

ORIGINAL ARTICLE

The effect of normothermic recirculation before cold preservation on post-transplant injury of ischemically damaged donor kidneys

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Conflicts of Interest

None to declare.

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Summary

Kidneys recovered from donation after cardiac death (DCD) are increasingly used to enlarge the deceased donor pool. Such renal grafts, especially those derived from uncontrolled DCD, have inevitably sustained profound warm ischemic injury, which compromises post-transplant function. Normothermic recirculation (NR) of the deceased donor's body before organ cooling could be an interesting approach to mitigate the detrimental effect of warm ischemia. To date, however, there is no evidence coming from preclinical studies to support the principle of NR in kidney transplantation. In this study, we subjected 48 Lewis rat kidneys to 15 or 30 min of warm ischemia, and subsequently 0, 1, or 2 h of NR. After 24 h cold storage, kidneys were transplanted into a recipient animal and 24 h later we measured the percentage of cortical necrosis, and determined gene expression of heme oxygenase-1, heat shock protein-70, transforming growth factor- β , kidney injury molecule-1, interleukin-6, hypoxia inducible factor-1 α , monocyte chemoattractant protein-1, and α -smooth muscle actin in kidney tissue. We found that NR had no significant influence on any of these markers. Therefore, we conclude that this animal study by no means supports the presumed beneficial effect of NR on kidneys that have been severely damaged by warm ischemia.

Introduction

To partially resolve the persistent donor organ shortage, kidneys recovered from donation after cardiac death (DCD) are increasingly used to enlarge the deceased donor pool. Compared to renal grafts recovered from donors after brain death, DCD kidneys have by definition sustained additional injury due to warm ischemia (WI) between cardiocirculatory arrest and cold organ perfusion. Although the duration of WI varies among the different types of DCD donors, recipients of such kidneys are known to have a substantially increased risk of delayed graft function and primary nonfunction, especially when

WI has been very profound such as in uncontrolled DCD [1].

Most established organ preservation protocols are based on rapid cooling immediately after cardiac arrest, followed by organ procurement and either static cold storage or hypothermic machine perfusion of the kidney graft [2]. To mitigate the detrimental effect of warm ischemia, some studies have suggested the use of normothermic recirculation (NR) before organ cooling is instituted. NR is an early organ preservation strategy, in which the deceased donor's body is artificially recirculated with warm oxygenized blood quickly after the declaration of cardiocirculatory death, for a limited period of

time such as 1 or 2 h. NR is typically administered through an extracorporeal membrane oxygenator, connected to a closed circuit with cannulae in the femoral vessels of the deceased donor [3]. A few studies have reported beneficial effects of this strategy on post-transplant graft function and survival. Most of these reports focus on NR prior to DCD liver transplantation [3–5]. So far, only one published clinical study presented results of NR in renal transplantation [6]. The authors reported a significant reduction of delayed graft function and an improved graft survival after transplantation when NR was compared with a protocol in which organs were immediately cooled. To our knowledge, hospitals in Barcelona, Madrid, Paris, St. Petersburg, and Taiwan are currently the only centers worldwide with an operational clinical NR protocol for potential DCD kidney and liver donors [3,7,8].

Before a novel preservation strategy such as NR can be widely implemented in human renal transplantation practice, more basic evidence is needed to quantify the magnitude of its presumed effect and to unravel the mechanism through which NR could be beneficial to a DCD kidney graft. To date, there is no evidence coming from mechanistic preclinical studies to support the principle of NR in kidney transplantation. We have conducted an animal study to investigate the potential of NR to reduce WI injury in a standardized renal transplantation model. The aim of the present study was to determine whether NR can reduce the amount of tubular necrosis after transplantation, and whether NR influences the expression of genes that are involved in renal damage, inflammation, interstitial fibrosis formation, cytoprotection, and tissue regeneration in kidneys that have sustained severe warm ischemic injury in the donor.

