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# Acute dietary zinc deficiency in rats exacerbates myocardial ischaemia-reperfusion injury through depletion of glutathione.

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1 **Acute dietary zinc deficiency in rats exacerbates myocardial ischaemia/reperfusion**  
2 **injury through depletion of glutathione**

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22  
23 **Running title: Dietary zinc deficiency and cardiac injury**

24 **Keywords:** Dietary zinc deficiency; Myocardial ischaemia/reperfusion injury; Glutathione;  
25 TPEN; Vascular function

27 **Abstract**

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

47

## 48 **Introduction**

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

57 Zn is important in cardiovascular physiology as it acts as an indirect antioxidant, serves to  
58 stabilise membrane structure and regulates metallothionein (MT) levels (reviewed in<sup>(5)</sup>). The  
59 antioxidant role of Zn is achieved through the requirement of Zn for superoxide dismutase 1  
60 (SOD1) activity, its protection of thiols and its ability to inhibit the Fenton reaction (which  
61 converts H<sub>2</sub>O<sub>2</sub> to hydroxyl radicals) by competing with iron<sup>(5)</sup>. Zn is also a physiological  
62 suppressor of apoptosis<sup>(6)</sup>. Given this essential role in maintaining cellular homeostasis,  
63 disruptions in cellular Zn levels (or alterations in intracellular signalling events involving Zn)  
64 could result in a loss of antioxidant capacity of tissues and may induce, or at least facilitate,  
65 the induction of apoptotic cell death. Consequently, disruption of Zn homeostasis is likely to  
66 play an important role in cardiovascular diseases.

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

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

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

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

108

## 109 **Methods**

### 110 *Animals*

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

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

130

### 131 *Dietary intervention study*

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

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

152

### 153 *Coronary artery occlusion/reperfusion in the isolated heart*

154 Rats were anaesthetised with pentobarbital sodium salt (100 mg kg<sup>-1</sup> i.p; Sigma Aldrich,  
155 Poole, Dorset, UK) and the heart rapidly removed and arrested in ice cold Krebs's Henseleit  
156 buffer (KHB; 119mM NaCl, 4.7mM KCl, 1.18mM KH<sub>2</sub>PO<sub>4</sub>, 2.41mM MgSO<sub>4</sub>, 25mM  
157 NaHCO<sub>3</sub>, 2.52mM CaCl<sub>2</sub> and 10.88mM Glucose; pH7.4). After placement of a ligature (6-0  
158 silk suture (W812), Ethicon, Edinburgh) around the left coronary artery, the aorta was  
159 cannulated for retrograde perfusion on a Langendorff apparatus (AD Instruments). Hearts  
160 were perfused with KHB at 37°C at a rate of 12ml/min and allowed to stabilize for 15  
161 minutes prior to drug administration and subsequent coronary occlusion. The coronary artery  
162 was occluded (CAO) by tightening the ligature to induce regional ischemia for 30 minutes  
163 after which the ligature was loosened and the myocardium reperfused for 2 hours. A surface  
164 electrocardiogram (ECG) was recorded via electrodes placed on the right atrium and left  
165 ventricle and coronary perfusion pressure (CPP) recorded via a pressure transducer (MLT844  
166 physiological pressure transducer; AD Instruments) connected to the mounting head of the  
167 Langendorff apparatus. Ventricular arrhythmias that occurred during the ischaemic period  
168 were analysed according to the Lambeth Conventions<sup>(28)</sup>. Heart rate (HR; calculated from the  
169 ECG), ECG, and CPP were all monitored continuously throughout the experimental period  
170 using a Power Lab data acquisition system via an Animal Bio Amplifier and Bridge  
171 Amplifier, respectively, and data subsequently analysed using Chart Software (all equipment  
172 and software from AD Instruments). Any hearts which developed spontaneous arrhythmias  
173 prior to CAO were excluded from the study. Hearts from rats included in the dietary  
174 intervention study were used to determine the effect of acute dietary Zn depletion on the  
175 outcome or I/R. To determine the impact of acute *in vitro* Zn depletion, either vehicle (0.01%  
176 DMSO; n=10) or TPEN (10µM; concentration chosen based on previously published work in  
177 isolated heart studies<sup>(14,29)</sup>) was infused (at a rate of 100µL/min) into isolated hearts via the

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

180

### 181 *Histological measurement of infarct size*

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

195

### 196 *Isometric myography*

197 Once hearts were mounted on the Langendorff apparatus and in the stabilisation period, the  
198 mesenteric arterial arcade was excised and placed in ice cold KHB. Third order mesenteric  
199 arteries were then dissected out, cleaned of perivascular fat, and stored in KHB overnight at  
200  $4^{\circ}\text{C}$ . Vascular function was then assessed in isolated mesenteric arteries mounted onto a two-  
201 channel wire myograph (Model 510A, Danish Myo Technology (DMT), Denmark)  
202 containing oxygenated (95%  $\text{O}_2$  & 5%  $\text{CO}_2$ ) KHB at  $37^{\circ}\text{C}$ . Vessels were normalised to  
203 achieve a transmural pressure of 100mmHg using the DMT Normalisation software.  
204 Isometric tension was recorded and displayed using a PowerLab and Chart Software (both  
205 AD Instruments). The viability of the smooth muscle was tested via the addition of an 80mM  
206 KCl solution. Following KHB washes, a cumulative concentration response was carried out  
207 with the thromboxane mimetic, U46619 (9,11-Dideoxy-11 $\alpha$ ,9 $\alpha$ -epoxymethanoprostaglandin  
208 F2 $\alpha$ ; Tocris Bioscience, UK). Vessels were then precontracted with a submaximal  
209 concentration ( $\text{EC}_{80}$ ) of U46619 and cumulative concentration responses carried out with



