both Angeli's salt and DEA/NO induce vasodilation via soluble guanylyl cyclase (sGC)-dependent signalling[12,20,21], activating sGC by interacting with its iron-containing haem protein, forming a ferrous ($Fe^{2+}$)-nitrosyl complex[22,23]. HNO is resistant to reactivity with reactive oxygen species (ROS) such as superoxide however, whereas NO• reacts readily with ROS to generate the highly reactive, cytotoxic species, peroxynitrite[24,25]. Both the acute phase of I-R-induced LV dysfunction[26], as well as the chronic HF that results from myocardial infarction (MI), are associated with increased levels of ROS and oxidative stress[27]. It is thus likely that NO• reactivity with ROS, and/or sGC oxidation as a result of concomitant oxidative stress, are potential contributing mechanisms to the I-R injury-induced loss of vasodilator responsiveness to NO• observed in the present study, whereas those of HNO were preserved. Indeed, we have previously reported that pyrogallol-induced increased oxidative stress alone is sufficient to selectively attenuate the vasodilator action to DEA/NO, yet spare that of Angeli's salt, in rat isolated aorta[28]. Further, the ability of nitrovasodilators to reduce systemic vascular resistance in patients with acute HF is impaired[29]. Hence the inability of NO•-based pharmacotherapies to limit vasoconstriction and unload the heart in the context of acute HF is a major limitation, one that HNO-based approaches may overcome.

Both Angeli's salt and dobutamine elicited comparable enhancement of LV contractile function in the normoxic rat isolated heart at the doses utilised in the current study (Figures 3 and 4).

The two agents mediate their positive inotropic effects via quite distinct mechanisms. Dobutamine is a synthetic catecholamine that exhibits relatively high selectivity for cardiac $\beta_1$-adrenoceptors (although modest concomitant activation of $\beta_2$ and $\alpha_1$-adrenoceptors may also be observed)[5]. Cardiomyocytes predominantly express $\beta_1$ (more so than $\beta_2$)–adrenoceptors, through which dobutamine activates cAMP/protein kinase A-dependent signalling to phosphorylate regulatory proteins involved in cardiac excitation-contraction coupling, including L-type $Ca^{2+}$ channels and phospholamban, essentially mobilising $Ca^{2+}$, to enhance LV contraction[30]. By contrast, HNO released from Angeli's salt directly targets cysteine residues of key sarcomeric proteins such as ryanodine receptors (RyR2), sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA), and its endogenous regulator, phospholamban[31,32]. As HNO enhances RyR2 and SERCA2a activity concomitantly, $Ca^{2+}$ transients (and hence cardiomyocyte contractile function) are enhanced, without affecting diastolic $Ca^{2+}$ or total sarcoplasmic reticulum $Ca^{2+}$ levels, independent of cAMP[33,34]. The HNO donor CXL-1020 similarly exerts enhancement of LV+dP/dt when normalised to instantaneous LVDP in sGC-deficient and wild type mice *in vivo*[35], suggesting this response is independent of sGC. Deficiency of sGC in this previous study however dramatically enhanced LVDP (to which LV+dP/dt was normalised). In contrast, our own evidence has suggested that pharmacological sGC inhibition shifts the dose-response curve to Angeli's salt rightwards in the rat isolated heart (implicating a contributing role for sGC), but this may be secondary to HNO-mediated coronary vasodilation[12].

Despite the contrasting mechanisms by which Angeli's salt and dobutamine elicit enhancement of LV contractile function, inotropic responsiveness to both was blunted by ~75% in the acute phase of I-R-induced LV dysfunction (Figures 3 and 4). Even the relatively short time-frame of I-R injury in the current study is sufficient to impair $Ca^{2+}$ uptake and release activity from the sarcoplasmic reticulum, and reduce levels of RyR2 and SERCA protein in the rat isolated heart[36-38]. Increased oxidative stress as a result of I-R injury has been implicated in this loss of sarcoplasmic reticulum function[36-38]. This blunted inotropic response to dobutamine in hearts after I-R is consistent with previous findings[39]. Sarcoplasmic reticulum function represents a convergence point of Angeli's salt and dobutamine actions; acute dysfunction at this point of convergence may hence be considered a contributing mechanism to loss of inotropic responsiveness in this context. Further, as $Ca^{2+}$ re-uptake by the sarcoplasmic reticulum is a key driver of cardiac relaxation, its impairment as a result of acute I-R injury can likely contribute to the loss of lusitropic responsiveness to both Angeli's salt and

dobutamine (Figure 4B and 4F). The exacerbated tachycardia observed at higher doses of dobutamine (presumably a cAMP/PKA-mediated response)[5], would likely preclude use of the $\beta_1$-adrenoceptor agonist post I-R injury; this potentially adverse effect was not evident however with the HNO donor, favouring its potential utility in this context. Indeed, no cardiac arrhythmias were previously observed with infusion of Angeli's salt in failing dog hearts *in vivo*[13], in contrast to adverse arrhythmic events observed with most current clinically-used inotropes[40].

In the current study, administration of Angeli's salt for the full duration of reperfusion improved post-ischaemic recovery of LV contractile function, with reduced duration of ventricular fibrillation. This cardioprotective effect is consistent with that previously observed by Pagliaro and colleagues[16]. This previous study speculated that this protective effect may have been mediated via pro-oxidative and/or nitrosative stress-related mechanisms, based on the sensitivity of the cardioprotection response to N-acetylcysteine. Given that N-acetylcysteine is a known HNO scavenger, in addition to its antioxidant properties[24], and that HNO possesses superoxide-suppressing actions in the heart and vasculature[41,42], the potential for a pro-oxidant mechanism of HNO cardioprotection remains unfounded.

Interestingly, the observation that Angeli's salt is cardioprotective in both our own *ex vivo* studies here, as well as the above previous report[31], potentially appears in conflict with observations from an *in vivo* regional I-R injury model in rabbits[17]. Ma and colleagues clearly demonstrated that the NO• donor S-nitrosoglutathione robustly enhanced recovery of LV+dP/dt and reduced the area of necrosis and myocardial myeloperoxidase activity, effects which were not shared by Angeli's salt. Although the HNO donor itself did not exacerbate any of these endpoints, the work of Ma and colleagues suggested that Angeli's salt infusion may have had a detrimental impact on the myocardium after I-R injury on two other endpoints, namely LVEDP (by ~2 mmHg) and plasma total creatine kinase (CK) activity (by ~29%; the cardiac-specific CK-MB isoform was not measured)[17]. We note however that in their *in vivo* model: (i) LVEDP was not significantly elevated by I-R alone; (ii) the elevated CK was ameliorated by a lower dose of Angeli's salt; and (iii) the study design employed by Ma and colleagues administered Angeli's salt in 0.9% saline (rather than its conventional NaOH vehicle). As described by DuMond and King, the decomposition of Angeli's salt is pH-dependent, with a reported rate constant of $4–5\times10^{-3}$/s at pH 4–8, and hence an alkaline vehicle is required for biologically-relevant availability of HNO from this donor[24,43]. Further studies of the balance between

the likely cardioprotective effect of Angeli's salt versus possible deleterious actions in the context of I-R injury *in vivo* are thus warranted.

***Study limitations***

We acknowledge that the Langendorff perfused heart studied *ex vivo* does not completely replicate the *in vivo* setting, where the heart works against preload and afterload, nor does it take into account contributions from circulating inflammatory cells such as neutrophils and monocytes. This approach does however enable examination of acute changes in coronary flow concomitantly with impact on cardiac contractile function, in a whole heart that is beating spontaneously. Our study design, performing multiple dose-response curves to increasing bolus doses of 3 different cardioactive agents, precluded assessment of I-R injury on their mechanisms of action, on sGC, calcium-handling proteins such as SERCA and RyR2, or on myofilament sensitivity to calcium (in which thiol reactivity plays a major contrubiting role). We have however previously implicated both sGC and thiol reactivity in the acute haemodynamic responses to Angeli's salt[12].

***Conclusions***

Our observations suggest that HNO offers haemodynamic advantages over either NO• (in terms of preserved vasodilator responsiveness) or dobutamine (for inotropic responsiveness) following I-R *ex vivo*. Although I-R tended to broadly suppress inotropic responsiveness, the contractile responses to Angeli's salt that remained after I-R are likely free of concomitant pro-arrhythmic events (in contrast to dobutamine). Further, continuous Angeli's salt infusion during reperfusion improved recovery of cardiac contractility, with a lower incidence of I-R-induced ventricular fibrillation. Hence HNOs superior vasodilator responsiveness to its redox sibling (permitting unloading of the myocardium), paired with concomitant inotropic effects with less pro-arrhythmic potential suggest HNO donors may offer haemodynamic advantages over existing pharmacotherapy in acute HF scenarios.

