Original article

Ischaemic conditioning reduces kidney injury in an experimental large-animal model of warm renal ischaemia

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Background: **Ischaemic conditioning, using short repeated sequences of intermittent ischaemia, is a strategy that may ameliorate ischaemia–reperfusion injury. The aim of the study was to assess the effects of direct and remote ischaemic conditioning in a porcine model of renal warm ischaemia–reperfusion injury.**

Methods: **Pigs (50 kg) underwent laparotomy and 60-min occlusion of the left renal pedicle followed by right nephrectomy. Animals were divided into three groups: untreated controls (*n* = 8); direct postconditioning involving six 15-s cycles of clamping then releasing of the left renal artery (*n* = 7); or remote periconditioning involving four 5-min cycles of clamping then releasing of the left common iliac artery (*n* = 8). After 7 days kidney tissue was harvested, and blood and urine samples were collected on postoperative days 1, 3 and 7.**

Results: **The direct postconditioning group had a lower area under the serum creatinine curve (mean(s.d.) 1378(157) *versus* 2001(1022) μmol/l · day respectively; $P = 0{\cdot}036$) and peak creatinine level (316(46) *versus* 501(253) μmol/l respectively; $P = 0{\cdot}033$) compared with values in control animals. There was a significant increase in serum levels of tumour necrosis factor α on day 1 in control animals but not in the conditioning groups ($P = 0{\cdot}013$). Urinary levels of neutrophil gelatinase-associated lipocalin increased over the study period in both the control and remote groups ($P = 0{\cdot}001$ for both), but not in the direct group ($P = 0{\cdot}176$). There was no mortality and no complications related to either conditioning technique.**

Conclusion: **In this *in vivo* large-animal model, direct renal artery ischaemic postconditioning protected kidneys against warm ischaemia injury. This straightforward technique could readily be translated into clinical practice.**

Surgical relevance

Ischaemic conditioning has been shown to improve outcomes in both experimental studies and clinical trials in cardiac surgery. Evidence from small-animal and human studies assessing ischaemic conditioning techniques in renal transplantation have not yet established the optimal technique and timing of conditioning.

In this study, a large-animal model of renal warm ischaemia was used to compare different conditioning techniques. Postconditioning applied directly to the renal artery was shown to reduce renal injury. Furthermore, new evidence is provided that shorter cycles of ischaemic postconditioning than previously described can protect against renal injury.

Evidence from a large-animal model is provided for different conditioning techniques. The beneficial postconditioning technique described is straightforward to perform and provides an alternative method of conditioning following renal transplantation, with potential for application in clinical practice.

Presented to the Annual Meeting of the Society of Academic and Research Surgery, Durham, UK, January 2015

Paper accepted 1 July 2015

Published online 12 August 2015 in Wiley Online Library (www.bjs.co.uk). **DOI:** 10.1002/bjs.9909

Introduction

Renal transplantation from donation after circulatory death (DCD) donors has increased nearly tenfold in the past decade[1]. DCD organs are exposed to a period of warm ischaemic injury before retrieval. Following transplantation the organ is reperfused and the pre-existing tissue injury is exacerbated[2]. The consequence of these events is ischaemia–reperfusion injury (IRI), a series of inflammatory and cellular processes that cause damage to the tissue. IRI involves the production of reactive oxygen species, upregulation of inflammatory mediators such as tumour necrosis factor (TNF) α, infiltration of leucocytes, and necrosis and apoptosis[3–7]. The resulting organ damage leads to delayed graft function (DGF), which increases the risk of early graft loss and acute rejection, causes significant patient morbidity, prolongs hospital stay and increases costs[7–13].

Therapies to improve outcomes from DCD transplants have the potential to increase the pool of available organs, prolong graft survival and reduce patient morbidity. Ischaemic conditioning (IC) is a technique that upregulates host defences against ischaemic injury[14]. The mechanism of protection involves mediators such as adenosine, bradykinin and opioids, which target end effectors such as mitochondrial K_{ATP} channels to protect against ischaemia[14–16]. IC involves short repeated cycles of ischaemia followed by reperfusion, and is performed by sequentially occluding and releasing a selected artery. Conditioning can be direct, during which the target organ has its blood supply modulated, or remote, where an organ or anatomical region distant from the target organ has its blood flow modulated. Furthermore, conditioning can be performed before (preconditioning), during (periconditioning) or after (postconditioning) target organ ischaemia.