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

217

#### 218 *Myocardial Cu/Zn-SOD activity*

219 Cu/Zn-SOD activity was measured in cardiac tissue samples (representing both ischaemic  
220 and non-ischaemic tissue) from all experimental groups using a Superoxide Dismutase Assay  
221 Kit (catalogue number: 706002; Cayman Chemical). Briefly, cardiac tissue was homogenised  
222 in ice cold 20mM HEPES buffer (pH 7.2) containing 1mM EGTA, 210mM mannitol, and  
223 70mM sucrose. The resulting homogenate was then subjected to multiple centrifugation steps  
224 to separate mitochondrial and cytosolic SOD, the latter of which was retained for subsequent  
225 analysis. Following protein quantification, protein concentrations were normalised to  
226 0.5mg/ml in all samples and Cu/Zn-SOD activity measured via the kit as per the  
227 manufacturer's instructions.

228

#### 229 *Myocardial glutathione (GSH) content*

230 Total GSH content was measured in cardiac tissue using a Glutathione Assay Kit (catalogue  
231 number: 703002; Cayman Chemical). Briefly, cardiac tissue (representing both ischaemic and  
232 non-ischaemic tissue) was homogenised in ice cold MES buffer (pH 6.0) containing 200mM  
233 2-(N-morpholino) ethanesulphonic acid, 50mM phosphate, and 1mM EDTA. The resulting  
234 homogenate was then centrifuged at 10,000g for 15 minutes at 4°C, the supernatant removed  
235 and subsequently deproteinated via the addition of MPA reagent (1.25M metaphosphoric  
236 acid). Samples containing MPA reagent were then centrifuged at 3,000g for 2 minutes at  
237 room temperature, the supernatant removed and TEAM reagent (4M triethanolamine) added  
238 before assaying for GSH content as per the manufacturer's instructions.

239

240

241

242 *Myocardial caspase-3 activity*

243 Caspase-3 activity in heart tissue was determined by measuring conversion of the caspase-3  
244 substrate N-Acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-Pna) to p-nitroaniline.  
245 Briefly, tissue samples (representing both ischaemic and non-ischaemic tissue) were  
246 homogenised in ice cold HEPES buffer (25mM; pH 7.4) containing 5 mM EDTA, 2 mM  
247 DTT and 0.1% CHAPS and centrifuged at 20,000g for 30 minutes at 4°C). The supernatant  
248 (5µl) was then incubated with 85µl HEPES buffer (50mM; pH 7.4, containing 1.0mM EDTA,  
249 10mM DTT, 0.1% Chaps, 100mM NaCl and 10% glycerol) and 10µl Ac-DEVD-Pna  
250 (200µM) at 37°C for 24 hours; the concentration of the product (p-nitroaniline) was read at  
251 405nm.

252

253 *Myocardial and plasma zinc levels*

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

262

263 *Statistical analyses*

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

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

295

## 296 **Results**

### 297 *Impact of dietary zinc depletion on physiological measures*

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

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

310

### 311 *Dietary Zn depletion and the outcome of myocardial ischaemia/reperfusion*

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

326

### 327 *Dietary Zn depletion and vascular function*

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

332

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

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

336 was similar in both groups ( $43\pm 3\%$  vs  $45\pm 3\%$  of LV area in control and TPEN-treated hearts,  
337 respectively). Treatment with TPEN did not induce any significant reduction in tissue Zn  
338 levels (Figure 5b). The induction of I/R itself cause a reduction in both GSH levels (Figure  
339 5c) and Cu/Zn-SOD activity (Figure 5d) when compared to sham hearts, whereas I/R did not  
340 alter caspase-3 activity (Figure 5e). There was no impact of TPEN treatment on GSH content  
341 or caspase-3 activity, however in hearts given TPEN there was a significant reduction in  
342 Cu/Zn-SOD activity compared to control I/R hearts (Figure 5d;  $P<0.05$ ). TPEN also  
343 markedly reduced the number of ventricular arrhythmias occurring as single VPBs, salvos  
344 and VT (Figure 6a), and significantly reduced the incidence of VT but had no effect on the  
345 development of ventricular fibrillation (VF; Supplementary Table 3). Prior to coronary  
346 occlusion, TPEN induced a rise in CPP (Figure 6b), which was maintained throughout the  
347 period of regional ischaemia (Figure 6c). In contrast, neither the administration of TPEN nor  
348 the induction of regional ischaemia significantly altered heart rate in any of the isolated hearts  
349 (Figure 6d).

350

#### 351 *Acute in vitro Zn depletion and vascular function*

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

359

## 360 **Discussion**

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

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

369

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

371 Induction of dietary Zn deficiency for 14 days led to a slowed increase in body weight  
372 resulting from the reduced food intake, leading to lower fat accumulation (reduced WAT;BW  
373 ratio) and an increase in heart:BW ratio. In ZD rats, plasma Zn was markedly reduced (by  
374 >50%), while other blood markers that could influence infarct size ( $\text{Cu}^{2+}$  and  $\text{Ca}^{2+}$ , lactate  
375 and CK levels; Supplemental Data Table 1) were unaffected; thus, any difference between the  
376 ZD rats and the ZA/PF rats can be attributed to alterations in plasma Zn status alone.  
377 However, dietary Zn deficiency did not significantly alter cardiac tissue levels of Zn which,  
378 although perhaps surprising, agrees with other studies<sup>(16,17)</sup>. Notwithstanding this, it is worthy  
379 of note that the tissue Zn levels reported here represent protein-bound Zn, as Zn levels were  
380 undetectable in deproteinated samples (lower detection limit of the assay was 0.5nmol per  
381 sample), indicating that there was no detectable free Zn to participate in Zn-dependent  
382 cellular process such as glutathione synthesis (see below).

