Novel cardioprotective strategies for the uraemic heart.

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NOVEL CARDIOPROTECTIVE STRATEGIES FOR THE URAEMIC HEART

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Abstract

Cardiovascular disease is the leading cause of death in patients with underlying chronic kidney disease (CKD). Up to one third of patients presenting with an acute coronary syndrome have CKD stage 3-5. Outcomes following acute myocardial infarction in patients with underlying CKD remain poor. CKD patients are routinely excluded from clinical trials in novel cardioprotective strategies resulting in a paucity of prospective data on which to base guidelines for clinical practice.

The aims of this work were to:

- Establish and characterise two models of chronic uraemia in rodents: the subtotal nephrectomy model and the adenine diet model.
- Determine the effects of underlying chronic uraemia on myocardial ischaemia tolerance.
- Examine pharmacological cardioprotective strategies in the context of underlying uraemia using a PARP inhibitor
- Investigate the cardioprotective effects of ischaemic conditioning in the context of uraemia. Ischaemic preconditioning and postconditioning protocols were used in both uraemic and non-uraemic animals in a model of acute myocardial infarction.
- Preliminary work, using standard molecular biological techniques, was carried out in order to confirm the putative survival pathways responsible for the effect of preconditioning.
- Investigate the effect of combining early and late remote ischaemic preconditioning to identify whether summation of these strategies could provide additional tissue protection in a model of acute kidney injury.

The results demonstrate that both models develop a uraemic phenotype. Subtotal nephrectomy animals exhibit reduced ischaemia tolerance. PARP inhibition as a pharmacological post conditioning agent was shown to be ineffective at conferring tissue protection, whereas both ischaemic preconditioning and postconditioning were effective cytoprotective strategies in both non-uraemic and uraemic animals. Furthermore, additional benefit was seen when early and late remote preconditioning were summated in a rodent model of acute kidney injury.

This work provides a basis for future clinical trials in cardioprotection in the context of underlying CKD.

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Statement

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Finally Conor Byrne: we developed the uraemic models together and investigated ischeamia tolerance of the uraemic heart and the impact of uraemia on ischaemic preconditioning as a team.
Any ideas or quotations from the work of other people are fully acknowledged in accordance with the standard referencing practices of the discipline.

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Chapter 1

Introduction

Introduction

Heart disease in the general population

Cardiovascular disease is the leading cause of death in the western world. Cardiovascular disease accounts for 1 in 2.8 deaths[5], and causes more deaths than cancer, lung disease, accidents and diabetes combined[5]. Death rates from cardiovascular disease rose year on year peaking in the 1980s, before falling by approximately 20% over the last 30 years[6]. The incidence of cardiovascular events increases with age: from 3 per 1000 men aged 35-44 to 74 per 1000 from men aged 85-94[7]. Coronary heart disease makes up over half of all cardiovascular events. Incidence rates of new coronary events in a middle aged American cohort are between 4 and 12 events per 100 person years[2, 8]. Death rates from cardiac events have fallen over the last 40 years due to medical interventions such as revascularisation therapies, heart failure treatments, and new pharmacological agents and also life style changes in the population[9]. However despite this, the one year mortality following an acute myocardial infarction (AMI) is between 8-14%[4, 6].

Risk Factors for coronary heart disease in the general population

Hypertension

Hypertension is the most prevalent risk factor for cardiovascular disease, with 30% of Americans having this diagnosis[10]. Risk factors for the development of hypertension include age, ethnicity, a family history of hypertension, obesity, and lower socioeconomic status[6]. Hypertension has been defined as the level of blood pressure, which is associated with increased morbidity and mortality when compared to the general population. Current guidelines for hypertension suggest that a clinic blood pressure of >140 systolic or >90 diastolic are indicative of hypertension in an adult without co-morbidities[11].

The 'HOT'[12] study demonstrated benefits in cardiovascular outcomes with BP lowering to a diastolic of 82.6mm/hg. However further studies have indicated that lower targets are associated with better outcomes in people with additional co-morbidities such as diabetes[12] and renal disease[13]. Furthermore, studies looking at the cardiovascular effects

of antihypertensive treatment demonstrate that cardiovascular protection may go beyond absolute BP reduction in patients with additional co-morbidities using specific agents such as ACE inhibitors[14] or angiotensin receptor blockers[15].

Smoking

Cigarette smoking results in a 2-3-fold risk of dying from CHD, and on average smokers die 14 years younger than non-smokers. If a person stops smoking within 5 years the excess cardiovascular risk is approximately halved, and after 10 years the risk approaches that of life long non smokers[16].

Dyslipidaemia

There is a correlation between serum cholesterol and mortality[17]. The Framingham cohort results report that cholesterol levels remain static in men with age. Women however have lower cholesterol levels until menopause, when cholesterol levels rise and over take men at the 6th decade[18]. Over the last 25 years it has be well established that lowering of the serum cholesterol (with particular emphasis on the LDL component) reduces cardiovascular mortality [19]

The relative risk of coronary heart disease associated with physical inactivity ranges from 1.5-2.4: an increase comparable with that of smoking, hypertension or dyslipidaemia [20], with 12.2 % of the global burden of CVD risk is attributable to physical inactivity [21].

Obesity

The obesity epidemic has yet to fully manifest itself: the prevalence of BMI-for-age values at or above the 95th centile in adolescents has increased from 6.1% to 17.6% over the last 30 years [22]. Data from the NHANES database reveal that obesity was attributable with 13% of CVD deaths [23]. Abdominal obesity is strongly associated with hypertension, dyslipidaemia and insulin resistance, which are themselves risk factors for cardiovascular disease[24].

Diabetes

Underlying diabetes predisposes to CVD[25] and worsens the outcome of CVD with 2-4 times the disk of cardiovascular death compared to non-diabetic patients[26]. Diabetes confers the same risk of death as 2 of the 3 major coronary risk factors: smoking, hypertension and dyslipidaemia[27]. Furthermore diabetes tends to co-localise with other risk factors such as obesity, hypertension and dyslipidaemia, acting synergistically to increase risk further[28] .

The uraemic heart

Chronic kidney disease

Chronic kidney disease is a condition caused by loss of renal function characterised by elevations in serum creatinine concentrations. However the kidney does not only excrete creatinine: it is involved in fluid balance, acid base balance, blood pressure homeostasis, regulation of calcium and phosphate homeostasis, regulation of haemoglobin synthesis and excretion of toxic metabolites. Because of these functions, patients with CKD display a wide variety of physiological perturbations impacting on almost every organ system.

End stage renal failure is said to occur when the kidney function declines to a level where the patient is severely symptomatic from renal failure and the initiation of dialysis is needed to alleviate the metabolic complications, without which the patient would die either from fluid overload or hyperkalaemia. CKD has been divided into 5 stages[29] by glomerular filtration rate (GFR), which is defined as the volume of fluid filtered by the glomeruli into Bowman's space in ml/hr. CKD stage 1 and 2 represents no or very minimal reduction in GFR, but with urinary abnormalities such as haematuria or proteinuria. Stages 3-5 CKD are defined solely by GFR: stage 3 CKD: GFR 30-60 ml/hr, stage 4: 15-30ml/hr and stage 5 CKD is GFR is <15ml/hr or on dialysis.

The incidence of CKD and ESRD has increased by 40% in the last 10 years[30]. The prevalence of CKD stages 1-5 is around 12-15% in a large western cohort [30]. The prevalence of CKD rises with age, from 8.5% of those under 40 to almost 40% of those over 60 years of age[31]. A higher prevalence of CKD is associated with socioeconomic

status, a lower educational status and ethnicity, with Mexican and non-Hispanic black people having over twice the prevalence of CKD compared to the white population[31].
Risk factors for CKD include many of the traditional risk factors for CVD such as hypertension, diabetes, age, male sex, smoking and vascular disease. However, even after adjusting for these risk factors, underlying CKD is associated with excess cardiovascular risk[32]. Thus patients with CKD are likely to be at high risk of CVD already, before taking into account additional CKD specific risk factors.

Outcomes of Chronic kidney disease

The 5 year survival on haemodialysis is 40.3%[30], which is worse than many of the most common cancers such as breast, colon and non-Hodgkins lymphoma. Lindner et al.[33] were the first group to report that patients with end-stage renal failure had excessive cardiovascular mortality. One third of CKD patients have evidence of atheroma both at autopsy[34] and on angiography[35]. However, even accounting for this, uraemic patients have a 4-5 times higher 1 year mortality following AMI than non uraemic patients[36].
Foley et al.[37] reported that in incident haemodialysis patients, 40% had a history of ischaemic heart disease and heart failure and 75% had LVH on echocardiography. Foley at al. found that after adjusting for race, gender and age, cardiovascular mortality was on average 10-20 times that for an age-matched control (see Figure 1). This increased risk was particularly strong in the younger cohort where the cardiovascular mortality was over 500 times that of an age-matched control.

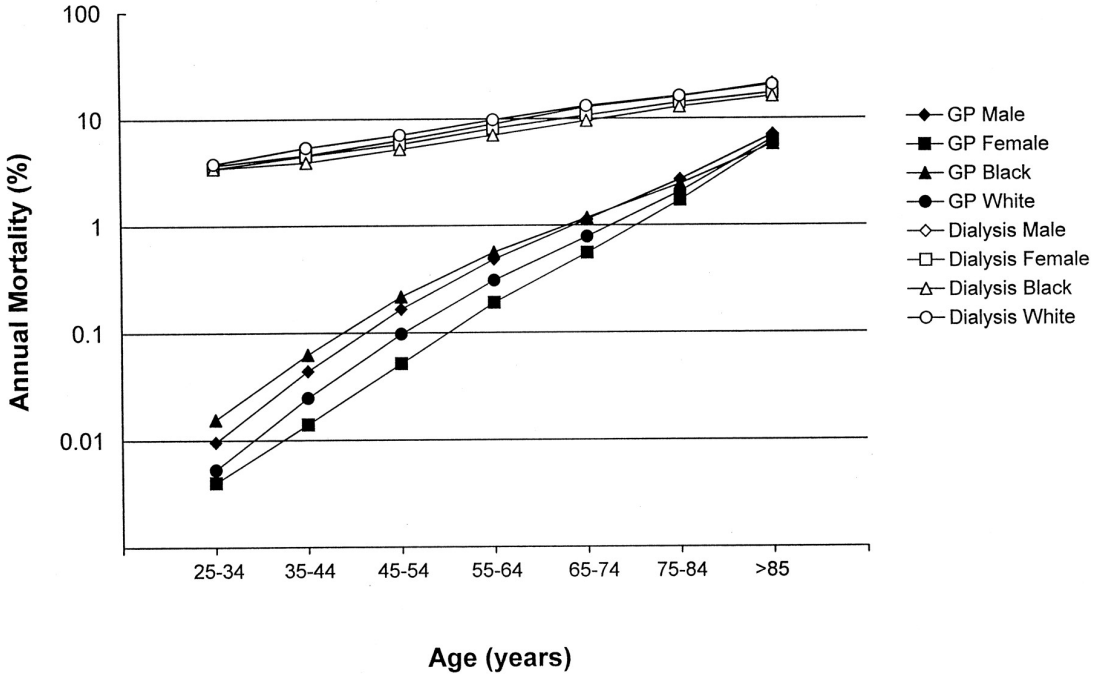


Figure 1. Seminal figure from work by Foley et al.[3] demonstrating the cardiovascular mortality in the general population compared to dialysis patients

Management of CVD in the CKD/ESRD population

Diagnosis of CVD in CKD/ESRD

Difficulties exist in the diagnosis of an acute myocardial event in a CKD/ESRD patient. Chest pain may be absent in the context of a diabetic patient, or may occur as a result of anaemia. A reduction in exercise tolerance may be due to anaemia, fluid overload or metabolic acidosis. Resting ECG abnormalities may be due to volume overload, LVH or electrolyte disturbances.

Cardiac enzyme rise, in the form of troponin, is one of the hallmarks of myocardial infarction. However, small elevations may be found on routine testing in patients with renal disease[38]. It is unclear whether these baseline elevations are due to low grade myocardial ischaemia/apoptosis or whether they represent reduced clearance of the troponin fragments which are detected by the troponin assay[39]. However, it is important

to note that in the context of an acute coronary syndrome (ACS), troponin concentrations predict death risk at 1 month regardless of creatinine clearance[40].

Given that the classical triad of symptoms, an abnormal ECG and raised cardiac enzymes may be less reliable in the context of CKD, along with the concern regarding contrast induced nephrotoxicity seen with diagnostic coronary angiograms, patients with CKD and CVD represent a diagnostic and management challenge.

Therapies for CVD in CKD/ESRD

CKD patients are known to have the highest incidence of CVD and have the worst outcomes post AMI. But this group of patients often receive fewer beneficial interventions than non-CKD patients. They receive less aspirin, beta-blockers, ACE inhibitors, glycoprotein IIb-IIIa receptor antagonists, thrombolysis, and percutaneous coronary interventions (PCI)[41-43]. This therapeutic nihilism is a result of physicians concern regarding bleeding risk, worsening renal failure, lack of evidence for certain drugs and the belief that CKD patients generally have poor outcomes. The safety data on glycoprotein IIb-IIIa inhibitors is limited in the context of renal failure, as most studies excluded CKD patients from clinical trials. However, a retrospective analysis of mild CKD patients undergoing PCI showed no increased bleeding risk with glycoprotein IIb-IIIa receptor antagonists[44].

While there is the concern of hyperkalaemia and worsening GFR with the use of ACE inhibitors in the context of CKD, ACE inhibitor use post AMI in a CKD population was associated with a reduction in 30 day mortality of 36%, which is similar to non CKD patients[43].

After a cardiac event, patients with CKD are also at much greater risk of heart failure, arrythmias and sudden cardiac death. Arrhythmias, leading to sudden cardiac death is the most common cause of cardiovascular death in the context of CKD [45]. Risk factors for the development of arrhythmias in dialysis patients are intradialytic blood pressure fall, dialysis vintage, time since prievious dislaysis session and the presence of diabetes[45].

Cardiac interventions

In hospital mortality following PCI is strongly correlated with renal function[41]. The role of PCI for AMI in the context of CKD is unclear. Early studies of angioplasty in

haemodialysis patients were disappointing: despite initial angiographic success patients had high re-stenosis rates of up to 81%[46]. Subsequently, procedural advances combined with the use of drug eluting stents reduced incidence of stent re-stenosis to 36%[47]. However a study by Dragu et al.[48] suggested that PCI may lead to inferior outcomes when compared to thrombolysis in CKD patients. The SWEDEHEART[49] study reported that the benefits of PCI over medical management diminished as the stage of CKD worsened, such that below a GFR of 30 ml/min there was no additional mortality benefit of PCI over medical management. It also suggested that patients with a GFR of <15ml/min tended to do worse in the PCI group. These data were explained by the increased rates of complications following PCI (bleeding and strokes) in the CKD stage 5 group.

Mortality following a CABG in dialysis patients is 3-4 times that of a non CKD patient[50, 51] and dialysis is an independent risk factor for poorer outcomes following CABG[52].

Accurate comparison between PCI and CABG outcomes are difficult as almost all trials are non-randomised. A report by Keely et al.[53] found that outcomes following PCI were superior to CABG, however, several other studies suggest that CABG may be superior to PCI in the CKD population[54-56]. The only randomised trial of PCI v CABG with a CKD cohort reported that a CABG resulted in reduced need for revascularisations but that there was no difference in mortality[57].

Historical perspective of acute myocardial infarction

The clinical and diagnostic picture of an AMI was first described almost exactly a century ago by two Russian authors, Obraztsov and Strazhesko in 1910[58] and by an American Herrick in 1912[59]. It was impressive how prophetic Herrick was when he wrote '*The hope for the damaged myocardium lies in the direction of securing a supply of blood through friendly neighbouring vessels so as to restore as far as possible its functional integrity'*. It was 30 years before Wilson et al.[60] made it possible to locate the infarct topographically using precordial electrocardiography. The diagnostic triad was completed in the 1950's with the introduction of enzymology into clinical practice, which was first reported by Ladue et al.[61] Although experimental models of coronary artery occlusion provided extensive descriptive accounts of the morphological changes associated with necrosis[62-64], they produced relatively few insights into the molecular mechanisms of cell death.

Therapeutic strategies evolved from observations that following a myocardial infarction, arrhythmias were one of the main causes of death within the first 72 hours[65]. Research began to focus on the use of pharmacological anti-arrhythmics and temporary pacemakers to improve immediate outcomes. In the 1970's, Brunwald et al.[66] produced a seminal paper, which highlighted the factors involved in myocardial oxygen consumption. This led researchers to focus on physiological and pharmacological interventions which could alter myocardial oxygen requirements to reduce necrosis of the myocardium[67]. At the same time, a novel concept was developed following work by Becker et al.[68], who demonstrated that in the absence of collateral circulation, ligation of the left anterior descending artery resulted heterogeneous injury. They found a central area of necrosis with surrounding non-necrotic areas. These non-necrotic areas, or border areas were felt salvageable by increasing oxygen supply and/or reducing oxygen consumption, by reducing the work of the heart. Much of the clinical research in this field during the 1970's focused on pharmacological manipulation of the oxygen supply/demand of the heart using vasodilators[69], calcium channel blockers[70], GTN[71] and β adrenoceptor blockade[72], with mixed success. A reason for the failure of clinical studies to report positive outcomes was that the so called border areas were shown subsequently not to exist[73, 74], and that researchers at the time had not appreciated that for optimal salvage, reperfusion must be employed.

Jennings and Reimer[75] coined the term 'wavefront' phenomenon to describe the progression of cell death over time during myocardial ischaemia and put forward the idea that the myocardium could be salvaged by reperfusion[76].

The idea of reperfusion to salvage ischaemic tissue was first translated into clinical practice by Chazov et al[77] in 1976, by administration of intracoronary streptokinase, and later by Gruntzig et al[78] in 1979, who reported the first successful use of angioplasty in humans to treat coronary artery stenosis.

Following a large transmural infarct, the myocardium undergoes a remodelling process characterised by ventricular dilation due to volume overload and scar formation in the necrotic area. A ventricular aneurysm may complicate dilatation with further impairment of systolic and diastolic function[79]. In the 1980's 2 new concepts developed: cardiac stunning and hibernation. The concept of stunning originated from experiments which demonstrated that brief (5-15 minutes) coronary artery ligation, did not cause cell death but depressed myocardial contractility. This effect could last for days after the ischaemic event[80]. Myocardial hibernation was first described in 1978[81] and describes the protective phenomenon of down regulation of myocardial contractility under ischaemic conditions. This serves to reduce oxygen demand during hypoxia, to preserve cell survival. Thus both myocardial stunning and hibernation are characterised by viable myocardium with depressed function. Stunned myocardium has normal blood flow whereas hibernating myocardium has diminished flow[82]. Over the last 40 years attention has focused on coronary reperfusion, with timely reperfusion seen as the most effective way to reduce infarct size[83]. However, restoration of blood flow is not a benign process; it is associated with additional reperfusion injury (see iPOST chapter page 184 for discussion on the nature of reperfusion injury).

Cardioprotection: a failure of translation

Historical aspects of cardioprotective strategies

It is 40 years since Maroko et al.[67] first suggested that infarct size can be modified by therapeutic manipulations during ischaemia. Since then, tens of thousands of animal studies have been performed to identify novel cardioprotective strategies. The vast majority of these studies have been positive. However, many treatments such as

magnesium, hyaluronidase, prostacyclin, corticosteroids, trimetazidine, nitrates and eniporide which were shown to be protective animal models, were subsequently translated into clinical trials with disappointing results[84].

Despite the huge number of potentially cardioprotective strategies, which appear to confer protection in-vitro, or in animal work, only a few agents have been shown to improve outcomes in humans, such as adenosine[85] and glucose-insulin-potassium treatment[86].

Preclinical barriers to translation

Several barriers exist which may account for the failure of translation. Firstly ex vivo/ in vitro models of disease lack physiological interactions such as an intact nervous system and a circulation required to ideally model ischaemia tolerance. Different species of animals display different tolerances to myocardial ischaemia[87]. Results of animal studies often rely on short term outcomes such as cardiac enzyme rise rather than a functional assessment of myocardial function at a later time point. This is an important failing as post infarct heart failure is a major determinant of morbidity and mortality following an AMI in humans.

Critically, the vast majority of animal studies use juvenile, healthy animals. These may be a poor representation of the elderly diabetic, hypertensive, vasculopathic patients who are at increased risk of AMI.

Clinical barriers to translation

Barriers at the clinical level include a lack of knowledge about the exact timing of ischaemia, the size of the area at risk, the extent of collateralisation of coronary vessels and variability in completeness of reperfusion, along with underlying co-morbidities and medication use in human patients. Furthermore, cardioprotective trials in the context of AMI are limited to treatments, which are given during ischaemia or at the point of reperfusion. Pre-treatment studies are impossible due to the unpredictable onset of an AMI.

Clinical studies are hampered by lack of a clear method to measure infarct size. Enzyme release may be confounded by reperfusion status and imaging studies such as SPECT can be altered by the timing of the acquisition of the images, requiring 24 hour access to a nuclear medicine department. Cardiac MRI[88] appears to provide better quantitative

data on tissue injury and also functional data, allowing non invasive longitudinal follow-up of patients, but this has yet to be implemented in routine care.

Clinical trials have also struggled to translate infarct size reduction into a clear clinical benefit, such as reduced mortality or improved functional status.

With current best management, short-term complications following AMI, while severe, are not very common, which requires large sample sizes to show an effect. To improve the ability of a study to access hard endpoints such as mortality, a large sample size is required which makes these clinical trials very costly.

Over the last 10 years several hugely expensive trials in the field of cardiovascular disease in the context of CKD (Choir[89], CREATE[90] TREAT[91] AURORA[92] and 4D[93]) have been published which have shown no benefit in terms of primary outcome. The exception to these dissapointeding studies is the recently published SHARP trial which demonstrated a positive outcome in terms of vascular events in a CKD cohort [94]. This may have led to a therapeutic nihilism from large pharmaceutical companies interested in translational research.

The impact of underlying comorbidities on ischaemia tolerance

Ischaemic heart disease in patients does not operate in isolation: it exists in the context of multiple co-morbidities such as LVH, diabetes, hypertension, senescence and dyslipidaemia, which affect the diseased state. Much of the characterisation of myocardial infarction and cardioprotection were performed in juvenile healthy animals with no underlying comorbidities. However, some data exists which explore these co-morbidities in more detail.

Left ventricular hypertrophy

LVH is a physiological adaptation of the myocardium in response to exercise. However, pathological conditions such as anaemia, hyperthyroidism, aortic stenosis, obesity and arterial hypertension lead to pathological LVH, with hypertension being the most common causative factor[95].

Accurate estimates of the prevalence of LVH in population studies have been hampered by difficulty in defining the normal limits of LV size. Historically, there was marked variation in the methods used for measuring LVH and these were of low sensitivity[96]. The prevalence of LVH in unselected hypertensive patients has been reported as 20%, but this figure rises both with severity of hypertension[97] and the age of the population studied. Tuzcu et al.[98] reported that in a cohort of nonagenarians referred to a cardiology clinic, over 80% had evidence of LVH. From analysis of the Framingham cohort, with the exception of age, LVH represents the strongest traditional independent risk factor for future cardiovascular events[99]. Hypertension accelerates the development of atherosclerosis in coronary arteries[100]. LVH is associated with changes in the density, structure, and vasodilatory capacity of the coronary vasculature so that although absolute coronary flow in hypertrophied hearts may be increased, there is reduced cross-sectional density of endomyocardial capillaries and reduced coronary reserve even in the absence of detectable coronary atherosclerosis[101, 102].

Following ischaemia and reperfusion, the hypertrophied heart develops hyper contracture (caused by intramyocyte calcium overload during reperfusion) earlier and has poorer blood flow[103]. In addition, the hypertrophied heart recovers contractile function more slowly and is associated with a greater enzyme release following

reperfusion[104]. Postulated reasons for this effect include altered mitochondrial energetics[105], changes in glucose metabolism during ischaemia[106] and increased reactive oxygen species (ROS) production[107].

LVH is associated with increased frequency of ischaemia-induced arrhythmias. This is possibly due to LVH rather than hypertension per se, as lowering blood pressure before ischaemia using a calcium channel blocker did not alter the probability of ventricular fibrillation[108]. LVH also makes hearts more susceptible to reperfusion arrhythmias. In DOCA-salt hypertensive rats Baxter et al.[109] demonstrated greater frequency and duration of reperfusion arrhythmias, an effect which resolved on resolution of the LVH by withdrawal of the DOCA-salt.

Despite LVH having an impact on the bioenergetics, altered blood flow, increased ROS production with more arrhythmias, there have only been a few studies of myocardial ischaemia tolerance in hypertensive animals. These studies did not demonstrate a reduced ischaemia tolerance in animal models of LVH[110-112].

Cardiac failure and remodelling

Patients with heart failure following a myocardial are at increased risk of arrhythmias and sudden death. In a dog model of healed myocardial infarction and superimposed heart failure, a very brief additional arterial ligation provoked fibrillation in 75% of animals. This could be abrogated by intrathecal clonidine, suggesting that increased sympathetic activity may be driving ischaemic arrhythmias[113].

Myocardial infarction can lead to cardiomegaly, with pathological structural changes such as fibrosis and microvascular damage[114]. These abnormalities would suggest that the failing heart would have a reduced ischaemia tolerance. However, data from Sharikabad et al.[115] show that in an in-vitro model of post infarct congestive heart failure, cardiomyocytes had in increased resistance to hypoxia-reoxygenation. In vivo data using pacing to induce heart failure in dogs also show that there was no reduction in ischaemia tolerance associated with heart failure. However, care must be used when interpreting this data as pacing itself has been shown to confer cardio-protection[116].

Hyperlipidaemia and atherosclerosis

Hyperlipidaemia and atherosclerosis are important risk factors for ischaemic heart disease. However, animal data regarding the impact of underlying hypercholesterolaemia on ischaemia tolerance is conflicting.

Rabbits fed cholesterol rich diets for 4[117] and 8[118] weeks have been shown to have reduced ischemia tolerance. This has been reported to be dependant on the inhibition of IL-1β and activation of caspase-1 and 3[118]. Experimental data show that acute hypercholesterolaemia in rabbits, induced by a 3 day high cholesterol diet, is associated with a reduction in ischaemia tolerance[119]. This effect was seen in the absence of increased atherogenesis.

Several studies suggest that the timing of the experiment is crucial: experiments using LDL receptor$^{-/-}$ mice on high cholesterol diets for 4 weeks showed a reduced ischaemia tolerance, however a 12 week high cholesterol diet resulted in an increased tolerance compared to controls[120]. Furthermore, other studies in rats[121] and rabbits[122] demonstrate that long term diets rich in cholesterol alter cardiac function but do not alter ischaemia tolerance.

Ferdinandy et al.[96] explain this conflicting evidence by suggesting that that long term animal models of hyperlipidaemia may lead to extra cardiac pathological conditions, such as liver failure, which may alter ischaemia tolerance.

Diabetes

Diabetes is a well recognised risk factor for the development of IHD. Ischaemic heart disease accounts for more than 50% of the deaths in diabetic patients in the Framingham cohort[123]. Moreover, diabetic patients were seen to have twice the mortality following an AMI when compared to non diabetic controls[124].

Several animal models have been used to study the effects of diabetes on myocardial function, with conflicting results. Tosaki et al.[125] reported that streptozotocin (STZ) treated rats were initially more resistant to ischaemia than non diabetic controls but that this protective effect had worn off by 6 weeks and by 8 weeks there was even a deterioration in post ischaemic myocardial function. A similar temporal relation was seen by other groups who found that 2 weeks after the STZ injection, there was an

increased tolerance to ischaemia, but several weeks later the effect had disappeared[126, 127]. Other models of type 2 diabetes such as the Goto-Kakizaki lean rat, which is an inbred strain of the Wistar rat, have shown similar increases in ischaemia tolerance compared to controls[128]. An explanation for the apparent increased ischaemia tolerance was suggested by Ma et al.[127] who found increased capillary density (in contrast to the published research demonstrating a reduced capillary density seen in the uraemic myocardium [129]), increased Akt phosphorlyation, and higher levels of nitric oxide and vascular endothelial growth factor in rats 2 weeks after STZ injection, with these effects disappearing by 6 weeks along with normalisation of ischaemia tolerance.

While the epidemiological data strongly suggest that diabetes is a potent risk factor for poorer outcomes following an AMI, ex-vivo tissue from human atrial appendages showed no difference in injury following ischaemia when compared to non diabetic tissue[130]. An explanation for the apparent differences between experimental data and epidemiological data could be that diabetes is associated with other known risk factors for IHD, such as hypertension, heart failure, obesity and CKD without itself being directly cardiotoxic. However, more recently subgroup analyses of the thrombolysis in myocardial infarction study group were able to demonstrate that following an AMI, diabetes remained a risk factor for 30 day and 1 year mortality after adjustment for other known risk factors[131].

Senescence

Epidemiological studies suggest that even in the absence of diabetes, hypertension and hypercholesterolaemia, age itself increases cardiovascular morbidity and reduces myocardial performance [132, 133].

Increased oxidative stress appears to be fundamental to the changes seen in the senescent myocardium[134]. Ageing leads to myocardial fibrosis[135] and altered purine metabolism leading to less adenosine production in response to ischaemia[136]. On a histological level, senescence is associated with a reduction in cardiomyocyte number and increased variability in cardiomyocyte size leading to reduced stress tolerance[137]. Senescent mitochondria form higher levels of free radicals[138], produce less ATP[139] and display reduced membrane potentials[140]. Following ischaemia, senescent rat hearts develop a greater degree of stunning[141], lower ATP levels[142], more ventricular arrhythmias and greater cardiac enzyme release[143]. However, Loubani at al.[144] reported

in an ex vivo human model of simulated ischaemia/reperfusion that no loss of ischaemia tolerance was seen in old hearts.

Pathogenesis of Uraemic cardiomyopathy

Traditional risk factors

Traditional risk factors such as hypertension, diabetes, dyslipidaemia and smoking are highly prevalent in the CKD population.

Hypertension occurs in >70% of ESRD patients. Many reasons exist for this: fluid overload due to the loss of urine output, activation of the renal-angiotensin system[145], high sympathetic activity[146], reduction in NO production[147] and as a result of EPO administration[148].

Diabetes is the leading cause of ESRD and the combination of diabetes and CKD appears to be synergistic in terms of cardiovascular mortality[30].

Smoking is a risk factor for progression of CKD[149]. Dialysis patients who smoke have a 22% greater risk in developing coronary artery disease than non smoking dialysis patients[150].

Dyslipidaemia is common in patients with CKD and ESRD, however unlike the general population, statins have not been shown to be effective in reducing cardiovascular endpoints in ESRD[93]. However more recently, a reduction in LDL cholesterol, using simvastatin and ezetimibe, has been shown to prevent aterosclerotic events in a CKD cohort[151]. However this did not translate into a survival advantage.

Despite the clustering of traditional risk factors around uraemia, it appears that these traditional risk factors alone do not account for the morbidity and mortality seen in the CKD population[152]. Over the last 15 years, work has focused on non-traditional risk factors, some of which may be particularly common or unique to CKD, to explain this increased risk.

Obesity

Obesity is an emerging risk factor for progressive renal disease. Its role goes beyond indirectly leading to CKD through colocalising with diabetes.

The percentage of obese incident dialysis patients is greater than that of the population average, with 63% of incident dialysis patients being classified as obese [153].

However somewhat paradoxically, in epidemiological studies raised BMI appears to be protective in ESRD patients [154]. Authors have suggested that BMI is not valid in a co-morbid patient group as a marker of cardiovascular mortality, due to the uneven distribution of fat, with visceral fat being more hazardous than peripheral fat [155]. Because of this the waist to hip ratio has been developed which has been shown to correlate better with cardiovascular disease in patients with co-morbidities than BMI [156].

The mechanism through which obesity leads to progressive CKD is thought to involve Adiponectin and 5'-AMP activated protein kinase (AMPK). Adiponectin is a protein secreted by adipocytes, which increases insulin sensitivity, reduces oxidative stress and inflammation [157] [158]. The concentration of adiponectin is inversely proportional to body fat [159], however the mechanism for this paradox is unclear. Epidemiological studies report that adiponectin and albuminuria are inversely correlated [160].

Several in-vitro and in-vivo studies by Sharma et al, implicate low adiponectin in the pathogenesis of obesity related renal disease. The authors demonstrated that adiponectin knock out mice developed proteinuria and a histological pattern similar to obesity related CKD seen in humans. This phenotype could be 'rescued' with exogenous adiponectin [161].

AMPK is a serine/threonine kinase which senses energy availability at the cellular level. In times of energy excess AMPK is inhibited [162]. In chronic energy excess persistent inhibition of AMPK has been shown to lead to histological changes similar to those seen in obesity related CKD [161]. The link between Adiponectin and AMPK has been shown by Kadowaki et al, who demonstrated that AMPK was stimulated by adiponectin [163]. In summary it is thought that excess calorific intake leads to adiposity, which in turn causes an inhibiton of adiponectin which is thought to lead to proteinuria, podocyte effacement and glomerulosclerosis through inhibition of AMPK.

Parathyroid hormone, calcium and phosphate metabolism

Phosphate

Phosphate has been shown to stimulate vascular calcification in vitro[164]. In humans serum phosphate levels have been correlated with coronary artery calcification[165], LVH[166] and mortality[167].

Calcium

Retrospective data suggest that high calcium levels are correlated with cardiovascular death in dialysis patients[168, 169], and that high calcium ingestion predisposes to coronary calcification which is an independent risk factor for CVD[170].

PTH

PTH has been implicated in the pathogenesis of LVH. In vivo, PTH causes trophic changes in myocytes and has been shown to be permissive in myocardial fibrosis[171]. Hyperparathyroidism has been shown in CKD patients to be an independent risk factor for cardiac events[172], and both systolic and diastolic dysfunction[173]. Several studies have shown improvement in LV function post parathyroidectomy [174-176].

Vitamin D

Left ventricular hypertrophy

It has been well established that LVH is a potent risk factor development of cardiovascular events and death. The vitamin D receptor (VDR) is present in the myocardium[177]. Treatment with activated vitamin D (calcitriol) has been shown to cause regression of LVH in dialysis patients[178] and reduce QTmax and QTc dispersion[179]; both electrocardiographic features associated with a risk of sudden death. VDR -/- mice develop hypertension and LVH due to loss of suppression of rennin biosynthesis [180]. In-vitro, vitamin D has been shown to inhibit myocyte proliferation and reduce apoptosis [181] and reduce myocyte hypertrophy in cultured cardiomyocytes [182].

Fibrosis

In vitro work by Artaza et al showed that incubation of a mesenchymal multipotent cell line with vitamin D reduced expression of collagens I and III, increased expression of bone morphogenic protein 2 and (both anti fibrotic factors), increased MMP8 (a collagen breakdown inducer) and increased follistatin (an inhibitor of the profibrotic factor myostatin) [181].

Atherosclerosis/inflammation

Inflammatory processes play an important role in the development of a vascular insult [183]. In-vitro vitamin D analogues have been shown to inhibit the production of proinflammatory cytokines (IL-2, IFN-γ) and cause up regulation of the anti-inflammatory cytokine of IL-10 in T cells [184]. The net result of this is to reduce macrophage activation, LDL oxidation, and reduce matrix metalloproteases, which reduces atheroma formation and plaque rupture. VDR agonists have also been shown to down regulate plasminogen activator inhibitor 1, which is a potent thrombogenic factor in human aortic smooth muscle cells [185].

Vascular calcification

The role of vitamin D and its analogues in vascular calcification is complex.

Studies show that vitamin D receptor agonists can cause hyperphosphataemia and vascular calcification in humans: both potent mortality risk factors in ESRD [169, 186]. High doses of oral vitamin D can induce vascular calcification in animals [187, 188].

However in populations at increased risk of ischaemic heart disease serum levels of active vitamin D were inversely related to the extent of vascular calcification [189].

Observational studies show that vitamin D analogues improve survival in dialysis patients [190, 191]. Work by Hsu et al reported that low doses of vitamin D, suppress aortic osteoblastic gene expression [192]. Calcritol inhibits proliferation of vascular smooth muscle cells by acute influx of calcium [193]. Matrix Gla protein, a potent

inhibitor of vascular calcification has been shown to be increased by calcitriol in-vitro [194].

Anaemia

Anaemia is a common consequence of CKD, due to a relative deficiency in erythropoietin production by the kidney. Epidemiological studies suggest that anaemia is an independent risk factor for the development of cardiac morbidity and mortality[195]. Anaemia results in a reduction in the oxygen carrying capacity of the blood and as such represents a reduction in ischaemia tolerance.

The use of recombinant erythropoietin has revolutionised the care of renal patients over the last 25 years. However, despite a historical general consensus that normalising haemoglobin should lead to improvement in cardiovascular outcomes, several large randomized controlled trials controversially have failed to show a benefit[89-91]. Criticisms of the trials have focused on iron supplementation and the use of very large doses of ESA to get patients to target. Over the last 5 years there has been a re-evaluation of target haemoglobin levels in dialysis and CKD patients downward from physiological levels to lower levels of 11-12g/dl.

Autonomic dysfunction

CKD is a state of tonic increase in sympathetic outflow[196], and this overactive sympathetic activity has been implicated in the excess cardiovascular events in dialysis patients[197]. The explanation for the increased cardiovascular mortality due to sympathetic over activity is suggested by data which implicate the sympathetic nervous system in the development of LVH[198], endothelial dysfunction[199] and arterial stiffness[200]. A recent pilot study in a hypertensive cohort reported that renal denervation not onlt resulted in a reduction in blood pressure, but also led to an improvement in glucose metabolism and increased insulin sensitivity [201].

Homocysteine

Raised levels of homocysteine contribute to vascular inflammation, smooth muscle proliferation and vascular thrombosis[202]. A linear relationship between homocysteine levels and poor cardio-vascular outcomes has been reported[203]. Homocysteine levels

are significantly higher in patients with renal failure than in the general population[204],with longitudinal studies suggesting an association between homocysteine levels and cardiovascular disease in dialysis patients [205]. However, intervention studies in the CKD and ESRD cohorts have yielded negative results [206, 207].

Inflammation and malnutrition

Malnutrition is common in CKD patients, both from restrictive diets imposed on patients to minimise phosphate, potassium and fluid intake, along with anorexia which is hallmark of advanced renal failure, which may occur prior to the initiation of dialysis[208]. Inflammation is also common in dialysis patients, with as much as 50% showing evidence of an inflammatory response in an American cohort[209].

Hypoalbuminaemia caused by inflammation or malnutrition, (or as often in the case in dialysis patients both) is an independent risk factor for death in dialysis patients[210]. CRP, a marker of inflammation has been shown to be an independent risk factor both for death on dialysis[211] and cardiovascular events in the CKD population[212]. IL-6 has also been shown to be an independent risk factor for cardiac mortality on dialysis[152].

A mechanism by which acute phase proteins can lead to vascular disease comes from data which show that CRP can bind to damaged endothelial cells and activate complement[213]. IL-6 may lead to endothelial injury resulting in lipid accumulation and proliferation of the vascular smooth muscle cells[214].

Oxidative stress and advanced glycation end products

The mitochondrial respiratory chain, present in all cell types, is the major producer of oxidants in the body. The generation of physiological oxidants is an important step in inflammation and tissue repair[215]. In ESRD there exists an imbalance between the pro- and anti-oxidant mechanisms[216]. This imbalance may due to deficiencies seen in ESRD patients of members of antioxidant defences such as Vitamins C and E, selenium and glutathione peroxidase[217, 218]. At the same time pro-oxidant activity is increased with haemodialysis, inducing repetitive bouts of oxidative stress though membrane bio-incompatibility[219]. Furthermore, chronic inflammation and uraemia itself is thought to contribute to oxidative stress[215].

Increased levels of oxidative stress have been associated with the development of atherosclerosis in a cross sectional study[220]. Vitamin E supplementation has been shown

to reduce the oxidative susceptibility to LDL in dialysis patients[221]. Vitamin E supplementation has also been shown to reduce all cause cardiovascular mortality in dialysis patients with known cardiovascular disease[222], and supplementation with the antioxidant acetylcysteine has been shown to reduce a composite cardiovascular end-point in a haemodialysis cohort[223].

Advanced glycation end products (AGE) are formed by the non-enzymatic oxidation and glycation of proteins and lipids[224]. AGEs normally accumulate with ageing but are also elevated in dialysis patients[225]. In vitro data suggest AGEs lead to progression of atherosclerosis through production of free radicals and inflammatory cytokines[226, 227]. AGEs are also associated with arterial stiffness and diastolic dysfunction[228]. However, evidence in favour of the correlation between elevated AGEs and mortality in dialysis patients is conflicting[229, 230].

Asymmetric dimethyl arginine

Asymmetric dimethyl arginine (ADMA) is the most potent endogenous inhibitor of NO synthase known[231]. It opposes the anti-atherosclerotic effects of NO[232] and leads to increased vascular resistance, hypertension, and vascular stiffness[233, 234].

Epidemiological studies demonstrate that elevated ADMA is an independent risk factor for all cause mortality and cardiovascular events[235, 236]. ADMA levels have been shown to correlate with LVH [232, 237] and elevated plasma levels of LDL cholesterol, triglycerides, glucose and homocysteine[238].

ADMA was first shown to be elevated in the context of dialysis by Vallance et al.[239], and subsequently increased ADMA concentrations have been demonstrated even in very early CKD[240].

Several reasons for the excess ADMA seen in CKD patients have been proposed. ADMA was first proposed to be excreted by the kidneys[239], however subsequently renal excretion has been shown to only play a minor role in ADMA excretion. ADMA is metabolised by the enzyme dimethyl-diaminohydrolase (DDAH), which is present in the kidneys (amongst other organs)[241]. The activity of DDAH may be reduced in the context of renal failure as it is inhibited by oxidative stress[215], homocysteine, hyperglycaemia, erythropoietin and smoking[232].

Carnitine

Carnitine is a water-soluble quaternary amine which plays an important role in myocardial energy metabolism. Carnitine deficiency is characterised by cardiac hypertrophy and heart failure[242]. Carnitine deficiency is common in dialysis patients[243], through loss of carnitine through the dialysis membrane [244]. Carnitine supplementation in haemodialysis has been shown to reduce inflammation[245] and LVH in dialysis patients[246] and there is also the suggestion that carnitine supplementation may reduce EPO requirements in the context of renal anaemia[247].

Aims of thesis

It has been established that cardiovascular disease in the context of CKD is distinct to that seen in the general population. It has a different aetiology, a different phenotype, with a different prognosis and a different response to treatments.

Because of this, there is a great unmet need for additional cardioprotective strategies in the context of uraemia to improve patient outcomes.

The aims of this thesis are to investigate novel cardioprotective strategies in the context of uraemia. This will be achieved through generation and characterisation of appropriate animal models of disease. Following this the models will be used to establish the role of underlying uraemia in myocardial ischaemia tolerance, ischaemic preconditioning, ischaemic postconditioning and pharmacological postconditioning using a PARP-1 inhibitor.

Chapter 2

Animal Models of Uraemia

Background

Uraemia is a multifaceted, heterogeneous syndrome involving, hypertension, LVH, disturbances in bone biochemistry, vascular calcification, dyslipidaemia and anaemia. There are several animal models that have been developed to attempt to reflect the complexity of this phenotype. The most widely used is renal reduction models.

Surgical models

Surgical models of chronic anaemia involve reduction in nephron mass, this was first described by Chanutin and Ferris in 1932[248]. The reduction in nephron mass may be achieved using different approaches. The infarction model uses a 2 stage procedure whereby following a uni-nephrectomy, the remaining kidney's renal arteries are ligated such that 2/3 of the kidney is infarcted and the subtotally infarcted kidney remains in place. The other more commonly used model is the 5/6 nephrectomy model (SNx). This employs a 2 stage subtotal nephrectomy model; 2/3 of one kidney is removed surgically followed, 2 weeks later, by a contralateral nephrectomy: leaving 1/6 of the remaining nephron mass in place.Histological examination of the remnant kidney reveals hypercellular glomerular tufts, with expansion of the glomerular volume and tubular atrophy[249]. However, when the infarction model is compared to the partial nephrectomy model (see below), the infarction model causes a much greater degree of variability in serum creatinine as a result of the greater technical difficulty than the SNx model[250] and so is a less desirable model in the rat. The SNx model is the most commonly used model of chronic uraemia in the rat.

Immune mediated CKD

This model relies on induction of glomerular sclerosis by injection of anti glomerular basement membrane (GBM) antibodies. To generate this model, rats are injected with rabbit anti GBM antiserum, with control animals injected with normal rabbit serum[251]. The a nti GBM serum is made by repeatedly inoculating New Zealand white rabbits over 2 weeks with purified rat GBM and an adjuvant. The rabbit serum (complete with anti GBM antibodies) is then injected intravenously into the rat[252].

This model was characterised by Nagano and colleagues[251] and results in progressive proteinuria and uraemia with evidence of growth restriction. Histological lesions appear

as early as day 4, with evidence of widespread segmental necrosis, followed by cellular crescents at day 11. At day 44 there is severe scarring with marked fibrosis with little active inflammation. Biochemical disturbances include a doubling of serum creatinine by day 30, with hypocalcaemia and hyperphosphataemia. However, there is still substantial variation in the creatinine levels after 3 weeks. The study did not examine the uraemic phenotype past 44 days, unlike other models of chronic uraemia. This model has not been widely adopted as a model of CKD, perhaps due to the complexity of the immune induction and due to the high variability of subsequent uraemia. There is no available data on the cardiac phenotype in this model.

Adenine Model

The use of supplemental adenine to generate a model of experimental uraemia was first described by Yokazawa and colleagues in 1986[253] and was based on previous observations by their group on the effects of exogenous adenine on renal function[254]. Following a diet supplemented with 0.75% adenine for 30 days, rats developed a uraemic phenotype. They found the rats kidneys to be larger and paler than their non-uraemic counterparts. On histological examination of the kidneys, granuloma around crystalline deposits were seen in the tubules and interstitium along with evidence of marked fibrosis. There was also evidence of microtubular obstruction and dilatation and not uncommonly, hydronephrosis[255].

Biochemical analysis of the rats revealed uraemia, with creatinine values over three times that of controls at the 30th day and rising to almost 5 times that of control animals by the 50th day. The animals died between the 40th-65th day. The mechanism of adenine induced renal failure stemmed from the understanding of the metabolism of adenine in rodents (see Figure 2). Adenine, unlike other purines, is metabolised to AMP via the enzyme APRT[254]. AMP may be metabolised via adenosine, inosine, hypoxanthine, xanthine, urate and finally excreted as alantion[254]. APRT can become saturated by excess adenine which is then oxidised to 2,8-dihydroxyadenine via xanthine oxidase[256]. The 2,8-dihydroxy-adenine is poorly soluble in the urine in the physiological pH range. The 2,8-dihydroxyadenine precipitates out in the urine causing a crystal tubulopathy with microtubular obstruction and granulomatous interstitial nephritis, leading to renal failure[256].

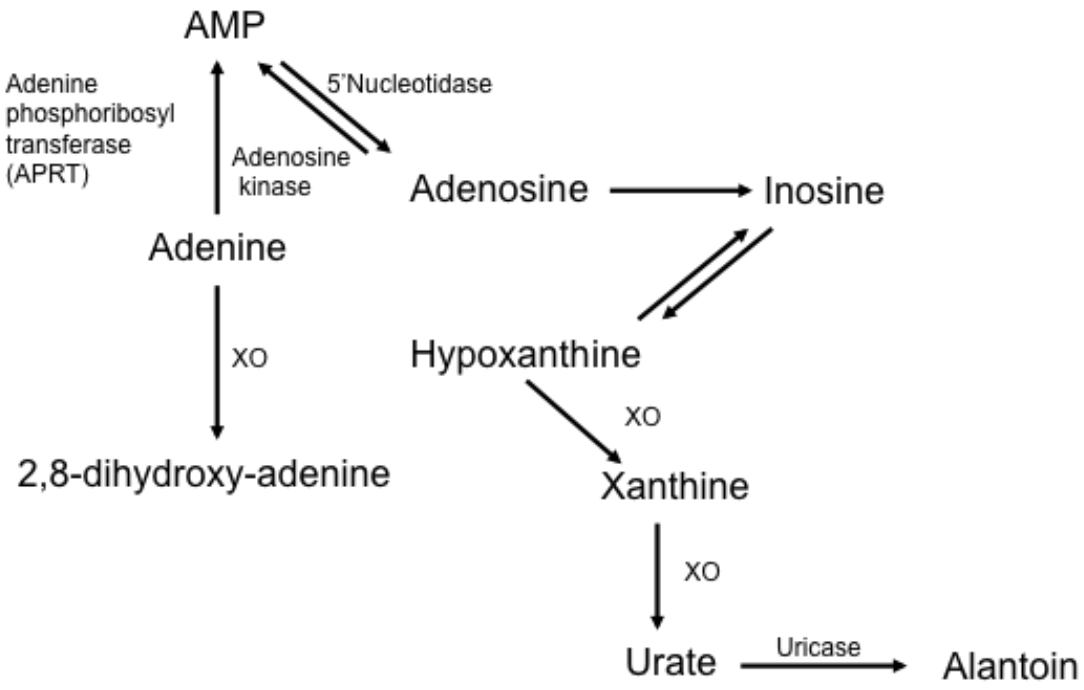


Figure 2. Metabolic pathways of adenine metabolism in the Rat [1, 2 4]

Human equivalent of the adenine model

The adenine model mimics a human disease, which causes progressive renal dysfunction. Adenine phosphoribosyltransferase (APRT) deficiency is an autosomal recessive inborn error of metabolism with a homozygous frequency of between 1 in 50,000 to 1 in 100,000[257]. It is characterised by stone formation and progressive renal decline. There are 2 forms of the disease. Type 1 results in the complete loss of APRT activity whereas type 2, seen more commonly in Japanese cohorts, results in a reduced affinity for adenine[258].

APRT is a purine salvage enzyme which catalyses the formation of AMP and pyrophosphate from 5-phosphoribosyl-1-pyrophosphate. Homozygotes for the mutation cannot salvage AMP from adenine and so instead, adenine is oxidized in a 2-stage process by xanthine oxidase to 2,8-dihydroxyadenine. 2,8-dihydroxyadenine is highly insoluble and when excreted in the urine forms crystals.

Comparison between Adenine diet and SNx models

Degree of uraemia

A SNx procedure typically generates a serum creatinine around 2-3 times that of sham operated controls[259-261]. The adenine diet however, causes a much more severe form of uraemia, with serum creatinine levels between 3 and 10 times that of the sham fed animals[262-264].

Time course of uraemia

Uraemia is induced earlier in the adenine diet compared to SNx, with significant uraemia seen within 2 weeks of the commencement of the adenine diet[265], whereas in the SNx model, a significant degree of uraemia is only seen 4 weeks after the second stage procedure[266].

Reproducibility

The subtotal nephrectomy model relies on the skill of the surgeon or technician to achieve a reproducible reduction in nephron mass. This model takes time to learn and

each animal requires several hours of labour during surgery and recovery. The adenine diet model requires no surgical skill, and often generates a more consistent degree of uraemia when compared to the SNx model[265].

Calcium

Serum calcium in SNx rats is similar to control rats[259, 267-269], however 4 weeks of adenine diet caused a reduction in serum calcium by up to 40% when compared to controls[263, 270, 271].

Phosphate

Hyperphosphataemia occurs earlier in adenine fed animals as compared to SNx animals and the degree of hyperphosphataemia appears to be greater in the adenine treated animals compared to SNx animals[265]. Adenine treated animals tend to have phosphate levels 2-3 times that of controls[262-264] with SNx animals having serum phosphate levels between 1.1 to 2.3 times that of controls[259-261].

Hyperparathyroidism

Both models of CKD develop marked hyperparathyroidism. SNx animals have PTH levels 3 to 17 times that of controls [260, 261, 268, 269], whereas adenine animals tend to have higher PTH levels ranging from 8.9 to 25 times that of control animals[262-264]. Furthermore, adenine treated animals had evidence of parathyroid gland hyperplasia at 4 weeks. Again this may be explained by the higher phosphate levels and greater degree of renal dysfunction. There is no evidence to suggest that for a given biochemical profile (creatinine, calcium and phosphate), adenine treated animals have greater degrees of hyperparathyroidism.

Vascular calcification

With the SNx model it is usually necessary to supplement the diet with additional phosphate or calcitriol to cause vascular calcification[272]. However, one study has shown vascular calcification in the SNx model on low phosphate diets but only after 24 weeks following the SNx procedure[273]. Adenine treated rats develop vascular calcification within 4 weeks of starting the diet without the need for high phosphate diets[274].

Dyslipidaemia

Both SNx[266] and adenine treated[253] animals have significant elevations in total cholesterol. Adenine treated animals do not develop hyper-triglyceridemia[253]. The data for SNx animals is conflicting, with some authors[249] noting elevation in triglyceride levels whereas others noted no change[265].

Anaemia

A paper by Okada et al.[275] reported that in rats, 2 weeks of adenine diet did not lead to a significant difference in haemoglobin when compared to a sham group. However, if both groups were then put on standard chow for a further 4 weeks, the adenine treated animals developed a significant degree of anaemia, with a 2g/dl difference in haemoglobin. This was despite almost total resolution in the renal dysfunction seen in the adenine treated group. Renal anaemia is characterised predominantly by a reduced production of erythrocytes due to relative deficiency of erythropoietin. The lag in development in anaemia is due to the life span of erythrocytes in the circulation. Rat erythrocytes have a half life of 14 days[276], thus during the 2 week adenine diet many of the red cells were formed before the diet was commenced and so were not affected by the uraemia of the adenine diet.

Four weeks of adenine diet has been shown to cause significant anaemia[275]. This anaemia was even more marked after a further 4 weeks off the adenine diet. Similar findings were seen with 6 weeks of adenine diet[275]. SNx animals are rendered anaemic following the 2 stage procedure, with a 20% fall in the haematocrit relative to non uraemic animals[277]. The SNx procedure causes anaemia through 2 ways; the first is blood loss during the initial 2/3 nephrectomy and the second is due to uraemia following a reduction in renal mass. No studies have directly compared the relative degrees of anaemia between the adenine model and the SNx model. However, a Japanese group performed 2 different experiments; 1 involving adenine induced uraemia[278] and one using the SNx model[279]: Six weeks following the second stage in the case of the SNx model and 6 weeks following a 4 week course of adenine diet in the case of the adenine rats, the rats fed adenine had much lower haematocrits than the SNx animals.

Iron status

There was no difference in serum ferritin, serum iron or transferrin in animals treated with 4 weeks of adenine diet when compared to controls, despite marked anaemia[280]. SNx animals however were found to have an upward trend in the levels of serum ferritin when compared to controls, which reached statistical significance at 15 weeks post 2nd stage procedure[249]. This was associated with a reduction in serum transferrin, suggesting an inhibition in iron trafficking from macrophages to erythroid cells, leading to a progressive rise in ferritin levels. These effects closely resemble 'Functional iron deficiency' seen in CKD patients[281].

Urine abnormalities

Yokozawa et al.[282] reported that urinary abnormalities develop rapidly in rats on the adenine diet. After 1 week on the adenine diet, rodents develop polyuria with no associated polydipsia. Adenine treated rats also develop naturia, calciuria and phosphaturia. Similar findings have subsequently been replicated in SNx animals[283]. Both adenine treated animals[284] and SNx rats[285] have been shown to develop significant proteinuria.

Blood pressure

Work by Ataka et al.[278] reported that 4 weeks of adenine diet causes marked elevation in serum creatinine, but did not cause hypertension. However, 6 weeks after the cessation of the diet, the uraemic animals began to develop significant hypertension. A study by Yokozawa et al.[286] reported that hypertension developed after 24 days of adenine diet. The degree of hypertension was more marked as the duration of the adenine diet was increased from 24 to 36 days. The same group also found a reduction in renal blood flow[284], with rising serum levels of ACE, angiotensin 2, and aldosterone in rats given the adenine diet. They hypothesised that the decreased renal blood flow triggered hypertension through activation of the renin-angiotensin system. SNx rats are significantly hypertensive at 4 weeks[266] and this persists through 12 weeks[266] to 25 weeks[248] after the second stage procedure.

LVH

The SNx model of uraemia is associated with LVH, both as measured by heart weight/body weight ratio[266] and on echocardiography[266]. Echocardiography in SNx rats reveals septal thickening and increased left ventricular posterior wall dimension. On histological examination of SNx hearts, cardiomyocyte hypertrophy was seen[266]. Cardiac hypertrophy was also demonstrated by Lacour et al.[287] who reported that hearts from SNx animals were heavier than those of adenine or sham animals, despite SNx animals weighing less than the other groups. This report also suggests that significant LVH was not seen in adenine treated animals.

Myocardial histology

SNx animals develop increased myocardial fibrosis, and reduced capillary density 12 weeks after the second stage procedure[266]. There is no data regarding cardiac histology following adenine diet.

Death rate

Death during induction of uraemia using the adenine diet is rare, whereas death is not uncommon following the 2 stage surgical procedure for SNx animals. In a study by Nagano et al.[265] none of the adenine treated animals died during the 4 week induction versus 20% of the SNx animals at 4 weeks. However, if the adenine diet was continued for an additional 2 weeks, over 60% of the animals died[275].

Growth restriction

Swapping animals to an adenine diet causes almost total cessation of food intake for up to 7 days[265]. Following 3 weeks of adenine diet, the animals were below their pre diet weights and were significantly lighter than the sham animals[287]. The protein intake of adenine rats over 4 weeks was reported to be only 65% of that of those on standard chow[288]. On cessation of the adenine diet the animals regained weight. The rate of weight gain depended on the duration of adenine diet. Animals who had 2 weeks of adenine diet regained weight rapidly such that after 4 weeks of standard diet, they approached the weight of the non-uraemic animals. Those animals that had 4 weeks of adenine diet only gained weight at the same rate as their non-uraemic controls, and 4

weeks later were still significantly lighter then controls. Those animals who had 6 weeks of adenine diet put on weight more slowly than controls and so over time, the weight differences between the sham animals and control animals widened[275]. SNx models of uraemia report growth restriction. The mechanism of growth restriction in the SNx model was reported by Mehls et al.[289] to be due solely to reduced food intake, as sham animals pair fed with the SNx animals were also found to be growth restricted compared to rats fed ad libitium. Work by Terai et al.[265] suggests that the mechanism of reduced food intake may not simply be due to the palatability of the adenine diet, because even when adenine was administered by gavage, a reduction in food intake was seen when compared with methylcellulose gavage.

Wash-out

One of the unique advantages of the adenine model is the ability to stop the diet and regain renal function. The ability of the animal to recover depends on the duration of the adenine diet[275]. There is evidence of at least partial recovery of renal function on cessation of the diet. Shuvy et al.[290] noted that 10 weeks after cessation of a 7 week high adenine/high phosphate diet, the renal function fell to almost to baseline, with the washout group having a mean serum creatinine of 115±13 mmol/l versus the control group 86± 16mmol/l. Some of histological features of adenine induced uraemia also resolved with cessation of the adenine diet, however, there was more prominent interstitial scaring and fibrosis in the washout group. This is unsurprising as the wash-out group was 10 weeks older than the 7 week old uninterrupted adenine group, and hence has more time to develop fibrosis and scarring. Adenine diet also caused an increase in apoptotic cells, which returned to baseline during the 10 week washout period.

Characterisation of 3 rodent models of chronic uraemia

Three different animal models of chronic uraemia were investigated: the subtotal nephrectomy model, the 4-week adenine diet model and the adenine washout model. All models were compared with a suitable non-uraemic control group.

The subtotal nephrectomy model is the most widely used model in the published literature but it is labour intensive to create with the outcomes dependant on the skill of the technician. The adenine and adenine washout models are attractive in that they do not rely on these factors and can create a more severe form of renal injury. The 3 models of uraemia were created so that a comparrison may be made of their different uraemic phenotypes.

Methods

Sub-total nephrectomy model

6-week-old Wistar rats (Charles River UK) were housed in an animal house with a 12-hour day night cycle and had free access to standard chow and water. All procedures were conducted using aseptic technique and all instruments were autoclaved before use. The subtotal nephrectomy model was created as previously described by Raine et al.[291].

Stage 1: Left subtotal nephrectomy

After 1 week acclimatisation, the rats were anaesthetised with inhaled 1.5% isoflurane (Baxter). The fur on the left flank wall was shaved and a 1cm skin incision was made just inferior to the lower margin of the rib cage. The flank musculature was blunt dissected and the parietal peritoneum was cut. The left kidney was then carefully externalised though the incision. The perinephric fat and renal capsule were removed. An arterial clamp was then placed around the renal artery and vein.

The amount of tissue removed was crucial to the development of progressive renal impairment. If too little tissue was removed then the animal would not generate a uraemic phenotype, whereas if too much tissue was removed, following the right nephrectomy, the animal would subsequently become severely uraemic and have to be humanely sacrificed to prevent further suffering. An algorithm was developed (see Figure 3) to estimate how much renal parenchyma should be removed to attempt to

improve reproducibility. To achieve this, the weight of the removed sections of the left kidney were weighed and expressed as a percentage of total body weight. It was found that if more than 0.25% of the kidney to body weight was removed then a significant number of animals died or had to be sacrificed due to suffering. If the animals did survive up to the time of LAD occlusion, they were obviously much more uraemic than their other uraemic litter-mates and when analysed, had serum creatinine values >300umol/l. While this succeeded in generating an advanced uraemic phenotype, the animals were often very sick. Not infrequently these animals suffered respiratory arrests post anaesthetic, and those that survived this were left hypotensive post-arrest making them unusable for the experiment. Thus a 250g rat would require approximately 0.5-0.62g of renal parenchyma to be removed. This value was used as a guide, because the absolute weight of renal tissue removed was not the only determinant of subsequent uraemia. The kidney is a heterogeneous organ, with the glomeruli concentrated at the cortex. Therefore the amount of glomeruli removed would be different depending on the exact location of the sections: removing the same weight of tissue from the upper and lower pole only would be likely to contain less glomeruli than removing many thin cortical sections from around the whole. Furthermore the more cuts that were made to the kidney in an attempt to get an exact amount of tissue removed the greater the likelihood of trauma to the remaining renal parenchyma and the greater the resultant degree of uraemia. Taking all these factors into account, 3 sections of renal parenchyma were removed: the upper pole, lower pole and the lateral surface of the kidney. The arterial clip was removed and pressure was applied to the cut surfaces to promote haemostasis. Once haemostasis was established, the kidney was replaced in the abdominal cavity. 1ml of sterile saline was instilled into the abdominal cavity to replace fluid losses. The muscular layers were stitched together with 4-0 polysorb braded suture (Syneture), and the skin was closed using a 'Precise' Disposable skin stapler (3M). 0.03ml of Vetegesic (Buprenorphine, Alstoe Animal health) was injected subcutaneously to provide post-operative pain relief. The rats were left to recover under a warming lamp and returned to their cages when fully awake.

