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Abstract

Zinc (Zn) plays an important role in maintaining the anti-oxidant status within the heart and helps to counter the acute redox stress that occurs during myocardial ischaemia and reperfusion. Individuals with low zinc (Zn) levels are at greater risk of developing an acute myocardial infarction, however the impact of this on the extent of myocardial injury is unknown. The present study aimed to compare the effects of dietary zinc depletion with *in vitro* removal of Zn (TPEN) on the outcome of acute myocardial infarction and vascular function. Male Sprague-Dawley rats were fed either a zinc adequate (ZA; 35mg Zn/kg diet) or zinc deficient (ZD; <1mg Zn/kg diet) diet for 2 weeks prior to heart isolation. Perfused hearts were subjected to a 30min ischaemia/2-hour reperfusion (I/R) protocol, during which time ventricular arrhythmias were recorded and after which infarct size was measured, along with markers of anti-oxidant status. In separate experiments hearts were challenged with the Zn chelator TPEN (10μM) prior to ischaemia onset. Both dietary and TPEN-induced Zn depletion significantly extended infarct size; dietary Zn depletion was associated with reduced total cardiac glutathione (GSH) levels, while TPEN decreased cardiac SOD-1 levels. TPEN, but not dietary Zn depletion also suppressed ventricular arrhythmias and depressed vascular responses to nitric oxide NO. These findings demonstrate that both modes of zinc depletion worsen the outcome from I/R but through different mechanisms. Dietary Zn deficiency, resulting in reduced cardiac GSH, is the most appropriate model for determining the role of endogenous Zn in I/R injury.

Introduction

Zinc (Zn) is the second most widely distributed trace element in the body after iron[1] and the most abundant intracellular trace element[2]. The storage capacity for Zn is low, and therefore healthy Zn levels are maintained through dietary intake, with around 2-3 mg Zn per day being required. However, the prevalence of nutritional Zn deficiency is estimated to be high, and current estimates suggest that over 2 billion people in the developing world have insufficient dietary Zn intake[3].. Moreover, in industrialized countries, elderly people represent a high-risk group as it is known that Zn intake decreases with age, with only ~40% of individuals aged 71 years or older having an adequate Zn intake[4].

Zn is important in cardiovascular physiology as it acts as an indirect antioxidant, serves to stabilise membrane structure and regulates metallothionein (MT) levels (reviewed in[5]). The antioxidant role of Zn is achieved through the requirement of Zn for superoxide dismutase 1 (SOD1) activity, its protection of thiols and its ability to inhibit the Fenton reaction (which converts H_2O_2 to hydroxyl radicals) by competing with iron[5]. Zn is also a physiological suppressor of apoptosis[6]. Given this essential role in maintaining cellular homeostasis, disruptions in cellular Zn levels (or alterations in intracellular signalling events involving Zn) could result in a loss of antioxidant capacity of tissues and may induce, or at least facilitate, the induction of apoptotic cell death. Consequently, disruption of Zn homeostasis is likely to play an important role in cardiovascular diseases.

Zn plays a protective role in the four key events that perpetuate myocardial injury following acute myocardial ischaemia and reperfusion (IR injury); studies have shown that cardiac Zn counters the acute redox stress that occurs in cardiomyocytes upon reperfusion, inhibits inflammatory processes that contribute to delayed injury, contributes positively to tissue healing and assists in maintaining cardiac stem cells involved in tissue repair (reviewed in[7]). Cardiac and plasma Zn levels are severely depleted in patients post-myocardial infarction, the extent of depletion correlating with MI outcome[8] and with the levels of enzyme markers (LDH and CK) of the extent of myocardial injury and the severity of arrhythmia[9], leading to the proposal that Zn supplementation given at the time of reperfusion may represent a cardioprotective approach. Indeed, studies have shown that administration of Zn pyrithione at the time of reperfusion in isolated hearts can ameliorate reperfusion-induced injury via the reperfusion injury salvage kinase (RISK) pathway[10], improve post-reperfusion contractile recovery and reduce reperfusion-induced ventricular fibrillation[11] and, in hyperlipidaemic hearts, restore the infarct reducing effect of preconditioning[12], while chronic Zn

supplementation prior to I/R reduces infarct size and increases cardiac glutathione (GSH) content [13]. Conversely, studies looking at acute Zn depletion, using the zinc chelator TPEN (N,N,N′,N′-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine), demonstrate a worsening of infarct size (reviewed in[7]) but reduced arrhythmia severity[14,15]. However, it is debateable how closely these experimental conditions mimic the conditions imposed by dietary Zn deficiency within the heart. Considering the prevalence of sub-optimal Zn intake in various populations, and the potentially detrimental impact this may have on the extent of cardiac injury sustained during an acute myocardial infarction, it is surprising that there is in fact a paucity of data on this topic. Beyond two experimental dietary Zn depletion studies that show increased cardiac lipid peroxide levels (but no impact on in infarct size) following sustained coronary occlusion without reperfusion[16], and a worsening of post-reperfusion recovery of contractile dysfunction associated with alterations in cardiac tissue anti-oxidant enzymes[17], very little is known.

In the vasculature, *in vivo* Zn deficiency is associated with an increased risk of atherogenesis (reviewed in[18,19]) due to a heightened pro-inflammatory status[20], and experimental studies have shown that Zn deficiency during foetal development[21] and growth[22] in rats induces changes in endothelial function that may contribute to the programming for the development of the hypertension observed in these animals. Dietary Zn deficiency also increases apoptosis in large arteries through a mechanism involving oxidative stress-induced induction of pro-apoptotic proteins[23], while *in vitro* studies show that cellular zinc depletion results in a reduction in endothelial cell barrier function[24] and an increase in vascular smooth muscle cell proliferation[25]. However, to date very little evidence exists as to what functional consequences these changes have on vascular contraction and relaxation.

The aim of the current study, therefore, was to explore the impact and underlying mechanisms of dietary Zn deficiency on the outcome (myocardial infarct size and incidence of arrhythmias) of an acute I/R insult, and on vascular function, in comparison with acute TPEN-induced Zn deficiency.

Methods

Animals

Male Sprague-Dawley rats (200-250g), were purchased from Harlan UK and housed in the University of Aberdeen Medical Research Facility. All animals were maintained at a

temperature of 21±2°C and humidity of 45±10%, with a 12-hour light/dark cycle and brought to the Biological Services Unit at the Robert Gordon University on a weekly basis and allowed to acclimatize before commencing the experimental protocol. All animals, except for those receiving a zinc deficient (ZD) diet and their pair-fed (PF) counterparts, were housed using standard caging and allowed free access to both food and tap water. Due to the requirement to accurately assess food intakes in the ZD and PF rats these animals were single housed in zinc-free cages with grid flooring; any discomfort caused by the grid flooring was minimised by the inclusion of zinc-free nesting tunnels/inserts in the cage. These animals were also housed on a reverse 12h light/dark cycle to allow for twice daily feeding and measurement of food intake. All studies were performed under an appropriate Project License authorized under the UK Animals (Scientific Procedures) Act 1986. All *in vivo* work is reported in accordance with the ARRIVE guidelines[26]. Group sizes were determined based upon power calculations performed on previous studies using isolated perfused hearts. Animals were allocated to dietary intervention groups based upon body weight so that pair-fed and zinc deficient animals were of a similar weight at the start of the intervention period. For the *in vitro* zinc depletion study, hearts were randomly assigned to control or TPEN groups.

Dietary intervention study

To determine the effects of acute zinc deficiency on the outcome of ischaemia/reperfusion and vascular function, rats (10 per group) were randomly allocated to a 14 day dietary intervention period of either a zinc-adequate (ZA) diet (35 mg Zn/kg diet) fed *ad libitum* or a ZD (<1 mg Zn/kg diet) diet provided twice daily to allow for measurement of food intake. Both diets have been described elsewhere[27] and were essentially based on the AIN-76A recommendations. Since the consumption of a Zn deficient diet results in cyclical feeding behaviour and reduced weight gain, a further group of 10 rats was included as pair-fed (PF) controls to determine the impact of reduced weight gain, as opposed to Zn deficiency, on any of the end-points. These rats were each weight matched to a ZD rat and fed the same quantity of ZA food consumed by the Zn-deficient rat the previous day, and body weights of all rats were monitored daily. The study parameters and humane end-points were set such that, if a ZD rat failed to consume any food on any one day, the PF rat would be provided with a specified quantity of food, and if the body weight of any ZD or PF animal varied by more than 30% from ZA controls fed *ad libitum* it would be euthanised by a Schedule 1

method. To mitigate against this the duration of dietary Zn restriction and pair feeding was restricted to 14 days and therefore neither intervention was required during the study. At the end of the dietary intervention period the rats were euthanised as described below for the isolated heart experiments. Prior to heart removal blood was withdrawn by cardiac puncture for biochemical measurements, and tissues (liver, white adipose tissue) removed and weighed.

