Ischemic Conditioning Protects the Uremic Heart in a Rodent Model of Myocardial Infarction

Running title: Byrne et al.; Ischemic conditioning in uremia

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Abstract:

**Background** - Outcomes following acute myocardial infarction in patients with chronic kidney disease are extremely poor. Ischemic conditioning techniques are amongst the most powerful cytoprotective strategies discovered to date. However, experimental data suggests that comorbidity may attenuate the protective effects of ischemic conditioning.

**Methods and Results** - We conducted investigations into the effects of chronic uremia on myocardial infarct size and the protective effects of ischemic preconditioning (IPC), remote ischemic preconditioning (RIPC) and ischemic post-conditioning (iPost) in two rodent models of chronic uremia. In addition, a limited investigation into the signaling mechanisms involved in cardioprotection following IPC was performed in both uremic and non-uremic animals.

Myocardial infarct size was increased in uremic animals but all three conditioning strategies (IPC, RIPC, iPost) proved highly efficacious in reducing myocardial infarct size (relative reduction [RR] 86% p<0.005, RR 39% p<0.05, RR 65% p<0.05, respectively). Moreover some protocols (IPC and iPost) appeared to be more effective in uremic than in sham (non-uremic) animals. Analysis of the signaling mechanisms revealed that components of both the RISK and SAFE pathways were similarly upregulated in both uremic and non-uremic animals following an IPC stimulus.

**Conclusions** - We conclude that conditioning strategies may present the best opportunity to improve outcomes for patients with chronic kidney disease following an acute coronary syndrome.

**Key words:** preconditioning, postconditioning, myocardial Infarction, renal insufficiency, ischemia
Introduction

Cardiovascular disease is the leading cause of death for patients with moderate to severe chronic kidney disease (CKD, Stages 3-5), and remains amongst the most common cause of death for recipients of renal allografts.1,2 Whilst most cardiovascular deaths are due to heart failure or sudden cardiac death, the rate of acute myocardial infarction (AMI) amongst patients with CKD is more than twice that of patients without CKD.3

There is a graduated risk of death for patients with kidney disease post AMI. Hospital mortality is in the order of 26-32%,4,5 some 15 times the rate for patients without CKD (2% in hospital mortality) and greater than twice the risk of diabetic patients without CKD. One year survival for dialysis patients post MI is a little over 40%6 and despite significant advances in the management of AMI over the last 30 years, there has been little change in the prognosis for patients with CKD post AMI.

Patients with CKD stage 3 and below make up between a quarter to nearly one third of patients presenting with an acute coronary syndrome (ACS).4, 7, 8 Despite making up a sizeable proportion of patients presenting with both ST elevation (STEMI) and non-ST elevation myocardial infarction (NSTEMI) such patients are routinely excluded from clinical trials9, 10 resulting in a dearth of prospective data on which to base clinical practice guidelines.

A consistent feature of observational studies has been the recognition that patients with CKD are less likely to receive reperfusion or revascularization therapies. The underutilization of such treatments, so called “therapeutic nihilism”, was thought to explain much of the excess mortality seen following AMI in patients with impaired renal function and indeed this maybe a contributing factor.4 However emergent data from a number of registry studies suggests that even when optimally treated, outcomes for patients with CKD receiving primary PCI for AMI remain
poor. Even those patients with relatively mild CKD (Stage 3 eGFR 30-59 ml/min) have roughly 7 times the rate of in hospital mortality than patients without CKD. Furthermore, reperfusion therapy seems to have little impact on in hospital mortality for patients with CKD and PCI for those with the most severe degrees of renal dysfunction may actually be harmful. However, in recipients of reperfusion therapy who survive to hospital discharge the long-term prognosis appears to be improved by primary PCI and early revascularization. Additionally in patients with stable coronary artery disease, PCI does not improve outcomes for patients with mild CKD over optimal medical management. The factors determining outcomes for patients with CKD remain largely unstudied.