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

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

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

401

#### 402 *Zn and I/R injury*

403 While there are substantial data to support the notion that supplementation with exogenous  
404 Zn in the setting of I/R is cardioprotective, relatively little is known about the importance of  
405 endogenous Zn in the development of myocardial injury<sup>(5)</sup>. Endogenous Zn has been reported  
406 to be both cardioprotective, by acting as an intracellular messenger that translates the  
407 signalling process in NO-mediated cardioprotection<sup>(35)</sup>, and detrimental, through activation of  
408 ERK/GSK3 $\beta$  to trigger cardiomyocyte death<sup>(36)</sup> in I/R. However very few studies have  
409 determined whether depletion of endogenous zinc levels can influence the outcome of I/R.  
410 In this study we have shown that both acute dietary Zn deficiency and acute *in vitro* Zn  
411 depletion increase infarct size following I/R injury in isolated hearts, which is consistent with  
412 the notion that Zn is cardioprotective. Since endogenous Zn is known to play an important  
413 role in maintaining redox status within tissues<sup>(37)</sup>, we also determined whether Zn deficiency  
414 upset the redox balance in cardiac tissue as a possible mechanism for the increase in tissue  
415 injury.

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

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

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

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

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



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

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

473

#### 474 *Zn and ventricular arrhythmias*

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

494

495 *Vascular effects of Zn depletion*

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

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

526

527 *Conclusions*

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

544

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549

550 **Conflict of interests**

551 None

552 **Author contributions:**

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

557

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- 706



707 **Figure Legends**

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

715

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

721

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

727

728 **Figure 4:** Responses of mesenteric arteries from ZA, PF and ZD rats, pre-contracted with  
729 U46618 (EC<sub>80</sub>), to the endothelium-dependent vasodilator methacholine (MCh; A) and the  
730 directly acting vasodilator sodium nitroprusside (SNP; B). Values are mean  $\pm$  s.e.m; n=10 per  
731 group.

732

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

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

742

743 **Figure 7:** The effects of TPEN (10 $\mu$ M; A & B) and the extracellular Zn chelator DPTA  
744 (10 $\mu$ M; C & D) on responses of mesenteric arteries to the endothelium-dependent vasodilator  
745 methacholine (MCh) and the directly acting vasodilator sodium nitroprusside (SNP). Values  
746 are mean  $\pm$  s.e.m; n=10 per group. P<0.05 compared to control.

747

#### 748 **Supplementary Table 1:**

749 The effect of a ZD diet, compared to a ZA and PF diet on plasma concentrations of Cu<sup>2+</sup> and  
750 Ca<sup>2+</sup>, and on plasma lactate and creatine kinase. Values are mean  $\pm$  s.e.m; n=10 per group.

751

#### 752 **Supplementary Table 2:**

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

757

#### 758 **Supplementary Table 3:**

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

763

764

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

<b><math>\Delta</math> Tension (mN/mm)</b>	<b>ZA</b>	<b>PF</b>	<b>ZD</b>
U46619	2.68 $\pm$ 0.59	2.41 $\pm$ 0.39	2.99 $\pm$ 0.56
<b><math>E_{max}</math> (%)</b>	<b>ZA</b>	<b>PF</b>	<b>ZD</b>
MCh	76.7 $\pm$ 9.7	81.6 $\pm$ 5.1	79.4 $\pm$ 9.1
SNP	95.9 $\pm$ 8.8	78.7 $\pm$ 4.7	76.6 $\pm$ 6.4
<b>pEC<sub>50</sub> (M)</b>	<b>ZA</b>	<b>PF</b>	<b>ZD</b>
U46619	6.48	6.46	6.51
MCh	7.39	7.51	7.27
SNP	7.45	7.69	7.81