Coronary artery occlusion/reperfusion in the isolated heart

Rats were anaesthetised with pentobarbital sodium salt (100 mg kg^{-1} i.p; Sigma Aldrich, Poole, Dorset, UK) and the heart rapidly removed and arrested in ice cold Kreb's Henseleit buffer (KHB; 119mM NaCl, 4.7mM KCl, 1.18mM KH_2PO_4, 2.41mM $MgSO_4$, 25mM $NaHCO_3$, 2.52mM $CaCl_2$ and 10.88mM Glucose; pH7.4). After placement of a ligature (6-0 silk suture (W812), Ethicon, Edinburgh) around the left coronary artery, the aorta was cannulated for retrograde perfusion on a Langendorff apparatus (AD Instruments). Hearts were perfused with KHB at 37^{o}C at a rate of 12ml/min and allowed to stabilize for 15 minutes prior to drug administration and subsequent coronary occlusion. The coronary artery was occluded (CAO) by tightening the ligature to induce regional ischemia for 30 minutes after which the ligature was loosened and the myocardium reperfused for 2 hours. A surface electrocardiogram (ECG) was recorded via electrodes placed on the right atrium and left ventricle and coronary perfusion pressure (CPP) recorded via a pressure transducer (MLT844 physiological pressure transducer; AD Instruments) connected to the mounting head of the Langendorff apparatus. Ventricular arrhythmias that occurred during the ischaemic period were analysed according to the Lambeth Conventions[28]. Heart rate (HR; calculated from the ECG), ECG, and CPP were all monitored continuously throughout the experimental period using a Power Lab data acquisition system via an Animal Bio Amplifier and Bridge Amplifier, respectively, and data subsequently analysed using Chart Software (all equipment and software from AD Instruments). Any hearts which developed spontaneous arrhythmias prior to CAO were excluded from the study. Hearts from rats included in the dietary intervention study were used to determine the effect of acute dietary Zn depletion on the outcome or I/R. To determine the impact of acute *in vitro* Zn depletion, either vehicle (0.01% DMSO; n=10) or TPEN (10µM; concentration chosen based on previously published work in isolated heart studies[14,29]) was infused (at a rate of 100µL/min) into isolated hearts via the

aortic cannula starting 5 minutes prior to ischaemia and terminating immediately before CAO.

Histological measurement of infarct size

Following completion of the ischaemia/reperfusion protocol, the ligature around the coronary artery was retied and Evans blue dye (2ml; 0.5% w/v) perfused through the heart to delineate area at risk. Hearts were then removed and stored at –20^{o}C prior to infarct size determination. Frozen hearts were sliced into 2-3mm slices from the apex to the base and allowed to defrost at room temperature. Myocardial tissue slices were then incubated in 1% triphenyltetrazonium chloride (TTC; Sigma Aldrich, UK) in phosphate buffered saline for 15 minutes at 37^{o}C to determine infarct size. Sections were then fixed in 10% buffered formal saline for 1 hour and imaged using an EOS 1100D digital SLR camera (Canon Inc., Tokyo, Japan) attached to a Leica S4E stereomicroscope (Leica Microsystems Ltd., Milton Keynes, UK). Left ventricular area, area at risk, and infarct size were determined using computerised planimetry (ImageJ software, National Institute of Health (NIH), Rockville Pike Bethesda, MD). Area at risk was expressed as a percentage of total left ventricular area, and infarct size was expressed as a percentage of area at risk.

Isometric myography

Once hearts were mounted on the Langendorff apparatus and in the stabilisation period, the mesenteric arterial arcade was excised and placed in ice cold KHB. Third order mesenteric arteries were then dissected out, cleaned of perivascular fat, and stored in KHB overnight at 4^{o}C. Vascular function was then assessed in isolated mesenteric arteries mounted onto a two-channel wire myograph (Model 510A, Danish Myo Technology (DMT), Denmark) containing oxygenated (95% O_2 & 5% CO_2) KHB at 37^{o}C. Vessels were normalised to achieve a transmural pressure of 100mmHg using the DMT Normalisation software. Isometric tension was recorded and displayed using a PowerLab and Chart Software (both AD Instruments). The viability of the smooth muscle was tested via the addition of an 80mM KCl solution. Following KHB washes, a cumulative concentration response was carried out with the thromboxane mimetic, U46619 (9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin F2α; Tocris Bioscience, UK). Vessels were then precontracted with a submaximal concentration (EC_{80}) of U46619 and cumulative concentration responses carried out with

either the endothelium-dependent vasodilator, metacholine (MCh) or the endothelium-independent vasodilator, sodium nitroprusside (SNP). Vessels from the dietary intervention groups were used to determine the impact of *in vivo* zinc depletion on vascular function. To determine the effect of acute *in vitro* Zn deficiency on vascular function either (0.01% DMSO), TPEN (10µM), or DPTA (10µM; extracellular zinc chelator,) was added to the myograph bath prior to performing cumulative concentration responses to either MCh or SNP.

Myocardial Cu/Zn-SOD activity

Cu/Zn-SOD activity was measured in cardiac tissue samples (representing both ischaemic and non-ischaemic tissue) from all experimental groups using a Superoxide Dismutase Assay Kit (catalogue number: 706002; Cayman Chemical). Briefly, cardiac tissue was homogenised in ice cold 20mM HEPES buffer (pH 7.2) containing 1mM EGTA, 210mM mannitol, and 70mM sucrose. The resulting homogenate was then subjected to multiple centrifugation steps to separate mitochondrial and cytosolic SOD, the latter of which was retained for subsequent analysis. Following protein quantification, protein concentrations were normalised to 0.5mg/ml in all samples and Cu/Zn-SOD activity measured via the kit as per the manufacturer's instructions.

Myocardial glutathione (GSH) content

Total GSH content was measured in cardiac tissue using a Glutathione Assay Kit (catalogue number: 703002; Cayman Chemical). Briefly, cardiac tissue (representing both ischaemic and non-ischaemic tissue) was homogenised in ice cold MES buffer (pH 6.0) containing 200mM 2-(N-morpholino) ethanesulphonic acid, 50mM phosphate, and 1mM EDTA. The resulting homogenate was then centrifuged at 10,000g for 15 minutes at 4^{o}C, the supernatant removed and subsequently deproteinated via the addition of MPA reagent (1.25M metaphosphoric acid). Samples containing MPA reagent were then centrifuged at 3,000g for 2 minutes at room temperature, the supernatant removed and TEAM reagent (4M triethanolamine) added before assaying for GSH content as per the manufacturer's instructions.

Myocardial caspase-3 activity

Caspase-3 activity in heart tissue was determined by measuring conversion of the caspase-3 substrate N-Acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-Pna) to p-nitroaniline. Briefly, tissue samples (representing both ischaemic and non-ischaemic tissue) were homogenised in ice cold HEPES buffer (25mM; pH 7.4) containing 5 mM EDTA, 2 mM DTT and 0.1% CHAPS and centrifuged at 20,000g for 30 minutes at 4°C). The supernatant (5µl) was then incubated with 85µl HEPES buffer (50mM; pH 7.4, containing 1.0mM EDTA, 10mM DTT, 0.1% Chaps, 100mM NaCl and 10% glycerol) and 10µl Ac-DEVD-Pna (200µM) at 37°C for 24 hours; the concentration of the product (p-nitroaniline) was read at 405nm.

Myocardial and plasma zinc levels

Cardiac tissue zinc levels were measured using a Zinc Assay Kit (catalogue number: MAK032; Sigma Aldrich UK). Samples were prepared as for the Cu/Zn-SOD activity assay and protein concentrations were normalised to 0.5mg/ml. Zn was measured via the kit as per the manufacturer's instructions, with the omission of the deproteination step to measure both free and protein-bound Zn. For determination of circulating trace metal levels plasma samples were analysed using a Unicam Solaar 969 atomic absorption spectrophotometer as described previously[30] and lactate dehydrogenase and creatine kinase levels were determined using a Konelab clinical analyser.

Statistical analyses

All data are expressed as mean ± s.e.m. and significance determined as $P<0.05$. For the acute zinc deficiency study, the ZD groups were compared to the PF group as the appropriate control to eliminate any impact of reduced energy intake on the end points. The impact of reduced energy intake on end points was determined by comparing the PF group with the ZA group. A two tailed Student's T-test was used to compare baseline CPP/HR values in the vehicle and TPEN treated hearts and compare the effect of TPEN on both CPP and HR. A one-way analysis of variance (ANOVA) & Dunnett's post-hoc test was used to compare pre-occlusion and post-occlusion CPP/HR values within experimental groups and a repeated measures ANOVA & Bonferroni post-hoc test used to compare post-occlusion CPP/HR

between the vehicle and TPEN groups. For the acute zinc deficiency study, a one-way ANOVA & Dunnett's post-hoc test was used to compare baseline CPP/HR values in the ZA, PF and ZD experimental groups and the pre-occlusion and post-occlusion CPP/HR values within the same experimental groups. A two-way ANOVA & Bonferroni post-hoc test used to compare post-occlusion CPP/HR values between the ZA, PF and ZD experimental groups. In both experimental studies, ventricular arrhythmias were determined from the ECG trace and classified according to the Lambeth Conventions (II)[28]. The effect of TPEN on the number of each type of ventricular premature beat (VPB) i.e. singles, salvos, ventricular tachycardia (VT) and total VPB count was analysed using a two-way ANOVA & Bonferroni post-hoc test. The effect of TPEN on the incidence of VT, reversible ventricular fibrillation (rVF), and irreversible (irrVF)/mortality was analysed using a Fisher's exact test. In the acute zinc deficiency study, the impact of the dietary interventions on the number of VPBs and incidences of VT, rVF and mortality were compared between groups using a two-way ANOVA & Bonferroni post-hoc test and Fisher's exact test, respectively. Nonlinear regression (using GraphPad Prism) was used to generate curves for all vascular data, and to calculate and compare pEC_{50} values for each group. Concentration responses between groups were compared via a repeated measures ANOVA and Bonferroni post-hoc test. E_{max} values (maximal relaxation as a percentage of induced tone) were compared using either a t-test (acute zinc deficiency study) or a one-way ANOVA & Dunnett's post-hoc test (*in vivo* zinc deficiency study). Similarly, food intake, BW, HW:BW, area at risk, infarct size, Cu/Zn-SOD activity, GSH content, caspase-3 activity, and plasma/cardiac zinc levels were all compared using either a t-test or where appropriate a one-way ANOVA & Dunnett's post-hoc test.