Stage 2: Right Nephrectomy

2 weeks after the subtotal left nephrectomy, rats were anaesthetised as before, a right flank incision was made and the right kidney was externalised in same way as the left. The right renal artery and vein were tied off and the entire kidney was removed distal to

the ties. The abdomen was sown up, opiates were given and the animal was left to recover as before.

The animals were then left for 4 weeks to develop a uraemic phenotype.

Sham SNx animals were created in a 2 stage procedure. This was identical to the SNx group, except that in the first stage no renal tissue was removed and in the second stage the right kidney was replaced back in the abdominal cavity after it was decapsulated.

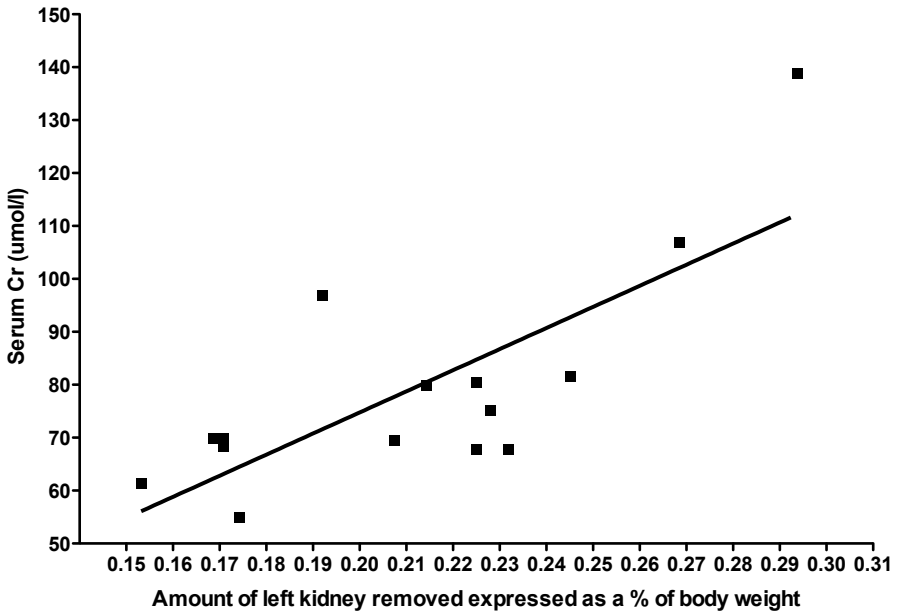


Figure 3 Impact of varying the amount of left renal tissue removal on subsequent development of renal dysfunction (r^2 0.58)

Adenine Diet

The adenine diet model as described by Yokozawa[253] was used. 6-week-old Wistar rats (Charles River UK) were housed in an animal house with a 12-hour day night cycle and had free access to water. After 1 week acclimatisation, during which time all animals were fed standard chow, animals were then either fed a standard pellet diet (Rat and mouse No.1 Maintenance diet: Special diet services UK) or the standard pellet diet with the addition of adenine 0.75% (Rat and mouse No.1 Maintenance diet +0.75% adenine: Special diet services UK). The animals were fed the adenine diet for 4 weeks to generate a uraemic phenotype. Animals were fed standard chow for 4 weeks to generate a non-uraemic control group.

Adenine Diet washout model

This model is identical to the standard adenine diet model, however at the end of 4 weeks using the 0.75% adenine diet, it is substituted for standard chow. The animals are then continued on standard chow for a further 2 weeks. Control animals are fed standard chow for a total of 6 weeks.

Characterisation of the uraemic phenotype

To characterise the uraemic phenotype of each model, animals were placed in metabolic cages. For the SNx and sham SNx groups, the animals were placed in metabolic cages 4 weeks after the second stage of the subtotal nephrectomy (or sham subtotal nephrectomy in the case of the sham SNx group). The adenine diet animals were placed in the metabolic cages 4 weeks after starting the adenine diet, or in the case of the control group, 4 weeks after starting the standard chow diet. The adenine washout group, were placed in the metabolic cages 2 weeks after switching from adenine diet to standard chow, the adenine washout controls were fed standard chow for 6 weeks.

All animals were placed in metabolic cages for 18 hours with free access to a known amount food and water. Food and water was weighed before and after the rats were placed in the metabolic cages, so that food and water intake could be calculated. Urine

was collected for measurement of volume. The urine was placed in a 10ml Falcon tube and was centrifuged at 500g for 5 minutes to remove particulate matter. The urine was then stored in 2ml Eppendorf tubes at -80 ^{o}C. After the animals were removed from the metabolic cages they were anaesthetised (for full details of anaesthesia see page 76) and underwent morphometry assessment (body length, tail length and tibial length). The rats then had venous and arterial lines inserted and a tracheostomy was performed (for full details of basic surgery protocol see basic surgery section p 76). Mean arterial pressure and heart rate was recorded (for full details on measuring cardiovascular parameters see page 77). 200µl of blood was withdrawn from the arterial line in a heparinised 1ml syringe and analysed using an ABL 77 blood gas analyser (Radiometer Copenhagen). A further 1ml of blood was withdrawn from the arterial line into a 2ml Eppendorf tube containing 30µL heparin (5000 iu/ml) and mixed, by repeated inversion. The sample was spun at 6000g for 3 min at 20^{o}C and the serum was aspirated into a new Eppendorf tube. The sample was then stored at -80 ^{o}C. A thoracotomy was performed (for full details of thoracotomy please see page 83) and the heart was quickly harvested.

Histological assessment of left ventricular hypertrophy

Increased heart weight to body weight ratio in the is an indirect measure of LVH. Histological analysis of uraemic and non-uraemic hearts was undertaken to confirm that the SNx model leads to LVH.

Hearts were harvested from SNx animals and ham SNx animals under deep anaesthesia and placed in 10% formalin for 10 days. The hearts were then processed by the histopathology department at the Royal London Hospital. The samples were embedded in paraffin, and sectioned into 5 um thick sections using a microtome. The hearts were stained using haematoxylin and eosin. The section through the widest point of the left ventricle was chosen as the point to measure the LV area. The cross sectional area of the left ventricle was calculated by subtracting the LV cavity area from the total area of the left ventricle in a blinded fashion using image-J software (NIH)[292]. This was repeated for each heart in the SNx group and sham SNx group.

Results

Subtotal nephrectomy model of chronic uraemia.

When compared to a sham procedure, the 2 stage subtotal nephrectomy procedure resulted in animals which were mildly growth restricted, with a 7% difference in mean weights, however there was no significance difference in body morphometry. Uraemic animals were polyuric and polydiptic, however no difference in food consumption was seen (see Table 1).

The SNx model resulted in a mean serum creatinine 2.4 times that of shams with a similar elevation in serum urea concentration. The uraemic animals were hypertensive, anaemic with greater heart weights and a greater heart weight to body weight ratio which has been used as a surrogate marker for cardiac hypertrophy[293].

On biochemical analysis, this model of uraemia was associated with hypercalcaemia and hypercholesterolaemia but was not associated with metabolic acidosis, hypoalbuminaemia, hypertriglyceridaemia, hyperkalaemia or hyperphosphataemia. Urine analysis of uraemic animals revealed significant proteinuria, natriuresis as evidenced by increased fractional excretion of sodium and increased urine sodium excretion.

	Sham SNx	SNx	p
Weight (g)	408 (29.5)	378 (33.4)	0.05
Water intake (ml/hr)	1.87 (0.40)	3.34 (0.40)	<0.0001
Urine output (ml/hr)	0.73 (0.21)	1.87 (0.47	<0.0001
Food intake (g/hr)	1.52 (0.20)	1.45 (0.29)	0.78
Tail length (cm)	19.4 (0.88)	18.8 (1.02)	0.18
Body length (cm)	25.2 (0.85)	24.4 (1.21)	0.08
Tibial length (cm)	5.89 (0.25)	5.87 (0.33)	0.89
Blood pressure (mm/Hg)	137 (26)	157 (19.6)	0.01
Pulse (BPM)	392 (51.6)	387 (44.7)	0.75
Heart weight (g)	1.14 (0.11)	1.31 (0.20)	0.04
Heart weight index*	2.85 (0.25)	3.44 (0.46)	0.003
Haematocrit (%)	37.8 (4.51)	27.1 (5.06)	< 0.0001
Plasma bicarbonate (mmol/l)	24.8 (4.1)	26.2 (3.4)	0.2
Plasma base excess (mEq/L)	1.77 (3.65)	2.42(6.0)	0.64
Serum albumin (g/l)	27.6 (2.24)	27.5 (1.90)	0.94
Serum urea (mmol/l)	6.2 (1.34)	17.4 (4.61)	<0.0001
Serum creatinine (µmol/l)	42.1 (5.41)	99.9 (30.1)	<0.0001
Serum cholesterol (mmol/l)	1.85 (0.37)	2.72 (0.50)	0.003
Serum triglycerides (mmol/l)	0.83 (0.50)	0.76 (0.43)	0.72
Serum sodium (mmol/l)	141 (9.73)	143 (1.76)	0.53
Serum potassium (mmol/l)	3.88 (0.54)	4.16 (0.41)	0.27
Serum phosphate (mmol/l)	2.50 (0.29)	2.29 (0.29)	0.11
Serum calcium (mmol/l)	2.52 (0.12)	2.75 (0.18)	0.003
Urine protein creatinine ratio**	1.4 (0.24)	4.25 (2.62)	0.0003
Urine sodium (mmol/l)	87.6 (27.3)	44.1 (11.0)	0.002
Fractional excretion of sodium (%)§	0.36 (0.10)	1.16 (0.58)	0.001
Urine sodium excretion (mmol/h/g)	0.15(0.04)	0.21 (0.04)	0.003

Table 1. Characterisation of the 2 stage subtotal nephrectomy model. Male Wistar rats underwent a 2 stage sham subtotal nephrectomy (sham SNx, n=10) were compared with rats which underwent a 2 stage subtotal nephrectomy model (SNx, n=12). All values presented as mean (SD), p values are results of 2-way unpaired t test using GraphPad software. Rows in bold type represent characteristics, which were significantly different between the 2 groups.
*Heart weight index was calculated by dividing the weight of the heart in grams by the weight of the animal in kilograms.**Protein creatinine ratio is calculated by dividing urine protein concentration by urine creatinine concentration. §Fractional excretion of sodium is calculated by dividing (urinary sodium concentration x plasma creatinine concentration) by (plasma sodium concentration x urinary creatinine concentration).

Adenine Diet model

4 weeks of 0.75% adenine supplementation resulted in significantly less weight gain than the control group, with a 31% difference in final weights (see Table 2). The adenine treated animals were polyuric but not polydiptic. Adenine fed animals ate just over 50% of the food compared to control animals.

Adenine diet was associated with growth restriction on morphometry, with a shorter tail, body and tibial length than controls. Adenine treatment resulted in significant uraemia with serum creatinine and urea concentrations 8 times that of the control groups, with a similar elevation in urea concentrations. The uraemic animals were not hypertensive, but were significantly more bradycardic than controls. Despite no difference in blood pressure, the heart weight index was significantly greater than the control group.

On biochemical analysis, this model of uraemia was associated with hypercalcaemia and hyperkalaemia but was not associated with metabolic acidosis, hypoalbuminaemia, hypercalcaemia, hypercholesterolaemia, hypertriglyceridaemia, or hyperphosphataemia. Adenine fed animals were also anaemic. Urine analysis of these uraemic animals revealed significant proteinuria and natriuresis as evidenced by increased fractional excretion of sodium and increased urine sodium excretion.

	Control	Adenine	p
Weight (g)	434 (25.6)	221(32.8)	<0.0001
Water intake (ml/hr)	2.66 (0.79)	2.63 (0.51)	0.91
Urine output (ml/hr)	0.8 (0.39)	1.59 (0.4)	<0.001
Food intake (g/hr)	1.50 (0.17)	0.82 (0.25)	< 0.0001
Tail length (cm)	20 (0.64)	17.5 (0.65)	< 0.0001
Body length (cm)	24.8 (0.75)	21.4 (0.98)	< 0.0001
Tibial length (cm)	5.91 (0.29)	4.99 (0.22)	< 0.0001
Blood pressure (mm/Hg)	137 (8.3)	129 (19.5)	0.19
Pulse (BPM)	397 (37.2)	296 (65.3)	< 0.0001
Heart weight (g)	1.15 (0.08)	0.78 (0.13)	< 0.0001
Heart weight index*	2.65 (0.24)	3.53 (0.42)	< 0.0001
Haematocrit (%)	42.3 (3.54)	27.3 (5.46)	< 0.0001
Plasma bicarbonate (mmol/l)	25.3 (3.4)	24.3 (1.6)	0.36
Plasma base excess (mEq/L)	2.81 (3.0)	1.83 (1.90	0.31
Serum albumin (g/l)	26.9 (2.23)	27.9 (2.30)	0.37
Serum urea (mmol/l)	5.87 (0.91)	59.3 (17.7)	< 0.0001
Serum creatinine (µmol/l)	33.5 (2.70)	266 (71.5)	< 0.0001
Serum cholesterol (mmol/l)	1.86 (0.37)	2.01 (0.16)	0.28
Serum triglycerides (mmol/l)	1.30 (0.86)	0.81 (0.71)	0.19
Serum sodium (mmol/l)	145 (2.36)	145 (1.93)	0.95
Serum potassium (mmol/l)	145 (2.36)	145 (1.93)	0.02
Serum phosphate (mmol/l)	2.86 (0.48)	2.66 (0.72)	0.47
Serum calcium (mmol/l)	2.22 (0.91)	2.57 (0.26)	0.02
Urine protein creatinine ratio**	1.71 (0.33)	4.57 (1.33)	< 0.0001
Urine sodium (mmol/l)	76.9 (30.6)	35.7 (6.0)	0.006
Fractional excretion of sodium (%)§	0.32 (0.07)	5.90 (1.69)	< 0.0001
Urine sodium excretion (mmol/h/g)	0.139 (0.04)	0.254 (0.07)	0.007

Table 2. Characterisation of the 0.75% adenine diet model. Male Wistar rats which were fed standard chow for 4 weeks (Control, n=12) were compared with rats which, were fed a diet containing 0.75% adenine for 4 weeks (Adenine, n=12). All values presented as mean (SD), p values are results of 2 way unpaired t test using GraphPad software. Rows in bold type represent characteristics which were significantly different between the 2 groups.
*Heart weight index was calculated by dividing the weight of the heart in grams by the weight of the animal in kilograms.**Protein creatinine ratio is calculated by dividing urine protein concentration by urine creatinine concentration. §Fractional excretion of sodium is calculated by dividing (urinary sodium concentration x plasma creatinine concentration) by (plasma sodium concentration x urinary creatinine concentration).

Adenine washout model

Four weeks of a 0.75% adenine diet followed by 2 weeks of standard chow resulted in rats which weighed only 73% of the control group (see Table 3). However, during two weeks of standard chow, the washout rats gained more weight than in the previous four and there was a reduction in difference in weight between the uraemic and non-uraemic animals (see Figure 4). The adenine treated animals were polyuric polydiptic, however no difference in food consumption was seen. The adenine washout group was associated with growth restriction, with a shorter tail, body and tibia length. The adenine washout model resulted in moderate uraemia with serum creatinine and urea concentrations 3 times that of the control. In addition, they were also significantly anaemic. The uraemic animals were not hypertensive and the bradycardia seen with adenine treatment had resolved. Despite no difference in blood pressure, the heart weight index was significantly greater than the control group.

On biochemical analysis, this model of uraemia was associated with hyperphosphataemia and hyperkalaemia but was not associated with metabolic acidosis, hypoalbuminaemia, hypercalcaemia, hypercholesterolaemia. Urine analysis of these uraemic animals revealed significant proteinuria, natriuresis as evidenced by increased fractional excretion of sodium and increased urine sodium excretion.

	Control	Ad washout	p
Weight (g)	477 (35.2)	348 (22.99)	<0.0001
Water intake (ml/hr)	1.88 (1.15)	3.89 (0.54)	0.008
Urine output (ml/hr)	0.89 (0.24)	2.39 (0.38)	0.003
Food intake (g/hr)	1.6 (0.08)	1.76 (0.29)	0.57
Tail length (cm)	20.2 (0.76)	17.9 (0.44)	< 0.0001
Body length (cm)	24.9 (0.74)	22.7 (0.54)	< 0.0001
Tibial length (cm)	5.42 (0.40)	4.79 (0.21)	0.005
Blood pressure (mm/Hg)	144 (31.1)	152 (26.6)	0.73
Pulse (BPM)	404 (12.0)	442 (33.6)	0.02
Heart weight (g)	1.43 (0.08)	1.43 (0.12)	0.95
Heart weight index*	3.02 (0.32)	4.36 (0.26)	< 0.0001
Haematocrit (%)	41.6 (1.82)	20.4 (5.19)	< 0.0001
Plasma bicarbonate (mmol/l)	31.6 (2.36)	28.9 (3.70)	0.23
Plasma base excess (mEq/L)	6.24 (1.36)	4.16 (3.05)	0.09
Serum albumin (g/l)	28.7 (1.00)	27.0(1.98)	0.11
Serum urea (mmol/l)	6.06 (0.69)	22.1 (11.1)	< 0.0001
Serum creatinine (µmol/l)	38.4 (3.01)	112 (54.4)	< 0.0001
Serum cholesterol (mmol/l)	2.18 (0.38)	3.13 (0.66)	0.009
Serum sodium (mmol/l)	142 (1.37)	144 (2.25)	0.08
Serum potassium (mmol/l)	4.02 (0.24)	5.44 (0.58)	< 0.0001
Serum phosphate (mmol/l)	2.34 (0.12)	2.65 (0.34)	0.05
Serum calcium (mmol/l)	2.51 (0.11)	2.58 (0.22)	0.43
Urine protein creatinine ratio**	1.86 (0.2)	11.7 (13.3)	0.04
Urine sodium (mmol/l)	0.44 (0.07)	3.23 (1.59)	<0.0001
Fractional excretion of sodium (%)§	0.44 (0.07)	3.23 (1.59)	0.002
Urine sodium excretion (mmol/h/g)	0.20 (0.04)	0.31 (0.08)	0.02

Table 3. Characterisation of the adenine washout model. Male Wistar rats fed standard chow for 6 weeks (Control, n=10) were compared with rats which were fed a diet containing 0.75% adenine for 4 weeks, followed by 2 weeks of standard chow (Ad washout, n=12). All values presented as mean (SD), p values are results of 2 way unpaired t test using GraphPad software. Rows in bold type represent characteristics, which were significantly different between the 2 groups.

*Heart weight index was calculated by dividing the weight of the heart in grams by the weight of the animal in kilograms.**Protein creatinine ratio is calculated by dividing urine protein concentration by urine creatinine concentration. §Fractional excretion of sodium is calculated by dividing (urinary sodium concentration x plasma creatinine concentration) by (plasma sodium concentration x urinary creatinine concentration).

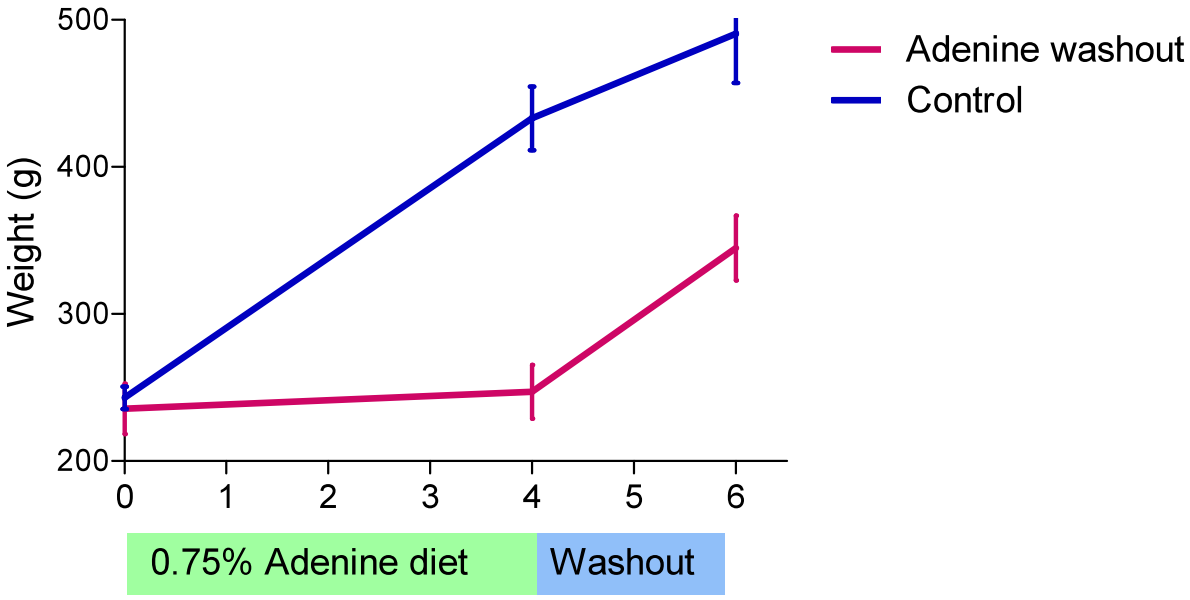


Figure 4. Results of weight gain in rats fed standard chow for 6 weeks (control) and rats fed a 0.75% adenine diet for 4 weeks followed by 2 weeks of standard chow (Adenine washout). Graph displays mean with error bars displaying SEM)

Comparison of 3 models of uraemia

Direct comparison of the weight gains of the 3 different models, is complicated because the adenine diet rats were 10 weeks old rather than 12 weeks for the SNx and washout models. However, the adenine washout group was still significantly lighter than the SNx group (see Figure 5, Figure 6 and Figure 7).

Both the adenine and adenine washout models resulted in a greater degree of polyuria and polydipsia than the SNx model ($p<0.01$).

The fractional excretion of sodium is the percentage of sodium filtered by the glomerulus, which is excreted in the urine. A FeNa of <1% in the context of uraemia suggests the cause of renal failure is hypoperfusion of the kidneys. The high FeNa compared to control animals seen in all 3 groups is consistent with tubular damage and reduction in nephron number. The adenine diet model is associated with a higher FeNa than adenine washout group ($p<0.001$), which in turn has a higher FeNa than the SNx model ($p<0.01$). The urine excretion of sodium is higher in the adenine washout group than the SNx group ($p<0.05$). This could be explained by the increased sodium intake in the washout group from the increased food intake. There was no significant difference in the degree of proteinuria between the 3 groups, despite a trend towards higher proteinuria in the adenine washout group. Both SNx and adenine models led to similar degrees of anaemia, but the adenine washout model was associated with significantly greater anaemia than the other 2 groups ($p<0.005$).

The adenine model resulted in a lower blood pressure than both the adenine washout and SNx models ($p<0.0001$). The pulse rate was also significantly lower in the adenine group than either the SNx group or the adenine washout group ($p<0.0001$). The adenine washout group also had a greater heart weight to body weight ratio than either the SNx model or adenine model ($p<0.001$). The SNx and adenine washout models resulted in a similar final concentration of creatinine and urea. However, the adenine model resulted in more severe uraemia with a creatinine which was over 2 times that of either of the other 2 models ($p<0.0001$). Both the adenine ($p<0.01$) and the adenine washout models ($p<0.001$) were associated with higher serum potassium concentrations than the SNx model.

There was no significant difference in serum calcium, sodium or phosphate concentrations between the 3 models. This was despite the adenine washout group

being the only group to develop hyperphosphataemia when compared to their individual controls.

Both the adenine washout and the SNx models had similar serum cholesterol concentrations. However, the adenine model had lower serum cholesterol than both the washout ($p<0.001$) and SNx models ($p<0.05$).

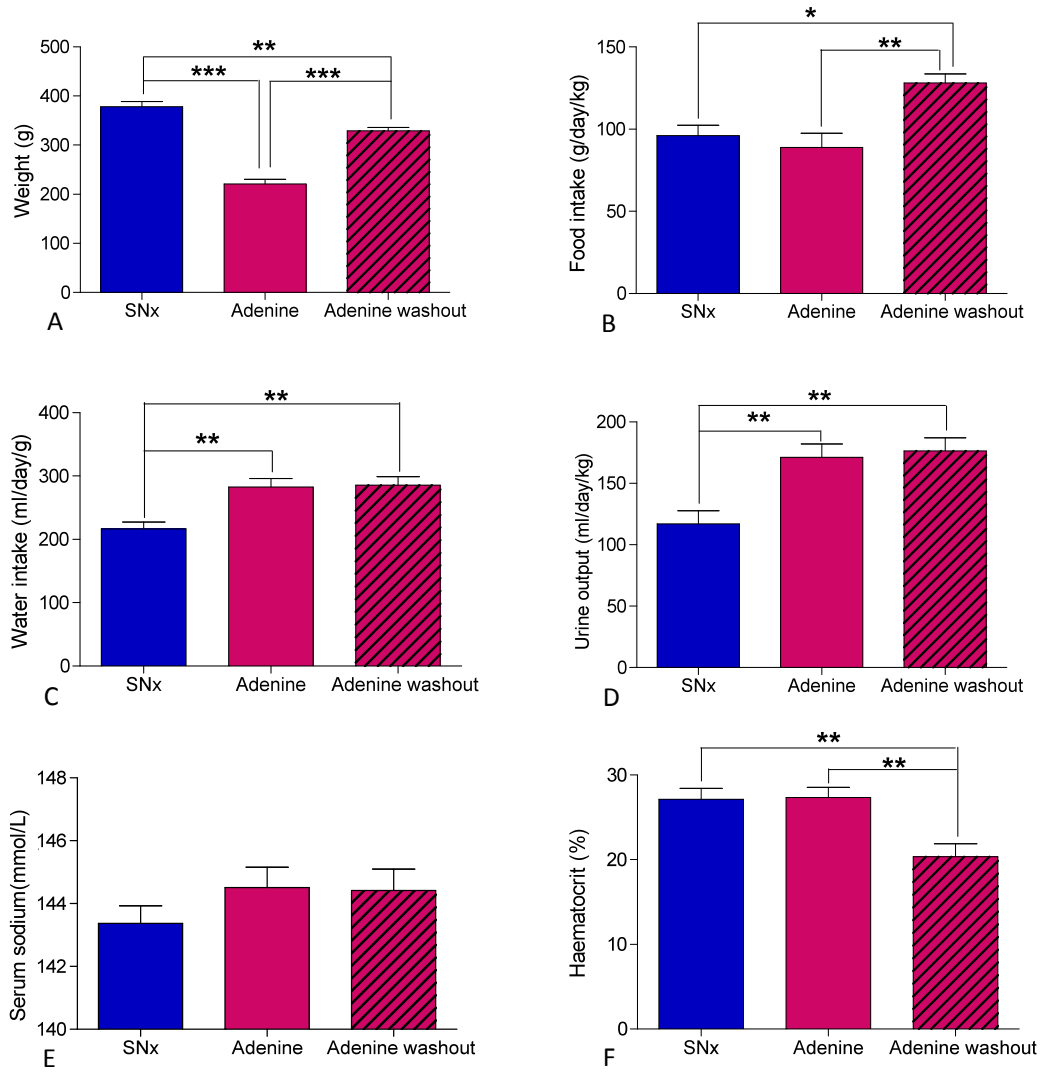


Figure 5. Comparison of the uraemic phenotype between different rodent models of chronic uraemia. A: Mean rat weight following a 2 stage SNx procedure, 4 weeks of 0.75% adenine diet, or 4 weeks of Adenine diet followed by a 2 week washout. B: Amount of food eaten per day, indexed for rat weight in Kg. C: Mean water intake per day indexed for rat weight in Kg. D: Urine output per day indexed for rat weight. E: Mean serum sodium concentration. F: Mean haematocrit values. In each group n=12. Graphs presented as mean with error bars as SEM. Results of 2 way ANOVA with “Bonferroni's Multiple Comparison Test" displayed as *,**,***. *p=0.01-0.05, **p=0.001-0.005, ***p<0.0001.

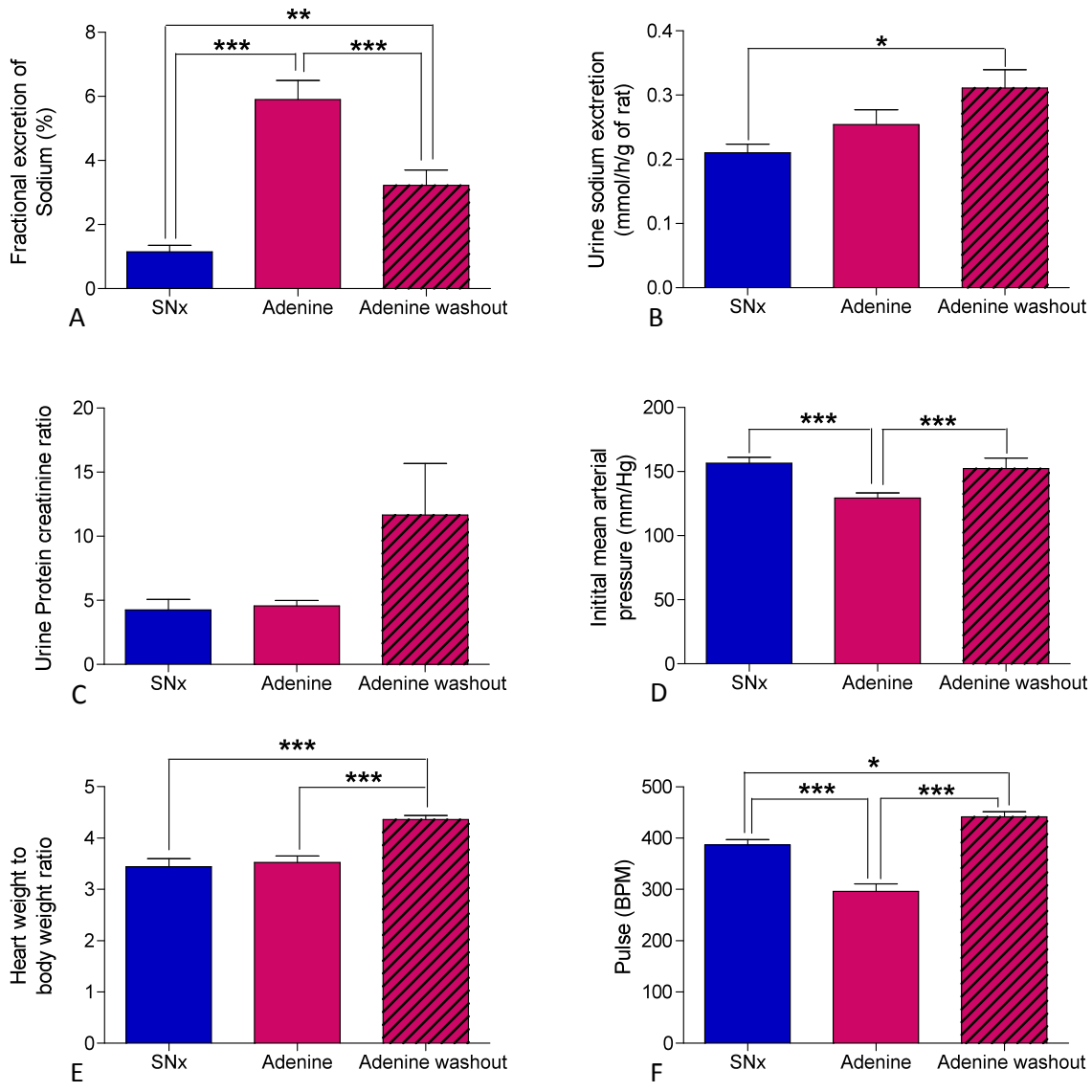


Figure 6. Comparison of the uraemic phenotype between different rodent models of chronic uraemia.

A: Urinary fractional excretion of sodium. B: Daily urinary sodium excetion indexed for weight of rat in g. C: Urine protein creatinine ratio. D: Mean arterial pressure as measured before throacotomy. E: Heart weight to body weight ratio calculated by dividing the heart weight in grams by the weight of the animal in Kg. F: Pulse rate measured beofre thoracotomy. In each group n=12. Graphs presented as mean with error bars as SEM. Results of 2 way ANOVA with "Bonferroni's Multiple Comparison Test" displayed as *,**,***. *p=0.01-0.05, **p=0.001-0.005, ***p<0.0001.

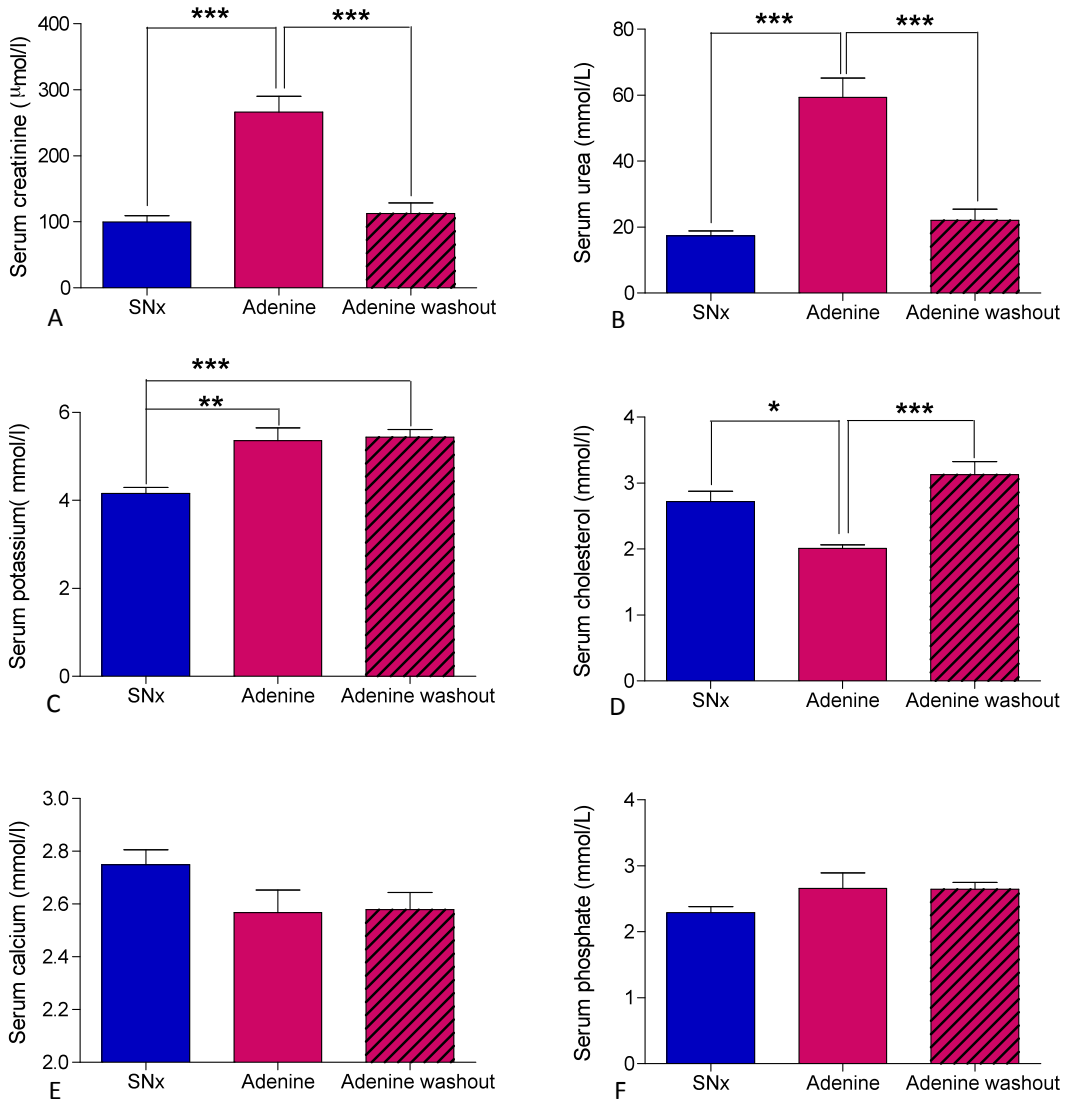


Figure 7. Comparison of the uraemic phenotype between different rodent models of chronic uraemia.

A:Mean serum creatinine following a 2 stage subtotal nephrectomy procedure (SNx), 4 weeks of 0.75% adenine diet (Adenine) or 4 weeks of adenine diet followed by a 2 weeks of standard chow. B:Mean serum urea in 3 models of uraemia. C:Mean serum potassium in 3 models of uraemia. D:Mean serum cholesterol in 3 models of uraemia. E: Mean serum calcium in 3 models of uraemia F: Mean serum phosphate in 3 models of uraemia.

In each group n=12. Graphs presented as mean with error bars as SEM. Results of 2 way ANOVA with "Bonferroni's Multiple Comparison Test" displayed as *,**,***. *p=0.01-0.05, **p=0.001-0.005, ***p<0.0001.

Histological assessment of left ventricular hypertrophy

On gross inspection it was clear which hearts came from uraemic animals and which did not. While the hearts were grossly the same size, the LV cavity was significantly smaller in the uraemic animals (see Figure 8, Image 1 and Table 4). On statistical analysis of the images, as assessed using image-J, the SNx group had a significantly larger LV surface area than the sham SNx group ($p=0.01$). When corrected for body weight, this difference became even more significant as the uraemic rats were lighter than the non uraemic animals ($p=0.003$).

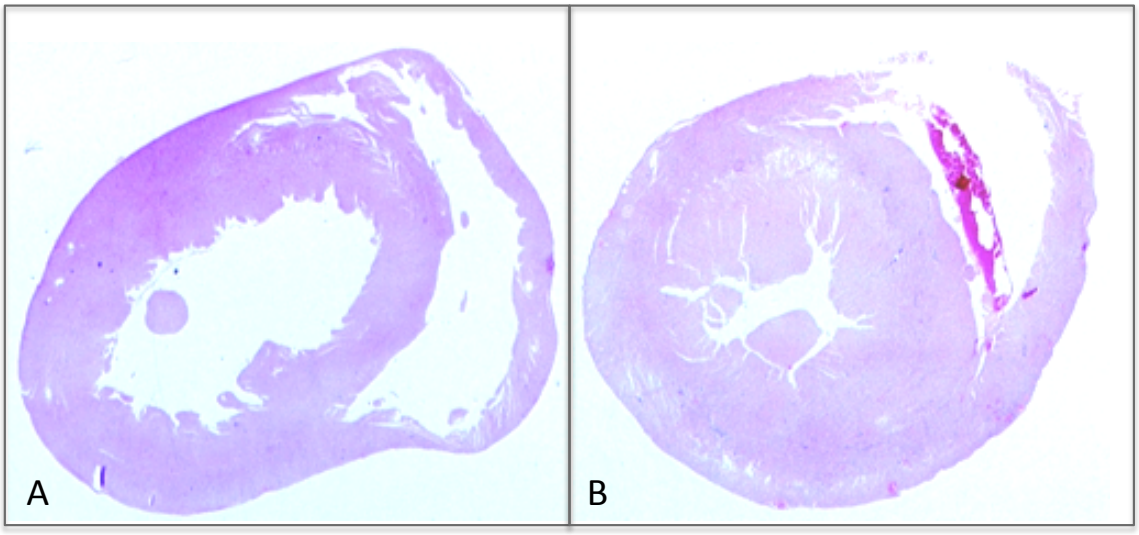


Image 1. Representative photographs of transverse sections through the non-uraemic sham SNx group (A) and the uraemic SNx group (B).

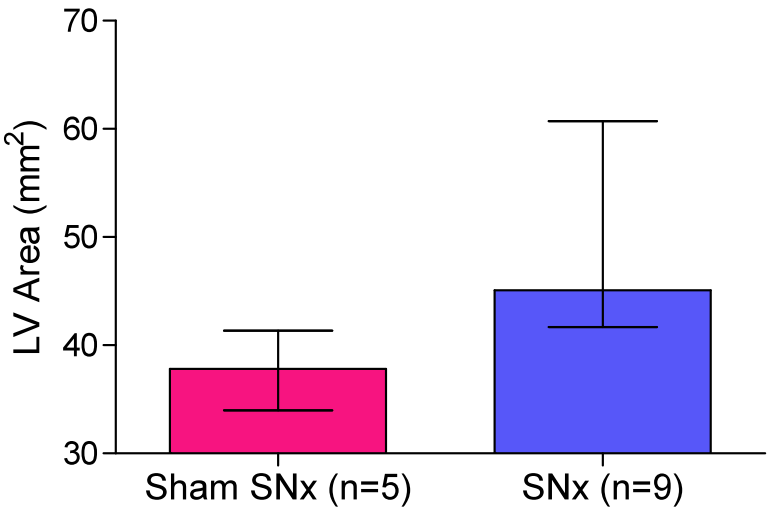


Figure 8. Graph showing the LV area in uraemic (SNx) and non uraemic (Sham SNx) rats. Colums reperesent median, with error bars IQR

	Sham SNx (n=5)	SNx (n=9)	p
LV area ($mm^{2)}$	37.8 (34-41.4)	45.1 (41.7-60.7)	0.01
LV area (mm^2/Kg)	92.7 (83.3-101.4)	19.3 (110.3-160.6)	0.003

Table 4. Table showing results of histological analysis of left ventricular surface area in uraemic (SNx) and non uraemic (Sham SNx) rats. The top row is the LV area, the bottom row is the LV area corrected fro body weight. Data shown as median (IQR). The displayed p value is the result of a 2 tailed Mann Whitney test using GraphPad software.

Discussion

All 3 models studied share several characteristics typical of a uraemic phenotype; anaemia, raised serum creatinine, raised serum urea, proteinuria, natriuresis, reduced weight gain and increased heart weight index. The heart weight index has been used as a surrogate marker for LVH. The histological study confirms the finding that the SNx model of chronic uraemia leads to the development of LVH. However the lack of adenine samples of myocardial histology to demonstatate presence of absence of LVH is a limitation.

Adenine animals have been shown to develop vascular calcification after 4 weeks[274]. Vascular calcification widens the pulse pressure[294], which may cause the MAP to fall despite elevated systolic pressures. Thus in the context of potential vascular calcification, a normal mean arterial pressure may miss combined systolic hypertension and relative diastolic hypotension.

An ideal animal model of chronic uraemia should reflect all aspects of the uraemic phenotype, and while these 3 models display many aspects seen in patients with CKD such as cardiac hypertrophy, growth restriction (commonly seen in the paediatric nephrology population), anaemia, proteinuria, loss of urinary concentrating ability (in the form of polyuria and a high FeNa), some important aspects are not represented in these models. Uraemia is associated with acidaemia caused by a reduction of hydrogen ion excretion by the renal tubules. None of these models were associated with a low plasma bicarbonate. Hypocalcaemia and hyperphosphataemia are hallmarks of uraemia leading to hyperparathyroidism, and vascular calcification. Only the washout model demonstrated significant elevations in serum phosphate. An explanation for this could be due to the increased food (and hence phosphate) intake during the washout phase. No model led to significant hypocalcaemia and interestingly, both the adenine and SNx models but not the adenine washout model was associated with hypercalcaemia. This is not a feature of the models in the published literature. A mechanistic explanation for the hypercalcaemia seen in these animals is difficult to explain, given the fact that both models showed similar levels of hypercalcaemia. The SNx animals had the same diet as the sham SNx animals effectively ruling out a dietary cause for the higher calcium. Tertiary hyperparathyroidism could lead to hypercalcaemia, however this is invariably associated with (and triggered by) hyperphosphataemia, which the rats did not display.

The development of a uraemic phenotype is dependant on 2 factors, the degree of uraemia and the duration to which the animal is exposed to uraemia. These factors may explain some of the differences seen in these models. While the 4-week adenine model represents the model with the highest serum creatinine, the haematocrit is similar to the SNx model. Relative erythropoietin deficiency caused by reduced nephron mass or damage to renal endothelial cells leads to a reduction in red cell production which in turn leads anaemia. Thus despite the SNx group having significantly better renal function, the animals have had underlying renal failure for longer. The duration of uraemia also explains the adenine washout group having the lowest haematocrit. The duration of uraemia may also explain the increased cardiac hypertrophy seen in the adenine washout group.

There are two possible explanations for the finding that 0.75% adenine diet leads to a reduction in food intake. Firstly it could be that as with humans, uraemia is associated with anorexia with resultant weight loss, or secondly, it could be that the adenine is unpalatable for the rats. If the lack of food intake was solely due to uraemia, then one would expect the food intake to gradually decrease over the 4 weeks of adenine diet and then gradually increase when placed back on standard chow as the uraemia partially resolves. However work by Terai et al.[265] reported that when rats were commenced on 0.75% adenine diet the rats stopped eating for up to 7 days. In addition during the washout experiment when the rats were placed back in cages with standard chow, despite being uraemic, they vigorously ate the standard pellets. This all suggests that adenine is very unpalatable and importantly part of the phenotype of the adenine diet may be related to starvation.

The bradycardia seen with the adenine diet model, which resolves after 2 weeks of standard chow, has not been reported in the published literature. This could explain both the bradycardia and its resolution on a normal diet. The cardiovascular effects of starvation has been studied in a human anorexic cohort. Starvation is known to cause bradycardia, and hypotension[295], which has been partly explained through an increased vagal tone[296].

Bradycardia could also be caused by increased levels of adenosine generated as adenine is metabolised (see Figure 2). Alterations in purine metabolism in the adenine model of chronic uraemia is investigated later (see section entitled: Investigation of the bioenergetics of the uraemic heart, page 118 for further details).

Future work

1) Replication of the cardiac histology study to include an adenine treated cohort and also an adenine washout group. This would provide confirmatory data on whether adenine induced uraemia (unlike the renal reduction model) lead to LVH. In additon further analysis of the histological specimens for presence of fibrosis and capillary density would provide valuable information on whether diefferences in the ischaemia tolerance of the models were linked to histological differences.

2) Further investigation of the apparent hypercalcaemia seen in the uraemic animals is needed. Additional work should include analysis of animla serum for PTH and FGF-23, as an explanation for hypercalcaemia.

3) Further investigation into the bradycardia seen with the adenine treated groups but not the SNx or Adenine washout groups. The bioenergetics of adenine treatment will be examined in a later chapter see p118. In addition to dissect whether adenine leads to bradycardia directly through chronotropic metabolites or indirectly through anorexia. Further experiments could be done comparing adenine treated animals with pair fed controls. If it were a direct effect of adenine supplementation then the pair fed animals would not be bradycardic relative to the adenine fed rats, whereas if the bradycardia was merely an effect of starvation then both groups would be expected to have similar heart rates.

Chapter 3

Reversible LAD Ligation as a Model of Myocardial Infarction in the Rodent

Methods

Basic Surgery

Six-week-old male Wistar Rats (Charles Rivers UK) were obtained and after 1 week acclimatisation in an animal house with a 12-hour day night cycle and free access to water and standard chow, they underwent protocols to generate uraemia (see methods section page 52 for details on creation of the SNx model of uraemia and page 55 for details on creation of the adenine model).

Rats were anaesthetised using an IP injection of Thiopental Sodium (Link Pharmaceuticals), the dose of anaesthetic required was optimised to ensure that the animals were anaesthetised rapidly, without causing respiratory depression and without requirement for additional anaesthetic. The dose used was 73mg/kg for the uraemic animals and 87mg/kg for the control animals.

Once the animals were anaesthetised they were placed on a homoeothermic control unit (Harvard Apparatus) and their temperature was measured using a rectal digital thermometer. Throughout the experiments the heating mat temperature was adjusted to maintain core temperature 37^0C +/- 1 ^{0}C. After removal of the skin over the anterior surface of the neck, the cervical musculature was blunt dissected to reveal the trachea. 2 sutures were placed around and behind the trachea 5mm apart. Between the 2 sutures approximately 70% of the circumferential length of the trachea was cut using Castroviejo angled spring scissors (Harvard Bioscience). The trachea was cannulated using Portex tubing (Smiths Medical Watford UK) internal diameter (ID) of 1.67mm and tied in place using the 2 sutures. If necessary any bronchial secretions were aspirated using a 10ml syringe connected to a length of Portex (ID 1.67mm) tubing.

The right carotid artery was exposed using careful dissection of the cervical musculature, a suture was tied at the cranial end of the artery and an arterial clip was placed at the caudal margin. Again using Castroviejo angled spring scissors, a small incision was made in the artery and the artery was cannulated using a length of Portex tubing (internal diameter 0.58mm) connected to a 2ml syringe filled with heparinised saline (15iu/ml). Once the catheter was in place the arterial clamp was removed and the catheter was tied in place. The external jugular vein was dissected out and tied off cranially using sutures as described above, and cannulated caudally using a length of Portex tubing (ID 0.4mm). The venous line was used for giving anaesthetic and also for

fluid administration. Rats were given saline at a rate of 1ml/kg every 30mins throughout the experiment.

Measurement of mean arterial pressure (MAP) and heart rate (HR)

The arterial line was connected to pressure transducers (BP domes MLA 844 ADI instruments), which were then connected to the Powerlab/85p system via bridge amps (all supplied by ADI instruments). The setup was calibrated at the start of each experiment using a mercury sphygmomanometer attached to the BP domes and inflated to pressures of 90 and 150 mmHg. The arterial pressure wave was displayed on a computer monitor using PowerLab Chart software. MAP and HR were recorded at regular times during the experiment, the precise timings varied depending on the experimental protocol. If in the rare circumstance the rat's MAP did not recover to higher than 80 mmHg post thoracotomy then the rat was humanely terminated, without proceeding to myocardial ischaemia.

Ventilation

Rats were ventilated using small animal ventilators (Harvard). The minute volume (defined as the ventilatory volume/minute required to maintain carbon dioxide homeostasis) that a rat requires is 575 cm^3 /min/kg (range 460-900 cm^3)[297]**.** Rats were ventilated at 70 breaths per minute (BPM) and the tidal volume (Vt) altered according to the weight. Thus a 300g rat would have a minute volume of 172.5 cm^3 and, when breathing at a rate of 70 BPM the Vt would be 2.46 cm^3.

Ventilator Calibration

To ensure that the Vt as displayed on the ventilators was actually the Vt given to the rats, each ventilator was calibrated. 1 litre measuring cylinders were filled with water and then quickly submerged under water. Tubing from the ventilators was placed under the water and the ventilators were turned on. Ventilators were set at varying rates (60, 70, 80 BPM) and at varying Vt and the amount of water displaced was recorded (see Figure 9). This was repeated three times and the average volume of water displaced was recorded. It was found that each ventilator gave slightly different Vt. These small differences in Vt would over the course of a lengthy experiment result in significantly different total ventilatory volumes. The delivered Vt was plotted against the measured

Vt and a normogram was plotted for each ventilator (see Figure 10). Different ventilators gave consistently different readings, thus the Vt on each ventilator depended on the weight of the animal and the ventilator used. Using this method, we were able to readily set the ventilatory properties to ensure the animals respiratory parameters were kept in the physiological range.

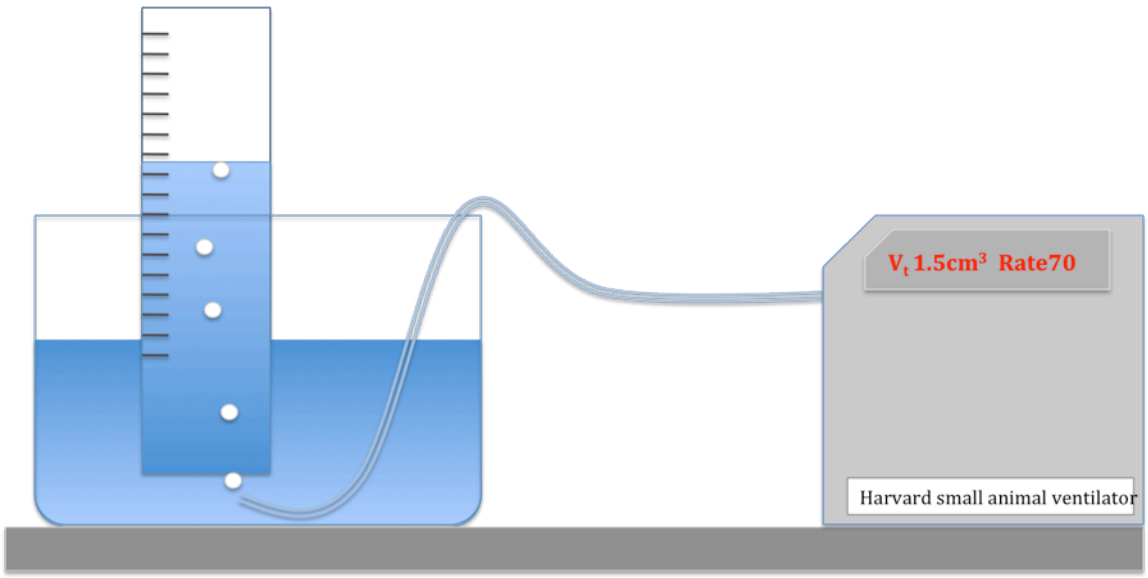


Figure 9. Ventilator calibration. The Harvard ventilator was set at 70BPM with varying Vt, and the volume of water displaced over 60s was recorded.

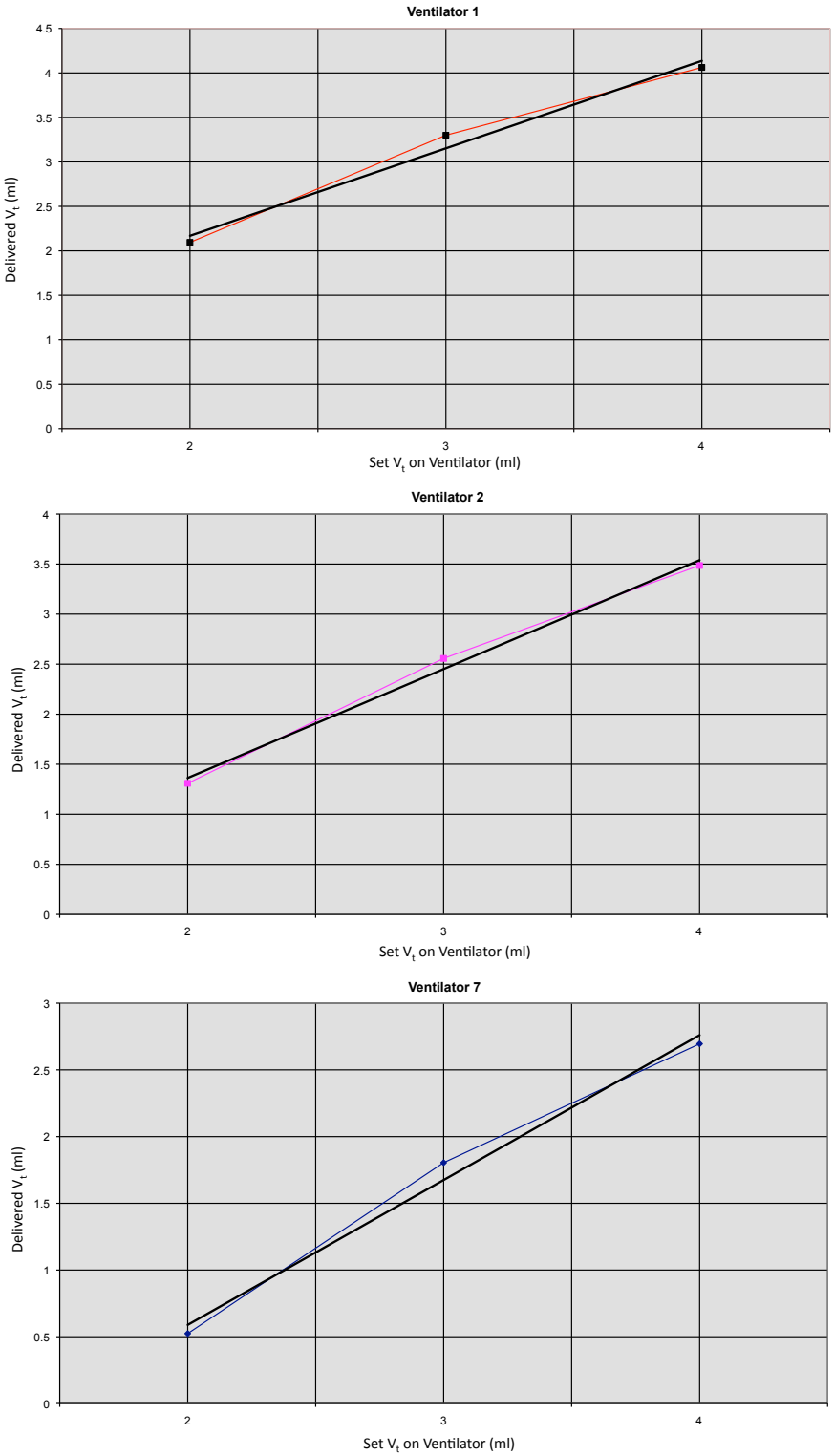


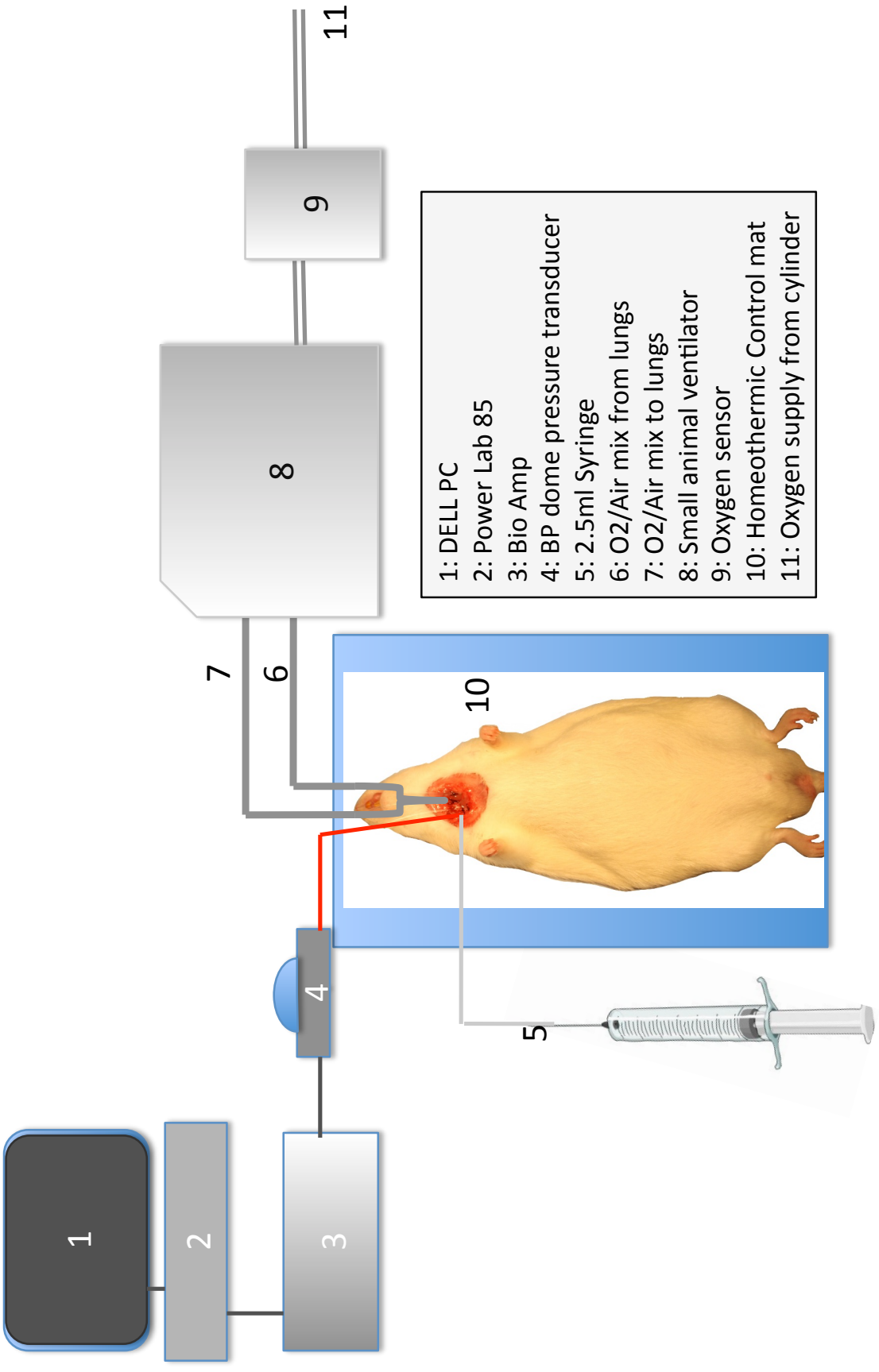
Figure 10. Individual normograms for 3 different Harvard small animal ventilators. Note should be made of the differing scales, thus to deliver a Vt of 2.5ml ventilator 1 was set to 2.4ml, ventilator 2 to 3ml and ventilator 7 was set to 3.8ml.

Individual normograms were created for each individual ventilator, which were verified on serial arterial blood gas (ABG) testing. After 15 minutes stabilisation on the ventilators, 200ul of arterial blood was withdrawn into a heparinised syringe and analysed using an ABL 77 blood gas analyser (Radiometer Copenhagen).

