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ORIGINAL ARTICLE

Improved GFR and renal plasma perfusion following remote ischaemic conditioning in a porcine kidney transplantation model

Peter Soendergaard,[1]* Nicoline V. Krogstrup,[1]* Niels G. Secher,[4] Kristian Ravlo,[1] Anna K. Keller,[5] Else Toennesen,[4] Bo M. Bibby,[6] Ulla Moldrup,[2] Ernst O. Ostraat,[2] Michael Pedersen,[3,5] Troels M. Jorgensen,[2] Henri Leuvenink,[7] Rikke Norregaard,[5] Henrik Birn,[1] Niels Marcussen[8] and Bente Jespersen[1]

1 Department of Renal Medicine, Aarhus University Hospital, Aarhus, Denmark
2 Department of Urology, Aarhus University Hospital, Aarhus, Denmark
3 MR Research Center, Aarhus University Hospital, Aarhus, Denmark
4 Department of Anaesthesiology and Intensive Care, Aarhus University Hospital, Aarhus, Denmark
5 Department of Clinical Medicine, Aarhus University, Aarhus, Denmark
6 Department of Biostatistics, Aarhus University, Aarhus, Denmark
7 Department of Surgery, University of Groningen, Groningen, the Netherlands
8 Department of Pathology, Odense University Hospital, Odense, Denmark

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Correspondence
Nicoline V. Krogstrup MD, Department of Renal Medicine, Aarhus University Hospital, Brendstrupgaardsvej100, 8200 Aarhus N, Denmark.
Tel.: +45 78 45 24 17;
fax: +45 78 45 24 30;
e-mail: nicoline.v.krogstrup@ki.au.dk

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*Both authors contributed equally to this work.

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Summary

Delayed graft function (DGF) complicates approximately 25% of kidney allografts donated after brain death (DBD). Remote ischaemic conditioning (rIC) involves brief, repetitive, ischaemia in a distant tissue in connection with ischaemia/reperfusion in the target organ. rIC has been shown to induce systemic protection against ischaemic injuries. Using a porcine kidney transplantation model with donor (63 kg) recipient (15 kg) size mismatch, we investigated the effects of recipient rIC on early renal plasma perfusion and GFR. Brain death was induced in donor pigs ($n = 8$) and kidneys were removed and kept in cold storage until transplantation. Nephrectomized recipient pigs were randomized to rIC ($n = 8$) or non-rIC ($n = 8$) with one kidney from the same donor in each group. rIC consisted of 4×5 min clamping of the abdominal aorta. GFR was significantly higher in the rIC group compared with non-rIC (7.2 ml/min vs. 3.4 ml/min; ΔGFR = 3.7 ml/min, 95%-CI: 0.3–7.2 ml/min, $P = 0.038$). Renal plasma perfusion in both cortex and medulla measured by dynamic contrast-enhanced magnetic resonance imaging (MRI) was significantly higher over time in the rIC group compared with non-rIC. This experimental study demonstrated a positive effect of rIC on early graft perfusion and function in a large animal transplantation model.

Introduction

The number of patients awaiting kidney transplantation is increasing [1]; the organ shortage necessitates improvement in the outcome of transplantation as well as strategies to increase the potential donor pool. This could be by extended donor criteria in donation after brain death (DBD) and donation after circulatory death (DCD). Marginal donors lead to a higher risk of delayed graft function (DGF) associated with impaired long-term graft function,

shorter graft survival, more rejections, prolonged hospital admissions and increased mortality [2]. Approximately 25% of all DBD kidney allografts develop DGF [3]. Small children receiving adult size kidneys are at increased risk of DGF and thrombosis, possibly as a result of their low cardiac output in relation to the transplanted organ [4,5]. Ischaemia-reperfusion injury is involved in the development of DGF and cellular lesions play a role in up-regulating the expression of class II MHC [6,7].

Remote ischaemic conditioning (rIC) involves brief, repetitive, nondamaging periods of ischaemia in a tissue, e.g. an arm, inducing systemic protection against ischaemic reperfusion injuries in distant organs [8,9]. Functional improvement has been proven in various organs following rIC [10], for instance the human heart [11]. Similarly, a protective effect of rIC on ischaemia-reperfusion injury in the kidney has been demonstrated [12–14]. A study in rats found improved renal function when performing rIC before ischaemic insult of the kidney [15]. Many studies, mostly animal studies, have investigated conditioning strategies in solid organ transplantation achieving generally positive results[16,17], but to our knowledge, none so far have found an improvement of renal graft function performing rIC in a large animal transplantation model or in humans.

The aim of this study was to investigate the effects of rIC on early renal graft perfusion and function when performed in the recipient prior to renal graft reperfusion. We used a porcine kidney transplantation model involving kidneys from large donors to small recipients and long cold ischaemia time resulting in a high risk of DGF.

Materials and methods

The study was approved by the Danish National Animal Ethics Committee (no. 2008-561-1584). Female Danish Landrace pigs were used. Donors corresponded in weight to human donors ($n = 8$, 63 ± 3 kg). The recipients corresponded in weight to small paediatric patients (15 ± 1 kg) and were randomised to either rIC ($n = 8$) or non-rIC ($n = 8$) before reperfusion of the graft. The recipients were transplanted simultaneously by experienced transplantation surgeons.

Prior to the experimental procedures, the animals fasted overnight, but had free access to water. The animals were sedated with an intramuscular injection (i.m.) of Azaperone (Stresnil™, 0.1 ml/kg) and Midazolam (0.5 mg/kg). Anaesthesia was induced with intravenous (i.v.) Midazolam (0.5 mg/kg) and Ketamine (5 mg/kg) before intubation. Atropine (0.02 mg/kg) was given i.m. Anaesthesia was continued with i.v. Mebumal (11.5 mg/kg/h) and Fentanyl (11.5 μg/kg/h). Mechanical ventilation was set to 40% oxygen and a tidal volume of 10 ml/kg. Expiratory CO_2 was kept between 4.5 and 5.5 kPa by adjusting respiration rate. Cefuroxime (750 mg) was administered i.v. at start and again after 3 h. The recipient pigs were infused with glucose monohydrate (1.0 g/h). The carotid artery and jugular vein were catheterized for blood pressure monitoring, blood sample collection and drug infusion; the urethra was catheterized for urine collection. Donors received 10 ml/h/kg Ringer-Acetate, recipients 15 ml/h/kg. In addition, the recipients were given 500 ml saline i.v. before surgery and 500 ml prior to reperfusion of the graft. Mean arterial blood pressure (MAP) was maintained above 60 mmHg by additional Ringer-acetate. A bolus of Adrenaline (0.05 mg) i.v. was given once or twice if fluid therapy could not maintain mean arterial pressure (MAP) >60 mmHg. Infusion with Dopamine (270 μg/kg/h) was introduced to maintain MAP >60 mmHg if other treatments failed. The animals were euthanized with a lethal dose of pentobarbital (80 mg/kg).