The cause of the excess mortality seen in patients with CKD following AMI is likely to be multifactorial. Individuals with CKD are at increased risk of bleeding and several studies have highlighted the risk of increased hemorrhagic complications post PCI associated with CKD. However, even when bleeding episodes are corrected for, the outcome for patients with CKD still remains significantly worse suggesting additional factors specific to patients with impaired renal function may be of importance. Patients with CKD are significantly more likely to experience post infarction heart failure and cardiac arrest.

Discovered by Murray and colleagues in 1986, ischemic preconditioning (IPC) describes the phenomenon whereby a brief episode/s of ‘sub-lethal’ ischemia (i.e. of insufficient duration to result in tissue damage) followed by reperfusion, confers resistance to a subsequent more prolonged or lethal episode of ischemia-reperfusion. Subsequently Przyklenk et al. reported that vascular beds adjacent to the preconditioned territory were also rendered resistant to ischemia reperfusion injury (IRI). It was later determined that this cytoprotective effect was also seen between organs and the term remote ischemic preconditioning (RIPC) was coined to
describe this phenomenon. Lastly Zhao et al. extended the scope of ‘conditioning’ when they discovered ischemic post-conditioning (iPost) in 2003. By interrupting reperfusion with three 30 s episodes of ischemia they were able to achieve a substantial reduction in myocardial infarct size.

Ischemic preconditioning, remote ischemic preconditioning and post-conditioning, which we will refer to together as ‘conditioning strategies’, are arguably the most powerful cardioprotective therapies yet discovered and may be considered benchmarks against which to determine the relative efficacy of potential drug therapies. There are data from both animal models and small scale human studies to suggest that the effect of conditioning may be attenuated by diabetes\textsuperscript{18-20} and senescence\textsuperscript{21, 22} as result of perturbations in the signal transduction mechanism.\textsuperscript{23} However, the effect of uremia on the response to conditioning stimuli is unknown.

We conducted a series of experiments to investigate the effects of chronic renal failure on the efficacy of a variety of conditioning protocols.

Methods

All experiments were approved by our institutional ethics committee and performed under license granted by The Home Office (UK) in accordance with the Animals (Scientific Procedures) Act 1986.

Male Wistar rats were used for all experiments (Charles River Laboratories UK, Margate, UK).

Models of chronic uremia

Sub-total nephrectomy

Animals first underwent a two stage sub-total nephrectomy (SNx) or a sham procedure in a
manner similar to that previously described. In brief; animals were anaesthetized with isofluorane (Animalcare, York, UK) and nitrous oxide (BOC, UK) with analgesia (buprenorphine, Reckitt Benckiser Healthcare (UK) Ltd, Hull, UK, 0.04 mg/Kg). Via a flank incision, the left kidney was decapsulated and approximately resected. Hemostasis was achieved with direct compression following which the kidney was returned to the retroperitoneal space. Before closure, 0.5 ml of saline was instilled into the peritoneal cavity. The incision was closed in layers, using 4/0 vicryl for the muscle layers and surgical clips (Precise Vista™, 3M, Bracknell, UK) to the skin. The animals were allowed 14 days to recover before undergoing the second stage of the procedure, right total nephrectomy, which was performed via a flank incision as above. Sham animals had the appropriate kidney decapsulated only. Creation of SNx animals was carried out by a single operator in order to reduce variability in serum creatinine.

The animals were allowed 4 weeks following the second stage (right total nephrectomy) to recover and develop the uremic phenotype before undergoing myocardial ischemia-reperfusion. Adenine Diet (AD)

Following a week’s acclimatization; a diet containing 0.75% (by weight) adenine (Special Diet Services, Essex, UK) was fed to 6 week old male Wistar rats for 4 weeks, as described previously by Yokozawa et al, at the end of which the animals had developed a marked degree of uremia and were used for LAD ligation experiments. A control group was fed normal chow for 4 weeks.

Myocardial ischemia-reperfusion

Myocardial ischemia-reperfusion was carried out in a manner similar to that previously described. Animals were anesthetized with an intra-peritoneal injection of sodium thiopental (73 mg/kg for SNx animals, 88 mg/kg for sham animals, LINK Pharmaceuticals, Horsham, UK),
a tracheostomy performed and an arterial line, to monitor pulse and blood pressure, inserted into the right carotid artery. A further line was inserted into the right jugular vein in order to administer maintenance anesthesia and fluids.

