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Inflammation and innate immunity in renal ischemia/reperfusion injury

Dorotty K. de Vries

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Dorotty K. de Vries

Inflammation and innate immunity in renal ischemia/reperfusion injury

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Inflammation and innate immunity in renal ischemia/reperfusion injury

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Inflammation and innate immunity in renal ischemia/reperfusion injury

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General Introduction

D.K. de Vries, A.F.M. Schaapherder, M.E.J. Reinders

Introduction

Kidney transplantation is the preferred treatment of end-stage renal disease, because of the recipient’s improved life expectancy, better quality of life, and the lower total healthcare costs, compared to dialysis treatment.\textsuperscript{1,2} Since the first successful kidney transplantation in 1954, patient and graft survival increased tremendously.\textsuperscript{1,4} Despite all the improvements in immunosuppressive drugs and surgical techniques, it appears that during the last decade a plateau in long term allograft survival has been reached.

As the demand for kidney grafts is ever increasing, the number of kidney transplantations is limited by the availability of organ donors. Almost half of all kidney transplantations are living donor kidney transplantations, whereas the others are transplantations with deceased donor kidney grafts.\textsuperscript{5} Deceased donor kidney grafts are retrieved from either brain dead donors or cardiac dead donors. Graft survival for living unrelated donor kidney transplantation is superior compared to that of deceased donor kidney transplantation, even though the average human leukocyte antigen (HLA) matching is worse in living unrelated transplantation.\textsuperscript{6} Therefore, the limited graft survival of deceased donor kidneys cannot be attributed exclusively to differences in immunogenicity; other causes of damage are probably more important. The most prominent of these causes is ischemia/reperfusion (I/R) injury, characterized by the exacerbation of tissue damage upon reestablishment of circulation after a period of ischemia. I/R injury is an important cause of delayed graft function, having a major influence on both graft function as well as graft survival.\textsuperscript{7}

Renal ischemia/reperfusion injury

Renal I/R injury occurs in a multitude of clinical situations. Periods of hypotension with impaired blood flow to the kidney can cause renal I/R injury, whereas more acute ischemia occurs in renal arterial thrombosis. In kidney transplantation renal ischemia is inevitable, and the duration of ischemia is often beyond control. Preventive and therapeutic measures in I/R injury would be required to reduce the severity of graft dysfunction and failure thus allowing safe expansion of the donor pool with marginal donor kidneys that have suffered more initial injury before organ retrieval. Unfortunately, current treatment for renal I/R injury is still primarily supportive, and experimental therapies aimed at minimizing I/R injury have been applied in animal models generally, not clinical trials. In order to design better therapeutics for clinical renal I/R injury, detailed knowledge on the pathophysiological mechanisms leading to ischemic acute graft injury after transplantation is required.
Chapter 1

Pathophysiology of renal ischemia/reperfusion injury

The pathophysiology of I/R injury is multifactorial and only partially understood. Inflammation however, is regarded the crucial event in the development of tissue injury and graft dysfunction in renal I/R injury. Based on animal experiments, many individual factors such as cytokines and complement have been suggested to be involved in the inflammatory response. However, intervention studies aimed at specific inhibition of a single factor have generally shown disappointing results. Cooperation, redundancy and interactions make the involved mechanisms more complex than previously thought. Pharmacological inhibition of the entire inflammatory cascade would appear a logical intervention, however the negative side-effects appear larger than the anticipated beneficial effects.

Although there may be differences in the exact pathophysiological mechanisms of I/R injury between different organs, some processes appear to play a universal role. The endothelium and microvasculature are very sensitive to hypoxia and are easily affected in I/R injury. Upon reperfusion, the vascular endothelial cell lining can undergo swelling which may lead to narrowing of the vascular lumen. Moreover, vasorelaxation can be impaired, together contributing to the no-reflow phenomenon. Endothelial injury can increase microvascular permeability which may lead to inflammatory cell trafficking into the reperfused kidney. There have been many reports of invading granulocytes, monocytes, dendritic cells (DC's) and lymphocytes into various tissues after reperfusion. Together with leukocytes, platelets can be activated by injured endothelium. In myocardial infarction, platelets mediate thrombotic occlusion and increase damage by causing microvascular occlusions, contributing to the no-reflow phenomenon. On the other hand, platelets are also able to invade tissue. This is essential since platelets can contribute to the inflammatory response through release of cytokines, chemokines and growth factors from their granules. In fact, platelets have been suggested to be involved in the inflammatory response of I/R injury in various organs. They are able to roll and adhere to post-reperfusion endothelium in a P-selectin dependent mechanism. In mouse myocardial tissue, the first activated platelets are present within two minutes after reperfusion, and then accumulate in the infarcted myocardium.

The ensuing inflammatory response which follows is considered to exacerbate damage. Both the innate as well as the adaptive immune system can be activated after reperfusion. Activation of the innate immune system is probably mediated by activation of pattern-recognition receptors such as toll-like receptors that recognize their endogenous ligands that are released upon tissue damage. The complement system is part of the humoral immune response and can play a role both as first line innate defense, but may also contribute to the adaptive immune response. In many animal experiments a role for
(terminal) complement activation in I/R injury has been suggested, although recent animal experiments doubt the involvement of the complement system itself in the initiation of injury. The role of complement activation in human I/R injury is even more complex. While a role of complement activation was suggested in human myocardial I/R injury, diverse anti-complement intervention studies did not lead to major improvements.

Ischemia-related metabolic adaptations and dysregulated mitochondrial homeostasis are thought to result in substantial release of reactive oxygen and nitrogen species (RONS) upon reintroduction of oxygen. The RONS overload can overwhelm the endogenous antioxidant system, resulting in oxidative damage. This may trigger secondary processes and contribute to the pro-inflammatory response upon reperfusion. Numerous animal studies clearly demonstrate that antioxidant therapy ameliorates I/R injury. Despite these findings, studies in humans consistently fail to show any clinically relevant effect. The basis for this discrepancy between human and animal studies is still unclear. Yet, it may suggest that the contribution of RONS to I/R injury in humans is less than commonly thought.

