

Effects of acute pre-treatment with ethanolamine on isoprenaline-induced myocardial infarction in adult Wistar rats

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INTRODUCTION

Ethanolamine (Etn) is a biogenic amine that plays an important role in mediating cellular growth and chemical signalling processes.^(1,2) In animals, Etn is found both as a component of membrane phospholipids and as a freely circulating organic compound in the blood. Consistent with its metabolic functions, the plasma levels of Etn vary with age and requirement for growth, with infants having much higher plasma concentrations of Etn than adults.⁽³⁾ Etn also exists naturally in many types of food, including those known to modulate cardiovascular tissue survival such as wine,^(4,5) grapes⁽⁶⁾ and chocolate.⁽⁷⁾

Physiologically, Etn has been implicated in regulation of cellular activity in cardiovascular and other tissues. In isolated fibroblasts,⁽⁸⁾ and isolated hepatocytes,⁽⁹⁾ Etn has co-mitogenic effects. It has also been shown to increase hepatocyte proliferation in regenerating livers in vivo.⁽¹⁰⁾ In the cardiovascular system, chronic Etn treatment in rats is associated with attenuation of hypercholesterolemia.⁽¹¹⁾ Furthermore, both chronic⁽¹²⁾ and acute⁽¹³⁾ pre-treatment with Etn protect the isolated rat heart from ischaemia-reperfusion injury. Despite these cardioprotective effects in isolated hearts, the effects of Etn on in vivo myocardial infarction (MI) are currently unknown.

Acute MI induced by the catecholamine isoprenaline (ISO), a synthetic analogue of epinephrine, results in infarct-like lesions similar to those found during human MI.^(14,15) High-dose ISO induces MI-associated pathology in this model after

ABSTRACT

Ethanolamine (Etn), a biogenic amine, protects isolated hearts against MI, but its effects in vivo are unknown. In this study, we investigated the effects of Etn pre-treatment on isoprenaline (ISO)-induced MI in vivo. Wistar rats were treated with either Etn (10mg.kg⁻¹, i.p.) or saline, prior to either ISO (67mg.kg⁻¹, s.c.) or saline. Haemodynamic- and electrocardiographic parameters were recorded under anaesthesia, 24 hours post-treatment with ISO. Infarct sizes were determined by triphenyltetrazolium chloride staining. Oxidative stress plasma parameters, conjugated dienes (CD) and thio-barbituric acid reactive substances (TBARS), were measured by spectrophotometric analysis. Etn decreased ISO-induced pathological Q-waves, but had no effect on ISO-induced low-voltage electrocardiogram. In addition, Etn decreased overall mortality induced by ISO treatment. However, Etn did not prevent ISO-induced infarction or the systolic- and diastolic hypotensive effects. Etn also enhanced the ISO-induced increase in heart-to-body weight ratio, and reversed the decrease in lungs-to-body weight ratio. Neither ISO nor Etn altered CD or TBARS. These results suggest that, despite a tendency to modulate cardiac electrical activity, Etn did not prevent ISO-induced MI or myocardial dysfunction in vivo. SAHeart 2016;13:112-118

24 hours.^(16,17) The current study investigated whether Etn can protect the heart in an in vivo model of ischaemic injury.

METHODS

Animals

Adult male Wistar rats (250 - 300g) were obtained from the University of Cape Town Animal Unit and allowed to acclimatise for 2 days under standard laboratory conditions (12 hour light/dark cycle 06h00 - 18h00, 300 lux, 22 ± 1°C) prior to experiments. Rats were fed standard rat chow (Afresh Vention 1, RSA) and had ad libitum access to food and water. Experiments were approved by the Faculty of Health Sciences Animal Ethics Committee at the University of Cape Town and performed in compliance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No.85 (23), revised 1996).

Experimental Design

Rats (n=53) were divided into 2 groups: Control and ISO. The ISO group were treated with a single dose of ISO

(67mg.kg⁻¹, s.c.) to induce MI.⁽¹⁷⁾ The Control group received equivalent volumes of 0.9% saline (3.3ml.kg⁻¹, s.c.). These 2 groups were further subdivided into Etn and vehicle treated groups (Figure 1) as follows: Control + Vehicle, Control + Etn, ISO + Vehicle, and ISO + Etn. Etn-treated rats were injected with Etn (10mg.kg⁻¹, i.p.) 2 hours prior to induction of MI. Vehicle-treated groups received equivalent volumes of saline 2 hours prior to induction of MI. A dose-response was established to determine the optimal dose and timing of Etn administration and a therapeutic dose of 10mg.kg⁻¹ of Etn was selected based on the ability to modulate MI-induced ECG disruptions.