The animals were ventilated using small animal ventilator (Harvard Apparatus, Kent, UK) and ventilatory parameters adjusted in response to arterial blood gas analysis (ABL77, Radiometer Ltd. Crawley, UK).

A left parasternal incision was performed using electrocautery (PromhoVet, Barcelona, Spain), the ribs and thymus retracted and the pericardium resected. A 6/0 silk suture was placed through the myocardium at the approximate level of the left anterior descending artery. A piece of polythene tubing (Portex™, Smiths Medical, Watford, UK), flared at one end, was placed over the free ends of the suture to form a snare.

**Determination of infarct size**

As previously described, \(^{28}\) at the end of reperfusion the LAD was re-occluded, Evan’s blue dye was injected via the right jugular vein in order to delineate the area at risk. The heart was excised and immersed in ice cold 0.9% saline, to achieve cardioplegia, and then sliced in transverse sections to the level of the suture. The right ventricle was dissected from the left and then the blue perfused portion of the LV was separated from the undyed, area at risk. The two portions of tissue were weighed, the ratio indicating that proportion of the LV that was “at risk”. The tissue from the ischemic zone was then diced into small pieces (~1mm\(^3\)) and incubated in nitro-blue tetrazolium (NBT, 0.5 mg/ml) for 30 min. NBT is reduced to a dark blue/purple azole in the presence of reducing compounds enabling the necrotic tissue to be distinguished from viable. The tissue was divided into two aliquots, on the basis of presence of purple staining. Infarct size was given by the ratio of the weights of these two quantities of myocardium.
Cardiac Troponin I (cTnI) Assay

A cardiac specific rat troponin I (cTnI) enzyme linked immunosorbent assay (Life Diagnositics, PA, USA) was used. Blood was drawn into a heparinized syringe after 2 h reperfusion. The blood was immediately separated in a centrifuge, the plasma aspirated and snap frozen in liquid nitrogen before being stored at -80°C until assayed. The assay was performed in accordance with the manufacturer’s instructions with the only modification being a 1:8 dilution of the plasma samples, performed using the ‘standard’ diluent contained in the kit. A further 1:4 dilution was performed using ‘plasma diluent’, as specified in the manufacturer’s instructions, giving a final dilution of 1:32. This was carried out in order to maintain the optical density within the range of the standard curve.

Conditioning protocols

(see Figure 1)

Ischemic preconditioning

1 or 3 cycles of 5 min LAD occlusion (ischemia) followed by 5 min reperfusion before either 25 min (standard) or 35 min of sustained ischemia (threshold experiment).

Remote preconditioning

The left femoral artery was carefully dissected out and separated from the femoral vein and nerve. A ligature was placed around the artery to assist in mobilization. A microvessel clip was used to occlude the artery. Pallor and a reduction in the temperature of the paw confirmed occlusion. Reperfusion was confirmed by hyperemia followed by restoration of normal color and temperature. Three cycles of 5 min ischemia followed by 5 min reperfusion were employed.

Post-conditioning

LAD occlusion (ischemia) for 25 min with 5 cycles of 10 s reperfusion/ 10 s ischemia upon
reperfusion then a further 1 h 58 min and 20 s reperfusion (i.e. 2 h reperfusion in total). At the end of the final reperfusion period the animals were sacrificed and the heart harvested for determination of infarct size.

**Immunoblotting and Protein extraction**

Hearts from sham operated or uremic animals, subjected to the IPC protocol detailed above were excised, washed once in ice-cold PBS, snap frozen in liquid N2 and stored at -80°C until further use. A total of 20 rats (10 SNx, 10 sham-operated controls), split into 4 groups of 5 were used to study the response to a preconditioning stimulus in the sub-total nephrectomy model. A further 15 rats were used to investigate signal transduction in the AD model (8 control animals split into 2 groups of 4 and 7 AD animals 3 of which were preconditioned).