Ultimately, when I/R injury to the cell is severe, various programs of cell death can be activated. Three major forms of cell death can be distinguished: necrosis, apoptosis, and autophagy. Besides acute cell death by necrosis or apoptosis during and directly after the ischemic period, cell death continues for several days following reperfusion. All three types of cell death can contribute to the continued loss of cells for days and even weeks in the reperfused tissue. In animal models, both necrosis and apoptosis continued after reperfusion with a maximum severity three days after reperfusion. Autophagy during the ischemic episode appears to keep cells viable and might play a protective role. It can be suggested, however, that activation of autophagy after reperfusion is detrimental. Indeed, a mouse model of myocardial I/R illustrates that protein levels of the autophagic mediator beclin can be greatly upregulated during reperfusion. Mice with reduced beclin levels exhibited smaller myocardial infarct sizes.

Long term impact
Although short term results of kidney transplantation are excellent, 5 year graft loss can be up to 30% in older recipients. Protocol biopsies obtained in the first years after transplantation have shown rapid increase in the prevalence of interstitial fibrosis/tubular atrophy (IF/TA). This finding has been correlated with later graft dysfunction and graft loss, mostly in cases of concomitant interstitial inflammation and fibrosis. Both allogen dependent and independent factors determine IF/TA. I/R injury is an important non-allogenecic factor and the duration of the cold ischemic period is directly correlated to delayed graft function and even graft failure. Even without allogenecic transplantation, I/R injury itself has been
shown to cause interstitial fibrosis and glomerulosclerosis in experimental models (Figure 1).\textsuperscript{71-73}

![Figure 1: Experimental renal I/R injury induces severe patchy renal fibrosis, although kidney function partially recovers. Sirius red staining shows A) normal mouse kidney and B) severe fibrosis 3 weeks after mice underwent 25 minutes of warm renal I/R injury. (Non published data)](image)

**Opportunities in studying renal I/R injury**

Until now, results of renal I/R experiments in small animal models have not been translatable into clinical kidney transplantation. The most probable reason is that the exact mechanism involved are probably different between species. Detailed knowledge on the pathophysiological mechanisms leading to I/R injury in human kidney transplantation is required to form a basis for experimental therapies.

Studying the pathophysiology of human renal I/R injury requires careful techniques that specifically assess the processes that occur in the kidney at the moment of reperfusion and thereafter. Two complementary approaches have been chosen in previous studies: assessment of processes in the intravascular compartment or assessment of changes in the renal tissue. Intravascular changes have been assessed frequently in renal I/R injury, and almost all studies measured changes in circulating factors by collecting sequential peripheral blood samples. In these peripheral blood samples however, the source of the released factors can never be ascertained to be the kidney. Even more since haemodynamics change upon reperfusion of the kidney graft, and the leg is reperfused simultaneously with the kidney upon removal of the iliac arterial clamp. Furthermore, release of factors into the circulation may be undetected because of their dilution in the total circulating volume. By collecting arteriovenous blood samples over the kidney during reperfusion, specific measurements can be done studying those factors that are released from the kidney, i.e. that have a higher concentration in renal venous blood compared to arterial blood. Moreover, the release of these markers can be assessed with higher sensitivity, since a small release will produce the
largest concentration difference in the efferent vein. Finally, by measuring time-series of these arteriovenous differences a dynamic and specific footprint of the processes occurring in the reperfused kidney can be reconstituted. In Figure 2 the technique of arteriovenous measurements over the transplanted kidney is illustrated. The renal artery and vein are selectively cannulated before reperfusion, and during the first half hour of reperfusion, timed and paired arterial and venous samples can be collected from the kidney. This technique is applied in many of the studies described in this thesis.

Figure 2: Schematic representation of the arteriovenous sampling method over the reperfused kidney by simultaneous blood collection from the renal artery (left) and renal vein (right). Illustration by Manon Zuurmond© (www.manonproject.com). Adapted from de Vries et al. 77

Aims of this thesis
The aims of this thesis were to explore the factors and processes involved in the pathophysiology of renal I/R injury in clinical kidney transplantation, in order to establish a basis for the development of specific therapies preventing and limiting renal I/R injury in kidney transplantation. Exact knowledge on the sequence of events by which graft damage is initiated after reperfusion in human kidney transplantation was still lacking. In chapter 2, 3 and 4, the release of pro-inflammatory cytokines from the reperfused graft is assessed and compared between living and deceased donor kidney transplantations. In chapter 5, an important actor of the innate immune system, the complement system, is assessed in human kidney transplantations. Whether endothelial activation and concomitant platelet activation are present in early reperfusion of transplanted kidneys is investigated in chapters 6, 7 and 8. Finally, oxidative damage, the most commonly mentioned process in the pathophysiology of I/R injury is carefully investigated in human kidney transplantation in chapter 9. Chapter
Chapter 1

10 shows new insights as I/R injury is approached from an unbiased, hypothesis generating angle, in which gene expression profiles are compared to assess changes upon reperfusion and baseline differences between different donor types. Chapter 11 and 12 summarize the findings in this thesis and review future perspectives in treatment of I/R injury.
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Early renal ischemia-reperfusion injury in humans is dominated by IL-6 release from the allograft


*Am J Transplant 2009;9:1574-1584*
Abstract

The pathophysiology of ischemia/reperfusion (I/R) injury is complex and current knowledge of I/R injury in humans is incomplete. In the present study, human living donor kidney transplantation was used as a highly reproducible model to systematically study various processes potentially involved in early I/R injury. Unique, direct measurements of arteriovenous concentration differences over the kidney revealed massive release of interleukin (IL)-6 in the first 30 minutes of graft reperfusion and a modest release of IL-8. Among the assessed markers of oxidative and nitrosative stress only 15(S)-8-iso-PGF$_{2a}$ was released. When assessing cell activation, release of prothrombin fragment 1+2 indicated thrombocyte activation, whereas there was no release of markers for endothelial activation or neutrophil activation. Common complement activation complex sC5b-9 was not released into the bloodstream, but was released into urine rapidly after reperfusion. To investigate whether IL-6 plays a modulating role in I/R injury, a mouse experiment of renal I/R injury was performed. Neutralizing anti-IL-6 antibody treatment considerably worsened kidney function. In conclusion, this study shows that renal I/R in humans is dominated by local IL-6 release. Neutralization of IL-6 in mice resulted in a significant aggravation of renal I/R injury.
Introduction