There is clinical and experimental evidence showing the benefit of IC in the cardiac setting, and evidence of renal protection in a small- and large-animal model, although follow-up time in the large-animal study was limited to 10 h of reperfusion[14–19]. There are two clinical studies in DCD transplant recipients that have examined two different conditioning techniques; the first[20] assessed direct IC and the second[21] considered remote IC, with conflicting results. Thus, the optimal technique of IC in renal warm ischaemia remains unclear. The aim of this study was to assess the role of both direct postconditioning (DC) and remote periconditioning (RP) in an *ex vivo* porcine kidney model of warm ischaemic injury.

Methods

Animal welfare

Animal welfare and experimental procedures were in adherence with Home Office codes of practice and the Animals (Scientific Procedures) Act 1986. Study design was ratified and ethical approval obtained through standard Home Office procedures. Female Landrace pigs (50 kg) were used following a minimum of 3 weeks' acclimatization at the experimental site. The Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines[22] were followed throughout the study. Mead's 1988 resource equation was used to determine sample size and an additional animal was added to each group to account for potential difficulties with the technique.

Study design

Pigs were allocated randomly into four groups: group 1, sham ($n=2$); group 2, control ($n=8$); group 3, DC ($n=8$); and group 4, RP ($n=8$) (*Fig. 1*). Control, DC and RP groups underwent the standard surgical procedure described below, with the same primary surgeons for all procedures. Sham animals had an identical surgical procedure without cross-clamping of the renal pedicle. After surgery, pigs were recovered for 7 days and then killed by lethal injection of intravenous pentobarbital.

Randomization

Administrative staff at the animal institution performed randomization and were independent of all staff directly involved with the study. A pseudo-random bodyweight stratification procedure, which yielded groups with approximately equal mean bodyweight, was used. There was no significant difference in the mean(s.d.) weights of the animals before surgery (sham 49·0(2·8) kg, control 40·2(3·3) kg, DC 40·3(3·9) kg, RP 41·1(4·9) kg; $P=0·883$). On each day the order of surgery was randomized so that the pigs received one of the three treatments.

Anaesthesia

Animals were sedated and anaesthetized following standard protocols for the study centre. Anaesthesia was maintained with isofluorane in oxygen via positive-pressure ventilation. All animals received intravenous Augmentin® 25 mg/kg (GlaxoSmithKline, Stevenage, UK) at induction. Remifentanil 2 μg/ml (GlaxoSmithKline) was given as a continuous intravenous infusion for analgesia and maintenance fluids were delivered at 2 ml per kg per h (Hartmann's solution;