Tissues for western blot were triturred with a mortar and pestle under liquid N2 and subsequently polytron-homogenised (3x 30 s bursts with 1 min intervals on ice) in a mammalian protein extraction buffer (GE healthcare; 10:1 v/w) supplemented with the following inhibitors: 1 mM EDTA, 0.5 mM DTT, 1% v/v protease inhibitor cocktail (Sigma), 1 mM NaF, 5 μM fenvalerate (Calbiochem), 1mM Na3VO4 and 1% v/v phosphatase inhibitor cocktails I and III (Sigma). The tissue homogenate was incubated for 10 min on ice and then centrifuged at 5,000 g for 10 min at 4°C. The supernatant was aliquoted and stored at -80°C 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 (40 μg of protein) were subjected to SDS-polyacrylamide gel electrophoresis using the NuPAGE electrophoresis system (Invitrogen) under reducing conditions as previously described. The ECLplus chemiluminescence detection kit (Amersham Pharmacia) was used to visualize protein bands. The following antibodies (supplied by Cell Signalling) were used: rabbit
anti-phospho-p44/p42 MAPK (Thr202/Tyr204), rabbit anti-p44/p42 (total ERK), mouse anti-
phospho AKT (Ser473), rabbit anti-AKT (total AKT), mouse anti-phospho-STAT3 (Tyr705),
rabbit anti-STAT3 (total STAT3) and peroxidase-conjugated secondary antibodies.

Densiometry was performed using Image J software on the immunoblots obtained. Method
as previously described.\textsuperscript{30}

Statistical analysis

Data was analyzed with GraphPad Prism software (San Diego, Ca, USA). Given the
relatively small sample size in some of the experiments, non-parametric statistical analysis was
used when possible and the data are presented as median with interquartile range (IQR). Two-
way ANOVA with Bonferroni post-test comparison was employed to analyze the results of
experiments with a 2x2 factorial design (immunoblot studies, RIPC and iPost), a two tail Mann-
Whitney U-test was used to test for significance in all other experiments.

Results

Determination of myocardial infarct size in uremic animals - reduced ischemia tolerance?

In our first series of experiments we sought to replicate previously published data
suggesting that uremic animals sustain larger myocardial infarctions than non-uremic sham
operated controls.\textsuperscript{31} We were able to demonstrate a modest but statistically significant increase in
myocardial infarct size in uremic rats compared with sham animals (\textbf{Figure 2}, sham 47.2\% [IQR
39.8-63.7\%] vs. SNx 62.3\% [53.5-69.5\%], a relative increase of 32\%, \textit{p}=0.03). The increase in
infarct size was remarkably similar to that reported by Dikow and colleagues (33\% relative
increase in infarct size).\textsuperscript{31} The area at risk was similar for both groups (Sham 50.9\% [43.5-
56.2\%] vs. SNx 44.8\% [42.2 – 51.4\%], \textit{p}=0.24).
Uremic (SNx) animals had a median serum creatinine, which was roughly three times that of the sham animals (91 μmol/l [83.7-116 μmol/l] vs. 34.4 [33.4-35.7 μmol/l]) and were significantly more anemic (SNx hematocrit 27% [24-31%] vs. Sham 39% [36.3-41%], p=0.0003).

Ischemic preconditioning (IPC) of uremic animals

Employing a pre-conditioning protocol consisting of 3 cycles of 5 min ischemia/ 5 min reperfusion we were able to achieve significant myocardial protection with an absolute reduction in infarct size of more than 50% (Figure 3A - Control 61.2% [54.2-70.5] IPC 7.7% [6-10%], relative reduction of 86% p=0.002), the area at risk (AAR) was similar between the two groups (Control 44.4 % [41.3-49.5%], IPC 42.2 % [37.5-47.3%], p=0.447) as was serum creatinine (Control 83.5 μmol/l [74-93.3 μmol/l] vs. IPC 89.7 μmol/l [87.6-92.2 μmol/l] p=0.211).