## ACKNOWLEDGEMENTS

### Grants

This work was supported in part by the Victorian Government's Operational Infrastructure Support Program. KYC was supported by a Baker IDI Bright Sparks PhD stipend and by the RMIT University School of Medical Sciences. RHR is an NHMRC of Australia Senior Research Fellow (ID472673 and ID1059960). LM was supported by an Endeavour Fellowship funded by the Australian Government.

### Disclosures

The authors declare that there are no conflicts of interest.

**Authorship contributions**: KYC, OLW and RHR conception and design of research; KYC, LM, CQX, NC performed experiments; KYC, CQX, OLW, RHR analyzed data; KYC, OLW and RHR interpreted results of experiments; KYC, OLW and RHR prepared figures; KYC, OLW and RHR drafted manuscript; KYC, LM, CQX, NC, OLW and RHR approved final version of manuscript; KYC, OLW and RHR edited and revised the manuscript.

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**FIGURE LEGENDS:**

**FIGURE 1:** Schematic diagrams of the experimental protocol. Hearts isolated from anaesthetised rats were Langendorff-perfused under constant pressure and were subjected to 30 min ischaemia followed by reperfusion as indicated. **A:** Following U46619 preconstriction of the coronary vasculature, 3 dose-response curves (DRC) were performed in randomised order, to determine dose-dependent haemodynamic responses to Angeli's salt, DEA/NO (both 0.001−1 μmol, to compare vasodilator responses) or to the inotrope dobutamine (0.1−100 nmol). An additional 10 μmol dose of Angeli's salt was included, to permit roughly equivalent inotropic responses to dobutamine. DRCs were performed under both normoxic and I-R injury as shown in the protocol. A 5 min washout (w/o) was performed after each DRC, followed by subsequent U46619 preconstriction just prior to the next DRC. Haemodynamic responses observed at timepoints indicated are shown in Table 2: $^{\#}$1 after equilibration; $^{\#}$2 after 25 min reperfusion in I-R treated hearts or 75 min normoxic perfusion; $^{\#}$3 after w/o following the first DRC, and $^{\#}$4 after w/o following the second DRC.

**FIGURE 2:** The vasodilator dose-response curves to **A:** Angeli's salt, **B:** DEA/NO and **C:** dobutamine in rat isolated hearts subjected to either normoxic sham perfusion (filled symbols, all n=8) or to I-R (open symbols, all n=7). *p<0.05 vs sham on two-way ANOVA with Sidak's *post-hoc* test for multiple comparisons. Data are expressed as percentage change from baseline, mean ± SEM.

**FIGURE 3:** Dose-response curves for LV contractile function indices LVSP and LVDP to each of Angeli's salt (Panels **A** and **B**), DEA/NO (Panels **C** and **D**), and dobutamine (Panels **E** and **F**), in rat isolated hearts subjected to either normoxic sham perfusion (filled symbols, all n=8) or to I-R (open symbols, all n=7). *p<0.05, ***p<0.001, ****p<0.0001 vs sham on two-way ANOVA with Sidak's *post-hoc* test for multiple comparisons. Data are expressed as percentage change from baseline, mean ± SEM.

**FIGURE 4:** Dose-response curves for LV+dP/dt and LV-dP/dt to each of Angeli's salt (Panels **A** and **B**), DEA/NO (Panels **C** and **D**), and dobutamine (Panels **E** and **F**), in rat isolated hearts subjected to either normoxic sham perfusion (filled symbols, all n=8) or to I-R (open symbols, all n=7). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs sham on two-way ANOVA with Sidak's *post-hoc* test for multiple comparisons. Data are expressed as percentage change from baseline, mean ± SEM.

**FIGURE 5:** Dose-response curves for LVEDP and Heart Rate to each of Angeli's salt (Panels **A** and **B**), DEA/NO (Panels **C** and **D**), and dobutamine (Panels **E** and **F**), in rat isolated hearts subjected to either normoxic sham perfusion (filled symbols, all n=8) or to I-R (open symbols, all n=7). **p<0.01, ***p<0.001 vs sham on two-way ANOVA with Sidak's *post-hoc* test for multiple comparisons. Data are expressed as change from baseline (in mmHg for LVEDP) or percentage change from baseline (for heart rate), mean ± SEM.

**FIGURE 6:** Angeli's salt (n=6), added to the perfusion buffer at the onset of reperfusion, attenuates the impairment in post-ischaemic recovery of LV function compared to vehicle-treated I-R (n=3). **A:** Time-course of recovery of LVSP; **B:** AUC analysis of the time-course of LVSP recovery; **C:** Time-course of recovery of LVDP, **D:** AUC analysis of the time-course of LVDP recovery; **E:** Time-course of recovery of LV+dP/dt, **F:** AUC analysis of the time-course of LV+dP/dt recovery, and **G:** Time-course of recovery of RPP, **H:** AUC analysis of the time-course of RPP recovery. *p<0.05 vs vehicle-treated I-R on two-way ANOVA with Sidak's *post-hoc* test for multiple comparisons; $^{\#}$p<0.05, $^{\#\#\#}$p<0.001, $^{\#\#\#\#}$p<0.0001 vs vehicle-treated I-R on one-way ANOVA with Dunnett's *post-hoc* test for multiple comparisons. Data are expressed as percentage change from baseline, mean ± SEM.

**FIGURE 7:** Total duration of ventricular fibrillation observed in the first 10 min of reperfusion, in the presence of Angeli's salt (n=6) or its vehicle control (n=3), added to the perfusion buffer at the onset of reperfusion. *p<0.05 vs vehicle-treated I-R on Student unpaired *t*-test. Data are expressed as mean ± SEM.

**TABLE 1:** Baseline haemodynamic characteristics of all hearts at the end of equilibration, prior to any intervention (dose-response curve or cardioprotection procotols). Data are expressed as mean ± SEM. All P=NS between hearts allocated to normoxic perfusion vs I-R.

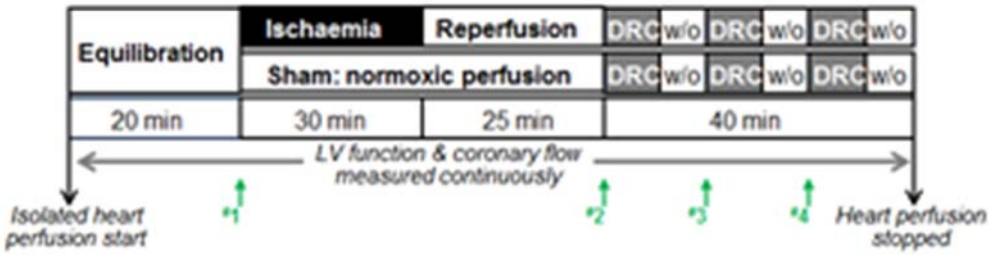
| **Parameter** | **Sham (n=11)** | **I-R (n=28)** |
|---|---|---|
| LVSP (mmHg) | 70 ± 4 | 64 ± 4 |
| LVEDP (mmHg) | 1.5 ± 2.3 | 0.5 ± 1.2 |
| LVDP (mmHg) | 68 ± 3 | 64 ± 4 |
| LV+dP/dt (mmHg/s) | 2359 ± 153 | 2453 ± 117 |
| LV-dP/dt (mmHg/s) | -1579 ± 146 | -1913 ± 91 |
| Heart rate (beats/min) | 257 ± 11 | 281 ± 8 |
| Coronary flow (ml/min) | 10.1 ± 0.3 | 10.7 ± 0.2 |

**TABLE 2:** Haemodynamic characteristics of rat isolated hearts allocated to the dose-response curve study protocol following I-R or normoxic perfusion, at the four timepoints indicated in Figure 1A, at the end of equilibration (#1), at the end of reperfusion, prior to U46619 infusion and construction of the first dose-response curve (#2), after washout following the first dose-response curve (#3) and after washout following the second dose-response curve (#4). Data are expressed as mean ± SEM. *P<0.05, **p<0.01, ***P<0.001 and ****P<0.0001 I-R vs sham, Student's unpaired *t*-test.