In vivo electrophysiological, haemodynamic and gross structural measurements

Of the 53 rats used in this study, 6 rats died within 24 hours of ISO treatment, without Etn pre-treatment and 1 died with Etn pre-treatment prior to induction of MI by ISO. The 7 rats that died were excluded from further analyses. The surviving rats (n=46) were anaesthetised with sodium pentobarbitone (60mg.kg⁻¹, i.p.) 24 hours after induction of MI. Rats were intubated and ventilated at a rate of 70 strokes.min⁻¹ (2.5ml room air.stroke⁻¹) on a rodent ventilator (Model 681, Harvard

Apparatus, USA). Body temperature, monitored using a rectal probe, was maintained at 37°C using a heating pad. The depth of anaesthesia was assessed using the pedal withdrawal reflex and, when required, a top-up dose of pentobarbitone (12mg.kg⁻¹, i.p.) was given. Heart rate was monitored from lead II of a 3-lead surface electrocardiogram (ECG) connected to the PowerLab system via an Animal Bio Amplifier (ML136, ADInstruments, Aus). Arterial blood pressure was measured in the right carotid artery using a heparinised, custom-made cannula attached to a pressure transducer (MLT0670, Lasec, RSA). Blood pressure readings were recorded by the PowerLab system via a Bridge Amplifier (ML221, ADInstruments, Aus). All electrophysiological and haemodynamic data recorded online by the PowerLab system were digitally acquired and analysed using the LabChart Pro 7 software (ADInstruments, Australia). After the recordings, a thoracotomy was performed and the heart was rapidly excised, flushed with cold saline (4°C) via the aorta, weighed, and stored at -20°C for staining.

Quantification of infarct size

Frozen hearts were cut transversely into 2mm slices from apex to base of the ventricles using a custom made Perspex mould and a stainless steel blade. The uppermost slice, consisting of the atria, was discarded. The slices were incubated in 1% sodium phosphate buffered triphenyltetrazolium chloride (TTC) solution (pH7.4) for 20min at 37°C and subsequently stored in 10% formalin solution at room temperature. After 24 hours, slices were placed on glass slides and digitally scanned. Infarct size was measured as the region of interest using ImageJ software (Version 1.44p, National Institute of Health, USA). Infarct size was quantified as the percentage of TTC-negative area to total ventricular area.

Quantification of organ weight to body weight ratio

The heart, lungs, kidneys, liver and adrenal glands were removed at the time of thoracotomy. Each organ was blotted dry of any external blood and weighed. The organ weight was expressed as a percentage of the starting body weight (i.e. the weight before any drug treatments) for each rat.

Lipid peroxidation assays

The concentration of conjugated dienes (CD) was determined using the assay described by Esterbauer, et al.⁽¹⁸⁾ Briefly, 100µL of plasma was added to 405µL chloroform:methanol (2:1). After centrifugation at 6 000g for 15min, the top aqueous layer was removed and the organic layer was isolated and dried under nitrogen. Cyclohexane (0.25ml) was added to solubilise the dry organic residue and the absorbance was read at 234nm on a spectrophotometer (Multiscan MS analyser, AEC Amersham, RSA). A molar extinction coefficient of 2.95x04/M/cm was used.

Thiobarbituric acid reactive substances (TBARS) were measured using the method described by Jentsch, et al.⁽¹⁹⁾ Briefly, to 50µL of plasma samples was added 6.25µL of 4mmol/L