If arterial blood gases demonstrated a significant respiratory acidosis or alkalosis, the minute volume was adjusted using the Vt or respiratory rate. After 15minutes the ABG was repeated to ensure normal physiological parameters.

Supplemental oxygen was delivered to the rats from an oxygen cylinder. The amount of oxygen delivered was controlled using a tap and measured using an oxygen sensor (TED 200T7 Teledyne industries). The oxygen flow rate was adjusted during the course of the experiment to maintain a pO_2 of 10-14 kPa as measured on ABG. ABGs were routinely taken at the following time points: 1) 15 minutes after ventilation, 2) Pre-occlusion and 3) at the end of reperfusion, at which time up to 1ml of blood was taken and stored for further analysis. Further ABGs were occasionally taken if a rat was unexpectedly hypotensive during the experiment (See Figure 11 for schematic representation of full experimental setup).

Figure 11. Schematic representation of experimental setup for the rat model of myocardial infarction



Thoracotomy

To ensure the rats were deeply anaesthetised their foot pad was squeezed using forceps to elicit a pain response. If there was any evidence of a pain response, a further 0.05ml of Thiopental Sodium was administered IV, using the internal jugular line. After a delay of several minutes, the procedure was repeated to ensure adequate anaesthesia before the thoracotomy was commenced.

An incision was made just inferior to the lower border of the sternum and the skin was blunt dissected off the anterior thoracic wall up to the superior margin of the sternum. The animals were placed on a metal conductive plate attached to an Electrosurgery system (PromhoVet). Using diathermy forceps and a diathermy electrode a left parasternal thoracotomy was performed involving the lower 6 ribs. The parietal pleura was then carefully dissected away to reveal the thoracic cavity. The cavity was held open using a rodent thoracic spreader (see Image 2). The thymus was lifted clear of the heart and held in place by an arterial clamp, while the pericardial membrane was carefully removed.

A 6-0 Mersilk round bodied curved needle suture (Ethicon) was used to snare the left anterior descending artery (see Image 3). The position of the suture was approximately 2mm inferior to the junction of the left atrium with the left ventricle.

Correct suture placement was critical: if the suture was placed too high it could snare the left main stem artery rather than the LAD. This would lead to a massive infarction, associated with fatal ischaemic arrythmias. If the suture was placed too low down the LV there was a greater chance of missing the LAD, which meant that when the occluder was tightened the distal myocardium was not rendered ischaemic. The correct placement of the suture was extremely challenging: the LAD was rarely visible, the heart was beating at 400 BPM with the chest cavity moving at 70 breaths/minute and the difference between placing the suture too high or too low was less than 5mm.

LAD occlusion

After the thoracotomy the rat was removed from the metal conductive plate and left to recover. A 2 cm section of Portex tubing ID1.67mm was cut. The tip of the section of tubing was held briefly over a candle flame so that the edge would flare out to make a suitable occluder.

The occluder was threaded over the suture and then pulled so that the LAD artery was occluded between the suture material and the occluder. The degree of pressure applied was critical; too little pressure and the LAD did not fully occlude, too much pressure may cause damage to the LAD, impairing reperfusion. To ensure the pressure was correct the tension was increased until the area at risk turned pale (see Image 5). At this point the occluder was locked in place using a straight mosquito forceps (Harvard). The area at risk (AAR) was regularly checked to ensure that the area of ischaemia remained distinct from the surrounding tissue.

At the end of ischaemic time, the mosquito forceps were released reopening the LAD artery. Reperfusion was confirmed by noting a prompt hyperaemic colour change in the previously ischaemic AAR (see Image 4) and reperfusion arrhythmias were noted. The animals were then left for the duration of the reperfusion time (generally 2 hours, but the exact reperfusion time depended on the individual experimental protocol). During this time the animals MAP, HR and temperature were recorded and saline boluses were given every 30 minutes.

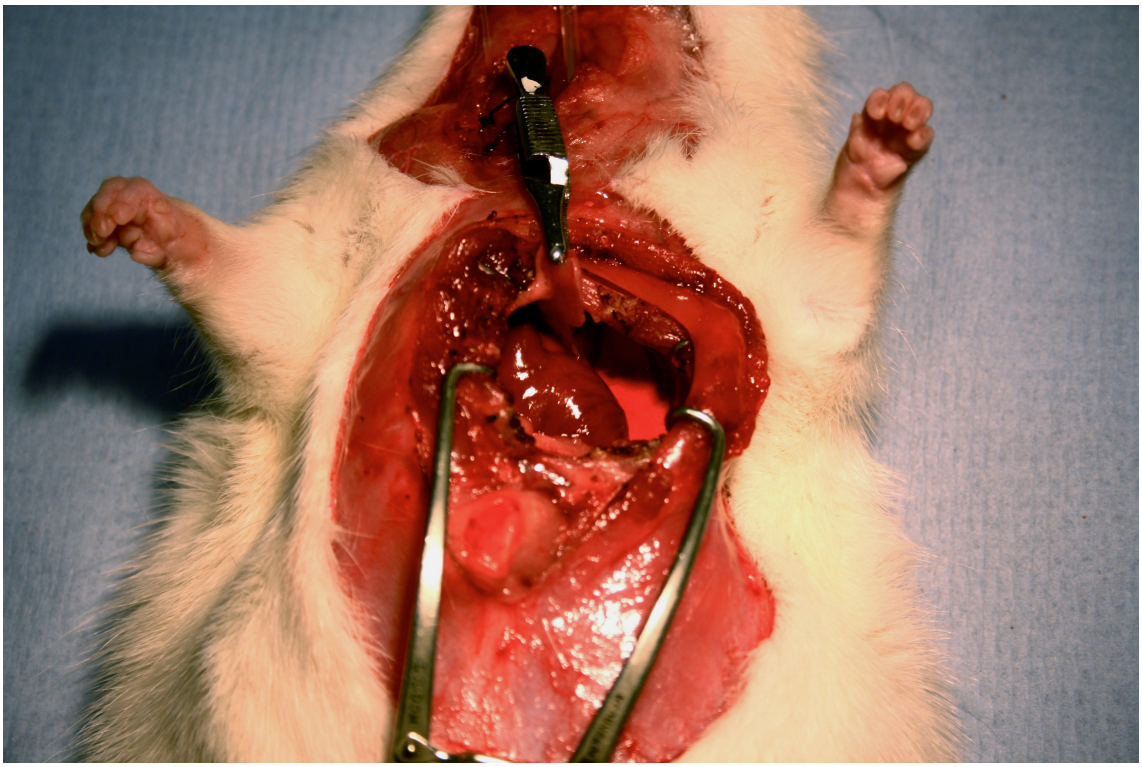


Image 2. Photograph of a rat post thoracotomy with pericardial membrane removed and thymus retracted

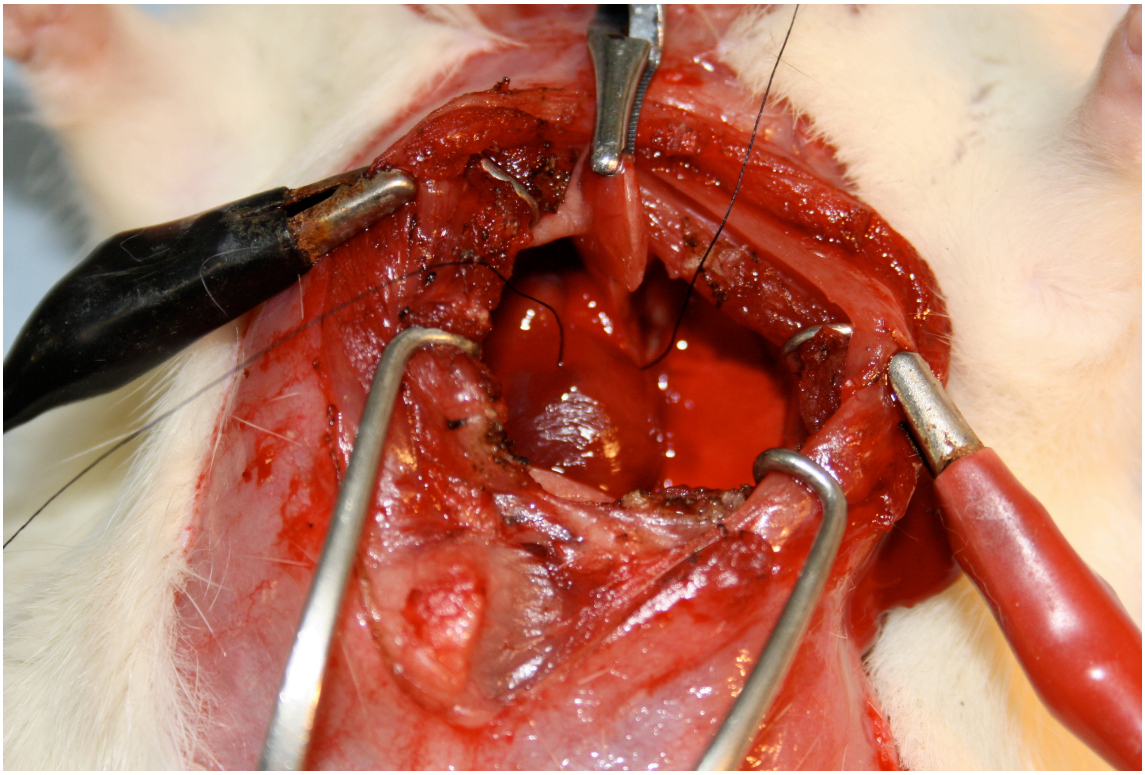


Image 3. 6-0 Mersilk suture placed 2-3mm inferior to atrio-ventricular junction . ECG electrodes attached to muscle wall for measurement of ischaemic changes and arrythmias.

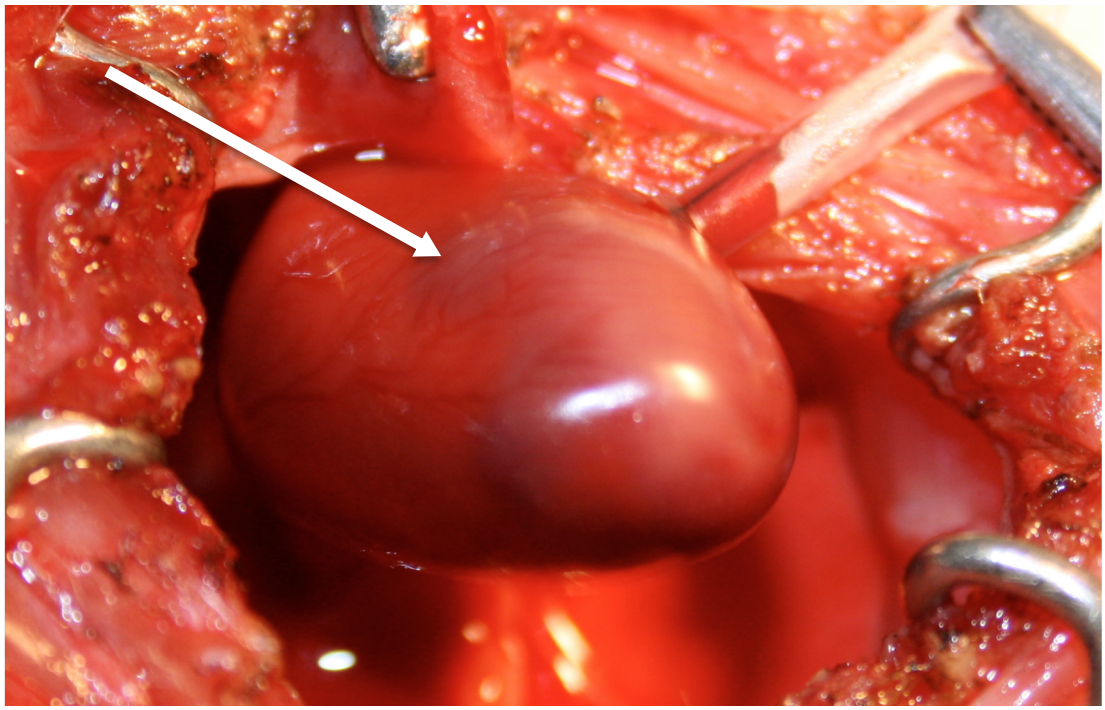


Image 5. LAD occluded using portex snare. The white arrow indicates the pale area representing the area at risk.

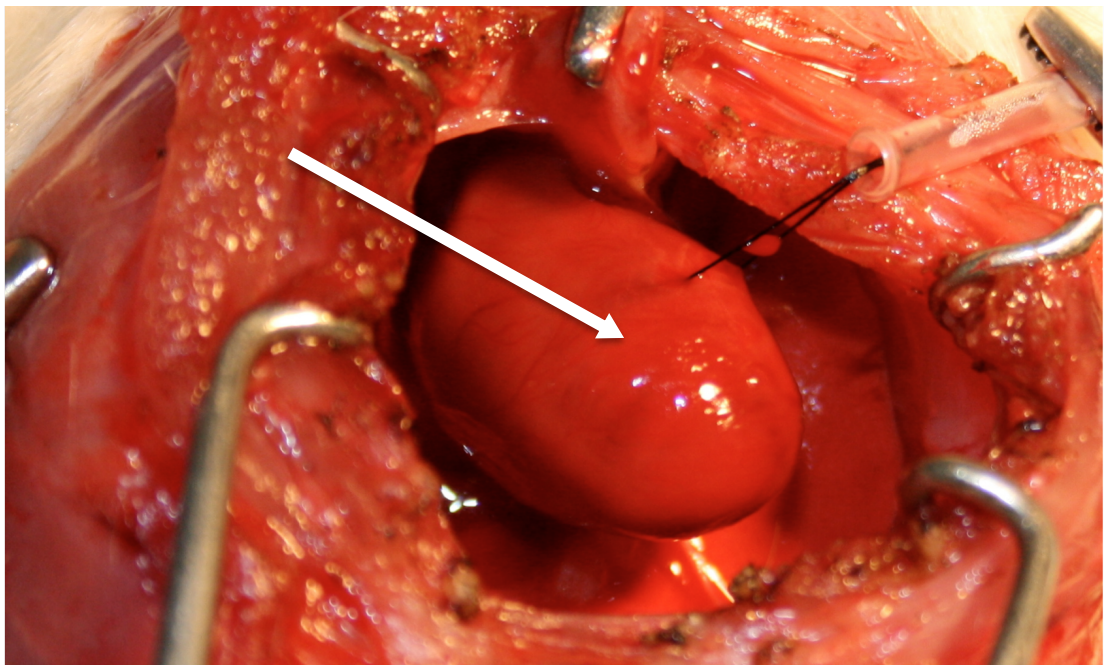


Image 4. After the tubing is unclamped the area at risk (white arrow) becomes markedly hyperaemic indicating reperfusion.

Harvesting

At the end of reperfusion, 1ml of blood was taken and spun in a centrifuge at 9900rpm for 4 minutes. The plasma was aspirated and frozen for future analysis. The LAD was briefly re-occluded as before. Following this, 2mls (2% w/v) of Evans blue dye (Sigma Pharmaceuticals) was then infused through the venous line until the whole animal developed a blue tinge. At this point, only the area of the heart which was ischaemic remained red. This was defined as the AAR. The heart was cut away from the animal and placed in ice-cold isotonic saline to ensure rapid cooling and termination of myocardial contractility. During this time the clamp remained on. After several minutes the heart was removed from the ice cold saline and the clamp was taken off. The heart was then placed inside a 2ml syringe whose end had been removed. Care was taken to ensure that the heart was orientated within the syringe so that the LV apex was towards the end of the syringe. The syringe plunger was then used to extrude the heart slowly so that 2 mm sections were exposed at the tip. These were then carefully sliced off and placed into a Petri dish containing ice-cold saline. This process continued until the point at which the suture was visible. The remainder of the heart was removed and weighed. For each section the right ventricle was removed using a scalpel and placed with the remainder of the heart above the suture point. After this the red areas of tissue (AAR) were cut from the blue stained tissue (perfused LV). These tissues were weighed to define the AAR expressed as a percentage of the total LV.

Determination of Infarcted tissue

Nitro Blue Tetrazolium (NBT) solution was made up using 5 mg of NBT (Sigma-Aldrich Gillingham UK) dissolved in 10ml Saline. NBT is reduced in the presence of an intact dehydrogenase enzyme system to form a dark blue formazan[298]. In infarcted tissue, these dehydrogenase pathways are not intact and so the tissue remains red/pink. In this way, viable and infarcted tissue may be distinguished from each other.

The AAR tissue was cut into 2mm cubes and incubated at 37^0C with 10ml of the NBT solution for 30 minutes. After 30 minutes the sections were then removed from the NBT solution washed in saline and separated according to their colour. Those samples that remained pink/red were considered infarcted tissue and those, which were stained blue, were considered viable. Both collections of tissue were weighed and the infarct was expressed as a percentage of the red to blue tissue by weight.

Detection of myocardial ischaemia and reperfusion using electrocardiography and blood pressure analysis.

During the LAD experiments, 3 electrocardiogram electrodes were attached to the rats allowing continuous recording of the ECG. The positions of the attachments were on the left and right side of the thoracotomy and on the left foot. The electrodes were connected to a Powerlab/85p system via bridge amps (ADI instruments), and displayed using Powerchart software (ADI instruments).

Analysis of the ECG trace assisted with determination of ischaemia and reperfusion.

ST segment elevation in response to infarction was first described by Kjekshus et al.[299], and is a very early indicator of myocardial ischaemia.

Measurements of the trace were made before LAD occlusion, to ensure that there was no evidence of myocardial ischaemia before the occlusion of the LAD artery and then during ischaemia and reperfusion. Typical ECG findings in a single rat during ischaemia and reperfusion are shown below (see Figure 12 A-I). Pre-occlusion (A), no ST segment changes were seen indicating that there was no progressive myocardial ischaemia during the thoracotomy or during the placement of the suture around the LAD artery. Two minutes after LAD occlusion (B), ST segment elevation developed indicative of myocardial ischaemia. Over the next 5 minutes (C), the ST segments rose to almost the same height as the QRS complex. Ten minutes after LAD ligation (D) the animal developed ventricular tachycardia, with widely spaced complexes on the ECG and loss of cardiac output, with the MAP and calculated pulse rate falling from 80 to almost undetectable levels. 1 minute later (E), there was evidence of ventricular fibrillation and after a further 4 minutes (F), in this animal there was spontaneous cardioversion to sinus rhythm with restoration of cardiac output. While in sinus rhythm there was persistent elevation of the ST segments (G), which persisted until reperfusion. At reperfusion (H), there was a further loss of cardiac output with a grossly abnormal ECG, which lasted for 30 seconds. Finally, 10 minutes after restoration (I) of flow in the LAD, there is normalisation of the ST segments indicating reperfusion.

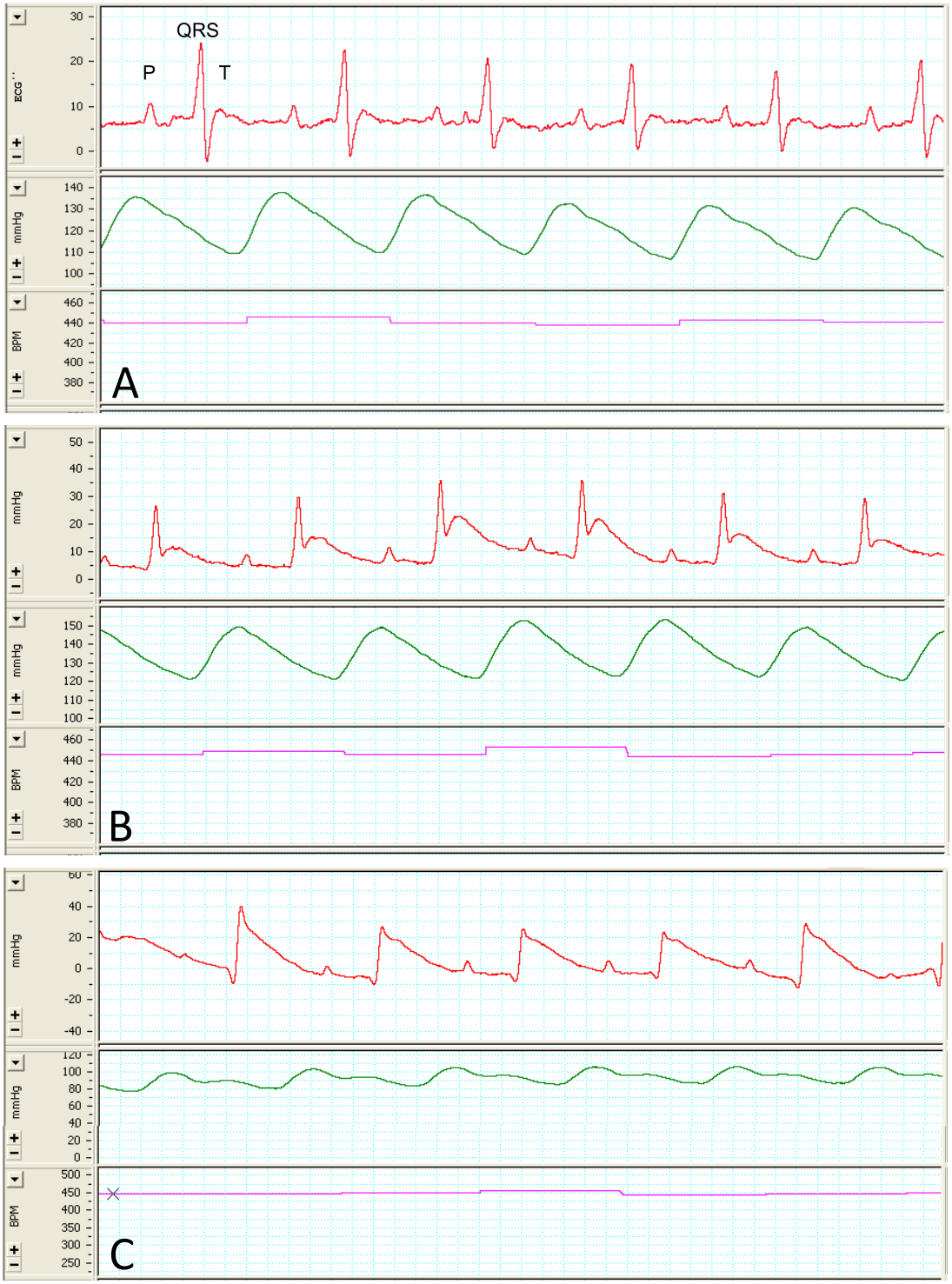
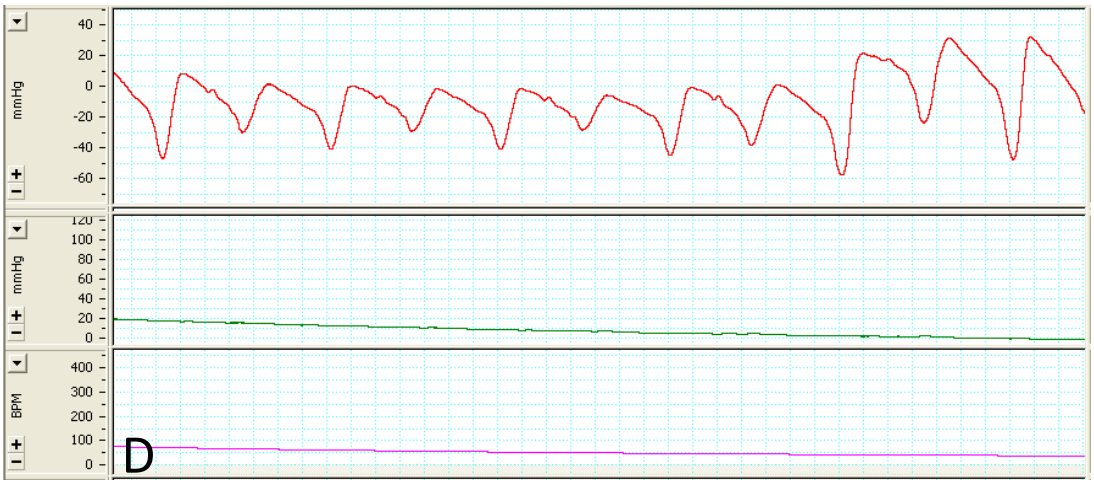


Figure 12. A-I. Recordings from Power Chart system during LAD ligation experiment. ECG trace is shown in red (top), MAP recording in the middle (green) and calculated pulse rate at the bottom (pink)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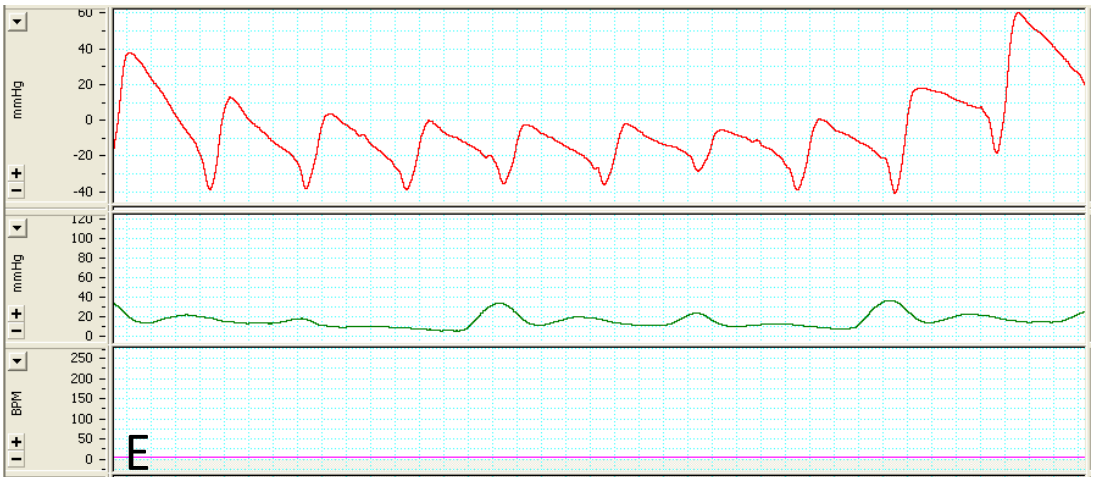
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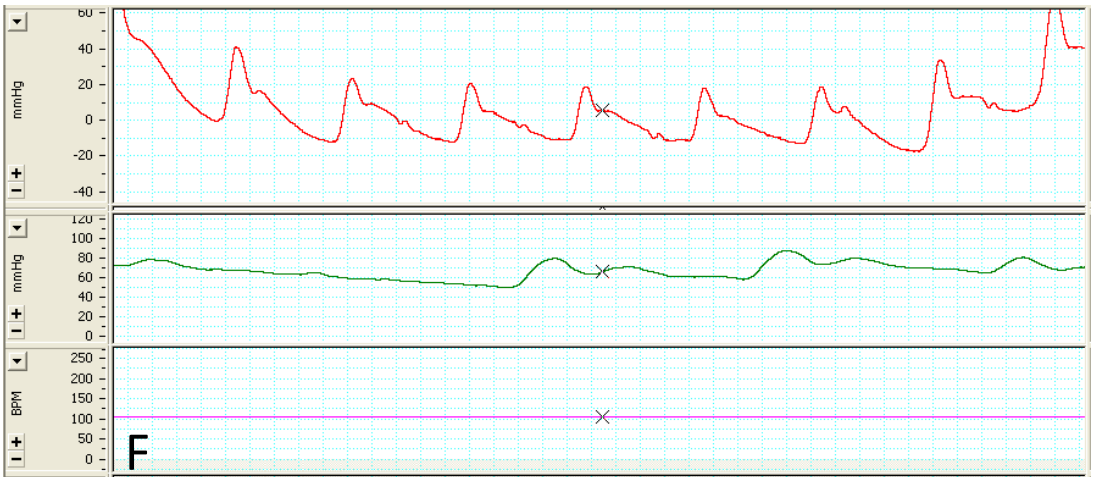
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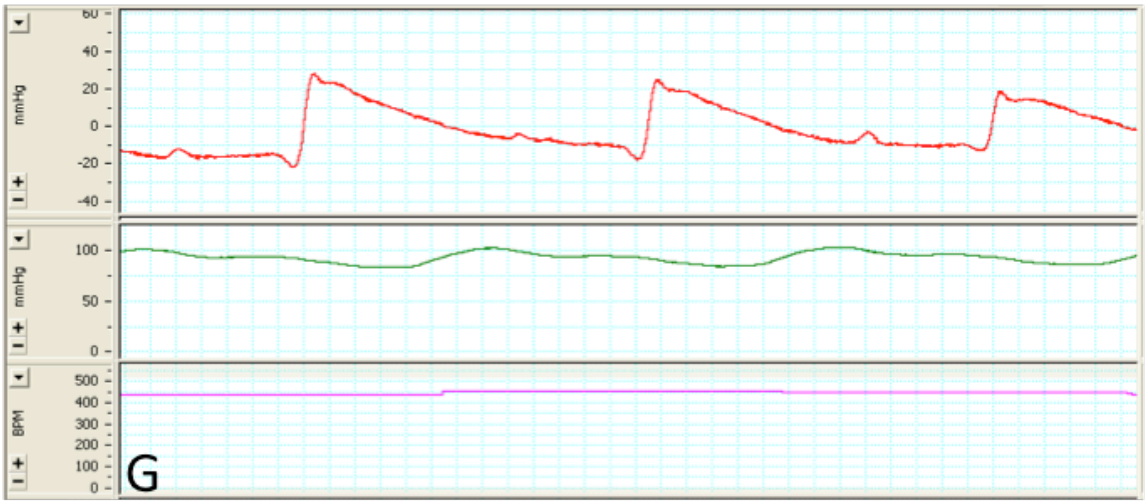
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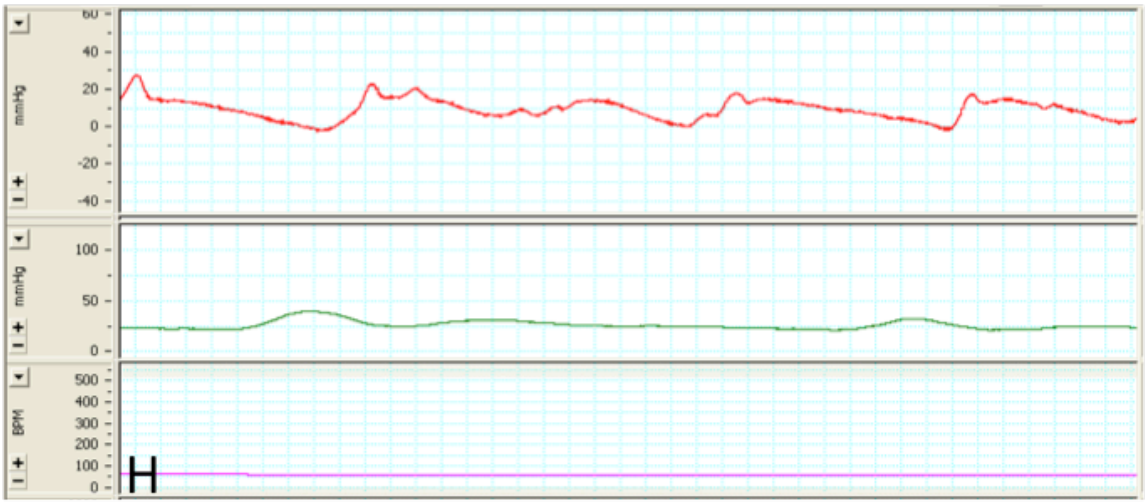
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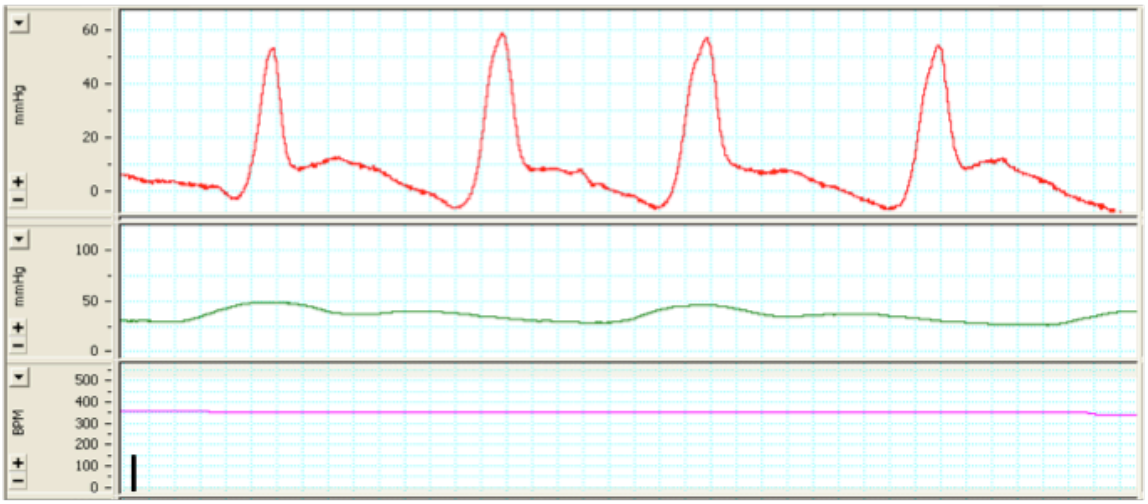
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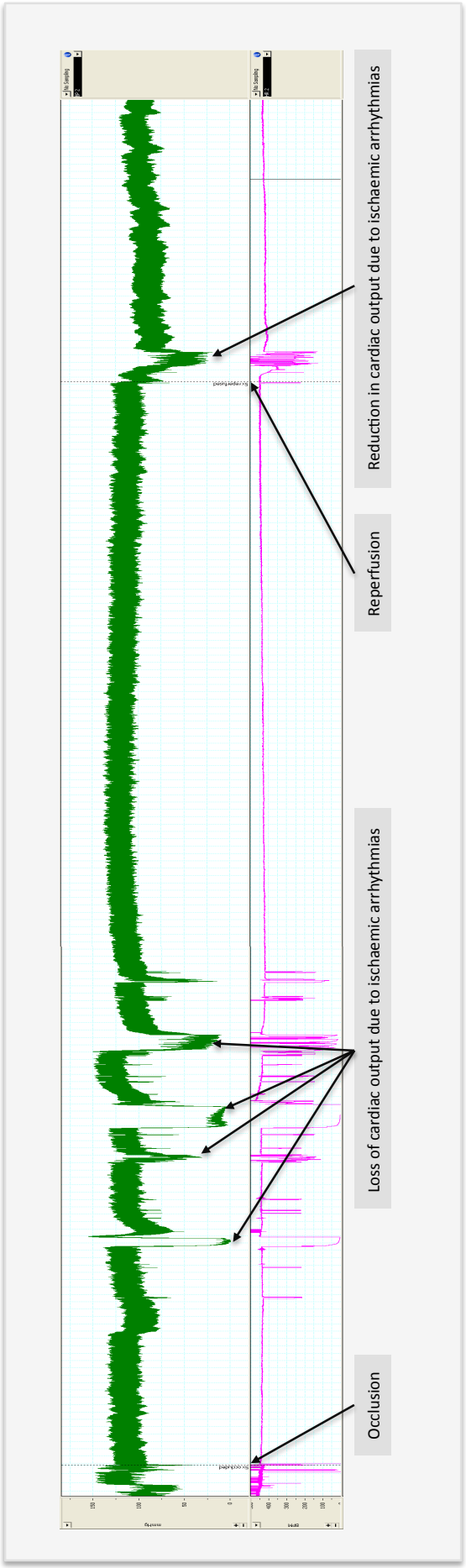


Figure 13. MAP and pulse recording during a single LAD ligation experiment. Note is made of the drop in MAP following occlusion of the LAD, the ischaemic arrhythmias which generally occur 4-7 minutes post occlusion and subside after 10 minutes, and the reperfusion arrhythmias which occur around 30 seconds after the occluder is released.

Observer bias and variability in the measurement of infarct size

Measurement of infarct size using NBT to stain viable myocardium black relies on a subjective decision by the investigator. Not infrequently, the tissue sections are not either dark blue/black nor are they pink, but somewhere in-between. In addition, an individual section of tissue may be heterogeneously stained. This is not surprising as within the myocardium, infarction is not a homogenous process but depends on the microenvironment and the diffusion distance between the capillaries and the myocytes. Other methods to measure infarct size includes such as triphenyltetrazolium chloride (TTC) staining and planometry[300], which stains viable tissue a reddish brown colour, leaving infarcted tissue pale. The advantage of planometry over staining using NBT is that the heart section images are stored as an image, which facilitates blinded scoring of the hearts. This still relies on observers deciding subjectively the staining of an area.

Factors that influence the accuracy of the estimation are the size and number of sections of tissue. Too few a number and each section is likely to contain both infarcted and viable tissue making correct allocation into non-viable or viable difficult. If this happens, the section of tissue can be cut divided again into viable and non-viable areas. However, if the tissue is sliced into very small sections, additional damage will be done during sectioning such that viable tissue may not stain darkly using NBT.

Because uraemic animals were visibly smaller than their non-uraemic counterparts, blinding between the groups was impossible. Furthermore, in later work using conditioning strategies the operator was performing the intervention so was not blinded to the outcome. To address this weakness throughout the course of the entire research project animals were chosen at random and re-counted by a blinded second researcher to ensure that there was no bias between the groups. As part of the quality control several samples were recounted by the same observer to ensure that any inter-observer variability was not due to intra-observer variability.

After the pieces of heart were counted and weighed, the sections were resuspended in saline and recounted; 7 results were recounted. The intra-observer variability in these experiments was small with a median absolute variability of 0.57% (IQR 0.3-1.1%) and a median relative variability of 1.7% (IQR: 1.5-5.2).

The inter-observer variability was calculated on the basis of 165 results. Several steps in this process could introduce bias. Firstly the calculation of the area at risk was considered. The area at risk was calculated by briefly drying and weighing the nonperfused tissue (red) and the remainder of the left ventricle (blue). The area at risk was expressed as percentage of total LV area. When the pieces of heart were dried before weighing, more vigorous drying could alter the weight and hence the AAR. The average absolute difference in weight of tissue between the 2 observers was 0.024g (SD=0.03); the relative difference between the 2 observers was 10.7% (sd=15.2). There was no tendency for one observer to dry out the hearts more than the other. In 73/165 occasions, one observer made the heart tissue heavier and in 85/165, the other observer made the heart heavier, with 7 occasions when both observers got exactly the same result.

Measurement of infarct size required 2 steps: a judgement as to whether the tissue was viable or non-viable. The second was weighing the groups of heart sections. There was no significant difference in the measurement of the infarct sizes between the 2 observers. The mean absolute inter-observer variability of infarct size estimation was 6% (SD=7.5) and the mean relative inter-observer variability was 13% (SD=20.1). There did not appear to be any consistent trend in observer bias, as observer 1 judged the infarct size greater than observer 2 in 52% of the samples, while observer 2 judged the infarct size greater in 47% of the samples, with both observers getting exactly the same infarct size to 2 decimal places was 1%.

To identify whether the variability depended on the degree of injury, the inter-observer variability was plotted against mean infarct size (see Figure 14 B). This shows that the absolute variability was highest when the infarct size was approximately 50%. This is not surprising, as when the infarct size approaches 50%, more sections of heart are likely to contain a mixture of viable and non viable tissue making the labelling of that section more challenging. If the infarct is very large or very small then there will be few pieces of tissue that contain a significant mix of viable and non viable tissue which explains why the absolute variability is reduced at extremes of infarct sizes. When the relative inter-observer variability (Figure 14A) is plotted against mean infarct size, variability decreases as infarct size increases. Again this is unsurprising, as at small levels of infarct, a single discrepancy may cause a large difference in infarct size estimation.

A second question regarding variability was: did the variability change over the 3 years of research? One would expect with researcher experience the degree of variability would narrow. However, (see Figure 14 C+D) there was no trend seen in either absolute or relative observer variability over time. This would suggest that it is an inherent error of the method of the model.

The intra-observer variability is approximately 8 times less than the inter-observer variability suggesting that despite multiple recounting, there is an intrinsic small error in this model and that there does not appear to be a bias in between observers. These results suggest that the lowest absolute difference in infarct sizes detectable is 6%. Therefore, any therapies which are known to have less than an absolute 6% effect size will not be detectable in this model.

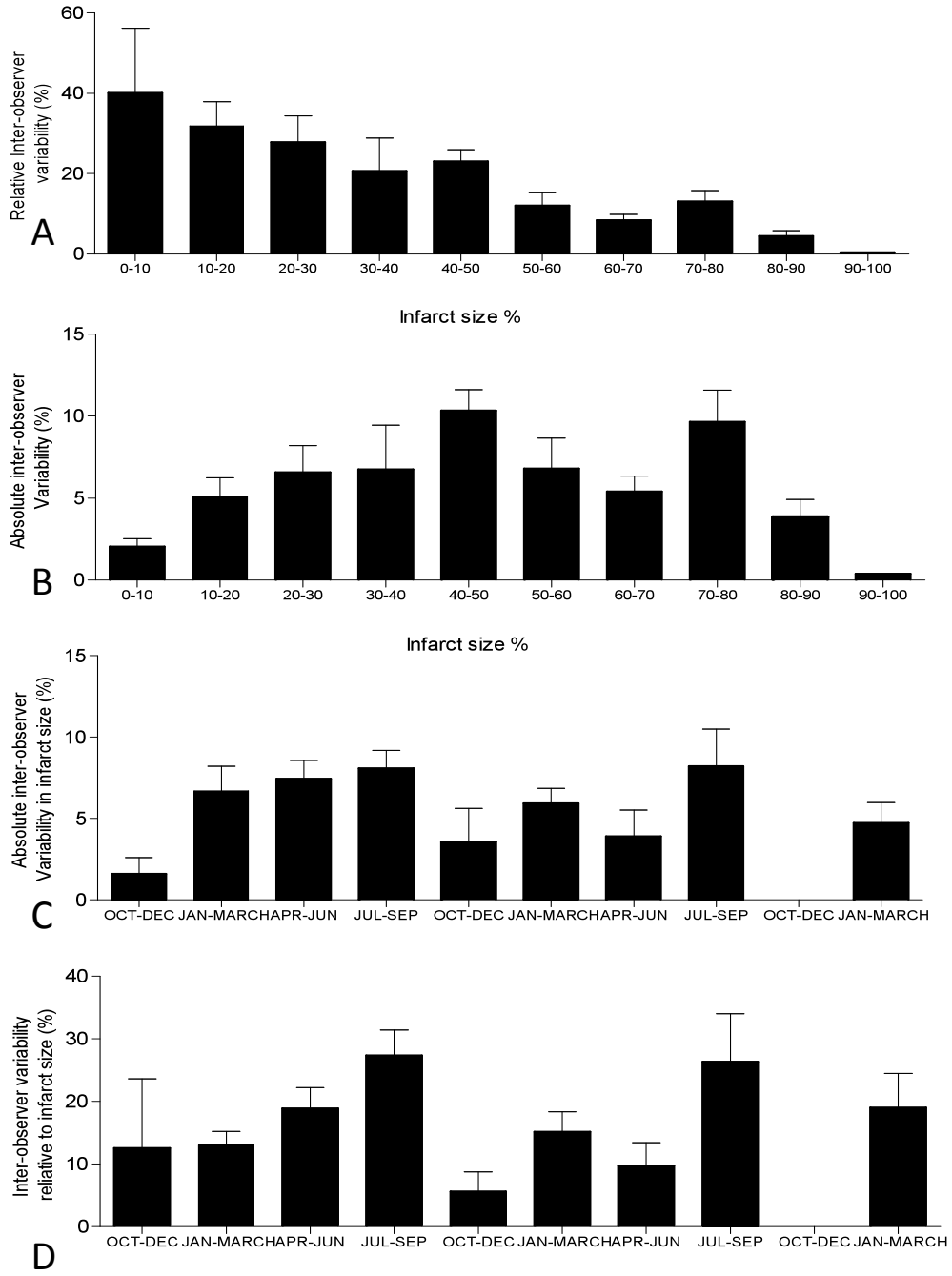


Figure 14. A: Graph showing how relative inter-observer variability varies with infarct size. B: Graph showing how absolute inter-observer variability varies with infarct size. C: Graph showing how absolute inter-observer variability varies over time. D: Graph showing how relative inter-observer variability varies over time. Graphs show mean with SEM as error bars.

Chapter 4

Ischaemia Tolerance of the Uraemic Heart

Background

It has been almost 40 years since Lindner et al.[33] suggested a link between renal function and increased cardiovascular mortality. Subsequently, there has been a great effort in the scientific community to describe the excess risk through epidemiological studies[37]. It is clear that CKD patients have a high incidence of acute myocardial events and following an acute event, CKD patients have much poorer outcomes than in the general population[301]. The increased incidence of CVD in CKD has been discussed in the introductory chapter, but one possible explanation for the very poor outcomes following a cardiac event is that for a given insult the uraemic heart generates a larger infarct size. This theory has been difficult to prove in humans as the infarct size is dependent on the ischaemic area or the area at risk (AAR)[302] and this has proved difficult to measure. Several strategies have been used to attempt to indirectly quantify the AAR including ^{99m}Tc-sestamibi[303], contrast echocardiography[304], angiographic scoring systems[305] and cardiac MRI[306]. While all methods have their limitations, gadolinium cardiac MRI appears to offer the best special resolution with good correspondence to histological necrosis[306].

In vivo animal studies afford the opportunity to measure the area at risk precisely allowing researchers to accurately investigate tolerance to ischaemia. In 2004, Dikow et al.[307] reported for the first time that experimental uraemia was associated with a reduced tolerance to ischaemia. They created a model of chronic uraemia using the standard 5/6 nephrectomy model and found that uraemic animals had a 33% greater infarct size than pair fed non-uraemic animals, with no differences in the AAR. They then went on to demonstrate that this increase was independent of hypertension, sympathetic activity and salt intake. Further work by the same group has replicated the data[308], however there has not been any further work published by other groups replicating these data or extending the findings to other models of chronic uraemia.

Abnormalities of the uraemic heart which confer a reduced ischaemia tolerance

Uraemic Left Ventricular Hypertrophy

Uraemia is associated with LVH. In a study by Greaves et al.[309] echocardiography was normal in only 37% of CKD and 29% of haemodialysis patients, with LVH being the

major abnormality. Levin et al.[310] undertook a prospective study using 175 CKD patients and found the prevalence of LVH increased from 26.7% in those with a GFR>50ml/min, to 45.2% in those with a GFR <25ml/min. Parfrey at al, [195], examined a incident dialysis cohort of 432 patients. They found that at initiation of dialysis only 16% of patients had a normal echocardiogram, with LVH being the most common abnormality. Furthermore those patients with incident LVH had an increased risk of subsequent heart failure and death.

LVH in uraemia has been independently associated with de novo IHD, heart failure and mortality[37]. LVH has been shown to increase with the dialysis vintage even in normotensive patients[311], and persists after transplantation[312].

The explanation of the high prevalence of LVH in CKD is multifactorial. CKD patients are anaemic, hypertensive, fluid overloaded, with sympathetic overactivity. Anaemia has been shown to be an independent risk factors for LVH in early renal disease[313] and ESA treatment used to raise haematocrit has been shown to partially reverse LVH[314]. Sodium and water retention or arterio-venous shunting from arterio-venous fistulas leads to volume overload and ventricular dilation. In addition, London et al.[294] proposed that loss of aortic elasticity leads to LVH directly due to the inherent stiffness of the vessels, increasing afterload and indirectly due to an increased pulse wave velocity. The pressure wave, which is generated by the heart, reflects off branching points in peripheral arteries and travels back towards the heart. In health, this wave travels more slowly and reaches the heart in diastole. However, when the aorta is stiff, the wave travels more rapidly and reaches the left ventricle during systole, increasing the after load[294, 315].

In contrast to the general population, the evidence for blood pressure as the driving force for LVH in CKD patients is unclear as it is known that CKD patients develop LVH even in the absence of hypertension[316]. Studies of patients with essential hypertension have shown LVH regression and mortality benefits with good blood pressure control[317], however these mortality benefits have not been shown in dialysis patients.

The difficulty of hypertension research in the field of ESRD comes from the difficulty in measuring a standard blood pressure in an ESRD cohort.

It is difficult to extrapolate the usual clinical BP measurements in haemodialysis patients to published research studies because usual systolic and diastolic pressures

both pre- and postdialysis are significantly higher by about 14/5 mmHg than if measured according to standardized American Heart Association criteria[318].

In addition BP varies between predialysis, intradialysis, post dialysis and interdialysis readings. It is currently unclear which BP data set is the best measure of LVH, however has been shown showed that 24 hour ambulatory BP recording on non dialysis days provided better prognostic information about future cardiac events [319] and better correlation with LVH [320] when compared to pre and post dialysis BP recordings.

Studies on the regression of LVH using antihypertensive therapy have been conflicting [321] [322] however more frequent HD[323] or accurate control of volume status[324] may lead to attenuation of progressive LVH associated with HD. In addition indirect evidence for good blood pressure control comes from work in the Tassin centre in France[325], where meticulous blood pressure control, along with multiple other interventions in a highly motivated patient group undergoing long hours of dialysis led to improved survival. An epidemiological study by Foley et al.[326] suggest that blood pressure control on dialysis and cardiovascular mortality have a U shaped relationship with those patients with low and high blood pressure having the greatest death rate. This is thought to be due to heart failure patients with low blood pressure and very high mortality rates confounding the data.

Coronary vascular disease

Uraemic patients are known to have a high prevalence of atheroma. However, in addition to intimal atheromatous disease, the media of the coronary arteries is significantly thickened in the context of uraemia, so that the lumen is narrowed both from atherosclerosis and increased medial thickness[327]. The plaques associated with uraemia differ in composition compared to those in non-uraemic patients, with evidence of increased calcification[328]. This calcification may partly explain the early poor outcomes of angioplasty in uraemic patients, with a 1 year re-stenosis rate of 70%[328]. The TIMI trial[329] demonstrated that the presence of coronary collaterals was associated with smaller infarct sizes. Sezer et al.[330] have shown that uraemia is associated with poor coronary collateral vessel development.

Uraemia is also associated with impairment of vasodilatation due to resistance to the vasodilatory effects of nitric oxide[331], and also in response to lower levels of NO

produced during the uraemic state[332]. Uraemia is also associated with increased production of endothelin-1 which triggers increases in intimal and medial thickness[333].
In addition to the changes seen in the coronary arteries, arterioles are also thickened in response to uraemia, an effect which appears to be independent of hypertension[334]. This proliferation appears to be dependant on renin-angiotensin[335] and endothelin[336]. The uraemic changes in the coronary vasculature may explain the reduction in the coronary reserve seen in patients with uraemia[337].

Capillary rarefaction, cardiomyocyte hypertrophy and myocardial fibrosis

In experimental uraemia there is a reduction in the density of capillaries in the myocardium[338]. There is also an expansion of the cardiomyocyte volume[339]. These findings suggest that capillary growth does not keep pace with the cardiomyocyte hypertrophy seen in uraemia, resulting in a greater diffusion distance of oxygen from the capillaries to centre of the myocyte. This distance is crucial because diffusion is related to the inverse square of the distance, thus small changes in distance have a large impact on ischaemia tolerance. This capillary mismatch appears to be specific to cardiac muscle with sparing of skeletal muscle[340].
The histological findings seen in experimental uraemia replicate those seen in humans. Post-mortem examination of dialysis patients' myocardium revealed capillary length density was only 50% of that seen with controls, with a myocyte diameter double that of controls, and volume density of non-vascular interstitium was 60% higher than controls[129]. The mechanism for these histological changes seen with uraemia is thought to involve the renin-angiotensin system, sympathetic over reactivity and endothelin, as pre-treatment of uraemic animals with an ACE inhibitor[341], moxonidine[341], metoprolol[342] and an endothelin receptor blocker[343] abrogated the histological changes associated with uraemia. Critically, these changes were in the absence of a significant reduction in blood pressure.
Uraemia (along with diabetes and hypertension) is associated with myocardial fibrosis, and was first noted in the context of uraemia over 60 years ago[344]. While myocardial fibrosis was reduced with calcium antagonists and ACE inhibitors in models of hypertension, this was not the case in uraemic models[345] suggesting that there may be different pathways which generate fibrosis in uraemia. In addition myocardial fibrosis

increases diffusion distance and has been shown to predispose to malignant arrhythmias[346].

Metabolic effects

Uraemic hearts display a marked reduction in myocardial energy supply, with a basal reduction in phosphocreatine/ATP ratio. During ischaemia, the phosphocreatine/ATP ratio falls[291]. The fact that uraemic hearts start out at a lower phosphocreatine ratio than controls may help explain the reason that uraemic hearts are less tolerant to ischaemia than control hearts.

During anaerobic glycolysis, more glucose is required to create high-energy molecules than under aerobic conditions. In uraemia, insulin mediated glucose uptake by cardiomyocytes is diminished[347]. Dikow et al.[308] showed that by restoring glucose uptake, using a glucose and insulin infusion, uraemic hearts regain their lost ischaemia tolerance. This effect was specific to uraemia, with no increase in ischaemia tolerance in sham-operated animals given glucose and insulin.

Anaemia and Erythropoietin

Erythropoietin (epo) production is stimulated by hypoxia and anaemia through hypoxia-inducible factor-1 [430]. However the relationship between epo levels and anaemia in the context of CKD is not straightforward. As GFR falls, minimal anaemia develops which leads to a compensatory increase in of epo levels such that in early CKD epo levels are inversely related to haemoglobin, however as GFR falls further epo production cannot keep pace with haemoglobin levels and the epo level does not rise further and in those patients with stage 4-5 CKD absolute epo levels may be lower for a given degree of anaemia than those with normal renal function [431].

Exogenous erythropoietin is known to have cardioprotective effects [432] and has been shown to confer cardioprotection by acting through the same pathways as classical ischaemic preconditioning: JAK/STAT [432], Akt [433] and PKC [434] (see p124 for full discussion of IPC). The role of endogenous epo in ischaemia tolerance is limited to remote ischaemic preconditioning (see p235 for more details on remote ischaemic preconditioning), as the myocardium does not produce epo unlike renal tissue. Erythropoietin has been implicated in remote ischaemic preconditioning of the heart

by the kidneys [435]. In addition hypoxic preconditioning has been shown to confer cardioprotection through epo dependent pathways [436].

Anaemia in the context of mild CKD could lead to a epo driven pro-survival state in which uraemic hearts are rendered resistant to subsequent injury, however against this is evidence from epidemiological studies which show that anaemia is a risk factor for poor outcomes following a cardiac event [437]. In addition longstanding anaemia is deleterious to the heart due the resultant LVH that leads to a reduced ischaemia tolerance. Therefore it is unlikely that a small pro-survival effect of increased epo levels in early CKD in response to anaemia outweighs the deleterious effects of anaemia, LVH and uraemic cardiomyopathy on the ischaemic tolerance of the uraemic heart.

Sympathetic overactivity

Uraemia is associated with an increase in sympathetic overactivity[348]. Increased sympathetic activity has been associated increased myocardial injury following AMI[67], with pharmacological blockade of β-adrenoceptors forming the cornerstone in acute cardioprotection following AMI over the last 40 years[349].

Inflammation

Inflammation, common in the context of CKD[209], has been widely reported to increase the incidence of cardiovascular disease but there is little evidence for the direct role of inflammation in the reduction of ischaemia tolerance. However, a study by Griselli et al.[213] demonstrated in a rat model of LAD ligation, that direct injection of human CRP resulted in a 40% increase in infarct size. This effect was thought to involve complement activation, as the increase was blocked in the presence of cobra venom, which depleted complement.

Aims

The aim of this work was to confirm the previously published work in the SNx model of chronic uraemia [307] and to determine whether this effect was seen in a more severe model of chronic uraemia, the adenine model.

Methods

Experiment 1: Myocardial Ischaemia tolerance in an adenine model of chronic uraemia.

Six-week-old male Wistar Rats (Charles Rivers UK) were obtained and after 1 week acclimatisation in an animal house with a 12-hour day night cycle and free access to water and standard chow, the animals were divided into 2 groups. The first group were fed a diet containing 0.75% adenine for 4 weeks, the second group were fed standard chow for 4 weeks to create a uraemic and non-uraemic group (for full details of the method for creation of the adenine model see page 55). At the end of the 4-week feeding, both groups underwent an experimental myocardial infarction procedure (see page 76-87 for full details on procedure). Briefly the rodents were anaesthetised, ventilated and underwent a thoracotomy following which, a suture was passed around the LAD artery. The LAD artery was occluded for 25 minutes to induce myocardial ischaemia, followed by 2 hours reperfusion. At the end of 2 hours reperfusion, the heart was harvested and the AAR and infarct size was calculated.

Experiment 2: Myocardial ischaemia tolerance in a subtotal nephrectomy model of chronic uraemia.

Six week old male Wistar Rats (Charles Rivers UK) were obtained and kept in an animal house with a 12 hour day night cycle and free access to water and standard chow for 1 week. All animals then underwent a 2 stage SNx procedure or sham SNx procedure (for full methods see 'Sub-total nephrectomy model' page 52)
4 weeks later, both groups underwent an experimental myocardial infarction procedure as above (see methods section on page 75 for full details).

Experiment 3: The effect of altering the duration of ischaemia on subsequent infarct size in a rodent model of experimental myocardial infarction.

Six week old male Wistar Rats (Charles Rivers UK) were obtained and housed in an animal house with a 12 hour day night cycle and free access to water and standard chow for 4 weeks.

Four weeks later, the rats underwent an experimental myocardial infarction procedure as above (see page 75 for full details on procedure). The animals were divided into 2 groups. The first group underwent 25 minutes of LAD ligation, whereas the second group underwent 145 minutes of LAD ligation. There was no reperfusion period. After 25 minutes or 145 minutes the hearts was harvested and the area at risk and infarct size was calculated.

Results

Myocardial ischaemia tolerance in an adenine model of chronic uraemia.

Adenine treated animals had similar blood pressures when compared to non-uraemic animals, however, they had lower pulse rates at all time points during the experiment (see Figure 15). Calculation of the area under the curve (AUC) for heart rate for each rat revealed a significant bradycardia when compared to non-uraemic animals ($p<0.002$). Pressure rate product (PRP), calculated by multiplying the heart rate by the mean arterial pressure, unsurprisingly had a similar result to the heart rate, with the AUC for the PRP being significantly lower in the adenine treated groups compared to the non uraemic group ($p<0.05$).

In keeping with previous work, 4 weeks of adenine diet resulted in animals which were growth restricted and weighed almost 50% less than their non uraemic counterparts ($p<0.0001$). They were anaemic ($p=0.0002$), with a creatinine 6 times that of the control fed animals ($p=0.0002$) (see Figure 16 and Table 5).

Despite this uraemic phenotype, following 25 minutes LAD ligation and 120 minutes reperfusion there was no significant difference in the infarct size when compared to non uraemic controls, with the adenine treated rodents and standard chow fed rodents having infarct sizes of 49.3% and 50.2% respectively. Both groups had similar AAR measurements of 47.1% and 42.2% in the control and adenine groups respectively.

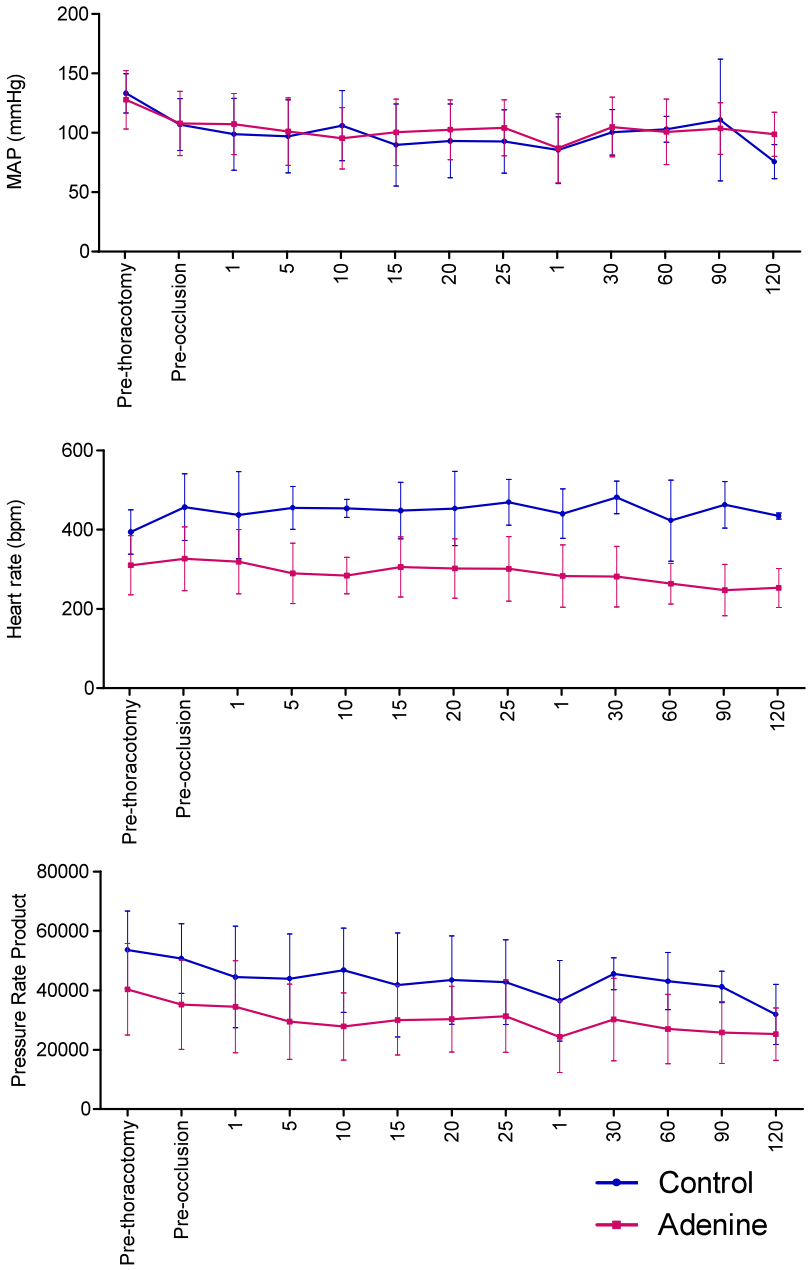


Figure 15. Graph showing the change in cardiovascular parameters during the course of the LAD ligation experiment in control and adenine fed rodents. The top graph shows mean arterial pressure changes over time. The middle graph demonstrates heart rate changes over time and the bottom graph demonstrates changes in pressure rate product over time. Data points represent mean with SEM as error bars.