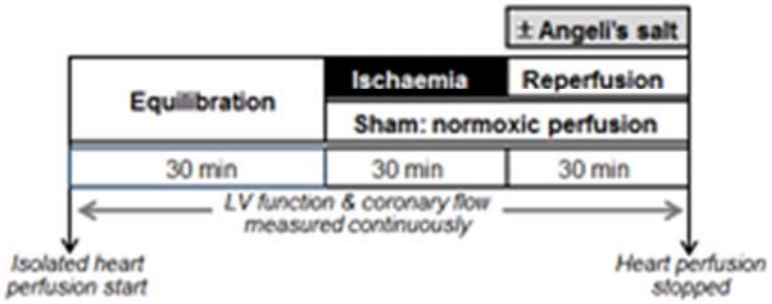
| **Parameter** | **End of Equilibration** (timepoint #1, prior to I-R) | | **End of Reperfusion** (timepoint #2) | | **End washout after 1st DRC** (timepoint #3) | | **End washout after 2nd DRC** (timepoint #4) | |
|---|---|---|---|---|---|---|---|---|
| | Sham (n=8) | I/R (n=7) | Sham (n=8) | I/R (n=7) | Sham (n=8) | I/R (n=7) | Sham (n=8) | I/R (n=7) |
| LVSP (mmHg) | 73 ± 5 | 86 ± 5 | 67 ± 10 | 119 ± 5 *** | 70 ± 8 | 123 ± 6 *** | 74 ± 11 | 118 ± 8 ** |
| LVEDP (mmHg) | 1.6 ± 3.1 | 5.9 ± 1.3 | 2.4 ± 5 | 77 ± 4 **** | 6.3 ± 2.4 | 76 ± 3.1 **** | 4.3 ± 2.3 | 75 ± 3.5 **** |
| LVDP (mmHg) | 71 ± 4 | 80 ± 6 | 68 ± 6 | 42 ± 8* | 66 ± 6 | 47 ± 7 | 71 ± 9 | 43 ± 9 * |
| LV+dP/dt (mmHg/s) | 2246 ± 126 | 2218 ± 158 | 2116 ± 113 | 971 ± 236*** | 2043 ± 132 | 1110 ± 217 ** | 2201 ± 249 | 1005 ± 254 ** |
| LV-dP/dt (mmHg/s) | -1391 ± 59 | -1661 ± 166 | -1258 ± 84 | -623 ± 129*** | -1362 ± 176 | -783 ± 109 * | -1566 ± 345 | -804 ± 190 |
| Heart rate (beats/min) | 253 ± 15 | 261 ± 7 | 229 ± 18 | 181 ± 30 | 240 ± 12 | 184 ± 15 * | 239 ± 14 | 195 ± 11 * |
| Coronary flow (ml/min) | 9.9 ± 0.3 | 10.1 ± 0.1 | 9.1 ± 0.7 | 1.7 ± 0.6**** | 8.3 ± 0.9 | 2.0 ± 0.5 **** | 7.9 ± 0.6 | 2.0 ± 0.5 **** |

**Figure 1**

**A** Dose-response curve study protocol




**B** Cardioprotection study protocol



## *Figure 2*

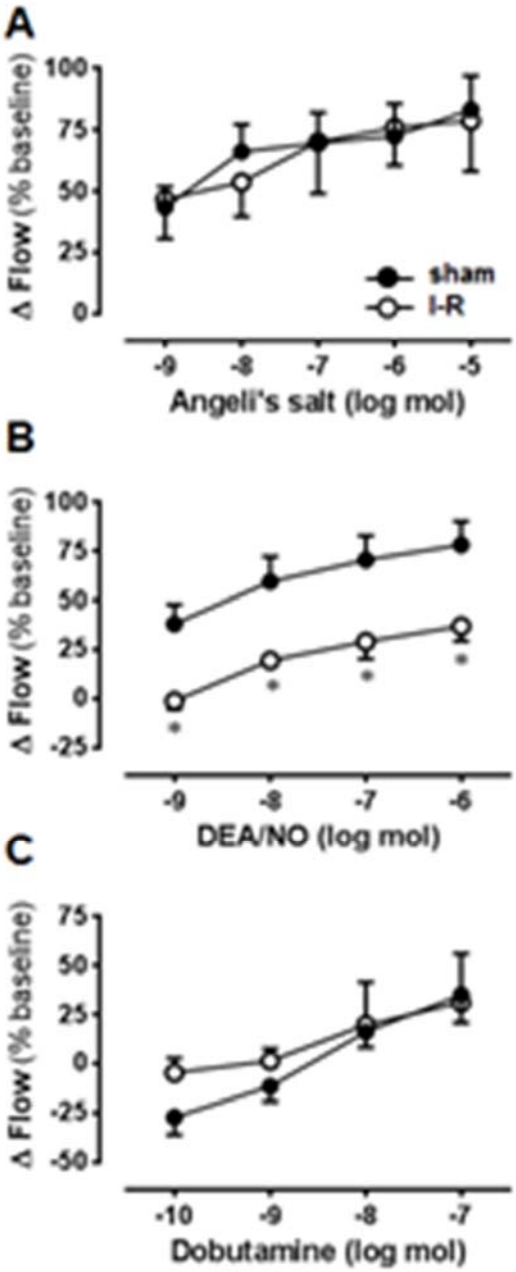

A
Δ Flow (% baseline)
100
75
50
25
0
sham
I-R
-9
-8
-7
-6
-5
Angeli's salt (log mol)
B
Δ Flow (% baseline)
100
75
50
25
0
-25
*
-9
-8
-7
-6
DEA/NO (log mol)
C
Δ Flow (% baseline)
75
50
25
0
-25
-50
-10
-9
-8
-7
Dobutamine (log mol)

**Figure 3**

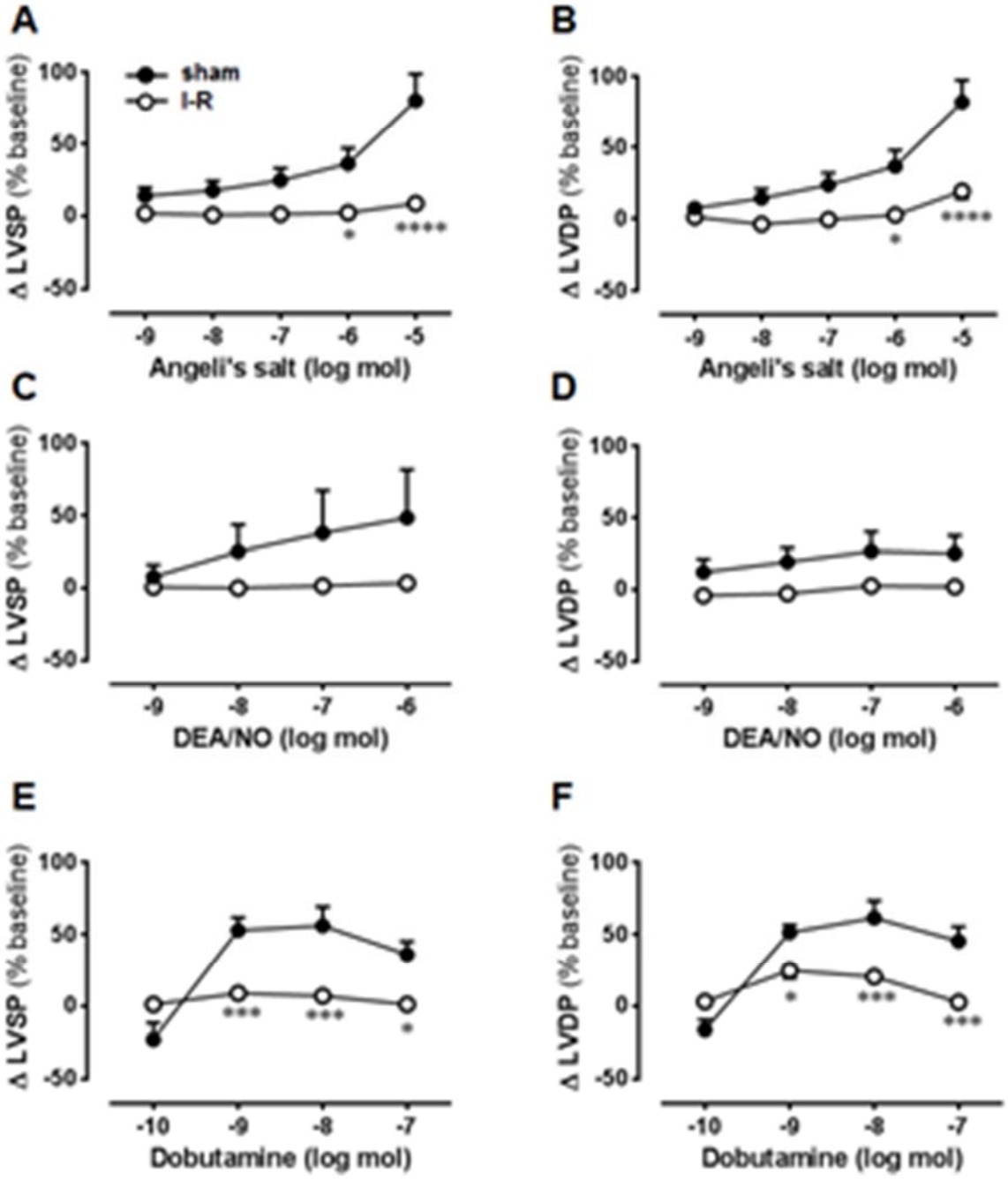

A
B
C
D
E
F
sham
I-R
Δ LVSP (% baseline)
Δ LVDP (% baseline)
100
50
0
-50
-9
-8
-7
-6
-5
-10
Angeli's salt (log mol)
DEA/NO (log mol)
Dobutamine (log mol)
*
***
****