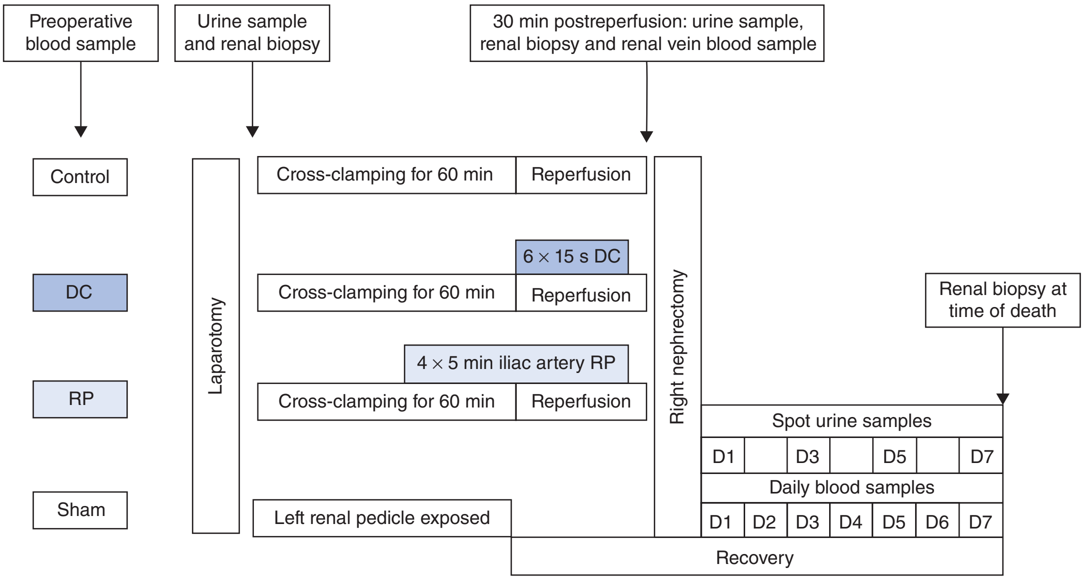

Fig. 1 Animals were anaesthetized and those in the sham group underwent midline laparotomy, exposure of the left renal pedicle and then right nephrectomy. The experimental groups underwent laparotomy, exposure of the left renal pedicle and then cross-clamping of the pedicle for 60 min. Animals in the DC group had 6 × 15-s cycles of occlusion and release of the renal artery, following reperfusion. RP animals underwent 4 × 5-min of occlusion and release of the common iliac artery 20 min prior to reperfusion. Following clamp release, a right nephrectomy was performed, the animal was reperfused, the abdomen closed and the animal was recovered for 7 days. Renal biopsies were taken before cross-clamping, 30 min after reperfusion and at autopsy, and serum and urine samples were collected during the recovery period. DC, direct postconditioning; RP, remote periconditioning; D, day

Aquapharm, Glasgow, UK) throughout surgery. Oxygen saturation, heart rate, respiratory rate, expired carbon dioxide, body temperature, electrocardiography and blood metabolic parameters were monitored throughout surgery.

Surgical technique

The pig was placed supine and the abdomen opened through a midline incision. The left renal pedicle was exposed and 5000 units of heparin administered. The renal vessels were then cross-clamped for 60 min. During the period of warm renal ischaemia a double-lumen cuffed silicone vascular access catheter (Tyco Healthcare, Rugby, UK) was placed in the right external jugular vein via surgical cut-down. The lumens of the central line were locked with 1·5 ml heparin, 1000 units/ml (Multiparin®; CP Pharmaceuticals, Wrexham, UK). After 60 min the left renal clamp was released and the kidney was reperfused. In the control group a right nephrectomy was then performed, and the renal artery and vein were suture-ligated with Prolene™ (Ethicon, Livingston, UK). The ureter was ligated and divided. The abdomen was mass-closed using loop PDS™ (Ethicon), and the skin was closed using Vicryl Rapide™ (Ethicon).

Direct postconditioning

Immediately after release of the vascular clamp occluding the left renal vessels, a sequence of six cycles of ischaemia for 15 s followed by reperfusion was performed. The non-traumatic vascular clamp used for the 60-min ischaemic period was removed repeatedly from the left renal vessels and then replaced for the 15-s cycles. The procedure then followed the same sequence described above.

Remote periconditioning

After 40 min of left renal ischaemia, the left common iliac artery was occluded for 5 min followed by 5 min of reperfusion. Four cycles of occlusion for 5 min followed by 5 min of reperfusion were performed. Removal of the clamp occluding the left renal vessels coincided with the end of the second cycle of conditioning. The procedure then followed the same sequence described above.

Postoperative care

Animal recovery was standardized. To ensure adequate hydration, Ringer's lactate solution (40 ml per kg per