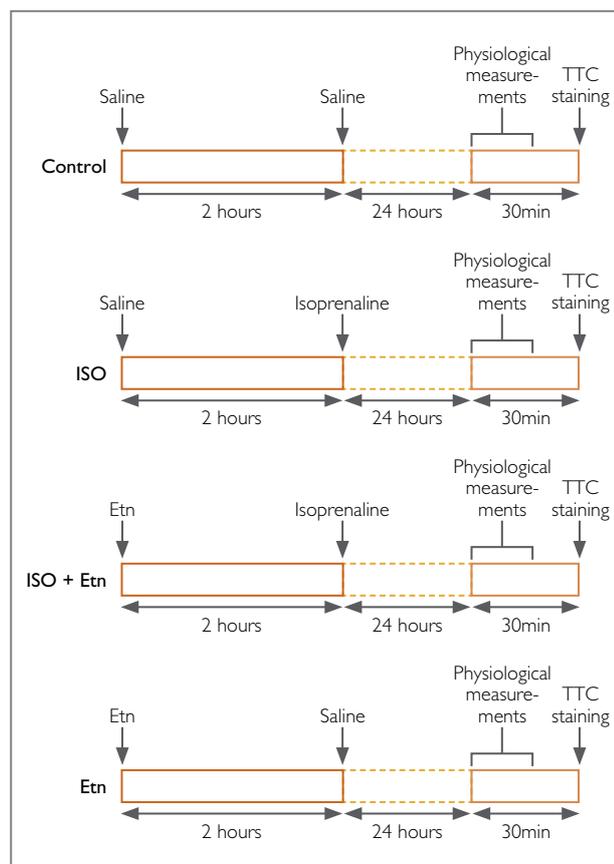


FIGURE 1: Timeline of treatments and measurements. Physiological measurements included ECG and arterial blood pressure.

Etn: Ethanolamine, MI: Myocardial infarction.

butylated hydroxytoluene/ethanol and 50 μ L of 0.2M ortho-phosphoric acid. The samples were then vortexed. TBA reagent (6.25 μ L), at 0.1 mol/L in 0.1 mol/L NaOH, was added and the mixture was microfuged at 3 000g for 2min to collect small volumes at the bottom of the Eppendorfs. The volumes were heated at 90°C for 45min, then placed on ice for 2min and then at room temperature for 5min before n-butanol (500 μ L) was added. Phase separation was enhanced by the addition of 50 μ L of saturated NaCl. The samples were vortexed and centrifuged at 12 000g for 2 min and 300 μ L of the top butanol phase was transferred into wells and read at A532nm on a spectrophotometer (Multiscan MS analyser, AEC Amersham, RSA). A molar extinction coefficient of 1.54 \times 10⁵M/cm was used. CD and TBARS measurements were performed in triplicate and the mean value was taken as the final result.

Drugs and chemicals

Sodium pentobarbitone was purchased from Kyron Laboratories (Johannesburg, RSA). Other drugs and chemicals were obtained from Sigma-Aldrich (Johannesburg, RSA).

Statistical analysis

All data are expressed as mean \pm standard error of the mean (SEM). Column statistics were conducted to check if data passed normal distribution with the D'Agostino and Pearson normality test. For normally distributed data, comparisons between groups were performed using a one-way ANOVA (Prism 5, Graphpad, USA) followed by a Tukey post-hoc test to compare group means. For data that was not normally distributed, a Kruskal-Wallis test was used for comparisons between groups, followed by a Dunns post-hoc test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Reduction of isoprenaline-induced mortality by acute pre-treatment with ethanolamine

Six of the 20 rats that were injected with ISO following a saline injection died, whereas only one of the 14 rats that were injected with ISO following Etn pre-treatment died. Therefore, mortality was reduced by 22.9% when rats, subjected to ISO-induced MI, were pre-treated with an acute dose of Etn.

Haemodynamic parameters are not improved by acute pre-treatment with ethanolamine

The administration of ISO caused arterial hypotension (Figure 2) as evidenced by a significant decrease in the systolic (Control: 147.7 \pm 4.9mmHg, ISO: 116.8 \pm 3.3mmHg; $p < 0.001$) and diastolic (Control: 119.5 \pm 3.5mmHg, ISO: 93.9 \pm 4.1mmHg; $p < 0.01$) arterial blood pressure, compared to Control rats. Arterial hypotension of a similar magnitude was seen in rats treated with ISO + Etn (systolic: 113.2 \pm 4.6mmHg; diastolic: 87.6 \pm 4.9mmHg; $p < 0.001$ vs. Control).

Ethanolamine improves some, but not all, electrophysiological disturbances

The average lead II ECG tracings in the different treatment groups can be seen in Figure 3 and Table I summarises the quantification of the ECG data for each treatment. The heart rate, PR-interval, QRS-interval, QT-interval and P-duration were unaffected by ISO or Etn treatment. When the QT-interval was corrected for heart rate (QTc) using the Bazett's formula, it was found that treatment with ISO + Etn resulted in a decreased QTc compared to control rats ($p < 0.05$ vs. Control), although this change was not observed in rats treated with ISO alone. There was no significant difference in QTc between ISO and ISO + Etn treated rats.