Brain death

Brain death was induced in the donors as described by Barklin *et al.* by increasing intracranial pressure though the inflation of a 22 Fr Foley urine catheter in the epidural space introduced through a hole in the cranium. Intracranial pressure was measured continuously. Brain death occurred when intracranial pressure was higher than the systolic blood pressure. This was accompanied by a 10 ml bolus of saline injected into the balloon to ensure incarceration [18]. To avoid muscle cramps, Rocuronium (130 mg) was administered i.v. and Mebumal infusion was discontinued at this point.

Kidney removal from donor

Both kidneys were exposed through a midline incision. A patch of the great abdominal vessels was taken in those having multiple renal vessels. Kidneys were removed in random order after at least 4 h of brain death. Immediately after removal, 19 ml saline with 5000 IU of heparin was injected into the renal artery and each kidney was cooled with arterial infusion of 1 l 4 °C Custodiol® and kept on frozen glucose. Warm ischaemia associated to removal was within 3–5 min. Kidneys were stored at 4 °C until transplantation.

Transplantation

A midline incision was made and native kidneys, aorta and vena cava were exposed retroperitoneally. Native kidneys were removed after ligation of artery, vein and ureter. A second part of the aorta was exposed further

distally and used for the rIC or non-rIC (sham) procedure in the two groups. Transplantation was performed by end-to-side anastomoses of the graft artery and vein to the aorta and vena cava respectively. Ureter was catheterized with an 8 Fr feeding tube and urine was collected directly from the kidney pelvis. Left and right donor kidneys were randomised, so both recipient groups received the same total number of left and right kidneys. Major lymphatic vessels surrounding the aorta and vena cava were ligated to prevent loss of fluids. Grafts were kept cold during the surgery with frozen glucose.

Remote ischaemic conditioning

In this study, we termed the procedure as remote ischaemic conditioning rather than the more well-known term preconditioning, as conditioning was induced in the recipient before reperfusion of the transplanted kidney, but after the ischaemic insult related to the removal of the organ from the donor.

Ischaemia was achieved in both hind legs and pelvine organs of the recipients by clamping the exposed abdominal aorta distal to the anastomosis. Non-rIC pigs had their aorta exposed as a sham procedure of equal length, but without clamping. The rIC protocol was performed during vein anastomosis and consisted of four periods of 5-min ischaemia separated by 5-min reperfusions. After 15 min of lower body reperfusion, the artery anastomosis was opened at $T = 0$ min.

Data collection

Blood and urine samples were collected every hour and blood pressure was noted. Arterial blood samples were drawn every hour to measure blood gases and haematocrit. Renal tissue was obtained after graft reperfusion (biopsy) and again just before euthanization. The biopsy specimens were either frozen in liquid nitrogen or kept in formalin. The renal tissue was examined histologically and graded for pathological rejection changes by an expert, using the same criteria as in the Banff Classification of human transplant pathology.

GFR

GFR was measured by a constant infusion clearance technique using ^{51}chrome-ethylenediamine tetraacetic acid (^{51}Cr-EDTA). Three hours before reperfusion of the graft, a bolus of 0.24 MBq/kg was given and infusion sustained at 0.12 MBq/kg/h. GFR was determined with 30-min intervals during T = 0–4 h and subsequently every hour. The activity in 0.5 ml plasma and urine samples was counted in a gamma ray detector (Cobra II, Packard, Meriden, CT) to a statistical accuracy of 1%. Adjustments were made for background radiation.

Renal plasma perfusion

The recipient pigs were placed in a clinical 1.5 T MRI scanner system (Philips Medical Systems, Best, The Netherlands) equipped with a 5-element radiofrequency-coil for data reception at 3, 5, 7 and 9 h after reperfusion. Perfusion-weighted data were acquired by injection of Gd-DTPA-BMA (Omniscan; GE Healthcare, Oslo, Norway) in parallel with a dynamic contrast-enhanced MRI sequence, using a temporal resolution of a1.5 s/frame. Sequence parameters were used according to Jørgensen *et al.* [19]. Data were analysed using a two-compartmental kinetic model and quantification of whole-kidney, cortical and medullary renal plasma perfusion (RPP) was estimated (ml/min/100 cm^3) [20].

Renal biomarkers in urine

Neutrophil gelatinase-associated lipocalin (NGAL) was measured using a NGAL ELISA kit, BioPorto Diagnostics. The activity of alanine aminopeptidase (Ala-AP) and N-acetyl-β-D-glucosaminidase (NAG) was measured using colorimetric assays [21]. NAG was measured by a modified enzyme assay according to Findlay [22] at pH 4.25 using p-nitrophenyl-N-acetyl-β-D-glucosaminide assubstrate. Ala-AP was detected with the modified enzymatic assay of Pfleiderer [23] using alanine-p-nitroanilide as substrate.

Haeme oxygenase 1 in renal tissue

Total RNA for quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was extracted from the cortex and inner medulla of the renal grafts using Nucleo Spin RNA II (Macherry-Nagel). Reverse transcription was performed using a high-capacity complementary DNA (cDNA) reverse transcription kit (Applied Biosystems). Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed using the Tagman gene expression assay system (Applied Biosystems). Serial dilution (1 ng–1 fg/mikroL) of cDNA was used as a template for the generation of a standard curve. The probes and nested primers were used to amplify standards and kidney cDNA samples: Ss03378516_u1 (Haeme oxygenase 1 (HO-1)) and Ss03388274_m1 (hypoxanthine phosphoribosyltransferase 1 HPRT1). A postrun melting curve analysis was performed identifying only one amplification product. Selected samples were subjected to gel electrophoresis confirming the expected size and specificity of the PCR-product (data not shown). HO-1 messenger RNA (mRNA)

expression was normalized against the housekeeping gene HPRT1 amplified from the same sample.