Ischemia/reperfusion (I/R) injury is the exacerbation of tissue damage upon reestablishment of circulation after a period of ischemia. I/R injury is an inevitable consequence of organ transplantation, and a major determinant of patient and graft survival. The pathophysiology of I/R injury is complex and incompletely understood. Numerous animal experiments and limited human studies have shown that free radicals, complement, neutrophils, and thrombocytes may contribute to I/R injury. To our best knowledge, none of the interventions targeting these processes had an appreciable impact on morbidity or mortality in humans, despite their beneficial results in animal models. Previous studies of I/R injury in humans mostly relied on measurements in peripheral blood. Unfortunately, peripheral blood measurements lack sensitivity and do not discriminate between the release of factors from the allograft and systemically released factors. We used a renewing approach to avoid the limitations of systemic measurements. Consecutive arteriovenous concentration differences of various factors were assessed directly over the reperfused organ to reveal the locally active processes in human I/R. Because of the maximal homogeneity in donor and patient characteristics, minimal variation in ischemia duration and the possibility to collect control blood and urine samples from the donor (i.e. over the same kidney that was studied after reperfusion in the recipient), it was decided to study I/R in living-donor kidney transplantation. Our results show that the early phase of reperfusion is dominated by IL-6 release from the kidney. Furthermore, immunoneutralization of IL-6 in mice resulted in aggravation of I/R injury.

Methods

Patient population
Twelve eligible patients undergoing renal allograft transplantation from living donors were recruited (Table 1). None of the donors had current illness and all were free of medication. For technical reasons (renal vein sampling) only patients receiving a left kidney were included. The study protocol was approved by the local ethics committee, and informed consent was obtained from each patient.
### Table 1: Donor, recipient and transplant characteristics

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Ischemia time (min)</th>
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<tbody>
<tr>
<td>Age</td>
<td>Gender</td>
<td>Disease</td>
</tr>
<tr>
<td>30</td>
<td>Male</td>
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</tr>
<tr>
<td>38</td>
<td>Male</td>
<td>glomerulonephritis</td>
</tr>
<tr>
<td>37</td>
<td>Female</td>
<td>IgA nephropathy</td>
</tr>
<tr>
<td>53</td>
<td>Male</td>
<td>reflux nephropathy</td>
</tr>
<tr>
<td>39</td>
<td>Female</td>
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<tr>
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<td>25</td>
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<tr>
<td>52</td>
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<td>renal failure with unknown cause</td>
</tr>
<tr>
<td>30</td>
<td>Male</td>
<td>renal failure with unknown cause</td>
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<tr>
<td>Mean</td>
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### Operation and materials

Living donor kidney transplantations were performed according to the local protocol. Prior to implantation of the graft, a 5 French umbilical vein catheter was placed in the renal vein through one of its side branches. At 30 sec., 1, 3, 5, 10, 20 and 30 min. after reperfusion (i.e., t=0) 10 ml blood aliquots were sampled. Paired arterial blood samples were obtained at 0, 3, 5, 10, 20 and 30 min. after reperfusion. Thirty minutes after reperfusion the abdominal wall was closed and the endpoint of sampling was reached.

Recipients were pre-treated with the following immune suppressants at the day of transplantation: basiliximab (20 mg), mycophenolate mofetil (1440 mg), cyclosporine (6 mg/kg in two doses) and prednisolone (100 mg in two doses). Donors underwent open nephrectomy. For cold perfusion and storage of the kidney, Custodiol® HTK solution (Tramedico, Weesp, The Netherlands) was used. Mean cold ischemic period was 177±17 min. The above described method was also used to obtain a control arterial and renal venous blood sample during donor nephrectomy prior to the induction of renal ischemia. All blood and urine samples were collected in tubes containing EDTA and immediately stored on ice. Blood and urine samples were centrifuged (1550 g, 20 min, 4°C) and the derived plasma/supernatant was re-centrifuged (1550 g, 20 min, 4°C) to deplete it from leukocytes and thrombocytes. Materials were aliquotted and immediately stored at -70°C until assayed.
A renal cortical biopsy was obtained after the period of cold storage. Control biopsies were acquired immediately (within the first minute) after completion of nephrectomy. Biopsies were fixed in formalin or immediately snap frozen in liquid nitrogen and stored at -70°C. The initial urine produced by the graft within 10 minutes after reperfusion was collected directly from the ureter. Control urine was collected from the urethral catheter of the donor during the nephrectomy procedure before kidney ischemia.

### Plasma measurements

The arteriovenous sampling method was validated by blood gas analysis of the arterial and venous samples. Kidney function directly after reperfusion was estimated by comparing the arterial and venous creatinine levels. Because cholesterol is not secreted, filtered or re-absorbed by the kidney, plasma cholesterol was measured to evaluate potential blood concentrating effect of the kidney. Lactate dehydrogenase (LDH) activity was determined as measure of cell lysis. Blood gases, creatinine, cholesterol and LDH activity were all measured in a certified facility, using routine laboratory assays.

We measured various markers for each of the processes potentially involved in I/R injury. Plasma was analyzed for biomarkers of oxidative and nitrosative stress. Total 15(S)-8-iso-PGF₂α was measured in plasma by gas chromatography-tandem mass spectrometry (GC-tandem MS) as described previously. Malondialdehyde (MDA) was measured in 100-µl aliquots of plasma by GC-tandem MS by a procedure similar to that reported previously for nitrite and nitrate. Thiobarbituric acid reactive substances (TBARS) were determined by a high-performance liquid chromatography (HPLC) method. As indicators of NO synthesis, its major metabolites nitrite and nitrate were measured by GC-MS as described previously. Nitrotyrosine in proteins was measured by ELISA in accordance with the manufacturer’s instructions (Biotech nitrotyrosine EIA, Oxis, Portland, Oregon, USA).

The soluble complement complex C5b-9 (sC5b-9) was measured by ELISA as described previously by Molness et al. reagents for sC5b-9 measurements were generously provided by professor T.E. Mollnes, Institute of Immunology, University hospital Oslo, Norway. sICAM-1 and sP-selectin levels were measured by a commercially available ELISA (human sICAM-1, DY720, human sP-selectin, DY137, R&D systems, Abingdon, UK) following the manufacturer’s instructions. Neutrophil activation was assessed by measuring lactoferrin, a neutrophil degranulation product. Lactoferrin concentrations in plasma were quantified by a human lactoferrin-specific ELISA as described by van Berkel et al. with minor modifications. A standard curve was constructed with recombinant human lactoferrin (a generous gift of Dr. H.A. van Veen, Pharming, Leiden, The Netherlands); the lower limit of detection was 25 μg/l of lactoferrin. Von Willebrand factor (vWF) antigen was measured by an in-house ELISA
using polyclonal rabbit antibodies to human vWF (Dako, Copenhagen, Denmark). Plasma concentration units were calculated as percentage of pooled plasma level. Prothrombin fragment (F) 1+2 was measured by ELISA conform manufacturer’s instructions (Enzygnost F1+2, Dade Behring, Marburg, Germany).