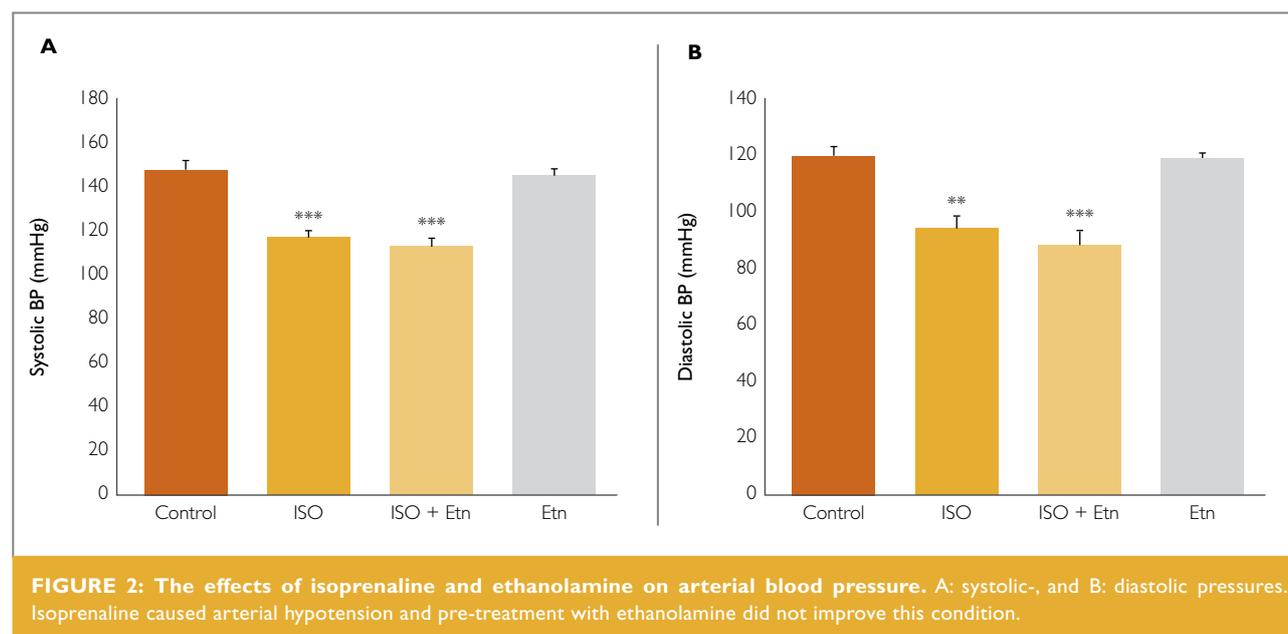


FIGURE 2: The effects of isoprenaline and ethanolamine on arterial blood pressure. A: systolic-, and B: diastolic pressures. Isoprenaline caused arterial hypotension and pre-treatment with ethanolamine did not improve this condition.

$n \geq 6$ ** $p < 0.01$, *** $p < 0.001$ (Treatment vs. Control).