Utilizing the same protocol, we performed an additional experiment (Figure 3B) employing an alternative model of chronic uremia (AD). These animals have significantly greater renal dysfunction (median serum creatinine ~8 fold higher than in controls c.f. SNx ~3 fold greater than sham animals). Yet despite this we still observed a relative reduction in median infarct size of almost 50% (Median infarct size of 50.6% [34.9-75.8%] in control animals, 29.4% [7.7-32.1%] in IPC group, p=0.014).

Threshold for Ischemic preconditioning of Uremic Animals

Having established convincingly that uremic animals could be preconditioned, we sought to determine if there was an increase in the threshold for eliciting cytoprotection with IPC in uremic animals. As near maximal protection was seen with 1 cycle of IPC and 25 min of sustained ischemia, we increased the period of sustained ischemia to 35 min for this series of experiments.
In contrast to diabetic and senescent animals, far from seeing a blunting of the effect of a single cycle of preconditioning, in uremic animals we demonstrated a significant reduction in the infarct size of uremic (SNx) animals receiving one cycle of IPC compared to non-uremic (sham) animals receiving a single cycle of IPC (median 18.1% [IQR 7.2-23.6] and 32.1% [IQR 22.9-46.6] respectively, p=<0.02, Figure 3C).

**Remote Ischemic Preconditioning (RIPC)**

RIPC markedly attenuated myocardial ischemia-reperfusion injury (p<0.0001, 2-way ANOVA). The absolute reduction in infarct size achieved with RIPC was comparable between the two groups (sham 32.3%, p<0.001 vs. SNx 30.9% p<0.0001, Bonferroni multiple comparison). However, relative reduction (RR) in infarct size was substantially but not significantly greater in non-uremic sham-operated rats (59.3% RR in sham vs. 48.3% RR in SNx, p=0.328, Mann-Whitney, see Figure 4). There was a trend towards an increased infarct size in uremic animals, which was of borderline statistical significance (p=0.062). No interaction between uremia and RIPC was demonstrated (p=0.572, 2-way ANOVA).

**Ischemic Post-conditioning (iPost)**

A post-conditioning protocol consisting of 5 cycles of: 10 s reperfusion/ 10 s ischemia commenced after a 25 min episode of sustained ischemia was employed (see Figure 5). As with all other conditioning protocols a substantial and statistically significant reduction in myocardial infarct size was observed (47% RR for non-uremic animals, 65% RR for SNx animals, p=0.0014, 2-way ANOVA). No evidence of an interaction was seen between renal function and response to iPost, i.e. iPost had an equivalent effect on infarct size reduction irrespective of the presence of uremia. Although uremic animals had a greater RR in infarct size, this was not statistically significant (Mann-Whitney). The area at risk was similar for all groups.
These results were mirrored by a reduction in the levels of cTnI in post-conditioned animals (p=0.0043, 2-way ANOVA. see Figure 5D). No interaction was observed (p=0.7312).

**Western blots**

In light of previous reports of altered signal transduction associated with co-morbidity\(^\text{18}\) and senescence,\(^\text{32}\) a limited study of signaling mechanisms was undertaken in both the SNx and AD models. A marked increase in both phosphorylated STAT3 (SNx p=0.001, AD p=0.02) and ERK1/2 (SNx were consistently demonstrated following 3 cycles (5 min ischemia, 5 min reperfusion) of IPC (see Figure 6 and Figure 7). The increase in phospho-STAT3 and phospho-ERK1/2 was similar in uremic and non-uremic animals (SNx p=0.034, AD p=0.002). However, in our hands we were unable to demonstrate a consistent result with respect to the phosphorylation of Akt in response to the preconditioning protocol.

**Discussion**

These data represent a comprehensive assessment of the effects of chronic uremia on ischemic conditioning and demonstrate that, unlike in diabetic,\(^\text{18-20}\) dyslipidaemic\(^\text{33, 34}\) and senescent animals,\(^\text{21, 22}\) chronic uremia does not appear to attenuate the effect of ischemic preconditioning, remote ischemic preconditioning or ischemic post-conditioning. Indeed uremic animals appear to derive greater protection than non-uremic animals from protocols that involve manipulating the blood supply of the target organ directly (i.e. IPC and iPost). In the context of chronic uremia, the IPC signal appears to be conducted through the same pathways.