Multiplex cytokine array (Evidence investigator cytokine array, Randox Laboratories, Crumlin, UK) was used to evaluate cytokine release in three patients’ arterial and venous sample taken 30 minutes after reperfusion. Detection limits were 0.9 ng/l for IL-1α, 1.6 ng/l for IL-1β, 4.9 ng/l for IL-2, 6.5 ng/l for IL-4, 1.0 ng/l for IL-6, 7.8 ng/l for IL-8, 1.8 ng/l for IL-10, 14.6 ng/l for vascular endothelial growth factor (VEGF), 4.6 ng/l for tumor necrosis factor α (TNF-α), 3.5 ng/l for interferon γ (IFN-γ) and 2.9 ng/l for endothelial growth factor (EGF). Based on the results of the cytokine array, all samples were analyzed for IL-1β, IL-6 and IL-8 by ELISA (PeliKine, Sanquin Reagents, Amsterdam, The Netherlands) and for TNF-α by high sensitivity ELISA (Quantikine HS, R&D systems, Abingdon, UK). Detection limits were 2 ng/l for IL-6 and IL-8, 3 ng/l for IL-18 and 0.12 ng/l for TNF-α.

Measurement of renal IL-6 mRNA content and IL-6 immunohistochemistry

All biopsy specimens were analyzed for IL-6 mRNA by semi-quantitative PCR (TaqMan method of Applied Biosystems, Perkin Elmer, Groningen, The Netherlands) by using a combination of a forward and a backward primer and a specific (6-carboxy-fluorescein/6-carboxy-tetramethyl-rhodamine [FAM/TAMRA]) double-labeled probe. Specific amplicons were chosen according to the manufacturer’s recommendations. IL-6 probes and primers were obtained from Isogen (Maarsen, The Netherlands) and the VIC labelled GAPDH primer-probe combination was purchased from Applied Biosystems (Nieuwekerk aan de IJssel, The Netherlands). Results were corrected for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Three kidney biopsies, obtained directly after the period of cold storage were stained for IL-6 as described before.

Animals and surgical procedure

To assess the dose of rat anti-IL-6 antibody (Ab) (MAB406, R&D systems, Abingdon, UK) needed to substantially inhibit the IL-6 response, a preliminary study in CRP knock-in mice was conducted. In 5 human CRP transgenic (huCRPtg) mice baseline CRP levels were measured (mean 5.4(3.3) µg/ml). Administration of recombinant murine IL-6 (cat no. 216-16; PreproTech Inc., New Jersey, USA) caused a significant increase in CRP levels to 17.1(9.0) µg/ml (measured 18 hours after administration), which was completely inhibited by administration of 100 ug anti-mouse IL-6 Ab per mouse i.p. (R&D systems, Abingdon, UK) Renewed IL-6 administration 30 hours after anti-IL-6 Ab administration did not cause an increase in CRP levels. This pilot study shows that the administration of 4 mg/kg anti-IL-6 antibody is sufficient in fully quenching the effect of IL-6 for at least 30 h.
Normal male (C57BL/6J/BALB/c/Cr1BR) F1 mice of 10 weeks old were used in this study. They were divided into the following three groups: 1) I/R pre-treated with anti-IL-6 Ab group (n=10); 2) I/R pre-treated with control Ab group (n=10); and 3) sham operated with control Ab treatment group (n=4). Mice in the pre-treated groups received either 4 mg/kg anti-IL-6 Ab or 4 mg/kg non-specific IgG1 Ab (R&D systems, Abingdon, UK) i.p. 24 hours preoperatively. Mice were anaesthetized with i.p Hypnorm®, midazolam mixture in a dose of 0.8 mg/kg fentanyl citrate, 25 mg/kg fluanisone and 12.5 mg/kg midazolam. Core body temperature was maintained at 37 °C using a thermic blanket. A baseline blood sample was obtained from the tail vein. Via a midline laparotomy the renal pedicles were identified and bilaterally clamped for 30 minutes using microvascular clamps (S&T, Neuhausen, Switzerland). In the sham group identical surgical procedures were used, except that clips were not applied. After the renal pedicle clamps were removed, the kidneys were observed for color change indicating reflow. One milliliter of warm saline was left in the abdominal cavity before the incision was sutured in two layers and the mice were allowed to recover from anesthesia. As analgesic 0.05mg/kg buprenorin® was administered s.c. After 48 hours the mice were anesthetized again. The final blood sample was obtained using cardiac puncture. Immediately after blood collection, the kidneys were removed and sagitally cut. One halve was snap frozen in liquid nitrogen and the other halve kept on formalin. Blood samples were collected in heparin coated capillary tubes. Plasma urea concentration was measured using the reflotron system (Roche diagnostics, Almere, The Netherlands) as indicator of kidney function at baseline and 48 hours after reperfusion. Throughout the whole experimental period the mice were maintained on standard diet and given water ad libitum. The study was approved by the veterinary authorities of the LUMC.

Histological evaluation
The kidneys were fixed in formalin for 24 hours and then embedded in paraffin for light microscopy and immunohistochemistry. Longitudinal sections of 4 µm thick were prepared and stained with Periodic Acid Schiff (PAS). Histological tissue injury was scored by an experienced pathologist (E.H.), blinded for the groups. Kidney injury was scored semi-quantitatively on amount of proximal tubule necrosis (score 0-3), band necrosis of cortex and medulla (score 0-1) and protein casts in the tubules (score 0-1).

Statistical analysis
Statistical analysis was performed using SPSS 14.0 statistical analysis software (SPSS Inc, Chicago, Ill). For the various outcome variables random coefficients mixed models were fitted with time as continuous predictor and with a categorical dichotomous predictor indicating whether the measurement was in a venous or arterial sample. This resulted in straight line models or parabolic curves where appropriate. It was tested whether the mean
venous curve as a whole was significantly elevated as compared to the mean arterial curve as a whole, instead of performing separate tests for the various points in time. Stated otherwise; it was tested whether there was an extra intercept for the venous compared to arterial curve.

Other variables were compared using the paired t-test and Mann-Whitney U test as appropriate. Histological score was tested by Chi-square test or Fisher’s exact test as indicated. Data are expressed as mean ± standard error of the mean (SEM). Graph error bars represent the SEM. A p-value of less than 0.05 was considered significant.