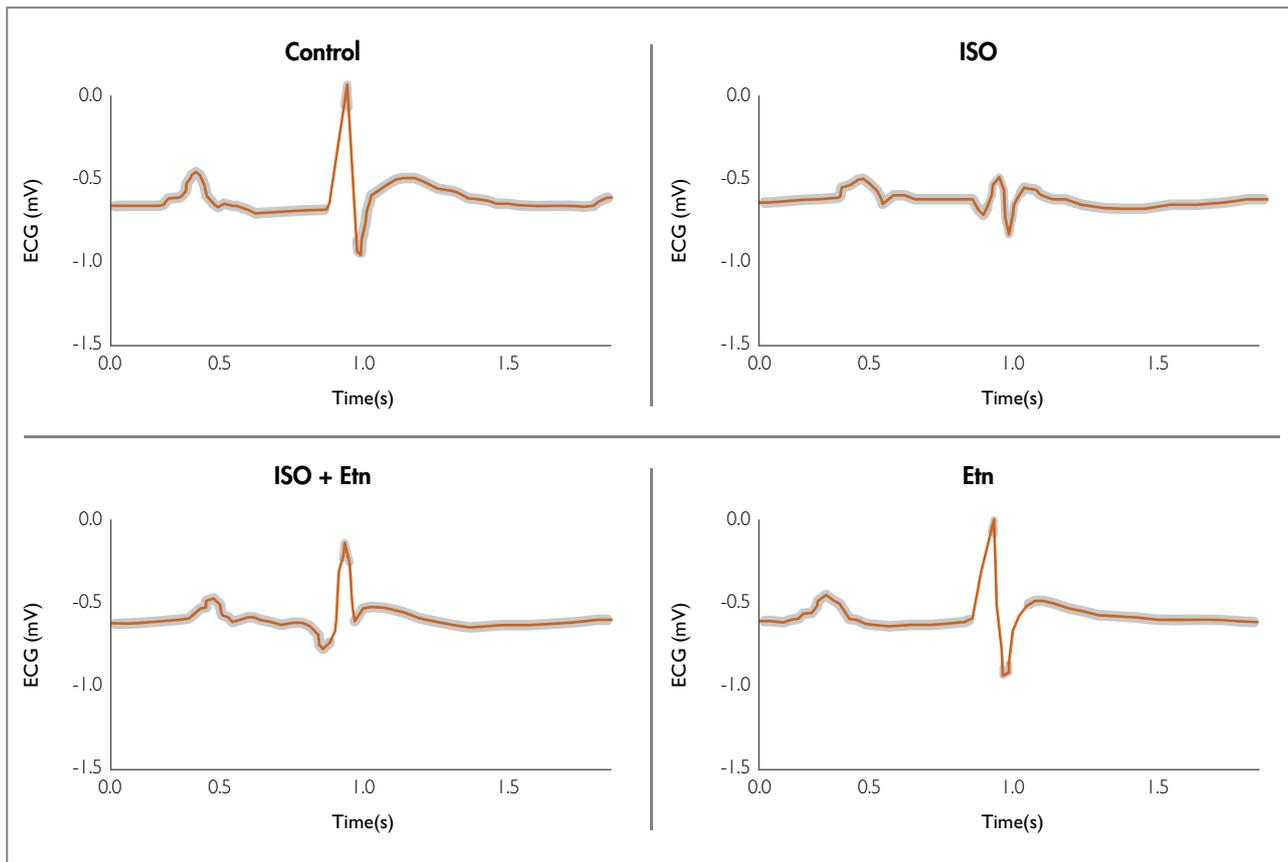


FIGURE 3: The effects of isoprenaline and ethanolamine on electrical activity. Superimposed original lead II ECG tracings from all 4 treatment groups.

Orange lines are the average tracing for Control: n=12 rats, ISO: n=14 rats, ISO + Etn: n=13 rats and Etn: n=7 rats. Grey lines indicate traces from individual rats.

TABLE 1: Summary of ECG characteristics for the effects of ethanolamine on myocardial infarction.

	Control	ISO	ISO + Etn	Etn
Heart Rate (beats.min ⁻¹)	403.7 ± 12.4	405.8 ± 10.9	398.5 ± 7.7	394.1 ± 10.6
QT-Interval (s)	0.055 ± 0.001	0.049 ± 0.005	0.044 ± 0.005	0.056 ± 0.001
QTc (s)	0.146 ± 0.002	0.128 ± 0.014	0.100 ± 0.010*	0.143 ± 0.004
T _{peak} -T _{end} (s)	0.030 ± 0.0	0.022 ± 0.002*	0.016 ± 0.002***	0.030 ± 0.0
Q-Amplitudex (mV)	-0.032 ± 0.008	-0.130 ± 0.027**	-0.093 ± 0.017	-0.018 ± 0.011
R-Amplitude (mV)	0.648 ± 0.036	0.167 ± 0.013***	0.225 ± 0.029***	0.588 ± 0.054
T-Amplitude (mV)	0.158 ± 0.014	0.044 ± 0.016***	0.054 ± 0.017**	0.120 ± 0.013

*p<0.05, **p<0.01, ***p<0.001 (Treatment vs. Control), n≥6.

In general, ISO-treated rats produced low-voltage ECG recordings as seen by decreased amplitudes of the R- and T-waves (p<0.001 vs. Control). Pre-treatment with Etn in ISO-treated rats did not restore either of these parameters to control values. ISO also induced pathologically large Q-waves

(p<0.01 vs. Control), an effect consistent with an evolving MI. Rats treated with ISO + Etn exhibited Q-waves that were not significantly different from those in Control rats. ISO treatment caused a shortening of the T_{peak}-T_{end} parameter (p<0.05 vs. Control). Treatment with ISO + Etn caused a further shortening of the T_{peak}-T_{end} (p<0.001 vs. Control).

Infarct size is not affected by acute pre-treatment with ethanolamine

Infarct size was determined in all hearts using TTC staining (Figure 4). The myocardium of the Control hearts was positive for TTC (dark red colour), whereas in ISO treated groups the intensity of TTC staining was decreased, indicating the presence of a global infarction. ISO administration significantly increased the infarct size compared to Control rats (14.7 ± 1.9% vs. 5.7 ± 0.6%, p<0.01). Hearts in the ISO + Etn group also had a significantly greater infarct size compared to Control (13.5 ± 5.3%, p<0.01). There was no significant difference between the infarct size of rats treated with ISO alone and those treated with ISO + Etn. Administration of Etn alone had no significant effect on infarct size compared to Control (5.8 ± 1.0%, p=n.s. vs. Control).