The HO-1 protein content in renal tissue was estimated by immunoblotting. Renal cortical tissues were homogenized and protein concentration was determined using the Bradford method. The homogenized tissue was loaded onto 12% gels according to protein concentrations followed by coomassie staining of the gel. The gel was scanned (Odessey 9120, LiCor Biosciences) and the intensity of individual lanes was determined. The final loading was adjusted according to the intensity of the scanned lanes. The gel was blotted onto PVDF membranes (Millipore) and incubated with a rabbit anti-HO-1 antibody (SPA-896, Stressgen). The fluorescent labelling was scanned and the intensity of the specific bands as well as the corresponding lanes of a similarly loaded coomassie stained gel were determined. The level of HO-l protein expression was determined relative to the intensity of the corresponding coomassie stained lanes with non-rIC expression levels defining 100%.

Statistics

Baseline characteristics, histology, haematocrit, kidney volume and HO-1 expression levels are presented as means with standard deviations or medians and ranges. Treatment groups are compared using t-tests when no clear deviations from the normal distribution were detected in the data. When not conforming to the normal distribution, treatment groups are compared using Wilcoxon's two-sample rank sum test.

GFR, renal plasma flow, urinary excretion rates of NGAL, Ala-AP and NAG, diuresis and MAP were analysed using a repeated measurement analysis of variance (ANOVA). More specifically, a linear mixed effects model was applied with treatment and time as fixed effects, and animal and donor as random effects. The larger (within and between) animal variation in the treatment group compared with the control group was taken into account by including a group specific standard error in the statistical model. Model diagnostics was performed by inspecting standardized residuals and by comparing observed and expected within animal correlations. Data were analysed in R 2.10.0 (R Development Core Team, 2009) and $P < 0.05$ was considered statistically significant.

Results

One donor developed ventricular fibrillation at the point of incarceration, but was resuscitated to sinus rhythm by defibrillation and adrenalin infusion. Diuresis was maintained and subsequent histological examination of the kidneys showed no sign of damage and this donor was thus not excluded. Two pigs in the non-rIC group died during the 10-h follow-up; the first during a hypotensive episode 3 h 22 min following reperfusion, and the second after 5 h 53 min, possibly because of arrhythmia during dopamine infusion initiated to maintain MAP >60 mmHg. Data obtained from these pigs until death were included in the study, but analyses excluding these data were performed as well. No deaths occurred in the rIC group.

No significant differences between the recipient groups were observed in basic physiological parameters, cold ischaemia time or fluid infusion (Table 1). During the 10-h follow-up, MAP decreased in both groups, though not significantly, $P = 0.60$ (Figure 1A). A nonsignificant tendency of a higher need for intravenous fluids was seen in the non-rIC group (Table 1). Four animals in the non-rIC group received adrenaline (0.1 mg) to stabilize MAP and two also required dopamine infusions. In the rIC group, one animal received adrenaline (0.1 mg) and two required dopamine.

The biopsies essentially revealed healthy tissue with only minor pathological changes. A slight interstitial inflammation at 10 h was observed in both groups. Interstitial

Table 1. Basic physiological parameters, cold ischaemia time and fluid replacement in the two groups.

	rIC pigs	Non-rIC pigs	*P*-value
Mean arterial blood pressure (mmHg)			
Baseline	108 (9)	114 (20)	0.44
At reperfusion	79 (15)	84 (21)	0.62
Arterial lactate (medians, mmol/l)			
Baseline	1.7 (range 1.1–2.8)	1.3 (range 0.7–4.1)	0.34
At reperfusion	3.1 (range 2.1–4.3)	2.3 (range 1.1–5.1)	0.37
Cold ischaemia time	21 h34 min (86 min)	21 h43 min (74 min)	0.83
Duration of brain death before explantation	4 h48 min (44 min)	4 h47 min (44 min)	0.96
Supplement fluid (median, ml)	589 (range 0–1800)	937 (range 0–1963)	0.32
Baseline weight (kg)	15 (1)	15 (1)	0.72

Baseline was defined as the beginning of anaesthesia. Data are presented as mean and standard deviation (SD), unless noted otherwise. No significant differences between the rIC and the non-rIC group were observed.