Furthermore we confirm the previously published observation of increased myocardial infarct size in uremic animals.\(^\text{31}\) Larger myocardial infarctions may in part explain the greater mortality, and in particular the increased incidence of heart failure, seen in patients with CKD.
post AMI. Left ventricular hypertrophy is a key feature of the uremic cardiac phenotype and is extremely common in individuals with ESRD$^{35}$ as well as experimental models of uremia.$^{36}$ However, in contrast to patients with left ventricular hypertrophy with normal renal function, LVH occurring in the context of uremia appears to result in a greater degree of intermyocyte fibrosis and capillary rarefaction.$^{37}$ Thus there is the potential for a simultaneous increase in oxygen demand (as a result of myocyte hypertrophy) and reduction in oxygen delivery consequent upon reduced capillary density and increased oxygen diffusion distance. The cardiomyocyte in the uremic heart is thus closer to the brink of ischemia.

The reduction in infarct size for uremic animals subjected to IPC was significantly greater than non-uremic animals subjected to IPC and a substantial difference, of borderline statistical significance, was seen in animals that received iPost. These observations might be the result of Type 2 error. However, it should also be noted that there was a non-significant trend towards a smaller area at risk in the SNx animals that received a single cycle of preconditioning. Alternatively, it is possible that as a result of a lower hematocrit, the uremic animals had less ‘no-reflow’ and thus the combination of either IPC or iPost, together with more extensive reperfusion, resulted in even smaller infarct sizes in these animals compared to the less anemic sham operated (non-uremic) animals. Of note uremic animals, treated with RIPC faired no better than their sham-operated counterparts. It is interesting to compare these results with those of Gritsopoulos et al.$^{38}$ who found that remote post-conditioning was a more potent cytoprotective strategy than classical post-conditioning, suggesting that further direct interference with the dependent vascular bed, which had experienced the index ischemic episode, was not entirely without consequence.
Given that ageing, diabetes and a variety of other metabolic disturbances have been acknowledged to reduce the efficacy of conditioning in both animal and human models. It is somewhat surprising that renal dysfunction does not appear to have a similar effect. We examined the effect of IPC in two different models of chronic uremia, the sub-total nephrectomy (SNx) model, the most commonly used model to study the pathophysiology of the uremic state, and adenine diet (AD) induced uremia. In our hands, SNx animals are typically hypertensive with LVH and have more significant anemia in comparison to those animals with renal dysfunction induced by AD. However, animals with AD may be considered to be more uremic in that the median serum creatinine is approximately 8 times that of controls, where as in SNx animals the median serum creatinine is around three fold greater. Despite this apparent disparity in the degree of renal dysfunction, AD animals that received preconditioning had infarct sizes that were nearly 50% smaller. We believe that this agreement in results obtained from experiments employing these two different models is mutually supportive.

In addition we have provided evidence that three cycles of IPC (5min ischemia, 5min reperfusion) is associated with a robust and reproducible increase in phospho-Stat3 and phospho-ERK1/2 representing key components of the two putative signal transduction mechanisms, the RISK pathway and the SAFE pathway.

Ischemic preconditioning is thought to be triggered by a number of factors including adenosine, bradykinin and endogenous opioids. Two major intracellular signaling cascades have been proposed as mediators of the preconditioning stimulus;

- The Reperfusion Injury Salvage Kinase (RISK) pathway
- The Survivor Activating Factor Enhancement (SAFE) pathway
In addition, both these pathways have also been implicated in mediating the infarct size limiting effects of iPost.

The RISK pathway is considered to consist of two arms, one arm involving MEK1/2 and ERK1/2 and the other PI3 kinase and Akt. Both arms are thought to be triggered by the binding of specific ligands with G-protein coupled receptors and terminate on glycogen synthase kinase-3-beta (GSK-3β) to reduce the likelihood of mitochondrial permeability transition pore (MPTP) opening and thus attenuate reperfusion injury.39

The SAFE pathway was first identified as the mediator of cardioprotection induced by tumour necrosis factor alpha (TNFα).40 However, JAK/Stat signaling, which is at the heart of the SAFE pathway, is now recognized as important in the transduction of a number of other infarct size limiting therapies and has been implicated in IPC (both the early and late phases41), iPost42 and RIPC. As with the RISK pathway, it is thought that inhibition of the MPTP is the end-effector of this pathway.