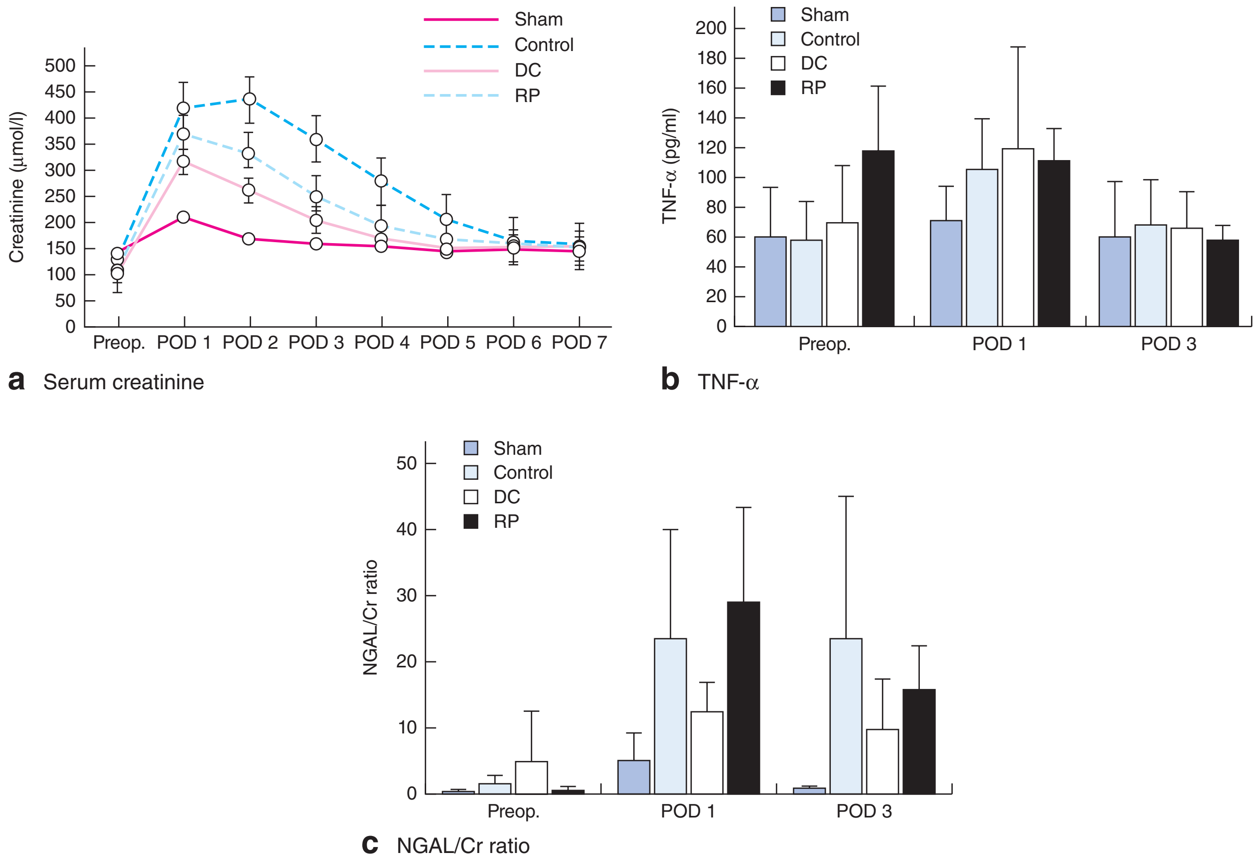

Fig. 2 **a** Mean(s.e.m.) serum creatinine levels before and after ischaemic injury in sham, control, direct postconditioning (DC) and remote periconditioning (RP) groups. Postoperative day (POD) 1, $P = 0{\cdot}007$; POD 2, $P = 0{\cdot}022$ (DC *versus* control group, *t* test). **b** Mean(s.d.) plasma levels of tumour necrosis factor (TNF) α before surgery and on POD 1 and 3 postischaemia. $P = 0{\cdot}013$ (control group on POD 1 *versus* preoperative value, *t* test). **c** Mean(s.d.) urinary neutrophil gelatinase-associated lipocalin/creatinine (NGAL/Cr) ratios before, and on POD 1 and 3. $P = 0{\cdot}001$, control and RP groups; $P = 0{\cdot}176$, DC group (POD 1 and 3 *versus* preoperative values, ANOVA)

24 h) (GlaxoSmithKline) was administered intravenously for 48 h after operation; animals had free access to water immediately and food was introduced on the first postoperative day. Intravenous buprenorphine 30 µg/kg (Schering-Plough, Welwyn Garden City, UK) was administered every 8–10 h for up to 4 days as postoperative analgesia. Additional analgesia was administered at the discretion of the veterinary surgeon responsible for care of the animals.

Sampling

Blood

Venous blood samples were taken before operation, at 30 min after reperfusion from the renal vein, and then daily from the venous catheter. Samples were centrifuged at 1000*g* for 15 min, and either analysed immediately or stored at −80°C.

Urine

Urine was collected where possible before surgery, and on days 1, 3, 5 and 7. This was achieved using either clean-catch sampling or temporary placement of the animals in a metabolic cage. Samples were centrifuged at 1000*g* for 15 min and stored at −80°C.

Renal tissue

A needle core biopsy (16 G) was taken before ischaemic injury, 30 min after reperfusion and at 7 days postischaemia at post-mortem examination, immediately after exsanguination. Samples were either fixed in 10 per cent formal saline or snap-frozen in liquid nitrogen and then stored at −80°C.