Results

Impact of dietary zinc depletion on physiological measures

Consumption of the zinc-deficient diet resulted in characteristic cyclical feeding behaviour associated with acute zinc depletion in rats in that food consumption reduced by approximately 50% every 2-3 days; the PF rats were therefore subjected to the same cyclical feeding (Figure 1a). Consequently, zinc deficiency resulted in a significantly reduced weight gain over the 14-day period of intervention compared to normal-fed ZA rats (Figure 1b). PF rats given a zinc adequate diet also exhibited a reduced weight gain, but to a lesser extent than the ZD rats. ZD rats had a higher HW;BW ratio (Figure 1c) and a lower white adipose

tissue (WAT) to BW ratio (indicative of altered body fat composition; Figure 1d). Plasma analysis confirmed that circulating zinc levels in the ZD rats were significantly lower than those in both ZA and PF rats (Figure 1e), while cardiac tissue Zn levels were similar across all groups (Figure 1f). All other plasma markers (Cu^{2+}, Ca^{2+}, lactate and creatine kinase) were unchanged (Supplementary Table 1).

Dietary Zn depletion and the outcome of myocardial ischaemia/reperfusion

Dietary zinc deficiency caused a significant increase in infarct size compared to the extent of damage seen in hearts from PF rats (Figure 2a; AAR 56±2% and 48±6% respectively); Infarct sizes in PF and ZA rats (AAR 43±3%) were not significantly different. PF rats exhibited a marked increase in cardiac caspase-3 activity compared to ZA rats while ZD rats exhibited similar myocardial caspase-3 activity to ZA rats (Figure 2b). Pair feeding had no impact on either GSH levels or Cu/Zn-SOD activity, while in ZD rats there was a marked reduction in GSH levels (Figure 2c) but no change in Cu/Zn-SOD activity (Figure 2d). Arrhythmia analysis revealed that PF animals exhibited a significantly fewer VPBs occurring as salvos (2-3 consecutive VPBs) and ventricular tachycardia (VT; 4 or more consecutive VPBs; Figure 3a), although the incidence of VT and total VF was unaffected (Supplementary Table 2); the arrhythmia profile in ZD rats was similar to that in PF rats. Baseline coronary perfusion pressures (Figure 3b) and heart rates (Figure 3c) were the same irrespective of dietary intervention, and the ischaemia-induced rise in perfusion pressure was similar in all groups, but only reached statistical significance in the ZD group.

Dietary Zn depletion and vascular function

Contractile responses to U46619 was unaffected by either pair feeding or zinc deficiency (Table 1). Similarly, blood vessels from both PF and ZD rats exhibited comparable endothelium-dependent (MCh; Figure 4a) and independent (SNP; Figure 4b) vasodilator responses, with no alterations in either E_{max} or pEC_{50} (Table 1).

Acute in vitro Zn depletion and the outcome of myocardial ischaemia/reperfusion

Perfusion with TPEN (10μM) prior to the onset of coronary artery occlusion caused a significant increase in infarct size compared to control hearts ($P<0.05$; Figure 5a); area at risk

was similar in both groups (43±3% vs 45±3% of LV area in control and TPEN-treated hearts, respectively). Treatment with TPEN did not induce any significant reduction in tissue Zn levels (Figure 5b). The induction of I/R itself cause a reduction in both GSH levels (Figure 5c) and Cu/Zn-SOD activity (Figure 5d) when compared to sham hearts, whereas I/R did not alter caspase-3 activity (Figure 5e). There was no impact of TPEN treatment on GSH content or caspase-3 activity, however in hearts given TPEN there was a significant reduction in Cu/Zn-SOD activity compared to control I/R hearts (Figure 5d; $P<0.05$). TPEN also markedly reduced the number of ventricular arrhythmias occurring as single VPBs, salvos and VT (Figure 6a), and significantly reduced the incidence of VT but had no effect on the development of ventricular fibrillation (VF; Supplementary Table 3). Prior to coronary occlusion, TPEN induced a rise in CPP (Figure 6b), which was maintained throughout the period of regional ischaemia (Figure 6c). In contrast, neither the administration of TPEN nor the induction of regional ischaemia significantly altered heart rate in any of the isolated hearts (Figure 6d).

Acute in vitro Zn depletion and vascular function

TPEN (10μM) significantly reduced the contractile response to U46619, which was used to pre-contract vessel rings to determine vasodilator responses (Table 2). Similarly, TPEN induced a significant shift to the right of the dose response curves and a reduction in maximum relaxant responses to both MCh (endothelium-dependent) and SNP (endothelium-independent) (Figure 7a,b & Table 2). In contrast DPTA (extracellular Zn chelator; 10μM) did not affect either the contractile response to U46619 or the vasodilator responses to MCh or SNP (Figure 7c,d & Table 2).

Discussion

The majority of studies determining the value of Zn in cardio- and vasculo-protection have focused on exogenous Zn supplementation, rather than considering the importance of endogenous Zn in maintaining a healthy and resilient cardiovascular system. This study aimed to determine the effects of endogenous Zn depletion, induced by two distinct methods (*in vivo* dietary deficiency and *in vitro* removal of intracellular Zn) to demonstrate the importance of maintaining adequate Zn levels to protect the heart in the event of an acute

myocardial infarction and also to determine the most physiologically relevant experimental model for further study.

Dietary deficiency vs acute depletion effects on zinc and blood/tissue marker status

Induction of dietary Zn deficiency for 14 days led to a slowed increase in body weight resulting from the reduced food intake, leading to lower fat accumulation (reduced WAT;BW ratio) and an increase in heart:BW ratio. In ZD rats, plasma Zn was markedly reduced (by >50%), while other blood markers that could influence infarct size (Cu^{2+} and Ca^{2+}, lactate and CK levels; Supplemental Data Table 1) were unaffected; thus, any difference between the ZD rats and the ZA/PF rats can be attributed to alterations in plasma Zn status alone. However, dietary Zn deficiency did not significantly alter cardiac tissue levels of Zn which, although perhaps surprising, agrees with other studies[16,17]. Notwithstanding this, it is worthy of note that the tissue Zn levels reported here represent protein-bound Zn, as Zn levels were undetectable in deproteinated samples (lower detection limit of the assay was 0.5nmol per sample), indicating that there was no detectable free Zn to participate in Zn-dependent cellular process such as glutathione synthesis (see below).

The mechanism by which cardiac tissue levels of Zn are maintained in the face of dietary deficiency is not clear but is likely linked to the tight control systems that maintain intracellular Zn homeostasis. The homeostatic mechanisms in the cardiomyocyte are as yet poorly defined[31] but in most cells intracellular Zn concentration is kept within a tight window by two families of zinc transporters (ZnT and ZIP). ZnT's promote efflux from the cell while ZIP's increase intracellular zinc by promoting transport into the cytoplasm (reviewed in[32]). Additional control is provided by metallothionein[33], which is largely a mechanism to protect the cell against excessive increases in intracellular Zn. ZnT2 and ZnT5, along with most of the ZIP transporters, are known to be expressed in cardiac tissue[34], and in non-cardiac cells ZnT2 is markedly downregulated in response to Zn deficiency while ZIP 2 and ZIP 4 are upregulated. If this is also the case in the cardiomyocyte this would result in reduced Zn efflux alongside increased entry, which could explain the preservation of the Zn levels in cardiac tissue despite dietary deficiency.

In vitro acute Zn depletion, achieved by treating hearts with TPEN, similarly did not alter total tissue Zn content. While alterations in zinc transporter activity may similarly explain preserved cardiac Zn levels in TPEN-treated hearts, we cannot rule out the possibility that the

effects of TPEN are due to removal of other cations (such as Ca^{2+}). However, at the concentrations used in the present study TPEN has a much higher affinity for Zn.

Zn and I/R injury

While there are substantial data to support the notion that supplementation with exogenous Zn in the setting of I/R is cardioprotective, relatively little is known about the importance of endogenous Zn in the development of myocardial injury[5]. Endogenous Zn has been reported to be both cardioprotective, by acting as an intracellular messenger that translates the signalling process in NO-mediated cardioprotection[35], and detrimental, through activation of ERK/GSK3β to trigger cardiomyocyte death[36] in I/R. However very few studies have determined whether depletion of endogenous zinc levels can influence the outcome of I/R. In this study we have shown that both acute dietary Zn deficiency and acute *in vitro* Zn depletion increase infarct size following I/R injury in isolated hearts, which is consistent with the notion that Zn is cardioprotective. Since endogenous Zn is known to play an important role in maintaining redox status within tissues[37], we also determined whether Zn deficiency upset the redox balance in cardiac tissue as a possible mechanism for the increase in tissue injury.