Methods

Animals and housing

Ninety-six adult male Lewis rats weighing 250–300 g, obtained from Harlan (Zeist, The Netherlands) were used as kidney donors ($n = 48$) and recipients ($n = 48$). Before surgery, animals were kept in standard polycarbonate housing (model 1354F, Tecniplast, Buguggiate, Italy), with a maximum of four animals together in one cage. After surgery, recipient animals were housed individually in the aforementioned polycarbonate housing. Throughout the experiment, all animals were allowed free access to a standard laboratory animal diet and acidified tap water. All experimental procedures were approved by the animal experiment committee of the University of Groningen, and the principles of laboratory animal care (NIH publication no. 85-23, revised 1985), as well as regula-

tions imposed by the Dutch law on animal experiments were followed.

Experimental design

We employed a syngeneic Lewis to Lewis rat renal transplant model with orthotopic transplantation of the left donor kidney, leaving the recipient's native right kidney *in situ*. Renal grafts in six experimental groups (eight transplants per group) received either 15 or 30 min of WI, followed by either no NR, 1 h NR, or 2 h NR, and subsequently 24 h cold storage (CS) preservation and transplantation into a recipient animal. Recipient animals were sacrificed exactly 24 h post-transplant. Experimental groups were as follows:

Group 1: no WI – no NR – 24 h CS – transplantation – 24 h reperfusion in the recipient

Group 2: 15 min WI – no NR – 24 h CS – transplantation – 24 h reperfusion in the recipient

Group 3: 30 min WI – no NR – 24 h CS – transplantation – 24 h reperfusion in the recipient

Group 4: 15 min WI – 1 h NR – 24 h CS – transplantation – 24 h reperfusion in the recipient

Group 5: 30 min WI – 1 h NR – 24 h CS – transplantation – 24 h reperfusion in the recipient

Group 6: 15 min WI – 2 h NR – 24 h CS – transplantation – 24 h reperfusion in the recipient

Group 7: 30 min WI – 2 h NR – 24 h CS – transplantation – 24 h reperfusion in the recipient

We chose 15 and 30 min for the duration of WI time in this animal model, as we had previously demonstrated that the combination of 15 min WI and 24 h CS results in a seriously damaged kidney graft, leading to delayed graft function after transplantation [9]. As NR is most interesting in the uncontrolled DCD (Maastricht categories I and II) [10] setting that will lead to severely damaged kidneys, we deliberately chose not to test the method on kidneys that have sustained only mild ischemic injury. We added the duration of 30 min WI to provide for an even heavier variant of this DCD animal model. We chose 1 and 2 h for the duration of NR, as these seem realistic times to apply NR in the human setting and are also comparable to the time periods that the Barcelona group reports for NR in their center.

Donor operation and organ preservation

After induction of inhalation anesthesia with 5% isoflurane/oxygen, donor animals received 250 IU heparin via the penile vein. Through a midline incision, the left kidney, both renal vessels, and the ureter were isolated. The left renal artery and vein were subsequently clamped for 15 or 30 min to induce WI. In stated experimental groups,

NR was induced by removal of the clamps and reperfusion of the left kidney for 1 or 2 h. Next, a ligature was placed around the aorta, superior to the right renal artery, to prevent flushing of the liver and intestine. The inferior caval vein was cut and both kidneys were flushed by inserting a 20 G needle into the aortic bifurcation and infusing 10 ml of 0.9% NaCl at 37 °C, directly followed by 10 ml of University of Wisconsin (UW) organ preservation solution at 4 °C. Glutathione (0.922 mg/ml) was freshly added to the UW solution. Immediately upon flushing, the left kidney was removed. Donor kidneys were preserved for duration of exactly 24 h by means of static CS at 0–4 °C, submerged into 25 ml of UW solution with added glutathione.