It is nearly a quarter of a century since Murray and colleagues16 made their seminal discovery of ischemic preconditioning and yet despite the widespread acknowledgement that ischemic conditioning represents the most powerful cytoprotective strategies yet discovered, with overwhelming animal data to attest to the benefit of conditioning, it has failed to gain acceptance within the broader medical community or to be adopted into routine clinical practice. This is possibly due to a perceived, comparative lack of efficacy of conditioning in humans. Human trials conducted to date have mostly been small scale, usually from a single center, with limited length of follow-up, thus being underpowered to demonstrate mortality differences. They have therefore employed surrogate outcome measures, such as the magnitude of rise in biomarkers of cardiac ischemia.43
Animal models that are used to evaluate cytoprotective strategies usually employ healthy, juvenile animals. This is in marked contrast to human disease, which rarely occurs in the absence of comorbidities. Such comorbid conditions may influence the efficacy of experimental treatments. Furthermore animal studies typically compare treated with untreated animals whereas in clinical trials; a new treatment, in addition to existing therapy, is compared to existing therapy alone.

Renal dysfunction is a common comorbidity in those presenting with AMI and engenders an adverse prognosis. It may also modulate the risk benefit relationship for existing therapies. The fundamental purpose of our studies was to examine the effect of impaired renal function on conditioning strategies.

It has been suggested that reperfusion therapy alone is sufficient to maximally limit infarct size for the majority of patients. However, it is estimated that one quarter of patients still sustain myocardial infarcts of >75% of the area at risk. In a recently published clinical trial of remote ischemic conditioning, for a given area at risk, patients that received remote ischemic conditioning had a smaller infarct size. This effect was most marked for those with the largest area at risk. A similar effect was seen in small clinical trial of iPost as an adjunct to primary PCI. Infarct size is a major risk factor for the development of post AMI heart failure and therefore long-term prognosis. With this in mind, we speculate that a major reason for the apparently disappointing results of human trials of conditioning may be due to poor patient selection. In that we should be targeting patients at the highest risk of an adverse outcome to receive adjuvant cardioprotective therapies. Therefore we would envisage that post AMI outcome might be improved for patients with CKD by the addition of a conditioning strategy to early reperfusion.
The higher event rate seen in patients with CKD and ESRD could in theory allow for trials, sufficiently powered to detect differences in mortality, to be conducted with a smaller sample size. It would be desirable to corroborate our findings in human subjects with CKD and ESRD.

In conclusion, we report for the first time that; conditioning strategies provide powerful cardioprotection in the setting of experimental uremia. There is an urgent and at present unmet need to improve outcomes for patients with CKD suffering AMI. The routine exclusion of individuals with impaired renal function from clinical trials impedes the advancement of the care of these patients. Patients with CKD may derive benefit from conditioning strategies over and above that seen in patients without CKD.

Conflict of Interest Disclosures: None

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**Figure Legends:**

**Figure 1.** Diagrammatic representation of conditioning protocols

**Figure 2.** Uremic rats sustain larger myocardial infarcts compared to sham-operated controls

(A) SNx animals sustained myocardial infarcts that were approximately 1/3rd larger than sham operated controls (32%, p=0.033,*). (B) The area at risk was similar for both groups (p=0.235).

(C) Uremic animals were significantly more anemic, as evidenced by a lower hematocrit, 27% as
opposed to 39% (p=0.0003,***). (D) Median serum creatinine was 34.4 μmol/l in the sham animals and 91.0 μmol/l in the uremic (SNx) animals (p<0.0001,****). Box and Whisker plots – Whiskers indicate minimum and maximum values, Box – the interquartile range (IQR), line – the median and ‘+’ the mean.