**Figure 4**

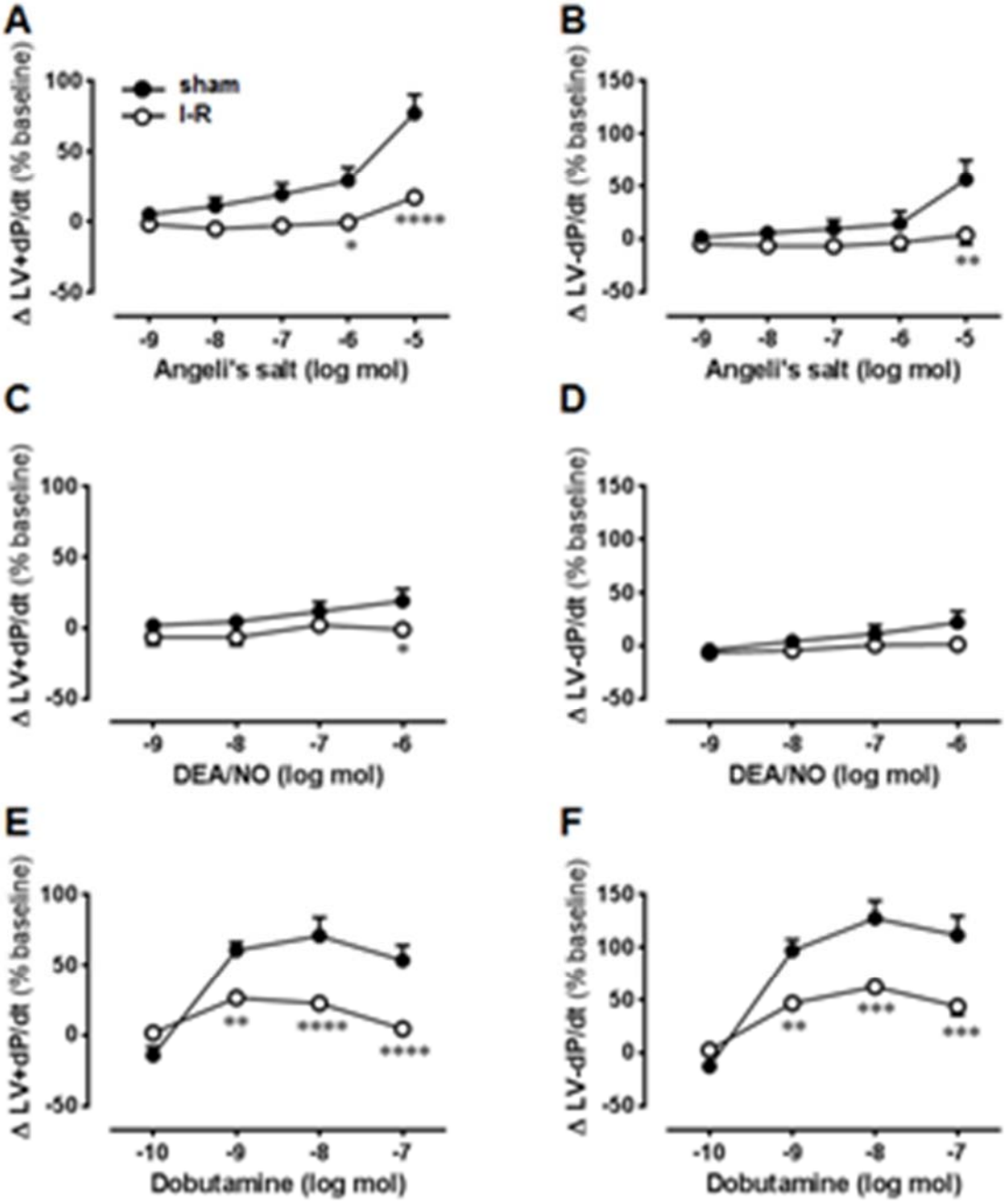

A
B
C
D
E
F
sham
I-R
Δ LV+dP/dt (% baseline)
Δ LV-dP/dt (% baseline)
Angeli's salt (log mol)
DEA/NO (log mol)
Dobutamine (log mol)