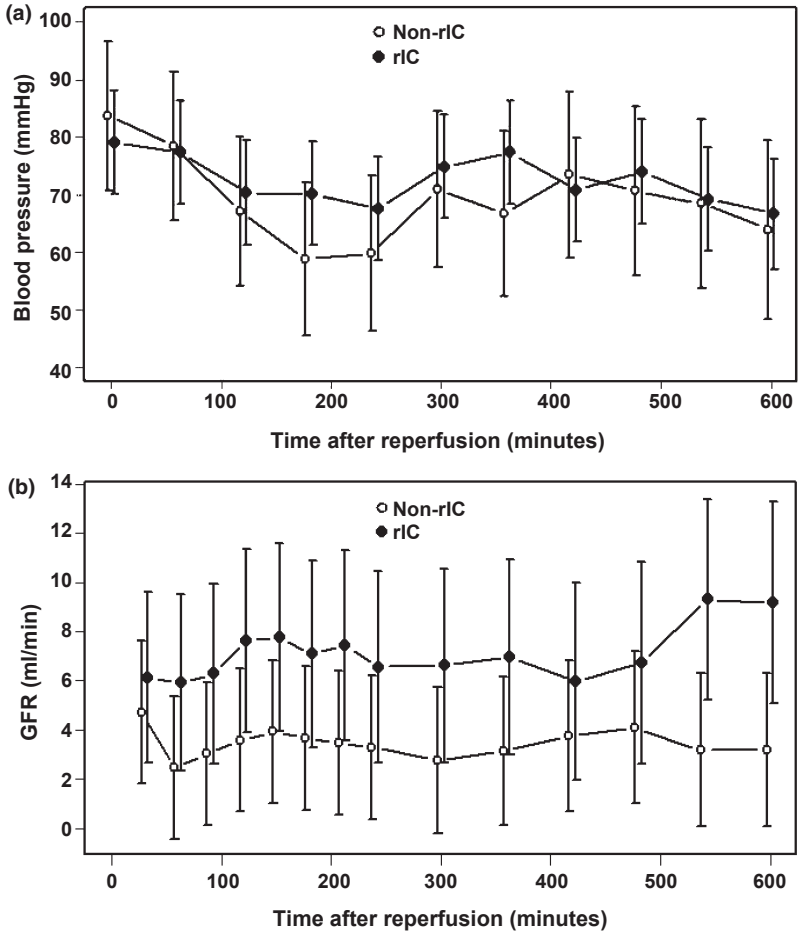

Figure 1 Mean arterial blood pressure (a) and GFR (b) of pigs receiving a renal transplant with or without remote ischaemic conditioning (rIC). There was no significant difference in blood pressure between the two groups ($P = 0.70$). Blood pressure significantly decreased over time ($P = 0.0003$). GFR of the rIC group was significantly higher than the non-rIC group ($P = 0.038$). There was no significant change in the difference over time ($P = 0.37$). Means and 95% confidence intervals are shown.

inflammation was not seen at 3 h and no difference was identified between groups. Only the glomerulitis score after 10 h was significantly different between groups being a little higher in the rIC group (Table 2).

GFR

The rIC group demonstrated a significantly higher GFR during the 10-h follow-up after graft reperfusion compared with non-rIC (ΔGFR = 3.7 ml/min, 95%-CI: 0.3–7.2 ml/min, $P = 0.038$, Fig. 1b). The difference was apparent from first measurement at 30 min and remained virtually unchanged ($P = 0.49$) during the 10-h observation period. GFR did not change significantly over time ($P = 0.37$). Analyses excluding the two recipients that died and adjustment for MAP were also performed (Table 3).

Renal plasma perfusion

As expected, perfusion was generally higher in cortex than medulla. RPP was significantly higher following rIC in both cortex and medulla after 7 and 9 h (cortex) and after 5, 7 and 9 h (medulla) respectively (Fig. 2). The difference depended on time and increased over time after reperfusion. *P*-values for the interaction between time and treatment: cortex: $P = 0.0006$ (excluding the two recipients that died $P = 0.001$) and medulla: $P = 0.047$ (excluding the two recipients that died $P = 0.024$)). Adjusting for MAP, these *P*-values changed to: cortex: $P = 0.0004$ (excluding the two recipients that died $P = 0.0009$) and medulla: $P = 0.033$ (excluding the two recipients that died $P = 0.012$). Despite successful randomization of left and right kidneys between the groups, kidney volume at first scan was slightly higher in the non-rIC group (data excluding the two recipients that died: non-rIC median 206 ml (range 199–235) and rIC median 194 ml (range 173–210), $P = 0.058$). This minor difference remained throughout all scans with $P = 0.032$–0.058. Within the groups, no change in kidney volume occurred over time (non-rIC $P = 0.90$ and rIC $P = 0.06$).

No differences between haematocrit values at 3 and 9 h were seen (non-rIC: median 0.23 vs. 0.22 $P = 0.10$ and

Table 2. Histological examination of renal tissue showing the Banff and PTC scoring for the non-rIC and rIC recipients 30 min and 10 h after graft reperfusion.

	Median (range)			
	Renal tissue 30 min		Renal tissue 10 h	
Banff score	Non-rIC	rIC	Non-rIC	rIC
Glomerulitis ('g')	0 (0–1)	0.5 (0–1)	1 (0–1)	1 (1–2)*+
Interstitiel inflammation ('i')	0 (0–0.5)	0 (0–1)	0.8 (0–2)+	1 (0–1)+
Tubulitis ('t')	0 (0–0)	0 (0–1)	0 (0–1)	0 (0–0.5)
Intimal arteritis ('v')	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
Peritubular capillaritis (PTC)	0 (0–1)	0 (0–0)	0 (0–2)	0 (0–1)

The star (*) indicates statistically significant when comparing non-rIC versus rIC recipients ($P < 0.05$). The plus (+) indicates statistically significant when comparing renal tissue samples 30 min versus 10 h after graft perfusion ($P < 0.05$).

Table 3. Difference in GFR (ml/min) between the rIC and non-rIC groups, mean with 95%-confidence intervals.

ΔGFR		All recipients ($n = 16$)	Recipients completing the 10 h follow-up period ($n = 14$)
Adjusted for MAP	No	3.7 (0.3;7.2), $P = 0.04$ ($n = 207$)	2.9 (−1.1;6.9), $P = 0.12$ ($n = 193$)
	Yes	3.0 (−0.6;6.7), $P = 0.09$ ($n = 174$)	2.2 (−2.0;6.3), $P = 0.25$ ($n = 162$)

Analyses were performed including or excluding the two recipients dying prior to the completion of the 10-h follow-up period. In all analyses, there were no significant changes over time ($P > 0.28$ in all cases). *n* indicates the number of observations in each analysis.

rIC: median 0.26 vs. 0.24 $P = 0.14$), and no differences between the groups were found (3 h: $P = 0.57$ and 9 h: $P = 0.48$).

Urinary output and excretion rate of renal biomarkers

Diuresis decreased markedly over time, $P < 0.0001$, and did not differ between the groups, $P = 0.70$ (Fig. 3a). No significant difference in urinary NGAL excretion was seen, $P = 0.17$ (Fig. 3b); this was also the case with NAG and AAP (data not shown). The excretion rates of all three urinary biomarkers followed the trend of the diuresis: initially high and followed by a decrease ($P < 0.0001$ in all cases).

Haeme oxygenase-1 mRNA and protein levels

HO-1 mRNA abundance tended to decrease in both cortex and medulla in response to rIC, but this was not statistically significant (Fig. 4a and b). Similarly, cortical HO-1 protein level after rIC was reduced to 71% of the levels of non-rIC (Fig. 4c). Tissue from dead animals was not included in the analysis.