In the case of dietary Zn deficiency, there was a marked reduction in total myocardial GSH levels of ZD rats compared to both ZA and PF rats. This provides a plausible explanation for the increase in infarct size since GSH is a powerful antioxidant that prevents ROS-induced tissue injury[38] and reduced GSH levels have been associated with a detrimental effect on tissue integrity following I/R injury[39]. Zn is an important co-factor in GSH synthesis as it increases the expression of glutamate cysteine ligase (GCL), the enzyme that catalyses GSH synthesis. Zn deficiency has been shown to reduce GSH levels in other tissues and cells such as liver[40,41], erythrocytes[42] and brain[43], but to our knowledge this is the first time this effect has been observed in cardiac tissue. However, while dietary Zn deficiency-induced GSH deficit has been linked to increased cleavage of GCL[44] resulting from activation of caspase-3[45], we did not see any increase in caspase-3 activity in hearts from ZD rats. Interestingly, hearts from PF rats exhibited higher caspase-3 activity, which is surprising since calorie restriction has been associated with caspase-3 inactivation in the heart[45], albeit for longer (15-35 weeks) and more severe (30% reduction in food intake) calorie restriction. Although Zn is similarly an important co-factor for the cytosolic superoxide scavenging

enzyme Cu/Zn-SOD (SOD-1) which, when applied exogenously to the heart upon reperfusion, is cardioprotective[46] we did not observe any changes in SOD-1 activity that could explain the impact of dietary Zn depletion on infarct size. However, this is consistent with the finding that endogenous SOD-1 deficiency does not influence the extent of reperfusion injury[47].

The only previous study of dietary Zn deficiency on myocardial injury, performed in rats subjected to permanent and sustained (48 hour) coronary occlusion, showed that despite a significant increase in cardiac lipid peroxide levels there was no effect on infarct size[16]. However, because lethal injury as a result of oxidative stress is induced upon reperfusion, and since Zn deficiency is known to diminish the ability of cells to respond to oxidative stress (reviewed in[37]), this supports the concept that endogenous zinc is important in moderating the extent of reperfusion, rather than ischaemic, injury. Indeed, our results with dietary Zn depletion are supported by findings that contractile recovery post I/R is significantly impaired in hearts from Zn deficient rats[17].

To correct for any influence of reduced food intake caused by cyclical feeding behaviour induced by the Zn deficient diet, we included PF animals as controls and found that pair feeding *per se* had no significant effect on the extent of I/R injury compared to ZA rats. Short term (7-14 days) caloric restriction has been associated with a cardioprotective effect in terms of post I/R recovery of cardiac function[48] and a reduction in infarct size following permanent coronary occlusion[49]. Therefore, since there was no expansion of infarct size in the PF hearts we can tentatively conclude that the worsening of I/R in the ZD rats is due to the lack of zinc rather than to reduced food, and therefore energy, intake.

Although *in vitro* endogenous Zn depletion with TPEN similarly resulted in an increase in infarct size, unlike dietary deficiency this was associated solely with a reduction in Cu/Zn-SOD activity since glutathione levels were preserved and there was no evidence of caspase-3 activation. Although, as mentioned above, endogenous SOD-1 has been shown to be an unlikely contributor to post-reperfusion tissue preservation, it is not the only Zn-dependent isoform of superoxide dismutase. Extracellular SOD (ecSOD; SOD-3), which is concentrated in the extracellular space between smooth muscle cells and the endothelium of the vascular wall, plays a critical role in regulating the vascular redox state[50] and evidence suggests that it is the interstitial levels of SOD-3, rather than SOD-1, that confers protection against I/R injury[51]. Since our assay measures total Cu/Zn-SOD (i.e. SOD-1 and SOD-3) then the

increase in infarct size is most likely due to an inhibition of SOD-3, rather than SOD-1 activity.

As with dietary Zn deficiency, very few studies have employed TPEN to determine the effects of acute endogenous Zn depletion on myocardial infarct size. While the expansion of infarct size in the presence of TPEN is compatible with numerous studies showing that Zn supplementation is cardioprotective (reviewed in [5]), the only other study to explore the role of endogenous Zn in infarct size using TPEN showed it to be cardioprotective when applied throughout reperfusion[36]. While this is difficult to reconcile with the present data, it is most likely explained by differences in experimental protocols (i.e. pre-ischaemia vs post-reperfusion administration), but certainly requires further investigation.

Zn and ventricular arrhythmias

In contrast to the effects on myocardial infarct size, dietary Zn deficiency did not have any worsening effect on the severity of ventricular arrhythmias but rather, when compared to ZA hearts, ZD hearts had a lower total arrhythmia count during the ischaemic period, predominantly due to a reduction in salvos, although the incidence of the more severe arrhythmias (VT and VF) were not changed. However, this effect is most likely explained by the reduced energy intake, as opposed to Zn depletion, in these animals as hearts from PF rats similarly exhibited fewer arrhythmias. Indeed, caloric restriction has been shown to induce bradycardia, prolong QT- and QRS-intervals and to reduce the arrhythmogenic response to adrenaline through a reduction in β-adrenoceptor density and binding[52]. Although we did not see any difference in baseline heart rates between ZA, ZD and PF hearts, these were measured in isolated hearts without the influence of sympathetic tone and so any β-adrenoceptor mediated impairment would not be detected. TPEN, on the other hand did exert a significant anti-arrhythmic effect by reducing total arrhythmia count and the incidence of VT during ischaemia. It is unlikely that this was related to the reduction in SOD-1/3 activity since neither superoxide generation nor superoxide scavenging has been associated with a pro- or anti-arrhythmic effect (respectively) in this model[53]. However, other studies describing the antiarrhythmic effects TPEN have ascribed these to inhibition of nitric oxide accumulation[14] and activation of the sarcolemmal Na^+/Ca^{2+} exchanger to prevent Ca^{2+} overload[15].

Vascular effects of Zn depletion

Zn deficiency/depletion is associated with adverse effects on both vascular smooth muscle[23] and endothelial[24] cells that would be expected to result in abnormalities of blood vessel function. Indeed, dietary Zn deficiency during foetal development[21] and growth[22] results in elevated blood pressure, and in adult hypertensive animals Zn deficiency exaggerates the elevated blood pressure[44], effects that are believed to be a consequence of increased oxidative stress resulting in endothelial impairment and reduced availability of nitric oxide. However, in normotensive adult animals subjected to dietary Zn deficiency blood pressure is unaffected and both endothelial nitric oxide synthase (eNOS) and SOD-1 expression is normal[55]. Although we did not measure arterial blood pressure in our dietary intervention study, the lack of effect of zinc deficiency on baseline coronary perfusion pressures, which gives an indication of vascular tone in the coronary resistance bed, and the normal responses to both and endothelium-dependent (MCh) and independent (SNP) vasodilator in blood vessels isolated from the ZD rats is consistent with the view that dietary Zn deficiency in normotensive adult animals does not impair vascular function, despite the pathological remodelling that has been demonstrated in previous studies[20,23].

While the impact of acute *in vitro* Zn depletion has been explored at the cellular level, very little has been done to determine the functional consequence in isolated intact blood vessels. Here we have shown that, in contrast to dietary intervention, exposure of mesenteric arteries to TPEN impairs both contractile and relaxant (endothelium-dependent and independent) responses, while the extra-cellular Zn chelator DPTA does not. Thus, in light of the observed impact of TPEN on cardiac SOD activity, the most likely cause of the reduced endothelium-dependent responses is the inhibition of SOD-1 (cytosolic isoform), since DPTA would only remove the zinc that would allow SOD-3 (located within the interstitial spaces) to operate, whereas TPEN would reduce Zn availability for both SOD-1 and SOD-3. This would also explain the reduced response to SNP, which donates a NO moiety that is as susceptible to degradation by ROS as endogenously produced NO. In relation to the attenuation of the contractile response seen with TPEN, although as a chelator it has a higher affinity for Zn than for other metals, it has been shown in other cell types to impair intracellular calcium release from the sarcoplasmic reticulum[56], albeit at much higher concentrations (50-500µM) that that used in the current study (10µM).

Conclusions

The principal findings from these studies are that both acute dietary Zn deficiency and acute *in vitro* Zn depletion worsen the extent of myocardial injury resulting from I/R while paradoxically protecting against severe ventricular arrhythmias. The mechanisms by which these effects are achieved, however, appear to differ. Dietary Zn deficiency results in a reduction in total cardiac GSH content, which predisposes the heart tissue to the widespread damaging effects of the oxidative stress induced during post-ischaemic reperfusion, while the predominant system that is affected by *in vitro* Zn depletion is the limited capacity to scavenge superoxide. Similarly, while *in vitro* Zn depletion has a marked effect on the ability of intact blood vessels to both contract and relax, dietary Zn deficiency does not. Again this appears to be related to the differing effects of the two interventions on SOD activity. Taken together these results have shown that dietary insufficiency of Zn presents a potential risk factor for a worse outcome following acute myocardial ischaemia, with the associated increased morbidity and mortality. Moreover, our findings with TPEN suggest that future studies into the role of zinc in cardiovascular physiology and pathophysiology should be carried out using a dietary intervention model, rather than an *in vitro* simulation of endogenous Zn depletion, to avoid misleading findings.