Recipient operation

After induction with 5% isoflurane/oxygen, maintenance inhalation anesthesia with 3% isoflurane/oxygen was used. Orthotopic kidney transplantation was performed on the left side: First, the native left kidney was removed after clamping both renal blood vessels. The graft renal artery was anastomosed end-to-end to the recipient's renal artery using eight interrupted Dafilon® 10-0 (B. Braun, Melsungen, Germany) non-absorbable sutures, and the graft renal vein was anastomosed to the recipient's renal vein with a running suture of the same material. Vascular anastomosis time was standardized to exactly 25 min for each procedure. The graft ureter was anastomosed end-to-end to the recipient ureter using four interrupted sutures. The abdominal fascia and skin were closed in layers with two separate absorbable Safil® 4-0 (B. Braun) running sutures. Analgesia was managed subcutaneously with buprenorphine: Animals received 0.01 mg/kg during surgery, 0.04 mg/kg immediately after transplantation, and 0.05 mg/kg 10–12 h postsurgery. An electric warming

blanket was placed under the cage floor to prevent hypothermia in the first hours after surgery. At exactly 24 h post-transplant, recipient animals were sacrificed by exsanguination under anesthesia. The transplanted kidney was harvested surgically before exsanguination.

Sample collection and analysis

Prior to termination of recipient animals, the donor kidney was collected and one tissue sample was fixed in 4% formalin for histologic examination. Another tissue sample was immediately snap frozen in liquid nitrogen. Histologic slices were stained by the periodic acid-Schiff (PAS) method and were quantitatively assessed for cortical necrosis. Digital images of each slice were taken and Aperio ImageScope software (Aperio, Vista, CA, USA) was used to calculate the percentage cortical necrosis as the quotient of the necrotic cortical area and the total cortical area. Figure 1 shows a representative example of the scoring method.

Real-time quantitative RT-PCR (qPCR) analysis of heme oxygenase-1 (HO-1), heat shock protein-70 (HSP-70), transforming growth factor- β (TGF- β), kidney injury molecule-1 (KIM-1), interleukin-6 (IL-6), hypoxia inducible factor-1 α (HIF-1 α), monocyte chemoattractant protein-1 (MCP-1), and α -smooth muscle actin (α -SMA) gene expression was performed to detect cytoprotection (HO-1 and HSP-70), tissue regeneration (TGF- β), renal tubular injury (KIM-1), aspecific inflammation (IL-6, HIF-1 α , and MCP-1), and early signs of interstitial fibrosis (α -SMA) 24 h after transplantation. Amplification primers (Table 1) were designed with Primer Express software (Applied Biosystems, Foster City, CA, USA) and validated in a six-step twofold dilution series. RNA was extracted from snap frozen tissue using TRIzol (Invitrogen, Breda, The Netherlands). Total RNA was treated

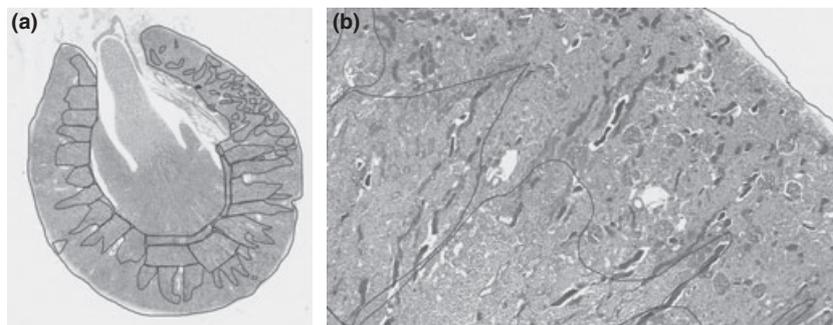


Figure 1 Representative example of the cortical necrosis scoring method. Histologic slices were stained by the periodic acid-Schiff (PAS) method and were quantitatively assessed. Digital images of each slice were taken and Aperio ImageScope software was used to calculate the percentage cortical necrosis as the quotient of the necrotic cortical area and the total cortical area. Overview of a kidney at 10 \times magnification (a), and a more detailed view at 50 \times magnification (b). The total cortical area and the necrotic sections are encircled with black lines. In panel (b), the necrotic area on the lower left hand side is bounded by a black line, on the other side of which vital cortical renal tissue can be seen.