Results

Patient and graft characteristics and ischemia times are summarized in table 1. Post-operative course was uneventful in all patients. One year patient and graft survival was 100%.

Arteriovenous measurements

Various valid markers were measured for each of the processes potentially involved in I/R injury (Figure 1). First, the arteriovenous sampling method was validated by blood gas analysis which reflected normal arterial and venous blood gas levels. Similar LDH levels in arterial and venous samples (p=0.73) excluded the release of intracellular constituents by cell lysis. Creatinine levels were consistently lower in the renal venous blood as compared to the arterial values (p<0.001), indicating immediate function of the kidney graft. Equal cholesterol levels (p=0.94) over the kidney excluded a blood concentrating effect of the kidney, therefore all results are presented without normalization.

Oxidative and nitrosative stress

We found a small but significant transrenal difference for 15(S)-8-iso-PGF$_{2\alpha}$ (p=0.003, Figure 2A). Arteriovenous measurements over the non-ischemic kidney in the donor revealed similar 15(S)-8-iso-PGF$_{2\alpha}$ levels showing its release is specific for I/R (p=0.69, Figure 2B). Urinary 15(S)-8-iso-PGF$_{2\alpha}$ levels did not change upon reperfusion (p=0.35, Figure 2C). In contrast, there was no transrenal MDA concentration difference after reperfusion as established both by GC-tandem MS (p=0.17) and the TBARS method (p=0.064). MDA levels (TBARS method) were similar in urine from recipients and in control urine (p=0.40). Measurement of nitrite and nitrate, indicators and measures of nitric oxide (NO) biosynthesis, as well as of nitrotyrosine, a biomarker of nitrosative stress, did not show arteriovenous differences (p=0.14, p=0.097 and p=0.93, respectively). Likewise, urinary nitrite and nitrate concentrations remained similar (p=0.057, p=0.69) after reperfusion.
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Figure 1: Schematic view of processes potentially involved in early I/R injury, showing the biomarkers measured in this study. Grey boxes indicate local release of F1+F2, 15(S)-8-iso-PGF_2α, sC5b-9, IL-6 and IL-8 from the human kidney, indicating involvement of thrombocyte activation, complement activation, and cytokines and chemokines in I/R injury.
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Figure 2 A: Significant differences between arterial and renal venous plasma concentrations of 15(S)-8-iso-PGF$_{2\alpha}$ during the first 30 minutes of reperfusion indicated local release from the graft (p=0.003, n=6);

B: Control arterial and renal venous plasma was collected from the donor during the nephrectomy procedure, before renal ischemia was induced. Concentrations of 15(S)-8-iso-PGF$_{2\alpha}$ did not reveal arteriovenous differences in non-ischemic conditions (p=0.69, n=6);

C: Concentration of 15(S)-8-iso-PGF$_{2\alpha}$ corrected for creatinine concentration in the initial urine produced within minutes after reperfusion did not differ from control urine collected from the donor during nephrectomy, before renal ischemia was induced (p=0.35, control: n=6, reperfusion: n=8).
Complement activation

To evaluate complement activation during reperfusion, we measured the common terminal end product of the three complement activation pathways. sC5b-9 concentrations were similar in arterial and venous samples (p=0.37). In contrast, sC5b-9 levels were significantly higher in urine produced immediately after reperfusion than in control urine from the donor (3.1 versus 0.11 U/mol creatinine, p<0.001, Figure 3).

Figure 3: Concentration of sC5b-9 complex, corrected for creatinine concentration, was significantly higher in the initial urine produced in the graft within minutes after reperfusion as compared to the control urine from the donor collected during nephrectomy, before renal ischemia was induced (p<0.001, control: n=6, reperfusion: n=9).

Cell activation

Prothrombin F1+2 levels showed a significant (p=0.001, Figure 4) concentration difference between arterial and renal venous blood, suggesting rapid thrombocyte activation during early I/R. Concentrations of sICAM-1 and sP-selectin in plasma were measured as indicators of endothelial activation. sICAM-1, sP-selectin and vWF levels were equal in arterial and renal venous blood (p=0.50, p=0.31 and p=0.12, respectively), providing no evidence for endothelial activation. In addition, there were no indications for neutrophil activation (p=0.12).
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Figure 4: Renal venous plasma concentrations of prothrombin fragment 1 and 2 (F1+2) were significantly higher as compared to arterial concentrations, demonstrating local release from the kidney during reperfusion (p=0.001, n=6).

Cytokine and chemokine response

The cytokine response after reperfusion was dominated by the local release of IL-6. IL-6 levels were significantly elevated in renal vein blood (p<0.001, Figure 5A), peaking directly after reperfusion. The IL-6 release was specific for I/R, as control measurements over the non-ischemic kidney in the donor did not show a transrenal difference in IL-6 levels (p=0.44, Figure 5B). This indicates that IL-6 release is not a consequence of manipulation of the kidney during surgery or a reaction to anesthetics. Urinary IL-6 release immediately after reperfusion was ten times higher than baseline release (41.2 vs 4.1 pg IL-6/μmol creatinine, p=0.003, Figure 5C).

To investigate whether IL-6 release from the reperfused kidney reflected enhanced IL-6 transcription before reperfusion or relates to release of pre-stored IL-6, we analyzed IL-6 mRNA levels and performed immunohistochemical IL-6 staining on kidney biopsies. This analysis demonstrated that IL-6 mRNA levels remained similar during the cold ischemic period (p=1.0, Figure 5D) but showed abundant granular cytoplasmatic IL-6 staining in the tubular epithelial cells of kidney biopsies collected before reperfusion. Glomeruli and interstitial cells were negative (n=3), (Figure 6).
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Figure 5 A: Arterial and renal venous plasma concentrations of IL-6 showed a substantial and continuous release of IL-6 from the reperfused kidney during the first 30 minutes of reperfusion (p<0.001, n=9); B: Control, pre-ischemic arterial and renal venous plasma concentrations of IL-6 in the donor during nephrectomy procedure were comparable, indicating IL-6 release is specific for I/R (p=0.44, n=5); C: Concentration of IL-6, corrected for creatinine concentration, was significantly higher in the first urine produced by the graft after reperfusion, as compared to control urine of the donor collected before renal ischemia was induced in the donor nephrectomy procedure (p=0.003, control: n=5, reperfusion: n=7); D: IL-6 expression relative to GAPDH expression in kidney tissue collected at the end of the cold preservation period (ischemia) did not differ from baseline expression in biopsies collected before ischemia in donor nephrectomy procedures (p=1.0, control: n=5, ischemia: n=6).