Table 1 Creatinine and urea values as markers of renal function in sham, control, direct postconditioning and remote periconditioning groups

	Sham	Control	DC	RP	P*
Creatinine					
AUC (μmol/l · day)	949(38)	2001(1022)	1378(157)	1704(357)	0·036
Peak (μmol/l)	210(8)	501(253)	316(46)	374(116)	0·033
T_{max} (days)	1(0)	1·5(0·1)	1(0)	1(0)	0·026
T_{crit} (days)	2·0(0)	4·5(1·3)	3·6(0·5)	4·3(1·3)	0·608
Urea					
AUC (μmol/l · day)	36·4(7·7)	72·2(34·3)	49·9(7·8)	59·9(12·5)	0·076
Peak (mmol/l)	8·9(1·8)	17·2(8·9)	11·9(3·1)	14·1(2·9)	0·041
T_{max} (days)	1·0(0)	2·0(0·8)	1·3(0·5)	1·8(0·7)	0·590
T_{crit} (days)	1·0(0)	4·4(1·1)	3·4(1·0)	3·9(1·1)	0·248

Values are mean(s.d.). AUC, area under the curve; T_{max}, time to peak level; T_{crit}, time for serum level to fall below 200 μmol/l for creatinine and 8 mmol/l for urea. DC, direct postconditioning; RP, remote periconditioning. *DC *versus* control group (*t* test).

Table 2 Plasma levels of endothelin 1, percentage superoxide dismutase inhibition and total nitric oxide in the renal vein 30 min after reperfusion in control, direct postconditioning and remote periconditioning groups

	Control	DC	RP	P*
Endothelin 1 (pg/ml)	2·6(1·9)	1·6(1·0)	2·0(1·2)	0·468
SOD (% inhibition)	59·0(4·0)	62·1(5·1)	57·8(3·5)	0·158
Total nitric oxide (pg/ml)	29·7(15·5)	19·4(16·1)	11·2(9·1)	0·060

Values are mean(s.d.). DC, direct postconditioning; RP, remote periconditioning; SOD, superoxide dismutase. *ANOVA.

Outcome measures

Analysis of urine samples

Urinary neutrophil gelatinase-associated lipocalin (NGAL) levels were determined using a pig NGAL sandwich enzyme-linked immunosorbent assay kit (BioPorto Diagnostics, Gentofte, Denmark). The samples were added in duplicate to the precoated wells and analysed according to the manufacturer's instructions.

Analysis of blood samples

TNF-α levels were determined by the quantitative sandwich enzyme immunoassay technique (R&D Systems, Minneapolis, Minnesota, USA). Measurement of endothelin (ET) 1 levels (EnzoLifescience, Exeter, UK), total nitric oxide colorimetric assay (BioVision, Milpitas, California, USA) and OxiSelect™ superoxide dismutase (SOD) activity assay (Cell Biolabs, San Diego, California, USA) were done in plasma samples taken directly from the renal vein 30 min after reperfusion. All assays were carried out in duplicate in accordance with the manufacturers' instructions.

Histology

Biopsies were fixed in formalin (10 per cent) and embedded in paraffin; 4-μm sections were cut and stained with haematoxylin and eosin. A consultant pathologist blinded to the group allocation and time points scored the sample to assess the level of injury. Biopsies were scored 0 (indistinguishable from normal), 1 (minor subjective changes, mostly tubular vacuolation) or 2 (definite tubular injury including dilatation of tubules and flattening of epithelium, with or without debris in the lumen).

Statistical analysis

Data are presented as mean(s.e.m.) or mean(s.d.). Multiple groups were compared using ANOVA. Comparison of two groups with normally distributed data was done with the unpaired *t* test with Bonferroni correction; $P < 0·025$ was considered significant. The Mann–Whitney *U* test was used for comparison of two groups of data that were not normally distributed. Categorical variables were compared with the χ^2 test. Statistical analysis was performed using InStat® and Prism® 6 software (GraphPad Software, San Diego, California, USA). $P < 0·050$ was considered statistically significant.