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Conflict of interests

None

Author contributions:

CL Wainwright designed the study and SK Walsh and JH Beattie contributed to the discussion; K Skene, SK Walsh, O Okafor & N Godsman performed the experimental work and data analysis; MJ Gordon performed the plasma analyses. CLW wrote the manuscript and SK Walsh, JH Beattie and K Skene reviewed and edited the manuscript.

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706

Figure Legends

Figure 1: Physiological alterations in response to a Zn-deficient diet in rats. Zn deficiency (ZD) resulted in a cyclical feeding pattern (A) that resulted in a slowed rate of growth (B). Pair feeding (PF) of rats a zinc adequate diet also slowed growth, but to a lesser extent. ZD also influenced heart (HW; C) and white adipose tissue (WAT;D) to body weight (BW) ratios. The impact of the ZD diet compared to PF and zinc adequate (ZA) on cardiac tissue (E) and plasma (F) Zn levels is also shown. Values are mean ± s.e.m; N=10 for panels A-D; n=4 for panels E&F. *P<0.05 compared to PF control animals.

Figure 2: The impact of a ZD diet on myocardial infarct size (A), caspase-3 activity (B), glutathione (GSH) content (C) and SOD-1 (Cu/Zn SOD) activity (D) in isolated hearts subjected t 30 min acute regional myocardial ischaemia and 2 h reperfusion. Values are mean ± s.e.m; n=10 per group. *P<0.05 ZD compared to PF controls; #P<0.05 PF compared with ZA controls.

Figure 3: The influence of a ZD diet on arrhythmia count (A), the incidence of ventricular tachycardia (VT and fibrillation (VF; B) and changes in coronary perfusion pressure (CPP: C) and hearts rate (HR; D) prior to and during the ischaemia/reperfusion protocol. Values are mean ± s.e.m; n=10 per group. #P<0.05 PF compared with ZA controls; *P<0.05 compared with pre-ischaemic ZD values.

Figure 4: Responses of mesenteric arteries from ZA, PF and ZD rats, pre-contracted with U46618 (EC_{80}), to the endothelium-dependent vasodilator methacholine (MCh; A) and the directly acting vasodilator sodium nitroprusside (SNP; B). Values are mean ± s.e.m; n=10 per group.

Figure 5: The effect of the Zn chelator TPEN (10μM) on myocardial infarct size (A), cardiac tissue Zn content (B), glutathione (GSH) content (C) and SOD-1 (Cu/Zn SOD) activity (D) and caspase-3 activity (B), in isolated hearts subjected to 30 min acute regional myocardial ischaemia and 2 h reperfusion. Values are mean ± s.e.m; n=10 per group. *P<0.05 compared to vehicle controls; #P<0.05 compared with sham controls.

Figure 6: The influence of TPEN (10μM) on arrhythmia count (A), the incidence of ventricular tachycardia (VT and fibrillation (VF; B) and changes in coronary perfusion pressure (CPP: C) and heart rate (HR; D) prior to and during the ischaemia/reperfusion protocol. Values are mean ± s.e.m; n=10 per group. *P<0.05 vs vehicle control.

Figure 7: The effects of TPEN (10μM; A & B) and the extracellular Zn chelator DPTA (10μM; C & D) on responses of mesenteric arteries to the endothelium-dependent vasodilator methacholine (MCh) and the directly acting vasodilator sodium nitroprusside (SNP). Values are mean ± s.e.m; n=10 per group. P<0.05 compared to control.

Supplementary Table 1:

The effect of a ZD diet, compared to a ZA and PF diet on plasma concentrations of Cu^{2+} and Ca^{2+}, and on plasma lactate and creatine kinase. Values are mean ± s.e.m; n=10 per group.

Supplementary Table 2:

The incidences of ventricular tachycardia (VT) and reversible (Rev VF) and irreversible (Irrev VF) ventricular fibrillation in rats fed either a ZA or a ZD diet, and in PF controls. Values are expressed as the number of animals from each group, with percentage incidences in parentheses).

Supplementary Table 3:

The incidences of ventricular tachycardia (VT) and reversible (Rev VF) and irreversible (Irrev VF) ventricular fibrillation in rat hearts treated with either TPEN (10μM) or vehicle prior to the onset of acute myocardial ischaemia. Values are expressed as the number of animals from each group, with percentage incidences in parentheses).

TABLE 1: Emax and EC50 values for responses to vasoactive agents in mesenteric arteries from ZA, ZD and PF rats. Values are mean ± s.e.m; n=10 per group.

Δ Tension (mN/mm)	**ZA**	**PF**	**ZD**
U46619	2.68±0.59	2.41±0.39	2.99±0.56
E_{max} (%)	**ZA**	**PF**	**ZD**
MCh	76.7±9.7	81.6±5.1	79.4±9.1
SNP	95.9±8.8	78.7±4.7	76.6±6.4
pEC_{50} (M)	**ZA**	**PF**	**ZD**
U46619	6.48	6.46	6.51
MCh	7.39	7.51	7.27
SNP	7.45	7.69	7.81

TABLE 2: Emax and EC50 values for responses to vasoactive agents in mesenteric arteries from ZA, ZD and PF rats. Values are mean ± s.e.m; n=10 per group.

		Δ Tension (mN/mm)	**E_{max} (%)**		**pEC_{50} (M)**	
		U44619	**MCh**	**SNP**	**MCh**	**SNP**
TPEN (1μM)	**Vehicle**	-	91.6±2.2	95.2±0.9	7.03	7.27
	TPEN	-	91.7±1.8	94.1±1.1	6.94	7.56
TPEN (10μM)	**Vehicle**	3.24±0.32	86.6±2.1	74.5±4.2	7.04	7.04
	TPEN	1.46±0.11*	68.2±3.4*	52.6±8.7*	6.52*	6.54*
DPTA (10μM)	**Vehicle**	5.84±0.83	72.4±6.9	87.8±3.5	7.01	7.18
	DPTA	5.87±0.90	80.1±6.2	86.4±4.6	6.86	7.32
*P <0.05 vs. vehicle						

FIGURE 1:

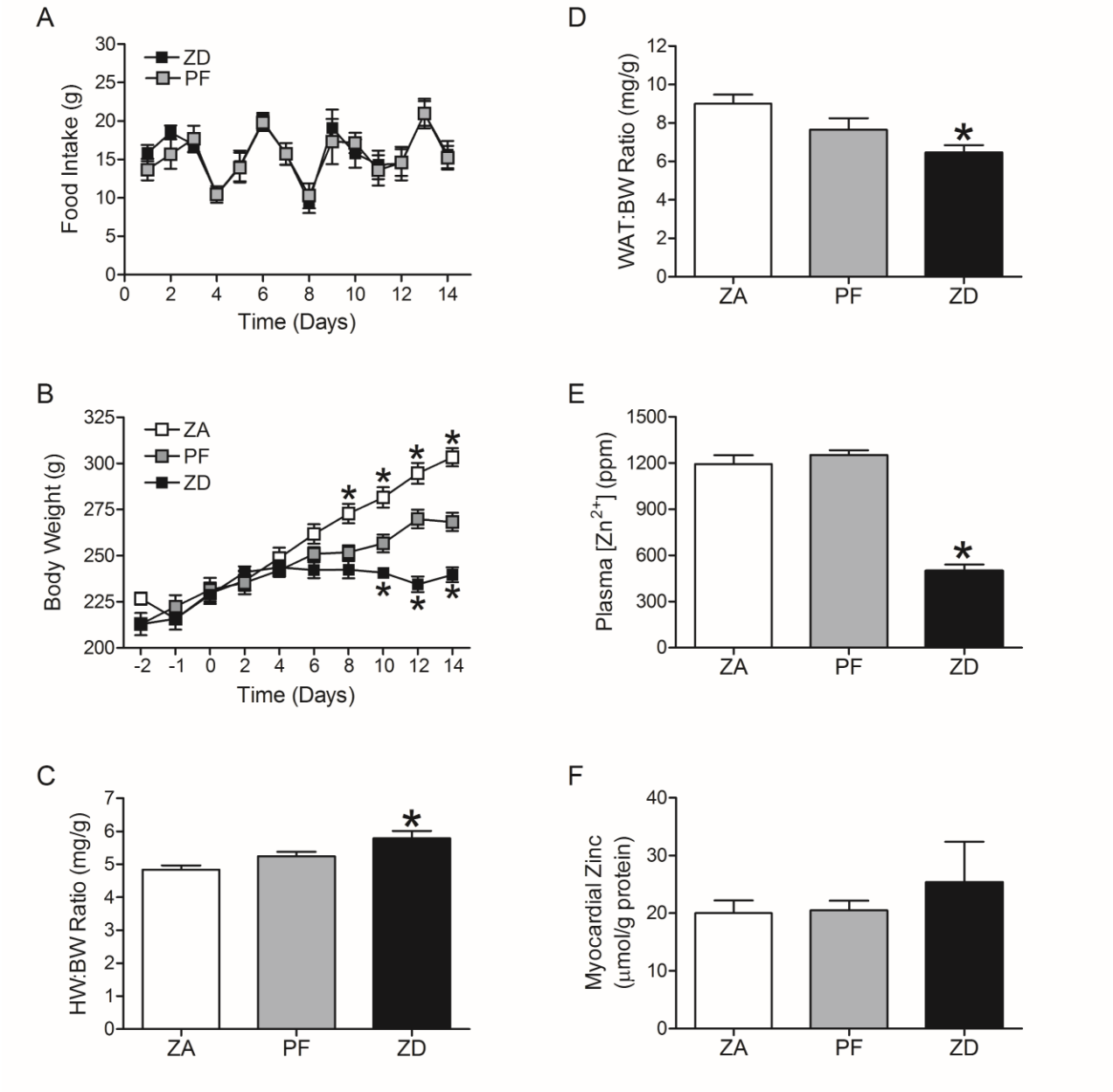

A
Food Intake (g)
ZD
PF
Time (Days)
B
Body Weight (g)
ZA
PF
ZD
Time (Days)
C
HW:BW Ratio (mg/g)
ZA
PF
ZD
D
WAT:BW Ratio (mg/g)
ZA
PF
ZD
E
Plasma [Zn2+] (ppm)
ZA
PF
ZD
F
Myocardial Zinc (µmol/g protein)
ZA
PF
ZD