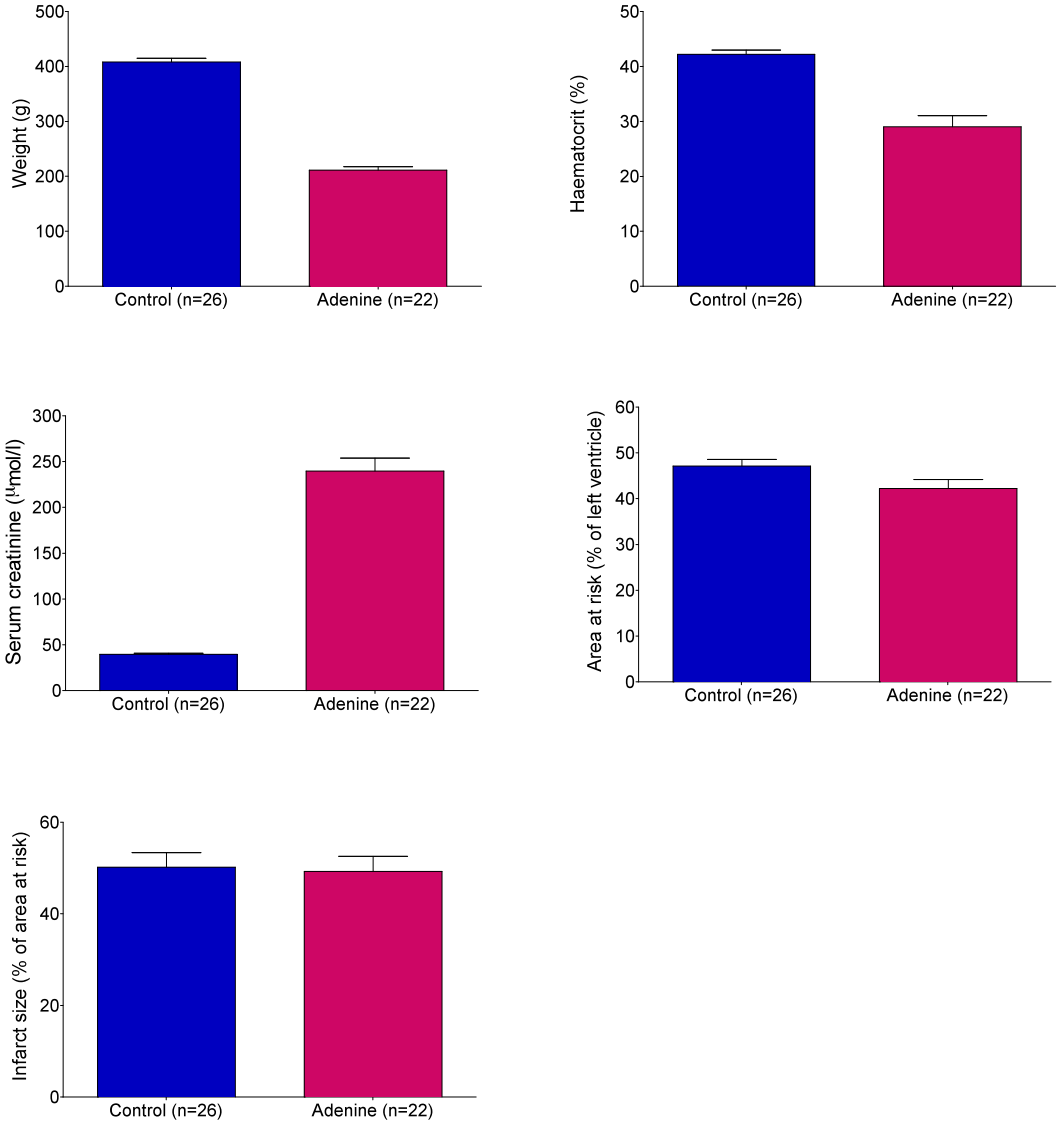


Figure 16. Results of experiment 1: Myocardial ischaemia tolerance in the Adenine model of chronic uraemia. These graphs show the effects of 4 weeks of adenine diet on the weight, haematocrit, and renal function of the rodents. Following 25 minutes of LAD ligation both groups had similar AAR and infarct sizes. Data displayed as mean with error bars as SEM.

	Control (n=26)	Adenine (n=22)	p
Weight (g)	408(30.9)	211.3 (16.4)	<0.0001
Hematocrit (%)	42.21 (3.31)	29 (5.42)	0.0002
Creatinine (µmol/l)	39.64 (4.86)	239.7 (37.62)	0.0002
Area at risk (%)	47.12 (7.3)	42.24 (9.14)	0.1
Infarct size (%)	50.16 (16.2)	49.27 (15.28)	0.847

Table 5. Tabular results of experiment 1: Myocardial ischaemia tolerance in the Adenine model of chronic uraemia. Data shown as mean with (SD). P values are the results of an unpaired t-test using GraphPad Software.

Myocardial ischaemia tolerance in a subtotal nephrectomy model of chronic uraemia

SNx animals tended to have a higher blood pressure during the experiment, however this did not reach statistical significance (see Figure 17). The uraemic animals also tended to have marginally higher heart rates during the experiment, and this too did not reach statistical significance. However, the uraemic animals did have a significantly higher PRP compared to the non-uraemic animals ($p=0.02$).

In keeping with previous work, the SNx model of uraemia resulted in rats that were growth restricted ($p=0.02$), anaemic ($p=0.0003$) and had a serum creatinine over 2.5 times that of sham operated animals ($p<0.0001$) (see Figure 18 and Table 6). Following 25 minutes of LAD ligation with 2 hours reperfusion, the sham operated group had a mean infarct size of 47.2% and the SNx group had a significantly higher mean infarct size of 62.3%, a relative increase of 32% ($p=0.03$). The AAR for both groups was similar.

The effect of altering the duration of ischaemia on subsequent infarct size in a rodent model of experimental myocardial infarction

25 minutes of ischaemia without reperfusion resulted in a small median infarct size of 3.5%. Increasing the duration of ischaemia to 145 minutes increased the infarct size to 82.8%, which despite the small group size was significant ($p=0.04$). Both groups had similar AAR (see Table 6).

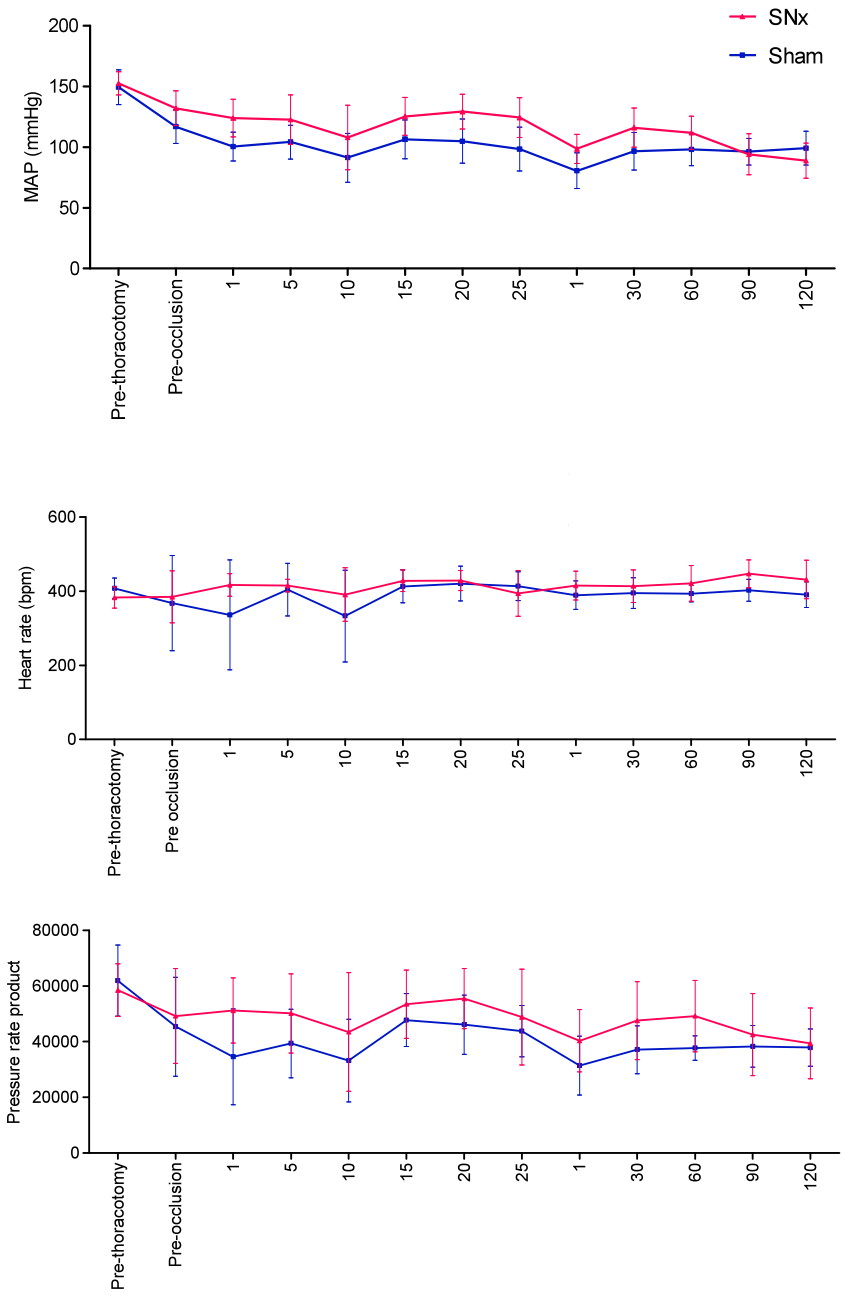


Figure 17. Graph showing the change in cardiovascular parameters during the course of the LAD ligation experiment in the SNx model of uraemia and sham operated non uraemic rodents. The top graph shows mean arterial pressure over time. The middle graph demonstrates heart rate changes over time and the bottom graph demonstrates changes in pressure rate product over time. Data points represent mean with SEM as error bars.

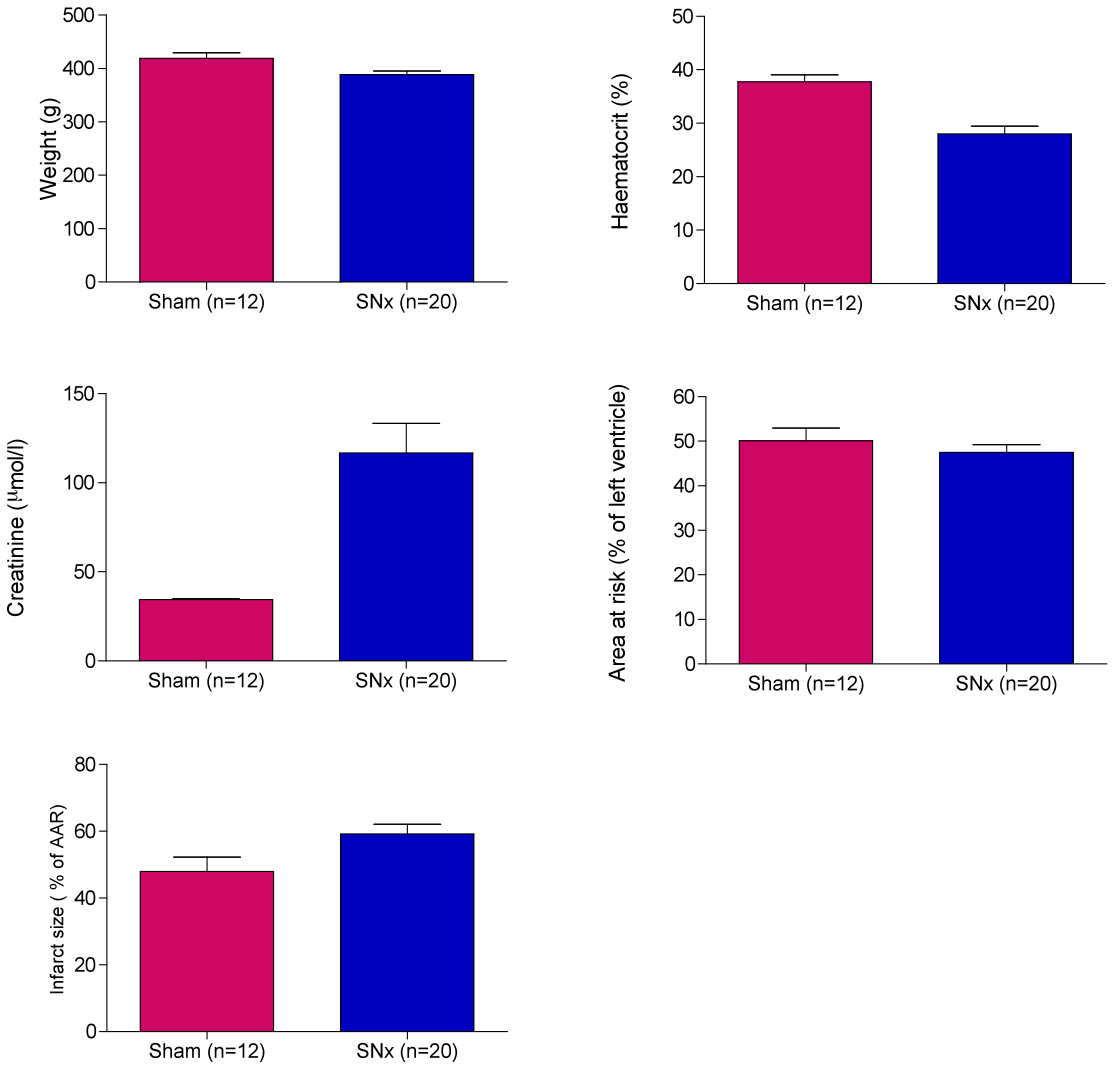
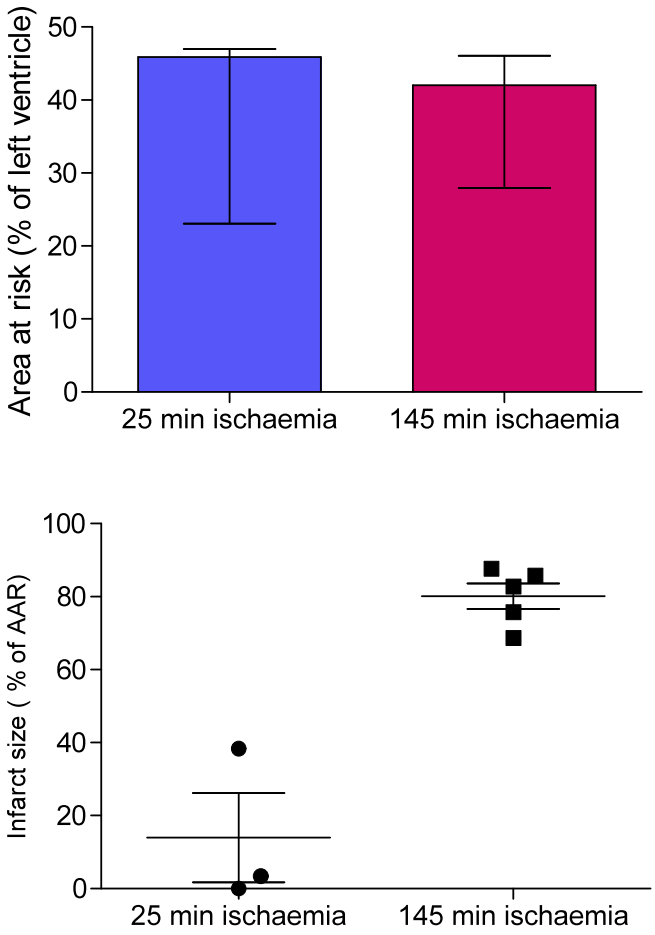


Figure 18. Results of experiment 2: Myocardial ischaemia tolerance in the SNx model of chronic uraemia. These graphs show the effects a subtotal nephrectomy procedure on the weight, haematocrit, and renal function of the rodents. Following 25 minutes of LAD ligation both groups had similar AAR and however the SNx group had a significantly larger mean infarct size ($p<0.05$). Data displayed as mean with error bars as SEM.

	Sham (n=20)	SNx (n=12)	p
Weight (g)	430 (391-450)	380 (370-410)	0.02
Hematocrit (%)	39 (36.3-41)	27 (24-31)	0.0003
Creatinine (µmol/l)	34.4 (33.4-35.7)	91.0 (83.7-116.3)	< 0.0001
Area at risk (%)	50.9 (43.5-56.2)	44.8 (42.2-51.4)	ns
Infarct size (%)	47.2(39.8-63.7)	62.3 (53.54-69.51)	<0.05

Table 6. Tabular results of experiment 2: Data shown as mean with (SD). p values are the results of an unpaired t-test using GraphPad Software.



	25min	145 min	p
Group size	3	5	
Ischaemia duration (min)	25	145	
Reperfusion duration (min)	0	0	
AAR (%)	45.9 (28.1-46.0)	42.0 (27.9-46.1)	ns
Infarct size (%)	3.5 (0-38.4)	82.8 (72.3-86.8)	0.04

Figure 19. Results of experiment 3: Top graph represents the area at risk, following LAD ligation for 25 minutes or 145 minutes. Data presented as median with IQR. Middle: graph shows the infarct size results from the 2 groups. Bottom: Table showing the AAR and infarct sizes, with p values displayed as results from 2 tailed Mann-Whitney test using GraphPad software.

Discussion

These experiments demonstrate that the SNx model of uraemia is associated with larger infarct sizes when compared with a non-uraemic group, and that the adenine model of chronic uraemia, while exhibiting a more severe uraemic phenotype, is not associated with a reduced ischaemia tolerance.

Several reasons exist which may explain these findings. Dikow et al.[307] reported that antihypertensive treatment in a SNx group lowered blood pressure to that of a non uraemic group, but did not alter the reduced ischaemia tolerance or the heart weight to body weight ratio. One could argue that the common finding in their work is that increased heart weight to body weight ratio, a surrogate marker for LVH, is the driving force behind the reduced ischaemia tolerance. Indeed the group of animals which had the highest infarct size, was the group of animals with the greatest heart weight to body weight ratio.

There is conflicting data on the effect of hypertension and LVH on ischaemia tolerance, with some authors reporting that it was hypertension per se[111] rather than LVH which was responsible. Others suggest that diastolic dysfunction in addition to LVH was required for a reduced ischaemia tolerance[350]. In humans, a hallmark of uraemic cardiomyopathy is LVH with diastolic dysfunction[351] and this phenotype has also been shown in the SNx rodent model[352]. It may be that diastolic dysfunction in addition to LVH is required to cause a reduction in ischaemia tolerance. There is no published work on the diastolic dysfunction in an adenine model of chronic uraemia.

Both the adenine treated animals and the uraemic SNx animals had evidence of increased heart weight to body weight ratio (see results section chapter 3). However this is only a surrogate marker of LVH, and while this marker was used in work by Dickow et al[307], their uraemic and non uraemic animals did not have as great a disparity in weight. It is likely that the adenine treated animal's heart weight to body weight ratio is schewed by their profound cachexia and they may be mislabelled as having LVH when infact they had a non-hypertrophied heart but were profoundly cachexic. It is a weakness of this experiment that LVH was not measured directly (either hisoloogically or using echocardiographic data) to confirm the presence of LVH in adenine treated animals.

Another possible explanation for the lack of increased infarct size in the adenine treated groups was directly starvation. It has been shown that switching rats to an adenine diet causes them to stop eating for up to 1 week[265]. Our previous data show that the adenine diet results in a reduction in the amount of food consumption and a failure to gain weight (see Figure 4). The adenine fed rats in this current experiment weighed 50% less than their non-uraemic counterparts. There is evidence that in the context of acute myocardial ischaemia, starved rats develop less ischaemic arrhythmias and better LV function on reperfusion[353], along with smaller infarct sizes than non starved controls[354], an effect which is thought to be due to increased levels of myocardial glycogen in response to starvation[353].

PRP is a measure of myocardial oxygen consumption[355]. In this work, the adenine treated rodents had a significantly lower PRP than their non uraemic controls, while the SNx treated rodents had a significantly higher PRP than their controls. Reducing myocardial oxygen demand during ischaemia/reperfusion had been one of the corner stones of cardiovascular research during the 1970's. Seminal papers demonstrated factors which increased oxygen demand led to larger infarcts and factors which reduced oxygen demand, such as β-blockade, were cardioprotective[66, 67, 69].

The third experiment while small in size is informative in two ways. Firstly, it would appear that 25 minutes of ischaemia by itself does not cause large amounts of irreversible damage to the myocardium. This is important because it leaves scope for subsequent cardioprotective strategies aimed at intervening at the point of reperfusion. The second important point of this experiment was that myocardial ischaemia for 145 minutes, in the absence of reperfusion, leads to infarct sizes in excess of 80%. The duration of ischaemia was chosen as it represented the total duration of the ischaemia and reperfusion durations. This protocol was chosen because there was a concern that despite release of the LAD occluder, occasionally the distal myocardial tissue did not reperfuse and was, in effect, rendered ischaemic for 145 minutes. The term 'no reflow' injury has been has been used to describe this phenomenon[356]. There are several causes of 'no reflow' injury. Firstly microthrombi and atheromatous plaque contents shower down-stream during plaque rupture, obstructing distal small arteries and arterioles[357]. The second mechanism is in-situ microvascular occlusion due to endothelial swelling, tissue oedema and neutrophil infiltration which occurs in response to ischaemia[357]. These processes may be enhanced by reperfusion[358]. A further, often temporary

phenomenon, is distal coronary arterial spasm which may occur as a direct result of embolisation of particulate matter[359] .

If no reflow occurs in this model, the subsequent infarct size is greater than 80%. This result may explain occasional results during the standard protocol of 25 minutes ischaemia and 120 minutes reperfusion where there is an unexpectedly large infarct size of 80-100%.

Investigation of the bioenergetics of the uraemic heart in 2 models of chronic uraemia

Background

ATP is required for all functions in the cell. In the heart, the amount of ATP present in the cell is enough only for a few beats[360] and therefore, myocytes continually re-synthesise large amounts of ATP. The concentration of ATP within a myocyte is maintained a constant level despite large changes in energy demand. Although the primary source of ATP re-synthesis is though fatty acid oxidation in the mitochondria, the myocardium responds to altered energy demands by utilising alternative pathways such as glycolysis, and phosphotransferase reactions from creatine kinase and adenylate kinase[360]. ATP generation from the reaction of phosphocreatine and ADP to form ATP and creatine, catalysed by creatine kinase, can generate ATP 20 times faster than fatty acid oxidation by mitochondria[361]. Because of this, phosphocreatine is considered the primary energy reserve compound in the heart[362].

Effect of co-morbidities on myocardial bioenergetics

The hypertensive failing heart

In both animals and humans, the hypertensive heart is associated with a fall in the ATP concentration, the total creatine pool and the phosphocreatine pool[363]. The phosphocreatine (PCr) falls more than ATP concentration, which leads to a fall in the PCr/ATP ratio. This reduction in the PCr/ATP ratio may lead to a reduction in ischaemia tolerance[363].

The uraemic heart

Raine et al.[291] studied the cardiac bioenergetics of uraemic rats using the SNx model. They found reduced basal phosphocreatine levels, with unchanged ATP levels. They concluded that the reduced phosphocreatine/ATP ratio indicated a reduced myocardial energy supply. They also found that ADP concentrations were doubled in uraemic animals. High levels of ADP have been shown to have an adverse effect on the myocardium[364] by inhibiting ATPase activity[365]. The group then went on to investigate whether this bioenergetic failure resulted in reduced ischaemia tolerance. They found in a model of simulated ischaemia, phosphocreatine fell to a lower level in uraemic hearts

than control hearts and more inosine was released by the uraemic hearts. Inosine release has been shown as a marker of ischaemic damage in vitro[366]. Therefore, they concluded that bioenergetic failure associated with uraemia led to a reduced ischaemia tolerance.

Adenine metabolism in the rat

A striking feature of the adenine treated rats is their bradycardia. There are 2 possible explanations for this bradycardia. The first is starvation[367], due to the unpalatability of the adenine diet, and the second is due to systemic effects of metabolites of adenine adenine diet. Starvation has been shown in human studies of anorexia to lead to bradycardia through increased vagal tone[296]. The uraemic rats consume excess adenine as part of their supplemented diet. Due to the saturation of the APRT, adenine is metabolised to the insoluble 2,8-dihydroxy-adenine (see Figure 2). The excess adenine initially gets converted to AMP. It is only when APRT is saturated that 2,8 dihydroxy-adenine will form, leading to deposition of insoluble crystals in the renal tubules and subsequent uraemia. AMP is metabolised into adenosine by 5' nucleotidase, and also to ADP by adenylate kinase.

The cardiovascular effects of increased adenosine are bradycardia, hypotension and coronary vasodilatation[368]. Exogenous adenosine has been shown, in an in vitro model, to increase both adenosine and ATP levels in isolated rat cardiomyocytes[369] and has been shown to be cardioprotective in human clinical trials[85, 370]. Furthermore, adenosine is involved in the tissue protection seen with ischaemic preconditioning[371-374], remote ischaemic preconditioning[375-377] and postconditioning [378-381].

Thus the consequences of excess adenine ingestion in the rat would be increased formation of AMP, adenosine and ADP before any 2,8 dihydroxy-adenine is formed. Therefore, this excess dietary adenine has 3 possible effects; uraemia from the insoluble 2,8 dihydroxy-adenine crystals, increased nucleotide pool leading to preserved bioenergetics and increased adenosine leading to bradycardia, relative hypotension and an improved ischaemia tolerance.

The aim of this experiment was to investigate these possibilities, which could explain the differences seen between the adenine model and the SNx model.

Methods

6-week-old male Wistar rats were divided into 4 groups. One group was fed standard chow for 4 weeks (control), the second group was fed 0.75% adenine diet, the third group underwent a sham SNx procedure and the fourth group underwent a SNx procedure. Four weeks after group 1 and 2 were started on their control or adenine diets, the animals were anaesthetised, ventilated and underwent a thoracotomy procedure as in the previous experiments. 15 minutes after the thoracotomy, the hearts were very rapidly harvested and immediately dropped in liquid nitrogen and were stored at -80^{O}C until used. 4 weeks after the second stage nephrectomy or sham procedure, groups 3 and 4 underwent myocardial harvesting and storage as above.

The hearts were processed by crushing them using a mortar and pestle under liquid nitrogen and the powdered sample was weighed. A solution of 9% perchloric acid (Sigma) and 1% cyclic xanthine monophosphate (cXMP) was added to the triturated tissue in the ratio of 1ml: 100mg tissue. The samples were then further agitated with a sonicator for 3 minutes and spun at 3000rpm in a centrifuge for 10 mins. The supernatant was aspirated and divided into 500 uL aliquots.

5 molar potassium carbonate (Sigma) was added in a 1:5 ratio to the aliquots to precipitate out protein-perchorate complexes (this ratio was chosen to reliably give a final supernatant pH of 7.5-8). The solution was spun at 13000 rpm for 10 minutes. The supernatant was then aspirated and transferred to 1.5 ml ependorf for storage.

Nucleotide, phosphocreatine and creatine concentrations were determined by high-performance liquid chromatography (HPLC). HPLC apparatus comprised of a PU-2089 Quarternary Low Pressure Gradient Pump and MD-2010 Photometric Diode Array UV/ Vis Detector (195-650nm) from Jasco Instruments (UK) Ltd. The pump was connected to an AS-2055 autosampler and separations were performed on a reversed phase Ace^{TM} C_{18} column (4.6 mm x 15 cm, 5 μm particle size) from Hichrom Ltd. (Theale, Reading, UK). Peak area was calculated by EZChrome Elite software and concentrations determined against reference material standard curves.

For ion-pair reversed-phased HPLC determination, the method of Perrett et al.[382] was used to assay the nucleotides, but slightly modified. Briefly, the mobile phase consisted of 2 % methanol, 98 % buffer (83.3 mmol/L orthophosphoric acid H_3PO_4 titrated to pH 5.8 with triethylamine). 10 μL of sample was injected onto the column with a flow rate

of 0.6 mL/min and a cycle time of 20 min. The column was cleaned at the end of each working day with 100 % methanol for 30 min and the autosampler injector was cleared with distilled water. Values were quantified against known standard concentrations of XMP. Each sample was processed twice and a CV of less than 10 % was accepted. The identification of each target molecule was confirmed by adding exogenous standard material (all from Sigma Aldrich Chemical Company) to samples.

Results

The hearts from groups 1 and 2 were crushed and analysed at the same time and the hearts from groups 3 and 4 were crushed and analysed at the same time. However, groups 1 and 2 were processed at a different time from groups 3 and 4 and therefore, groups 1 and 2 were considered separately from groups 3 and 4.

In this experiment, the SNx model (n=10) and sham SNx model (n=8) were similar in terms of myocardial adenine concentration, phosphocreatine/ATP, total adenine nucleotide pool, ATP/ADP, phosphocreatine/creatine and hypoxanthine (see Table 7A). Adenine treated animals (n=9) and control fed animals (n=9) were similar in terms of myocardial adenine concentration, total adenine nucleotide pool, ATP/ADP, phosphocreatine/creatine and hypoxanthine (see Table 7B). However, phosphocreatine/ ATP in the adenine group was higher in the adenine treated group, which approached significance (p=0.06).

A	Sham SNx (n=7)	SNx (n=10)	p
Adenine (μM)	3.16 (2.64-3.88)	2.83 (2.23-3.87)	0.89
pCr/ ATP (AU)	2.35 (1.64-2.67)	2.39 (2.19-2.58)	0.74
Total Adenine Nucleotide Pool (mM)	3.16 (2.65-3.88)	2.83 (2.28-3.87)	0.89
ATP/ ADP (AU)	2.49 (2.32-2.65)	2.74 (2.36-3.39)	0.16
pCr/ Total Cr (AU)	0.43 (0.31-0.49)	0.48 (0.45-0.51)	0.19
Hypoxanthine (μM)	1.14 (0.91-1.67)	0.98 (0.84-1.22)	0.47

B	Control (n=9)	Adenine (n=9)	p
Adenine (μM)	21.3 (17.2-25.4	18.3 (15.9-27.1)	0.54
pCr/ ATP (AU)	2.71 (1.67-3.81)	4.66 (2.68-7.73)	0.06
Total Adenine Nucleotide Pool (mM)	21.3 (17.2-25.4)	18.3 (15.9-27.1)	0.54
ATP/ ADP (AU)	3.65 (2.90-3.99)	2.12 (1.35-3.34)	0.16
pCr/ Total Cr (AU)	0.69 (0.60-0.82)	0.69 (0.67-0.73)	1
Hypoxanthine (μM)	4.74 (3.70-5.82)	6.04 (4.55-7.0)	0.09

Table 7. Results of bioenergetic study of sham SNx and SNx animals (A) and control fed and adenine fed rats (B). Data presented as median (IQR) with p values the results of a Mann-Whitney test using GraphPad software.

Discussion

The most striking differences in this experiment is the significant differences between groups 1 and 2 and groups 3 and 4. These differences are unlikely to be explained by the fact that the sham SNx animals were 2 weeks older than the control fed animals. It is more likely that this represents a difference in harvesting, storage and processing of the samples. Hearts from the first 2 groups were analysed at a different time from hearts from group 3 and 4, with different durations at being stored at -80^{O}C. It may be that despite storage in a -80^{O}C, there was degradation of the high-energy phosphates in groups 1 and 2.

The sham SNx and SNx groups were similar in all measures of myocardial bioenergetics. This goes against previous data[291], which reported a reduced phosphocreatine/ATP ratio and an increase in ADP concentration in an SNx model. Measurement of adenosine is challenging due to its short half-life. Therefore, in this experiment, hypoxanthine a down stream metabolite of adenosine was used as a surrogate measure. In these experiments, the SNx and sham SNx groups had similar levels of hypoxanthine. The adenine group had a no significant elevation of hypoxanthine compared to the control fed group ($p=0.09$). This result does not fully exclude small differences in adenosine concentrations between the adenine and control fed animals because it is presumes that the metabolism of adenosine, through inosine to hypoxanthine, is the same in both groups. Adenosine is also metabolised to AMP via adenosine kinase. Significant increases in adenosine would be expected to lead to increased AMP concentrations. High levels of AMP would lead to increased ADP formation at the expense of ATP by the adenylate kinase reaction[383]. This would also lead to an increase in the total adenine nucleotide pool, with a reduction in the ATP/ADP ratio. However, these results do not support this; the total adenine nucleotide pool and the ATP/ADP ratio were similar between the groups. Taken together, these results do not support the hypothesis that adenine diet leads to increased adenosine levels, suggesting that the bradycardia seen with the adenine diet is caused by starvation.

There was only a trend towards a higher phosphocreatine/ATP ratio in the adenine group, which did not reach statistical significance. These data do not suggest that the differences in ischaemia tolerance seen in the SNx and adenine treated animals are due to altered cardiac bioenergetics.

Chapter 5

Ischaemic Preconditioning of the Uraemic Heart

Background

Following AMI, the size of an infarct is a critical determinant of prognosis[384]. The need for novel cardioprotective strategies is particularly important in patients with underlying additional risk factors such as diabetes, the metabolic syndrome or renal failure. Diabetic[385] and metabolic syndrome patients[386] have twice the mortality following AMI or CABG compared to controls. CKD patients are at even greater risk, with up to 15 times the in-hospital mortality following AMI compared to patients with normal renal function[41, 387].

Ischaemic preconditioning was first reported in a landmark paper by Murry et al[388]. It had followed on from earlier research which found that, contrary to expectations, repeated brief episodes of ischaemia and reperfusion did not lead to necrotic injury but resulted in a retardation in the fall of ATP when the heart was subsequently rendered ischaemic[389]. Murry et al reported that in anesthetised, open-chest dogs, four cycles of 5 minute coronary artery occlusion followed by 5 min of reperfusion, before the onset of 40 min of coronary occlusion and 4 days of reperfusion, resulted in a reduction in infarct size of 75% compared to controls. Ischaemic preconditioning (IPC) has become the 'gold standard' to which other cardioprotective strategies are compared[390]. The work by Murry et al. has generated a huge research effort over the last 25 years to elucidate the mechanisms involved and translate these findings into clinical practice.

The phenomenon of IPC exerts robust and reproducible protection and appears to be a ubiquitous endogenous protective mechanism. It has been shown to confer cardioprotection in rodents, pigs, rabbits, ferrets, guinea pigs, sheep and man, and in a diverse variety of organs including the brain, gut, skin, skeletal muscle, retina, bladder and liver[391]. In addition to its anti-apoptotic and anti-necrotic tissue protective effects, IPC also reduces ischaemic arrythmias[392, 393], attenuates endothelial dysfunction[394] and reduces cardiac stunning[395].

The IPC stimulus elicits 2 distinct windows of cardioprotection. The first results in immediate protection which wanes over several hours, and the second 'delayed window' starts 12-24 hours later and lasts for 2-3 days[396].

Mechanism of the cardioprotective effects of IPC

Reperfusion injury salvage kinase (RISK) pathway.

The RISK pathway was first described in the mid 1990s, as a collection of pro-survival, anti-apoptotic protein kinases which were specifically activated at the time of myocardial reperfusion to confer profound cardioprotection[397]. The main constituents of the RISK pathway are the PI3K-Akt and the MEK 1/2-Erk 1/2 signalling cascades. These may be activated by many disparate stimuli, of which IPC is one[398]. Over 30 other substances have been shown to activate the RISK pathway such as insulin, erythropoietin, atorvastatin, adenosine, angiotensin II, glucagon-like peptide-1, volatile anaesthetics, opiates and atrial natriuretic peptide[399].

Activation of the PI3K-Akt or the MEK1/2-Erk 1/2 pathways results in a number of responses which led to cell survival, such as inhibition of proapoptotic proteins, inhibition of mitochondrial cytochrome c release, caspase inactivation and activation of eNOS and PKC.

Inhibition of proapoptotic proteins

Activation of the RISK pathway leads to phosphorylation of the pro-apoptotic protein BAD, which results in its sequestration from its mitochondrial target, thus preventing apoptosis[400]. Bax is a pro-apoptotic protein which translocates to the mitochondria in response to an apoptotic stimulus to cause cytochrome c release either directly, or via opening of the mitochondrial permeability transition pore, a crucial end effector in cell death[401]. Activation of the RISK pathway inhibits the mitochondrial translocation of Bax, preventing apoptosis[402]. The pro-apoptotic protein BIM is upregulated when Erk 1/2 or PI3K-Akt are inhibited[403]. Activation of the RISK pathway also leads to degradation of the pro-apoptotic protein p53[404].

Inhibition of mitochondrial cytochrome c release

Cytochrome c release from mitochondria is a potent apoptotic stimulus. Work by Kennedy et al.[405] showed that Akt inhibited this release and prevented cell death.

Inhibition of caspases

Caspases are a collection of intracellular enzymes, which are crucial to effective apoptosis. Both ERK1/2[406] and Akt[407] have been shown to inhibit caspase activation.

Activation of eNOS

Nitric oxide is a cytoprotective molecule which acts through inhibition of the mitochondrial permeability transition pore (mPTP) opening[408]. Akt has been shown to activate eNOS[409].

Activation of protein kinase C

Protein kinase C, a potent cytoprotective enzyme family, is activated following a preconditioning stimulus to inhibit the mitochondrial permeability channel (mPTP)[410].

The Survivor Activating Factor Enhancement (SAFE) pathway

Over the last decade it has become clear that activation of the RISK pathway is not the only way to lead to tissue protection through preconditioning. TNFα deficient mice were able to precondition in response to exogenous adenosine, but were unable to respond to IPC[411]. This indicates that there are other pathways which involve TNFα, that can confer tissue protection. The involvement of the pro-inflammatory TNFα in preconditioning is somewhat surprising as TNFα has been thought to contribute to reperfusion injury, and is expressed in cardiomyocytes following ischaemia[412]. There is conflicting evidence for the role of TNFα in myocardial protection. Smaller infarct sizes have been seen in TNFα deficient mice [413] and the addition of TNFα neutralising antibodies reduces infarct size in rabbits[414]. However, other work has demonstrated that exogenous TNFα protected myocytes in vitro[415] and that mice deficient in TNFα and its cell surface receptors develop larger infarct sizes[416]. Furthermore, it has been shown that an IPC protocol leads to increased expression of TNFα[411].

TNFα is thought to exert its role by binding to one of the two TNFα receptors on the cell surface[417], and possibly the mitochondria[418], suggesting a pathway capable of delivering the TNFα from the surface to the mitochondria. The multiple receptors for TNFα may explain the apparent conflicting results of its role in cell survival, as it is known that the 2 cell surface receptors have differing actions, with receptor 1 activation leading to increased cell death, and receptor 2 activation leading to cytoprotection[419].

TNFα preconditioning activates the sphingolipid pathway, protein kinase C and the mitochondrial potassium ATP dependent channel[420] and finally the mPTP[421].

The signal transduction pathway of TNFα involves TNFα binding to its receptor which in turn trans-phosphorylates 2 Janus kinases (JAK). The activated JAK proteins then activate STAT proteins. The STAT family consists of several different members which are activated by JAK to form homo and heterodimers which translocate to the nucleus

and alter gene transcription[422]. Evidence for the role of the JAK-STAT pathway in IPC comes form experiments which demonstrate that IPC activates the JAK-STAT pathway and that inhibition of this pathway abolishes the tissue protection seen with IPC[423]. Furthermore STAT-3 cardiomyocyte specific knock out mice cannot respond to an IPC stimulus[424]. Downstream targets of STAT activation, in the context of IPC, are the activation of antiapoptotic gene Bcl-2, decreasing transcription of the pro-apoptotic gene Bax and phosphorylation and inactivation of the proapoptotic factor Bad. These effectors are thought to inhibit mPTP opening[425].

The effect of STAT activation following IPC to confer tissue protection may be too rapid a response to rely on de-novo transcription of antiapoptotic factors. The mechanism and targets for these non-genomic effects is currently unknown. Furthermore, these 2 paths may not be entirely separate entities and there may exist some cross-talk between the pathways. Evidence in favour of this comes from experiments which show that STAT-3 phosphorylation is dependant on PI3K activation in a model of pharmacological preconditioning[426] and that STAT-3 knock-out mice did not phosphorylate Akt in response to an IPC stimulus[424].

Mitochondrial potassium ATP channel

The mitochondrial potassium ATP (K_{ATP}) channel appears to be both a mediator and effector of the cardioprotection seen with IPC, as antagonism of the mitochondrial K_{ATP} channel before the IPC stimulus abolished the cardioprotective effect and antagonism of the mitochondrial K_{ATP} at reperfusion also abolished the effect of IPC[427]. The mechanism by which opening of the K_{ATP} channel leads to tissue protection is unclear. The effect may involve a reduction in calcium overload seen during ischaemia[428] or an indirect antiapoptotic effect through the reduction in cytochrome c release, caspase activation and cleavage of poly(ADP-ribose) polymerase[429].

The mitochondrial permeability transition pore

The downstream effectors of the RISK pathway, and perhaps the SAFE pathway, converge on the mPTP. The mPTP was first characterised in the late 1970s by Haworth and Hunter and is thought to be a voltage and Ca2+ dependant high conductance channel located in the inner mitochondrial membrane[438, 439].

During an episode of myocardial ischaemia and reperfusion, the fate of the cardiomyocyte is dependant on preserving the function of the mitochondria, as they are

the critical mediators of survival and death in this setting[440]. The major effector pathway of cardiomyocyte cell death following IR injury is through mitochondrial dysfunction arising from the formation of the mPTP. The opening of the mPTP at the time of reperfusion triggers a series of events which culminates in apoptotic or necrotic cell death[425].

Opening of the channel causes a rapid increase in permeability to solutes up to 1.5KDa[441]. This leads to a collapse in the inner mitochondrial membrane potential causing ATP depletion, respiratory inhibition and eventual swelling and rupture of the mitochondrial membrane. This results in cytochrome C leaking into the cytoplasm[425, 442] and triggering apoptosis[425].

At reperfusion the intracellular pH is rapidly restored which together with a burst in ROS and regeneration of ATP, remove the physiological inhibition of mPTP opening. The removal of the inhibitors combined with the continued presence of high calcium levels promote optimal conditions for mPTP opening[443].

The role of the mPTP in preconditioning was suggested by work by Hausenloy et al.[444] who showed that pharmacological inhibition of the mPTP opening at the point of reperfusion resulted in a significant reduction in infarct size. Subsequently, ischaemic preconditioning has been shown to inhibit mPTP opening to confer tissue protection[445]. The precise mechanism though which IPC results in inhibition of mPTP opening is not fully elucidated. IPC may inhibit mPTP opening indirectly by producing an intracellular milieu which favours mPTP closure, or directly via members of the RISK pathway.

IPC had been shown to reduce cytosolic and mitochondrial calcium concentrations in rat hearts[446]. ROS generation during myocardial ischaemia and a further ROS burst at reperfusion have also been shown to trigger for mPTP opening[83].

Myocardial ischaemia leads to cellular ATP depletion and ADP production, which in turn leads to mPTP opening. IPC was first described as a protocol which preserves myocardial ATP levels during ischaemia[388]. There is indirect evidence that IPC can alter the pH normalisation on reperfusion, because infusion of alkaline sodium bicarbonate at the time of reperfusion abolishes the beneficial effect of IPC[447]. Opening of the mPTP may also be inhibited directly by components of the RISK pathway such as PKC[448], mitochondrial K-ATP channel[449], glycogen synthase kinase-3β[450] and eNOS (though PKC-ε)[451]. The effect of inhibition of mPTP opening in the context of myocardial infarction has been shown in humans. Piot et al.[452] reported that

administration of ciclosporin (a known mPTP inhibitor) in the context of an acute myocardial infarction led to a reduction in subsequent infarct size.

Ischaemic conditioning in human studies

Epidemiological data suggest that IPC may be effective in the context of myocardial injury. Patients with underlying chronic angina (which could be thought of as an endogenous IPC protocol) have less injury following CABG surgery than those with no angina[453]. A possible explanation for this, above and beyond transient reduction of flow, is that coronary microembolisation resulting from plaque rupture has been shown to trigger a preconditioning response several hours later in a TNF-α dependant mechanism[454].

The major drawback of IPC is that the timing of the myocardial ischaemia must be known, which limits its clinical translation to elective surgical procedures. The first human study confirming the protective effect of IPC was reported in 1993 by Yellon et al.[396] in a small study (n=14) in patients undergoing cardiopulmonary bypass. Yellon et al. found that following the bypass procedure the IPC group had higher levels of tissue ATP than the control group. Since the work by Yellon et al. there have been 32 clinical human clinical trials using ischaemic preconditioning to confer cardiac protection. These trials have invariability shown beneficial effects. However, these trials tend to be small, with the largest individual trial including only 86 patients[393]. Unfortunately, these trials have lacked the duration of follow up and size required to be powered for mortality outcomes and it is these outcomes which are needed cause change in routine clinical practice. Pharmaceutical companies are unlikely to fund large trials in ischaemic conditioning if there is no profit for their shareholders as it is impossible to patent IPC.

There have been no human clinical trials in IPC in the heart over the last 7 years. At first this suggests that interest in preconditioning, as a translational strategy, may have waned. However, clinical trials in preconditioning have moved from ischaemic preconditioning to pharmacological preconditioning, remote ischaemic preconditioning and post conditioning, as each additional expansion of the field lends to greater clinical translatability.

Effect of underlying co-morbidities on IPC

Hypercholesterolaemia

Hypercholesterolaemia is one of the most prevalent risk factors for coronary artery disease. Hypercholesterolaemia has been noted to block the pacing induced cardioprotection in rabbits fed high cholesterol diets. When these animals were placed back on a standard diet, the serum lipids normalised with a return of the effectiveness of IPC[455]. However Ilodromitis et al.[456] have shown that IPC was still effective in hypercholesterolemic rabbits. In rats, hyperlipidaemia has been shown to abolish the effect of IPC in the isolated rat papillary muscle[457]. Hypercholesterolaemia has been shown to abolish the effect of delayed IPC by preventing the upregulation of tetrahydrobiopterin, an essential cofactor for activation of nitric oxide synthase[458]. Another suggested mechanism of the IPC limiting effect of hypercholesterolaemia is suggested by work which shows that IPC decreases the formation of peroxynitrite[459] and hypercholesterolaemia enhances the formation of peroxynitrite[460]. Thus excessive generation of peroxynitrite by hyperlipidaemia may lead to a resistance to IPC. Hypercholesterolaemia also blocks preconditioning induced inhibition of cardiac matrix metalloproteinase-2[461] and has been shown to inhibit the opening of the mitochondrial ATP sensitive potassium channel (K_{ATP})[462], the opening of which is a major mechanistic component of IPC.

The hypercholesterolaemia-induced abrogation of the cardioprotective effect of IPC was also observed in a clinical study in which hypercholesterolaemia attenuated the anti-ischemic effect of preconditioning in humans during coronary angioplasty[463].

Hyperglycaemia

Conflicting evidence exists as to whether the diabetic myocardium can be preconditioned. Some studies report that diabetes is not a barrier to IPC[464, 465], but the majority of studies report that the diabetic heart[466, 467] and the acutely hyperglycaemic heart[468] are resistant to IPC. Tsang et al.[128] reported that diabetic hearts could be preconditioned, but that they had a higher threshold for preconditioning. The diabetic animals required 3 cycles of IPC to achieve significant tissue protection, whereas the non-diabetic controls required only 1 cycle. A lower basal level of phosphorylated Akt in the diabetic animals was postulated as the cause for the resistance to IPC. Administration of anti-diabetic agents has been reported to restore protection in a

genetic model of diabetes[469]. IPC was abolished in a diabetic sheep model of myocardial stunning which was reported to be due to K_{ATP} channel dysfunction[470]. Underlying diabetes has been shown to inactivate eNOS, generate ROS leading to endothelial dysfunction which, may also be responsible for the loss of response to IPC[471].A human ex vivo study by Ghosh et al.[130] reported that hearts from patients with diabetes, or those with poor cardiac function, were unable to respond to preconditioning and this effect appeared to be mediated through a failure of mitochondrial ATP channel opening.

Post infarction remodelling

Ischaemic preconditioning has been shown to fail to reduce infarct size in post-infarcted remodelled hearts[472]. However, pharmacological preconditioning using the K_{ATP} channel opener diazoxide remained unaffected in the contest of remodelled hearts, suggesting that the impairment to preconditioning in the context of post infarction/remodelling was upstream of the K_{ATP} channel.

Hypertension

There is conflicting evidence for the role of hypertension in resistance to IPC.
Some authors report that hypertensive rats could still be preconditioned[112, 373], however, more recently others report that hypertension abolished the cardioprotective effects of IPC[473] and cardiac hypertrophy attenuated the response to IPC in guinea pigs[474]. The mechanism by which hypertension may abolish the effects of IPC are unknown.

Ageing

The ageing heart has a diminished functional and adaptive reserve capacity and a reduced ischaemia tolerance[475]. There is conflicting evidence for the impact that ageing has on the ability of the myocardium to respond to IPC. Przyklenk et al.[476] reported that there was no loss of the effect of IPC in old rabbits, although other authors report that IPC did not minimise the post-ischemic myocardial dysfunction in senescent, isolated perfused rat hearts[477], nor the necrotic cell death and contractile dysfunction in hearts from 22 month old rats[478]. In humans, a study in 'elderly' patients (70-90 years) undergoing cardiac surgery reported that this group had no increased resistance to IPC[144].

In further evidence of the effects of age on IPC potency, Schulman et al. reported that 'middle aged' rats had a resistance to IPC, which could be overcome with an increased number of cycles of IPC, however, elderly rats were unable to be preconditioned[479]. Tani et al.[480] explored the mechanism of resistance to IPC in year old rats. They found that these rats were resistant to IPC and this effect was mediated by a reduction in PKC activation.

Obesity

Zucker rats display a blunted response to an IPC signal, an effect which was mediated through enhanced oxidative stress and reduced activation of mitochondrial K_{ATP} channels[481].

Hyperhomocysteinemia

Hyperhomocysteinemia has been associated with the pathogenesis of many cardiovascular pathologies[482]. Hyperhomocysteinemia has been associated with a resistance to IPC[483]. This effect is thought to be mediated through loss of opening of the K_{ATP} channels in response to IPC[484].

Medication use

Concomitant use of medication may alter the effect of IPC. Indeed many common medications frequently used by patients presenting with an acute myocardial infarct have been shown to interact with the RISK pathway including statins[485], insulin[486], EPO[487] and glucagon-like peptide-1 agonists[488].

Aims

There is no published literature on the effects of underlying uraemia on the ability of the heart to respond to a preconditioning signal. The rat models of chronic uraemia share several of the co-morbidities which have been associated with resistance to IPC, such as hypertension, LVH and hyperlipidaemia.

The ability of the uraemic heart to respond to a preconditioning signal is a vitally important issue to address. Up to one third of patients presenting with an acute coronary syndrome have CKD stages 3-5[41, 49, 489], and yet patients with CKD are routinely excluded from trials in cardioprotection[490, 491].

This study seeks to answer 2 questions:

Can the uraemic heart be preconditioned?

If the uraemic heart can be preconditioned, is there an increased threshold to preconditioning seen in the context of underlying chronic uraemia?

This study seeks to investigate the effects of cardioprotection through IPC in 2 different rats models of chronic uraemia, and in addition to investigate the possible mechanisms involved.

Methods

Myocardial ischaemic preconditioning in the subtotally nephrectomised rat

Six week old male Wistar Rats (Charles Rivers UK) were obtained and after 1 week acclimatisation in an animal house with a 12 hour day night cycle and free access to water and standard chow. All animals then underwent a 2 stage SNx procedure (see Reversible LAD ligation as a model of myocardial infarction in the rodent page 75). Four weeks after the second stage procedure, the animals were anaesthetised, ventilated and underwent a thoracotomy procedure (see page 76-83). 15 minutes following the thoracotomy, the animals were divided into 2 groups. The first group (control) were left for a further 30 minutes before undergoing a reversible 25-minute LAD ligation (see page 83- 87 for full details of LAD ligation, harvesting and infarct measurement). The second group (IPCx3) underwent 3 cycles of 5 minutes LAD ligation followed by 5 minutes reperfusion as a preconditioning stimulus, before finally undergoing a further 25 minutes LAD ligation. After the 25-minute LAD ligation, both groups were reperfused for 120 minutes. At the end of the reperfusion, the hearts were harvested and infarct size was quantified using Evans blue to delineate the area at risk (AAR) and NBT to stain non-infarcted tissue (see page 87 for methods).

Myocardial ischaemic preconditioning in the adenine diet treated rat

Six week old male Wistar Rats (Charles Rivers UK) were placed on 0.75% adenine diet (Rat and mouse No.1 Maintenance diet +0.75% adenine: Special diet services UK) for 4 weeks. At the end of 4 weeks, the animals underwent a similar experimental protocol as above, with the animals split into 2 groups. The IPC group underwent 3 cycles of 5 minutes ischaemia and reperfusion immediately before 25 minutes LAD ligation and the control group did not undergo the IPC protocol.

Resistance to myocardial ischaemic preconditioning of the subtotal nephrectomised rat

Rats that had undergone a 2 stage subtotal nephrectomy or a sham SNx procedure, to create a group of uraemic and non uraemic animals, underwent basic surgery and

thoracotomy in the standard manner. Both groups underwent an IPC protocol consisting of 1 cycle of 5 minutes LAD ligation and 5 minutes reperfusion before undergoing 35 minutes of myocardial ischaemia. After two hours reperfusion, the heart and infarct size was measured as before.

Resistance to myocardial ischaemic preconditioning of the adenine diet treated rat

In this experiment rats were divided into 2 groups. The first group of animals were given standard chow for 6 weeks. The second group, adenine 'washout', were given 0.75% adenine diet for 4 weeks, at the end of this 4 week period they were placed back on standard chow for a further 2 weeks. At the end of the 6-week period, each group underwent either 1 cycle of IPC or a sham IPC procedure. Subsequently, they underwent 25 minutes of myocardial ischaemia followed by 2 hours reperfusion. At the end of this time, the hearts were harvested and infarct size was measured as before.

Analysis of the mediators of IPC in uraemic and non uraemic animals

To investigate the underlying mechanisms a further set of experiments were performed (see Figure 24). Male Wistar rats were divided into 4 groups. The first group underwent a sham 2 stage subtotal nephrectomy procedure to generate a non-uraemic control group. The second group underwent a 2-stage subtotal nephrectomy procedure. The third group were placed on standard chow for 4 weeks and the fourth group were treated with 4 weeks of 0.75% adenine diet. All animals underwent basic surgery, a thoracotomy procedure, and a needle was passed around the LAD artery as in previous experiments. Each group then underwent an IPC protocol or a sham procedure. At the end of the IPC or sham protocol the LAD was briefly occluded and Evans blue dye was perfused via the venous line to delineate the area of the heart exposed to IPC (or sham IPC). The heart was rapidly removed from the chest cavity and cooled in ice-cold saline for 20 seconds. The myocardial territory exposed to IPC was quickly cut away from the rest of the heart and rapidly snap frozen in liquid nitrogen and stored at -80^{o}C until further use.

Tissues for western blot were ground with a mortar and pestle under liquid nitrogen and subsequently polytron-homogenised (3x 30sec bursts with 1min intervals on ice) in a mammalian protein extraction buffer (GE healthcare; 10:1 v/w) supplemented with the

following inhibitors: 1mM EDTA, 0.5mM DTT, 1% v/v protease inhibitor cocktail (Sigma), 1mM NaF, 5μM fenvalerate (Calbiochem), 1mM Na_3VO_4 and 1% v/v phosphatase inhibitor cocktails I and III (Sigma). The tissue homogenate was incubated for 10 minutes on ice and then centrifuged at 5000g for 10 minutes at 4^oC. The supernatant was aliquoted and stored at -80^oC until further use. Protein concentrations were determined using the Bicinchoninic Acid assay (BCA, Pierce), with bovine serum albumin (BSA, Sigma) as the protein standard.

Lysates (40μg of protein) were subjected to SDS-polyacrylamide gel electrophoresis using the NuPAGE electrophoresis system (Invitrogen) under reducing conditions as previously described[492]. The ECLplus chemiluminescence detection kit (Amersham Pharmacia) was used to visualise protein bands. The following antibodies (from Cell Signalling) were used: rabbit anti-phspho-p44/p42 MAPK (Thr202/Tyr204), rabbit anti-p44/p42 (total ERK), mouse anti-phospho AKT (Ser473), rabbit anti-AKT (total AKT), mouse anti-phospho-STAT-3 (Tyr705), rabbit anti-STAT-3 (total STAT-3) and peroxidase-conjugated secondary antibodies.

Arterial blood gas analysis

During the experimental procedures, arterial blood gas (ABG) measurements were taken at 3 time points. Additional ABGs were occasionally taken if an animal was unexpectedly hypotensive during the experiment. The baseline ABG was taken after the animals had been put on the ventilator to ensure that the animals were receiving the correct minute volume and fractional inspired O_2. The second routine ABG was taken during pre-occlusion to ensure that there had not been a change in ventilatory requirements after the thoracotomy, when the compliance of the lungs was altered due to loss of mechanical resistance from the thorax. Finally, an ABG was taken at the end of the experiment to access further changes in ventilatory, acid base and haemostasis parameters.

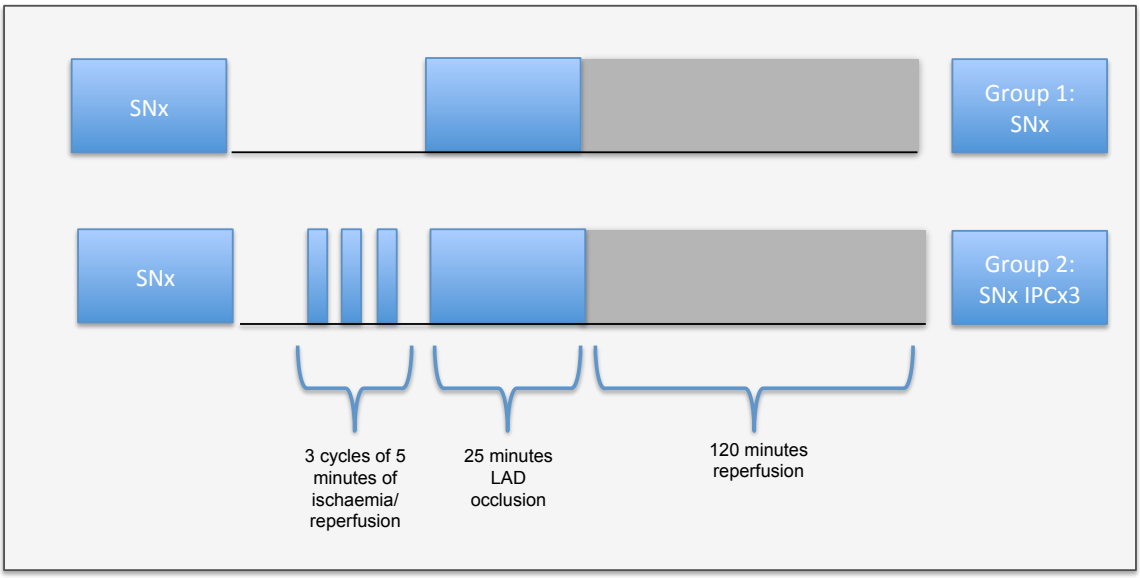


Figure 20. Schematic representation of experiment 1: Myocardial ischaemic preconditioning of the Subtotally nephrectomised rat

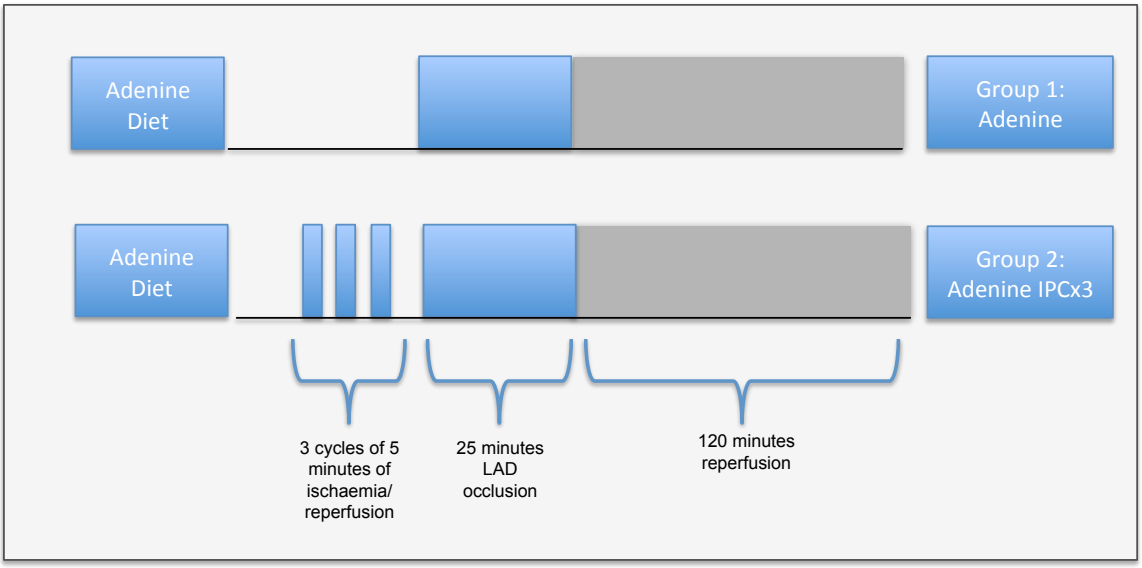


Figure 21. Schematic representation of experiment 2: Myocardial ischaemic preconditioning of the Adenine diet treated rat.