Figure 6: Typical example of immunohistochemical staining for IL-6 in human kidney biopsies collected before reperfusion showing abundant granular cytoplasmatic IL-6 staining in the tubular epithelial cells, whereas the interstitial cells and glomeruli are negative. Magnification × 20, inset magnification × 80, the bar measures 100 μm.
The findings of the multiplex cytokine screening assay (table 2) were validated by individual ELISA measurements. Besides IL-6, also IL-8, TNF-α and IL-1β concentrations were assessed. There was a small but significant arteriovenous difference in IL-8 levels over the kidney (p<0.001, Figure 7A). Control arteriovenous measurements over the non-ischemic kidney did not indicate IL-8 release (p= 0.95, Figure 7B). Urinary IL-8 levels after reperfusion were equal to pre-ischemic concentrations (p=0.11). Plasma TNF-α levels did not show transrenal arteriovenous concentration differences (p=0.81). Plasma IL-1β, and urinary IL-1β and TNF-α levels were all below the detection limits of the assay.

Figure 7  
A: Arterial and renal venous plasma concentrations of IL-8 during the first 30 minutes of reperfusion showed a modest but significant IL-8 release from the graft (p<0.001, n=9); B: Pre-ischemic arterial and renal venous plasma concentrations of IL-8 were similar in the samples obtained from the donor during nephrectomy procedure (p=0.95, n=4).
Table 2: Pilot measurements of plasma cytokine concentrations using multiplex cytokine array.

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<th>IL-4</th>
<th>IL-6</th>
<th>IL-8</th>
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<: lower than the detection limit

Anti-IL-6 treatment

Given the dominance of IL-6 release in the arteriovenous measurements, we investigated the role of IL-6 in the pathophysiology of I/R injury in a mouse experiment. The day after undergoing bilateral renal ischemia and reperfusion, one control antibody treated mouse and two anti-IL-6 treated mice had died. Autopsy did not reveal any bleeding or other obvious cause of death. Baseline urea concentration was identical in all mice, however at 48 hours after reperfusion, urea concentration in anti-IL-6 treated mice was significantly higher than in control antibody treated mice (p=0.046) (Figure 8). These results concerning kidney function are supported by the histological analysis of the kidneys at 48 hours after reperfusion. There seemed to be considerably more kidney injury in the anti-IL-6 antibody treated group as compared to the control antibody treated group (Figure 9). However, the difference in semi-quantitative histological score was not statistically significant. The histological score of sham operated mice was 0 for all three categories (Figure 10).

Figure 8: Plasma urea levels measured 48 hours after kidney reperfusion were significantly higher in anti-IL-6 treated mice compared to untreated mice. * p=0.046, ** p=0.004, *** p=0.20. The box runs from the lower to upper quartile, the whiskers indicate the 5 to 95 percentile, while the horizontal line is set at the median.
Figure 9: The effect of anti-IL-6 treatment on renal histology, 48 hours after kidney reperfusion.
G: glomerulus, †: protein casts, #: proximal tubule necrosis.
A: Specimen from sham operated mouse showing normal renal cortex; B: Sham operated mouse showing intact renal medulla; C: Cortex of control antibody treated mouse showing protein casts; D: Medullar necrosis in control antibody treated mouse; E: Cortex section of anti-IL-6 antibody treated mouse, showing numerous protein casts and proximal tubule necrosis; F: Medulla section showing extensive band necrosis and protein casts. All sections show a representative image of the group, magnification × 20, PAS stain. The bar measures 10 μm.
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Figure 10: Semi-quantitative histological damage score for kidneys 48 hours after reperfusion. Differences between anti-IL-6 Ab treated and control Ab treated groups did not reach statistical significance. Sham group scored 0 for all measures. BN=band necrosis, PTN=proximal tubule necrosis, PC=protein casts, sum=sum of the three scores. Co Ab (n=8) vs anti-IL-6 Ab (n=7); BN p=0.49, PTN p=0.11, PC p=0.29, sum p=0.35 (BN, sum: chi-square test; PTN and PC: Fisher’s exact test).

Discussion

Although many studies have focused on I/R injury, the pathophysiological mechanisms underlying this phenomenon are only partially understood. Studies in humans performed thus far largely rely on peripheral measurements, which have a limited sensitivity and validity. Instead, measurement of arteriovenous concentration differences over the graft provides a unique and accurate means to assess local processes implicated in human I/R injury. In the present study, living donor kidney transplantation was used as a highly reproducible clinical model of I/R. The results of this study show that the early phase of reperfusion is dominated by the release of soluble mediators, predominantly IL-6. Immunoneutralization of IL-6 in mice resulted in aggravation of kidney I/R injury. We therefore hypothesize that the renal IL-6 response upon reperfusion is protective in human I/R injury.

Free radicals, and oxidative and nitrosative stress have been considered the pivot of I/R injury for years. However, interventions using antioxidants show contradicting results.\textsuperscript{10,11,21,30} To assess the involvement of oxidative and nitrosative stress in renal I/R injury, we measured a combination of independent markers. Since assessment of oxidative and nitrosative stress in biological samples is difficult, measurement of multiple factors is an established method to guarantee the validity of the results.\textsuperscript{20} Therefore, representative markers of oxidative...
and nitrosative stress, i.e. 15(S)-8-iso-PGF$_{2a}$, MDA, 3-nitrotyrosine, nitrite and nitrate were measured in arteriovenous plasma samples. Apart from a small but significant release of 15(S)-8-iso-PGF$_{2a}$ from the reperfused kidney, we found no evidence for oxidative or nitrosative stress. In the absence of release of other biomarkers for oxidative/nitrosative stress from the kidney, it can be doubted whether the observed release of 15(S)-8-iso-PGF$_{2a}$ indeed reflects increased oxidative stress levels. Moreover, the 15(S)-8-iso-PGF$_{2a}$ plasma concentrations measured in our patients are close to those measured in plasma of healthy humans$^{23}$ and the urinary excretion of 15(S)-8-iso-PGF$_{2a}$ did not change after reperfusion. It is known that 15(S)-8-iso-PGF$_{2a}$ can be formed by cyclooxygenase (COX)-1 activity in thrombocytes as well.$^{31-34}$ As we found indications for thrombocyte activation in the early phase of I/R, 15(S)-8-iso-PGF$_{2a}$ production directly after reperfusion is more likely to relate to COX-1 activation in thrombocytes,$^{31}$ rather than to oxidative or nitrosative stress mediated production. The thrombocyte activation itself could be explained as a result of activation upon crossing disrupted endothelial cell lining in the graft.$^{35,36}$