Discussion

We have established a porcine model mimicking transplantation of adult size kidney grafts into paediatric recipients and with a long cold ischaemia time, a high-risk situation of DGF. The model successfully produced DGF without thrombotic events. Single kidney GFR (skGFR) of healthy 15–16 kg Danish Landrace pigs has been found to be in the range 18–23 ml/min [24,25]. In our model with ischaemically injured, size-mismatched kidneys, skGFR values in the range 0–22 ml/min appear to be suitable for the study of intervention effect. Our results showed that rIC improved early GFR and is associated with increased renal graft plasma perfusion in both cortex and medulla. The positive effect of rIC appeared to be associated with a trend towards low HO-1 and was not associated with significant changes in the early excretion of urinary acute kidney injury biomarkers.

Maintaining an adequate MAP and diuresis after renal transplantation into paediatric recipients is a well-known challenge [4]. In the present experiment, this was accentuated by the need for anaesthesia during the entire 10-h post-transplant observation period. Trends towards lower MAP and need of extra fluids were seen in the non-rIC group, but this, however, could not explain the differences in RPP observed between the groups. It can be hypothesized that rIC has systemic effects stabilizing haemodynamics. Studies have previously shown that rIC improves parameters reflecting the function of several different organs [14,26]. A positive, systemic effect may also be indicated by the survival of all pigs exposed to rIC, whereas two non-rIC pigs died during the observation period.

GFR improved significantly after rIC, in fact, the observed difference of 3.7 ml/min represents a 109% increase

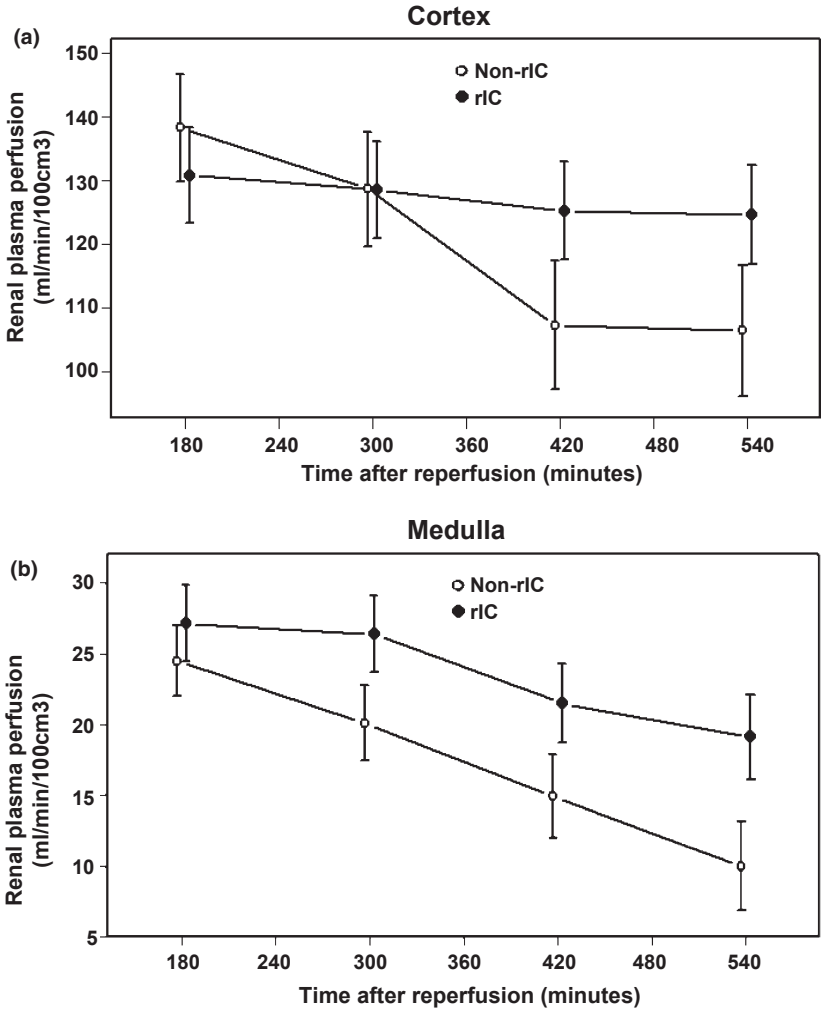

Figure 2 Renal plasma perfusion estimated with magnetic resonance imaging (MRI) of pigs receiving a renal transplant with or without remote ischemic conditioning (rIC). Means and 95% confidence intervals are shown. Renal cortical plasma perfusion (a) was significantly higher at 7 h ($P = 0.012$) and 9 h ($P = 0.013$). Renal medullary plasma perfusion (b) was significantly higher at 5 h ($P = 0.005$), 7 h ($P = 0.006$) and 9 h ($P = 0.0008$).

compared with non-rIC. An increase in GFR of 3–5 ml/min is likely to be clinically relevant by preventing the need for acute dialysis following transplantation and improving both short- and long-term graft function. The significant increase in RPP following rIC suggests this to be a mechanism for the positive effect of rIC. In addition, improved graft perfusion may prevent early graft thrombosis and attenuate post-perfusion ischaemia, and thus adhesion of dendritic and other cells initiating the immune response [27,28]. The difference between the groups increased over time, suggesting the positive effects of rIC may persist beyond the 10-h follow-up in this study. Haematocrit and kidney volumes were unaffected over time and cannot explain the increased plasma perfusion after rIC. The slightly higher kidney volumes found in non-rIC recipients could be as a result of mild oedema secondary to ischaemic lesions.

None of the tested biomarkers, NGAL, NAG or AAP, were able to predict renal graft outcome measured as GFR after 10 h, nor were any differences observed between the groups. The initially high but subsequently decreasing excretion rates following the pattern of the diuresis could suggest a wash out effect. Our analyses were hampered by missing measurements because of low urinary output possibly introducing a bias as anuria is associated with worse renal outcome.