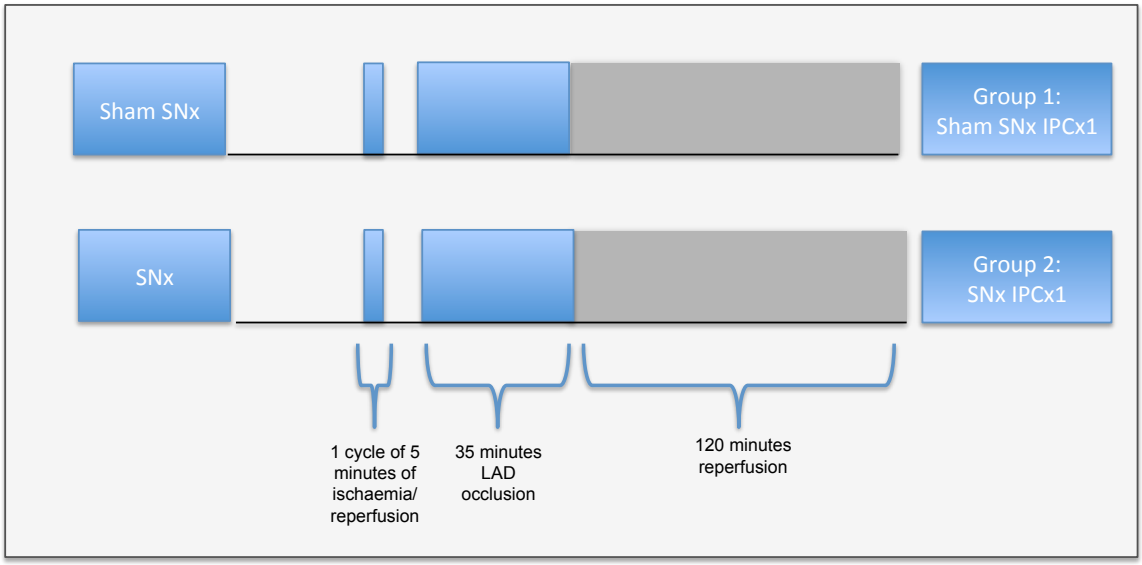


Figure 22. Schematic representation of experiment 3: Resistance to myocardial ischaemic preconditioning of the subtotally nephrectomised rat

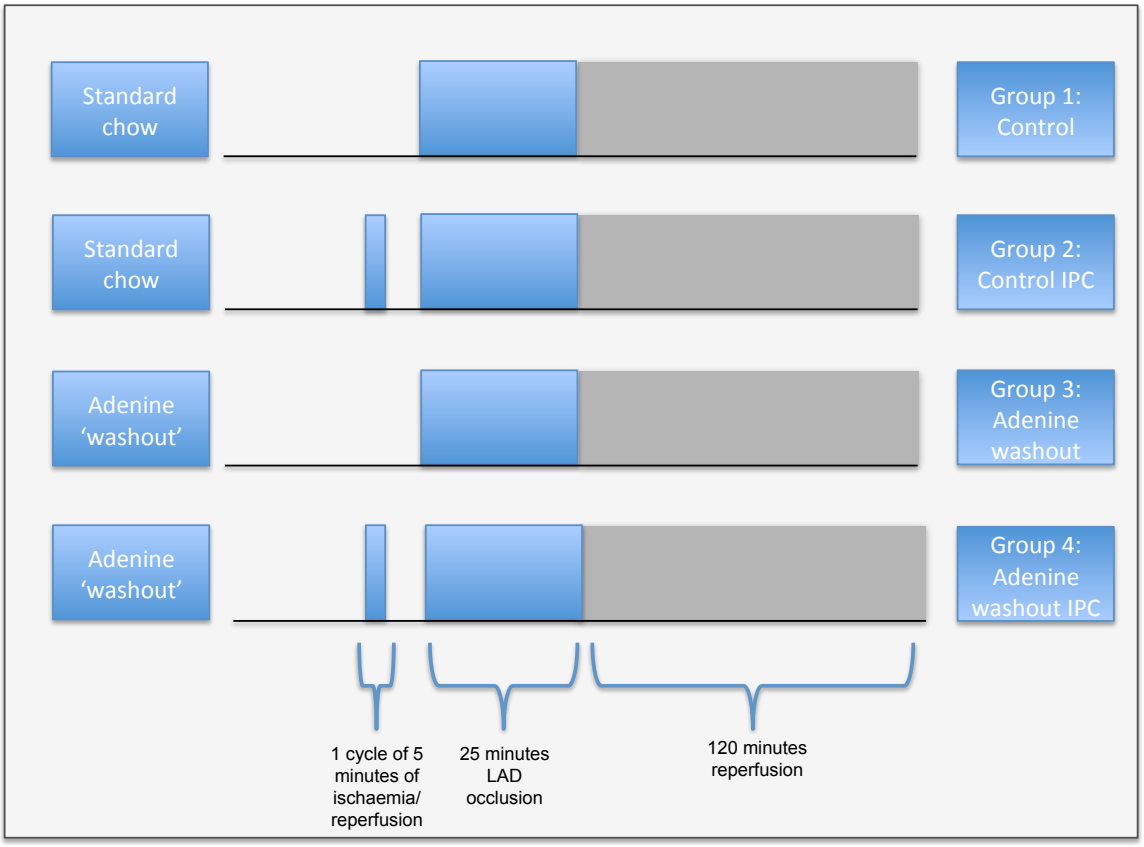


Figure 23. Schematic representation of experiment 4: Resistance to myocardial ischaemic preconditioning in the adenine diet treated rat.

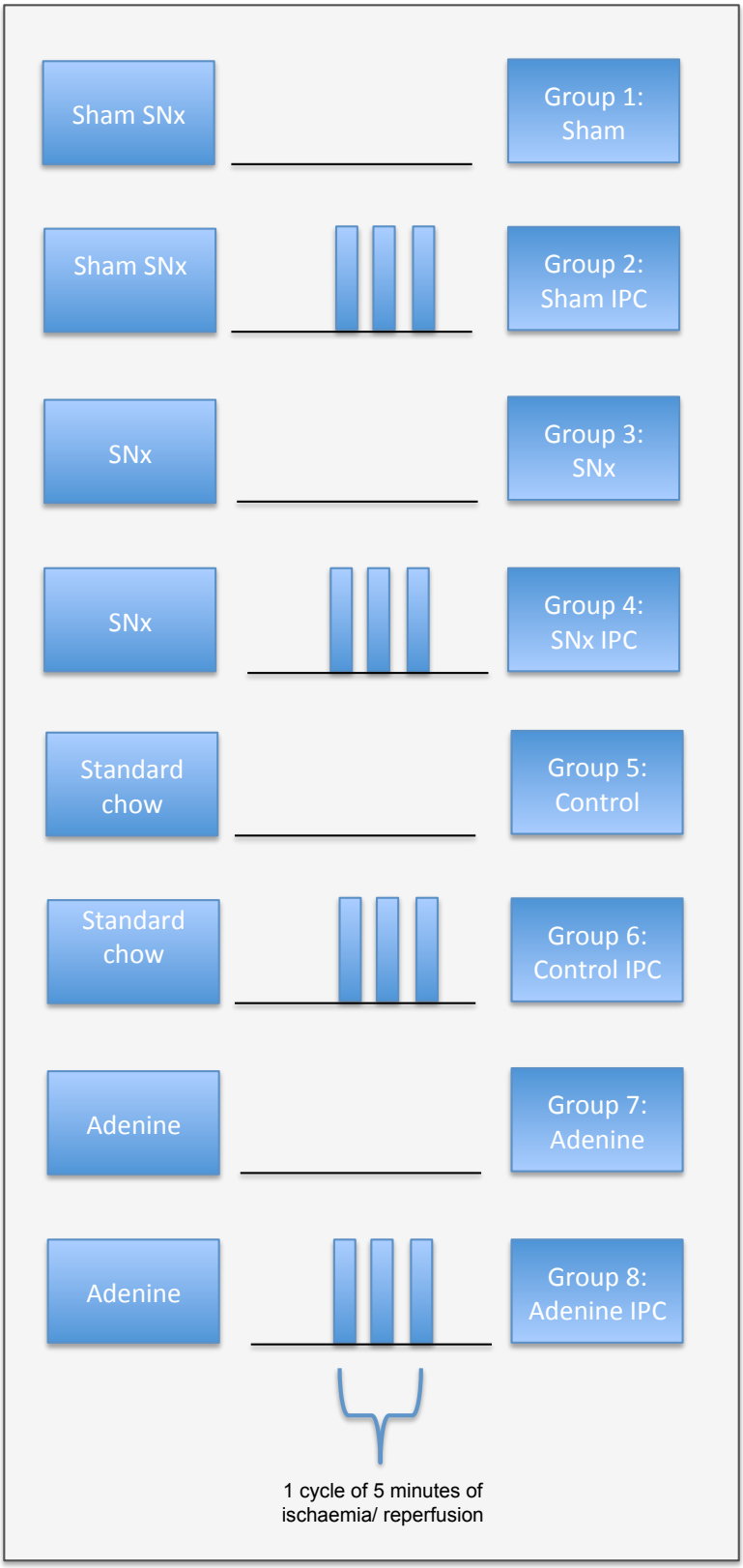


Figure 24. Schematic representation of experiment 5: Analysis of the mediators of IPC in uraemia

Results

Experiment 1. Myocardial ischaemic preconditioning in the subtotal nephrectomised rat

Cardiovascular parameters

3 cycles of preconditioning did not significantly alter mean arterial pressure, pulse rate or rate pressure product (see Figure 25).

Arterial blood gas analysis

Both groups were similar in terms of plasma pH, pCO_2 and base excess during the course of the experiment. However, the pO_2 was significantly higher in the control group than the IPC group. Haematocrit (HCT), pO_2, pCO_2 and pH measurements remained stable during the experiment, however, the base excess fell during the experiment from a median baseline of 4 and 3.1 mEq/l in the SNx control and SNx IPCx3 groups respectively to -0.1 and -2.6 mEq/l at the end of reperfusion in the SNx control and SNx IPCx3 groups respectively. This result was highly significant on Kruskall-Wallis testing ($p<0.0001$). Furthermore, the baseline HCT was higher in the IPC group compared to the control group. This difference was not seen in the pre occlusion or end reperfusion samples (see Figure 26).

Infarct size

The 2-stage subtotal nephrectomy procedure results in moderate uraemia with serum creatinine measurements approximately 2.5 times that of historical non uraemic controls. The 2 groups were similar in weight, creatinine and AAR. However, 3 cycles of IPC led to an 88% reduction in median infarct size ($p=0.002$), (see Figure 27 and Table 8).

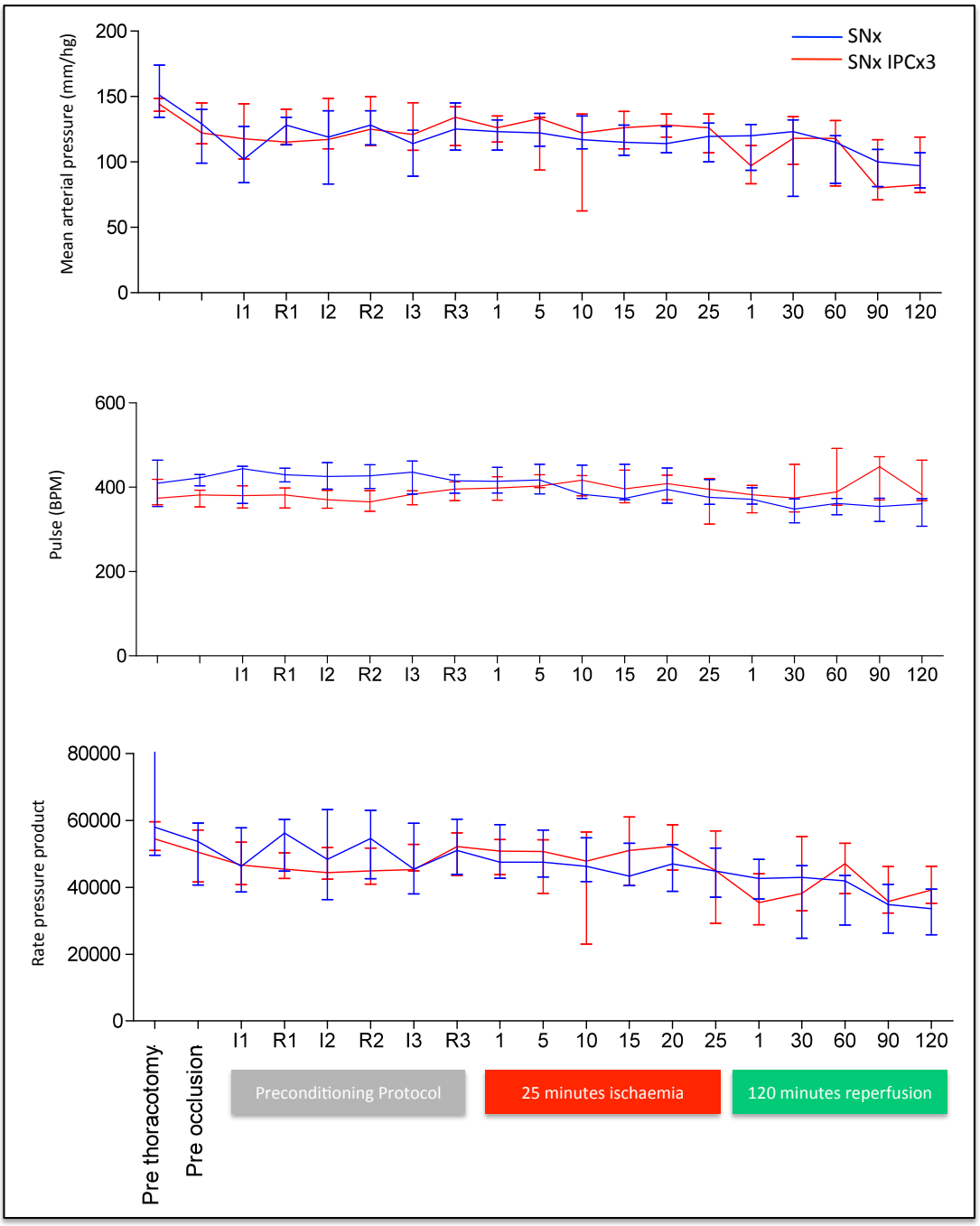


Figure 25. Analysis of cardiovascular parameters of experiment 1. Graph shows how mean arterial pressure (top), heart rate (middle) and rate pressure product, calculated by multiplying blood pressure by pulse (bottom) vary during the course of the experiment in uraemic animas with and without an IPC protocol. Data points represent median with error bars as IQR.

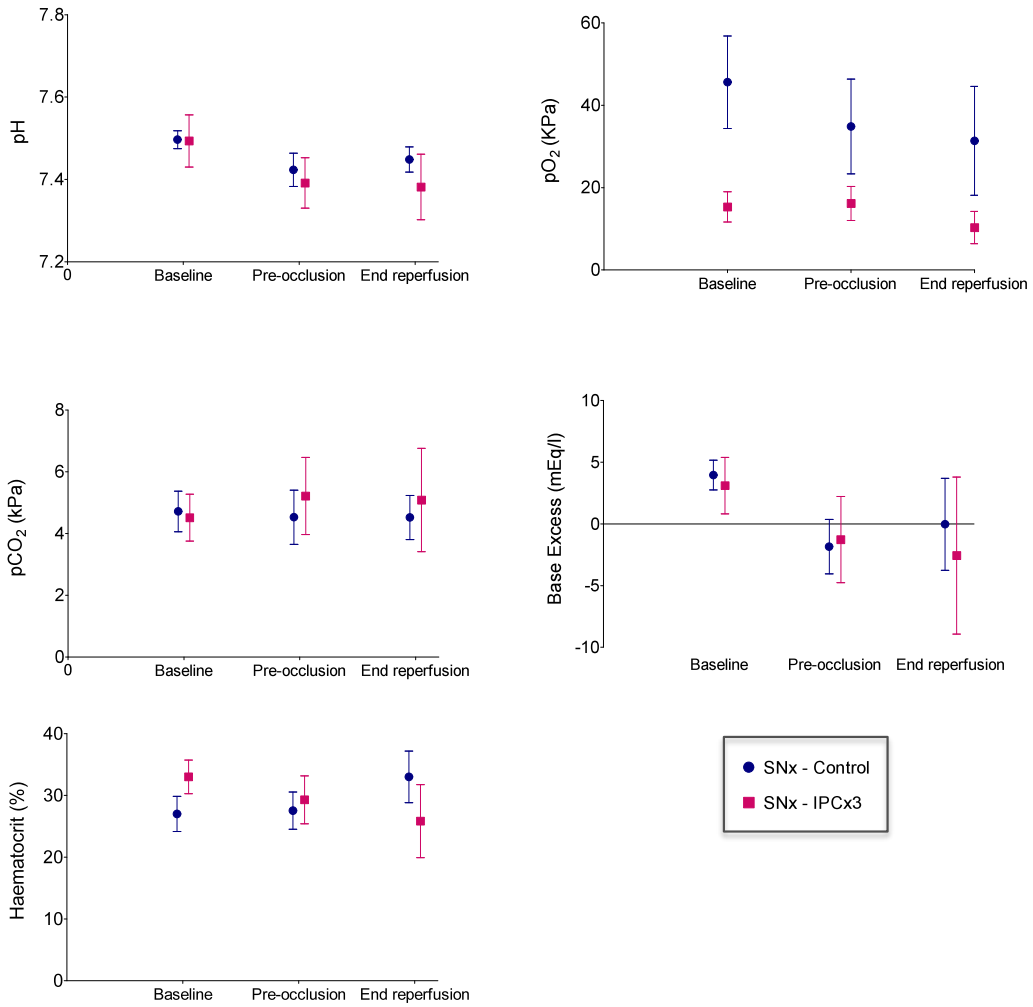


Figure 26. Graph demonstrating changes in arterial blood gas measurements during experiment 1.
The 2 experimental groups were SNx animals which underwent 3 cycles of IPC (SNx IPCx3) or did not (SNx control). Plasma pH, pO_2, pCO_2, Base excess and haematocrit were measured at 3 time points.: baseline, pre occlusion and at the end of reperfusion. All data expressed as medians with error bars representing IQR, calculated using GraphPad software.

	SNx	SNx IPCx3	p
Animals (n)	10	4	
Weight (g)	375 (370-383)	335 (320-395)	0.11
Hematocrit (%)	27 (25.5-28.5)	33.5 (28.8-36.8)	0.03
Creatinine (µmol/l)	83.5 (74-93.3)	88.7 (85-90.9)	0.18
Area at risk (%)	44.4 (41.3-49.5)	37.5 (33.9-46.4)	0.31
Infarct size (%)	61.1 (54.2-70.5)	7.1 (6.0-8.7)	0.002

Table 8. Results of experiment 1. Data presented as median (IQR). Statistical significance is presented as the results of a Mann-Whitney test using graphPad software.

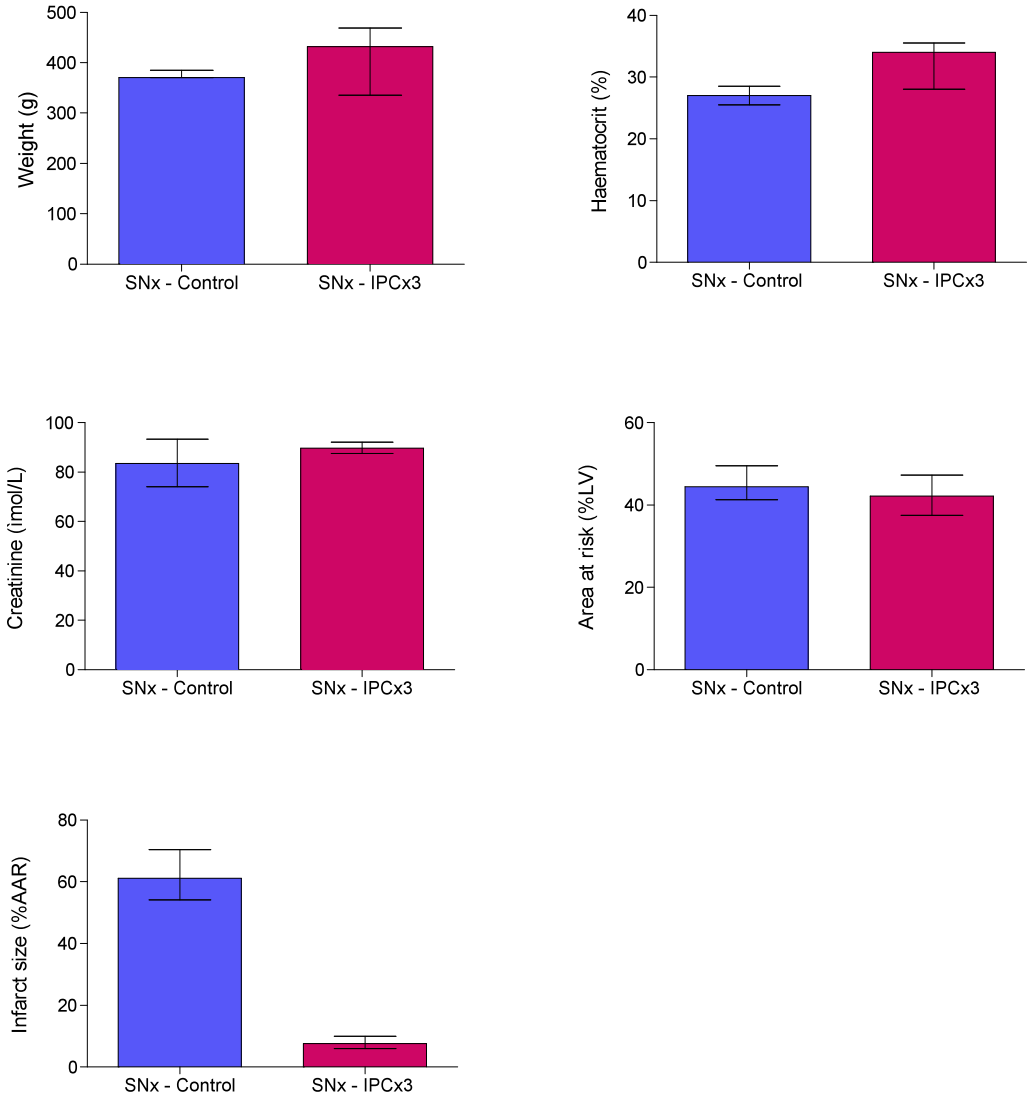


Figure 27. Results of experiment 1. Columns represent medians with IQR as error bars.

Experiment 2: Myocardial ischaemic preconditioning in the adenine diet treated rat

Cardiovascular parameters

At baseline, both uraemic groups were similar in terms of blood pressure, pulse and PRP (see Figure 28). The adenine groups which had undergone 3 cycles of IPC had a tendency to have higher blood pressure and PRP, however, during reperfusion this did not reach statistical significance ($p=0.25$).

Arterial blood gas analysis

Due to a defective arterial blood gas analyser at the time of this experiment, there was an incomplete data set for experiment 2 (see Figure 29). Only the baseline arterial blood gas parameters were available for analysis. Given that at baseline both groups of animals has been treated identically, it is unsurprising that both groups were similar in terms of plasma pH, pO_2, pCO_2, base excess and haematocrit.

Infarct size

4 weeks of 0.75% adenine diet resulted in severe uraemia in both groups, with serum creatinine values 7 times that of historical controls (see Figure 30 and Table 9).
3 cycles of 5 minutes LAD ischaemia/reperfusion before 25minutes LAD ligation, led to a reduction in median infarct size by 33% when compared to no IPC ($p<0.05$). The median area at risk was similar in the non-preconditioned and preconditioned groups ($p=0.4$). The group that underwent preconditioning were heavier than the non-preconditioned group ($p=0.05$). However, there was no significant difference in the degree of anaemia or serum creatinine in the groups.

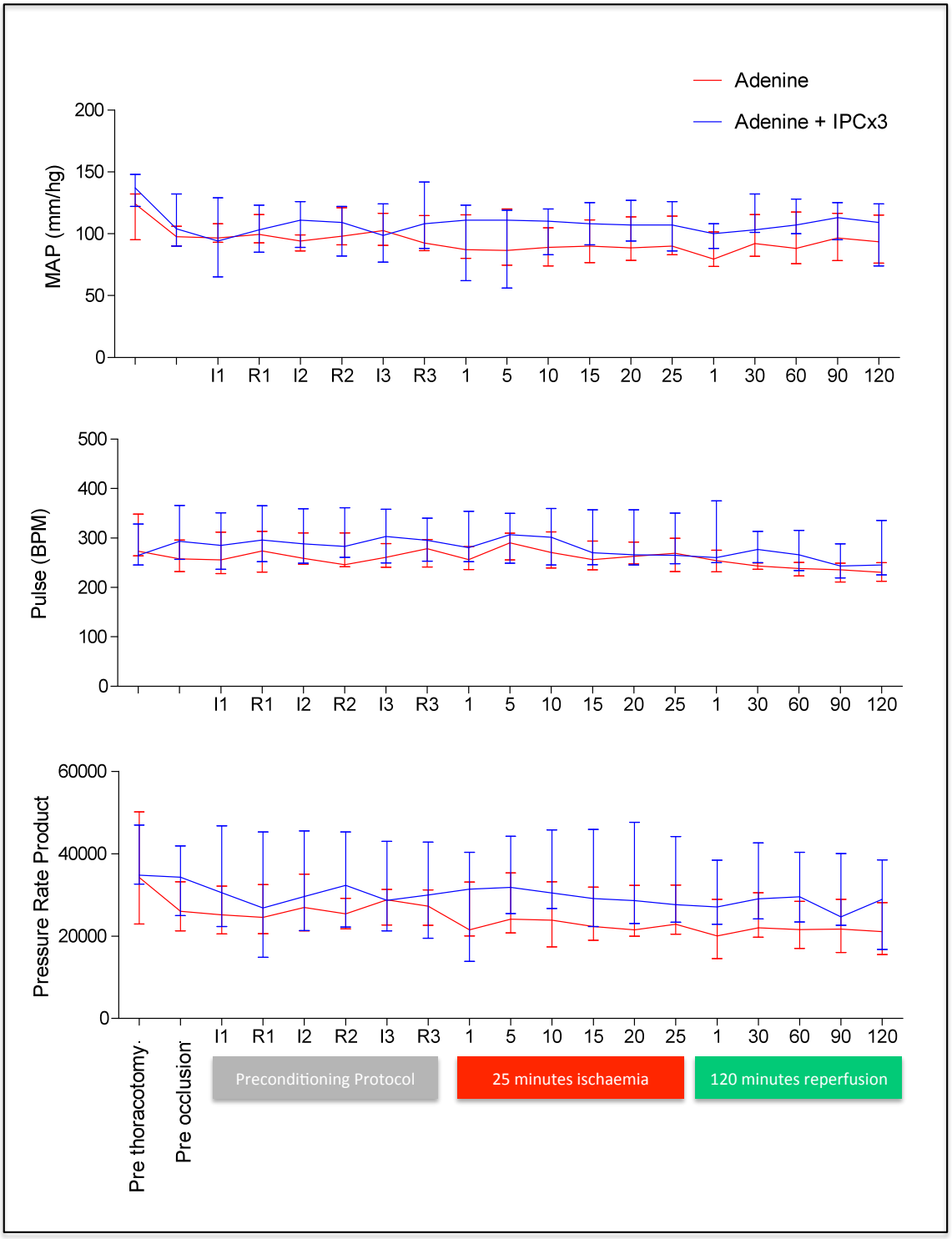


Figure 28. Analysis of cardiovascular parameters of experiment 2. Graph shows how mean arterial pressure (top), heart rate (middle) and rate pressure product (bottom) vary during the course of the experiment, in Adenine treated animals undergoing an IPC or sham protocol. Data points represent median with error bars as IQR.

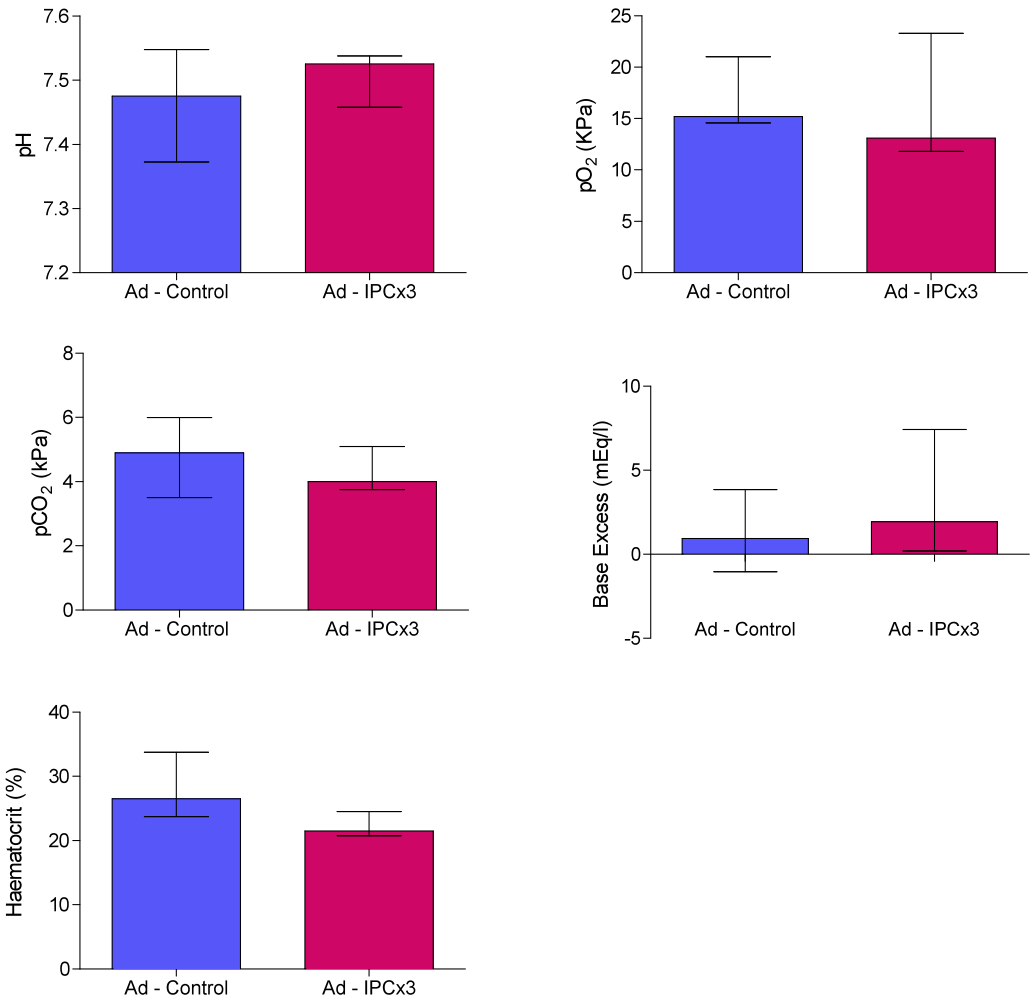


Figure 29. Graph showing baseline ABG measurements in experiment 2. Data expressed as median with IQR as error bars.

	Adenine	Adenine IPCx3	p
Animals (n)	8	8	
Weight (g)	220 (203-225)	238 (213-243)	0.05
Hematocrit (%)	24 (23-33)	22 (21-25)	0.07
Creatinine (µmol/l)	250 (235-267)	285 (180-336)	0.9
Area at risk (%)	46.1 (36.4-52.3)	40.0 (33.5-46.3)	0.4
Infarct size (%)	43.7 (28.5-69.5)	29.4 (7.7-32.1)	<0.05

Table 9. Results of experiment 2. Data presented as median (IQR). Statistical significance is presented as the results of a Mann-Whitney test using graphPad software.

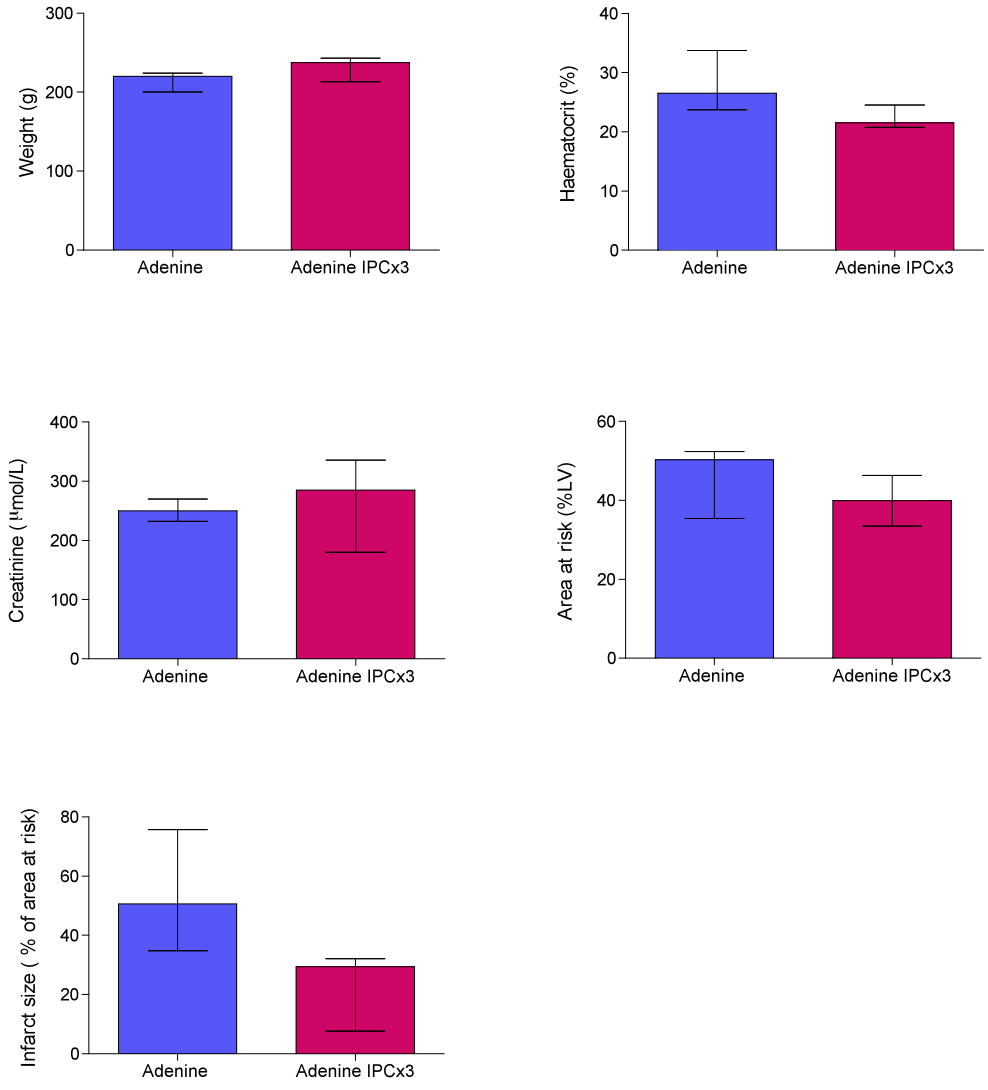


Figure 30. Results of experiment 2. Columns represent medians with IQR as error bars.

Experiment 3:Resistance to myocardial ischaemic preconditioning of the subtotal nephrectomised rat

Cardiovascular parameters

Analysis of heart rate, blood pressure and pressure rate product revealed no significant differences between the preconditioned and non-preconditioned uraemic animals (see Figure 31).

Arterial blood gas analysis

There was no significant difference in plasma pH, pO_2, pCO_2 or base excess between the sham SNx group and the SNx group during the course of the experiment (see Figure 32). At baseline testing, the non-uraemic group had a significantly higher haematocrit than the uraemic group, although this difference disappeared during the course of the experiment. There was no significant change in ventilatory parameters over time during the experiment, however plasma base excess fell during the experiment, from a median of 2.2 and -0.3mEq/l in the sham SNx and SNx groups respectively at baseline to -5 and -6.7 mEq/l in the sham SNx and SNx groups at the end of reperfusion. This was highly significant on Kruskall-Wallis testing ($p<0.0001$).

Infarct size

Compared with a sham procedure, a 2 stage subtotal nephrectomy resulted in a uraemic phenotype similar to previous experiments (see Figure 33 and Table 10). The uraemic animals had 2.5 times the serum creatinine concentrations that of sham animals ($p= 0.0003$) and they were anaemic ($p<0.01$). Following 1 cycle of IPC and 35 minutes LAD ligation, both groups had similar area at risks ($p=0.16$). However, the uraemic group had a significantly smaller median infarct size compared to the non-uraemic group ($p<0.01$).

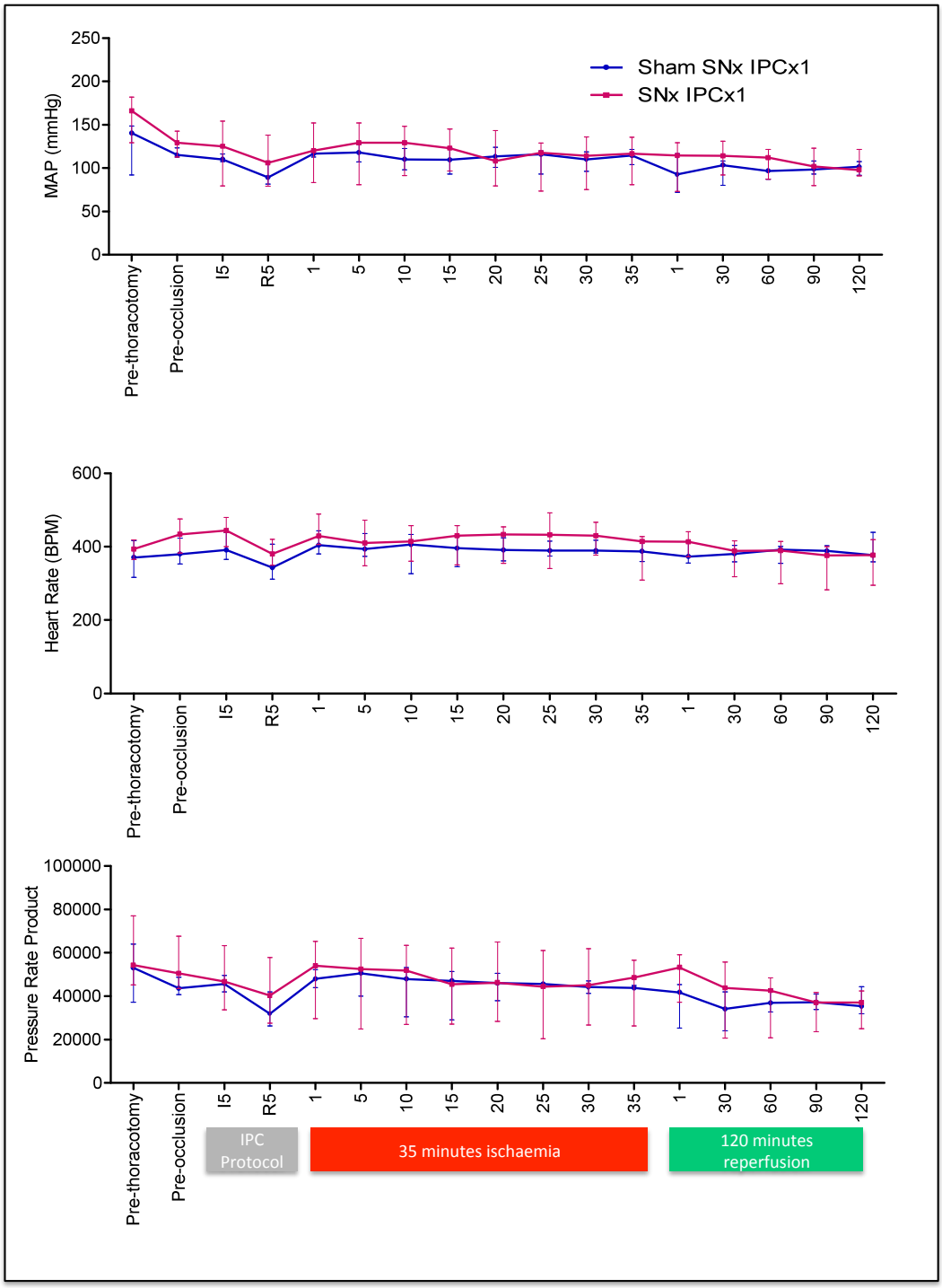


Figure 31. Analysis of cardiovascular parameters of experiment 3. Graph shows how mean arterial pressure (top), heart rate (middle) and rate pressure product, (bottom) vary during the course of the experiment involving uraemic (SNx) and non uraemic (Sham SNx) animals undergoing an IPC protocol. Data points represent median with error bars as IQR.

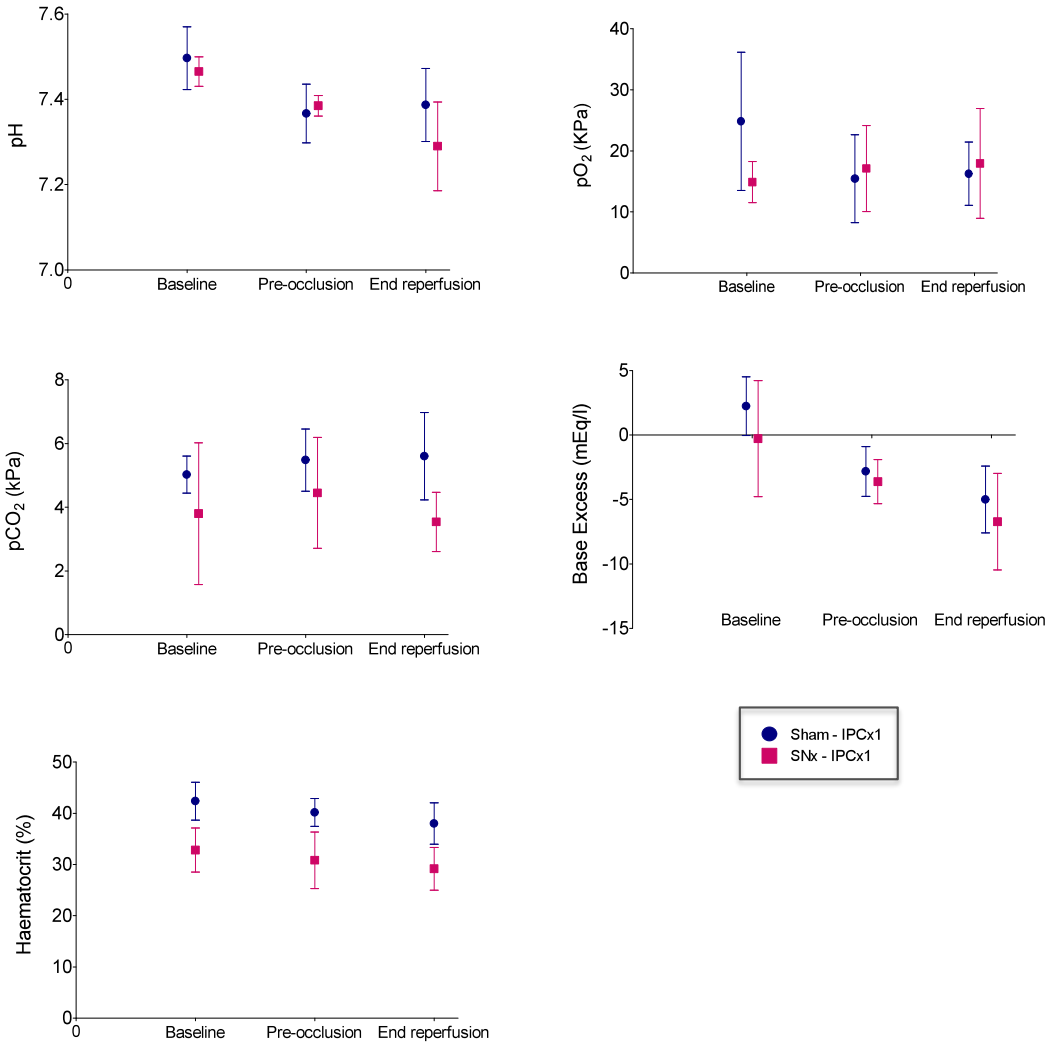


Figure 32. Graph demonstrating changes in arterial blood gas measurements during experiment 3. The 2 experimental groups were SNx animals and sham SNx animals, both groups underwent a single cycle of IPC. Plasma pH, pO_2, pCO_2, Base excess and haematocrit were measured at 3 time points.: baseline, pre occlusion and at the end of reperfusion. All data expressed as medians with error bars representing IQR, calculated using GraphPad software.

	Sham IPCx1	SNx IPCx1	p
Animals (n)	9	9	
Weight (g)	370 (332.5-386.5)	337.5 (316.3-352.5)	0.13
Hematocrit (%)	47 (44-49)	37.8 (36.5-40)	<0.01
Creatinine (µmol/l)	37.9 (32.3-42.7)	93.2 (89.6-117.9)	0.0003
Area at risk (%)	45.4 (35.2-51.8)	37.2 (32.6-42.9)	0.16
Infarct size (%)	32.1 (22.9-46.6)	18.1 (7.2-23.6)	<0.01

Table 10. Results of experiment 3. Data presented as median (IQR). Statistical significance is presented as the results of a Mann-Whitney test using graphPad software.

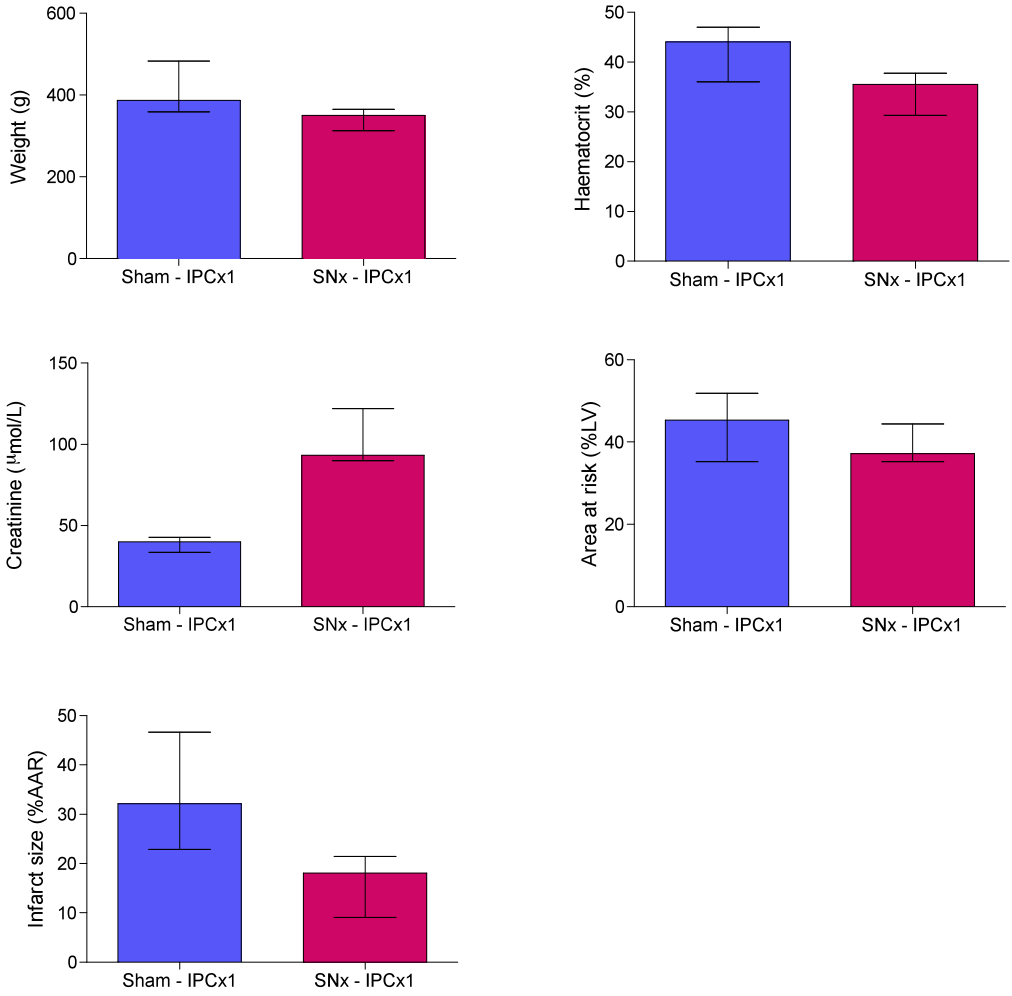


Figure 33. Results of experiment 3. Columns represent medians with IQR as error bars.

Experiment 4: Resistance to myocardial ischaemic preconditioning of the adenine diet treated rat

Cardiovascular parameters

Analysis of heart rate, blood pressure and pressure rate product revealed no significant differences between any of the 4 groups (see Figure 34). 1 cycle of preconditioning did not significantly alter the cardiovascular parameters of MAP, pulse rate or PRP, nor did underlying uraemia.

Arterial blood gas analysis

All groups had similar pH concentrations, with the exception of the control group (see Figure 35) which remained more alkalotic during the course of the experiment with a larger AUC for pH ($p<0.02$). There was no significant change in the partial pressure of oxygen or CO_2 during the course of the experiment and there was no significant difference in pO_2 or CO_2 between the 4 groups. As in the other experiments, a fall in base excess was seen following thoracotomy. However, in this experiment the results did not reach statistical significance. All groups had similar degrees of base excess at each time point during the experiment. There was no change in haematocrit over time in any of the individual groups. Both non-uraemic groups had similar haematocrit levels to each other, as did both uraemic groups. However, when the adenine groups were compared to both the control groups, adenine treatment was associated with a significant degree of anaemia ($p<0.0001$).

Infarct size

Animals treated with 4 weeks of 0.75% adenine diet followed by 2 weeks of standard chow were over 30% lighter than animals fed standard chow for 6 weeks (see Figure 36 and Table 11). The adenine washout groups were also significantly more anaemic with a reduction of over 45% in haematocrit ($p<0.0001$). Adenine washout resulted in serum creatinine concentrations that were over three times that of standard chow fed animals ($p=0.0006$).

Within the uraemic and non-uraemic groups respectively, there was no difference in weight, HCT or serum creatinine. At the end of the experiment, all 4 groups had similar area at risks. In non-uraemic animals, 1 cycle of IPC led to a reduction in the median infarct size of 50%. In the adenine washout groups, 1 cycle of IPC reduced infarct size by 31%. To establish how much of the difference seen between the 4 groups was attributable to underlying renal failure and how much was attributable to preconditioning, a 2 way ANOVA was performed using Graphpad software. The results indicated that the effects of IPC were independent of uraemia and that underlying uraemia accounted for <0.1% of the total variance, whereas IPC counted for 39% of the total variance.

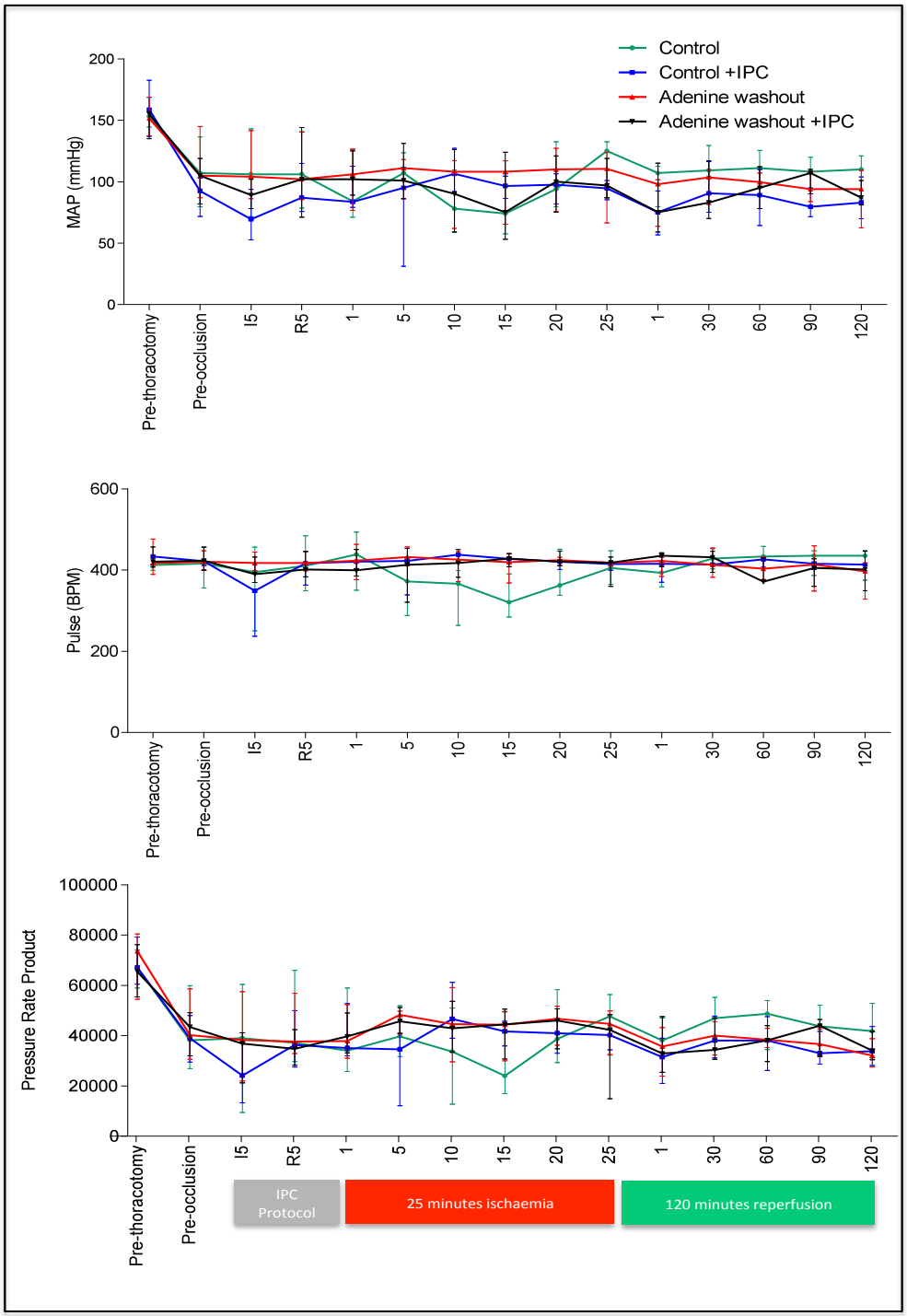


Figure 34. Analysis of cardiovascular parameters of experiment 4. Graph shows how mean arterial pressure (top), heart rate (middle) and rate pressure product,(bottom) vary during the course of the experiment. Experimental groups are adenine diet washout groups with and without IPC, and standard chow fed animals with and without IPC. Data points represent median with error bars as IQR.

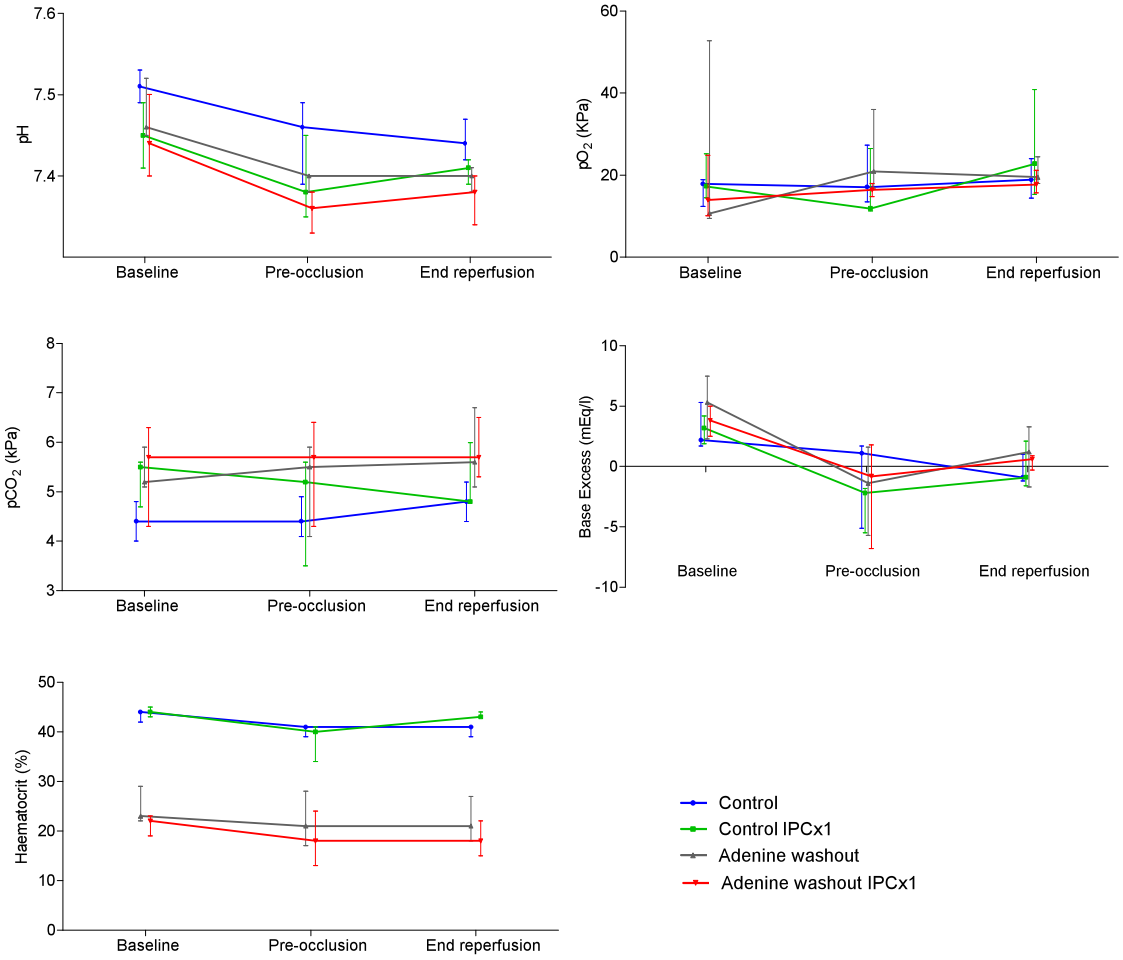


Figure 35. Graph demonstrating changes in arterial blood gas measurements during experiment 4.
The 4 experimental groups were: animals fed standard chow for 6 weeks (control), animals fed standard chow for 6 weeks and has 1 cycles of IPC (Control IPCx1), animals fed 0.75% adenine diet for 4 weeks followed by 2 weeks of standard chow (adenine washout) and finally animals fed 0.75% adenine diet for 4 weeks followed by 2 weeks of standard chow who had 1 cycle of IPC. All animals then underwent 25 minutes of LAD ligation and 2h reperfusion. Plasma pH, pO_2, pCO_2, Base excess and haematocrit were measured at 3 time points: baseline, pre occlusion and at the end of reperfusion. All data expressed as medians with error bars representing IQR, calculated using GraphPad software

	Control	Control IPCx1	Adenine washout	Adenine washout IPCx1	p
Animals (n)	11	7	11	7	
Weight (g)	498 (453-517)	510 (490-550)	342 (322-350)	350 (318-364)	<0.0001
Hematocrit (%)	44(42-45)	43 (41-44)	23.5 (22-27.5)	22 (19-23)	<0.0001
Creatinine (µmol/l)	40.5 (38.6-41.8)	39.6 (34.7-41.7)	137.1 (86-150.2)	119.7 (83.8-162.5)	0.0006
Area at risk (%)	52.6 (45.8-66.5)	49.8 (37.6-59)	43.1 (38.6-60.5)	47.2 (37.3-68.8)	0.69
Infarct size (%)	61.8 (47.8-73.8)	31.7 (9.1-37.7)	61.6 (52.8-67.9)	42.5 (12.4-48.8)	0.002

Table 11. Results of experiment 4. Data presented as median (IQR). Statistical significance is presented as the results of a Kruskal-Wallis test using GraphPad software.