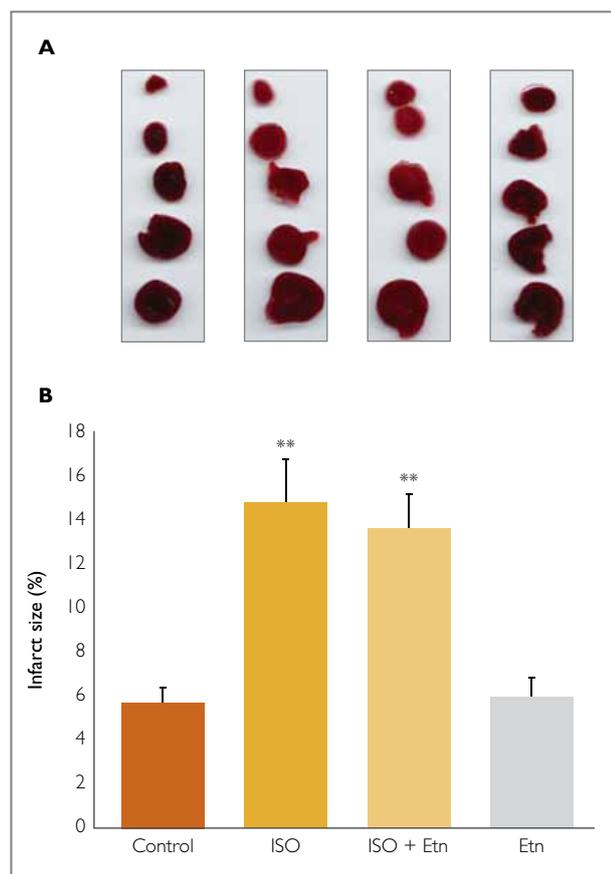


FIGURE 4: The effect of ethanolamine on isoprenaline-induced MI. A: Representative cross sections of ventricular myocardium from each group stained with TTC. B: Infarct size expressed as a percentage of total ventricular area.

$n \geq 6$ ** $p < 0.001$ vs. Control.

Heart weight to body weight ratio is increased by acute pre-treatment with ethanolamine

Hearts were weighed immediately after flushing with saline and their weight was expressed as a percentage of the initial body weight of the rat (Figure 5). Rats treated with ISO displayed an increased heart weight to body weight (HW/BW) ratio ($4.8 \pm 0.2 \text{ mg} \cdot \text{g}^{-1}$, $p < 0.0001$) compared to saline-treated rats ($3.3 \pm 0.1 \text{ mg} \cdot \text{g}^{-1}$). Pre-treatment with Etn further increased this change ($5.1 \pm 0.2 \text{ mg} \cdot \text{g}^{-1}$, $p < 0.0001$ vs. Control; $p < 0.05$ vs. ISO alone). Treatment with Etn alone did not affect the HW/BW ratio ($3.3 \pm 0.0 \text{ mg} \cdot \text{g}^{-1}$, $p = \text{n.s.}$ vs. Control).

Isoprenaline and ethanolamine do not affect lipid peroxidation

Treatment with ISO caused no significant difference in the quantity of CDs in the plasma ($p = \text{n.s.}$ vs. saline; Table II). Similar results were seen when rats were treated with ISO + Etn. The quantities of TBARS were also not significantly altered by ISO or ISO + Etn administration compared to saline-treated rats. Treatment with Etn alone had no significant effect on the concentration of CDs or TBARS produced.

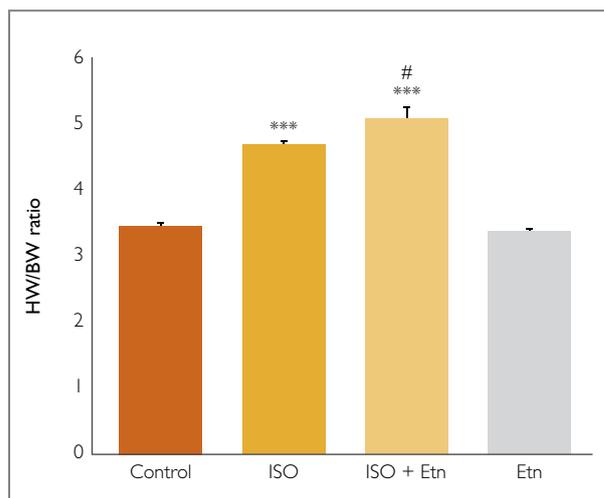


FIGURE 5: Changes in heart weight to body weight (HW/BW) ratio.