HO-1 is a stress responsive enzyme that acts during inflammatory responses as the first and rate-limiting step of haeme degradation, producing CO, biliverdin and ferrous iron [29]. In experimental organ transplantation, HO-1 expression is increased in the graft during acute and chronic rejection, and during ischaemia-reperfusion injury [30,31]. Up-regulation of HO-1 has been associated with protection against ischaemia-reperfusion injury and could thus provide another mechanism for the observed protective effect of rIC [32]. However, we demonstrated a nonsignificant decrease of HO-1 expression both at the mRNA and at protein level in the rIC group 10 h after transplantation. Although an increased

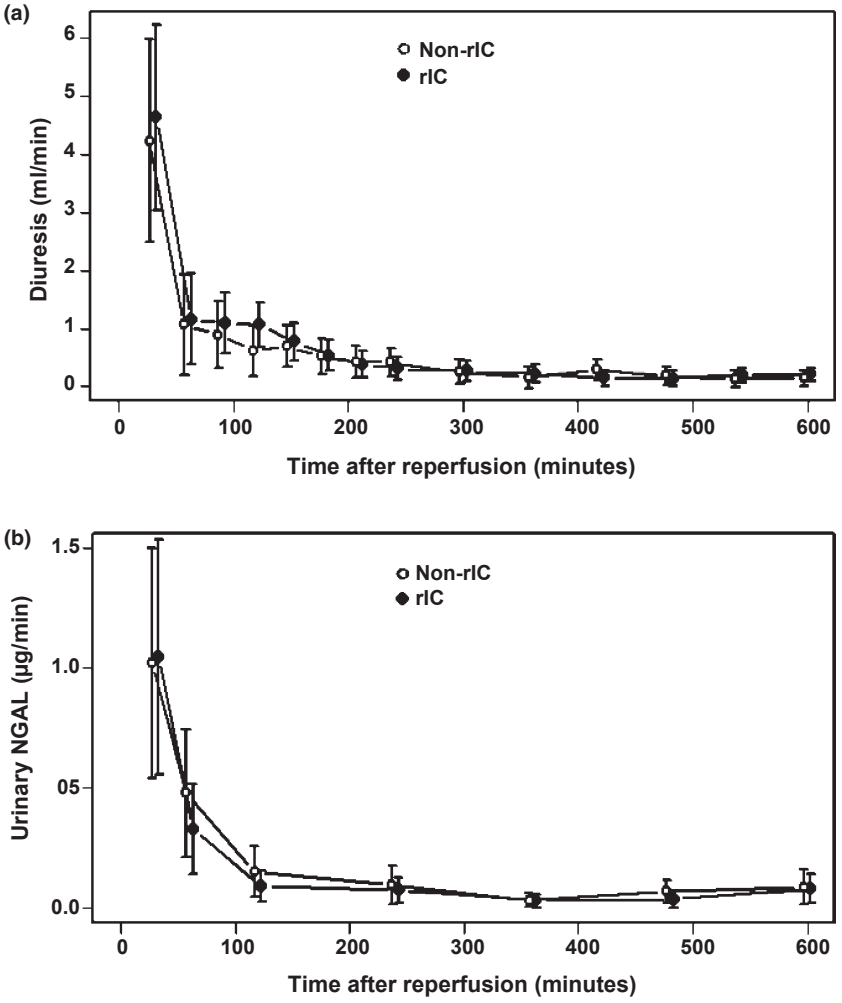

Figure 3 Diuresis (a) and urinary excretion rate of neutrophil gelatinase-associated lipocalin (NGAL) (b) of pigs receiving a renal transplant with or without remote ischaemic conditioning (rIC). Diuresis and NGAL both decreased significantly over time ($P < 0.0001$). No significant difference was found between the groups. Means and 95% confidence intervals are shown.

expression of HO-1 very early in the post-transplant observation period after rIC cannot be fully excluded, our findings may suggest that the protective effect of rIC does not involve up-regulation of HO-1. The trend towards a decreased HO-1 expression following rIC may in fact reflect reduced injury from ischaemia-reperfusion. In human liver allografts, higher HO-1 mRNA levels were associated with poor initial graft function [33]; in humans, renal HO-1 protein levels shortly after allograft reperfusion were closely and inversely related with initial graft function [34] in accordance with our observations.

In this study, we investigated the potential renoprotective effect of rIC in the recipient. Our setup allowed comparison of the effect on kidneys from the same donor exposed to either rIC or non-rIC. The two recipients were transplanted in parallel and the surgery times including time for the anastomoses were similar. Conditioning of the recipient prior to reperfusion is easily implemented in the clinical situation and allows optimal timing with respect to reperfusion. Our study does not exclude additional protective effects from preconditioning of the donor prior to organ removal. Jia *et al.* investigated the effect of rIC on the donor in renal transplantation in rats [12] showing no significant effects on functional renal markers, but a decrease in renal tubular, histological injury. To understand the mechanisms of rIC in transplantation, donor and recipient may be studied separately, but rIC performed on both donor and recipient may also have an added, positive effect evaluated in the ongoing REPAIR study (http://repair.lshtm.ac.uk/).

The organ protecting mechanisms behind rIC still remain unclear; it may release different biochemical messengers into the bloodstream that can reduce oxidative stress and for instance modulate mitochondrial function. An effect on neurogenic pathways and modulation of gene expression has also been suggested [35,36]. Ravlo *et al.* studied dendritic cells from the animals of this study, but found no modulation by rIC during the 10-h follow-up [28].