767

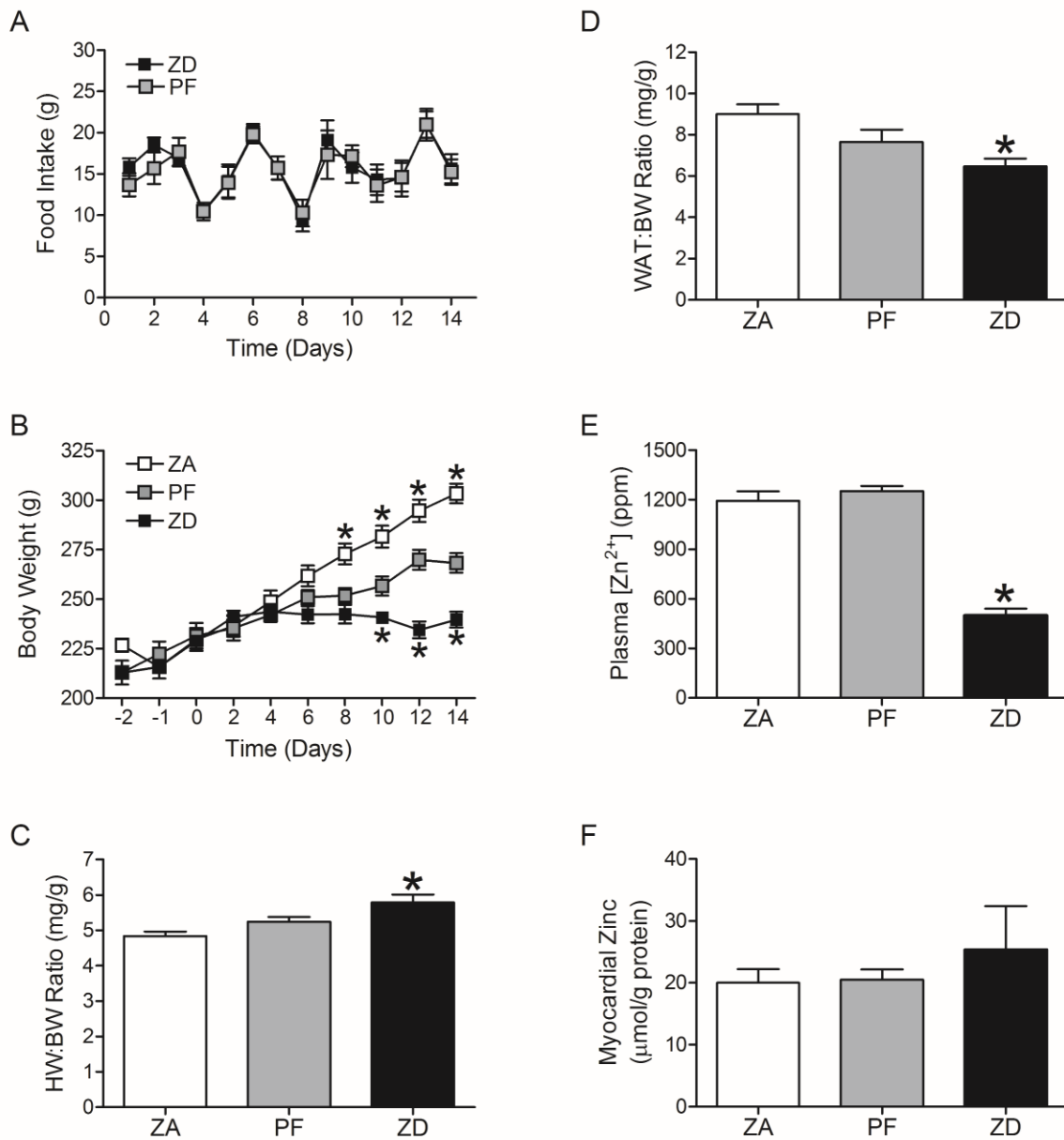
768

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

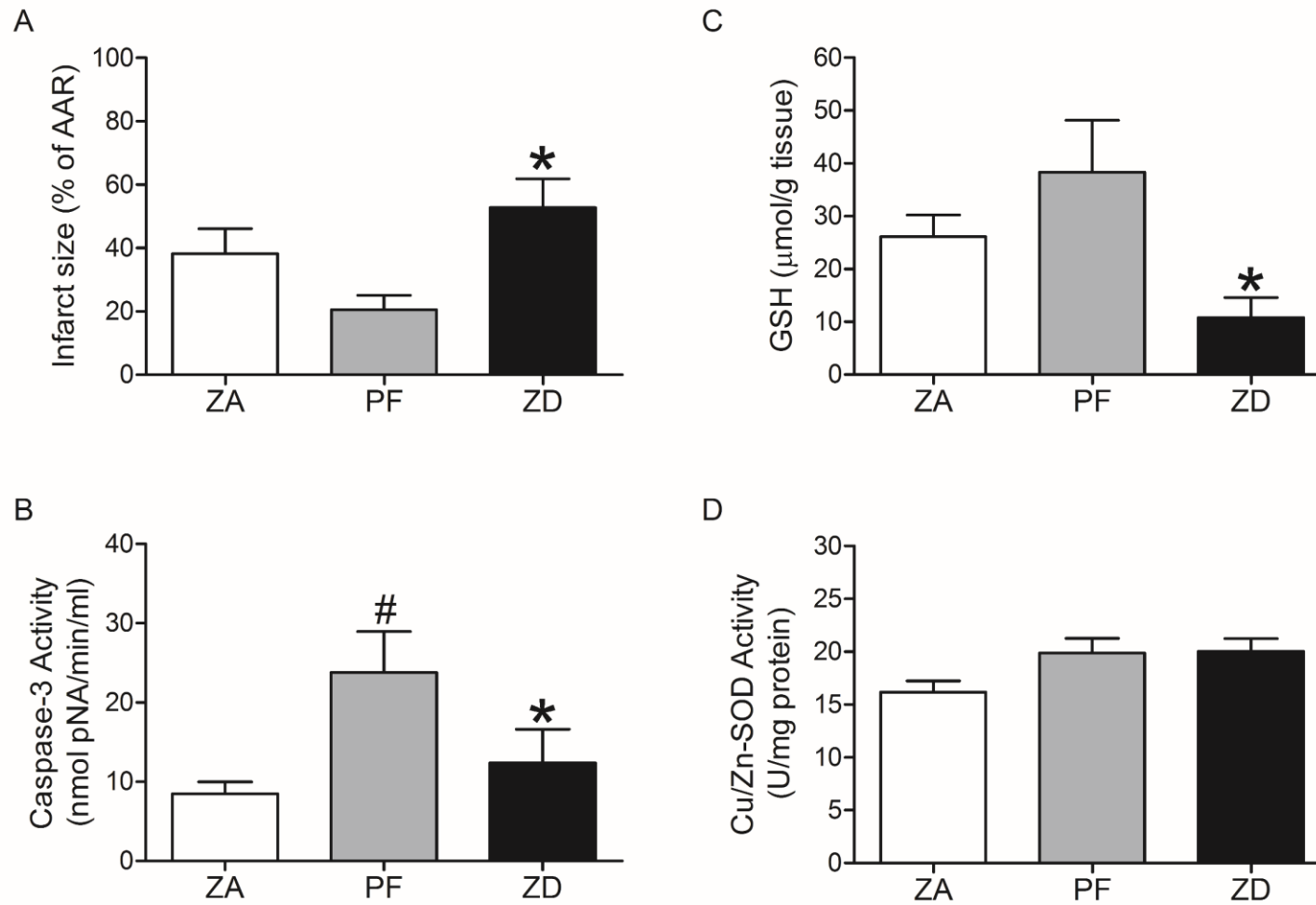
		<b><math>\Delta</math> Tension (mN/mm)</b>	<b><math>E_{max}</math> (%)</b>		<b>pEC<sub>50</sub> (M)</b>	
		<b>U44619</b>	<b>MCh</b>	<b>SNP</b>	<b>MCh</b>	<b>SNP</b>
<b>TPEN (1<math>\mu</math>M)</b>	<b>Vehicle</b>	-	91.6 $\pm$ 2.2	95.2 $\pm$ 0.9	7.03	7.27
	<b>TPEN</b>	-	91.7 $\pm$ 1.8	94.1 $\pm$ 1.1	6.94	7.56
<b>TPEN (10<math>\mu</math>M)</b>	<b>Vehicle</b>	3.24 $\pm$ 0.32	86.6 $\pm$ 2.1	74.5 $\pm$ 4.2	7.04	7.04
	<b>TPEN</b>	1.46 $\pm$ 0.11*	68.2 $\pm$ 3.4*	52.6 $\pm$ 8.7*	6.52*	6.54*
<b>DPTA (10<math>\mu</math>M)</b>	<b>Vehicle</b>	5.84 $\pm$ 0.83	72.4 $\pm$ 6.9	87.8 $\pm$ 3.5	7.01	7.18
	<b>DPTA</b>	5.87 $\pm$ 0.90	80.1 $\pm$ 6.2	86.4 $\pm$ 4.6	6.86	7.32