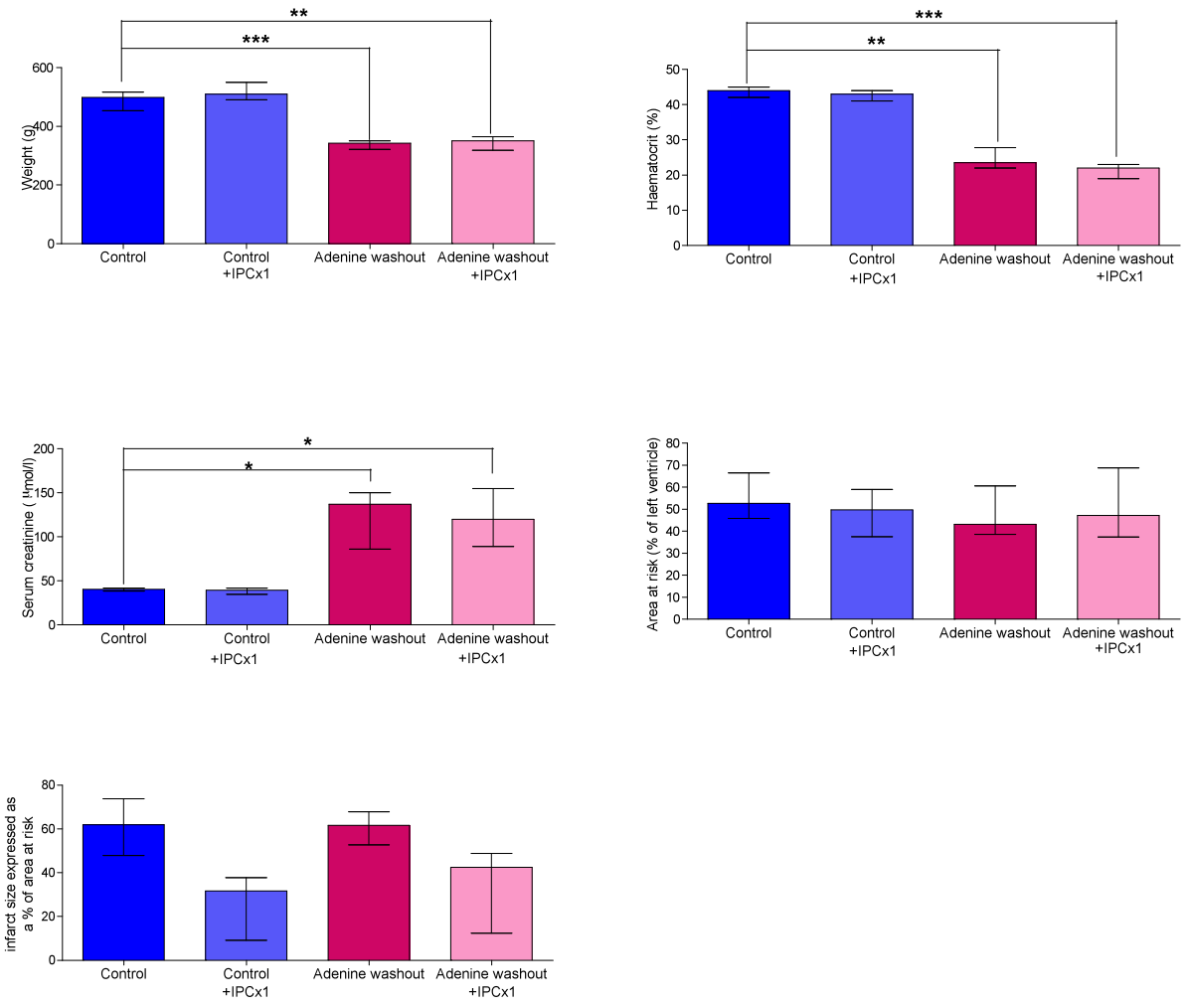


Figure 36. Graphical representation of experiment 4. Columns represent medians with error bares IQR. Significance testing results of Dunns post testing following a 2 way Kruskall Wallis test. P values: * $p<0.05$, ** $p<0.01$, *** $p<.0001$.

Investigation of the signalling pathways of myocardial IPC

The SNx model of chronic uraemia

This experiment had 4 groups of rodents. Sham SNx animals, sham SNx animals that had undergone IPC, SNx animals and SNx animals that had undergone IPC. Heart homogenates were analysed for components of the RISK and SAFE pathways and for each group, 5 homogenates were processed.

When the SNx model is compared to the sham SNx model, there is no difference in the basal activity of components of the RISK pathway or STAT activation. Preconditioning leads to significant ERK phosphorlyation in both the sham SNx animals and SNx animals in all 5 replicates. A similar effect is seen with STAT-3 in both groups, an effect which is consistent across replicates. There is no clear pattern across the 5 replicates studied for the impact of IPC on Akt phosphorylation. In group 1, IPC appears to lead to Akt phosphorylation in the sham animals but a relative reduction in phosphorylation in the SNx animals. In group 2, Akt does not appear to be affected by IPC in either the sham or SNx animals. In groups 3 and 4, IPC inhibits phosphorylation of Akt in the sham animals and activiates in the SNx animals. In group 5, IPC appears to lead to increased phosphorylation of Akt in both the SNx and sham groups (see Figure 37 and Figure 38).

The adenine model of chronic uraemia

This experiment had 4 groups: non uraemic control animals, control animals with IPC, adenine animals and adenine animals which had undergone IPC. 16 rats were used in the experiment with 4 animals in each group. There was no difference in the basal activity of ERK1/2 or STAT-3 phosphorylation between the uraemic and non uraemic rats. IPC consistently resulted in increased phosphorylation of ERK and STAT in both uraemic and non-uraemic rats (see Figure 39).

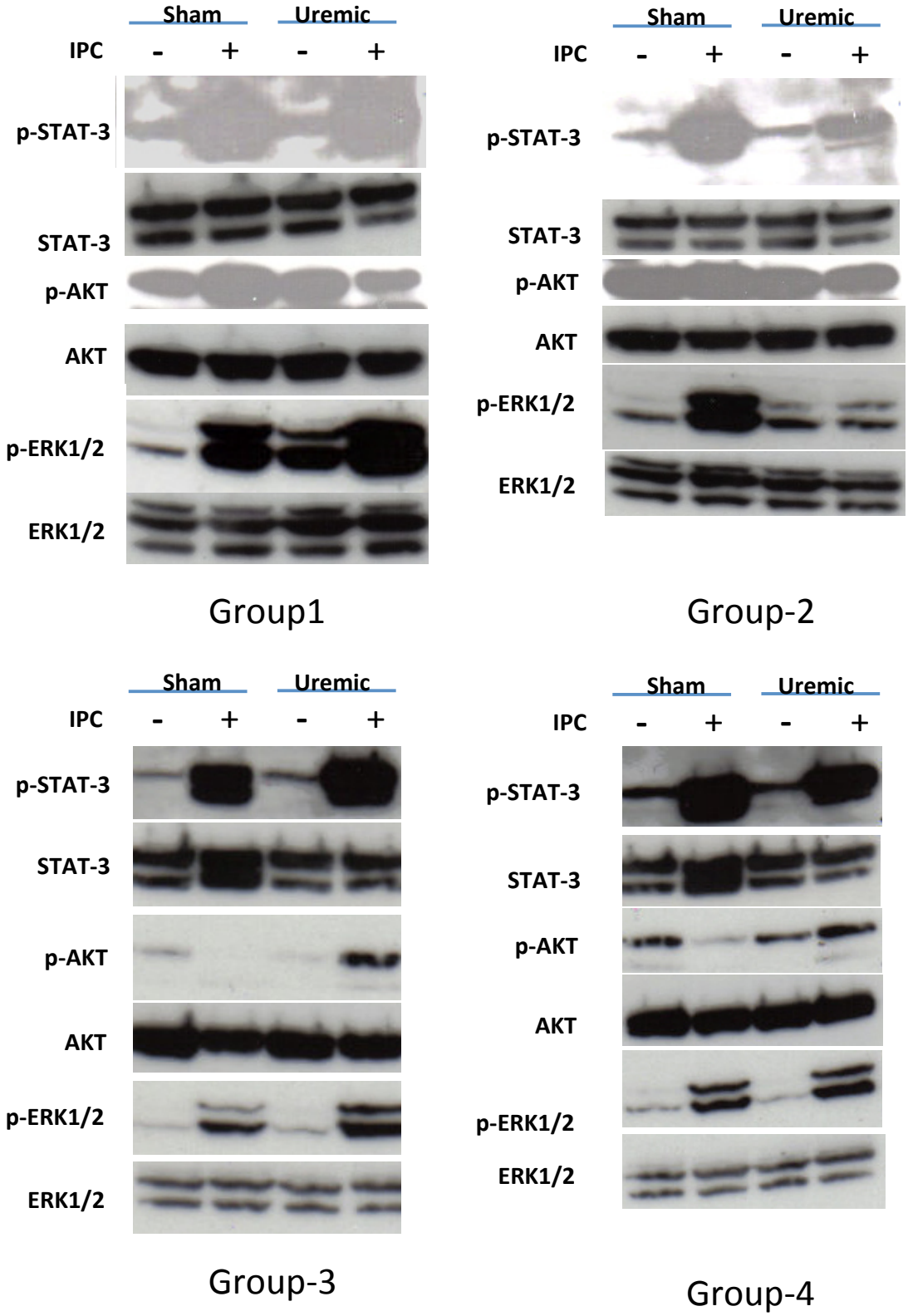


Figure 37. Results of western blots investigating the effects of IPC in SNx (ureamic) and non uraemic sham SNx animals (Sham).. Gels were probed for STAT-3, pSTAT-3, Akt, p-Akt, p-ERK 1/2 and ERK 1/2. Each experiment was repeated 5 times. This figure represents the first 4 replicates (see for the 5th replicate)

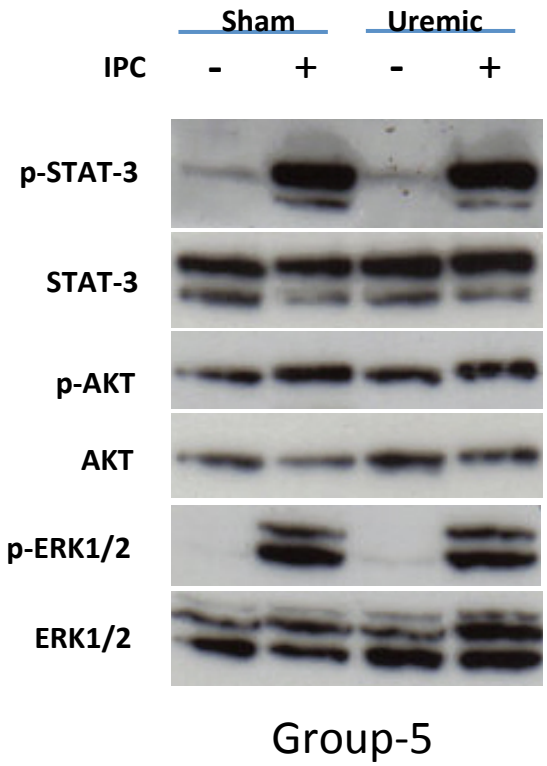


Figure 38. Results of western blots investigating the effects of IPC in uraemic and non uraemic animals. Gels were probed for STAT-3, pSTAT-3, Akt, p-Akt, p-ERK 1/2 and ERK 1/2., this figure represents the fifth replicate. See the previous figure for replicates 1-4.

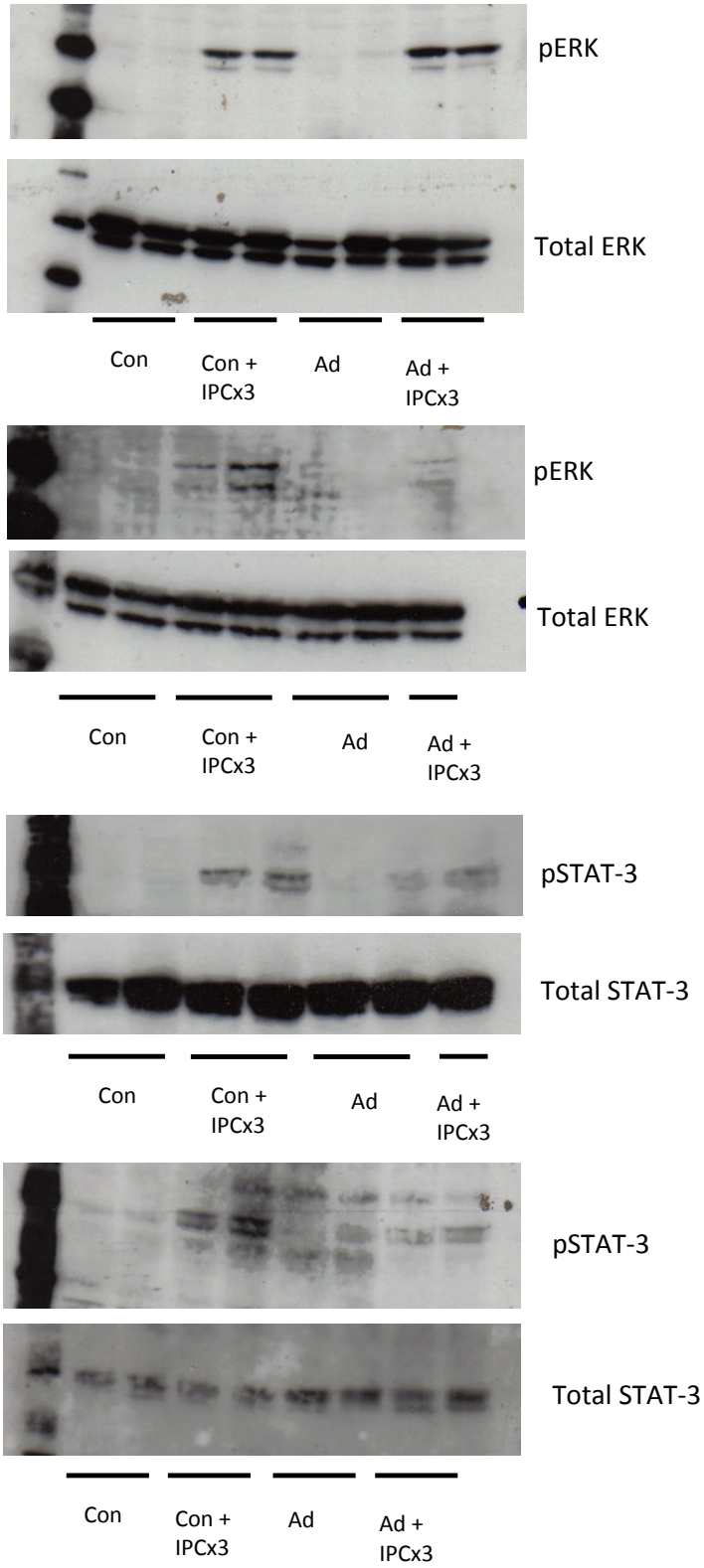


Figure 39. Results of western blots investigating the effects of IPC in adenine (Ad) and control fed (Con) animals. Gels were probed for p-ERK 1/2, ERK 1/2, pSTAT-3 and STAT-3. Each experiment was repliciated 4 times, with each lane representing a different homogenate.

Discussion

This is first time that the effect of uraemia on cardioprotection from IPC has been studied. The results show that IPC is a potent cardioprotective strategy in both non uraemic and uraemic rats, with the suggestion of even greater tissue protection in the context of the SNx model of uraemia. 3 cycles of IPC reduced infarct sizes by 88% in the SNx model and 33% in the adenine model, with 1 cycle of IPC reducing infarct size by 31% in the adenine wash out group.

In experiment 3, the duration of the ischaemic insult was lengthened from 25 to 35 minutes. It is known that the duration of the ischaemic insult is critically important when investigating the effects of preconditioning, too small an injury and the beneficial effects of preconditioning are not seen, however too long an insult may cause irreparable damage which cannot be modified by ischaemic conditioning[493]. The reason for lengthening the ischaemic period was an attempt to generate a larger infarct in the control group, such that any small beneficial effect of only 1 cycle would be more easily demonstrable. Despite the longer duration of ischaemia, both groups displayed significant reductions in infarct sizes when compared to historical controls. Interestingly, despite a reduction in ischaemia tolerance the uraemic animals appeared to develop a smaller infarct size in response to 1 cycle of preconditioning when compared to the non-uraemic animals. The reason for this is unclear. There is no difference in the basal activity of cardio-protective mediators (Akt/ERK/STAT) in the SNx group. One could argue that due to the lower haematocrit of the uraemic group, less thrombus is generated during manipulation of the LAD artery during the IPC protocol leading to less 'no reflow' injury during reperfusion. However, the adenine washout group had a much lower haematocrit than the SNx animals and those animals did not show greater cardio-protection than their control fed partners. When comparing the relative effects of IPC between the adenine and SNx groups, it appears that IPC confers greater protection to the SNx group. The relative reduction in infarct size following 3 cycles of IPC is almost two and a half times that in the SNx group compared to the adenine group. This may be due to a greater degree of uraemia conferring resistance to the beneficial effects of IPC. However, from this work even 1 cycle of IPC was enough to significantly protect all groups studied.

During the first experiment (SNx v SNx IPCx3) the pO_2 was significantly higher in the control group than the IPC group. This unexpected result may have been due to

difficulty in accurately titrating the delivered oxygen to the animals. The amount of oxygen delivered was controlled by a gas tap, which was difficult to finely adjust. The result is also surprising as at any one time, 2-3 rats were being operated on, with each rat receiving the same FiO_2. Furthermore, during each experimental day both IPC and non IPC animals were operated on. The impact of supra-physiological oxygen is unlikely to be cardio-protective and it may enhance oxidative stress leading to an increased oxidative burst[494] and tissue injury. The high partial pressure of oxygen in the control group could have contributed to injury. This may make the impact of IPC in this experiment more significant than it actually is. However, it is unlikely to have made such a large impact as to alter the conclusions from this experiment, and in all other experiments there was no significant difference in the oxygenation between any of the groups and yet protection was still seen.

These results show that across all experiments, ventilatory parameters were generally similar between groups and were not altered by any cardio-protective strategies. However, across all groups, the base excess fell progressively during the course of the experiments. This effect is seen during all LAD ligation experiments and is not surprising given the inherent tissue damage caused during thoracotomy along with the associated fall in blood pressure, which is likely to be due to altered respiratory physiology due to removal of the rib cage.

Analysis of the signalling mechanisms involved in IPC

All eight experimental groups (sham SNx, sham SNx IPC, SNx, SNx IPC, control, control IPC, adenine and adenine IPC) had myocardial homogenates run on western blots for ERK 1/2, Akt and STAT-3. However, the Akt and p-Akt gels for all 4 adenine groups did not demonstrate specific binding for Akt or p-Akt and therefore were excluded from the analysis.

This work fits nicely with the published literature on the effect of IPC on the RISK and SAFE pathways of myocardial protection. STAT-[423], ERK1/2[398] and Akt[398] have all been shown to be activated, following an IPC protocol, leading to tissue protection. This current series of experiments have shown that the signalling cascades appear to be unaffected by underlying uraemia, with both uraemic and non uraemic models phosphorylating ERK and STAT in response to IPC.

The results of Akt phosphorylation are difficult to interpret and do not show a clear trend in either the non uraemic or uraemic groups. It is important to remember that the function of Akt is not solely as a link in signal transduction chain for IPC from the cell surface to the mPTP. A possible explanation for the variability in the p-Akt results is that phosphorylation of Akt (as with other mediators) is a dynamic phenomeon. Akt phosphorylation is inhibited during ischaemia[398], and rises on reperfusion such that 5-10 minutes after the IPC protocol, the p-Akt is significantly higher than at baseline[495]. It could be that the variability of the results is due to the differential speed of phosphorylation of Akt during the brief reperfusion or that uraemia alters the rate of Akt phosphorylation during reperfusion or de- phosphorylation during ischaemia. This is speculative and further work needs to be done on the dynamics of Akt phosphorylation in the context of uraemia during IPC, with a greater delay between the end of preconditioning and harvesting to ensure more homogenous results.

In both uraemic models, SNx and adenine treated rats did not differ in terms of basal levels of phosphorylated STAT-3 or ERK 1/2. SNx rats had no significant basal increase in phosphorylated Akt (p-Akt) when compared to sham animals. This is in contrast to work by Tsang et al.[128] which showed that compared to non diabetic rats, diabetic rats had lower levels of p-Akt at baseline. They also found that p-Akt rose with subsequent IPC cycles. Tsang et al. proposed that lower basal levels of phosphorylated cardio-protective mediators in diabetic rats could explain the apparent resistance to IPC seen in such animals. This work was repeated by the same group in an ex vivo model, using human atrial appendages in diabetic and non diabetic patients with similar results[496].

The finding that underlying uraemia does not alter the basal levels of cardioprotective mediators concurs with their hypothesis because the uraemic rats did not have lower levels of p-Akt and did not display a resistance to preconditioning.

Conclusions

These experiments demonstrate that in multiple models of chronic uraemia, renal dysfunction, unlike diabetes, dyslipidaemia or senescence, does not lead to a resistance to the cardio-protective effects of IPC.

This work paves the way for translational research in the field of cardio-protection in CKD patients, who have high rates of cardiovascular disease, with poor outcomes following AMI and in whom standard therapies do not appear to be as efficacious or perhaps even deleterious. This cohort represents the ideal group to study, as their event rate following AMI is much higher than the general population meaning that smaller studies could still be powered for event based out comes.

In essence, CKD patients have the most to lose from CVD and this work suggests that they may have the most to gain from preconditioning strategies.

Future work

Future work should investigate the effect of underlying uraemia on the ability of the myocardium to respond to a remote ischaemic preconditioning signal. This work is currently the subject of on going research by my colleague Dr Conor Byrne.

Chapter 6

The Anti-arrhythmic effect of Preconditioning in Uraemic Models of Myocardial Ischaemia

Background

The anti-arrhythmic effect of conditioning strategies have been widely documented in the published literature[497-502]. The mechanism through which preconditioning inhibits arrhythmias is unclear. Work by Matejikova et al.[503] suggest that the effect is not dependant on a functioning PI3K/Akt system. They report that administration of a PI3K/Akt inhibitor abolished the cardioprotective effect of preconditioning but did not alter the anti-arrhythmic effects of preconditioning. Miura et al.[504] found that inhibition of bradykinin, prostaglandin or adenosine did not affect the anti-arrhythmic effects of IPC. However, subsequently work by Drimov et al.[505] reported that bradykinin antagonists abolished the anti-arrhythmic effects of IPC, that infusion of bradykinin mimicked the anti-arrhythmic effect of IPC and that both these effects could be abrogated by a sarcolemmal specific K_{ATP} inhibitor. The fundamental difficulty with identifying a specific mechanism for the anti-arrhythmic effects of IPC is that interventions which may lead to increased ischaemia tolerance may indirectly lead to a reduction in reperfusion arrhythmias by virtue of the fact that there is less tissue damage.

Patients with CKD have a high prevalence of cardiac arrhythmias. Sudden cardiac death attributed to arrhythmias is the single largest cause of death in dialysis patients[506]. The explanation for this is thought to involve fluid and electrolyte shifts during dialysis, combined with underlying coronary vascular disease, LVH, myocardial fibrosis and heart failure[507-509].

It is unknown whether underlying uraemia has an impact on the anti arrhythmic effects of IPC.

Methods

During the IPC experiments all animals had continuous monitoring of cardiovascular parameters using the Powerlab/85p system (ADI instruments), which were displayed on a monitor using LabChart software (ADI instruments). The timing of each intervention during the experiment was recorded on the LabChart software for later analysis.

After the experiments the blood pressure and heart rate trace could be analysed for evidence of arrhythmias. For the purposes of analysis, an arrhythmia was defined as a disturbance in the mean arterial pressure (MAP) trace lasting greater than 2 seconds. This time period was chosen so as to exclude occasional ectopic beats, which were unlikely to alter tissue perfusion. Asystole was defined as any period whereby the blood pressure was unrecordable (MAP <20mm/hg) for 2 seconds or greater. This may be caused by true asystole (no mechanical activity in the heart) or pulseless ventricular tachycardia or ventricular fibrillation. True asystole was uncommon and was almost invariably a terminal event.

Arrhythmias occurring during the first 30 seconds following LAD occlusion were excluded from analysis, as brief arrhythmias were not uncommon immediately after occlusion. These were often due to repositioning of the heart in the thoracic cavity using forceps to get good visualisation of the area at risk, rather than due to ischaemic arrhythmias.

The duration of arrhythmias (including asystole) occurring during preconditioning, reperfusion or during the index ischaemia were recorded. In addition, the delay between the onset of ischaemia/reperfusion and the onset of arrhythmias were recorded for each animal.

Results

In the SNx model of uraemia, during experimental myocardial infarction, 3 cycles of IPC resulted in a reduction in the duration of arrhythmias during the 25 minute index ischaemia (i25). This result approached marginal statistical significance (p=0.06). There was a significant reduction in the duration of reperfusion arrhythmias following IPC, from a median of 20 seconds to 9.5 seconds in the SNx IPC group (p=0.03). IPC did not alter the time to develop the first ischaemic arrhythmia, the duration of asystole, the time to develop the first reperfusion arrhythmia or the total duration of all arrhythmias (see Table 12 and Figure 40).

In the adenine model of chronic uraemia, 3 cycles of IPC resulted in a reduction in the duration of arrhythmias during i25 (p=0.05) and led to a trend in reduction in reperfusion arrhythmia duration (p=0.06). IPC had no effect on the time to develop ischaemic or reperfusion arrhythmias, nor did it have any effect on the duration of asystole or the total duration of arrhythmias (see Figure 41 and Table 12).

Next, the effect of underlying uraemia on the anti-arrhythmic effect of IPC was considered (see Table 12 and Figure 42). SNx animals and sham SNx animals did not differ in the response to an IPC protocol in terms of the duration of ischaemic arrhythmias, reperfusion arrhythmias or total duration of all arrhythmias, nor did uraemia have an effect on the delay in development of ischaemic arrhythmias following IPC.

Finally, the anti-arrhythmic effect of IPC was investigated in an adenine washout model of uraemia with non uraemic controls (see Figure 43 and Table 13). The adenine washout group had significantly less arrhythmias than the control animals following 1 cycle of IPC (p=0.001). When asystole duration was considered, no adenine washout animals developed asystole during ischaemia. This result was highly significant using Dunn's multiple comparison after a Kruskall-Wallis test (p<0.0001). Furthermore, IPC reduced the duration of asystole in the control group (p<0.005). Neither uraemia nor IPC appeared to alter the delay between the start of ischaemia and the first ischaemic arrhythmia.

The duration of the ischaemic, reperfusion and combined arrhythmias was investigated in the Adenine washout experiment (see Table 13). On Kruskall-Wallis testing, all 3 durations were significantly different. To establish the individual impact of uraemia or

IPC or both on these durations, a further 2 way ANOVA was performed. IPC reduced the duration of i25 arrhythmias in both uraemic and non uraemic animals ($p=0.04$) and there was no interaction between IPC and uraemia. Uraemic animals had a shorter duration of ischaemic arrhythmias when IPC was accounted for and this result approached borderline significance ($p=0.08$). Similar results were seen with reperfusion arrhythmias. IPC reduced the duration of reperfusion arrhythmias in both uraemic and non uraemic animals ($p=0.04$) and there was no interaction between IPC and uraemia. Uraemic animals had a shorter duration of reperfusion arrhythmias after IPC was accounted for ($p= 0.02$). Finally, when all arrhythmias were considered together, IPC did not alter the duration of these arrhythmias ($p=0.1$). When IPC was accounted for, uraemia was still associated with a reduction in total duration of arrhythmias when compared to control fed animals ($p=0.003$).

	SNx	SNx IPCx3	p
Duration of preconditioning arrhythmias (secs)	na	53 (24.5-79)	
Time to develop first arrhythmias during i25 (secs)	325 (250-360)	380 (60-700)	1
Duration of i25 arrhythmias (secs)	58 (22.25-112.5)	0 (0-50)	0.06
Duration of Asystole during i25 (secs)	0 (0-6)	0 (0-1.5)	0.38
Time to develop first reperfusion arrhythmias after i25 (secs)	21 (14.75-24)	22.5 (20-25)	0.68
Duration of reperfusion arrhythmias (secs)	9 (7.25-17)	0 (0-7)	0.03
Total duration of all arrhythmias (secs)	20 (8-48)	9.5 (4-47)	0.43

	Ad	Ad IPCx3	p
Duration of preconditioning arrhythmias (secs)	na	30.25 (7-50)	
Time to develop first arrhythmias during i25 (secs)	362 (150-442.5)	150 (150-720)	0.94
Duration of i25 arrhythmias (secs)	12 (4.5-40.5)	0 (0-9.5)	0.05
Duration of Asystole during i25 (secs)	0 (0-13)	0 (0-0.25)	0.43
Time to develop first reperfusion arrhythmias after i25 (secs)	27 (2-30.5)	25 (5-26)	0.61
Duration of reperfusion arrhythmias (secs)	3 (0-16)	0 (0-2)	0.06
Total duration of all arrhythmias (secs)	15 (9-29)	14 (6.5-22.5)	0.62

	Sham IPCx1	SNx IPCx1	p
Duration of preconditioning arrhythmias (secs)	97 (38.5-160)	42 (29-99)	0.46
Time to develop first arrhythmias during i35 (secs)	390 (300-480)	280 (195-365)	0.53
Duration of i35 arrhythmias (secs)	5 (0-38.5)	12 (3-27.5)	0.71
Duration of Asystole during i35 (secs)	0	0	
Total duration of all arrhythmias (secs)	17.5 (9.5-48.5)	26.5 (12-36.25)	0.78

Table 12. Tables showing impact of IPC strategies on arrhythmia duration during reversible LAD occlusion. Top table: The anti-arrhythmic effect of 3 cycles of IPC in an SNx model of chronic uraemia. Middle table: The anti-arrhythmic effect of 3 cycles of IPC in an Adenine model of chronic uraemia. Bottom table: The effect of underlying uraemia on the anti-arrhythmic effects of IPC. Definitions: preconditioning arrhythmias: all arrhythmias which occur during the preconditioning cycles, i25/i35 arrhythmias: arrhythmias which occur during the 25 or 35 minute ischaemic period, reperfusion arrhythmias: arrhythmias which occur on reperfusion following 25 or 35 minutes ischaemia. Results presented as median (IQR). p values are expressed as results of Mann-Whitney test using GraphPad software.

	Control	Control IPCx1	Adenine	Adenine IPCx1	p
Duration of preconditioning arrhythmias (secs)	na	132 (103-305)	na	15 (7-60)	0.001
Time to develop first arrhythmias during i25 (secs)	260 (180-312)	234 (39.8-372)	380 (312.5-435)	109 (20-198)	0.01
Duration of i25 arrhythmias (secs)	87 (47-128)	3 (0-7)	16 (4-53)	0 (0-4)	0.0007
Duration of Asystole during i25 (secs)	18 (13-147)	0 (0-1)	0 (0-0)	0 (0-0)	0.0001
Time to develop first reperfusion arrhythmias after i25 (secs)	25 (10-32)	27 (10-34.5)	21 (19-21)	15 (7-21)	0.5
Duration of reperfusion arrhythmias (secs)	15 (9-22)	9 (0-17)	5 (0-15)	0 (0-7)	0.02
Total duration of all arrhythmias (secs)	37 (13-101)	15 (4-83)	15 (7-26.5)	8 96-34.75)	0.03

Table 13: Anti-arrhythmic effect of 1 cycle of IPC in control and adenine washout groups. Results presented as median (IQR). p values are expressed as the results of a 2-tailed 1 way ANOVA except in row 1 when the Mann-Whitney test was used.

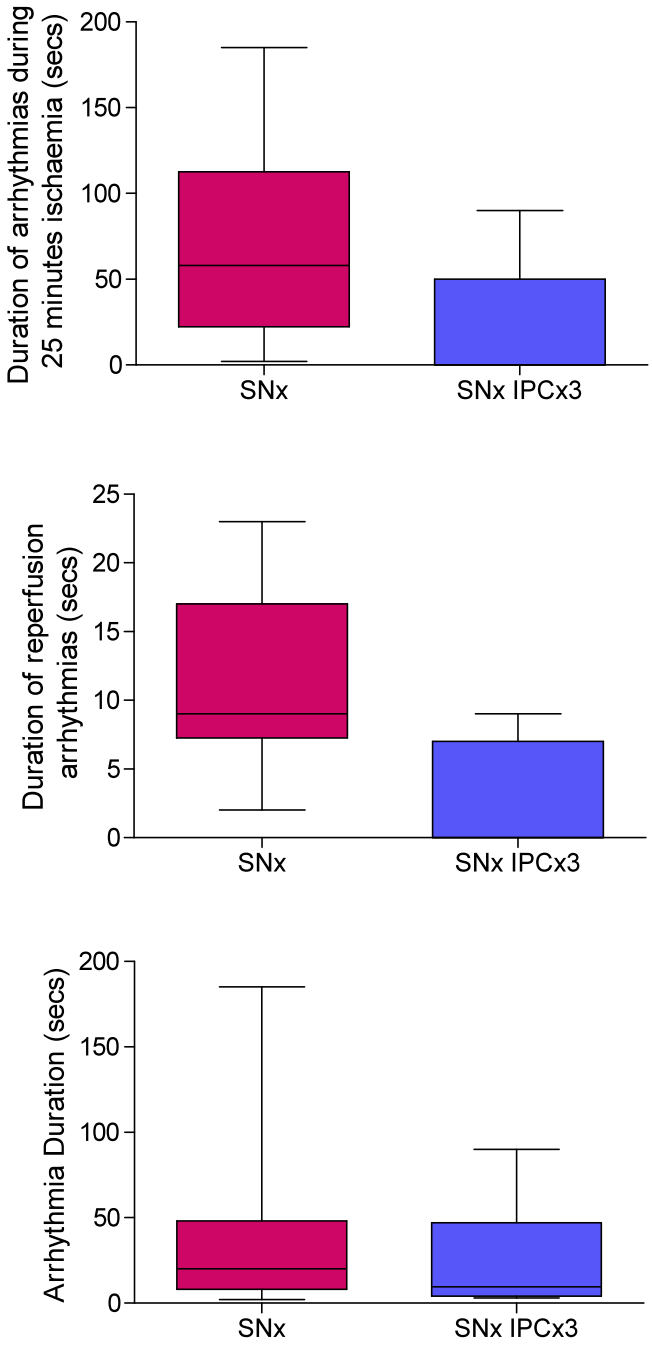


Figure 40. Box (IQR) and whisker plots (max/min) showing the anti-arrhythmic effect of 3 cycles of IPC in an SNx model of chronic uraemia. Figures show the effects of IPC on the ischaemic arrhythmias (top), reperfusion arrhythmias (middle) and total duration of all arrhythmias (bottom).

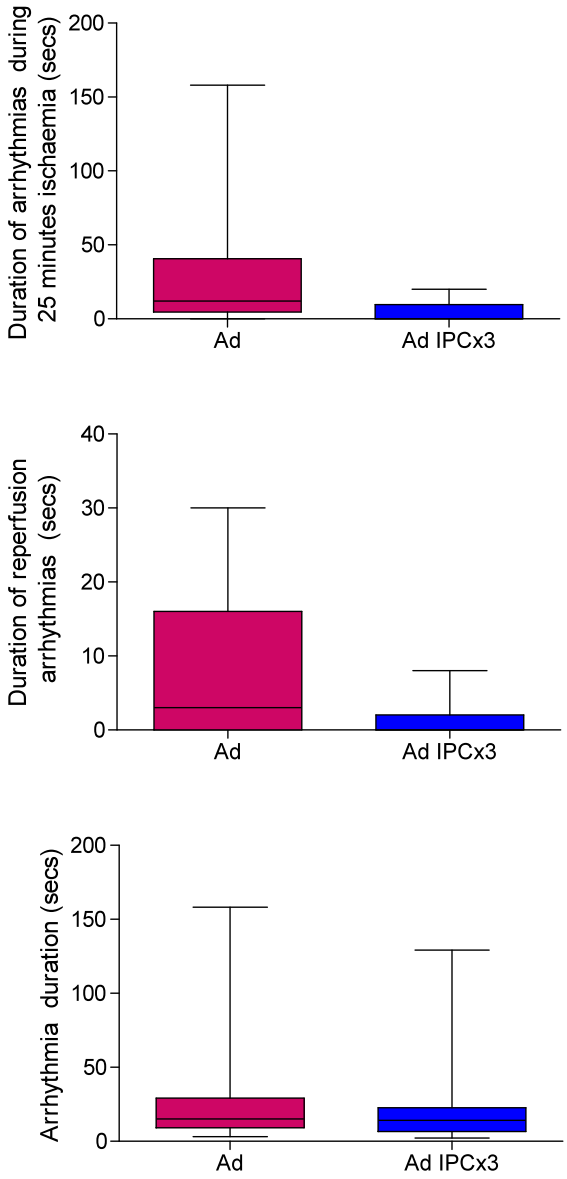


Figure 41. Box (IQR) and whisker plots (max/min) showing the anti-arrhythmic effect of 3 cycles of IPC in an adenine model of chronic uraemia. Figures show the effects of IPC on the ischaemic arrhythmias (top), reperfusion arrhythmias (middle) and total duration of all arrhythmias (bottom).

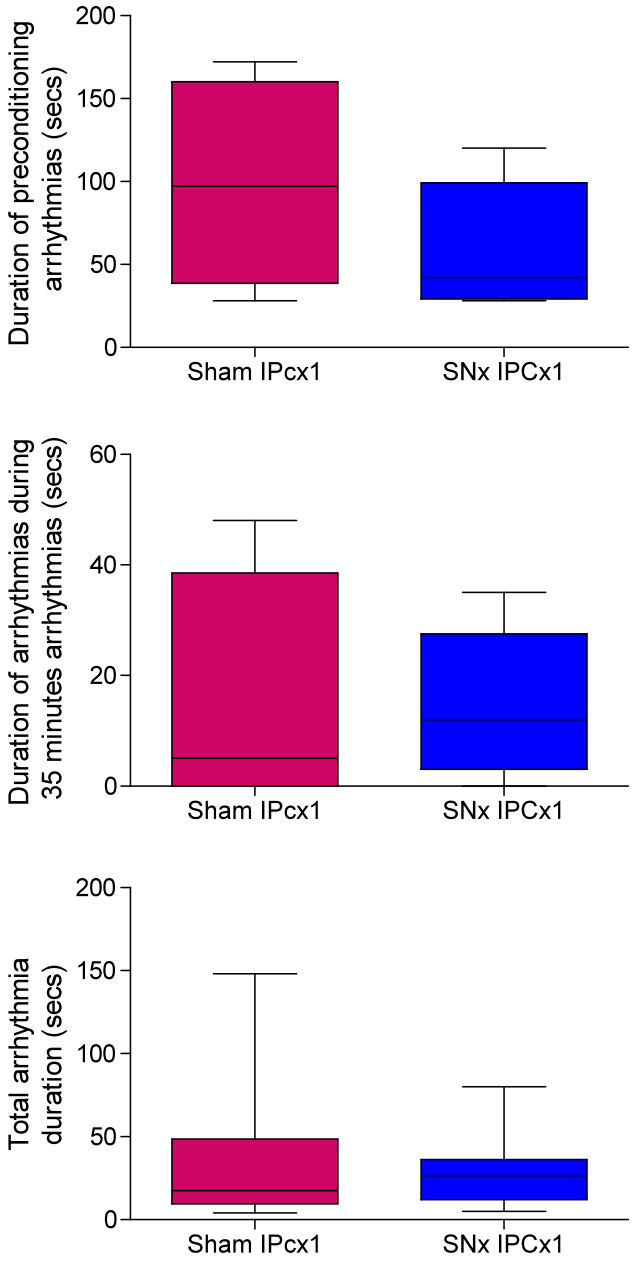


Figure 42. Box (IQR) and whisker plots (max/min) showing the effect of underlying uraemia on the anti-arrhythmic effects of 1 cycle of IPC. Figures show the effects of underlying uraemia on the duration of preconditioning arrhythmias (top), index ischaemic arrhythmias (middle) and total arrhythmia duration (bottom).

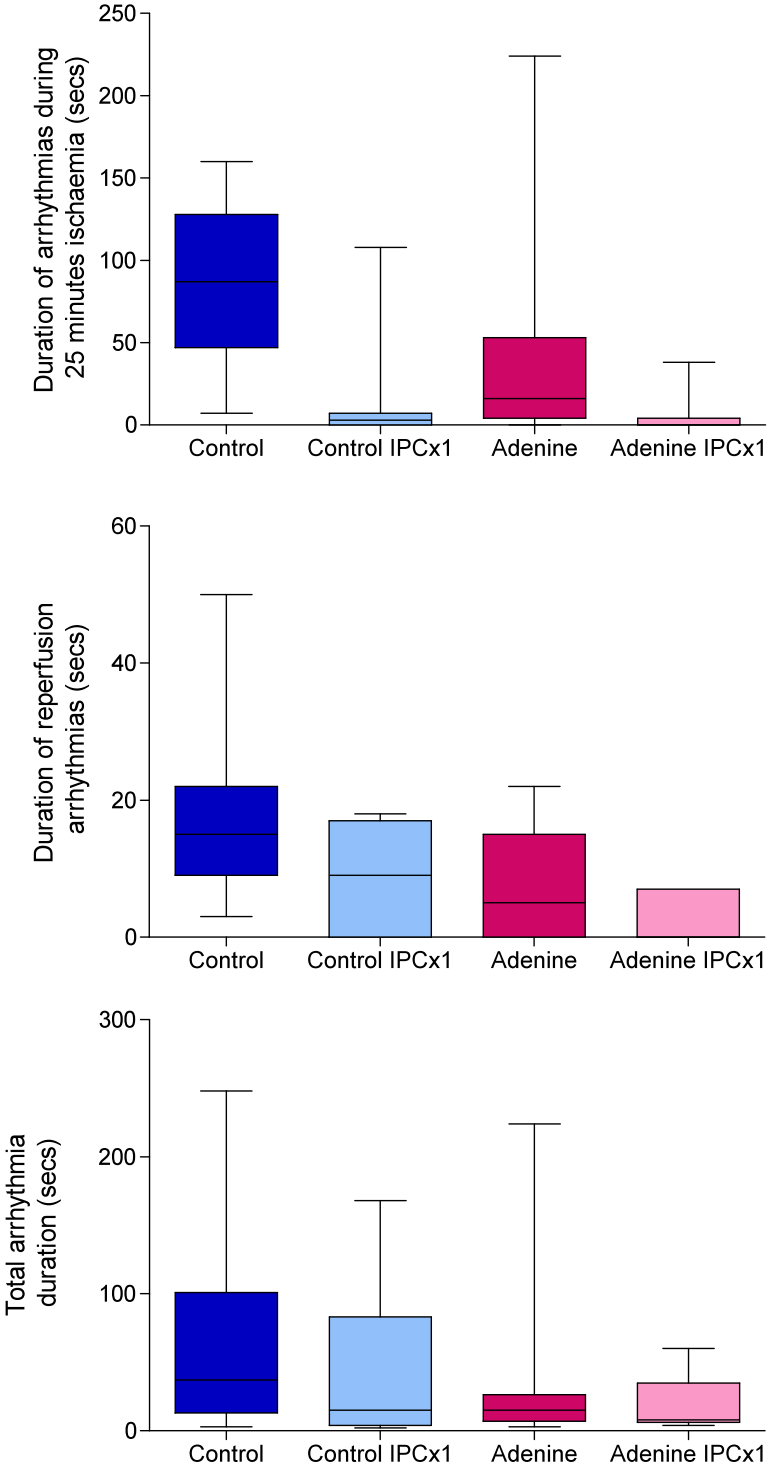


Figure 43. Box (IQR) and whisker plots (max/min) showing the effect of one cycle of IPC and underlying uraemia on the anti-arrhythmic effects of 1 cycle of IPC. The adenine groups had 4 weeks of adenine diet followed by a 2-week washout period; the control groups had 6 weeks of standard chow. The Figures show the effects of uraemia and IPC on the duration of ischaemic arrhythmias (top), reperfusion arrhythmias (middle) and total duration of arrhythmias (bottom).

Discussion

This analysis confirms the published literature that ischaemic preconditioning is anti-arrhythmic, both in reducing the duration of ischaemic and reperfusion arrhythmias and reducing the duration of asystole.

IPC did not delay the appearance of ischaemic arrhythmias during any of the experiments, nor did underlying uraemia appear to have an effect on this delay.

Underlying uraemia does not appear to be a barrier to the anti-arrhythmic properties of IPC, with both the SNx and adenine treated groups developing significantly shorter durations of arrhythmias following IPC when compared to uraemic animals, which did not undergo IPC.

With regards to the anti-arrhythmic effects of IPC in the published literature, authors have not analysed the extent or duration of the arrhythmias which occur during the preconditioning protocol itself. During IPC, particularly after the first ischaemic episode, long periods of arrhythmias are common. These were often in excess of that seen during subsequent ischaemic or reperfusion arrhythmias. It is known that inducing tachycardia[510] or myocardial dys-synchrony[511], with the use of external pacing, can induce cardio-protection through preconditioning. Clearly induction of arrhythmias is not the only mechanism through which hearts can be protected, but it is interesting to note that IPC produces lengthy periods of reduced or desynchronised cardiac output caused by VF/VT during the cycles of ischaemia/reperfusion. This loss of output may render the myocardium transiently ischaemic which may directly contribute to the reduction in infarct size. When all arrhythmias are considered, not just those that occur during the index ischaemic event, IPC no longer reduces the total duration of arrhythmias.

Therefore, this work suggests that IPC does not reduce the total arrhythmia duration, but brings arrhythmias forward in time to a point before lethal injury where they can potentially act as an additional preconditioning stimulus.

In the SNx model, uraemia does not appear be pro-arrhythmogenic in the context of IPC when compared with sham operated animals. Both uraemic and non uraemic animals develop similar durations of arrhythmias in response to 1 cycle of IPC followed by 35 minutes of LAD ligation. The adenine washout model, however, appears to confer a resistance to arrhythmias with a shorter duration of ischaemic arrhythmias, reperfusion arrhythmias and total duration of arrhythmias seen when compared to

control fed animals. This occurs both with and without an IPC protocol and appears to be independent of IPC. A possible explanation for this result could be due to the starvation or anorexia seen with the adenine model. Starvation has been shown in a rat model to increase myocardial glycogen content, protect against ischaemic arrhythmias[353] and led to a reduction in infarct size[354]. However, the model used a washout period of 2 weeks following the adenine diet, during which time the animals gain weight faster than their non uraemic counterparts. This points against the animals being in a starvation state. To investigate this apparent effect, further studies could be performed with longer washout periods to ensure that the metabolic effects of the adenine supplementation have resolved.

Chapter 7

Ischaemic Postconditioning of the Uraemic Heart

Background

17 years after Murry et al.[388] reported their seminal finding of IPC, Zhao et al.[512] reported, in an open-chest dog model of reversible LAD ligation, that interruption of reperfusion by 3 cycles of 30 seconds LAD occlusion/reperfusion could reduce the subsequent infarct size by 44%. In addition, they found that postconditioning (iPOST) caused a reduction in tissue oedema, neutrophil accumulation and endothelial damage.

This had followed on from work performed by their group showing that gradual reperfusion reduced infarct size[513, 514]. Other groups showed that maintaining a lower pH by staged reperfusion led to reduced stunning[515]. This finding had huge potential to overcome the main limitation of IPC, which was that the preconditioning stimulus had to take place before the ischaemic insult, thus limiting clinical translation of IPC to elective operations.

The prerequisite for rescuing ischaemic myocardium and reducing mortality is the early reinstitution of coronary flow. However, the idea that reperfusion itself contributes to lethal injury was first proposed by Jennings et al.[76] in 1960. At the time, this was considered highly controversial with different authors arguing that reperfusion merely augmented the effects of lethal ischaemic injury[516, 517]. Evidence that reperfusion itself can cause lethal injury is suggested by many experiments which show that pharmacological interventions, such as administration of PARP inhibitors[518], adenosine[519] bradykinin[520] or opiates[521] at the onset of reperfusion, can lead to a reduction in infarct size.

Factors which determine the response to iPOST are the duration of the index ischaemia, the delay between the beginning of reperfusion and the onset of iPOST, the duration and number of reocclusions. If the ischaemic period is too long[522] or too short[523], iPOST loses its protective effect.

There is even data suggesting that when the index ischaemic time is short, iPOST may be directly injurious and lead to higher infarct sizes[493]. These data suggest there is a certain degree of injury, which may be insult and species specific, which is amenable to cell salvage through iPOST.

Data suggest that the time delay from the point of reperfusion before commencement of iPOST is crucial to ensure cardioprotection. In rats[524], a 60 second delay following reperfusion abolished protection. However, this appeared to be species specific with rabbits still gaining a reduction in infarct size after a delay of up to 60 seconds[525].

Larger animals appeared to be more resistant to a delay in the initiation of iPOST; a 60 second delay still lead to cardio-protection in dogs[526] and a 180 second delay still lead to cardioprotection in pigs[527].

Too few cycles and/or too brief a cycle may reduce the 'dose' of iPOST. The precise duration and number of cycles again appears to be species specific. In pigs, 4 cycles of iPOST did not lead to cardioprotection, however when the number was increased to 8 cycles, protection was seen[528], while in rabbits 3 cycles of post conditioning with duration of 20 seconds but not 10 seconds, resulted in cardioprotection[529]. It is unclear whether the need for a longer cycle duration was because the number of cycles was only 3.

The beneficial effects of iPOST are, like IPC not limited to the heart. iPOST has been shown to confer tissue protection in the brain[530], kidney[531] and liver[532].

Mechanisms

The mechanisms of tissue protection from iPOST has relied on knowledge gained from research into the mechanisms of IPC, with multiple simultaneous endogenous (and exogenous) extracellular triggers acting in an autocrine/paracrine fashion to activate cytoprotective pathways resulting in tissue protection. The general approaches which have been used to elucidate the mechanism of protection include the assessment of the effectiveness of ischaemic postconditioning under specific pharmacological antagonism or inhibition, modification or loss of postconditioning in receptor or autacoid deficient mice and the induction of protection by exogenous autacoids or by selective synthetic agonists when given immediately prior to, or at the onset of reperfusion[533].

Triggers of postconditioning

Adenosine

Adenosine receptor activation by endogenous or exogenous adenosine to confer cytoprotection through iPOST has been demonstrated using a combination of pharmacological antagonism and gene deletion studies[533, 534].

Opioids

Zatta et al.[521] reported that iPOST led to opioid production, that opioid inhibitors blocked iPOST and exogenous opioids mimicked the effects of iPOST.

Bradykinin

Exogenous bradykinin given at reperfusion mimics iPOST[520]. Bradykinin inhibitors abrogate the effects of iPOST[535] and bradykinin knockout mice do not respond to an iPOST signal[536].

Acidosis and calcium overload

Reperfusion leads to rapid normalisation of myocardial pH at the expense of Na^+ and Ca^+ overload[537]. Postconditioning had been shown to delay pH recovery in rats[538] which has been shown to inhibit mPTP opening[539].

Effectors of postconditioning

RISK pathway

The RISK pathway has been shown to be involved in tissue protection following IPC. The RISK pathway was initially thought not to be involved in iPOST because the components of the RISK pathway were believed to be activated before ischaemia rather than during reperfusion. However, Hausenoly et al.[398] showed that inhibiting components of the RISK pathway during the first 15 minutes of reperfusion abolished the protection seen with IPC. Further evidence for the role of the RISK pathway comes from data which show that mice given an ERK inhibitor (but not a PI3 inhibitor) were rendered refractory to iPOST[540]. The importance of the RISK pathway in iPOST has also been shown in humans. Using an ex vivo experiment in human atrial tissue, Yellon's group were able to show that hypoxic postconditioning improved recovery of contractile function, which was dependant on intact PI3 kinase and MEK1/2 pathways[541].

Skyschally et al.[542] showed that the intracellular signal transduction of iPOST may involve non RISK pathways. They reported that an iPOST protocol in pigs conferred cardioprotection but did not result in a greater degree of up regulation in components of the RISK pathway (Akt, ERK and GSK-3β) when compared to a sham iPOST protocol. Furthermore, the protective effects of iPOST may remain even in the face of inhibition of the PI3 kinase or ERK1/2 pathways[543].

Survivor Activating Factor Enhancement Pathway

The survivor activating factor enhancement (SAFE) pathway comprises STAT-3 and TNFα. Lacerda et al.[544] have shown that TNFα administered at the time of reperfusion

confers subsequent cardioprotection, which is mediated via binding of TNFα to the TNF receptor-2, and is independent of the RISK pathway. However, this protection was dependant on STAT-3 activation. The JAK-STAT pathway conveys extracellular stress signals from the cytokine receptors on the plasma membrane to the nucleus, resulting in transcription of proteins involved in cardioprotection. Inhibition of the STAT pathways at the point of reperfusion has been shown to abolish the effects of iPOST[545].

Both the SAFE and RISK pathways appear to converge on mitochondria to achieve their cytoprotective effects[546].

Protein kinase C and G

Both inhibition of protein kinase C[547] and inhibition of protein kinase G have been shown to abolish the effects of iPOST, however the signalling mechanisms remain unclear.

Mitochondrial permeability transition pore

Like preconditioning, the mPTP is thought to be the end common effector for the cardioprotective pathways in iPOST[548, 549].

Inflammation

Postconditioning may also inhibit reperfusion injury by reducing neutrophil adherence to the vascular endothelium, inhibit activation of the coronary endothelium, reduce production of ROS and pro-inflammatory cytokines[512, 550].

Human trials in iPOST cardioprotection

The first work to translate the animal data into human trials was a small proof of concept study by Staat et al.[302], which showed a 36% reduction in the area under the curve of CK release in an iPOST cohort when compared to controls. They also found that there was a significantly higher 'blush grade' in the iPOST group, indicative of less 'no reflow', which is in line with the original findings by Zhao et al[512] who found that iPOST had a protective effect on endothelial function.

The benefits of iPOST have been shown to persist for up to 1 year. A small open label randomised study (n=38), demonstrated that 4 cycles of balloon inflation, in the context

of primary PCI, resulted in a 40% reduction in infarct size leading to an improved ejection fraction at 1 year[551]. These findings are in agreement with retrospective data which report that in 433 patients who underwent four or more balloon inflations in the context of AMI had smaller infarct sizes than those with 3 or less[552].

There have been 15 human clinical trials in cardioprotection with iPOST. However they have been small with an average size of 58 patients. Four have been in children undergoing cold cardioplegia for correction of tetralogy of Fallot and all have shown less inotrope requirement and shorter ITU stay[553]. Three of these studies looked at cardiac enzymes as a marker for myocardial injury and all have shown a benefit[554-556]. Of the 11 trials in adults, 7 have only looked at short-term outcomes such as ST resolution[557], cardiac apoptosis[558] or cardiac enzyme rise[302, 559, 560]. One used cardiac MRI at 1 week to access infarct size[561] and another used SPECT at 1 week[562]. Three studies had echo data at 8 weeks[563-565], and 1 study used SPECT at 6 and 12 weeks and an echo at 1 year to confirm cardiac protection[551].

Comorbidities

The published literature, almost without exception, shows that when iPOST is delivered in a timely manner with an appropriate study protocol, it is a potent cytoprotective strategy. While the published human clinical trials have shown benefit, the trials have generally been small, with little long term outcome data and were not powered to show a mortality benefit, in addition they have also not demonstrated the same magnitude of tissue protection seen in the animal literature. A possible reason for the failure of widespread translation from the animal literature to clinical practice could be that patients who present with a cardiac event differ from juvenile healthy animals. Patients who present with an AMI are likely to be elderly, dyslipidaemic and with vascular disease, and many are diabetic.

Gender

The role of gender in iPOST is unclear. One study in rats indicated that the effect of iPOST was smaller in female than male rats[523]. However, in another, the effect of iPOST depended on gender and duration of ischaemia; female rats did not postcondition when the ischaemia duration was 25 minutes, but did respond when the duration was 20 minutes, unlike male rats who were protected at both time points[566].

Dyslipidaemia

Underlying dyslipidaemia has been shown to attenuate the protective effects of iPOST. A study by Iliodromitis et al.[456] reported in a rabbit model that underlying hypercholesterolaemia attenuated the protective effects of iPOST. Hypercholesterolaemia has also been found to attenuate the effects of iPOST in the pig[527] and rat[567].

Senescence

Aging appears to cause resistance to the effects of iPOST. Isolated perfused hearts from older mice (20-24 months old) could not respond to an iPOST protocol unlike adult (3-4 month) mice. The mechanism by which senescence resulted in a loss in ability to respond to iPOST was investigated by Przyklenk et al.[540]. They showed that older mice did not phosphorylate ERK in response to an iPOST stimulus and had higher basal levels of MAP kinase phosphatase-1 (MKP-1), which is thought to have a primary role in ERK dephosphorylation. Due to increased dephosphorylation of ERK by MKP-1 in old mice, iPOST was not able to confer protection. The role of MKP-1 in iPOST was confirmed by showing that inhibiting MKP-1 reinstated the cytoprotective effects of iPOST in older mice[540].

Diabetes

Streptozotocin treated mice were unable to respond to an iPOST protocol due to a loss of iPOST mediated ERK phosphorylation. However the beneficial effects of iPOST were regained if they were rendered euglycaemic with a islet cell transplant 2 weeks before the cardiac ischaemia[568].

Obesity

Ob/ob mice, characterised by hyperglycaemia, hypercholesterolaemia, left ventricular hypertrophy and higher body weight were found to have both a reduced ischaemia tolerance and a failure to respond to iPOST[569].

Coronary artery stenosis

30% of patients who present with AMI already have significant coronary artery disease. Oikawa et al.[570] reported that rats with experimental coronary artery stenosis were unable to respond to iPOST, due to a failure to upregulate ERK and Akt.

Medication use

Patients who present with AMI are often on multiple medications, which may have an effect on the ability to postcondition. Chronic use of statins[571], beta-blockers[572] and drugs which block the mitochondrial K_{ATP} channel[573], have been shown to render the heart resistant to iPOST. Furthermore, nicorandil, a K_{ATP} channel opener and a common drug used in patients with ischaemic heart disease, has been shown to mimic postconditioning when given in the context of AMI[574]. It is unknown whether chronic treatment with nicorandil may render the heart resistant to iPOST via the K ATP pathway. Up to 30% of patients presenting with ACS have underlying CKD stage 3-5[41, 49, 489]. The impact of CKD on resistance to iPOST is currently unknown.

Methods

40 male Wistar rats, which had undergone a 2 stage, subtotal nephrectomy procedure or sham procedure (as described in the methods section) to generate uraemic and non uraemic groups were used. 4 weeks after the second stage, the rats underwent reversible LAD occlusion (see methods section for full details of experimental protocol). After 25 minutes of LAD occlusion, the occluder was loosened to begin reperfusion. The animal then either underwent a postconditioning protocol comprising 5 cycles of 10 seconds ischaemia followed by 10 seconds reperfusion, or they did not. This meant that there were 4 experimental groups. Group 1 (sham control) were animals which had undergone a sham SNx procedure and no iPOST. Group 2 (sham iPOST) were animals which had undergone a sham SNx procedure and iPOST. Group 3 (SNx control) were animals which had been made uraemic with an SNx procedure, but had not undergone an iPOST protocol. Group 4 (SNx iPOST) were animals which had been made uraemic with an SNx procedure and had undergone an iPOST protocol. Following this, the animals underwent a 2-hour reperfusion period at the end of which blood was taken for analysis and the heart was harvested and processed for infarct size determination in the standard manner (see methods section for full details of harvesting and infarct size quantification).

Exclusions

During the experiment, one animal died during basic surgery (uraemic group). 3 animals had no area seen at the end of the experiment and were excluded (2 uraemic,1 sham). 1 animal (sham) died during ischaemia, 6 animals died during reperfusion (4 had not undergone iPOST of which 3 were uraemic, 2 had undergone iPOST of which one was uraemic). This left group 1 with 9 animals, group 2 with 8 animals, group 3 with 6 animals and group 4 with 6 animals for analysis.

Measurement of arrhythmias

Arrhythmias were measured in the same was as in the work in IPC (see methods section the arrhythmia experiments in the IPC chapter).

Quantification of Serum Troponin by ELISA

Serum troponin was quantified using a high sensitivity rat cardiac troponin-I (cTni) ELISA kit (cat no 2010-2-HSP Life diagnostics, Pennsylvania USA).

Technique

Before the thoracotomy and at the end of reperfusion, 1ml of blood was withdrawn from the arterial line into a 2ml Eppendorf tube containing 30μL heparin (5000 iu/ml) and mixed by repeated inversion. The sample was spun at 6000g for 3 min and the serum was aspirated into a new Eppendorf tube. The sample was then stored at -80^{O}C.

After equilibration of the kit components to room temperature, cardiac troponin stock solution was prepared and serial dilutions were made to generate standards which were carefully pipetted into the wells. Serum samples were prepared, in the case of the pre-thoracotomy samples, by diluting 100μl of the sample with 3 volumes of plasma dilutent. It was found, after some trial and error, that the post reperfusion samples required a 1 in 32 dilution step before further dilution with plasma dilutent, to avoid going over the top of the standard cure, due to much greater troponin levels post-reperfusion. 100μl of the appropriately diluted pre and post samples were added to the wells. 100μl of troponin HRP conjugate was added to each well and left on an orbital shaker (150 RPM) at room temperature for 60 minutes. The incubation mixture was then emptied from the wells and the wells washed with a provided wash solution. This was repeated 6 times to ensure all non bound HRP conjugated antibodies were removed. 100μl of tetramethylbenzidine, a HRP substrate, was added to all wells and the plate was again incubated at room temperature on an orbital spinner at 150 RPM for 20 minutes. The reaction was terminated with a stop solution of HCL, turning the wells from blue to yellow. A Dynex revelation 4.25 plate reader was used to quantify the absorbance at 450nm.

Results

Baseline characteristics

The uraemic animals (SNx) had 4 times the serum creatinine and serum urea compared the non uraemic (sham) animals (see Table 14). The SNx groups were growth restricted ($P<0.0001$), anaemic ($p<0.0001$), hypoalbuminaemic ($p=0.004$), hypertensive ($p=0.03$), acidaemic ($p=0.02$) and hypercalcaemic ($p=0.009$). While the heart weights between the two groups did not differ significantly, the LV mass index (a surrogate marker for LVH) was higher in the uraemic groups ($p=0.005$) .

Cardiovascular Parameters

Over the course of the experiment, the blood pressure fell in all groups, as a result of anaesthesia, blood loss during thoracotomy, mechanical ventilation with impairment of cardiac output during ischaemia and cardiac stunning during reperfusion parameters (see Figure 44). There was a further, temporary fall in the blood pressure 5-10 minutes after the beginning of ischaemia and at reperfusion, which was caused by arrhythmias. Pulse rate did not change significantly during the experiment, with the exception of during arrhythmias. The rate pressure product fell, in line with blood pressure, during the experiment. There was no significant difference between the cardiovascular parameters of the 4 groups during the experiment.

Arrhythmias

In all groups, ischaemic arrhythmias developed around 5 minutes after the start of myocardial ischaemia (see Figure 45). The groups did not differ in terms of duration of ischaemic arrhythmias or duration of asystole during ischaemia. iPOST did not appear to delay the onset of reperfusion arrhythmias, which occurred 20 seconds after the beginning of reperfusion. There was no difference in the duration of reperfusion arrhythmias or duration of reperfusion associated asystole.