The strengths of the current experimental study include accurate estimates of GFR and RPP, close monitoring

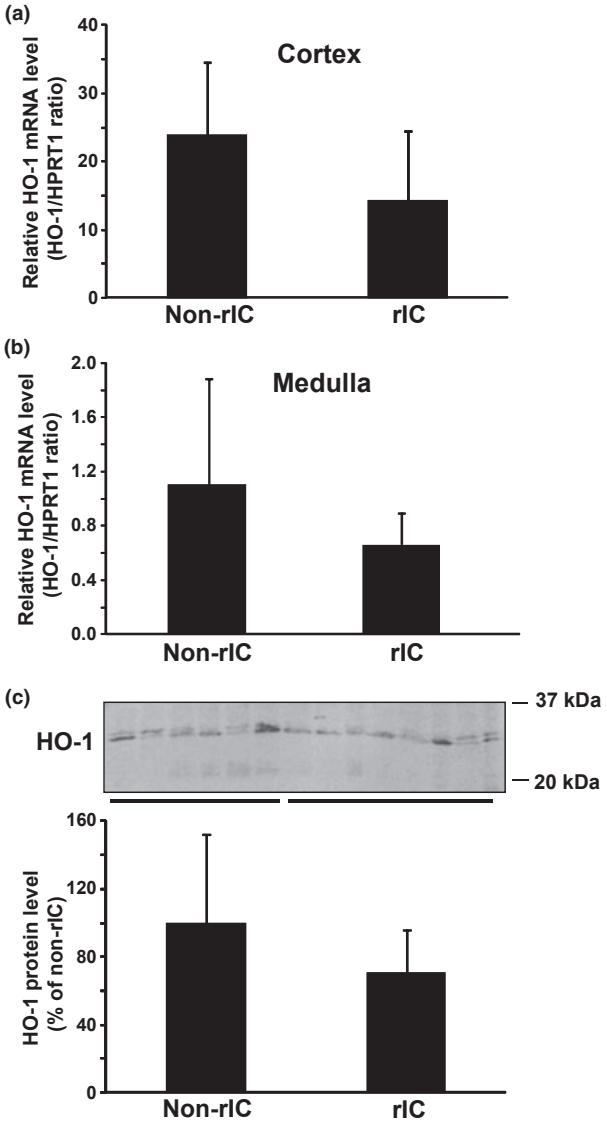

Figure 4 HO-1 mRNA and HO-1 protein levels in renal tissue of transplanted pig kidneys from recipients exposed to rIC (n = 8) or non-rIC (n = 6). Animals that died prior to the completion of the 10-h observation period (n = 2) were excluded. (a) Relative HO-1 mRNA level (HO-1/HPRT1 ratio) in renal cortical tissue. There was no significant difference (P = 0.13). (b) Relative HO-1 mRNA level (HO-1/HPRT1 ratio) in renal medullary tissue. There was no significant difference (P = 0.18). (c) Immunoblotting identifying HO-1 (MW ~ 34 kDa) in renal cortical tissue. The bands were semi-quantitated by scanning and the intensities related to total protein content as determined by a similar loaded, coomassie stained gel. The levels following rIC were 71 ± 25% of levels in non-rIC (100% ± 52%), nonsignificant (P = 0.19).

during the early post-transplant period, establishment of an animal model resembling transplantation in humans and a paired setup where a recipient in each group received a kidney from the same donor pig. The weaknesses include the short follow-up, nonblinded intervention, usage of opioids, which are considered a potential trigger of rIC, and rIC by an invasive technique. Uremia could also influence the effect of rIC and recipient animals were not uraemic when transplantated. The short follow-up allowed us to examine the effect of rIC without the use of immunosuppressive medication, as none of the recipients were preimmunized. Although essential in human transplantation, immunosuppressants may interfere with early graft function, making observations of the isolated effects of rIC more difficult. The influence of immunosuppressive treatment on the effects of rIC remains unknown, and the positive findings of this study need to be confirmed in human renal transplantation. To our knowledge, there are currently two ongoing clinical studies investigating the effect of rIC in kidney transplantation. One is the multicenter REPAIR study examining the effect of different conditioning approaches of both the donor and the recipient in living-donor kidney transplantation; the other is the multicenter CONTEXT study initiated by our group based on the present results and applying rIC to the recipient in DBD transplantation.

In conclusion, this study showed that four cycles of 5 min hind leg ischaemia separated by 5 min reperfusion before renal graft reperfusion in the recipient can improve initial renal plasma perfusion and GFR in a renal graft exposed to several risk factors of developing DGF. Such findings have not previously been described in a large animal transplantation model or in humans and suggest rIC to prevent DGF.

Authorship

NVK: collected and analysed data, drafted the paper. PS: designed and performed the study, collected and analysed data, edited the paper. NGS and KR: designed and performed the study. AKK: designed and assisted in performing the study. ET: designed the study, anaesthesiologist. BMB: analysed data, biostatistician. UM and EOO: designed and performed the study; surgeons. MP: MRI scanning technique and scan analysis, edited the paper. TMJ: designed the study, surgeon. HL: assisting in biomarker measurements. RN and HB: measured biomarkers, edited the paper. NM: pathologist. BJ: sponsor, responsible of overall project design, edited the paper.