\* $P < 0.05$  vs. vehicle

**FIGURE 1:**

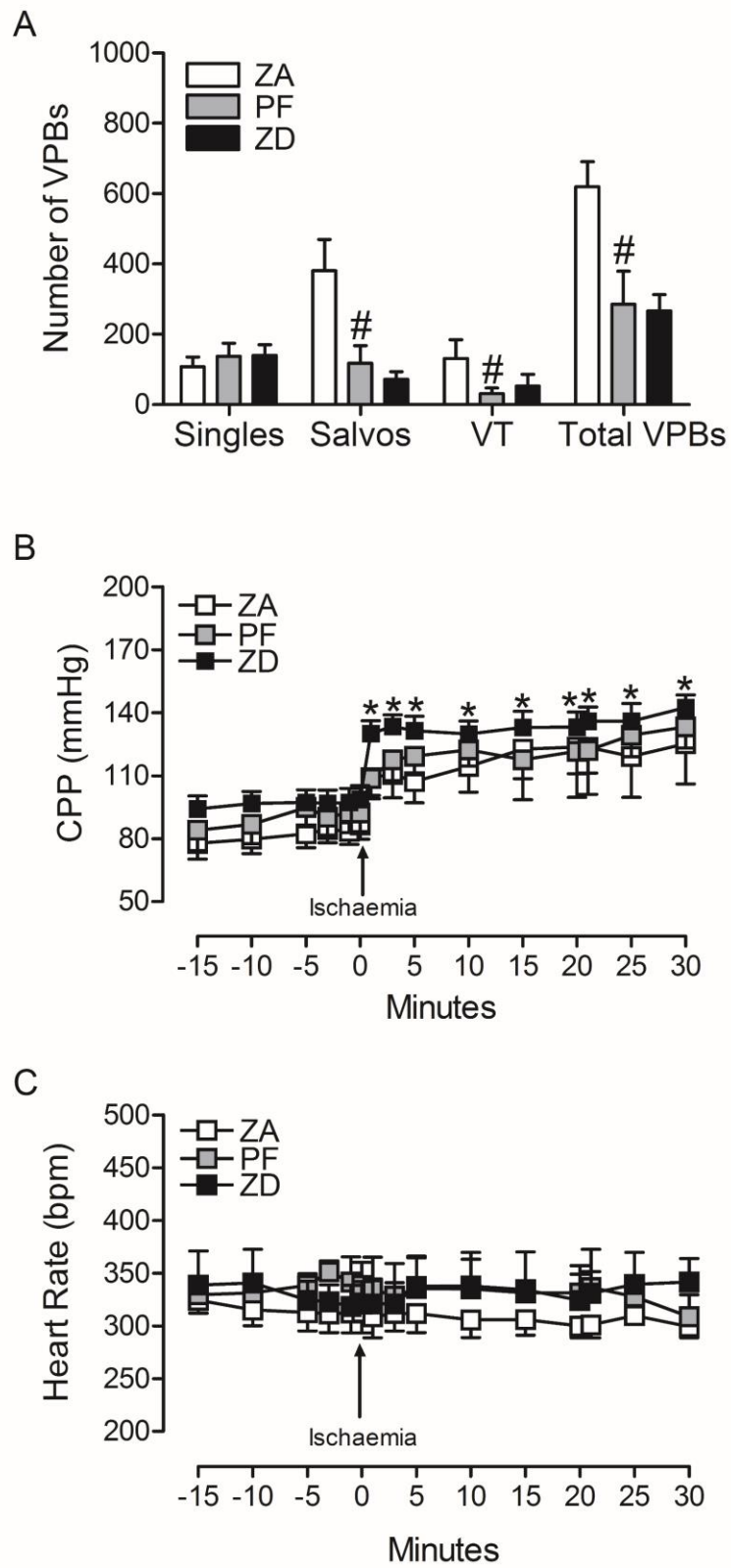