**Figure 3.** Ischemic preconditioning markedly reduced infarct size in uremic animals and was more effective in uremic than non-uremic animals. (A) Three cycles of IPC (5 min ischemia/ 5 min reperfusion) is highly effective in protecting the uremic heart (SNx model) against ischemia-reperfusion injury (p=<0.0001,****). Area at risk (p=0.305) and median creatinine were similar for the two groups (84 and 90 μmol/l respectively, p=0.182). (B) The same protocol proved efficacious in an alternative model of chronic uremia (adenine diet model) despite substantially worse renal function. The median creatinine was the similar for both groups (250 and 285 μmol/l respectively, p=0.902). (C) A single cycle of IPC was more effective in limiting myocardial infarct size in uremic animals after 35 min ischemia (2 h reperfusion) than in sham operated control animals (p=0.031). Box and whisker plots: whiskers indicate maximum and minimum, box interquartile range, line the median and ‘+’ indicates the mean.

**Figure 4.** RIPC of the hind limb reduces myocardial infarct size in experimental chronic uremia. (A) RIPC was associated with a significant reduction in infarct size (p<0.0001,****). The relative reduction in infarct size was similar for both uremic and non-uremic animals (p=0.456, unpaired t-test). (B) The area at risk was similar for all four groups (p=0.466, Kruskal-Wallis). (C) Plasma creatinine was significantly higher in SNx animals (p<0.0001,****). Hematocrit was significantly lower in SNx animals (p<0.0001,****), there was no within group difference. Box
and whisker plots: whiskers indicate maximum and minimum, box interquartile range, line the median and ‘+’ indicates the mean.

**Figure 5.** Ischemic post-conditioning (iPost) reduces infarct size in uremic animals.

(A) iPost resulted in a significant reduction in myocardial infarct size in both uremic and non-uremic animals (p<0.0001,****, 2-way ANOVA). (B) Area at risk was similar for all four groups (p=0.444, Kruskal-Wallis). (C) Plasma creatinine was significantly higher in the SNx animals compared to sham-operated controls (p<0.0001,****). There was no difference in Creatinine between the SNx groups (p>0.05, Bonferroni multiple comparison). (D) Plasma troponin following 2 h reperfusion was significantly less in post-conditioned animals (p=0.0043,**). Box and whisker plots: whiskers indicate maximum and minimum, box interquartile range, line the median and ‘+’ indicates the mean.

**Figure 6.** Immunoblots for pERK, tot-ERK, pSTAT3 and tot-STAT3. (A) Representative immunoblot. (B) A significant increase in pERK was seen in both uremic and non-uremic animals following 3 cycles of IPC (p=0.034,*). (C) No differences in total ERK levels were seen in either uremic or non-uremic animals. (D) The ratio of pERK/ tot-ERK was significantly increased following IPC but did not differ in magnitude between uremic and non-uremic animals (p=0.013,*). (E) Levels of phosphorylated STAT3 were significantly increased by IPC (p=0.0014,**). (F) There was no significant difference between the four groups in the levels of total STAT3. (G) The ratio of phosphorylated to total STAT3 was significantly increased following IPC but did not differ between uremic and non-uremic animals (p=0.0001,***). Bar charts show mean with SEM. N=5 for each group.
Figure 7. Immunoblots of pERK, tot-ERK, pSTAT3 and tot-STAT3 in AD animals. (A) Representative immunoblot. (B) A significant increase in pERK was seen in both uremic and non-uremic animals following 3 cycles of IPC (p=0.018,**). (C) No differences in total ERK levels were seen in either uremic or non-uremic animals. (D) The ratio of pERK/ tot-ERK was significantly increased following IPC but did not differ in magnitude between uremic and non-uremic animals (p=0.0077,**). (E) Levels of phosphorylated STAT3 were significantly increased by IPC (p=0.02,*). (F) There was no significant difference between the four groups in the levels of total STAT3. (G) The ratio of phosphorylated to total STAT3 was significantly increased following IPC but did not differ between uremic and non-uremic animals (p<0.0001,****). Bar charts show mean with SEM. N=4 for each group except AD-IPC where N=3.
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