Table 1. Primers used for qPCR analyses.

Gene	Forward primer	Reverse primer
β -actin	5'-GGAAATCGTGCGTGACATTA-3'	5'-GCGGCAGTGGCCATCTC-3'
HO-1	5'-CTCGCATGAACACTCTGGAGAT-3'	5'-GCAGGAAGGCGGTCTTAGC-3'
HSP-70	5'-GGTTGCATGTTCTTTGCGTTA-3'	5'-GGTGGCAGTCTGAGGTGT-3'
TGF- β	5'-GCTCTTGTGACAGCAAAGATAATGTAC-3'	5'-CCTCGACGTTGGGACTGAT-3'
KIM-1	5'-AGAGAGAGCAGGACACAGGCTTT-3'	5'-ACCCGTGGTAGTCCCAACA-3'
IL-6	5'-CCAACCTCCAATGCTCTCTAATG-3'	5'-TTCAAGTCTTCAAGAGTTGGAT-3'
HIF-1 α	5'-GAACATGATGGCTCCCTTTTC-3'	5'-CCTGGTTGCTGCAGTAACGTT-3'
MCP-1	5'-CTTTGAATGTGAACCTGACCCATAA-3'	5'-ACAGAAGTGTGAGGTGGTTGT-3'
α -SMA	5'-GAGAAAATGACCCAGATTATGTTTGA-3'	5'-GGACAGCACAGCCTGAATAGC-3'

HIF-1 α , hypoxia inducible factor-1 α ; HO-1, heme oxygenase-1; HSP-70, heat shock protein-70; IL-6, interleukin-6; KIM-1 kidney injury molecule-1; MCP-1, monocyte chemoattractant protein-1; α -SMA, α -smooth muscle actin; TGF- β , transforming growth factor- β .

with DNase I, Amp Grade (Invitrogen). cDNA synthesis was performed from 1 μ g total RNA using T₁₁VN oligos and M-MLV Reverse Transcriptase, according to supplier's protocol (Invitrogen). Amplification and detection were performed with the ABI Prism 7900-HT Sequence Detection System (Applied Biosystems) using emission from SYBR Green (SYBR Green master mix, Applied Biosystems). All assays were performed in triplicate. After an initial activation step at 50 °C for 2 min and a hot start at 95 °C for 10 min, qPCR cycles consisted of 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Gene expression was normalized with the mean of β -actin mRNA content and calculated relative to healthy controls. Results were expressed as $2^{-\Delta\text{CT}}$ (C_T threshold cycle).

Statistics

To minimize the number of animals required per group, a 3 \times 3 between-subjects factorial design was constructed in which the levels of two independent variables (WI time 0, 15, or 30 min, and NR time 0, 1, or 2 h) were varied among the seven groups. Using Mead's formula for sample size estimation in factorial designs [11], we calculated that a minimum of five animals per group would be required to obtain adequate statistical power. Some interaction between WI time and NR time could be assumed, and variances could be different among the six groups. In addition, prior experience showed that in 15–20% of the transplants a technical complication would occur. Therefore, we determined that the initial number of animals per group should be eight. Statistical analyses were performed with SPSS software, version 18 (IBM, Armonk, NY, USA). One-way ANOVAs were performed which tested whether the dimensions WI time and NR time significantly influenced each of the nine dependent variables (cortical necrosis, and the expression of eight genes) after transplantation, and whether there was any significant interaction between WI time and NR time. In case a

significant effect of WI or NR time was found for a certain dependent variable, we used Turkey's *post hoc* test to determine between which of the three levels of these variables the significant difference existed. As none of the independent variables were normally distributed, all values were transformed to ranks before being entered into the analyses. Two-sided *P*-values below 0.05 were considered to indicate statistical significance.