Troponin concentration

Baseline serum troponin was higher in the uraemic animals ($p=0.02$) compared to non-uraemic animals (see Figure 46). The baseline level of serum troponin was strongly

correlated (r=0.72) with underlying renal function. 25 minutes of ischaemia with 2h reperfusion resulted in a 2000 fold increase in troponin compared to baseline. There was no significant difference in serum troponin levels between uraemic and non uraemic animals following myocardial infarction. Troponin release was significantly correlated with infarct size (r=0.4755,p=0.0079)

Postconditioning was associated with a reduction in serum troponin, both in the uraemic and non uraemic groups. There was a 75% reduction in the median serum troponin level in the non uraemic group following iPOST (p=0.02) and in the uraemic group, there was a 69% reduction (p=0.04).

Infarct size

The median area at risk was similar between all groups; sham control (40.1%), sham iPOST (43.1%), SNx control (45.1%) and SNx iPOST (47.3%). In non uraemic animals, iPOST resulted in a significant reduction (P<0.01) in infarct size of 49% (median infarct size 61.4 and 31.2 in the control and iPOST groups respectively). In uraemic animals, iPOST caused a 69% reduction in infarct size (p<0.01), with median infarct sizes of 63.7% and 19.4% for the control and iPOST groups respectively. Comparing the infarct size of non uraemic and uraemic animals following iPOST revealed that uraemic animals had lower infarct sizes, a result which approached borderline statistical significance (p=0.051). To confirm that underlying uraemia had no impact on the ability of the heart to respond to a preconditioning stimulus, a 2 way ANOVA was performed, which confirmed that iPOST (in either uraemic or non uraemic models) led to a highly significant degree of tissue protection (p<0.0001), but that uraemia per se did not affect the result (p=0.29), with uraemia accounting for less than 2% of variability seen between the groups.

	Sham (n=17)	SNx (n=12)	p
Weight (g)	435 (24.7)	392 (20.9)	<0.0001
Initial MAP (mm/hg)	144 (14.6)	158 (22.0)	0.03
Initial pulse (BPM)	398 (28.3)	365 (70.4)	0.07
Haematocrit (%)	40.3 (4.21)	28.1 (6.41)	<0.0001
Plasma HCO3 (mmol/l)	28.3 (3.46)	25.3 (3.31)	0.02
Creatinne (umol/l)	33.4 (10.7)	143 (89.4)	<0.0001
Urea (mmol/l)	5.3 (1.13)	26.7 (22.7)	<0.0001
Albumin (g/l)	25.4 (1.88)	23.1 (2.31)	0.004
Phosphate (mmol/l)	1.97 (0.24)	1.88 (0.44)	0.45
Calcium (mmol/l)	2.39 (0.16)	2.55 (0.17)	0.009
Heart weight (g)	1.20 (0.15)	1.51 (0.73)	0.1
LV index	0.28 (0.03)	0.51 (0.67)	0.005

Table 14. Baseline characteristics, in uraemic (SNx) and non uraemic (sham) male Wistar rats. Values presented as mean (SD), p value calculated using unpaired t-test.

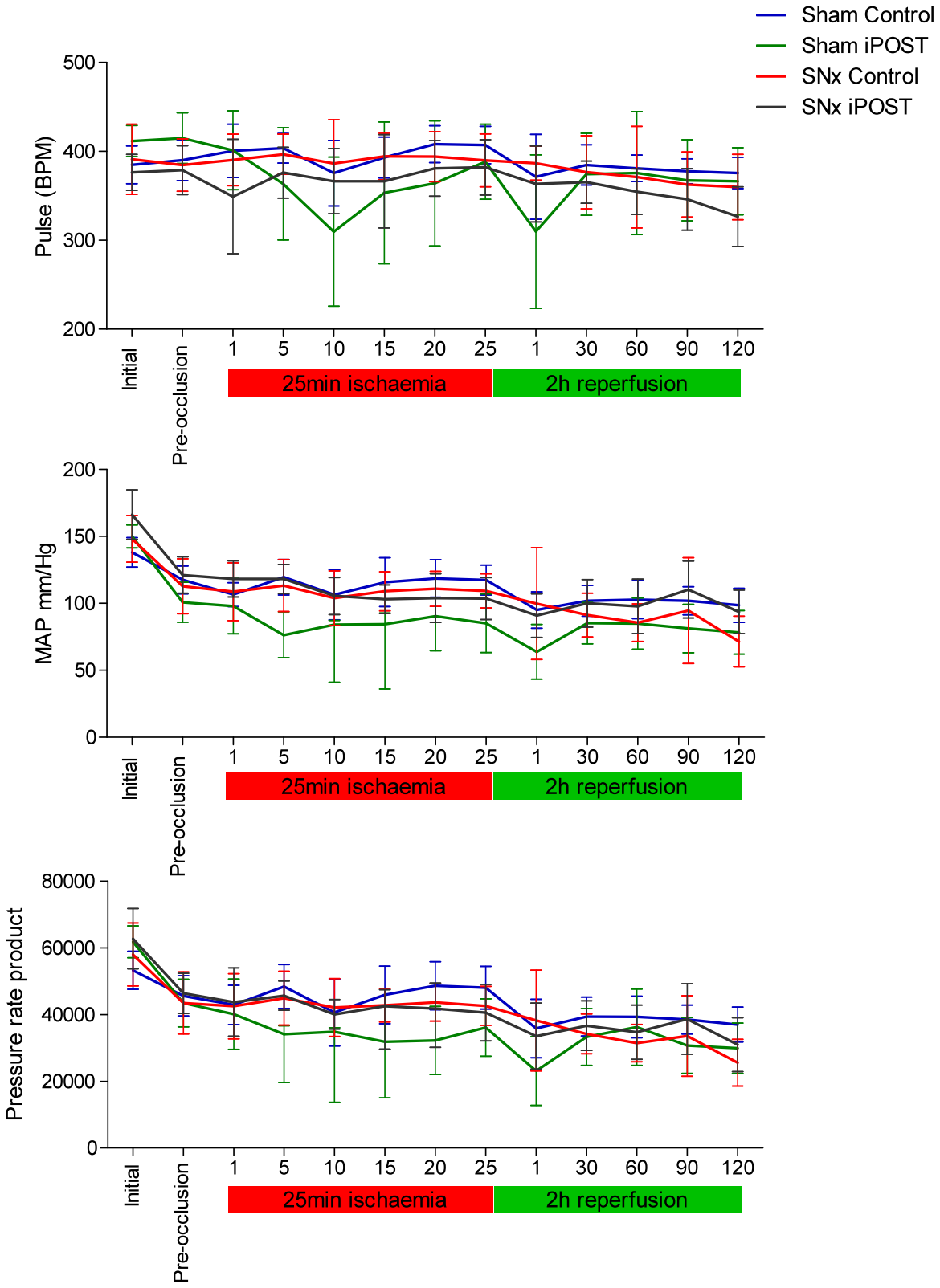


Figure 44. Figure showing the change in mean heart rate (top), blood pressure (middle) and pressure rate product (bottom) over time during the experiment in the 4 groups. Data points represent mean with error bars representing SEM.

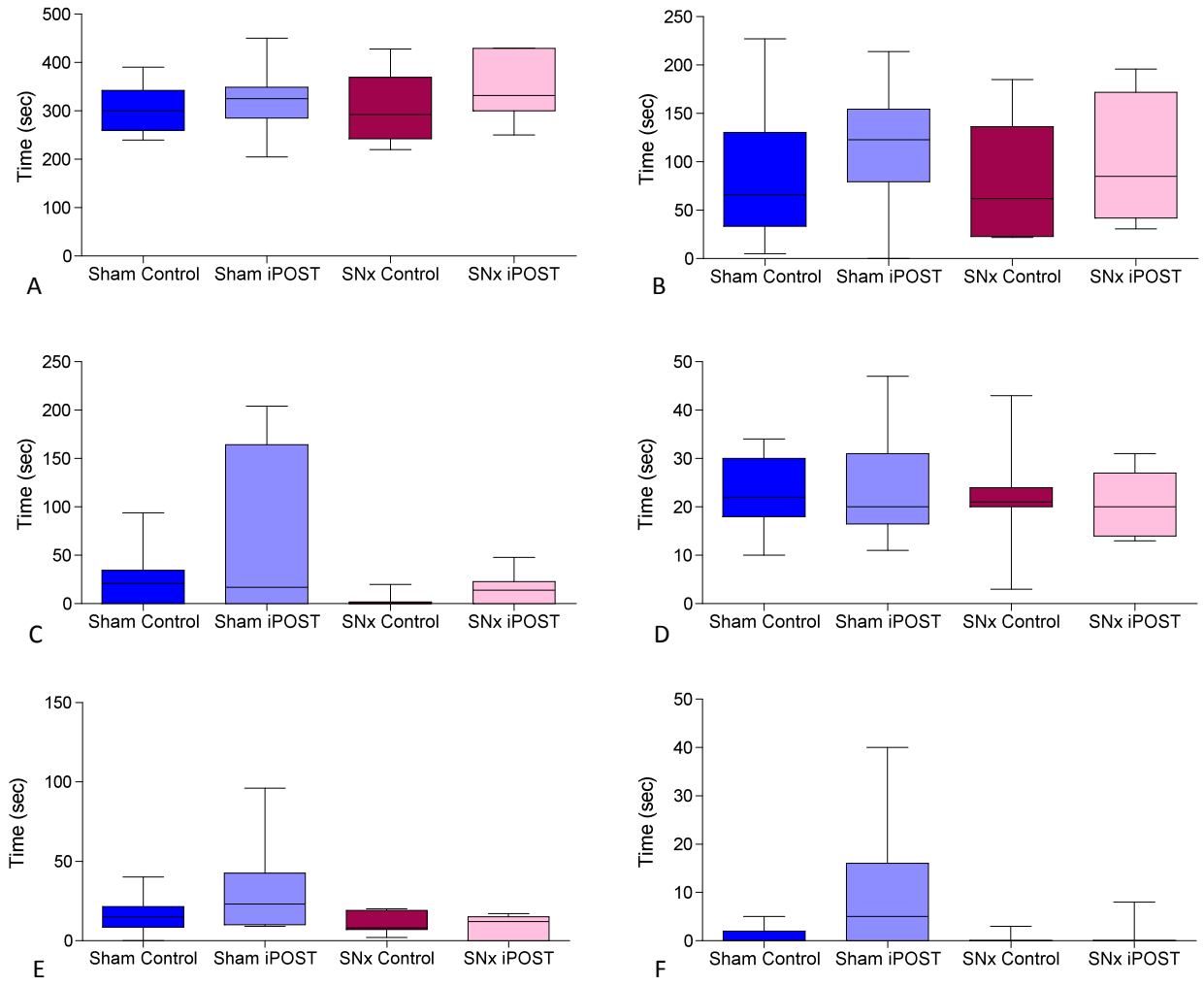


Figure 45. Figure showing the results of analysis of arrhythmias during myocardial ischaemia/reperfusion. Box and whisker graph with line (median), box (IQR) and whiskers (Max/Min). A: Time taken after the beginning of ischaemia to develop the first arrhythmia. B: Total duration of all arrhythmias during 25 minutes of ischaemia. C: Duration of asystole during 25 minutes of ischaemia. D: Time taken to develop 1st reperfusion arrhythmia. E: Total duration of reperfusion arrhythmias. F: Duration of reperfusion asystole. One way ANOVA testing (Kruskall-Wallis) revealed no significant variability in the medians between the 4 groups.

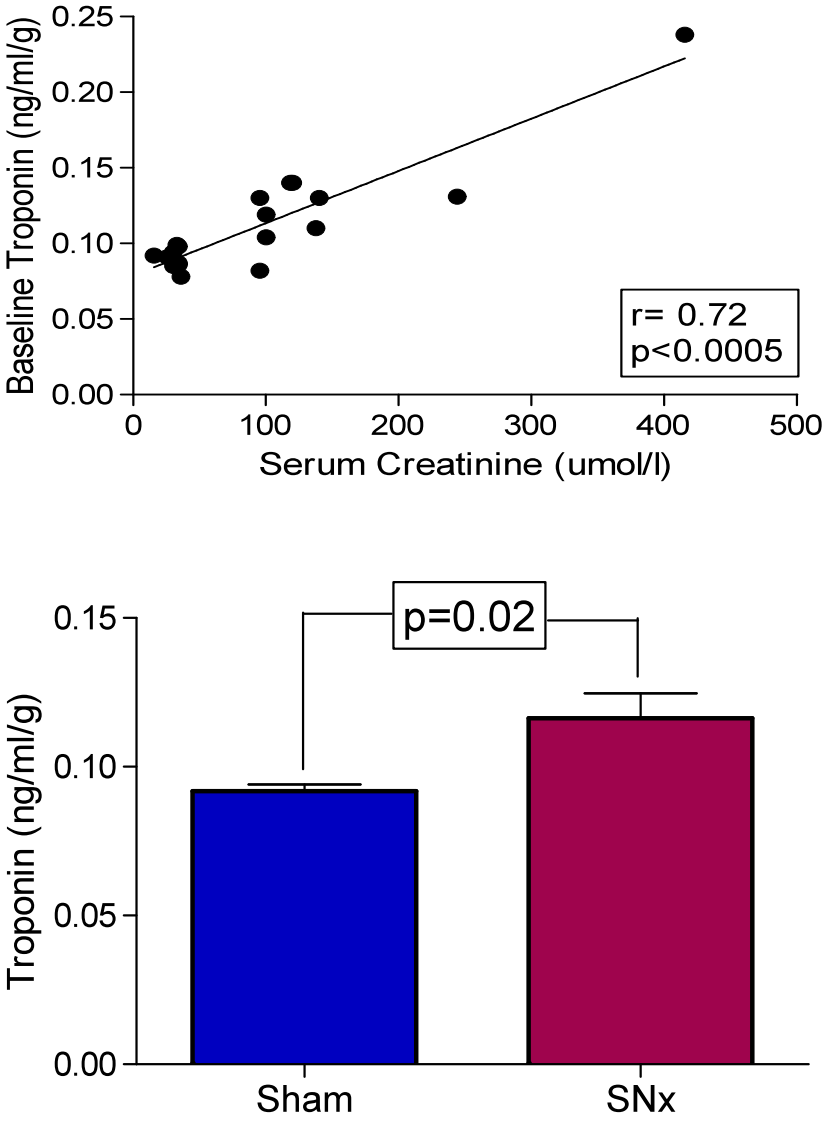


Figure 46. Results of baseline troponin quantification.

The top figure shows the correlation between baseline serum troponin levels and underlying renal function. R value is the Spearman's rank correlation coefficient, with p value to result of a student t-test. The bottom figure shows pre-ischaemia (baseline) troponin levels in non uraemic (sham) and uraemic (SNx) rats. p value results of MannWhitney test calculated using GraphPad software.

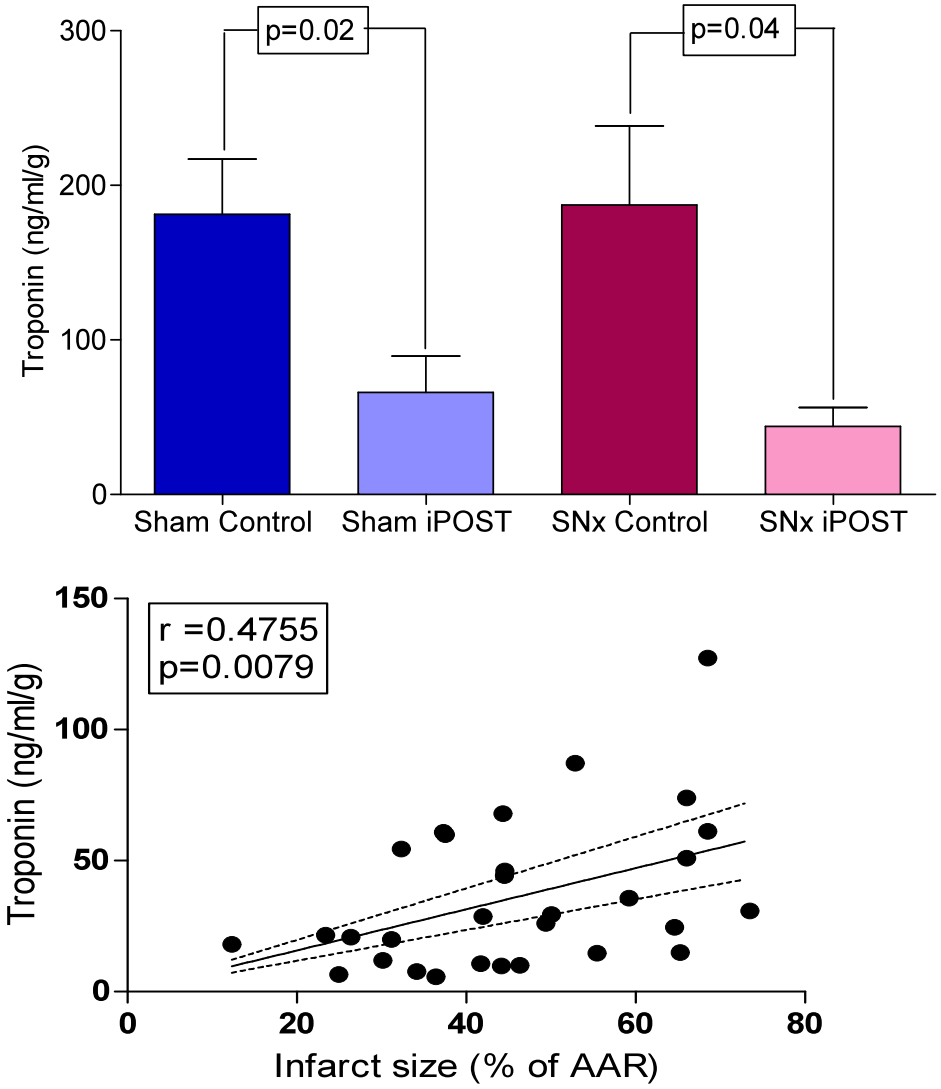


Figure 47. Results of serum troponin levels. Top figure shows the effect of iPOST on serum troponin levels following myocardial ischaemia in both non uraemic and uraemic animals, with a 1 way ANOVA (Kruskall-Wallis) statistic of $p<0.01$. The effect of iPOST on serum troponin levels was compared within the uraemic and non uraemic groups and displayed on the figure, as results of a Mann-Whitney test. Bottom figure showing correlation between infarct size as measured using NBT assay and serum troponin levels. r value is the Spearman's rank correlation coefficient, with p value to result of a student t-test.

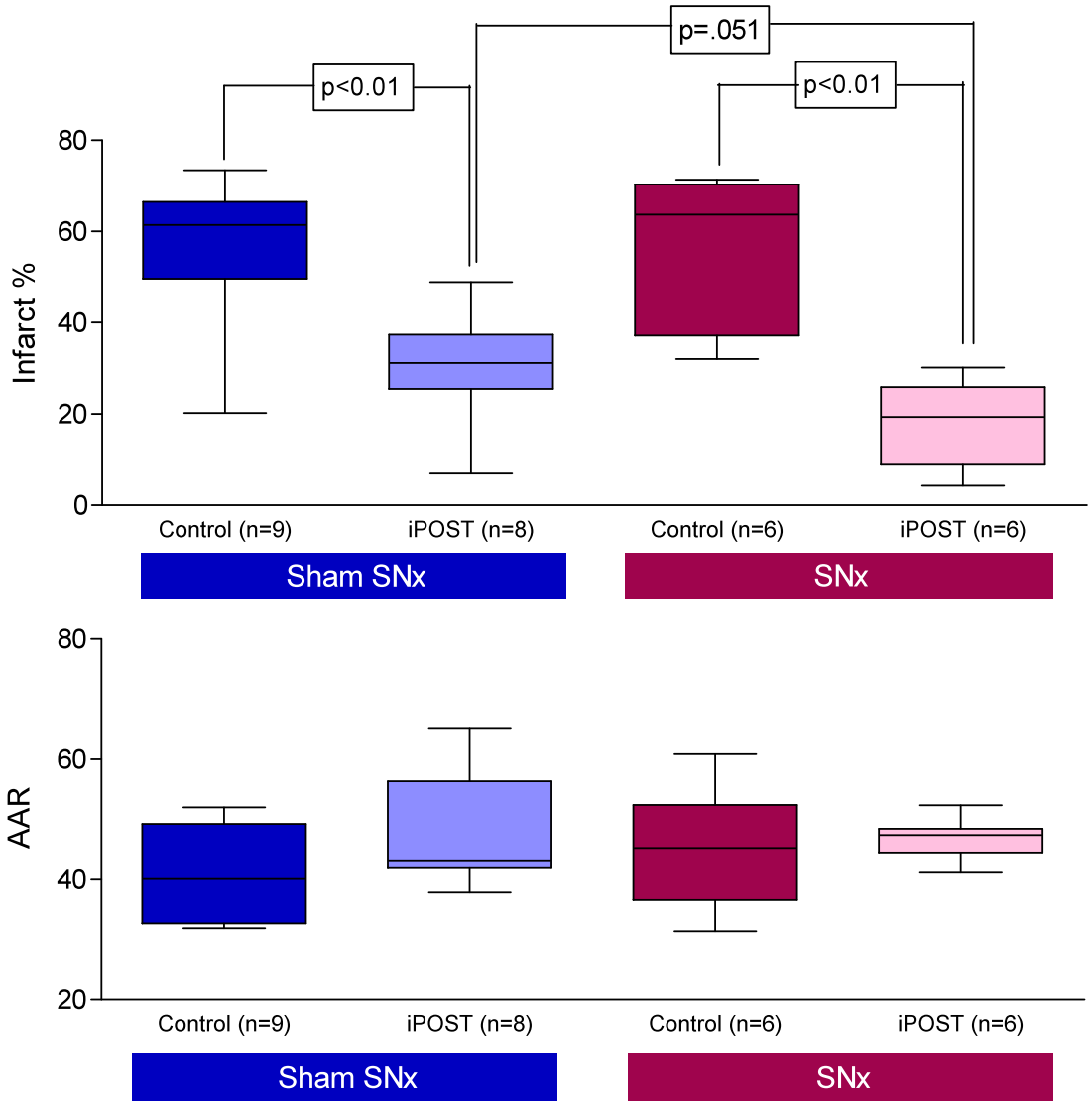


Figure 48. Box and whisker plot showing median (line within box), IQR (box) and whiskers (Max/Min values). Infarct size, expressed as a % of AAR (top) and area at risk (bottom) following reversible LAD ligation in the 4 groups. p values result from the Mann-Whitney test calculated using GraphPad software.

Discussion

This is the first time that the impact of underlying chronic uraemia on the ability to gain cytoprotection from iPOST has been studied. This study used the SNx model as the model of uraemia. The reason this was chosen over the adenine model is that the SNx model of uraemia is much more widely published, in addition we had already demonstrated that the SNx model had a reduced ischaemia tolerance, and therefore may more closely resemble the human cardiac phenotype.

This study confirms that unlike diabetes, senescence or obesity, underlying uraemia did not abolish the effects of postconditioning, as measured by infarct size and troponin release.

The use of serum troponin as a cardiac biomarker is useful, as unlike the method for assessing infarction using NBT to identify an intact LDH system, it allows for assessment of tissue injury without requiring the myocardium to be destroyed. This would enable additional measurements to be made on the heart, such as histology.

However, serum troponin estimation appears to have a greater degree of variability than infarct size estimation using the NBT method. The interquartile ranges expressed as a percentage of the median for the troponin data are around 4 times that of the infarct size data.

To help improve the correlation between troponin concentrations and infarct size, it is possible to perform repeated troponin measurements over time to generate a graph, the integral of which may be a better marker of infarct size than a single troponin estimation.

In this experiment there was no significant difference in the infarct size of the sham SNx control group and the SNx control group. This is in contrast to previous work which shows that uraemia leads to a reduced ischaemia tolerance. The reason for this is that this experiment used much smaller groups than in the experiment specifically investigating the role of uraemia in ischaemia tolerance. Smaller groups were used in this experiment because the expected effect size of iPOST was much greater than the effect size of underlying uraemia. This meant that this experiment was under powered to detect a difference in the ischaemia tolerance of uraemia per se.

The results of this work suggest that uraemic animals may derive even greater benefit from postconditioning than non uraemic animals. The uraemic iPOST group had a lower infarct than the non uraemic iPOST group ($p=0.051$). This effect has also been

seen with preconditioning in SNx animals by me (see Table 10). A possible explanation for this is that uraemic animals are significantly more anaemic than their non uraemic counterparts. Direct manipulation of the vessel, during pre or postconditioning with interruption of flow down stream, is likely to cause microvascular occlusion and no reflow injury. Because the uraemic animals have a lower haematocrit, they may be less likely to generate as much microvascular thrombus, leading to less no reflow injury. Furthermore, iPOST is not a totally benign procedure. If the ischaemia time is too low, iPOST may actually increase infarct size[493]. It could be that this is specific to short ischaemic times or it could be that the deleterious effects of iPOST are only uncovered when there is a small index ischaemia injury, and hence little for iPOST to salvage in its traditional role.

Arrhythmias

iPOST has been shown to inhibit reperfusion arrhythmias in dogs[550] and rats[501], although the mechanism for this effect is unknown. However, it does not appear to be mediated via the MPTP, PI3-Kinase, K_{ATP} or adenosine pathways[575]. The results from this work do not support this. However the variability in the timing, duration and severity of the arrhythmias may have overshadowed a small difference.

Future directions

1) This work suggests that uraemia is not an absolute barrier to postconditioning. However, it is still unknown whether uraemia results in a relative resistance to iPOST. Future work should address this issue by reducing the number of postconditioning cycles to the minimum number, which confers protection in the non uraemic animals, and then repeat that protocol in uraemic animals to identify whether underlying uraemia causes any resistance to iPOST.
2) Remote ischaemic post conditioning has been shown to be beneficial in rats[380], however the role of underlying CKD has not been studied.
3) These results suggest that there may perhaps be some additional benefit, or perhaps loss of harm, when the response to iPOST is compared in uraemic and non uraemic animals. This finding needs further testing to confirm its presence. However, a

possible explanation for this could be that the lower haematocrit in the uraemic animals leads to less no reflow injury following iPOST. The hypothesis that anaemia in isolation results in less injury is difficult to test, since venesection to render a rat anaemic will lead to up regulation of erythropoietin which is known to be tissue protective [576]. Rendering an animal chronically anaemic will also lead to LVH, which may alter the hearts response to ischaemia.

One solution to identify whether a low haematocrit results in reduced microvascular occlusion during the postconditioning protocol would be to harvest the heart for histology and quantify microthrombi in both non uraemic and uraemic animals following myocardial infarction with and without iPOST.

Additionally, the adenine model of CKD could be used to assess this effect because in earlier work in IPC, adenine animals did not appear to derive greater protection from preconditioning than non uraemic controls.

4) This novel finding that uraemic animals, unlike diabetic or dislipidaemic animals, can respond to postconditioning is important when designing future clinical trials in postconditioning and this work suggests that patients with underlying CKD should not be excluded from clinical trials in postconditioning.

5) Additional work into cardiac biomarkers other than troponin for evaluation of tissue injury in the context of myocardial infarction would help to identify whether these would provide a more powerful surrogate marker for tissue damage in the rat than troponin. Rat serum should be analysed for myosin light chain and fatty acid binding protein and the results compared with troponin

6) In this current work, only SNx animals were studied. Future work could compare the effects of different models of underlying uraemia on the ability of the uraemic heart to respond to a preconditioning signal.

Chapter 8

Evaluation of the effect of AZ12785452: a PARP-1 Inhibitor in a SNx Rodent Model of Myocardial Infarction

Background

Poly (ADP-ribose) polymerase 1 (PARP-1) is the most abundant isoform of the PARP family[577]. PARP-1 is a DNA damage sensor and signalling molecule. It binds to damaged DNA and catalyzes the cleavage of NAD^+ into nicotinamide and ADP-ribose to form long branches of ADP-ribose polymers on glutamic acid residues of a number of target proteins including histones and PARP-1 itself [578].

Until recently it was thought that PARP activation was primarily triggered by recognition of DNA breaks[577, 579]. However, studies have provided evidence that PARP-1 activity can be modulated by other factors such as vitamin D[580], caffeine[581] as well as calcium, magnesium, histones and ATP[582]. Furthermore, the degree to which PARP is activated by DNA breakage may be regulated by other factors such as ERK1/2[583].

All cells exposed to DNA damaging agents can enter three major pathways based on the intensity of the trigger[579]. Moderate injury causes PARP-1 activation leading to DNA repair by signalling cell cycle arrest and by interacting with DNA repair enzymes. This allows cells to survive without passing on mutated genes. More severe DNA damage triggers an apoptotic cell death pathway during which caspases inactivate PARP-1 by cleaving it into two fragments, preventing the loss of cellular ATP, which is required for the process of apoptosis. Very extensive DNA damage causes an over activation of PARP. Activated PARP rapidly depletes the cell of NAD^+, eventually resulting in ATP depletion and death by necrosis. Thus PARP activation, in the face of different severities of insult, can act to repair the cell, send the cell on an apoptotic pathway or through over activation, cause necrotic cell death. Pharmacological inhibition of PARP could switch cells from a necrotic to an apoptotic death or if the injury was only moderate, prevent apoptosis occurring[578]. PARP-1 also facilitates the action of apoptosis inducing factor (AIF). AIF is translocated from the mitochondria to the nucleus and induces DNA fragmentation[584].

PARP is also thought to increase expression of proteins involved in inflammation. The absence of functional PARP-1 (either genetic or pharmacological) leads to a reduction in pro-inflammatory mediators, including cytokines, chemokines, adhesion molecules, and enzymes (e.g., iNOS)[585]. PARP is also involved in the JNK, p38 MAP-kinases and STAT-1 signalling cascades[578].

PARP-1 inhibitors in myocardial ischaemia reperfusion injury

Reperfusion injury is associated with a surge of reactive oxygen species and reactive nitrogen species[586] along with local activation of leucocytes leading to further inflammation and ROS generation to form a so-called 'respiratory burst'[587]. The burst of reactive oxygen and nitrogen leads to coronary endothelial dysfunction, myocardial stunning, cellular calcium overload and cell death[588]. In the rat, inhibition of PARP in the context of myocardial ischaemia/reperfusion has been shown to be cytoprotective in several studies[589-591].

Animal data

The cytoprotective effect of PARP inhibition in the context of AMI has been studied in several species: mouse[592], rabbit[593], pig[594] and rat (discussed below). A study Zhao et al.[595] used 3-aminobenzamide (3-AB) as a PARP inhibitor in a LAD ligation model in a rodent. They found that the inhibitor reduced PARP activation and reduced AIF expression. These results are interesting in 2 ways; firstly they used a permanent ligation model, so that there was no reperfusion phase, which is the phase thought to trigger PARP activity. Furthermore, the PARP inhibitor was given IP, *after* occlusion and it is unclear how the drug acted on the ischaemic myocardium, since by definition, there was no blood supply to that area. However, Bowes et al.[589] reported a significant reduction in infarct size, in an ex vivo rodent model of reversible LAD occlusion using the same drug as an inhibitor of PARP. Liaudet et al.[591] demonstrated in a rat model of reversible myocardial ischaemia, PARP activation in the reperfused myocardium, which persisted for 23h. The PARP activity was reduced by 3-AB, which lead to a significantly lower infarct size. Wayman et al.[596] demonstrated that 5-aminoisoquinolinone inhibited PARP activity in vitro and in vivo in a rat model of reversible LAD occlusion. They also found that administration of 5-aminoisoquinolinone 1 minute before reperfusion resulted in a reduction of infarct size of 50% when compared to control animals.

Human studies

Toth-Zsamboki et al.[597] analysed serum, plasma and leucocyte samples from patients presenting with a STEMI, and followed their progress through angioplasty to recovery.

They reported that following successful angioplasty (reperfusion) there was a rapid increase in reactive oxygen/reactive nitrogen species, oxidative DNA damage, activation of PARP-1 and AIF translocation from the mitochondria to the nucleus. These observations support the theory that ischaemia/reperfusion injury in acute myocardial infarction is capable of activating PARP-1.

Morrow et al.[598] reported the first human clinical trial on PARP inhibitors in the context of myocardial infarction. The study was a phase II randomised single blind multicentre placebo-controlled trial in patients with STEMI undergoing primary PCI, using the drug INO-1001 as a PARP inhibitor. This was a small (n=40) trial with only a short follow up (30 days). Importantly, renal disease (defined as a serum creatinine of >2mg/dl) was an exclusion criteria. The group found that INO-1001 was safe and efficacious at inhibiting PARP in an ex vivo experiment. There was also a trend towards reduced inflammatory response in the form of lower CRP and IL-6 levels.

The role of PARP in preconditioning

Liaudet et al.[599] reported that PARP-1 deficient mice had smaller infarcts following LAD ligation than wild type animals. They showed that an ischaemic conditioning protocol did not confer cardioprotection in the PARP-1 deficient mice, unlike wild type and even appeared to be deleterious with a 60% *increase* in infarct size seen following preconditioning in the PARP-1 deficient mice.

These findings were replicated using 3-AB as a pharmacological inhibitor of PARP-1 in rats with similar results. In addition they found that both 3-AB and IPC reduced PARP activation in the myocardium of rats that underwent myocardial infarction. However when 3-AB was combined with IPC, no inhibition of PARP activity was seen. IPC alone was associated with generation of reactive nitrogen species and a small elevation in PARP activity. The authors speculated that the small elevation in PARP after IPC might be involved in the mechanism of IPC.

There are several hypothesises which can be used to explain the effects of IPC on PARP. Liaudet et al.[599] hypothesise that IPC triggers low level PARP activity which through auto-ribosylation, leads to auto-inhibition. Evidence in support of this hypothesis comes from their data showing that 3-AB given before IPC nullifies the cytoprotection of IPC. This hypothesis also explains the finding that IPC alone activates PARP and that 3-AB given after IPC does not reduce the cytoprotective effects of IPC.

Another hypothesis to explain the effect of IPC on PARP is that purines formed during the myocardial ischaemia of IPC, such as inosine and hypoxantine, are present at concentrations which can inhibit PARP directly[600]. Garnier et al.[601] reported in an in vitro model of cerebral ischaemia, that 'chemical ischaemia', a model of IPC, led to PARP-1 cleavage with resultant cytoprotection. The authors report that IPC triggers caspase activation which in-turn cleaves PARP-1 into an inactive form, rendering the cells resistant to subsequent injury.

Exclusion of CKD patients from clinical trials

The sole clinical trial using PARP inhibitors in the setting of cardiovascular disease excluded patients with underlying renal dysfunction[598]. Clinically, PARP inhibition has been studied more widely in the field of cancer and there have been several trials published. In these trials patients were either excluded unless they had 'adequate renal function'[602-604], of if their GFR <50%[605]. In others renal function data was not reported[603].

PARP inhibition and CKD

Swierczynski's group have published several papers on N-methyl-2-pyridone-5-carboxamide (2PY). 2PY is one of the end products of NAD degradation and is normally excreted in the urine. In health, the serum concentration of 2PY is low, however in uraemic patients, the serum level of 2PY is elevated by as much as 20 fold[606, 607]. The concentration of 2PY is found to negatively correlate with GFR. Another potential reason for elevated levels of 2PY in uraemia is through excessive metabolism of NAD[608]. The authors suggest that increased oxidative stress associated with renal failure leads to greater PARP activation metabolising more NAD into nicotinamide and subsequently to 2PY. The group also found increased levels of 2PY in the serum and tissues in rats that had been rendered uraemic by a SNx procedure[609].
Interestingly the group found that 2PY was able in inhibit PARP-1 in an in vitro assay in a dose dependant manner[607]. Furthermore, when 2PY was added to endothelial cells treated with peroxynitrite to simulate oxidative stress, there was a reduced depletion of NAD and ATP when compared to controls, indicative of PARP inhibition[610].

Aims

The role of underlying CKD in the outcome of PARP inhibition in the context of myocardial ischaemia reperfusion is unknown. This experiment sought to investigate the role of PARP inhibition as a pharmacological conditioning agent in the context of uraemia.

Methods

Six week old male Wistar Rats (Charles Rivers UK) were obtained and after 1 week acclimatisation in an animal house with a 12 hour day night cycle and free access to water and standard chow. All animals then underwent a 2 stage SNx procedure (see methods section for details on subtotal nephrectomy procedure page 52). Four weeks after the second stage procedure, they were anaesthetised, had venous and arterial lines inserted and had a tracheostomy performed and were placed on a ventilator (see methods section for full details of basic surgery and ventilation page 76). The animals then underwent a reversible LAD ligation procedure (see page 83 for full details of reversible LAD ligation technique). The duration of ischaemia was 30 minutes with a 2-hour reperfusion period.

5 minutes before the end of the 30 minutes ischaemia, AZ12785452 (Astra Zeneca UK), a PARP inhibitor or vehicle (10% DMSO) was infused. AZ12785452 was made up in 10% DMSO at a concentration of 1mg/ml. The infusion was initially at a rate of 1ml/kg/min for 1 minute followed by 0.12ml/kg/min for the remainder of the experiment (124 minutes). At the end of the experiment the infarct size and area at risk were measured as before.

Quantification of serum troponin

For method of measurement of serum troponin (see methods section page 193)

Preparation of peripheral blood mononuclear cell (PBMC) sample

At the end of the reperfusion period 1 ml of non-heparinised blood was drawn via the arterial line into an EDTA tube (max 5mM final concentration). One ml of blood/EDTA was then transferred from the EDTA tube to a 1.5 ml Eppendorf tube and mixed, by repeated inversion, with 0.25 ml of solution D (1.6 volumes of OptiPrep™ (Sigma) to 1 volume of solution B (0.85% {w/v} NaCl, 30 mM Tricine NaOH, pH 7.4)). A volume of 100 µL of Solution C (0.85% (w/v) NaCl, 10 mM Tricine NaOH, pH7.4) was layered on top and the Eppendorf tube was spun at 1300g for 30 min at 20^{O}C. Approximately 600µL of PBMCs were collected from the semi clear overphase and transferred to a new 2ml Eppendorf tube. The collected material was washed by diluting with 2 volumes of cold phosphate buffered saline and spun at 500g for 10 min

at 4°C. The supernatant was poured off and the PBMC cell pellet was frozen and stored at -80°C. The samples were sent to AstraZeneca for quantification of PARP activation.

Histological evaluation

12 animals, 6 in each group, had hearts harvested for histology. At the end of the 2 hour reperfusion period, the hearts were quickly excised and placed in ice-cold saline for 2 minutes. The organs were then immersed in 10ml of Formalin (Sigma) for storage. The samples were sent to AstraZeneca R+D for histological analysis and quantification of PARP activation.

The hearts were sectioned and stained with haematoxylin and eosin, and underwent immunohistochemistry for PAR (the product of PARP activation). The antibody used was mouse anti rat PAR at 1.25ug/ml (Abcam Cambridge UK). The sections were also stained for with an iostype specific control antibody, mouse IgG3 (Abcam Cambridge UK). The histology sections were analysed by an independent histopathologist in a blinded fashion.

Analysis of PARP activity in PBMC pellets

PBMC pellets were lysed in extraction buffer. Extraction buffer (7ml) was prepared using 6.694ml of PBS, 1 mini-complete protease inhibitor tablet (Roche), 70µl of 1% igepal CA-630 (Sigma), 1.4ul of 200µM DTT (Sigma), 100µl of PI cocktail 1 (Sigma) and 100µl of PI cocktail 2 (Sigma). The cell lysate (5µg of protein) was then incubated in PCR 8-strip tubes (VWR 3925-550-000), in a cooling block with 2µl of reaction buffer (250µl of 1M Tris, 1µl 1M DTT, 20µl of 1M magnesium chloride, 729µl MilliQ water) and 2µl of 250µM PARG inhibitor (Calbiochem 118415), the main enzyme responsible for degrading PARP. MilliQ water was added to ensure a final volume of 8µl/sample.

1µl of 1mM NAD (Sigma) solution was mixed with 1µl of DNA stock solution. DNA stock solution was made using 2 oligonucleotides which have been shown to activate PARP[611]. The DNA stock solution was made by adding equal amounts of the oliconucleotides to a buffer (100mM NaCl, 10mM Tris pH=7.4, 1mM EDTA pH=8)

and then diluted to a final concentration of 100ng/µl. The solution was then boiled and left to cool at room temperature.

The NAD/DNA mix was added into the lids of the PCR strip, the lid was closed and the strip spun down to start the reaction. The strip was then transferred to a pre-cooled PCR block, which was heated up to 30^{O}C for 5 minutes and then cooled to 4^{O}C ready for use. 150µl of ELISA buffer (PBS with 2% BSA) was added and mixed.

A commercial ELISA kit (HT PARP in vivo Pharmacodynamic Assay II, Trevigen cat# 4520-096-K) was then used to quantify PARP. Serial dilutions from a supplied PAR standard were performed to generate PAR standards. 50µl of the standards were added in triplicate to the first 3 rows of the plate. Then 50µl of samples and 50µl of Jurkat Cell lysate standards (as a positive control, and to monitor assay drift between experiments) were added to the plate in triplicate. The plate was covered with sealing film and left overnight to equilibrate. The plate was then washed 4 times with PBST solution and 50µl of diluted 'PAR polyclonal detecting antibody' was added to each well and the plate was incubated for 2h. After incubation, the plate was washed 4 times in PBST. 50µl of diluted goat anti-rabbit IgG-HRP conjugate was then added to each well and the plate was incubated at room temperature for 60 minutes. After this incubation the plate was washed a final time. 'PeroxyGlow' A+B solutions (supplied with the kit) were mixed and 100µl of this solution was added to each well and the chemiluminescent readings were taken immediately.

Results

Baseline characteristics

The groups did not differ in terms of weight, serum creatinine, serum urea, serum phosphate, serum calcium, serum albumin, haematocrit and plasma bicarbonate (see Figure 51 and Table 15). There was no difference in initial blood pressure or heart rate between the 2 groups. Both groups were similar in terms of left ventricular weight and LV weight expressed as a percentage of body weight, which is a surrogate marker for LVH.

Infarct size and troponin estimation

There was no difference in tissue injury as measured by infarct size or troponin quantification between the 2 groups (see Table 15 and Figure 49). Infarct size was 52.0% and 53.3% for the vehicle and AZ12785452 treated groups respectively. While the AAR was not significantly different, there was a trend towards a larger AAR in the AZ12785452 treated group (48.0 v 53.01, p=0.07). Serum troponin concentration corrected for weight of infarcted tissue was similar between the 2 groups, with means of 437 ng/ml/g and 402 ng/ml/g in the vehicle and AZ12785452 groups respectively.

Cardiovascular parameters

There were no significant differences between the MAP, heart rate or pressure rate product between the 2 groups (see Figure 50).

Histology

The histological changes associated with AMI reperfusion injury in both vehicle and AZ12785452 treated animals were analysed (see Image 6). There was evidence of neutrophil infiltration, oedema accumulation, fibrin accumulation, myocyte necrosis and haemorrhage. The samples were scored qualitatively on 5 aspects of myocardial injury (see Table 16). All samples had evidence of all variables.

Samples were also stained using immunohistochemistry for the presence of PAR (see Image 7). Neutrophils, both intravascular and intermyocyte, were seen to stain strongly for PAR in both groups. There was a light staining of myocytes in areas of infarction/inflammation but this was also seen in the negative isotype control

indicating non-specific binding. Areas of leak, oedema and intravascular thrombi/red blood cells showed some light staining but again, this was seen in both the positive and the negative sections. There was no difference in PAR staining between the vehicle and AZ12785452 treated groups.

Analysis of PARP activity in PBMC sample

PAR is the product of the PARP enzyme, and its concentration in this assay is proportional to underlying PARP activity. In this experiment (see Figure 49 A), there was a trend in reduction in PAR concentration in the AZ12785452 treated animals compared to vehicle treated animals (mean 2.85 v 2.03 pg/ml), but this did not reach statistical significance (p=0.33).

	Vehicle (n=13)	AZ12785452 (n=16)	P value
Weight (g)	415.2 (24.4)	406.4 (24.5)	0.35
MAP (mm/hg)	151.6 (22.2)	155.5 (19.0)	0.62
Pulse (BPM)	398.5 (34.6)	422.4(48.5)	0.16
Hamatocrit (%)	31.77 (3.24)	32.25 (2.67)	0.65
Bicarbonate (mmol/l)	28.47 (4.08)	27.42 (3.00)	0.44
Creatinine (µmol/l)	86.86 (18.18)	82.61 (16.93)	0.54
Urea (mmol/l)	17.88 (3.50)	17.68 (4.33)	0.9
Albumin (g/l)	25.1 (1.38)	24.91 (2.01)	0.78
Calcium (mmol/l)	2.6 (0.11)	2.61 (0.145)	0.84
Phosphate (mmol/l)	1.84 (.018)	1.80 (0.23)	0.57
LV weight (g)	1.45 (0.20)	1.40 (0.15)	0.17
LV weight/body weight	0.359 (0.05)	0.345 (.043)	0.41
Infarct size (%)	52 (17.5)	53.3 (22.2)	0.86
Area at risk (%)	48 (10.51)	56.01 (12.07)	0.071

Table 15. Results of reversible LAD ligation a SNx rodent model. All values given as mean (SD). All samples normally distributed as confirmed by the Shapiro-Wilk normality test and the D'Agostino & Pearson omnibus normality test. p value presented as results of unpaired 2 tailed T test using GraphPad Prism 5 software.

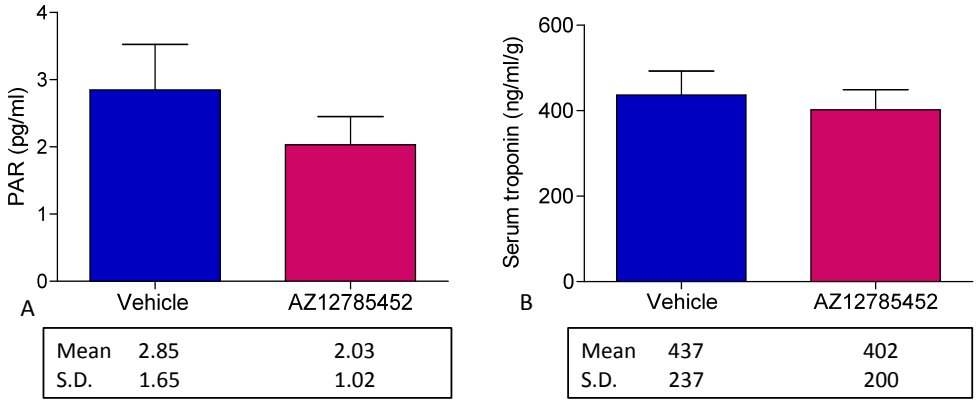


Figure 49. A: results of PAR ELISA, corrected for protein loading. B results of Troponin ELISA corrected for weight if infarcted tissue.

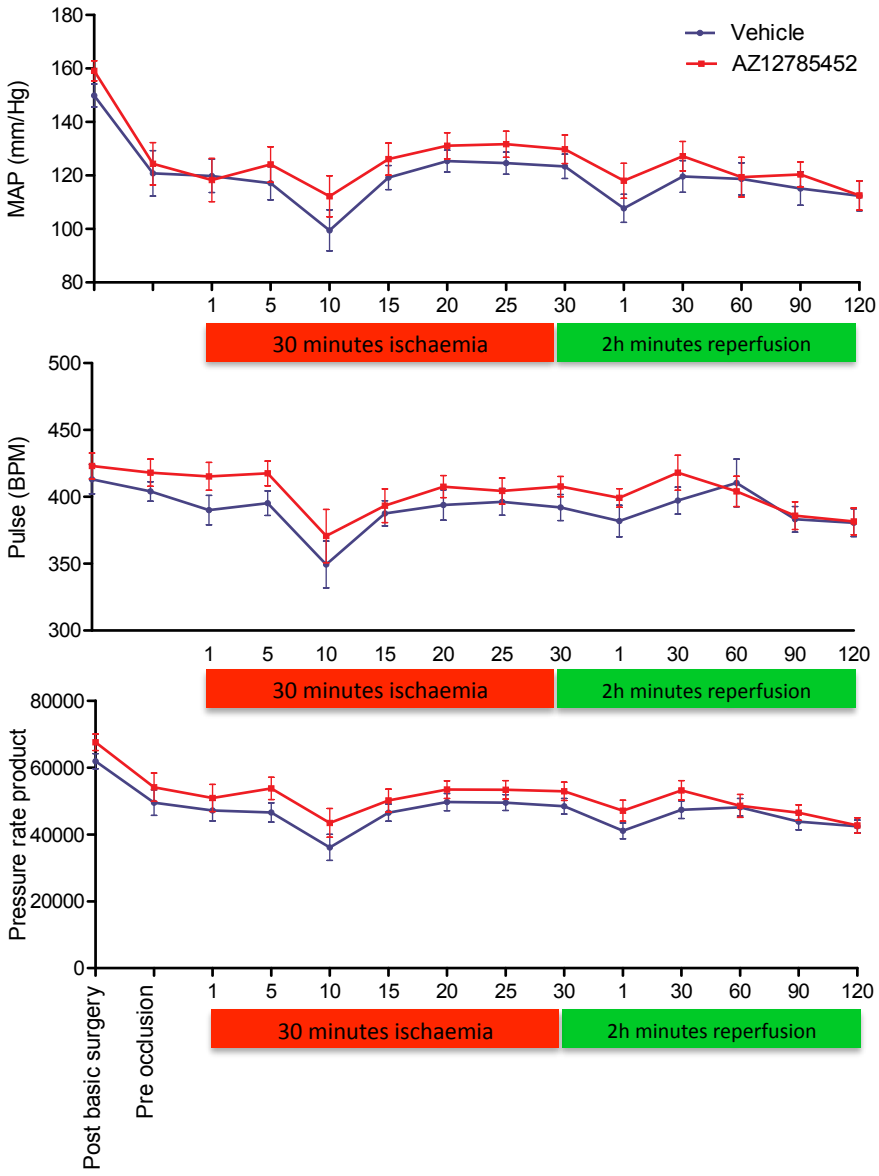


Figure 50. Graphs showing differences in MAP (top), heart rate (middle) and pressure rate product (bottom) over time between the vehicle treated group and the AZ12785452 group. Graph shows mean with SEM as error bars.

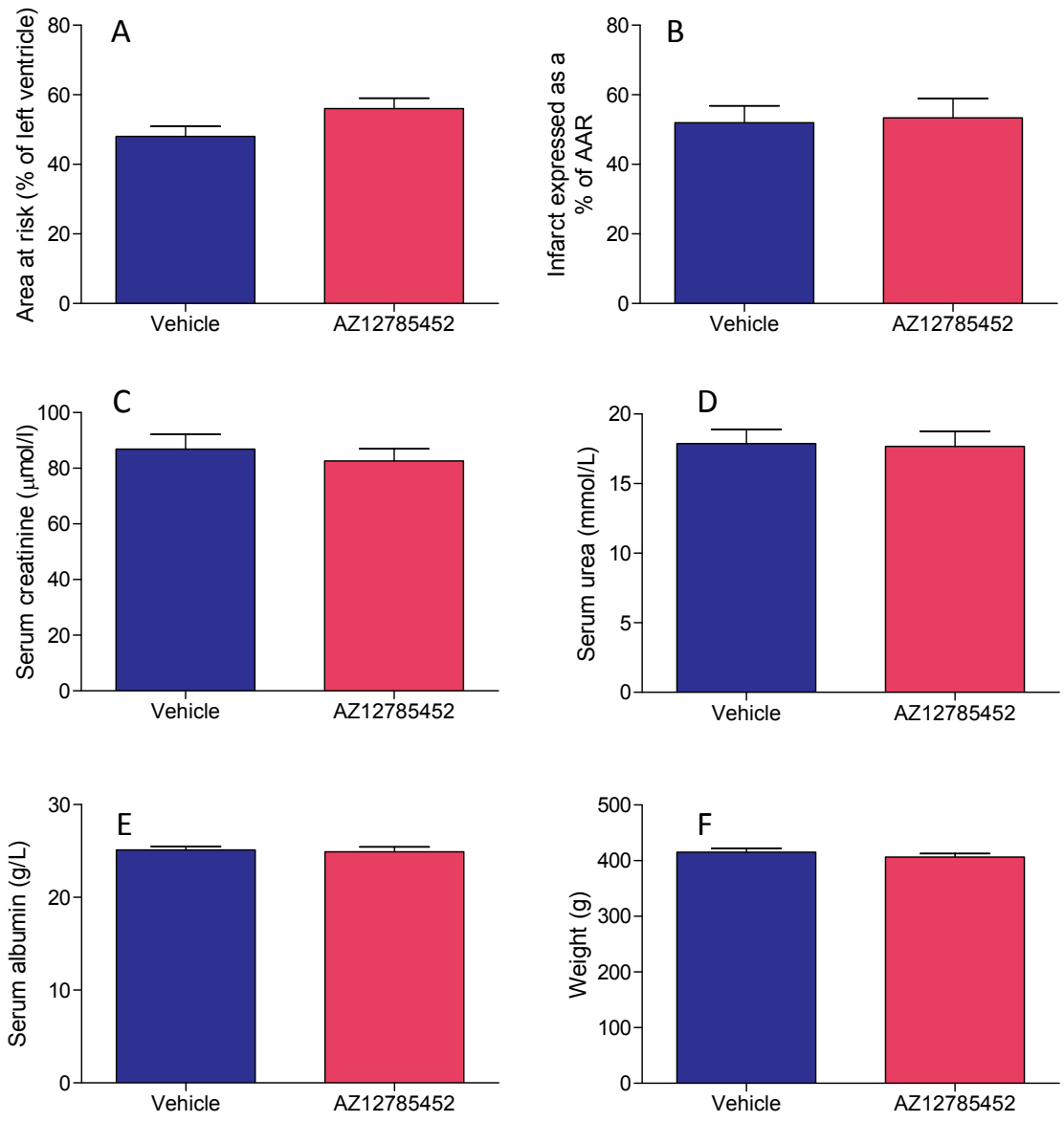


Figure 51. Results following reversible LAD ligation in a uraemic rodent model of acute myocardial infarction. Rats were either given AZ12758452 (n=13) or vehicle (10% DMSO) (n=16).
Bar graphs showing mean with SEM as error bar.
A: The AAR at risk is similar between the 2 groups. B: Infarct size is similar between the 2 groups. C: Serum creatinine measured before myocardial ischaemia is size is similar between the 2 groups. D: Serum urea measured before myocardial ischaemia is size is similar between the 2 groups. E: Serum albumin measured before myocardial ischaemia is size is similar between the 2 groups.
F: There is no difference in weight between the 2 groups.

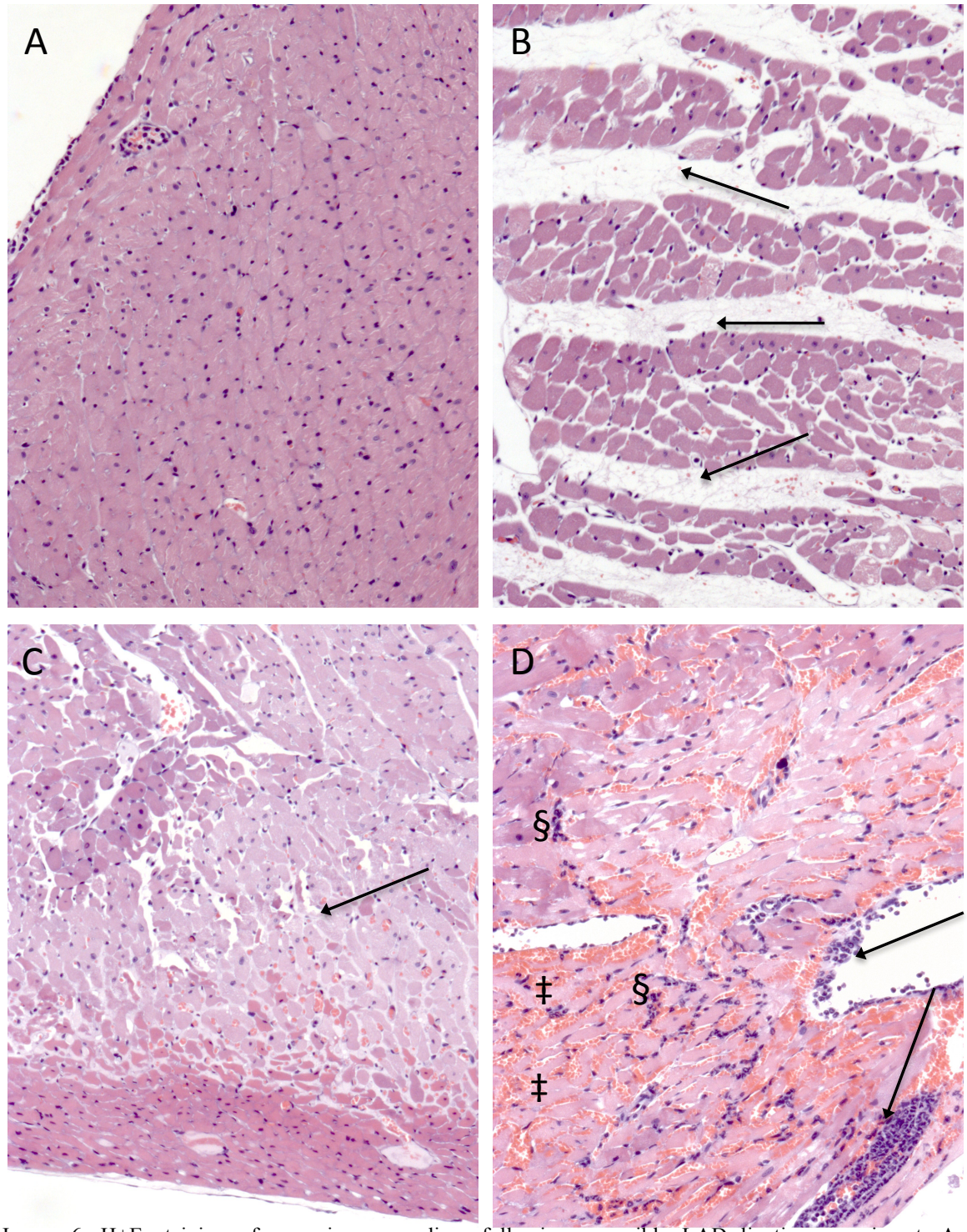


Image 6. H+E staining of uraemic myocardium following reversible LAD ligation experiment. A: Pericardial neutrophilia in right ventricular wall. B: → indicate areas of oedema and fibrin accumulation. C: → indicates areas of myocyte necrosis. D: → indicate neutrophils in blood vessels, § indicate intermyocyte neutrophils, ‡ indicate areas of haemorrhage.

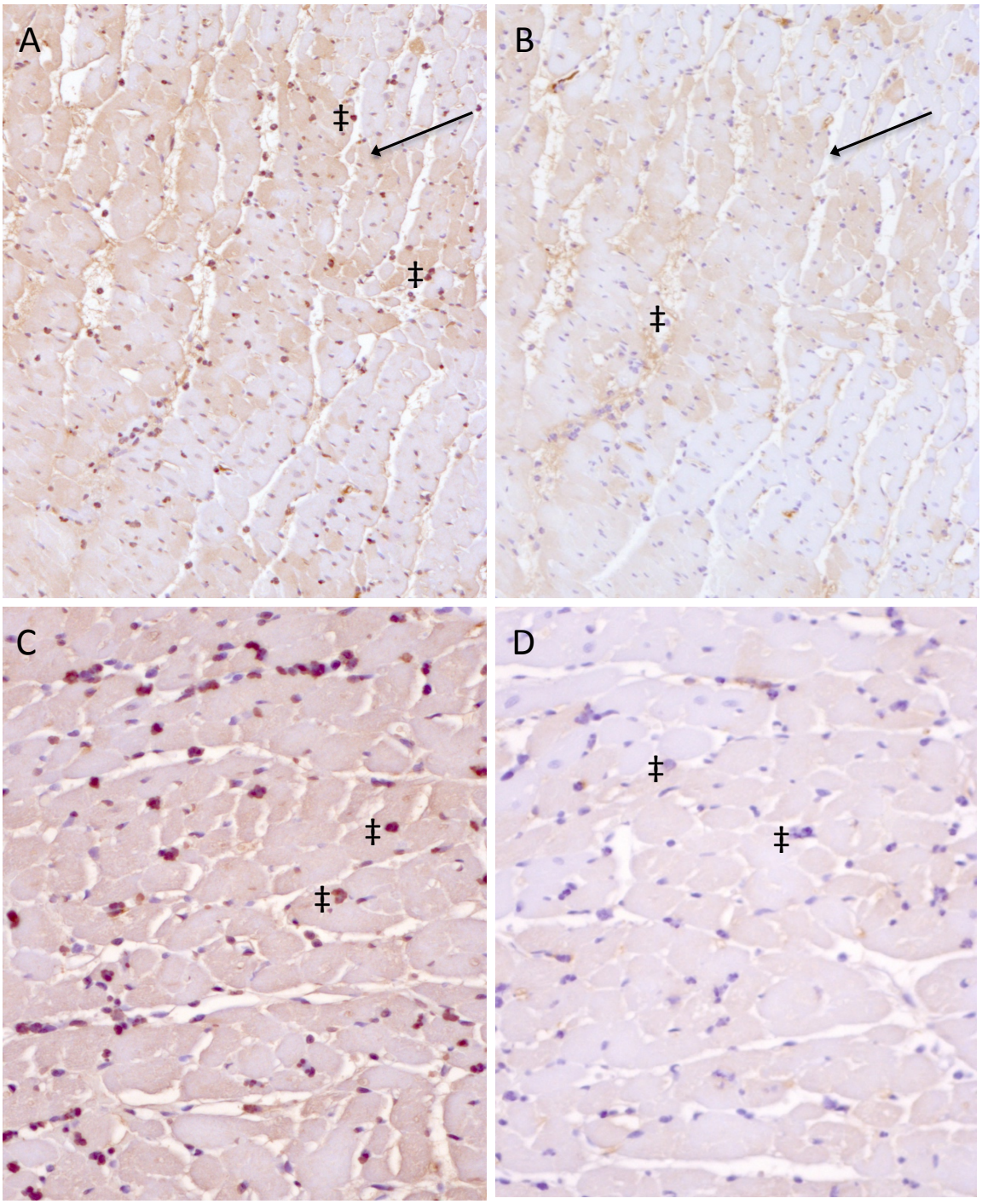


Image 7. Myocardium stained using immunohistochemistry for PAR. A: Low power view of myocardium stained for PAR: ‡ inflammatory cells staining strongly for PAR, → weak staining of myocytes. B: Low power view of myocardium stained with isotype control.‡ No staining of inflammatory cells, → weak staining of myocytes. C: High power view of myocardium stained for PAR. ‡ inflammatory cells staining strongly for PAR. D: high power view of myocardium stained with isotype control.‡ No staining of inflammatory cells.