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References

1. Solomon H. Opportunities and challenges of expanded criteria organs in liver and kidney transplantation as a response to organ shortage. *Mo Med* 2011; **108**: 269.
2. Humar A, Ramcharan T, Kandaswamy R, Gillingham K, Payne WD, Matas AJ. Risk factors for slow graft function after kidney transplants: a multivariate analysis. *Clin Transplant* 2002; **16**: 425.
3. Halloran PF, Hunsicker LG. Delayed graft function: state of the art, November 10-11, 2000. Summit meeting, Scottsdale, Arizona, USA. *Am J Transplant* 2001; **1**: 115.
4. Salvatierra Jr O, Singh T, Shifrin R, *et al.* Successful transplantation of adult-sized kidneys into infants requires maintenance of high aortic blood flow. *Transplantation* 1998; **66**: 819.
5. Pape L, Offner G, Ehrich JH, de Boer J, Persijn GG. Renal allograft function in matched pediatric and adult recipient pairs of the same donor. *Transplantation* 2004; **77**: 1191.
6. Shackleton CR. Upregulation of major histocompatibility complex-expression under ischemic conditions in experimental models. *Transplant Proc* 1998; **30**: 4264.
7. Bidmon B, Kratochwill K, Rusai K, *et al.* Increased immunogenicity is an integral part of the heat shock response following renal ischemia. *Cell Stress Chaperones* 2012; **17**: 385.
8. Przyklenk K, Bauer B, Ovize M, Kloner RA, Whittaker P. Regional ischemic 'preconditioning' protects remote virgin myocardium from subsequent sustained coronary occlusion. *Circulation* 1993; **87**: 893.
9. Goto M, Liu Y, Yang XM, Ardell JL, Cohen MV, Downey JM. Role of bradykinin in protection of ischemic preconditioning in rabbit hearts. *Circ Res* 1995; **77**: 611.
10. Kanoria S, Jalan R, Seifalian AM, Williams R, Davidson BR. Protocols and mechanisms for remote ischemic preconditioning: a novel method for reducing ischemia reperfusion injury. *Transplantation* 2007; **84**: 445.
11. Botker HE, Kharbanda R, Schmidt MR, *et al.* Remote ischaemic conditioning before hospital admission, as a complement to angioplasty, and effect on myocardial salvage in patients with acute myocardial infarction: a randomised trial. *Lancet* 2010; **375**: 727.
12. Jia RP, Zhu JG, Wu JP, Xie JJ, Xu LW. Experimental study on early protective effect of ischemic preconditioning on rat kidney graft. *Transplant Proc* 2009; **41**: 69.
13. Lee HT, Emala CW. Protective effects of renal ischemic preconditioning and adenosine pretreatment: role of A(1) and A(3) receptors. *Am J Physiol Renal Physiol* 2000; **278**: F380.
14. Ali ZA, Callaghan CJ, Lim E, *et al.* Remote ischemic preconditioning reduces myocardial and renal injury after elective abdominal aortic aneurysm repair: a randomized controlled trial. *Circulation* 2007; **116**: I98.
15. Wever KE, Warlé MC, Wagener FA, *et al.* Remote ischaemic preconditioning by brief hind limb ischaemia protects against renal ischaemia-reperfusion injury: the role of adenosine. *Nephrol Dial Transplant* 2011; **26**: 3108.
16. Selzner N, Boehnert M, Selzner M. Preconditioning, postconditioning, and remote conditioning in solid organ transplantation: basic mechanisms and translational applications. *Transplant Rev (Orlando)* 2012; **26**: 115.
17. Koetting M, Frotscher C, Minor T. Hypothermic reconditioning after cold storage improves postischemic graft function in isolated porcine kidneys. *Transpl Int* 2010; **23**: 538.
18. Barklin A, Larsson A, Vestergaard C, *et al.* Does brain death induce a pro-inflammatory response at the organ level in a porcine model? *Acta Anaesthesiol Scand* 2008; **52**: 621.
19. Jørgensen B, Keller AK, Radvanska E, *et al.* Reproducibility of contrast enhanced magnetic resonance renography in adolescents. *J Urol* 2006; **176**: 1171.
20. Aumann S, Schoenberg SO, Just A, *et al.* Quantification of renal perfusion using an intravascular contrast agent (part 1): results in a canine model. *Magn Reson Med* 2003; **49**: 276.
21. van den Eijnden MM, Leuvenink HG, Ottens PJ, *et al.* Effect of brain death and non-heart-beating kidney donation on renal function and injury: an assessment in the isolated perfused rat kidney. *Exp Clin Transplant* 2003; **1**: 85.
22. Findlay J, Levvy GA, Marsh CA. Inhibition of glycosidases by aldonolactones of corresponding configuration. 2. Inhibitors of beta-N-acetylglucosaminidase. *Biochem J* 1958; **69**: 467.
23. Pfleiderer G. *Particle Bound Aminopeptidase from Pig Kidney. Methods in Enzymology*. London: Academic Press, 1970: 514 pp.
24. Friis C. Postnatal development of renal function in piglets: glomerular filtration rate, clearance of PAH and PAH extraction. *Biol Neonate* 1979; **35**: 180.
25. Eskild-Jensen A, Rehling M, Nielsen AS, Nielsen LE, Frøkiaer J. Use of plasma clearance of technetium Tc 99m pentetate to estimate renal clearance during postnatal development in pigs. *Am J Vet Res* 2002; **63**: 1203.
26. Cheung MM, Kharbanda RK, Konstantinov IE, *et al.* Randomized controlled trial of the effects of remote ischemic preconditioning on children undergoing cardiac surgery: first clinical application in humans. *J Am Coll Cardiol* 2006; **47**: 2277.
27. Weis M, Schlichting CL, Engleman EG, Cooke JP. Endothelial determinants of dendritic cell adhesion and migration: new implications for vascular diseases. *Arterioscler Thromb Vasc Biol* 2002; **22**: 1817.

28. Ravlo K, Koefoed-Nielsen P, Secher N, *et al.* Effect of remote ischemic conditioning on dendritic cell number in blood after renal transplantation - flow cytometry in a porcine model. *Transpl Immunol* 2012; **26**: 146.
29. Tenhunen R, Marver HS, Schmid R. The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. *Proc Natl Acad Sci USA* 1968; **61**: 748.
30. Katori M, Busuttil RW, Kupiec-Weglinski JW. Heme oxygenase-1 system in organ transplantation. *Transplantation* 2002; **74**: 905.
31. Otterbein LE, Soares MP, Yamashita K, Bach FH. Heme oxygenase-1: unleashing the protective properties of heme. *Trends Immunol* 2003; **24**: 449.
32. Soares MP, Bach FH. Heme oxygenase-1 in organ transplantation. *Front Biosci* 2007; **12**: 4932.
33. Geuken E, Buis CI, Visser DS, *et al.* Expression of heme oxygenase-1 in human livers before transplantation correlates with graft injury and function after transplantation. *Am J Transplant* 2005; **5**: 1875.
34. Ollinger R, Kogler P, Biebl M, *et al.* Protein levels of heme oxygenase-1 during reperfusion in human kidney transplants with delayed graft function. *Clin Transplant* 2008; **22**: 418.
35. Tapuria N, Kumar Y, Habib MM, Abu Amara M, Seifalian AM, Davidson BR. Remote ischemic preconditioning: a novel protective method from ischemia reperfusion injury–a review. *J Surg Res* 2008; **150**: 304.
36. Nielsen TT, Støttrup NB, Løfgren B, Bøtker HE. Metabolic fingerprint of ischaemic cardioprotection: importance of the malate-aspartate shuttle. *Cardiovasc Res* 2011; **91**: 382.