$n \geq 7$ *** $p < 0.0001$ vs. Control, # $p < 0.05$ vs. ISO alone.

TABLE II: The effects of isoprenaline and ethanolamine on the products of lipid peroxidation.

	Control	ISO	ISO + Etn	Etn
CD ($\mu\text{mol/L}$)	45.8 ± 5.1	60.3 ± 2.4	55.3 ± 6.4	44.9 ± 8.4
TBARS ($\mu\text{mol/L}$)	4.16 ± 0.20	4.12 ± 0.20	3.90 ± 0.56	4.88 ± 1.03

$p = \text{n.s.}$, $n \geq 6$.

TABLE III: Alterations in body weight (BW) and organ weights in Control, isoprenaline and ethanolamine treated rats.

	Control	ISO	ISO + Etn	Etn
BW Lost (%)	-0.01 ± 1.8	$-3.53 \pm 2.1^{**}$	$-5.58 \pm 3.2^{***}$	-0.62 ± 1.4
Liver/BW Ratio	50.59 ± 1.40	45.73 ± 1.41	46.74 ± 1.06	48.95 ± 1.49
Lungs/BW Ratio	7.33 ± 0.12	$6.44 \pm 0.09^{**}$	6.93 ± 0.19	7.14 ± 0.42
Kidneys/BW Ratio	10.73 ± 0.12	10.29 ± 0.14	10.44 ± 0.11	10.9 ± 0.31
Adrenals/BW Ratio	0.37 ± 0.01	0.39 ± 0.01	0.42 ± 0.02	0.36 ± 0.03

** $p < 0.01$, *** $p < 0.01$ (Treatment vs. Control), $n \geq 6$.

Effects of isoprenaline and ethanolamine on non-cardiac structures

ISO caused a 3.52% loss in body weight compared to Control ($p < 0.01$) and treatment with ISO + Etn resulted in a further loss in body weight by 2.05% ($p < 0.001$ vs. Control, Table III). ISO + Etn appeared to protect against the ISO-induced decrease in the lungs/BW ratio (Table III). ISO neither affected the gross structural appearance of the liver, kidneys and the adrenal glands nor impacted the weight of these organs.

DISCUSSION

In this study, we demonstrated that pre-treatment with Etn ($10 \text{ mg} \cdot \text{kg}^{-1}$) given 2 hours prior to induction of MI does not

decrease the infarction caused by ISO but does reduce ISO-induced mortality, although significantly increasing the HW/BW ratio. Furthermore, there were significant changes in electrocardiographic parameters in ISO + Etn treated hearts compared to Control hearts, which were not seen in hearts treated with ISO alone. Incidentally, ISO + Etn pre-treatment reversed the ISO-induced decrease in lungs/BW ratio.

ISO caused severe disruption to the electrical activity of the heart after 24 hours. ISO administration produced low voltage R-waves. The R-wave characterises ventricular depolarisation and abnormalities may represent myocardial oedema and poor R-wave progression (PRWP), which is indicative of an anterior MI.⁽²⁰⁾ Treatment with Etn did not restore R-wave values. ISO also induced pathologically large Q-waves which is an effect consistent with an evolving MI. Pre-treatment with Etn restored the Q-wave value near to control levels, which may have positively altered the evolution of the MI. ISO also affected the $T_{\text{peak}}-T_{\text{end}}$ parameter which provides an indication of transmural dispersion of repolarisation.⁽²¹⁾ Treatment with Etn appeared to worsen this parameter, suggesting that Etn may have altered the architecture of the myocardium. This suggestion is supported by the increased HW/BW ratio induced in the ISO + Etn group. The amplitude of the T-wave was decreased when ISO was administered and was not improved with Etn pre-treatment. This may be indicative of disturbances in electrolyte balance, left ventricular hypertrophy or left bundle branch block.⁽²²⁾ Pre-treatment with Etn caused a shortening of the QTc compared to control rats, an alteration that was not viewed in ISO-only treated rats.