Results

In 9 of 53 transplants (17%), technical complications occurred, which were mostly related to inadequate vascular flushout in the donor and/or leakage or occlusion of the vascular anastomosis in the recipient. These nine cases were excluded from further analysis. Exclusions occurred in all groups with WI and led to a median of seven animals per experimental group. In the remainder of procedures, all recipient animals survived until sacrifice at 24 h after transplantation.

Cortical necrosis

Figure 2a shows a plot of the percentage cortical necrosis for each individual transplant, categorized per experimental group. In all groups that had sustained WI, we found profound cortical necrosis 24 h after transplantation with an overall median of 65% (interquartile range 37–86%) of the total renal cortical area. In kidneys that had sustained 15 min of WI, the median cortical necrotic area was 43% (interquartile range 34–71%), whereas renal grafts with 30 min of WI in the donor had a significantly higher median cortical necrotic area of 82% (interquartile range 43–95%; *P* = 0.03 vs. 15 min of WI). Kidneys without WI in the donor had a significantly lower median percentage of cortical necrosis 24 h after transplantation (18%, interquartile range 11–21%; *P* = 0.01 versus 15 min of WI and *P* < 0.005 vs. 30 min of WI). In contrast, NR did not have

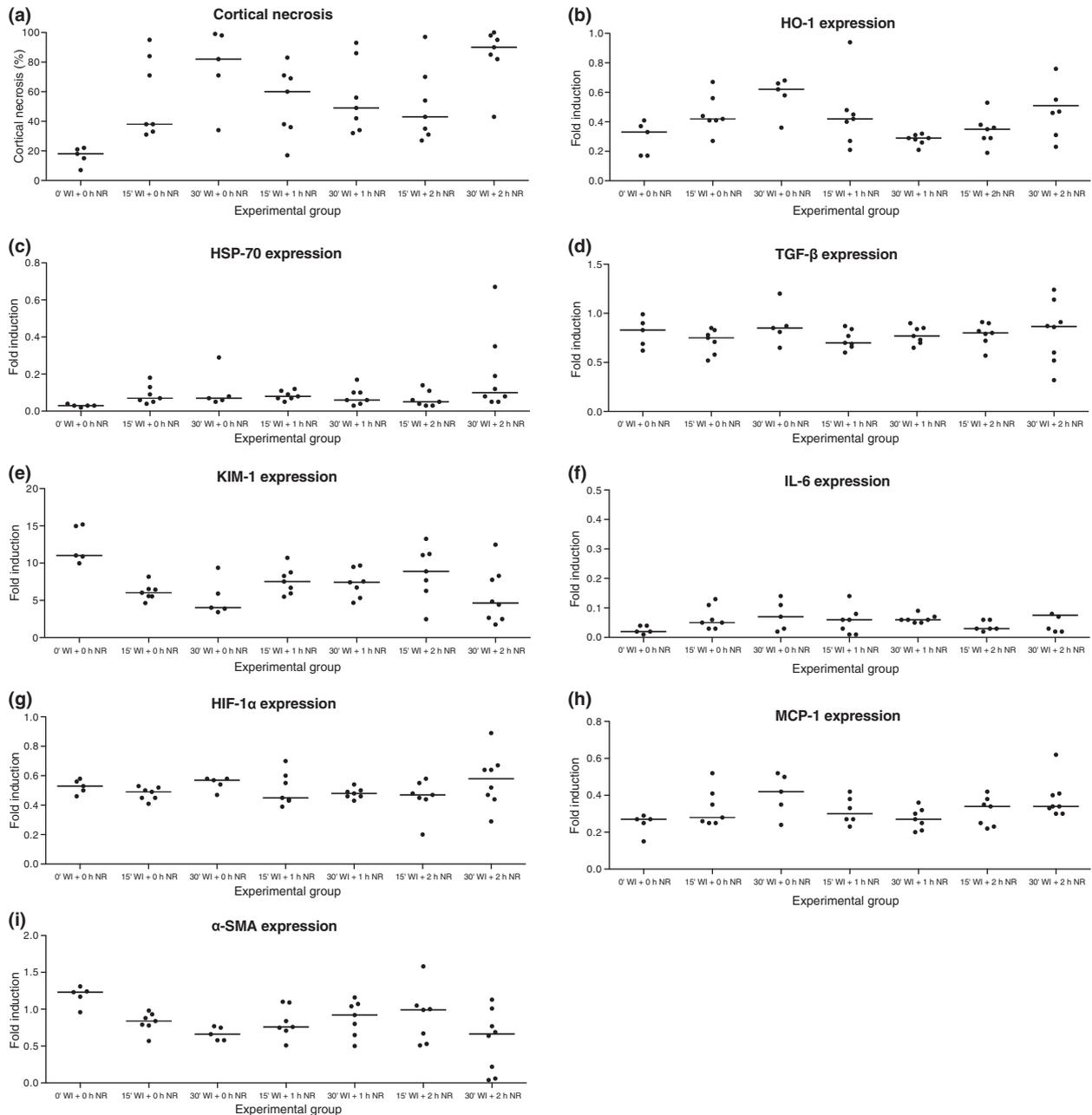


Figure 2 Percentage of cortical necrosis in kidney grafts, 24 h after transplantation (a), and the expression of heme oxygenase-1, heat shock protein-70, transforming growth factor- β , kidney injury molecule-1, interleukin-6, hypoxia inducible factor-1 α , monocyte chemoattractant protein-1, and α -smooth muscle actin 24 h after transplantation (b–i). Each black dot represents a single case, and horizontal lines indicate median values per experimental group.

any significant effect on the percentage of cortical necrosis after transplantation: In WI injured kidneys that underwent no NR the median cortical necrotic area was 71% (interquartile range 35–92%) 24 h post-transplant, and for kidneys that were treated with 1 or 2 h of NR this figure was 53% (interquartile range 36–74%) and 76% (inter-