**Figure 5**

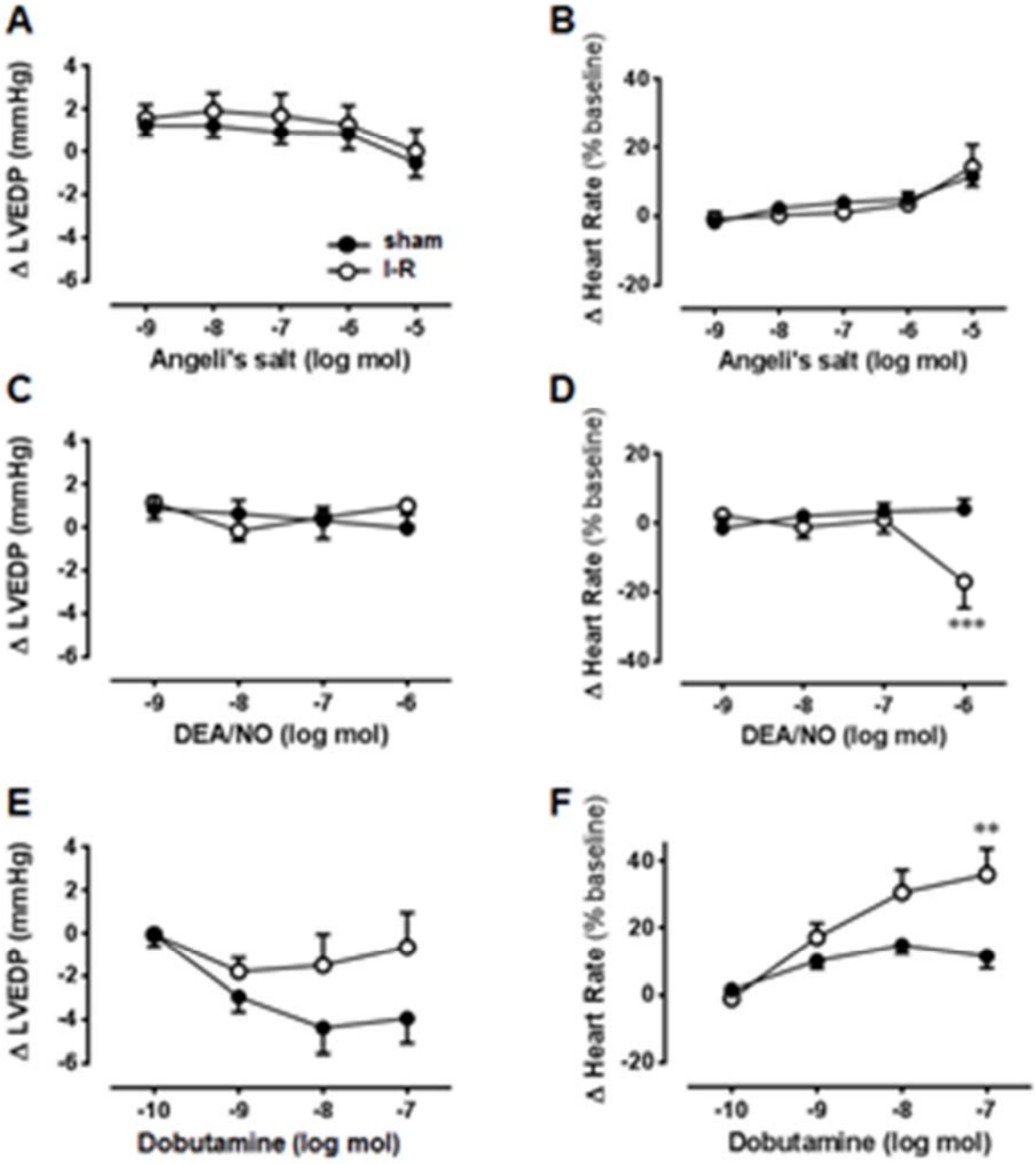
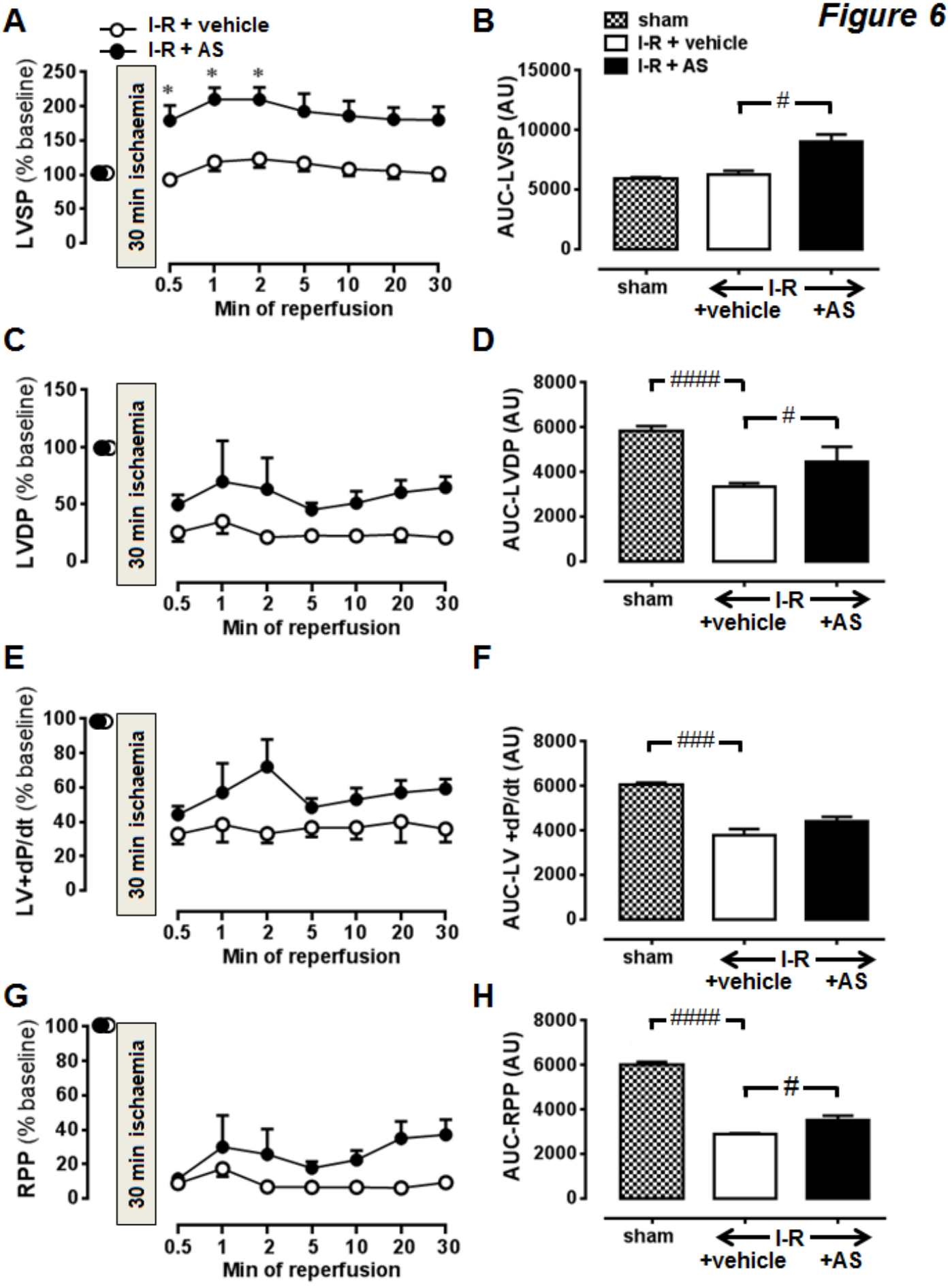
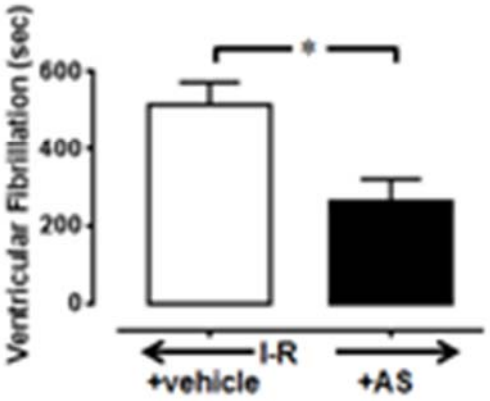

A
Δ LVEDP (mmHg)
4
2
0
-2
-4
-6
sham
I-R
-9
-8
-7
-6
-5
Angeli's salt (log mol)
B
Δ Heart Rate (% baseline)
40
20
0
-20
-9
-8
-7
-6
-5
Angeli's salt (log mol)
C
Δ LVEDP (mmHg)
4
2
0
-2
-4
-6
-9
-8
-7
-6
DEA/NO (log mol)
D
Δ Heart Rate (% baseline)
20
0
-20
-40
***
-9
-8
-7
-6
DEA/NO (log mol)
E
Δ LVEDP (mmHg)
4
2
0
-2
-4
-6
-10
-9
-8
-7
Dobutamine (log mol)
F
Δ Heart Rate (% baseline)
**
40
20
0
-20
-10
-9
-8
-7
Dobutamine (log mol)