**FIGURE 2:**





**FIGURE 3:**



**FIGURE 4:**

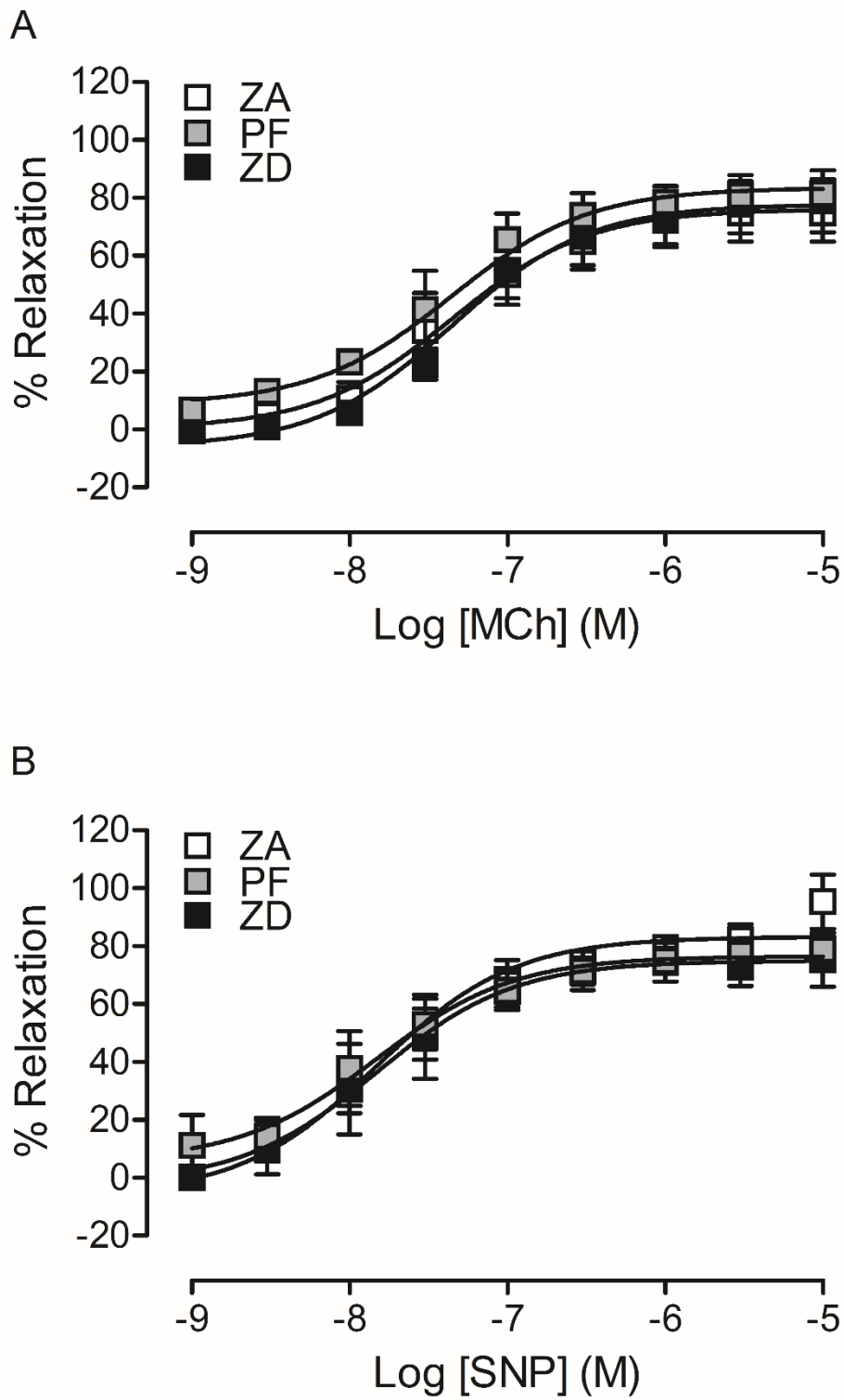