quartile range 41–95%), respectively ($P = 0.28$). There was no significant interaction between WI time and NR time ($P = 0.24$) for the dependent variable cortical necrosis. Table 2 lists P -values resulting from the one-way ANOVAS which tested whether WI time, or NR time significantly influenced each dependent variable.

Table 2. *P*-values resulting from the one-way ANOVAS which tested whether either warm ischemic time, or normothermic recirculation time significantly influenced each dependent variable.

Dependent variable	<i>P</i> -value for WI	<i>P</i> -value for NR
Cortical necrosis	<0.005	0.28
HO-1	0.01	0.01
HSP-70	0.005	0.98
TGF- β	0.37	0.66
KIM-1	<0.005	0.14
IL-6	0.03	0.81
HIF-1 α	0.20	0.57
MCP-1	0.06	0.18
α -SMA	0.004	0.47

HIF-1 α , hypoxia inducible factor-1 α ; HO-1, heme oxygenase-1; HSP-70, heat shock protein-70; IL-6, interleukin-6; KIM-1, kidney injury molecule-1; MCP-1, monocyte chemoattractant protein-1; NR, Normothermic recirculation; α -SMA, α -smooth muscle actin; TGF- β , transforming growth factor- β ; WI, warm ischemia.

qPCR results

Figure 1b–i present the results of qPCR analyses for each individual transplant, categorized per experimental group. WI time had a statistically significant influence on the expression of KIM-1, HSP-70, α -SMA, HO-1, and IL-6 in the ANOVAS. In the *post hoc* tests, this significant effect was mostly explained by the difference between 0 and 15 min and/or 0 and 30 min of WI, and not by the difference between 15 and 30 min of WI. Overall, the expression of genes that are involved in cytoprotection, tissue regeneration, tubular injury, inflammation, and interstitial fibrosis was minimally influenced by NR time. Only for HO-1 NR time had a significant influence on gene expression 24 h after transplantation ($P = 0.01$), but there was also a significant interaction between WI time and NR time in this model ($P = 0.005$). For all other markers tested, NR did not significantly influence gene expression in the kidney graft 24 h post-transplant (Table 2).