FIGURE 2:

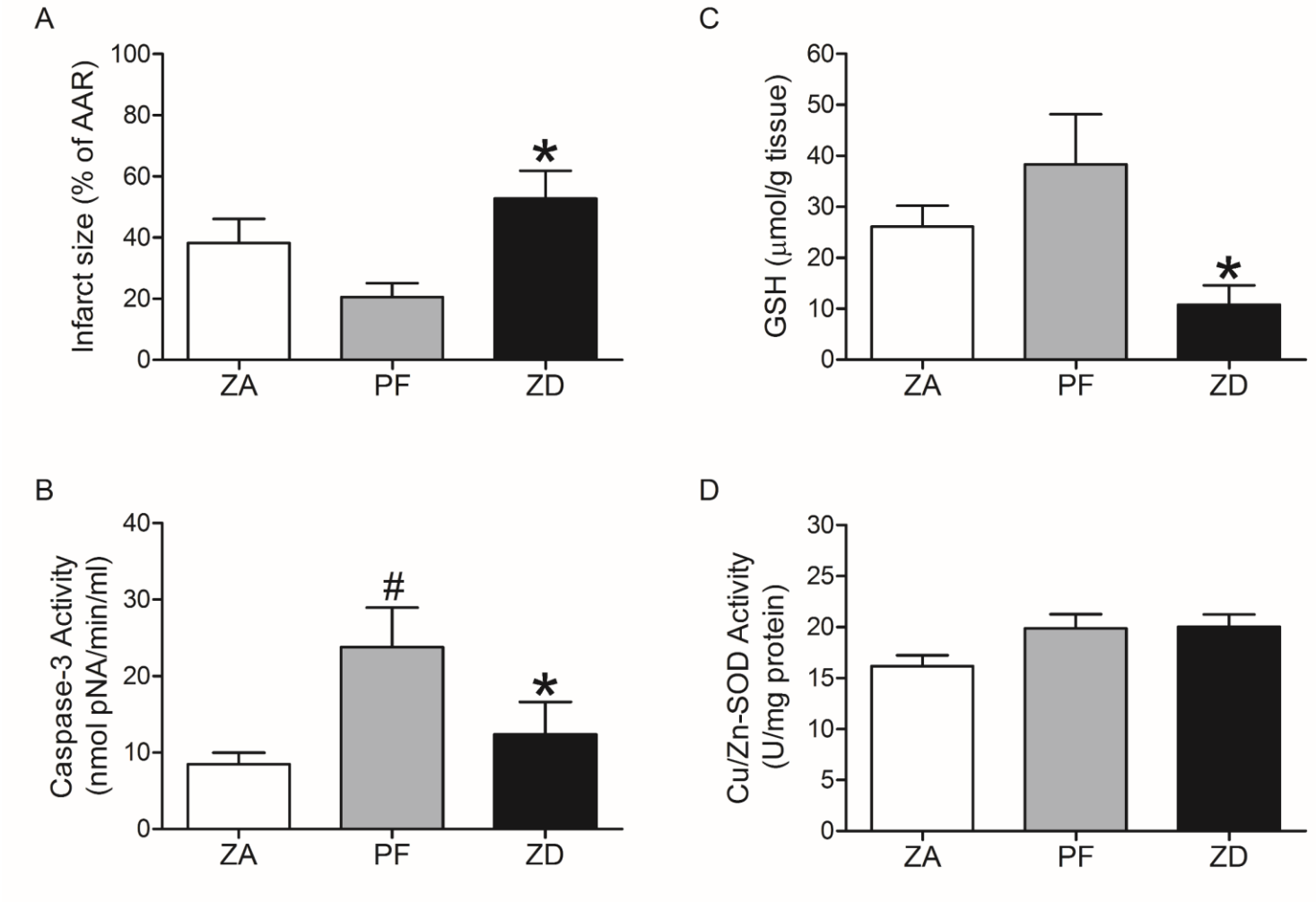

A
Infarct size (% of AAR)
100
80
60
40
20
0
*
ZA
PF
ZD
C
GSH (µmol/g tissue)
60
50
40
30
20
10
0
*
ZA
PF
ZD
B
Caspase-3 Activity
(nmol pNA/min/ml)
40
30
20
10
0
#
*
ZA
PF
ZD
D
Cu/Zn-SOD Activity
(U/mg protein)
30
25
20
15
10
5
0
ZA
PF
ZD

FIGURE 3:

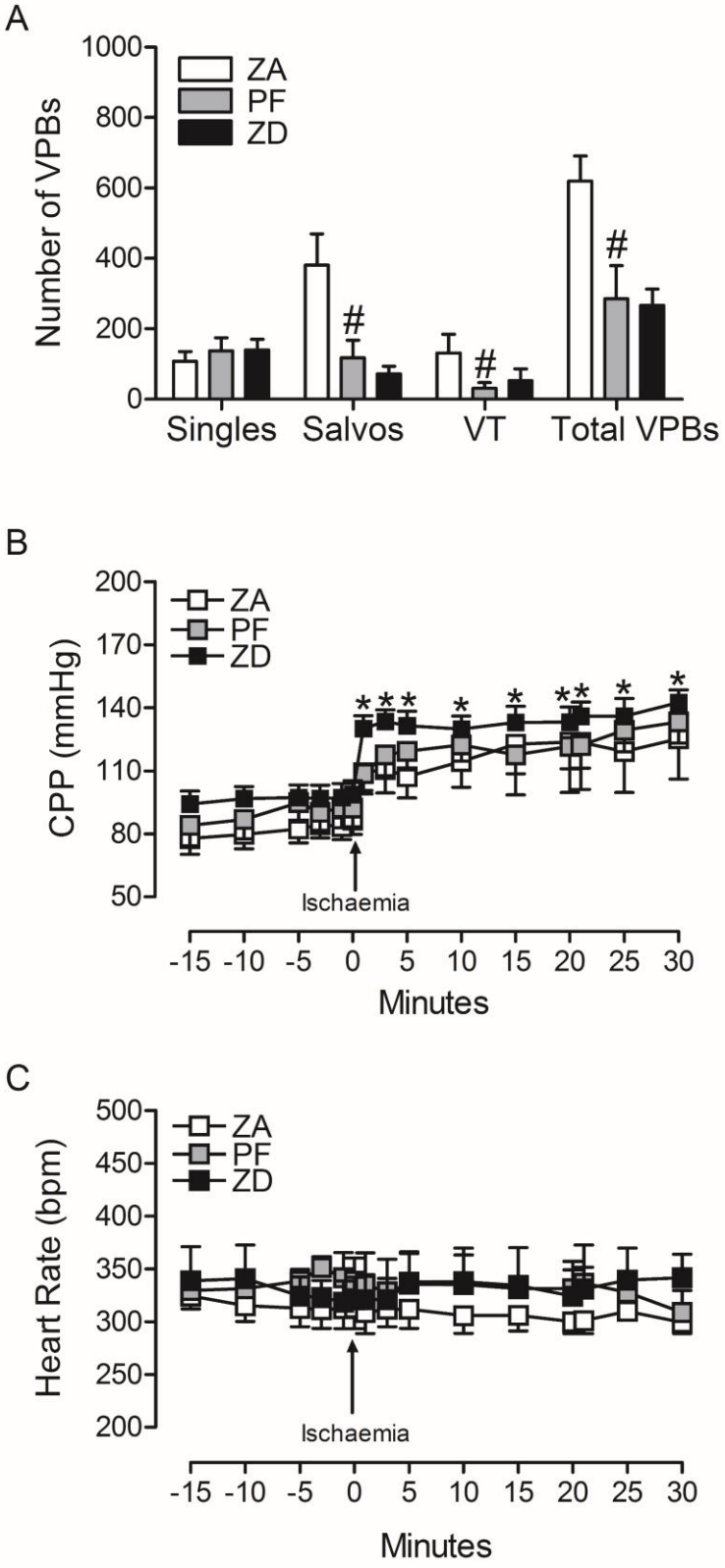

A
Number of VPBs
1000
800
600
400
200
0
ZA
PF
ZD
#
#
#
Singles
Salvos
VT
Total VPBs
B
CPP (mmHg)
200
170
140
110
80
50
ZA
PF
ZD
Ischaemia
-15 -10 -5 0 5 10 15 20 25 30
Minutes
C
Heart Rate (bpm)
500
450
400
350
300
250
200
ZA
PF
ZD
Ischaemia
-15 -10 -5 0 5 10 15 20 25 30
Minutes