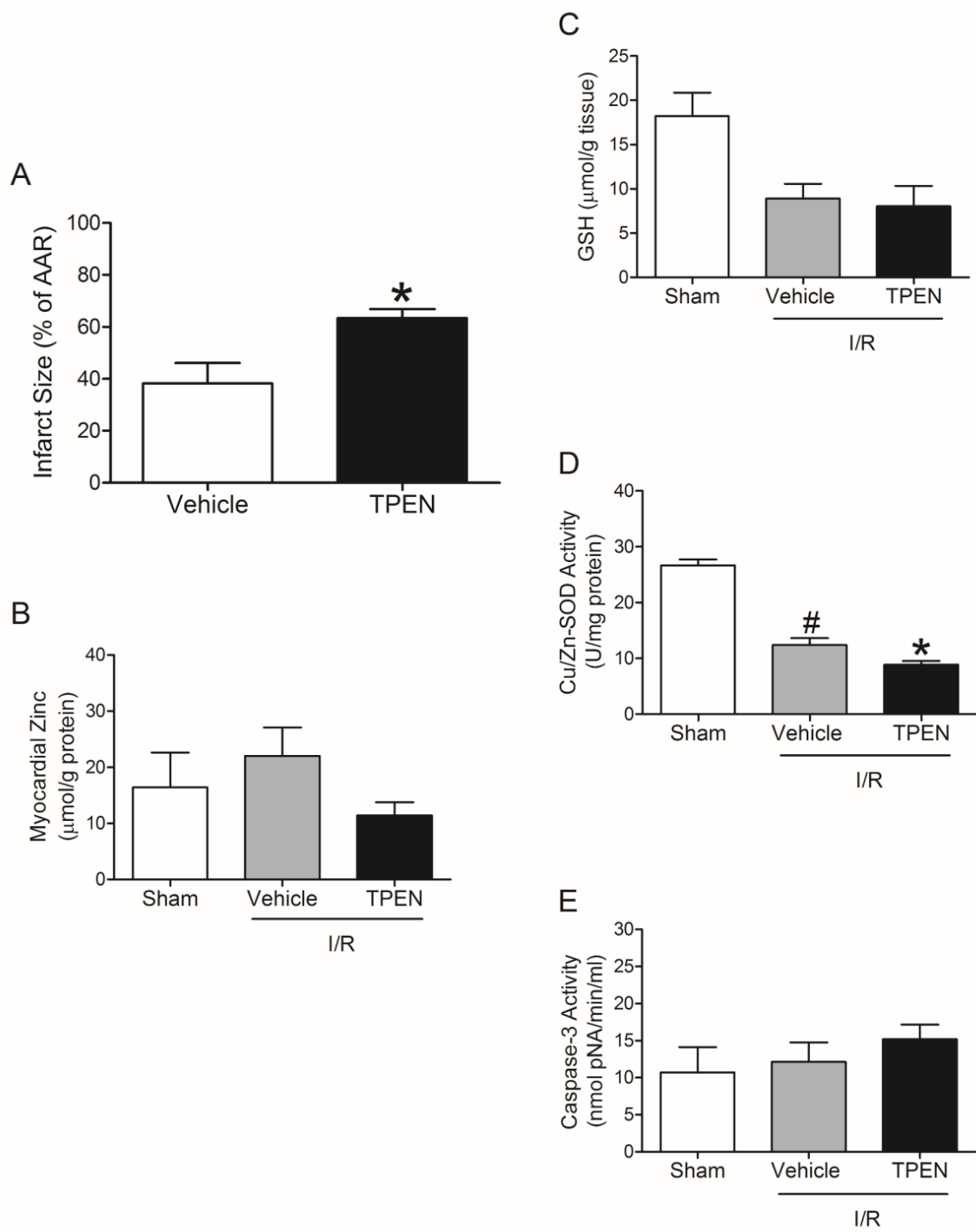
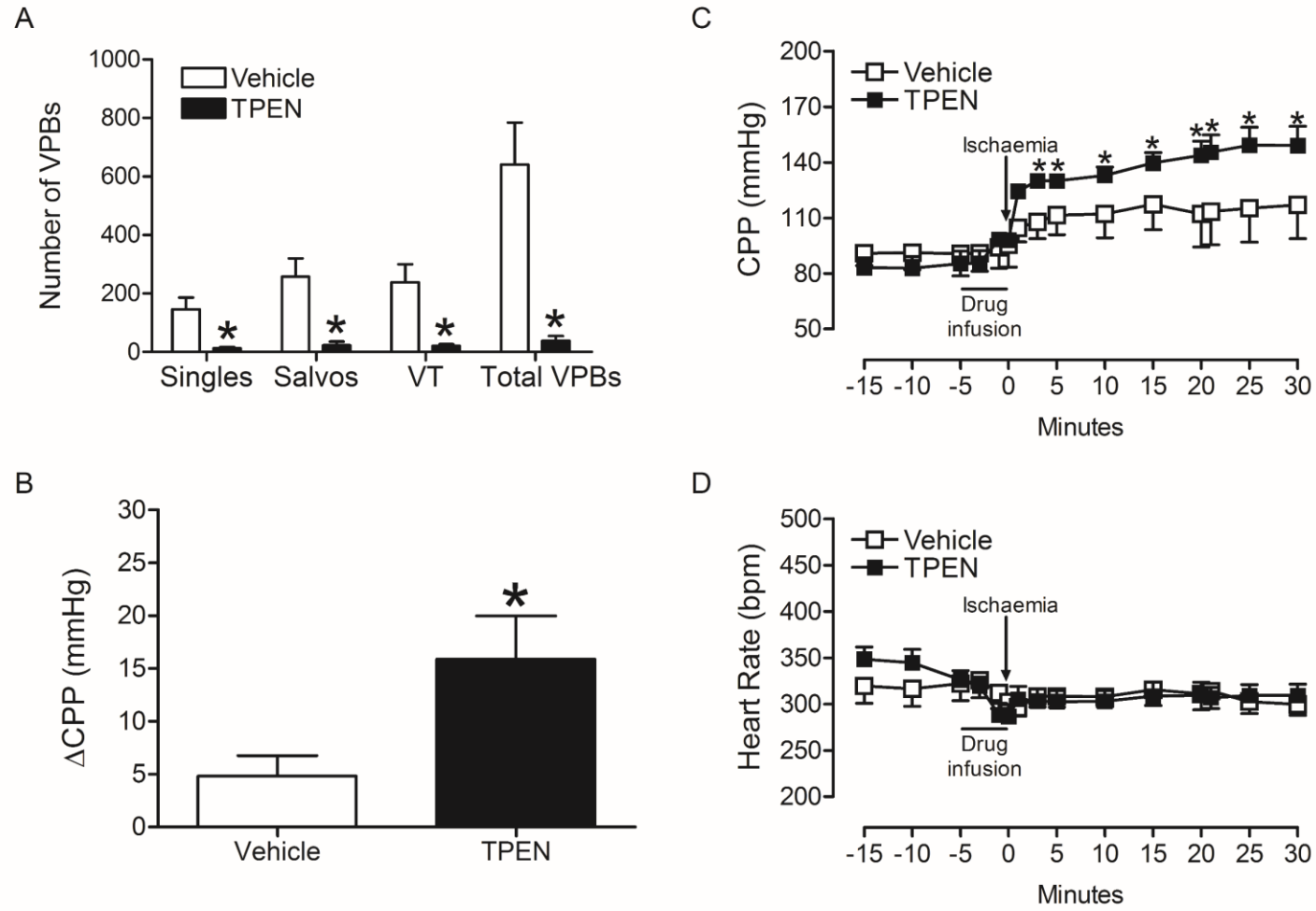