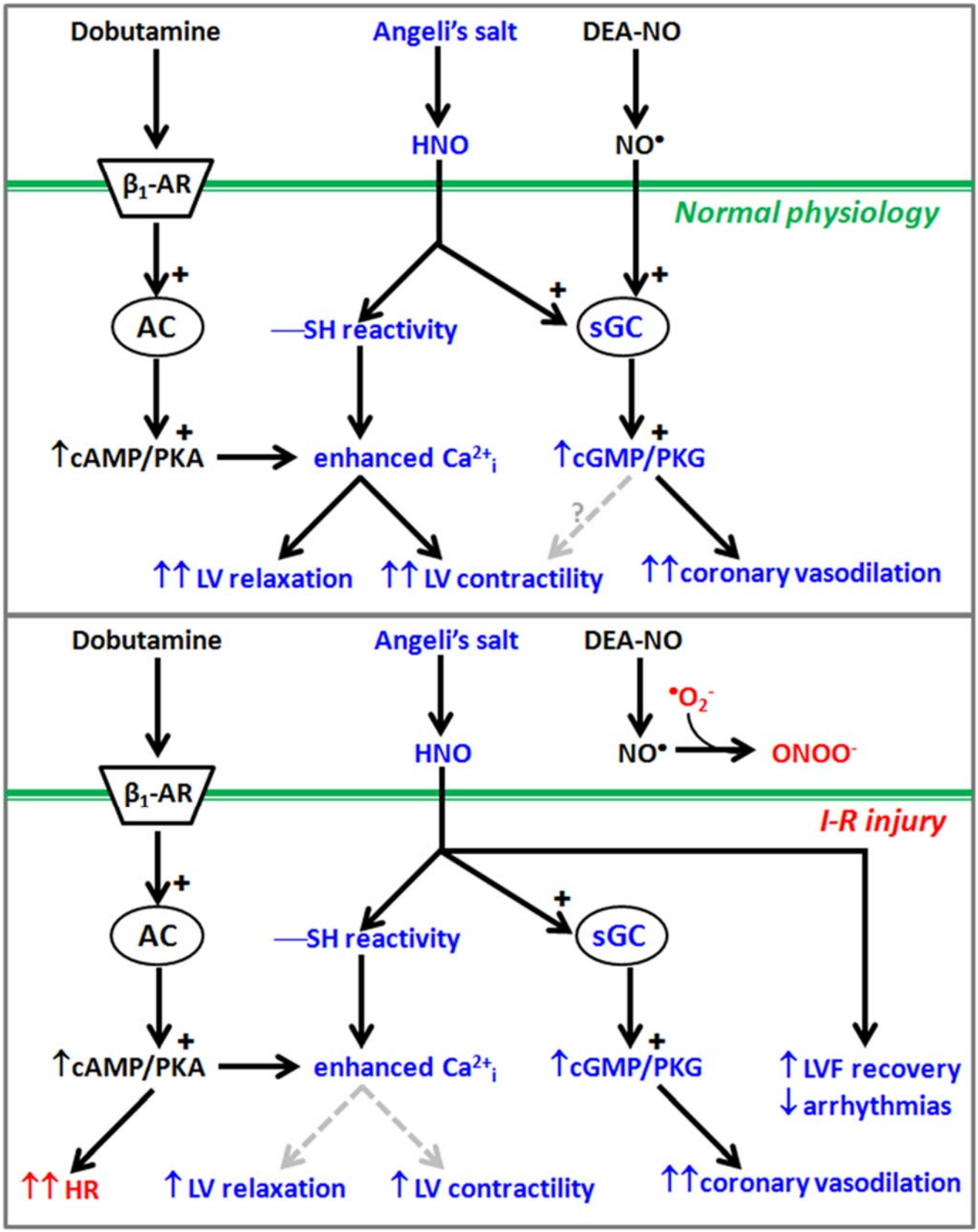
**Figure 6**


A
I-R + vehicle
I-R + AS
LVSP (% baseline)
30 min ischaemia
Min of reperfusion
B
sham
I-R + vehicle
I-R + AS
AUC-LVSP (AU)
sham
I-R
+vehicle
+AS
C
LVDP (% baseline)
30 min ischaemia
Min of reperfusion
D
AUC-LVDP (AU)
sham
I-R
+vehicle
+AS
E
LV+dP/dt (% baseline)
30 min ischaemia
Min of reperfusion
F
AUC-LV +dP/dt (AU)
sham
I-R
+vehicle
+AS
G
RPP (% baseline)
30 min ischaemia
Min of reperfusion
H
AUC-RPP (AU)
sham
I-R
+vehicle
+AS

## *Figure 7*



**GRAPHICAL ABSTRACT:** In contrast to the $\beta_1$-adreneoceptor ($\beta_1$-AR) agonist dobutamine and the NO• donor diethylamine NONOate (DEA/NO), the vasodilation properties of the nitroxyl (HNO) donor Angeli's salt were preserved after ischaemia-reperfusion (I-R) injury. Although I-R injury blunted the inotropic effects of both Angeli's salt and dobutamine, the HNO donor did not increase heart rate (HR). Improved recovery of left ventricular function (LVF) and reduced incidence of arrhythmias was also observed. HNO donors may thus offer haemodynamic advantages over existing pharmacotherapy in acute heart failure. AC, adenylyl cyclase; $\bullet O_2^-$, superoxide; $ONOO^-$, peroxynitrite; PKA, protein kinase A, PKG, protein kinase G; sGC, soluble guanylyl cyclase, SH, thiol. Blue and red text represent beneficial and detrimental effects, respectively. Grey arrows indicate more modest effects than black arrows.



# The HNO donor Angeli's salt offers potential haemodynamic advantages over NO• or dobutamine in ischaemia-reperfusion injury in the rat heart *ex vivo*

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## DATA SUPPLEMENT

## SUPPLEMENTARY MATERIALS AND METHODS

### *Isolation and cannulation of the rat heart for Langendorrf perfusion*

Following the induction of anaesthesia, a lack of responsiveness with respect to pedal or blink reflexes indicates anaesthesia has been achieved. Working promptly, the chest is opened and the heart removed and plunged into ice-cold Kreb's buffer, to arrest beating. Whilst continuing to work on ice, any extraneous tissues are removed (e.g. thymus, lungs). Using fine forceps, the aorta is cannulated onto the aortic cannula, using a 4.0 suture tied with a tight double knot. The left atrial appendage is removed, and a drain inserted. The balloon is then inserted into the LV, and coronary flow is commenced, at 10 ml/min. The LV balloon is slowly inflated using a water-filled syringe, to attain an LV End Diastolic Pressure (EDP) of ~5mmHg. LV Systolic Pressure (LVSP) should be >120mmHg. Using the STH Pump Controller of the ADInstruments Langendorff System® (ADInstruments Pty, Ltd., Bella Vista, Australia) to maintain constant perfusion pressure, coronary flow is then continuously detected. The ADInstruments PowerLab data acquisition system is used to acquire LVSP, LVEDP, LV developed pressure (LVDP) and the first derivatives of LV pressure (LV ± dP/dt), as well as coronary perfusion pressure and coronary flow[1].

### *Impact of NO• on I-R injury*

Hearts isolated from anaesthetised male Sprague-Dawley rats were Langendorff-perfused and assigned to either sham normoxic perfusion for a further 60 min after equilibration, or to I-R injury (30 min global ischaemia with 30 min reperfusion, as illustrated in Supplementary Figure 1). The source and catalogue number of all reagents is detailed in Supplementary Table 1. The NO• donor sodium nitroferricyanide (sodium nitroprusside, SNP) was dissolved in Kreb's buffer. The Kreb's perfusion buffer during reperfusion was randomly allocated to supplementation with either SNP (1 μM,

commencing 10 min prior to ischaemia, to mimic the treatment time observed to limit simulated I-R injury in isolated cardiomyocytes *in vitro*)[2], or its Kreb's vehicle control. The impact of SNP on recovery of LV function post I-R injury was assessed, expressing each of LVSP, LVDP, LV+dP/dt and RPP as the percentage change from pre-ischaemic values. The AUC for the time-course of post-ischaemic recovery of these parameters of LV function in the presence and absence of SNP during reperfusion was calculated using Graphpad Prism®. Total duration of ventricular fibrillation during the first 10 min of reperfusion was calculated. All results were expressed as mean ± SEM, with the incidence of arrhythmias in normoxic versus I-R hearts compared using Student's unpaired *t*-test. The time-course of post-ischaemic recovery of LV function in the presence and absence SNP during reperfusion was also compared using two-way ANOVA with Sidak *post hoc* analysis for multiple comparisons. AUC for recovery of LV function during reperfusion was compared using one-way ANOVA with Dunnett's *post hoc* analysis for multiple comparisons. $P<0.05$ was considered statistically significant.

## SUPPLEMENTARY RESULTS

### *Representative traces of haemodynamic responses after I-R in rat isolated hearts*

Supplementary Figure 2 shows the time course of coronary flow in representative rat isolated hearts subjected to dose-response curves constructed after normoxic sham perfusion or I-R injury. The three dose-response curves were performed in randomised order, following U46619 preconstriction, with a washout period between each curve. The parallel changes in contractile parameters LVDP and LV+dP/dt are shown in Supplementary Figure 3. Both figures also serve to illustrate the duration of each dose-response curve, and the haemodynamic stability of the hearts over the full timecourse of the study.

### *Impact of NO• on I-R injury*

Continuous infusion with the NO• donor SNP (n=5), for the full duration of reperfusion, failed to offer any significant benefit compared to its Kreb's buffer vehicle, particularly on LVDP, LV+dP/dt and RPP, as shown in Supplementary Figure 4. This was in direct contrast to the cardioprotective effects seen with the HNO donor, Angeli's salt. Furthermore, the duration of ventricular fibrillation in the first 10 min of reperfusion with SNP was comparable to its Kreb's buffer vehicle (Supplementary Figure 5).