Results

Surgical outcome

One animal in the DC group failed to progress as expected after operation, and autopsy at day 6 showed a thermal bowel injury; the animal was therefore excluded from the study. One animal in the RP group suffered a splenic injury with 500-ml blood loss requiring splenectomy, and was included in the study as recovery was unaffected. All other animals survived to day 7 postischaemia.

Renal function

Creatinine

There was no significant difference in the baseline serum creatinine level between the groups ($P = 0·100$, ANOVA)

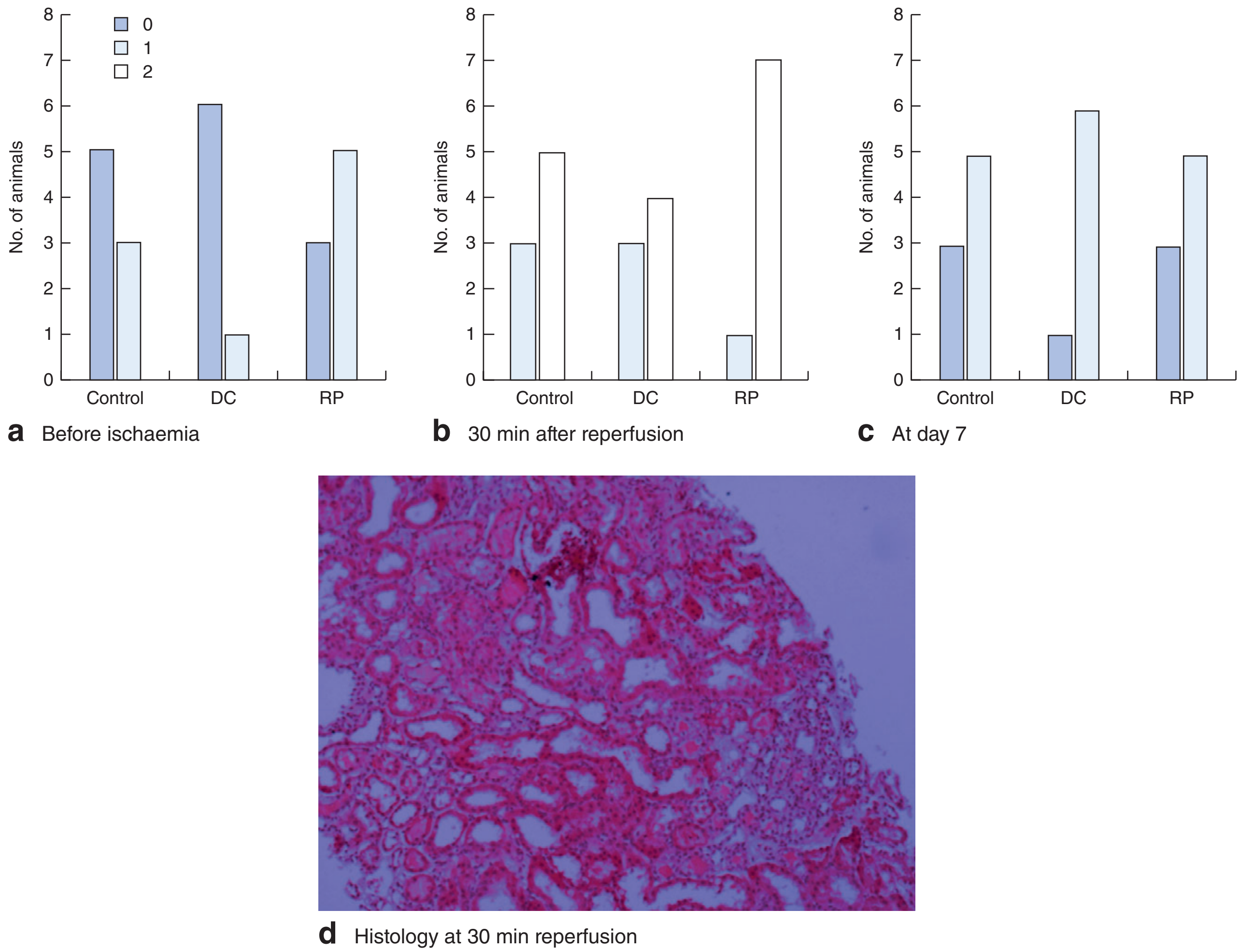

Fig. 3 a–c Histograms showing histology scores from renal biopsies taken **a** before ischaemia, **b** 30 min after reperfusion and **c** at 7 days from animals in control, direct postconditioning (DC) and remote periconditioning (RP) groups. Biopsies were scored 0 (indistinguishable from normal), 1 (minor subjective change, mostly tubular vacuolation) or 2 (definite tubular injury including dilatation of tubules and flattening of epithelium, with or without debris in the lumen). **d** Haematoxylin and eosin-stained section of kidney from a control animal after 30 min of reperfusion, showing tubular vacuolation