Figure 5



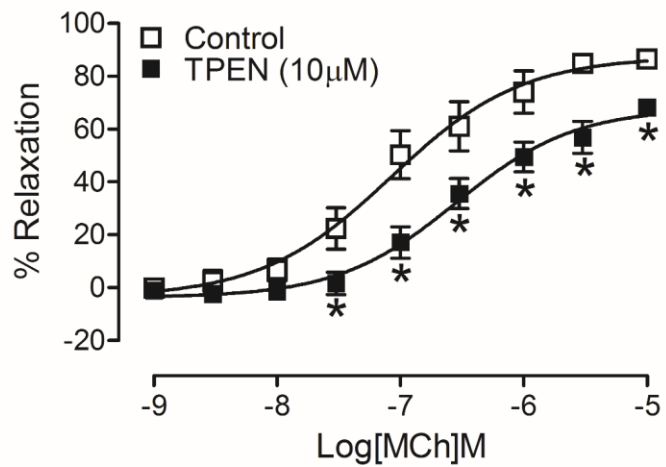
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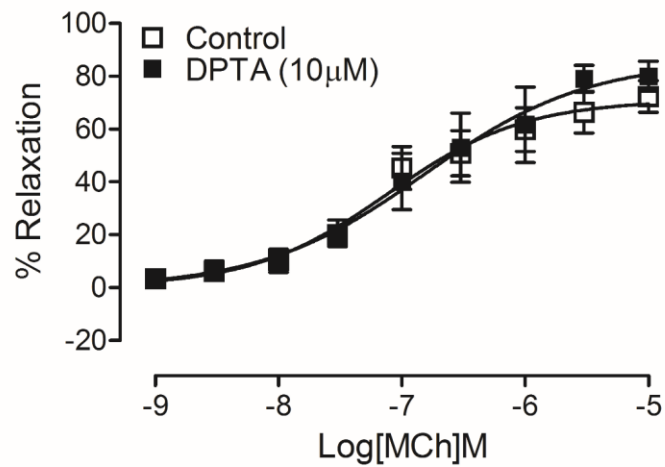


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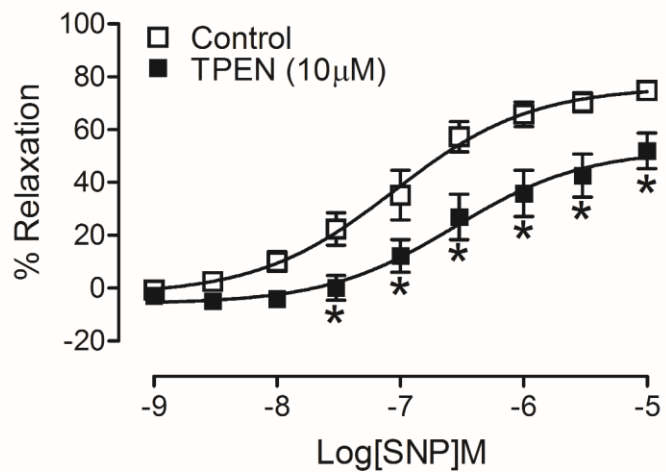
**A**



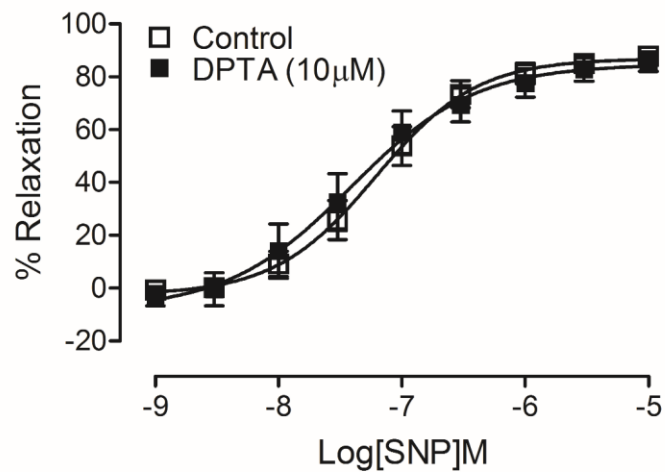
**C**



**B**



**D**



**SUPPLEMENTARY TABLE 1:**

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

**SUPPLEMENTARY TABLE 2:**

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

**SUPPLEMENTARY TABLE 3:**

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