## REFERENCES


44. **Chin KY, Qin C, Cao N, Kemp-Harper BK, Woodman OL, Ritchie RH**. The concomitant coronary vasodilator and positive inotropic actions of the nitroxyl donor Angeli's salt in the intact rat heart: contribution of soluble guanylyl cyclase-dependent and -independent mechanisms. *Br J Pharmacol* 171: 1722-34. 2014
45. **Garreffa AM, Woodman OL, Cao AH, Ritchie RH**. Sodium nitroprusside protects adult rat cardiac myocytes from cellular injury induced by simulated ischemia: role for a non-cGMP-dependent mechanism of nitric oxide protection. *J Cardiovasc Pharmacol* 47: 1-8. 2006

**SUPPLEMENTARY TABLE 1:**

| Reagent | Source | Catalogue # | PubChem CID |
|---|---|---|---|
| Sodium chloride (NaCl) | Sigma-Aldrich (St. Louis, USA) | S7653 | 5234 |
| Potassium phosphate monobasic ($KH_2PO_4$) | Sigma-Aldrich (St. Louis, USA) | P9791 | 516951 |
| Sodium bicarbonate ($NaHCO_3$) | Sigma-Aldrich (St. Louis, USA) | S5761 | 516892 |
| Ethylenediaminetetraacetic acid (EDTA) | Sigma-Aldrich (St. Louis, USA) | E6511 | 9902403 |
| D-glucose | Sigma-Aldrich (St. Louis, USA) | G8270 | 5793 |
| L-LDH | Sigma-Aldrich (St. Louis, USA) | 10107085001 | N/A |
| Sodium pyruvate | Sigma-Aldrich (St. Louis, USA) | P8574 | 23662274; |
| Sodium phosphate monobasic monohydrate ($NaH_2PO_4•H_2O$) | Sigma-Aldrich (St. Louis, USA) | S9638 | 516949 |
| Nicotinamide adenine dinucleotide (NADH) | Sigma-Aldrich (St. Louis, USA) | N6005 | 44134852 |
| Dobutamine | Sigma-Aldrich (St. Louis, USA) | D0676 | 36811 |
| Sodium nitroferricyanide (III) dihydate (sodium nitroprusside dehydrate, SNP) | Sigma-Aldrich (St. Louis, USA) | 228710 | 11953895 |
| Sodium trioxodinitrate (Angeli's salt) | Cayman Chemical Company (Ann Arbor, USA) | 82230 | 10129945 |
| Diethylamine NONOate (DEA/NO) | Cayman Chemical Company (Ann Arbor, USA) | 82100 | 9571404 |
| U46619 | Cayman Chemical Company (Ann Arbor, USA) | 16450 | 6610266 |
| Sodium hydroxide (NaOH) | Merck (Darmstadt, Germany) | 106462 | 14798 |
| Potassium chloride (KCl) | Merck (Darmstadt, Germany) | 529552 | 4873 |
| Magnesium sulphate hepta hydrate ($MgSO_4•7H_2O$) | Merck (Darmstadt, Germany) | 105886 | 24843 |
| Calcium chloride ($CaCl_2$) | Merck (Darmstadt, Germany) | 102382 | 5284359 |

**SUPPLEMENTARY FIGURE LEGENDS:**

**SUPPLEMENTARY FIGURE 1:** Schematic diagram of the experimental protocol to examine potential NO• cardioprotection. Hearts isolated from anaesthetised rats were Langendorff-perfused under constant pressure and were subjected to 30 min ischaemia followed by reperfusion as indicated. SNP (1 μM, commencing 10 min prior to ischaemia) or its Kreb's buffer vehicle were added to the perfusion buffer.

**SUPPLEMENTARY FIGURE 2:** Time course of coronary flow in representative rat isolated hearts subjected to dose-response curves constructed after normoxic sham perfusion or I-R injury. The three dose-response curves were performed in randomised order, following U46619 preconstriction, with a washout period between each curve. The x-axis shows time in minutes or perfusion duration.

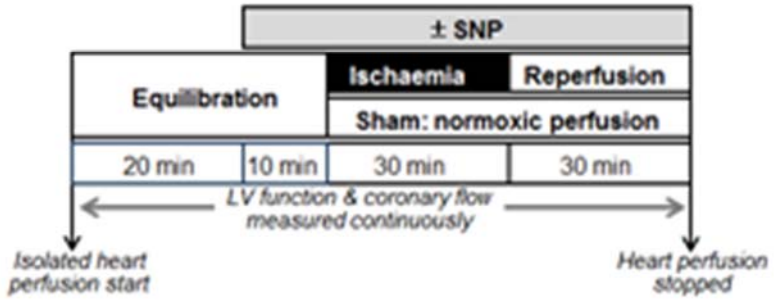
**SUPPLEMENTARY FIGURE 3:** Time course of LVDP and LV+dP/dt in representative rat isolated hearts subjected to dose-response curves constructed after normoxic sham perfusion or I-R injury. The three dose-response curves were performed in randomised order, following U46619 preconstriction, with a washout period between each curve. The x-axis shows time in minutes or perfusion duration.

**SUPPLEMENTARY FIGURE 4:** SNP (n=5), added to the perfusion buffer 10 min prior to I-R and continued for the duration of reperfusion, does not impact the impairment in post-ischaemic recovery of LV function compared to Kreb's buffer vehicle (n=7). **A:** Time-course of recovery of LVSP; **B:** AUC analysis of the time-course of LVSP recovery; **C:** Time-course of recovery of LVDP, **D:** AUC analysis of the time-course of LVDP recovery; **E:** Time-course of recovery of LV+dP/dt, **F:** AUC analysis of the time-course of LV+dP/dt recovery, and **G:** Time-course of recovery of RPP, **H:** AUC analysis of the time-course of RPP recovery. $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, $^{\#\#\#\#}p<0.001$ vs vehicle-treated I-R on one-way ANOVA with Dunnett's *post-hoc* test for multiple comparisons. Data are expressed as percentage change from baseline, mean ± SEM.

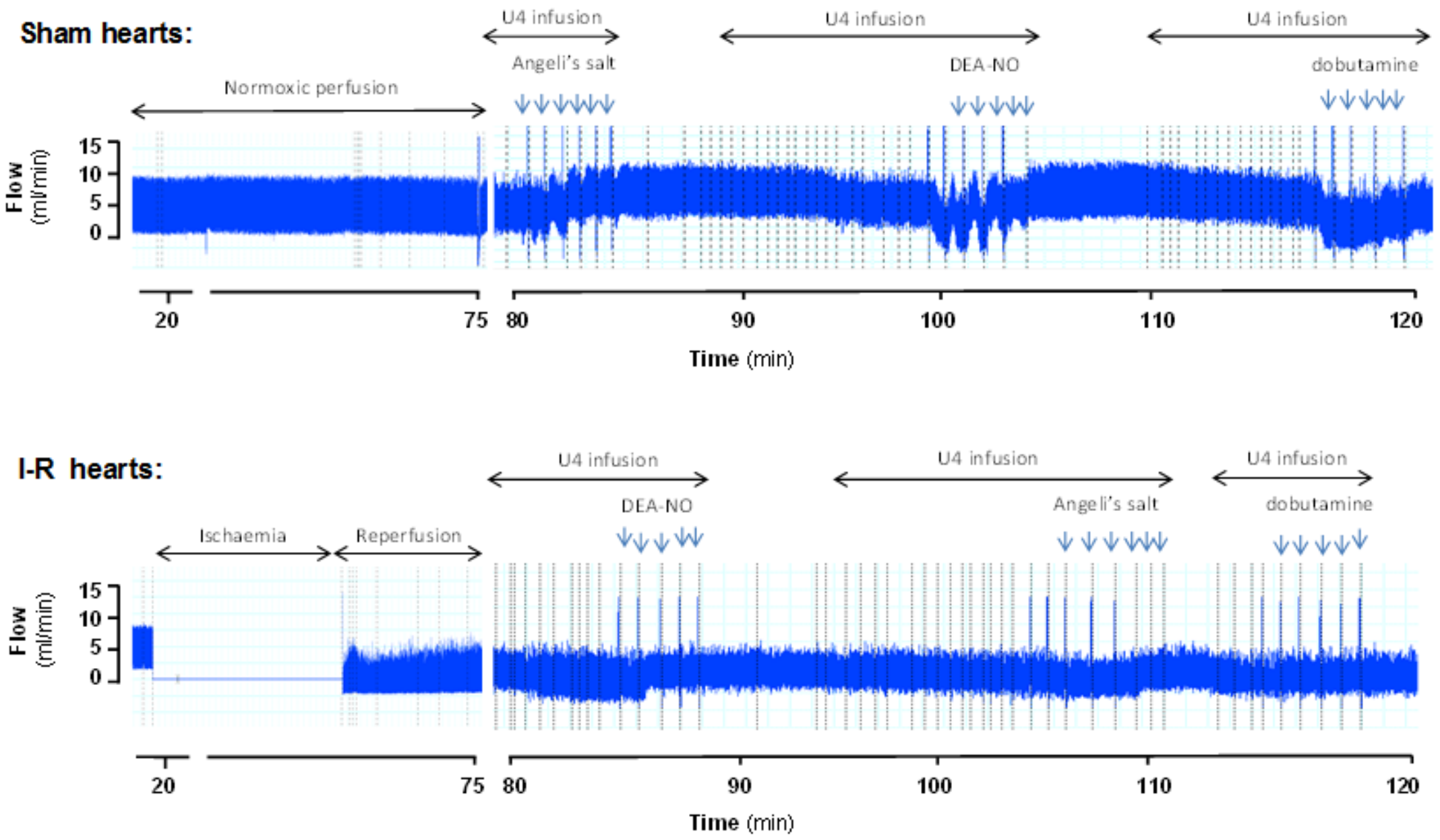
**SUPPLEMENTARY FIGURE 5:** Total duration of ventricular fibrillation observed in the first 10 min of reperfusion, in the presence of SNP (n=5) or its vehicle control (Kreb's buffer, n=7), added to the perfusion buffer 10 min prior to I-R and continued for the duration of reperfusion (P=NS, Student's unpaired *t*-test). Data are expressed as mean ± SEM.