Despite some of the modulations on electrical activity mentioned above, in the current study pre-treatment with Etn failed to affect the infarct size in an ISO-induced model of MI in vivo. In contrast, Etn pre-treatment has previously been shown to decrease infarct size in a Langendorff perfused model of ischaemia-reperfusion injury.⁽¹³⁾ The possible reasons for these differences include large variations in infarct size seen in the groups treated with ISO, suggesting that individual rats responded differently to ISO and therefore the infarct was not reproducible. In addition, the dose ($10\text{mg}\cdot\text{kg}^{-1}$) and time (2 hours prior to ISO) of administration of the drug may not have been optimal since the global nature of the infarct in this study was different from regional infarcts induced in isolated hearts. If the dose of Etn administered in this study remained in the blood, it would amount to a concentration of $3\mu\text{M}$ (working on a blood volume of $64\text{ml}\cdot\text{kg}^{-1}$), which may not have been sufficient to elevate the plasma concentration of Etn above its physiological levels of approximately $30\mu\text{M}$.⁽³⁾ Furthermore, it is unlikely that the Etn remained in the blood since it is often incorporated into phospholipid membranes.⁽²³⁾

ISO causes cardiac-specific dysfunction, being a synthetic catecholamine that targets β -adrenergic receptors in the heart; however ISO also affects other organs.^(14,24) In this study we

found that ISO caused a decrease in the lungs/BW ratio. The effect of ISO on lungs/BW ratio has not been extensively researched however, being a β -adrenergic agonist, ISO can increase Na^+ active transport, which may cause fluid clearance in the lungs.^(25,26) This may explain the decrease in the lungs/BW ratio after 24 hours. Pre-treatment with Etn prevented the ISO-induced decrease in lungs/BW ratio which may suggest an interaction of Etn with Na^+ active transport.

The increased HW/BW ratio could be indicative of hypertrophy and/or oedema, fibroblast proliferation and infiltration of inflammatory cells. Etn has not previously been linked to oedema, however it is possible that Etn could exacerbate hypertrophy and fibroblast proliferation in a damaged organ, particularly as Etn has been shown to act as a co-mitogen^(8,27,28) and several mitogens are upregulated and/or released in response to MI, for example; myostatin,⁽²⁹⁾ vascular endothelial growth factor⁽³⁰⁾ and hepatocyte growth factor.^(31,32) Furthermore, Etn induced cardioprotection in isolated hearts is mediated by STAT-3 activation.⁽¹³⁾ The JAK/STAT pathway plays a pivotal role in the development of cardiac hypertrophy.⁽³³⁾ The fact that Etn alone did not affect HW/BW ratio suggests that it is unlikely that administration of Etn in vivo, in an undamaged heart, may have interacted with STAT-3 to cause hypertrophy. However, the increase in HW/BW ratio seen in the ISO + Etn group may suggest that stimulation of STAT-3 by Etn in an already damaged heart, or some drug-drug interaction between ISO and Etn, may facilitate structural alterations of the heart.

Contrary to several previous studies, our data suggest that ISO did not increase oxidative stress. Karthikeyan, et al.,⁽³⁴⁾ Patel, et al.,⁽³⁵⁾ and Mukherjee, et al.⁽³⁶⁾ reported that ISO causes an increase in oxidative stress markers. However, there were several critical differences between these studies and the current study: (1) animals in the previous studies received repeated doses of ISO, as compared to the single dose administered in the current study, (2) measures of lipid peroxidation and oxidative stress were measured two or more days after the initial dose of ISO, as compared to 24 hours after the initial dose in the current study, (3) all 3 of these studies analysed homogenised myocardium, not circulating plasma. Interestingly, in support of our findings R oth, et al.⁽³⁷⁾ demonstrated that although tissue levels of TBARS were elevated after cardiac injury induced by coronary artery ligation, they had returned to basal levels by 24 hours after ISO administration. Future studies should include an analysis of the effects of ethanolamine on tissue levels of lipid peroxidation indicators in ISO induced MI. Furthermore, bearing in mind that the signalling pathways involved in the global MI induced by ISO may not be the same as those involved in the regional MI, repetition of this study in a model of coronary artery ligation may provide further insights into the protective nature of Etn.

In conclusion, we have shown that Etn pre-treatment minimises ISO-induced mortality, but does not directly reverse the co-

induced global MI. The mechanisms by which this improved survival occurs have not been identified in this study and require further investigation. Despite the lack of protection against MI, Etn still potentially modulates the heart, possibly via architectural changes in the heart as shown by diminished ECG disruptions and increased HW/BW ratio.

ACKNOWLEDGEMENTS

CG was supported by the Oppenheimer Memorial Trust grant and UCT Masters Research Scholarship, RK-L by the UCT URC/Carnegie Research Development grant, the South African Heart Association grant, the UCT Health Sciences Faculty Research Committee and the National Research Foundation (NRF Grant No 85768), and AG by the UCT URC/Carnegie Research Development grant, NRF (Grant No 91514) and ADInstruments (Australia) grant. We thank Dr Kishor Bugarith for insightful discussions and Mr Henri Carrara for statistical advice.

Conflict of interest: none declared.

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