We did not observe an arteriovenous concentration difference for the sC5b-9 complex, but sC5b-9 levels in recipients’ urine after reperfusion were more than ten times higher than in control urine. With the absence of detectable sC5b-9 release in donor urine we show that sC5b-9 release is specific for I/R, and does not relate to anesthesia or manipulation of the kidney during surgery. Besides urinary release, local complement depositions may be formed in the kidney that could not be demonstrated in this experimental set-up. An explanation for the selective urinary excretion of sC5b-9 could be the local production and release of sC5b-9 in renal tubular epithelium upon I/R. This is supported by a mouse experiment showing C9 deposition mainly in tubular epithelium after reperfusion.$^{37}$ Remarkably, in their animal model elevated renal C5b-9 formation was not detected up to 12 hours after reperfusion, in contrast to the immediate urinary release we observed in the present study.

Our results show a substantial local release of IL-6 from the kidney during the first 30 minutes of reperfusion. Control arteriovenous measurements immediately prior to donor nephrectomy did not show any release of IL-6 from the kidney in non-ischemic conditions, indicating that renal IL-6 release is specific for I/R, rather than a response to anesthesia or surgery. IL-6 release upon reperfusion is not specific for kidney tissue. IL-6 was found to be released locally from the gastrointestinal tract,$^{38,39}$ the liver,$^{40}$ the coronary sinus,$^{41,42}$ and the lower extremity$^{43}$ after reperfusion. Sources of the released IL-6 in the kidney may be circulatory or resident renal cells. Renal tubular epithelial cells have the potential to produce numerous cytokines and chemokines, including IL-6.$^{44}$ Furthermore, IL-6 can be generated by many different types of circulating cells.$^{45}$ The low baseline arterial IL-6 levels in the recipients are an important argument supporting the release of IL-6 from resident
kidney cells (Figure 5A and 5B). It is known that immunosuppressive medication, especially corticosteroids, are able to suppress cytokine response. This difference in baseline concentrations of IL-6 suggests that the immunosuppressive regimen the recipients are pre-treated with is able to fully suppress the procedure related systemic IL-6 response. Since the transplanted kidney itself is not exposed to immunosuppressive agents before reperfusion it maintains the potential to produce and or release IL-6. Immunohistochemical staining of IL-6 in kidney biopsies collected immediately before reperfusion showed abundant presence of IL-6 in tubular epithelium in a granular pattern, suggesting that the kidney itself is a potential source of the released IL-6. Semi-quantitative realtime PCR did not indicate enhanced IL-6 transcription during cold storage, suggesting that IL-6 is released from pre-existing storage pools.

IL-6 is a pleiotropic cytokine produced by various types of cells under various circumstances. It has been described as having both pro- as well as anti-inflammatory effects. IL-6 binds to a membrane bound IL-6 receptor (IL-6R) and this complex associates with two molecules of signal transduction protein gp130. While gp130 is present on almost all cells, IL-6R is not. However, a soluble form of IL-6R (sIL-6R) can bind its ligand IL-6 and this complex can activate gp130 and induce ‘trans-signaling’ in cells lacking IL-6R.

The protective role of IL-6 in I/R injury is demonstrated in studies of liver and myocardial I/R injury. Several studies show that IL-6 plays a protective role in liver I/R injury by reducing cell damage and stimulating hepatocyte proliferation. Furthermore, infarcted area and myocardial apoptosis after coronary occlusion and reperfusion were reduced by IL-6/sIL-6R complex. In kidney tissue, the protective effect of IL-6 has been described as well. Nechemia et al. concluded from a murine model of HgCl2 induced acute kidney injury that IL-6 first mediates early kidney damage by an inflammatory response. Subsequently, when more sIL-6R becomes available, IL-6 is responsible for resolving the tissue damage via trans-signaling. Moreover, Homsi et al. showed that IL-6 stimulated tubular regeneration in a time-dependent pattern early after glycerin induced acute renal failure. After 24 hours, there was a predominance of proliferative activity in the renal medulla that shifted to the cortical zone at 72 hours. However, the role of IL-6 in kidney I/R injury is not fully univocal. The studies by Patel et al. and Kielar et al. have demonstrated that IL-6 -/- mice are protected against renal I/R injury. Concerning anti-IL-6 Ab treatment, Patel et al. and Kielar et al. reported conflicting results. Moreover, the value of a knockout model for IL-6 can be questioned as redundancy may play a role. Because IL-6 belongs to a family of cytokines that all use the same receptor gp130 subunit for signal transduction, they may induce similar responses to IL-6 signaling and therefore knockout mice may functionally overcome IL-6 deficiency.
A hypothesis explaining the difference between our findings and those by Patel et al. and Kielar et al. concerning the effects of neutralizing anti-IL-6 Ab can be derived from studies on non-ischemic kidney damage. Both the studies by Nechemia et al.\textsuperscript{52} and Homsi et al.\textsuperscript{53} demonstrated a time-dependent effect of IL-6 in kidney injury. Consequently, the discrepancy between the results of Patel et al. and Kielar et al. and our results could be due to differences in timing of the experiment. These groups evaluated the degree of kidney injury at 24 hours after reperfusion, whereas the endpoint in our study was 48 hours after reperfusion. It can be suggested that between 24 and 48 hours after reperfusion the protective effect of IL-6 becomes apparent. Moreover, it should be noted that Patel et al. did not ensure that the administered antibody completely inhibited the IL-6 response; whereas in the study of Kielar et al., it was actually shown that the treatment only partially inhibited the IL-6 response. Finally, we can not rule out that the differences relate to different immunological responses in the mice strains used. In both prementioned studies C57BL/6J mice were used, which have a different inflammatory response than the C57BL/6J/BALB/c F1 mice used in this study.\textsuperscript{56}

Beside IL-6, IL-8 also showed an arteriovenous difference. Control measurements did not show any release of IL-8 under non-ischemic conditions and urinary IL-8 excretion did not change upon reperfusion. Nevertheless, as the absolute arteriovenous differences in IL-8 concentration over the graft were rather small, the clinical relevance of IL-8 in kidney transplantation appears limited. This is supported by the lack of evidence for neutrophil activation and degranulation, a main action of IL-8.\textsuperscript{57} Although neutrophils contain large amounts of IL-8, in the absence of lactoferrin release it is highly unlikely that they are responsible. Alternatively, thrombocytes are an established source of IL-8,\textsuperscript{58} and activated thrombocytes could also be proposed as a source of IL-8 release in renal I/R injury.