(*Fig. 2a*). Serum creatinine levels were significantly lower on postoperative day (POD) 1 and 2 in the DC group compared with the control group ($P = 0{\cdot}007$ and $P = 0{\cdot}022$ respectively) (*Fig. 2a* and *Table 1*). The area under the curve (AUC) for serum creatinine was significantly lower in the DC than in the control group ($P = 0{\cdot}036$). Numerically, the AUC for serum creatinine and peak creatinine levels were also lower in the RP group compared with the control group, although these differences were not statistically significant ($P = 0{\cdot}920$ and $P = 0{\cdot}218$ respectively). In both DC and RP groups, serum creatinine levels peaked at POD 1 in all animals. In the control group, levels peaked on day 1 in five of eight animals ($P = 0{\cdot}026$) (*Table 1*).

Urea

The peak urea concentration was significantly lower in the DC group than in the control group ($P = 0{\cdot}041$).

Tubular injury and inflammation

Tumour necrosis factor α

Systemic plasma levels of TNF-α were significantly higher in the RP group before surgery ($P = 0{\cdot}011$) (*Fig. 2b*). Compared with preoperative levels, a significant increase was observed in the control group at POD 1, followed by normalization by POD 3 ($P = 0{\cdot}013$). There was no significant increase in the DC or RP group (*Fig. 2b*).

Neutrophil gelatinase-associated lipocalin
Urinary level of NGAL was used as a biomarker of kidney injury. There was no increase in urinary NGAL from preoperative levels to POD 3 (NGAL/Cr ratio) in the DC group ($P = 0{\cdot}176$) (*Fig. 2c*). However, levels did increase over the study interval in both control and RP groups ($P = 0{\cdot}001$ for both) (*Fig. 2c*).

Renal vein samples

Endothelin 1
There was no difference in levels of ET-1 between the three groups ($P = 0{\cdot}486$) (*Table 2*).

Superoxide dismutase
There was no difference in percentage SOD inhibition between the three groups ($P = 0{\cdot}158$) (*Table 2*).

Nitric oxide
There was no significant difference in the levels of total nitric oxide between the three groups, although levels were numerically lower in the RP group ($P = 0{\cdot}060$) (*Table 2*).

Histology

Kidney histology scores showed no significant differences between the groups (*Fig. 3*; *Table S1*, supporting information). In biopsies collected 30 min after reperfusion, definite tubular injury including dilatation of tubules and flattening of epithelium was observed, with or without debris in the lumen in five of eight animals in the control group, four of seven in the DC group and seven of eight in the RP group. At POD 7, cellular injury had recovered to baseline values.

Discussion

This study shows that direct ischaemic postconditioning (DC) preserves renal function and may protect against tubular injury in a large-animal model of renal warm ischaemia. In addition, both DC and remote ischaemic periconditioning (RP) reduced the level of some markers of inflammation, such as TNF-α.

The extent of renal injury sustained after 60 min of left renal warm ischaemia and right nephrectomy in pigs provides sufficient deterioration in function to demonstrate a difference between experimental groups, but with no deaths from renal failure[23]. The DC group demonstrated the greatest degree of renal protection, with a significant reduction in serum creatinine and blood urea levels, reduced time to peak creatinine concentration, and reduction in AUC for creatinine. The RP group had numerically lower serum creatinine levels, which did not reach statistical significance. TNF-α is a well validated early marker of inflammation in IRI[24–26], and a significant increase in serum TNF-α concentration in the control group was observed, suggesting that both DC and RP reduce inflammation. Furthermore, during the study period urinary levels of NGAL, a biomarker of tubular injury, increased in both the RP and the control group, but not in the DC group, suggesting that DC may protect against tubular injury. There was no significant difference in renal vein levels of ET-1, nitric oxide and percentage inhibition of SOD, although overall the levels were numerically higher in the control group.