	Vehicle (n=6)	AZ12785452 (n=6)	p
Pericardial neutrophilic inflammation	6	6	ns
Myocardial neutrophilic inflammation, periphery of left ventricle wall	6	6	ns
Myocyte degeneration and necrosis, left ventricle wall	6	6	ns
Intermyocyte oedema and fibrin accumulation	3	6	0.18
Haemorrhage, left ventricle wall	6	6	ns

Table 16. Qualitative measurement of histological features of myocardial injury following a reversible LAD experiment. Data are shown as number of rats (n=6 in each group). There was no significant difference between the groups using a 2 tailed Fishers exact test, performed by GraphPad software.

Discussion

This experiment sought to use a PARP-1 inhibitor as a pharmacological postconditioning strategy to reduce myocardial reperfusion injury in the context of uraemia.

Histology

Myocardial infarction generated neutrophilic inflammation in the pericardium and in the myocytes of the left ventricular wall, along with degeneration and necrosis of the myocytes in the left ventricular wall. Pre-reperfusion infusion of AZ12785452 did not appear to affect this pathology. Anti-PAR antibodies stained the inflammatory cells strongly but not the myocytes. Non-specific staining of the inflammatory cells was excluded using the isotype control antibody. Infusion of AZ12785452 did not appear to affect the staining of PAR.

PAR ELISA

AZ12785452 infused at a rate of 1mg/ml resulted in a non-significant reduction in PAR concentration. The failure of the PAR ELISA to detect a significant inhibition of PARP could be due to inherent variability associated with the technique, which requires multiple complex steps resulting in high levels of variability and leading to a type 2 error. This could be improved with greater sample numbers. It was not possible to generate multiple PBMC samples from the experiment because the delineation of the area at risk using Evans blue dye requires a significant cardiac output. If large volumes of blood were withdrawn before harvesting, the cardiac output and blood pressure would be too low to satisfactorily perfuse the myocardium and hence stain the perfused area. This would lead to an overestimation of the non perfused area which would in turn lead to an underestimation of the infarct size, since some perfused tissue would be misclassified into viable non-perfused tissue. As a result, only relatively small volumes of blood could be withdrawn before harvesting and this in turn meant that only 1 small PBMC pellet was produced from each rat.

The lack of PARP inhibition could also be because AZ12785452 did not inhibit PARP. However, the same batch of AZ12785452 was used by Kapoor et al. in our

lab (see Figure 52), in their unpublished work on non uraemic animals, which resulted in tissue protection and therefore, this is an unlikely explanation.

This experiment suggests that AZ12785452 infused at a concentration of 1mg/ml did not confer cardioprotection, as measured by serum troponin, histological scoring or infarct size in a uraemic rodent model of reversible LAD ligation. There are several possible explanations for this unexpected result. The first is that the concentration of AZ12785452 given may not have been optimal. The dose used was chosen based on the best available evidence. AZ12785452 has been shown in our lab to be cardioprotective in a rat model of reversible LAD ligation. Kapoor et al. investigated AZ12785452 induced cardioprotection in the non-uraemic rat. Several concentrations of AZ12785452 were given to identify the optimal cytoprotective protocol. Infusion of 0.04mg/ml and 4mg/ml did not result in cardioprotection. Concentrations of AZ12785452 of 0.2mg/ml and 1mg/ml protected the myocardium with 1mg/ml giving the greatest degree of protection. Therefore, this was the reason the concentration of 1mg/ml was chosen in the experiments involving uraemic rats. However, the experimental protocol and rat phenotype used by Kapoor et al. differed from this work (see Table 17). The rats used in work done by Kapoor et al. were younger (6-7 weeks vs. 11-12 weeks) and weighed less (285g vs. 425g). They had not undergone a 2 stage SNx procedure, and were therefore not uraemic. The duration of ischaemia was 25 minutes in work done by Kapoor et al, rather than the 30 minutes as in this work. The initial plan for the experimental protocol had been to employ a 25-minute ischaemic phase with a 120-minute reperfusion period. However, analysis of the first 6 uraemic rats that underwent LAD ligation (5 treated with vehicle and 1 with AZ12785452) revealed a median infarct size of 46.4 (IQR: 24.9-67.8) with a median AAR of 55.2% (IQR: 46.6-65.9). This infarct size was lower than in past experiments. Given that 5/6 rats were given vehicle and that the hypothesis was that AZ12785452 would lead to cardioprotection it was felt that to have the best chance of detecting a protective effect of AZ12785452 the injury should be increased. To do this, the ischaemia time was lengthened to 30 minutes.

Kapoor et al. demonstrated that there is a complex relationship between AZ12785452 concentration and cardioprotection. At small doses, no cardioprotection was seen. It is easy to surmise that at these dosages not enough PARP was inhibited at the time of reperfusion to cause a difference in protection. At higher doses, more PARP was inhibited and cardioprotection was seen. However when AZ12785452 was given at

the highest concentration of 4mg/ml, the cardioprotection was lost. It is difficult to imagine how inhibiting PARP more fully would lead to less protection. Unless at high concentrations *despite* inhibition of PARP, AZ12785452 led to increased injury due to a different toxic effect. Thus it may be that in uraemic animals, the threshold for a toxic dose is lower than in non uraemic animals, or the threshold for a cytoprotective response is higher than in non uraemic animals. To fully explore the tissue protective effects of AZ12785452, further studies should be considered using different concentrations of AZ12785452.

A further possible explanation of this result could be due to N-methyl-2-pyridone-5-carboxamide (2-PY), which has been shown to be an endogenous inhibitor of PARP whose concentration is inversely proportional to renal function. This would suggest that renal failure is associated with a state of endogenous PARP inhibition, and that any additional exogenous PARP inhibition may not be as effective. This could provide a further reason why the dose response relationship for AZ12785452 may be different in uraemia. There are several problems with this argument. Firstly, the thrust cardiovascular research in the context of CKD over the last 40 years has repeatedly demonstrated that underlying CKD is potent risk factor for cardiovascular disease. Additionally, if 2-PY significantly inhibited PARP at concentrations seen in the SNx model then it might be expected that the uraemic myocardium would have a greater ischaemia tolerance than the non uraemic myocardium; a phenomenon which has been disproved by us (see results section page) and others[307]. Finally, despite a much larger sample size in the uraemic animal experiment (13 and 16 in each arm) compared to work done by Kapoor (n=8 in each arm), there was much greater variability in the baseline characteristics of the uraemic animals. The blood pressure, pulse and PRP data had significantly greater variability in the uraemic group (see Table 17). There was also significantly greater variability in the AAR and infarct sizes in the uraemic animals when compared to the non uraemic animals. Kapoor et al. found that at a concentration of 1mg/ml, AZ12785452 caused a relative reduction in infarct size by 21.3%. If AZ12785452 conferred an equal reduction in uraemic animals, then at this level of variability the number of animals (m) needed in each arm to have an 80% power to detect a relative risk reduction of 21.3%, with a p value of 5% is given by the formula[612]

$$m = \frac{2 \times [z_{(1-\alpha/2)} + z_{(1-\beta)}]^2}{\Delta^2}$$ Where Δ is given by the formula $$\Delta = \frac{\delta}{s}$$

Where δ is the effect size (absolute difference in infarct size =11.08%), s is the standardised difference, $Z_{(1-\alpha/2)}$ is the significance level (1.96) and $Z_{(1-\beta)}$ is the power (0.8416), m= 50.4.

Thus to demonstrate a 21% relative infarct size reduction with a mean SD of 19.9 would require over 100 rats, which is prohibitive in terms labour and expense.

This variability strikes at the heart of translational research. As research seeks to better represent human disease through animal models, more complex development of animal models are needed. This complexity generates variability, requiring a much larger sample size to show a difference. This variability in baseline characteristics rather than being a hindrance in animal work, perhaps is merely an indication of a better model of human disease, with the same inherent variability seen in clinical studies.

Future directions for study

1) A different concentration of AZ12785452 should be tried, to exclude a different dose response effect in uraemic animals.
2) Use of different PARP inhibitors in the SNx model, such as 3-aminobenzamide, which has been widely reported to inhibit PARP and cause cardioprotection in rodents, or INO-1001which has been studied in a phase 2 clinical trial in humans in the setting of AMI. This would confirm that that the loss of cardioprotection is not specific to AZ12785452.
3) The impact of chronic PARP administration to SNx animals. Given that the SNx procedure leads to an inflammatory state with renal fibrosis and glomerulosclerosis, PARP inhibition may protect against this progressive cardiac phenotype. This would be akin to the work done in the diabetic animal models [613-616].

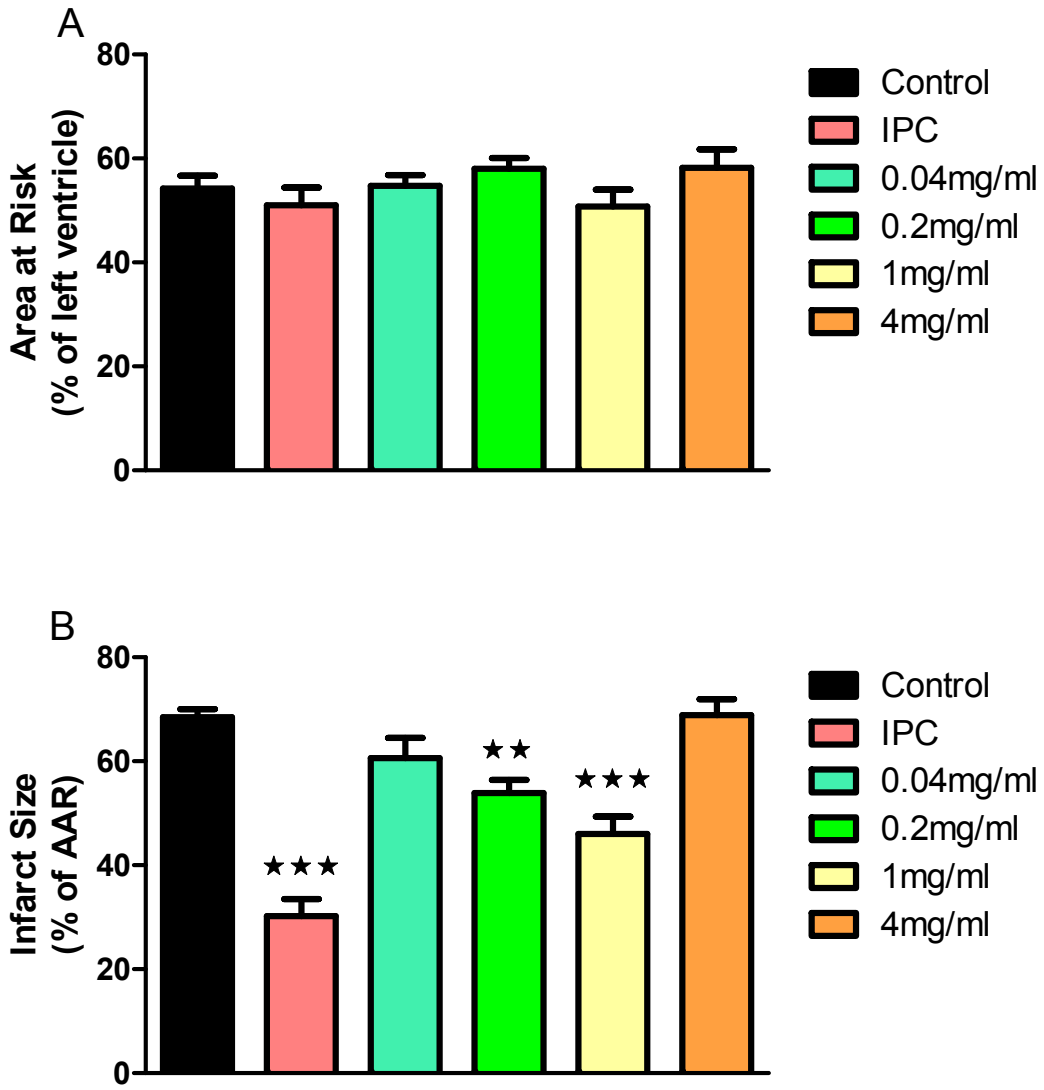


Figure 52. Results of unpublished work performed by Dr Kapoor, Dr Patel and Professor Thiemermann: researchers in our group. (A)Area at risk and (B) infarct size of rats subjected to regional myocardial ischaemia (25min) and reperfusion (2h) and pre treated with either vehicle (control, 10 % DMSO 1ml/kg i.v.,(n = 8), or 2 cycles of IPC (5 min) followed by LAD occlusion (25min) and reperfusion (2 h) and pre-treated with vehicle (IPC, 10% DMSO 1 ml/kg i.v., n = 8), or rats subjected to regional myocardial ischaemia (25 min) and reperfusion (2 h) and pre-treated with AZ12785452 (0.04 mg/ml i.v., n = 8); AZ12785452 (0.2 mg/ml i.v., n = 8); AZ12785452 (1 mg/ml i.v., n = 8) or AZ12785452(4mg/ml i.v., n=8). P<0.01 vs. control Group, P<0.001 vs. control Group.

A

	Kapoor et al (n=48)	SNx Animals (n=29)	p	F test
Weight (g)	282 (40)	410 (24)	<0.0001	**
MAP (mm/hg)	107 (24.5)	122.7 (36.68)	0.02	**
Pulse (BPM)	420 (36.7)	411(38.7)	0.28	ns
PRP	57951 (28995)	51826 (17608)	0.26	**
AAR (%)	54.5 (8.3)	52.4 (11.9)	0.37	*

B

	Kapoor et al (n=8)	SNx animals*	p	F test
Vehicle	68.5 (4.4)	52 (17.51)	0.02	**
AZ12785452	53.88 (7.2)	53.3 (22.2)	0.948	**

Table 17.
A: comparison of Kapoor et al. data with SNx data in terms of weight, MAP, pulse, pressure rate product and area at risk. SNx animals are heavier and have higher MAP (at occlusion). Importantly there is greater intra-group variability in the SNx animals AAR, MAP, PRP. There is a greater variability in weight in the Kapoor et al data.

B: comparison of Kapoor et al data with SNx Data. Kapoor et al: infarct size of non uraemic 6-7 week old male Wistar rats undergoing 25 minute LAD ligation, with infusion of either vehicle (n=8) or AZ12785452 (n=8) with 2h reperfusion. SNx animals: infarct size of 11-12 week old male Wistar rats who have undergone a 2 stage SNx procedure to generate a model of chronic uraemia who then underwent 30minutes of LAD ligation , with infusion of either vehicle (*n=13) or AZ12785452 (*n=16) with 2h reperfusion. The 2 groups treated with AZ12785452, had similar infarct sizes, however the non uraemic vehicle treated group had a significantly larger infarct size than the SNx group. There was significantly greater variability of infarct size in the SNx groups compared with the Kapoor et al. data.

Data in cells represent mean (SD), p value shown is a result of an unpaired 2 tail T-test. F test results show if the standard deviations are significantly different. Statistical analysis performed by GraphPad Prism 5.

Summary and Conclusions

Summary

The results of described in this thesis can be summarised as follows:

Characterisation of animal models of uraemia

3 models of rodent models of chronic uraemic were developed: the SNx model, the adenine diet model and the adenine washout model. All 3 models displayed many features of the human uraemic phenotype; anaemia, raised serum creatinine, raised serum urea, proteinuria, natriuresis, reduced weight gain, increased heart weight index and growth restriction.

Myocardial ischaemia tolerance of the uraemic heart

The SNx model, but not the adenine diet model of chronic uraemia resulted in reduced myocardial ischaemia tolerance in a model of reversiable LAD ligation. No underlying differences in bioenergetics were found between these models to explain the observed difference in ischaemia tolerance.

Ischaemic preconditioning of the uraemic heart

This work demonstrated that an IPC protocol confers a profound reduction in infarct size and arrhythmias. This experiment reported the novel finding that unlike dyslipidaemia and senescence, underlying uraemia did not abolish the effects of postconditioning as measured by infarct size and troponin release in 3 different rodent models. In addition, underlying uraemia did not confer an increased resistance to IPC. The role of the RISK and SAFE pathways in the intracellular signal transduction cascade of IPC were confirmed, along with the finding that underlying uraemia did not appear to significantly alter these pathways.

Ischaemic postconditioning of the uraemic heart

This experiment reported the novel findings that unlike senescence and diabetes, underlying uraemia did not abolish the effects of postconditioning as measured by infarct size and troponin release.

The effect of the effect of AZ12785452: a PARP-1 inhibitor in an SNx rodent model of myocardial infarction

Infusion of AZ12785452 immediately before reperfusion such that it acts at the point of reperfusion and as such acts as a pharmacological postconditioning mimetic, in a uraemic rodent model of AMI did not result in an alteration of reperfusion injury as measured by NBT staining, troponin release or histological analysis.

The failure and future of translation of ischaemic conditioning

For an intervention to have a good chance of clinical translation, an intervention should be effective in all species studied and can be replicated in different laboratories and in different experimental models. Conditioning strategies (IPC, RIPC, iPOST) represent ideal interventions, which fulfil all of the above criteria, and yet in the 25 years since IPC was reported, these procedures have not become part of routine clinical practice.

The apparent simplicity of the induction of ischaemic conditioning is a double-edged sword. As ischaemic conditioning cannot be patented by a pharmaceutical company, researchers employing ischaemic conditioning can save time and money because there are no associated drug costs and less pharmaceutical regulations are involved. This has lead to a rapid translation of small, single centre trials, which while they have almost been universally positive, have lacked the duration and size to provide clinicians with the evidence they need to change their practice.

Pharmaceutical companies are unlikely to fund large trials in ischaemic conditioning if there is no profit for their shareholders. Without this industry funding, ischaemic conditioning trials have generally been small and underpowered for the mortality outcomes needed to force a change in routine clinical practice.

This thesis suggests that the ideal cohort to study novel therapeutic cardioprotective strategies are CKD patients. They are a group of patients hugely burdened by cardiovascular disease, with much poorer outcomes following cardiovascular events and who have been routinely excluded from clinical trials in cardioprotective strategies. Through reduced ischaemia tolerance, they are at risk of greater tissue injury for a given insult. Several standard therapies used for the general population have been shown to be less effective or even harmful in the CKD/ESRD cohort.

This thesis concludes that underlying CKD is not a barrier to ischaemic preconditioning or ischaemic postconditioning, making it an attractive intervention in the context of CKD.

Future work should focus on optimisation of ischaemic conditioning protocols to achieve greater tissue protection, including the use of combinations of conditioning strategies such as IPC combined with iPOST or pharmacological conditioning combined with IPC. I have performed preliminary work in this area using 2 RIPC

signals 24 hours apart to investigate whether summation of early and late RIPC can achieve greater tissue protection than early RIPC alone in a model of AKI (see supplementary data page 234-254).

Finally future work should focus on translation of these findings into a human CKD and ESRD cohort. The first step in the widespread translation of ischaemic conditioning needs to involve a small 'proof of concept' trial in CKD and ESRD patients. A suitable model would be the forearm flow medicated vasodilatation model which is relatively simple and non invasive. This can be followed by larger intervention studies aimed at recruiting haemodialysis patients undergoing an elective PCI intervention followed by investigation of the role of iPOST in the setting of acute myocardial events. These trials could be much more powerful than trials in non CKD patients because CKD patients have 7 times the event rate following AMI, and thus smaller trials could still be powered for cardiovascular mortality.

Taken together, CKD patients have the most to lose from cardiovascular disease and this thesis suggests that through novel cardioprotective strategies they may also have the most to gain.

Supplementary Data

The use of Summation Remote Ischaemic Preconditioning to Protect the Kidney from Ischaemia/Reperfusion injury

Background

Ischaemic preconditioning was first described by Murry et al[388] as a phenomenon which conferred tissue protection immediately after the preconditioning stimulus. This cytoprotective effect was temporary, with loss of protection seen if there was a delay of several hours between preconditioning protocol and injury. 6 years after Murry's seminal paper, Marber et al.[396] published work which demonstrated a 'second window' of tissue protection, which began 12-24 hours after the preconditioning stimulus and lasted for 24-48 hours. Remote ischaemic Preconditioning (RIPC) was first described by Przyklenk et al in 1993[617]. They described the phenomenon whereby several brief episodes of ischaemia and reperfusion in the circumflex artery in the dog could confer subsequent tissue protection on the territory supplied by the LAD artery. This form of intramyocardial tissue protection was subsequently extended to non cardiac organs, such as the kidney[377], small intestine[618], liver[618], skeletal muscle[619] and brain[620]. Li et al.[621] also showed that the second window exists for remote preconditioning. The group showed that the heart could be protected from lethal ischaemic injury using a RIPC protocol involving 3 cycles of femoral artery occlusion and reperfusion 24 hours before the index ischaemia. This protection is broadly similar in magnitude to an IPC protocol (employing 3 cycles of 5 minutes LAD occlusion and reperfusion) employed immediately before the index ischaemia[622]. Delayed remote preconditioning has notable benefits over standard IPC because the target organ does not need to be rendered ischaemic to confer protection, and because the window of protection is larger, the precise timing of the injury is less crucial.

Mechanisms of delayed tissue protection from remote preconditioning

The mechanism by which RIPC confers tissue protection is not as fully understood as that of IPC or iPOST, and even less is understood about the different aspects of early and late remote preconditioning. The signal transduction of RIPC stimulus is complex. The signal has to get from the remote organ to the tissue of interest and then from the cell surface to the mitochondria. There are 2 different mechanistic pathways which are thought to exist to transfer the signal from the remote organ to the organ of interest; the humoral and neural pathway.

Humoral Pathway

The humoral pathway consists of a mediator, which is produced in response to several sub lethal episodes of ischaemia in a distant tissue bed, which is then transported via the circulation to the target organ. Evidence in favour of this pathway comes form work which shows that reperfusion is required to 'washout' the substance to generate a signal[623], along with work by Dickenson et al.[624], which showed that the coronary effluent from an isolated preconditioned rabbit could reduce infarct size in an untreated isolated rabbit heart. Studies have sought to identify the humoral mediator, or mediators, responsible for the signal transduction of RIPC. Evidence from proteomic analysis of tissue effluents suggest that the factor is between 3.5-8kDa[625, 626]. While the composition of the mediator still remains unknown, several candidate molecules have been implicated in the inter organ signal transduction of RIPC, including opioids[627], endocannabinoids[628], noradrenaline[629] and angiotensin[630].

Neural Pathway

Evidence in favour of a neural pathway comes from work by Gho et al.[618] who demonstrated that myocardial protection following anterior mesenteric artery RIPC could be abolished by hexamethonium, a ganglion blocker. It was proposed that compounds released in response to brief periods of IR, stimulate afferent nerve fibres to transmit the signal to the target organ. Compounds which stimulate afferent nerves in response to brief periods of ischaemia leading to tissue protection, are thought to include adenosine, bradykinin and calcitonin gene-related peptide (CGRP). The role of adenosine as a mediator in the neural pathway comes from data by Ding et al.[375] who showed that when the kidney was used to precondition the heart there was increased afferent renal nerve discharge. This was blocked by a selective adenosine antagonist, abolishing cardiac protection. Furthermore, sectioning the renal nerves before renal preconditioning also blocked the cardioprotective effect of renal RIPC on the heart. Liem et al.[376] reported that local administration of adenosine into a mesenteric vascular bed conferred subsequent cardioprotection, which was blocked by hexamethonium. The role of bradykinin in RIPC is suggested by data which show that intra-mesenteric administration of bradykinin acted as an RIPC mimetic and that this effect was abolished in the presence of hexamethonium[631].

Evidence for the role of CGRP in transduction of the RIPC signal comes from work which shows that RIPC stimulates sensory nerves in the intestinal vasculature to secrete CGRP into the blood-stream where it is carried to the heart[632, 633]. To summarise, mediators may be released into the blood stream and carried to a distant organ, mediators may be generated locally which stimulate afferent nerves to send a pro-survival signal to the distant organ or finally preconditioning stimulates nerves to release factors into the blood stream.

Intracellular signal transduction cascade

Once the mediators reach the cell surface, it is thought that they bind to G-protein cell surface receptors. Unlike IPC or iPOST, the intracellular signal transduction cascade from cell surface to the mPTP is unclear, but it appears to involve a similar cohort of compounds including the K_{ATP} channel[634], nitric oxide[635], ROS[636], PKC[633] and finally the mPTP[637].

Delayed RIPC

There is very little data specifically concerning late RIPC. However, it appears to be effective in humans in the flow mediated vasodilation model of endothelial function[638]. In rats, hind limb RIPC attenuated myocardial injury 24 hours later[622]. In the kidney, there is evidence that pharmacological late preconditioning can reduce tissue injury during experimental renal transplantation using the K_{ATP} channel opener, ciclosporin[639].

Summation preconditioning strategies

Early and late pathways of preconditioning are thought to be mechanistically distinct, because of this research has been undertaken to identify whether these 2 stimuli can be combined to produce even greater tissue protection. Stambaugh et al.[640] showed that an in vitro model of simulated myocardial ischaemia, combining early and late 'preconditioning' strategies using adenosine agonists and K_{ATP} channel openers to achieve early preconditioning and monophosphoryl lipid A to induce late PC, resulted in greater cellular protection than early PC alone.

In vivo work by Sato et al.[641] showed that combining iPOST with delayed IPC resulted in greater tissue protection following myocardial infarction than with either alone, and that the additional protection seen with delayed IPC could be abolished with a COX 2

inhibitor. Mullenheim et al.[642] found that employing an IPC protocol to a rabbit heart resulted in tissue protection 24 hours later. This protective effect could be improved with the addition of sevoflurane, an agent known to precondition, via opening of the K_{ATP} channel. This effect was abolished with the K_{ATP} channel blocker, 5-hydroxydecanoate[642]. Furthermore, they reported that combining early and late IPC protocols protected the myocardium better than either alone[643]. Xin at al. reported that combining iPOST with remote preconditioning provided greater protection than either alone[644].

Clinical translation of Summation preconditioning

All published work on summation preconditioning has been focused on the myocardium as the target tissue. There has been no work on whether the kidney can achieve extra benefit through summation. This question is of clinical relevance particularly in the field of renal transplantation. During harvesting, the kidney is exposed to both warm ischaemia during retrieval, and cold ischaemia during transportation of the organ and storage before it is exposed to reperfusion injury in the recipient following restoration of perfusion[645]. Ischaemia reperfusion injury at the time of transplantation is an important factor in both the short term outcome, with respect to delayed graft function[646], and also long term graft outcome[645-647]. Remote preconditioning has been shown in a small trial to be effective in reducing renal injury in the transplant setting, with evidence of a sustained benefit in renal function to 24 months[648]. Human clinical trials are in progress to investigate the effect of remote preconditioning in the context of cadaveric organ donation with the aim of reducing the effect of IRI to the kidney inherent in the procedure of renal transplantation.[649] The Repair Trial[648], a randomised multicentre trial currently recruiting, is investigating the effects of early, late and combined early and late RIPC on the outcome of live kidney donation. It is currently unknown whether the additional renal protection can be achieved by adding delayed remote preconditioning to classical early remote preconditioning.

Aims

The aim of this experiment was to establish whether combining early and late RIPC strategies provided additional benefit over early RIPC alone in a rat model of AKI.

Methods

Forty five, six week old male Wistar Rats (Charles Rivers UK) were housed in an animal house with a 12 hour day night cycle and free access to water and standard chow for 1 week.

All animals were anaesthetised using 100mg/kg ketamine (Fort Dodge animal Health) and 10mg/kg xylazine, (Rompun Bayer) injected intraperitoneally together with 0.01mg/kg of Vetergesic S/C. All surgery was carried out under full aseptic conditions, on a homoeothermic control mat (Harvard).

Once anaesthetised the fur over the left groin was shaved and a 5mm incision was made over the left femoral area. Blunt dissection through the muscle layers revealed the femoral neurovascular bundle. The femoral artery was isolated without damage to the nerve or vein, and a sterile tie (3-0 Sofsilk Syneture) was placed round the artery for easy identification at a later stage. The animals then were split into 3 groups (see Figure 53). Group 1 and 2 were left for 30 minutes before the skin was closed using skin staples (3M Precise). This was a sham preconditioning protocol. Group 3 underwent a preconditioning protocol; the femoral artery was reversibly occluded using an arterial clip for 5 minutes with 5 minutes reperfusion. This procedure was repeated 3 times. Ischaemia to the hind leg was confirmed with the observation that the left foot became paler and colder than the right and upon reperfusion there was a dramatic reactive hyperaemia of the foot. All rats were then left to recover. After 48 hours, the rats were then anaesthetised as before. Group 1 underwent a sham preconditioning protocol as before, group 2 and 3 underwent a preconditioning protocol, using 3 cycles of 5 minutes left femoral artery occlusion and reperfusion. Following this, all animals had their fur over their abdomen shaved and a midline laparotomy incision was carried out using blunt tipped scissors. The small intestine was carefully pushed towards the animals left flank to expose the right kidney. The right kidney was decapsulated and mobilised. Sterile ties were placed around the renal pedicle and tied off. The right kidney was then rapidly removed. The left kidney was then exposed. The renal artery and vein were carefully dissected away from the surrounding tissue and an arterial clamp was used to occlude the renal artery. The left kidney underwent 45 minutes of ischaemia, which was confirmed with the observation of renal pallor. At the end of 45 minutes, the clamp was released and reperfusion was confirmed with the observation of

prompt hyperaemia of the kidney. The abdominal musculature was then closed using suture (4-0 Polysorb Synture) and the overlying skin stapled with skin sutures. The animals were then left to recover, with close monitoring for signs of distress or suffering.

After a further 48 hours the animals were anaesthetised using an IP injection of sodium thiopental at a dose of 58mg/kg (Link Pharmaceuticals). This was a smaller dose of anaesthetic than in previous experiments because the animals were more uraemic. Once the animals were anaesthetised, they were placed on a homoeothermic control unit (Harvard) and their temperature was measured using a rectal digital thermometer. A tracheostomy and arterial line was inserted as in previous experiments. The animals were not routinely ventilated; the tracheostomy was inserted as a precaution in case of respiratory arrest due to the anaesthetic. Blood pressure and pulse readings were collected. 200ul of blood was taken in a heparinised syringe for arterial blood gas measurement. Additionally, 1.5ml of blood was taken and spun at 6000g for 3 minutes in a centrifuge, to separate the serum from the cells. The serum was removed and placed in liquid nitrogen for storage. The serum was sent for biochemical analysis (Vet Lab services UK).

Histological evaluation and TUNEL staining

At the end of the experiment, the left kidney was quickly removed and cut in into two halves which were fixed in immersion in 10% (wt/vol) formaldehyde in PBS (0.01 M; pH 7.4) at room temperature. After dehydration using graded ethanol, pieces of kidney were embedded in Paraplast (Sherwood Medical, Mahwah, NJ), cut in fine (8μm) sections and mounted on glass slides. Sections were then deparaffinized with xylene, counterstained with hematoxylin and eosin, and viewed under a light microscope (Dialux 22; Leitz, Milan, Italy). A scoring system was performed as previously described by Sharples et al.[650].

One hundred intersections were examined for each kidney, and a score from 0 to 3 was given for each tubular profile: 0, normal histology; 1, tubular cell swelling, brush border loss, and nuclear condensation with up to one third nuclear loss; 2, as for score 1, but greater than one third and less than two thirds tubular profiles showing nuclear loss; and 3, greater than two thirds tubular profile showing nuclear loss. The histological score

for each kidney was calculated by addition of all scores, with a maximum score of 300. Sections were also examined for inflammation, with the number of polymorphonuclear (PMN) cells infiltrating the tissue counted per high power field.

Sections were assessed quantitatively for apoptotic nuclei and graded for severity and extent of nuclear changes. TUNEL assay was performed by a pathologist who was blinded to the treatment animals had received. The TUNEL assay was conducted using a TUNEL detection kit according to the manufacturer's instruction (HRP kit DBA; Apotag, Milan, Italy).

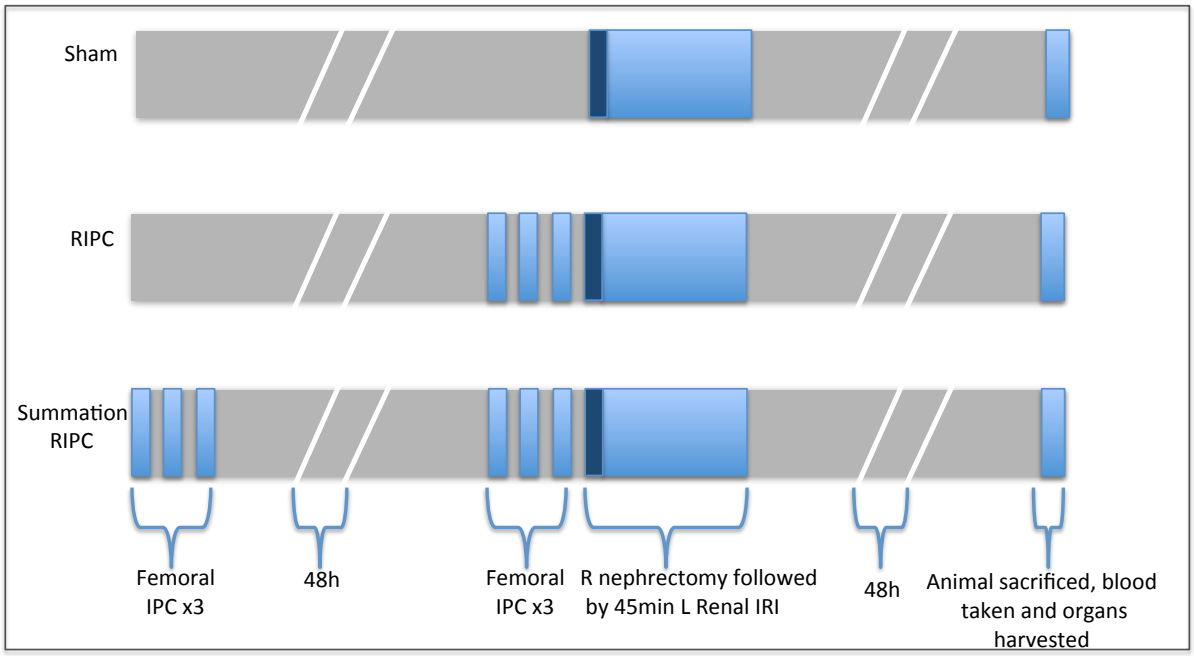


Figure 53. Schematic representation of the experimental protocol.

Results

45 male Wistar rats were used in the experiment. 8 animals died before the organs were harvested and therefore were excluded from the analysis. No animals died during the femoral surgery or in the perioperative period following the renal IRI. All 8 animals died between 36 hours and 48 hours after the renal IRI, presumably as a result of acute renal failure. 1 animal in the summation group died, 3 died in the RIPC group and 4 died in the control group. This left 11, 12 and 14 animals included for analysis in the control, RIPC and summation RIPC groups respectively.

At the beginning of the experiment, the 3 groups were similar in weight (see Table 18 and Figure 54). 48 hours following renal IRI, the anaesthetised rats in the 3 groups had similar cardiovascular parameters, haematocrit, serum sodium and calcium levels. On arterial blood gas analysis there was a stepwise trend in a lower pH in the control group versus the RIPC group versus the summation RIPC group, which approached statistical significance ($p=0.07$). In parallel with the pH, there was a significant stepwise improvement in the base deficit seen between control, RIPC and summation RIPC groups ($p=0.05$). Serum potassium was significantly higher in the control group than the RIPC or summation RIPC group ($p<0.0001$). Serum phosphate was significantly lower in the summation groups compared to the control or RIPC groups ($p<0.001$). The mean urea was 73.6, 62.8 and 44.5mmol/l in the sham, RIPC and summation RIPC groups respectively. Both the sham and RIPC groups had significantly higher urea concentrations than the summation RIPC group. A similar trend was seen in serum creatinine concentrations in the different groups. The mean creatinine was 506, 400 and 276 μmol/l in the sham, RIPC and summation RIPC groups respectively. There was a stepwise fall in serum creatinine across the groups ($p<0.001$). The RIPC group had a significantly lower mean creatinine than the control group ($p<0.05$) and a significantly higher mean creatinine than the summation RIPC groups ($p<0.05$).

Histological analysis

The mean ATN score was 112, 123 and 23 out of a possible 300 for the control, RIPC and summation RIPC groups respectively. Both the control group and the RIPC group had similar ATN scores and both were significantly higher than the summation RIPC group (see Image 8 and Table 18).

Polymorphonuclear cell infiltration into renal tissues

The mean number of PMN seen per high power field was 9.8, 11.4 and 3.5 in the control, RIPC and summation RIPC groups respectively. Both the control group and the RIPC group had similar numbers of PMN infiltration and both had significantly greater infiltration than the summation RIPC group (see Table 18 and Image 9)

The mean number of TUNEL+ve cells seen per high-powered field was 3.30, 3.61 and 0.81 in the control, RIPC and summation RIPC groups respectively. Both the control group and the RIPC group had similar numbers of TUNEL+ve cells seen per high-powered field and both had significantly greater infiltration than the summation RIPC group.

	Control	RIPC	Summation RIPC	p
Weight (g)	298 (10.5)	300 (6.33)	293 (11.32)	0.38
BP (mm/Hg)	128 (9.6)	131 (11.1)	137 (12.2)	0.23
Pulse (BPM)	413 (48.6)	416 (69.1)	399 (46.5)	0.79
Plasma pH	7.29 (0.048)	7.32 (0.072)	7.35 (0.053)	0.07
Plasma HCO3 (mmol/l)	22.2 (3.63)	20.3 (3.26)	23.1 (2.95)	0.26
Base deficit (mEql/l)	6.24 (4.19)	4.83 (3.94)	2.01 (3.35)	0.05
Haematocrit (%)	37.6 (2.41)	36.7 (2.06)	38.9 (3.59)	0.22
Sodium (mmol/l)	138 (3.08)	140 (1.66)	139 (3.47)	0.42
Potassium (mmol/l)	7.01 (1.05)	5.04 (1.09)	4.55 (1.16)	<0.001
Calcium (mmol/l)	2.17 (0.28)	2.20 (0.18)	2.33 (0.25)	0.29
Phosphate (mmol/l)	5.82 (0.40)	5.46 (1.03)	3.46 (1.01)	<0.0001
Urea (mmol/l)	73.6 (8.9)	62.8 (16.4)	44.5 (12.6)	<0.0001
Creatinine (umol/l)	506 (66)	400 (96)	276 (131)	<0.0001

Table 18. Results of summation preconditioning experiment. Data presented as mean (SD), with p value presented as the results of a 1 way ANOVA using GraphPad software.

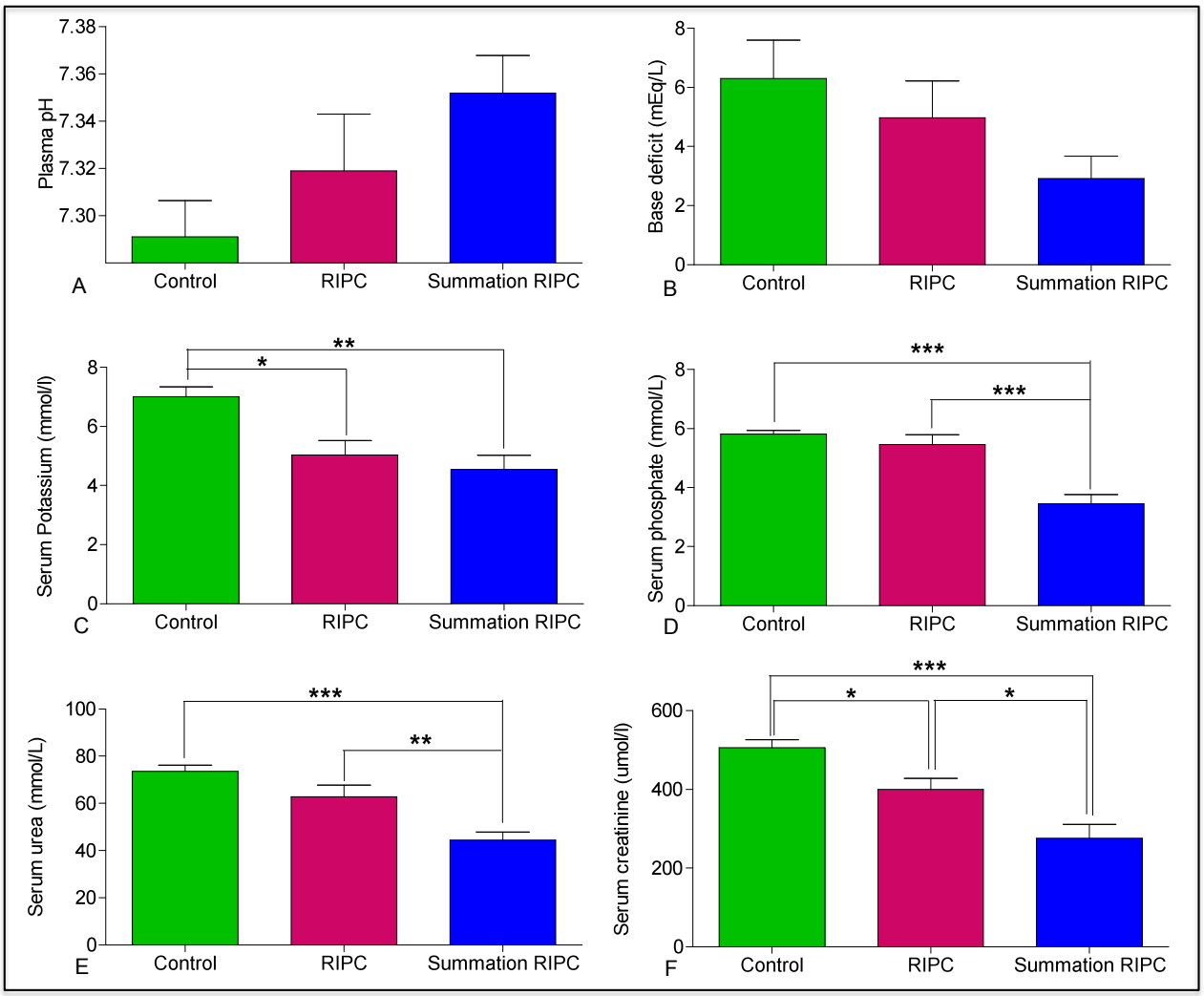


Figure 54. Biochemical analysis of results following control (n=11), RIPC (n=12) or summation RIPC(n=14) protocols. Data presented as mean with SEM. Statistical analysis data presented as results of one way ANOVA with Bonteferroni's multiple comparison testing as a post test. All statistical analysis calculated using GraphPad software. * $p<0.05$, **$p<0.01$ *** $p<0.001$

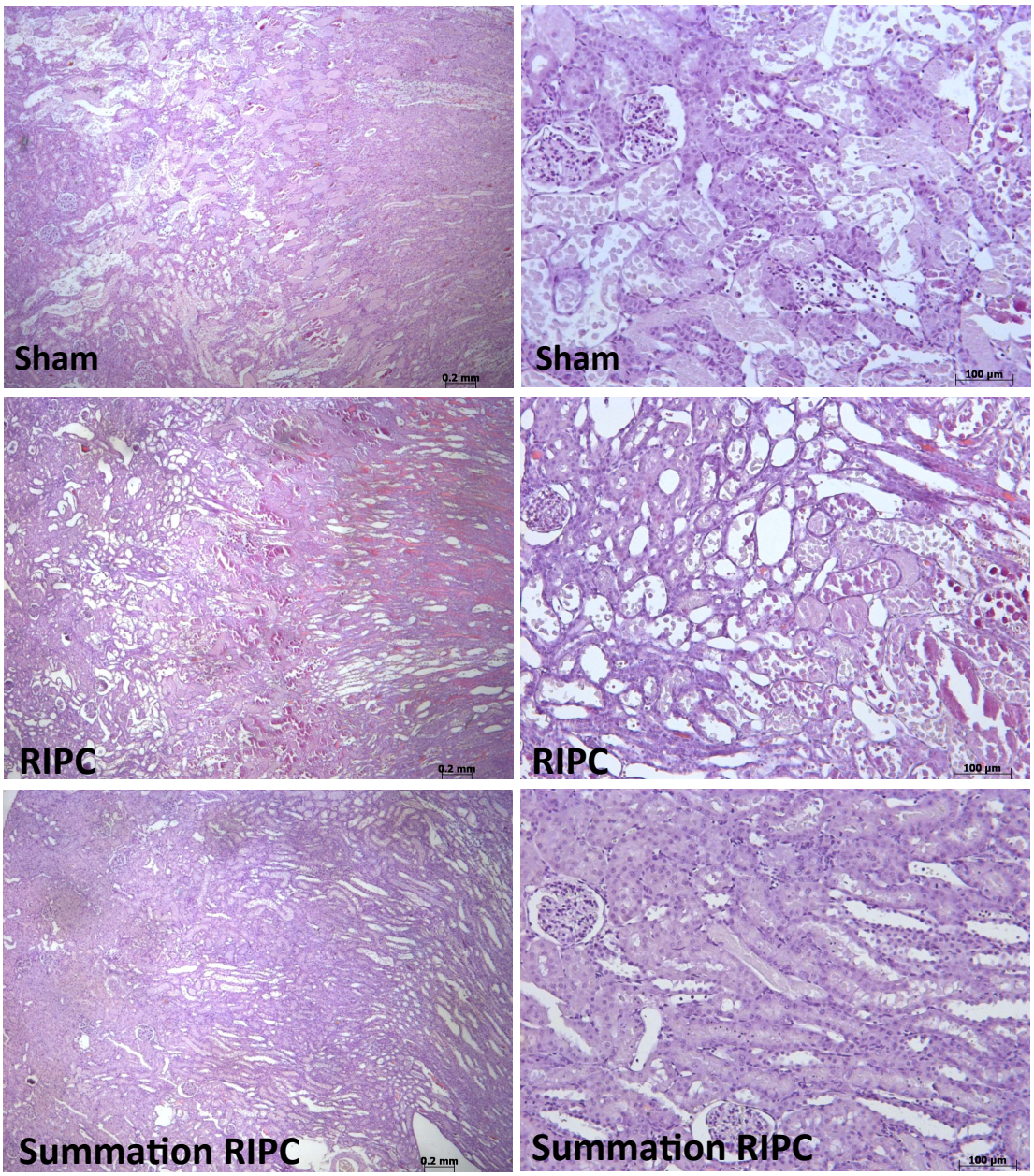


Image 8. Representative photographs of renal histopathology stained with hematoxylin and eosin. The top row is from the control group, the middle row is from the RIPC group and the bottom row is from the summation RIPC group. The left column shows renal sections at low power, the right column shows the sections at high power.

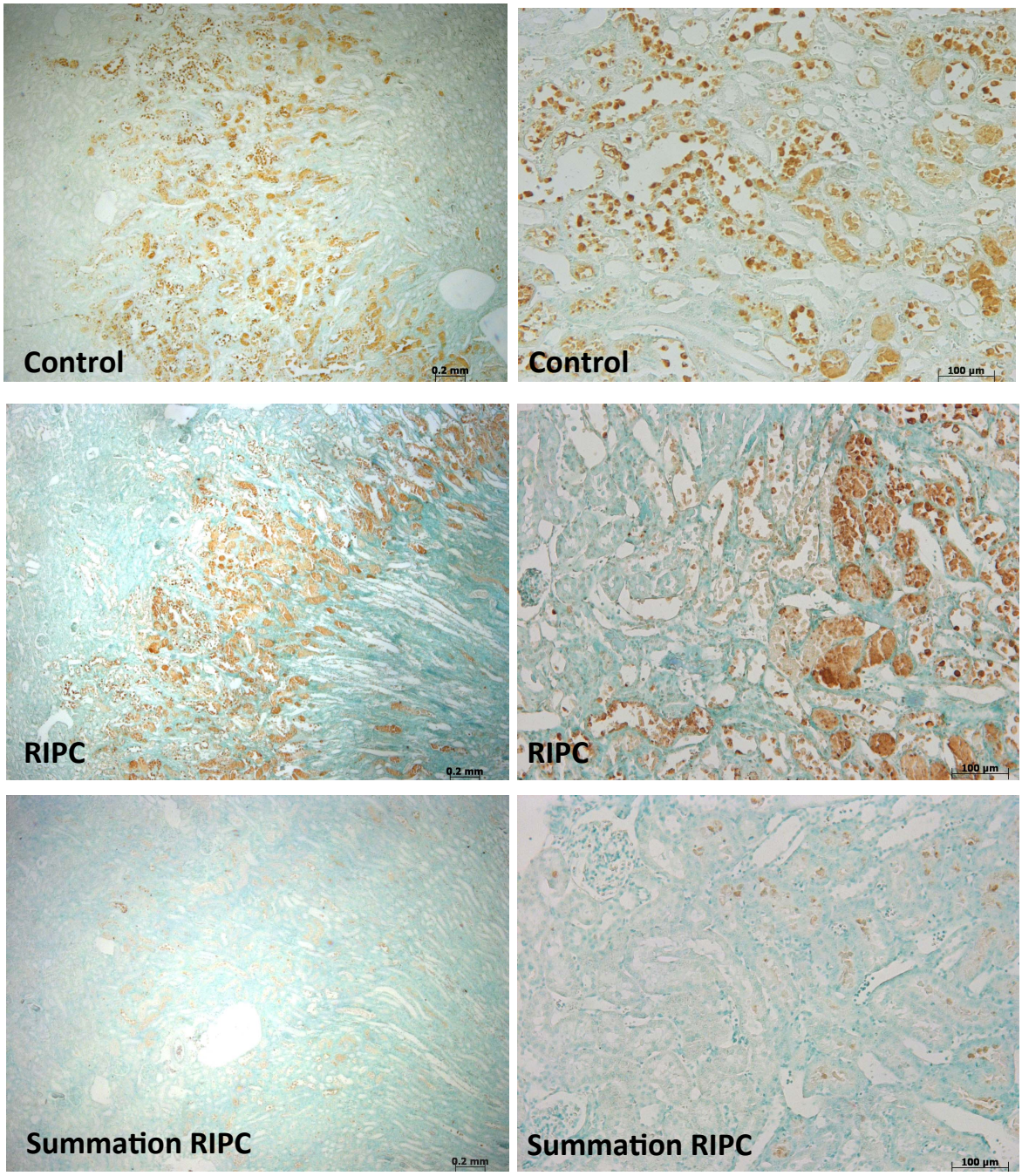


Image 9. Representative photographs of renal histopathology stained for TUNEL positive nuclei.
The top row is from the control group, the middle row is from the RIPC group and the bottom row is from the summation RIPC group. The left column shows renal sections at low power, the right column shows the sections at high power.

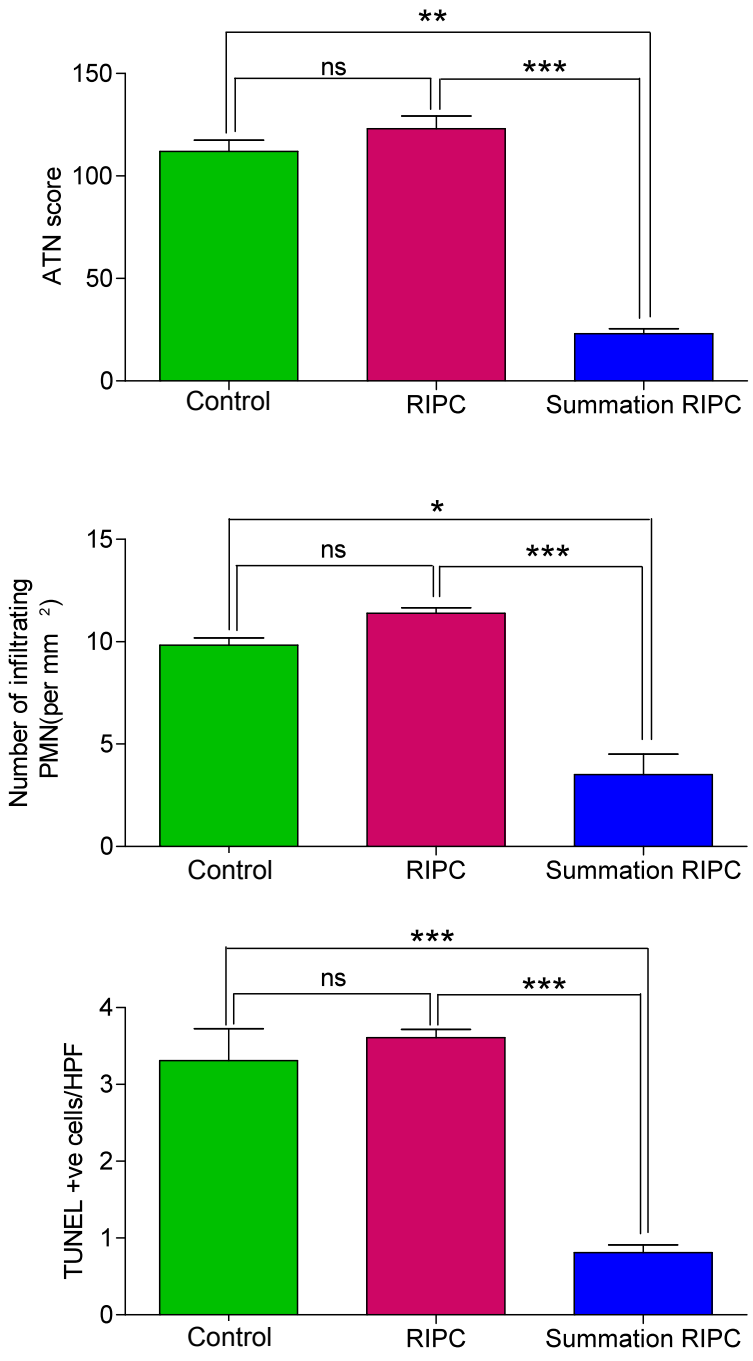


Figure 55. Histological comparison of animals undergoing a sham RIPC protocol followed by renal IRI: control (n=10), animals undergoing an RIPC protocol followed by renal IRI: RIPC (n=10) and animals undergoing a summation RIPC protocol and renal IRI: summation RIPC (n=10). Top: the semi-quantative ATN score of the 3 groups. Middle: the number of polymorphonuclear cells seen at high power in the 3 groups. Bottom: the number of TUNEL positive cells pre high power field in the 3 groups. Graphs display mean with error bars representing SEM. Statistical analysis was performed using the one way ANOVA test, with Bonteforri post test for comparison of each pair of columns. *=$p<0.05$, **=$p<0.01$, *** =$p<0.001$.

Discussion

Acute renal failure is associated with a high risk of in hospital mortality, in excess of >30%[651], with those patients who develop the most severe degree of acute renal failure having both the highest short and long term mortality. Despite many advances in medical practice, the in hospital mortality following acute renal failure has not significantly altered in several decades[652]. Furthermore, the incidence of AKI appears to be increasing over time[653-656]. There is a great unmet need to develop cytoprotective strategies to prevent AKI and improve patient outcomes. There is no published literature on combining any form of conditioning strategy to improve outcomes following renal IRI.

Acute renal failure in humans is characterised by various perturbations of plasma biochemistry; hyperkalaemia, hyperphosphataemia, acidaemia in the context of an elevated serum creatinine and urea levels. Anaemia is not a feature of AKI, it is more commonly seen as a marker of chronicity in kidney disease.

48 hours after unilateral nephrectomy and contralateral renal ischaemia reperfusion injury, the male Wistar rats developed a phenotype which shares many features of acute kidney injury seen in patients; hyperkalaemia, hyperphosphataemia, acidosis, uraemia, with an elevated serum creatinine but with no changes in serum sodium, calcium plasma haematocrit. Indeed, the mean potassium in the control group was 7, which carries with it a high risk of lethal cardiac arrhythmias, which could explain the higher rate of animals death in this group, as compared to the RIPC and summation RIPC group.

The choice of unilateral nephrectomy followed by contralateral renal IRI, rather than the more commonly used model of bilateral renal IRI, was chosen for 3 reasons. Firstly, performing a unilateral nephrectomy before contralateral renal IRI enables harvesting of the kidney for future work on identifying the mechanism of immediate and delayed RIPC. At the point of harvesting, the kidney taken from the control group will not have been exposed to an RIPC signal, allowing the tissue to act as a baseline in future mechanistic work. The kidney removed from the RIPC group will have been exposed to an immediate RIPC signal from the hind limb preconditioning protocol and the kidney removed from the summation RIPC group will have been exposed to both an immediate and a delayed RIPC signal from the 2 episodes of femoral RIPC 48 hours apart.

Secondly, earlier attempts at this work were performed using bilateral renal ischaemia and in approximately 10% of cases one of the 2 kidneys failed to fully reperfuse. This was seen as a dusky colouration of the whole kidney, or part of the kidney, on release of the clamp, rather then a prompt hyperaemia seen in normal reperfusion. The mechanism for this likely to be due to traumatic damage to the endothelium during the occlusion of the artery or embolisation of thrombus formed at the time of ischaemia down the main renal artery leading to no reflow injury. The effect of this would be development of a greater degree of acute renal failure in those rats which had not been fully reperfused, which was independent of duration of ischaemia or preconditioning protocol used. This would contribute to increased variability, leading to an increased risk of a type 2 error. By removing 1 kidney and occluding the other, it halved the chances of partial reperfusion and so led to a reduced intra group variability.

A further reason for performing a unilateral nephrectomy before a contralateral renal IRI was so generate a more severe form of renal injury than bilateral renal IRI. The hypothesis was that summation RIPC would provide greater tissue protection than RIPC alone, and much greater protection than no RIPC. If the model generated only a moderate injury, then immediate RIPC alone may have maximally salvaged renal tissue and so summation RIPC may not have any additional shown benefit.

Finally, there was a theoretical concern that during bilateral renal ischaemia, unless the clamps on each renal artery were removed at the same time, one of the kidneys may have been exposed to a form of remote post-conditioning from reperfusion of the contralateral kidney, potentially leading to some degree of protection from injury for the second kidney to be reperfused. This again could lead to increased variability in the tissue injury seen 48 h later. The potential effect of remote iPOST was abolished by unilateral nephrectomy before renal IRI.

This is the first time that the same preconditioning protocol modality has been summated to achieve tissue protection. Other published work, almost without exception in the heart, has combined different modalities such as pharmacological preconditioning with IPC or IPC with iPOST. Combining identical stimuli is the most elegant way to establish the effects of summation, because other studies have looked at different triggering stimuli it may result in different intracellular signal transduction cascades being activated by the different stimuli. A speculative explanation for the ability of delayed RIPC to provide additional benefits over early RIPC is that delayed RIPC leads to de-novo synthesis of anti-apoptotic proteins and members of the cytoprotective

signalling cascades. This has been shown to occur in delayed IPC for compounds such as mitochondrial manganese superoxide dismutase (mMSOD)[657, 658], heat stress proteins 60, 70 and 72[396], iNOS [659, 660] and the RISK pathway itself [661].

Histology

The semi-quantitative scoring system used to characterise the degree of ATN has been used extensively by our lab in the past[650, 662-664]. Acute tubular necrosis is the sine qua non of ischaemia reperfusion injury to the kidney. PMN infiltration is both a cause and effect of renal IRI, with neutrophil infiltration triggered by cytokine release during ischaemia followed by further inflammation triggered by neutrophil mediated ROS production.

TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labelling) staining is a method for detecting DNA fragmentation that results from apoptotic signalling cascades, and as such is considered an indirect histological marker for apoptosis[665].

The histological analysis shows that summation RIPC resulted in significant tissue protection over delayed RIPC alone, as evidenced by a lower ATN score, less PMN infiltration and less apoptosis. Interestingly, no statistical difference was seen between the sham and RIPC groups. This is surprising given that the biochemical data supported the stepwise effect of protection seen between the 3 groups, and that the renoprotective effect of immediate RIPC has been shown in both human and animals models[666, 667]. This result is unlikely to be due to sampling error as 100 fields were analysed for each kidney sample. Further work with larger group sizes needs to be carried out to investigate the apparent discordance between the biochemistry and histology data in the sham and RIPC groups.

Future work

1: Further experiments are required to investigate possible mechanisms for the tissue protection seen in both the RIPC and summation RIPC. Candidate markers for the early RIPC are the K_{ATP} channel, PKC, NO and ROS. The mediators involved in delayed RIPC are unknown, however a suitable place to start would be to look at mediators which are known to play a role in delayed IPC: HSP 60, 70 and 72, mSOD and iNOS.

2: Investigation of the apparent discordance between the biochemical outcome data and the histological outcome data in the summation and RIPC groups.

3:Further work should also investigate the effect of delayed RIPC alone in the context of renal IRI. Ideally the current study should have included delayed RIPC alone as a fourth group. The reason for this is that all the initial studies on RIPC employed the RIPC strategy immediately before the prolonged injury. The concept of delayed RIPC is only a recent development and has yet be investigated as fully as early RIPC. Because of this, early RIPC is the default protocol for RIPC. It was because of this that the study planned to answer the question: could the effect of RIPC (early RIPC) be improved upon to confer greater protection? It was not how summation RIPC would differ from early or late RIPC alone. In hindsight, answering the second question would have provided an insight into the contribution of delayed RIPC versus early RIPC.

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