FIGURE 4:

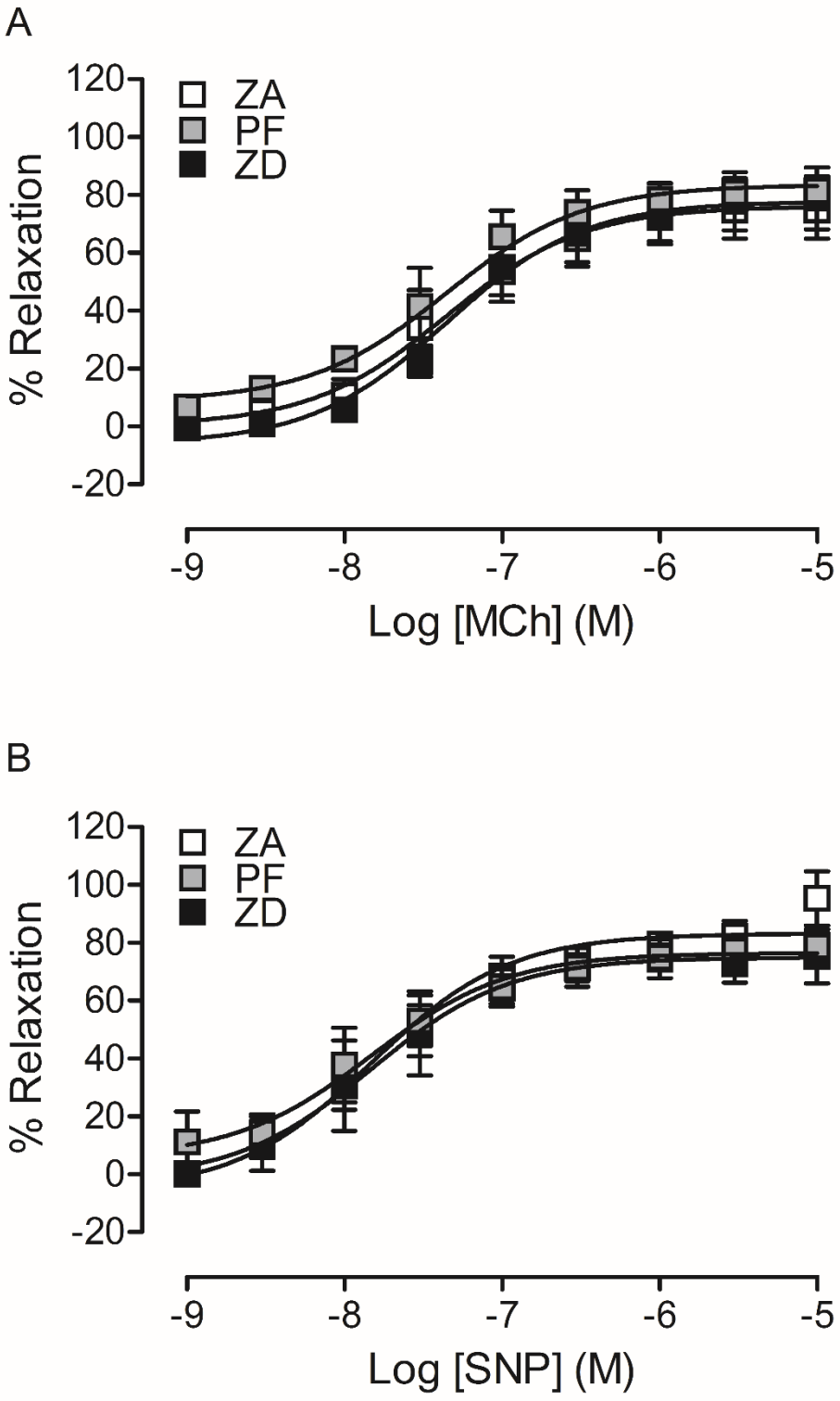

A
120
100
80
60
40
20
0
-20
% Relaxation
ZA
PF
ZD
-9
-8
-7
-6
-5
Log [MCh] (M)
B
120
100
80
60
40
20
0
-20
% Relaxation
ZA
PF
ZD
-9
-8
-7
-6
-5
Log [SNP] (M)

Figure 5

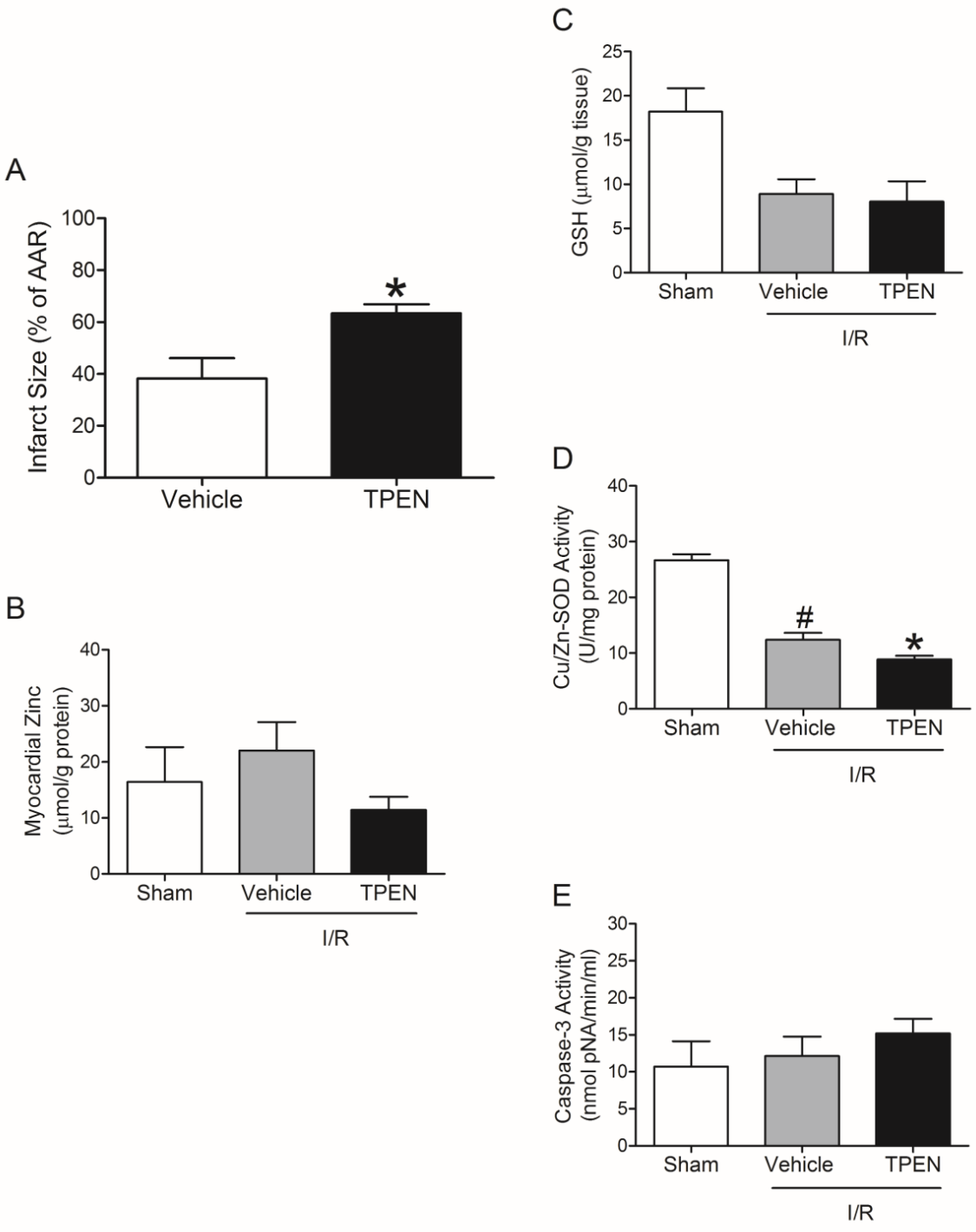

A
Infarct Size (% of AAR)
100
80
60
40
20
0
*
Vehicle
TPEN
B
Myocardial Zinc (μmol/g protein)
40
30
20
10
0
Sham
Vehicle
TPEN
I/R
C
GSH (μmol/g tissue)
25
20
15
10
5
0
Sham
Vehicle
TPEN
I/R
D
Cu/Zn-SOD Activity (U/mg protein)
40
30
20
10
0
#
*
Sham
Vehicle
TPEN
I/R
E
Caspase-3 Activity (nmol pNA/min/ml)
30
25
20
15
10
5
0
Sham
Vehicle
TPEN
I/R

FIGURE 6:

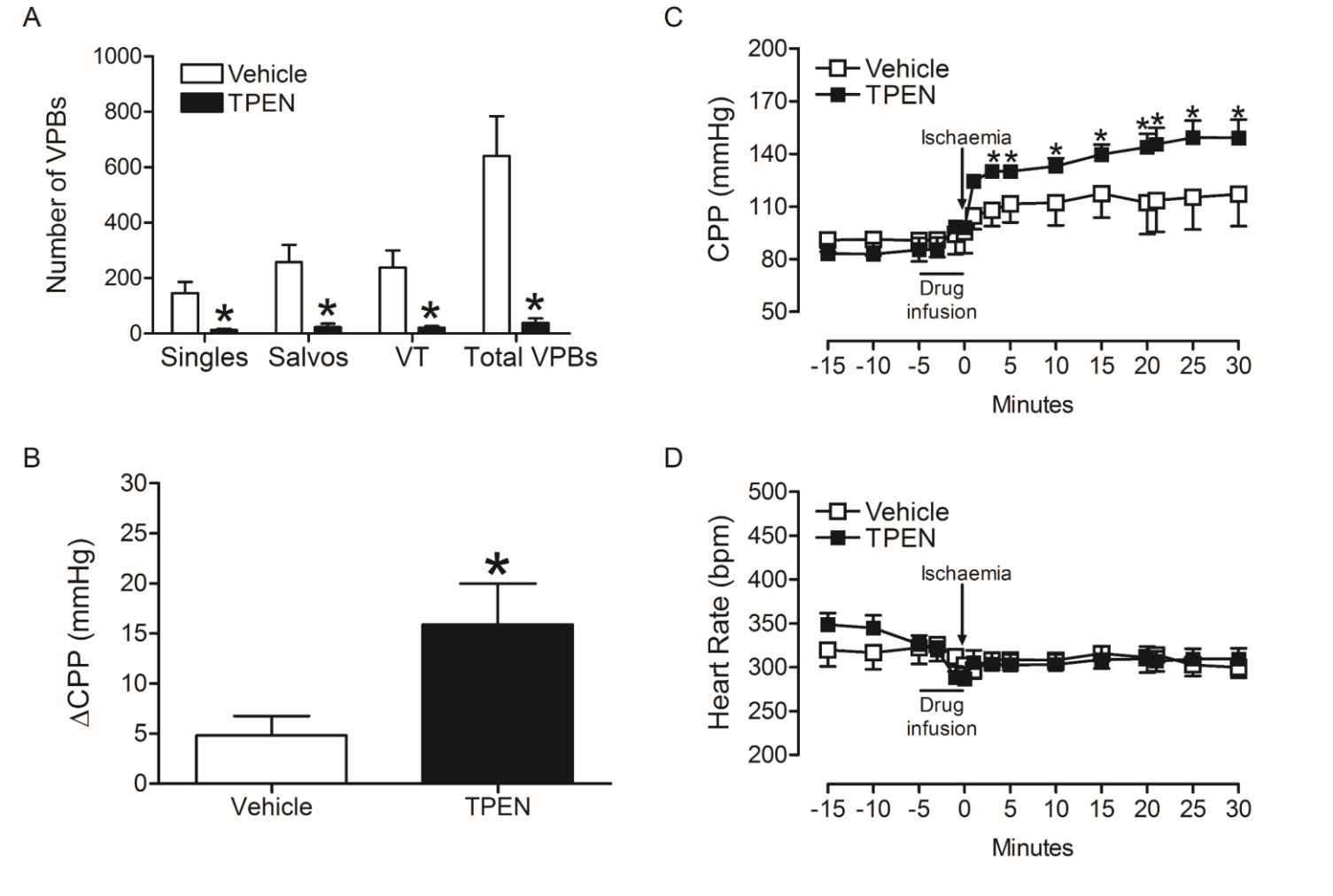
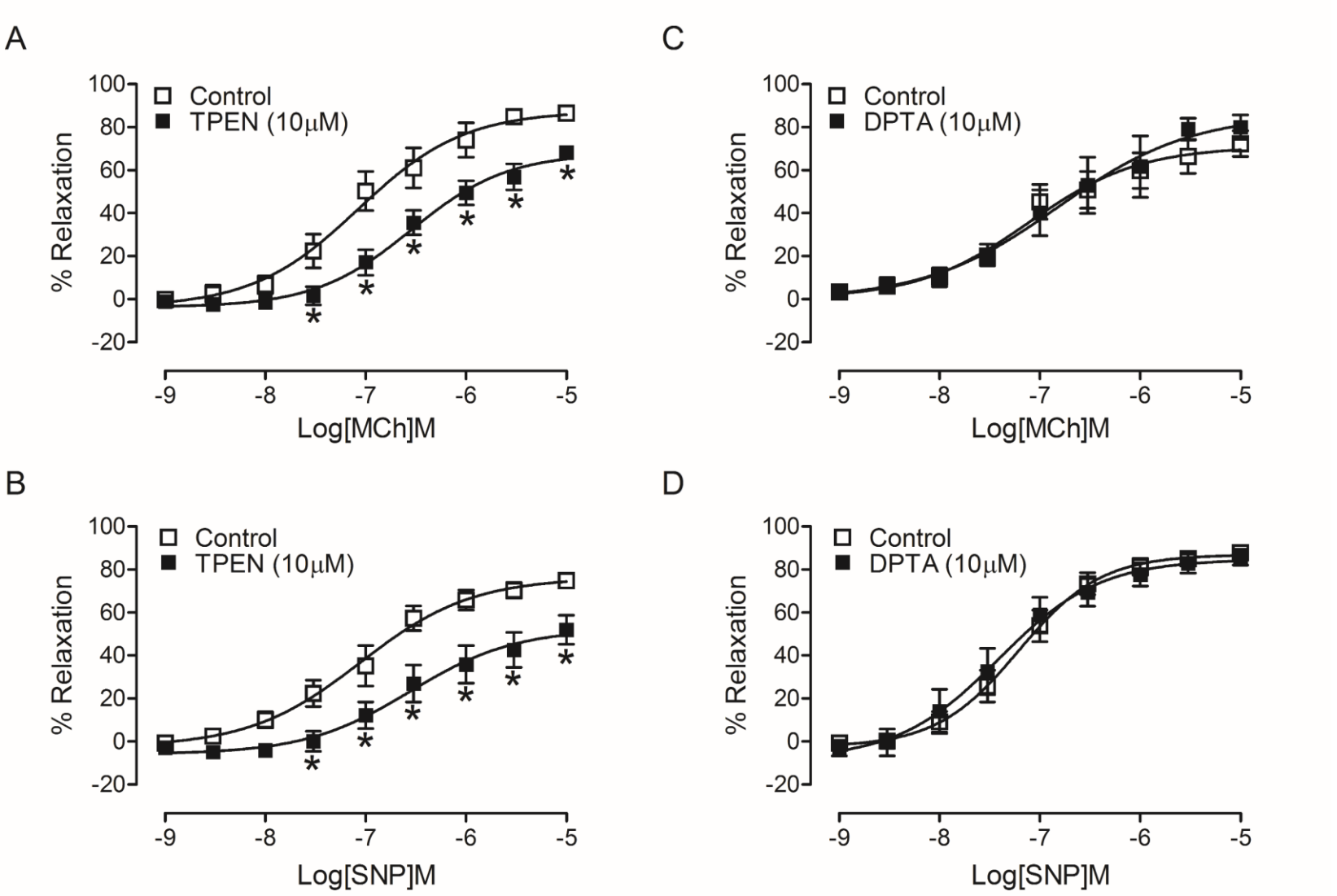

A
Number of VPBs
1000
800
600
400
200
0
Vehicle
TPEN
*
Singles
Salvos
VT
Total VPBs
B
ΔCPP (mmHg)
30
25
20
15
10
5
0
*
Vehicle
TPEN
C
CPP (mmHg)
200
170
140
110
80
50
Vehicle
TPEN
Ischaemia
Drug infusion
-15 -10 -5 0 5 10 15 20 25 30
Minutes
D
Heart Rate (bpm)
500
450
400
350
300
250
200
Vehicle
TPEN
Ischaemia
Drug infusion
-15 -10 -5 0 5 10 15 20 25 30
Minutes

FIGURE 7:


A
Control
TPEN (10μM)
% Relaxation
Log[MCh]M
C
Control
DPTA (10μM)
% Relaxation
Log[MCh]M
B
Control
TPEN (10μM)
% Relaxation
Log[SNP]M
D
Control
DPTA (10μM)
% Relaxation
Log[SNP]M

SUPPLEMENTARY TABLE 1:

	ZA	PF	ZD
Cu^{2+} (ppb)	675±26	670±36	677±13
Ca^{2+} (ppb)	94±5	86±4	86±3
Lactate (mmol/L)	4.21±0.82	3.42±0.60	2.02±0.66
Creatine Kinase (U/L)	454±109	714±86	914±213

SUPPLEMENTARY TABLE 2:

Incidence	ZA	PF	ZD
VT	6/7 (86%)	5/8 (63%)	6/9 (67%)
Rev VF	0/7 (0%)	0/8 (0%)	0/9 (0%)
Irrev VF	2/9 (22%)	0/8 (0%)	0/9 (0%)

SUPPLEMENTARY TABLE 3:

Incidence	Vehicle	TPEN
VT	9/10 (90%)	3/8 (38%)*
Rev VF	1/10 (10%)	0/8 (0%)
Irrev VF	1/11 (9%)	0/8 (0%)
*P <0.05 vs. vehicle		