***Suppl Figure 1***

**SNP cardioprotection study protocol**

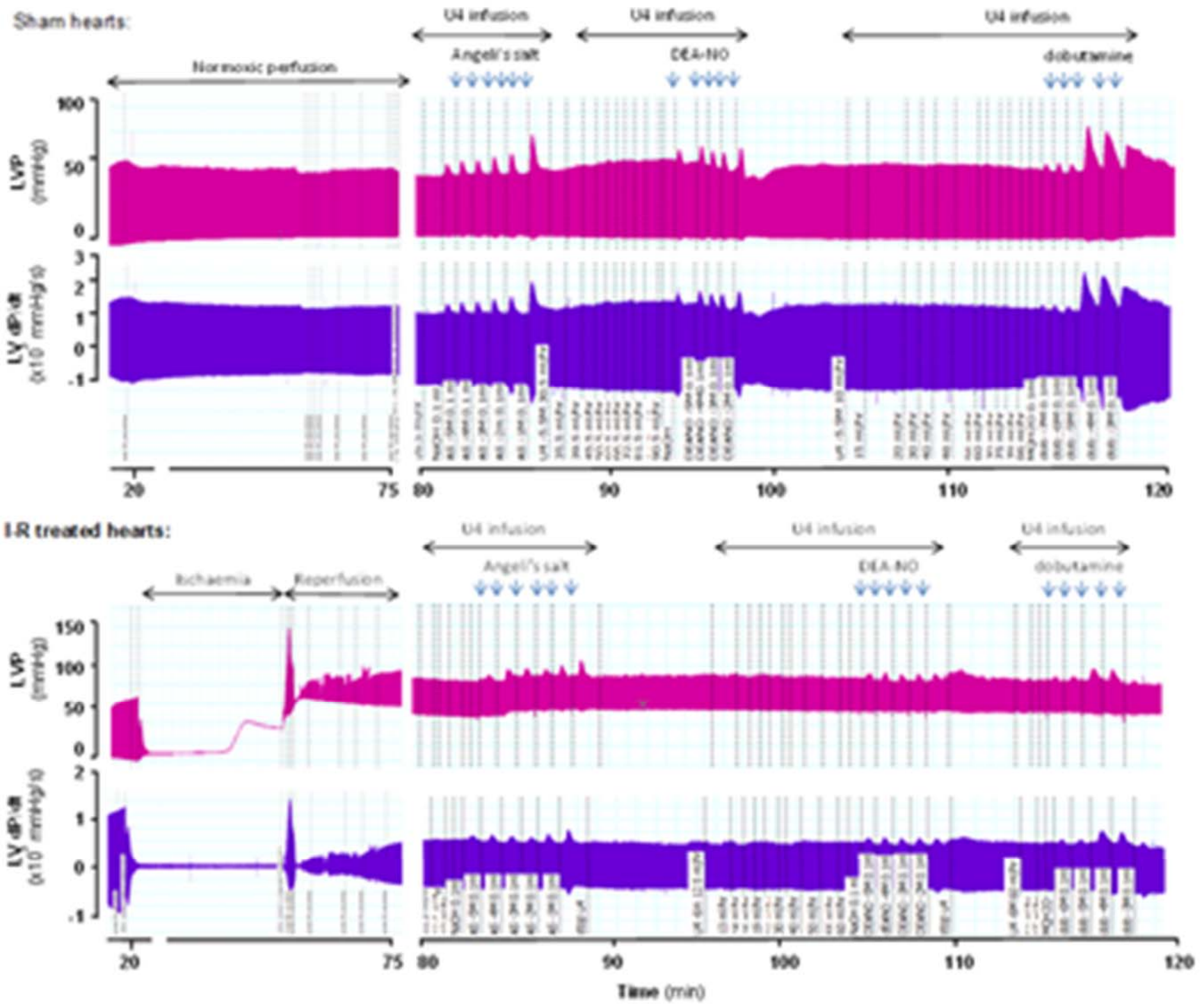


# *Suppl Figure 2*

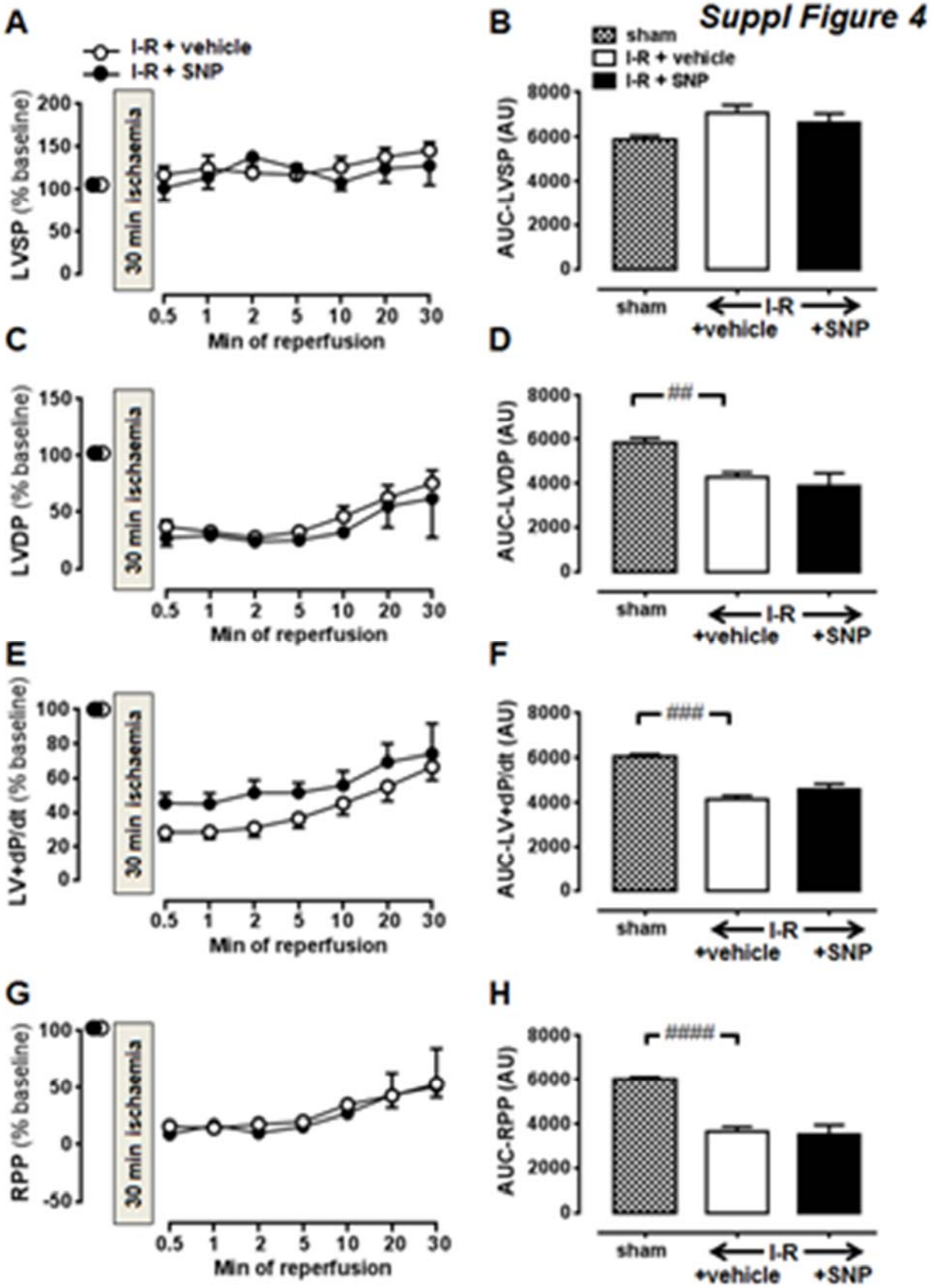


**Suppl Figure 3**

## Cardiac Contractile Function



**Suppl Figure 4**



## *Suppl Figure 5*