Soendergaard and colleagues[19] demonstrated an improvement in glomerular filtration rate (GFR) in pig kidneys exposed to remote preconditioning using a porcine donation after brain death transplant model. Paired kidneys with 20 h of cold ischaemia time and 3–5 min of warm ischaemia time were allocated to control or IC in which the external iliac artery (EIA), distal to the transplant, was occluded for four 5-min intervals during the venous anastomosis. They showed an improvement in GFR and renal perfusion in the experimental group, although the animals were recovered for only 10 h. These authors also measured haem oxygenase 1 and NGAL excretion and inflammation markers, but demonstrated no difference between the groups, which may be due to the short recovery period[27]. Two randomized studies[28,29] comparing living donor transplant recipients have shown that IC has no effect on renal function. This could be explained by the minimal ischaemic injury sustained by the organs.

There have been two clinical studies[20,21] assessing IC in deceased donor renal transplantation. Van den Akker and co-workers[20] carried out a single-arm pilot study of postconditioning in DCD kidney recipients to assess safety and feasibility of the technique. They applied a Dietrich (vascular) clamp to occlude the EIA around the renal transplant arterial anastomosis and used three 1-min occlusions at organ reperfusion. This technique rendered both the transplanted organ and lower limb ischaemic during the clamp placement. These authors compared the group with a historical control group and, although the proportion of DGF was greater in the experimental group (17 of 20 *versus* 25 of 40 patients), the difference did not reach statistical significance ($P = 0{\cdot}07$). There were also two major vascular complications including injury to the renal vein during clamp removal, resulting in blood loss of 800 ml and an additional 10 min of warm ischaemia time, and renal artery thrombosis. Wu and colleagues[21] randomized paired

DCD kidneys to either a control group or a remote preconditioning group, during which the EIA was clamped for three 5-min cycles before organ reperfusion. They demonstrated an improvement in renal function, reduction in DGF and reduction in urinary NGAL in the IC group. Interestingly, there was no incidence of DGF or acute rejection in the IC group, which is surprising in recipients of DCD kidneys. These authors reported no complications associated with the technique.

There were no complications associated with the non-traumatic bulldog clamp that was applied to the renal artery in the present study, although the authors acknowledge that in clinical practice surgeons may be hesitant to clamp the transplant renal artery, which may be technically difficult and undesirable for fear of arterial injury. However, as discussed above, the evidence shows that clamping the EIA around the anastomosis is not without risk[20]. The non-traumatic bulldog clamp is smaller than a Dietrich clamp and could be suitable for assessment in a DCD transplant study. In addition, direct digital pressure, occluding the renal artery between index finger and thumb, is an alternative technique that could be used in clinical practice.

The shortened period of 15-s occlusion used in the present study limits the potential ischaemic injury but is sufficient to show an improvement in renal function in comparison with the longer periods used previously[20]. The variation in methods of IC and terminology makes direct comparison of studies difficult. The present method used in the DC group conditions the organ directly rather than the host lower limb, and may explain why results were more favourable in the DC group than in the RP group.

The present study was a preclinical assessment of IC and focused on the functional outcome of warm ischaemia rather than mechanisms of action. As a result, potentially confounding factors that would be of importance in a transplant model, such as cold ischaemia and immunosuppression, were not included. Although the exact mechanism of action is unclear, IC upregulates mediators such as bradykinin and adenosine, which reduce endothelial injury by decreasing prostacyclin and nitric oxide production, thereby minimizing vasoconstriction and thrombosis. In addition, IC appears to activate mitochondrial K_{ATP} channels, which inhibits permeability transition pores leading to a reduction in mitochondrial swelling and cell death[14–16].

This study has demonstrated that using short 15-s cycles of IC applied directly to a target organ at reperfusion can improve renal function, and may reduce tubular injury and inflammation in a large-animal model of renal warm ischaemia. The findings support assessment of direct ischaemic postconditioning in a DCD transplant model, ideally as a randomized clinical trial.

Acknowledgements

This project was funded from charitable donations to the Transplant Group, University of Leicester, Leicester, UK.
Disclosure: The authors declare no conflict of interest.

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Supporting information

Additional supporting information may be found in the online version of this article:

Table S1 Histology scores in control, direct postconditioning and remote periconditioning groups before ischaemic injury, 30 min after reperfusion and 7 days after ischaemic injury (Word document)