Discussion

Interventions that aim at better preserving donor organ quality prior to transplantation are becoming increasingly important in an era with more marginal deceased donor grafts in the pool [12,13]. Normothermic recirculation immediately after cessation of cardiopulmonary resuscitation measures was initially instituted by the group of the *Hospital Clinic* in Barcelona, Spain, to gain extra time and obtain the compulsory judicial permission for uncontrolled (Maastricht category I and II) DCD organ donation [3]. As a side effect, clinicians observed an improved early function of those renal grafts that had been subjected to NR in the donor, compared with kidneys that

came from donors whose organs were immediately cooled when cardiopulmonary resuscitation was stopped, with 12.5% versus 75% delayed graft function incidence [6]. These findings, together with favorable results of DCD liver transplantation after NR (post-transplant course of uncontrolled DCD livers after NR comparable with that of livers recovered from DBD donors without NR), have led to the hypothesis that NR may somehow resuscitate a DCD donor kidney that has been exposed to severe WI injury [14]. However, to date, the mechanism as well as the magnitude of its postulated effect remains to be unraveled. Interestingly, NR is already clinically utilized at a small scale, although there is no convincing preclinical evidence which supports its principle and/or effectiveness for kidneys. The aim of the present study was to provide a first piece of such evidence. It needs to be stressed that we did not set out to exactly mimic the human DCD and NR situation. Instead, the present study makes several simplifications and specifically focuses on standardized severe WI injury to rat kidneys and the question whether a period of warm, oxygenized reperfusion with autologous blood before cold storage will have any effect on cortical necrosis and gene expression post-transplant. Much to our surprise, we could not find any indication that such an intervention will somehow protect or resuscitate renal grafts that have sustained profound WI injury in this rodent model. Therefore, the present animal study does not support the scarce published evidence which suggested that NR could have a beneficial effect on kidneys recovered from uncontrolled DCD.

Apart from merely being a preclinical study in a standardized animal model, this study has a few other relevant limitations that should be considered when translating our findings to the human clinical setting. First, in our model WI injury was induced by clamping the renal vessels after systemic heparinization, which is not fully comparable with cardiac arrest followed by cardiopulmonary resuscitation and cessation of such measures as it occurs in human uncontrolled DCD [15]. We chose not to employ a genuine cardiac arrest model, because we wanted to focus on the effect of NR after a clear-cut duration of real WI, avoiding the more complex situation of slowly worsening hypoxia, hypotension, and the associated systemic neurologic and humoral responses that could all have their own isolated effect on the kidney graft. In addition, we needed a physiologically intact circulation in the donor animal for NR after WI. We employed an auto-NR model, as artificial warm and oxygenated recirculation of small rodents is technically challenging and therefore likely to introduce more variation in our model. A second limitation is that our study lacks functional end points in terms of renal function or graft survival after transplantation. To obtain such data, recipient

animals need to stay alive for at least a few weeks post-transplant and the native contralateral kidney of the recipient would have to be removed at the same moment when the donor graft is implanted, or shortly after, to be able to measure early renal function of only the transplanted kidney. Normally, with donor kidneys that have sustained only minimal injury, an orthotopic renal transplant model with native contralateral nephrectomy is easily applicable and rather stable [16]. However, for the present study donor kidneys needed to be severely damaged. Hence, most kidneys would develop delayed graft function in the first days after transplantation. Without a native contralateral kidney *in situ*, most animals would die of uremia soon after transplantation, or become unacceptably ill in those first days [9]. Dialysis of small rodents is technically very complex and would introduce too much variation in our data. With a native contralateral kidney *in situ*, as in our model, reliable isolated measurement of graft function is impossible. As a consequence, this study lacks functional end points.

Another limitation of this study could be that we have possibly chosen a too heavy model of WI injury, which rendered most kidneys in a condition that was beyond any recovery. Thus, we may have missed a potential beneficial effect of NR because renal grafts in our study sustained too much damage to be repaired by this intervention. Although the cortical necrosis results do suggest that those kidneys with WI injury were severely damaged versus the control group without WI, gene expression patterns were not very different in the experimental groups with WI compared with the control group without WI. This finding suggests that WI injured kidneys in our study were still capable of gene expression within a normal range. Hence, if NR would have any influence on gene expression 24 h post-transplant, our model should have detected it. It can also be argued that the 24 h of cold ischemia that all kidneys in the present study have sustained might have been too long, leading to irreversibly damaged kidneys at transplant. However, our group has positive prior experience with this length of cold storage in Lewis rat kidneys, leading to still viable kidneys post-transplant. Even though NR might show some measurable effects in kidneys that are only minimally injured, it is a logistically challenging and rather costly method in humans. We feel that its application is most relevant when NR would lead to a significant improvement of severely damaged donor grafts, which would otherwise not be sufficiently suitable for transplantation.

An earlier preclinical study by Net *et al.* showed that, in DCD liver transplantation, the effect of NR could be mediated by a form of ischemic preconditioning [4]. Although proven to be protective against ischemia/reper-

fusion injury in liver transplantation, ischemic preconditioning does not seem to have such an effect on kidney grafts [17]. This may in part explain why NR does reduce ischemia/reperfusion related injury in DCD liver grafts, but the method does not significantly protect and/or resuscitate ischemically damaged kidneys.

It is well established that although ischemia itself does lead to tissue injury, subsequent reperfusion will cause even more damage through a multitude of pathways including acute aspecific inflammation and the detrimental effect of reactive oxygen species [18–20]. A donor procedure with NR, followed by transplantation of the kidney will follow the sequence WI – warm oxygenized reperfusion – cold ischemia – warm oxygenized reperfusion and therefore has two instead of just one potentially detrimental episodes of reperfusion. As a consequence, NR might even lead to more instead of less ischemia-reperfusion related kidney injury. In our study, donor kidneys after 30 min of WI and 2 h of NR had significantly more cortical necrosis than renal grafts that had also sustained 30 min of WI, but underwent only 1 h of NR. This finding carefully supports the hypothesis that a long period of NR after profound WI could actually be detrimental to a kidney graft. In addition, in the human setting NR would most likely reperfuse a substantial part of the donor's body, all of which has endured WI. In contrast to our animal model in which only the kidney sustained WI, a human DCD kidney would also be exposed to circulating inflammatory mediators and oxygen free radicals that are released upon warm reperfusion of the intestine and the liver.

In conclusion, the present animal study could not show any beneficial effect of NR in terms of more cytoprotection, elevated tissue regeneration, less interstitial fibrosis formation, a lower level of aspecific inflammation, or a decreased percentage of tubular necrosis in transplanted kidneys that had sustained severe warm ischemic injury in the donor. Our data do have several relevant limitations which preclude a direct translation to the human clinical setting. Nevertheless, this study by no means supports the concept of NR for DCD kidneys. We feel that more preclinical evidence is needed to decide whether this method should be implemented in human uncontrolled DCD or not, as neither the mechanism has been elucidated in animal studies, nor has its effectiveness been shown in randomized controlled studies so far.

Authorship

CM: designed the experiment, was involved in most experimental procedures, supervised qPCR analyses, and wrote the first and subsequent drafts of the manuscript. GvR: performed all measurements of tubular necrosis and

took part in the writing of the manuscript. RP and HL: supervised the experimental work and proofread the manuscript.

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