In the present study we investigated I/R injury in living donor kidney transplantation. We intentionally chose this model because of the excellent homogeneity in donors and ischemia times, thereby minimizing the influence of donor state and variations in cold ischemia time. As the goal of this study was to assess the basic pathophysiological processes of I/R injury instead of correlating findings to clinical outcome, small patient numbers were sufficient. For ethical reasons our study was limited to a sample collection time of 30 minutes, which was the timeframe needed to anastomose the ureter to the bladder. I/R injury is generally considered an acute process that is initiated directly after reperfusion.\textsuperscript{20} However, we can not exclude that processes, other than those identified in this study, play a role in I/R injury after this time period we were limited to.
We used a mouse model of renal I/R injury to assess the effects of IL-6 neutralization. Results show that inhibition of IL-6 significantly aggravates renal I/R injury in mice, suggesting that IL-6 may play a protective role in the post-reperfusion phase. Obviously, it should be noted that an animal model can only approximate the human situation and that some components might be different, i.e. in the animal model the kidney is not cooled whereas in clinical transplantation the grafts sustain a period of cold ischemia. Since the animal experiment was primarily intended to determine the effect of IL-6 inhibition, the pathophysiological mechanisms remain to be elucidated in consecutive studies.

In summary, in this study we present a renewing approach of arteriovenous measurements in human clinical I/R during kidney transplantation. Our results show that thrombocyte activation, inflammation and complement activation are potentially involved in human renal I/R injury. Systematic measurement of a series of biomarkers revealed that early renal I/R is dominated by a local release of IL-6. Immunoneutralization of IL-6 in a mouse model resulted in a significant aggravation of renal injury.

**Acknowledgments**

The authors thank Peter Nibbering for lactoferrin measurements. The statistical assistance by Ron Wolterbeek is gratefully acknowledged. The authors thank Frank-Mathias Gutzki for performing GC-MS and GC-MS/MS analyses and Lee Bouwman for initial data acquisition. The excellent technical assistance of Maria-Theresa Suchy and Adri Mulder is gratefully acknowledged. The authors thank Professor T.E. Mollnes for the kind gift of reagents for the sC5b-9 measurements. Robert Kleemann is acknowledged for performing the IL-6 dosage pilot experiments and Dorien Peeters for facilitating urea measurements.
Chapter 2

References


IL-6 and its potential protective role in I/R injury


Interleukin-9 release from human kidney grafts and its potential protective role in renal ischemia/reperfusion injury

D.K. de Vries*, K.A. Kortekaas*, M.E.J. Reinders, E. Lievers, J. Ringers, J.H.N. Lindeman, A.F.M. Schaapherder * Both authors contributed equally to this work

Abstract

Background: The pathophysiology of ischemia/reperfusion (I/R) injury is dominated by an inflammatory response. In the identification of new therapeutic agents, the role of individual cytokines may be essential. Interleukin (IL)-9 is a pleiotropic cytokine recently identified to be involved in various immune responses. In this study, the role of IL-9 in renal I/R injury was assessed.

Methods: We performed repeated direct measurements of arteriovenous IL-9 concentration differences over the reperfused graft in human kidney transplantation.

Results: Substantial renal IL-9 release was observed from deceased donor kidneys ($P = 0.006$). In contrast, living donor kidneys, which have a more favourable clinical outcome, did not release IL-9 during early reperfusion ($P = 0.78$). Tissue expression of IL-9 did not change upon reperfusion in both living and deceased human donor kidneys. To assess the role of IL-9 in I/R injury, an experimental study comprising IL-9 inhibition in mice undergoing renal I/R was performed. Although there was no difference in kidney function, structural damage was significantly aggravated in anti-IL-9 treated mice.

Conclusions: Deceased donor grafts show a substantial IL-9 release upon reperfusion in clinical kidney transplantation. However, inhibition of IL-9 aggravated kidney damage, suggesting a regulating or minor role of IL-9 in clinical I/R injury.
Introduction

Ischemia/reperfusion (I/R) injury is an inevitable consequence of organ transplantation and a major determinant of patient and graft survival. I/R injury induces delayed graft function, which complicates around 20%\(^1,2\) of deceased donor kidney transplantations and has a major influence in graft function and survival.\(^3\) The pathophysiology of I/R injury is complex and incompletely understood, although both preclinical\(^4-6\) and clinical studies\(^7-9\) have shown that inflammation is an important mediator of I/R injury. The exact functions of the various cytokines involved in regulating the complex inflammatory events after I/R injury are not fully unraveled yet.\(^10\) Insight in the role of individual cytokines may be essential in the identification of new therapeutic agents.

Interleukin (IL)-9 is a pleiotropic cytokine recently discovered to be involved in various immune responses. Studies on the etiology of asthma and allergies have demonstrated an evident pro-inflammatory role of IL-9.\(^11,12\) IL-9 is produced by T helper cells\(^13\), regulatory T cells\(^14\), and mast cells\(^15\) and is able to modulate their production of various other cytokines.\(^12\) In a preceding study, we described for the first time a renal release of IL-9 after kidney transplantation.\(^10\)

The aim of the present study was to explore the potential pathophysiological role of IL-9 in renal I/R injury. IL-9 release during reperfusion of both living and deceased human donor kidney grafts was assessed, and the effects of IL-9 inhibition on renal I/R injury were evaluated in an animal experiment.

Methods

Patient population

Twenty-four patients undergoing renal allograft transplantation were included; of these, eight patients received a kidney from a living donor and sixteen patients received a kidney from a deceased donor (nine brain-dead donors and seven cardiac-dead donors). Kidney transplantations were performed according to the local standardized protocol.\(^16\) In living donors open nephrectomy was performed and Custodiol\(^10\) histidine-tryptophan-ketoglutarate solution (Tramedico, Weesp, The Netherlands) was used for cold perfusion and storage of the kidney. Brain-dead and cardiac-dead donor kidneys were perfused and stored with either University of Wisconsin solution or Custodiol\(^10\) histidine-tryptophan-ketoglutarate solution. The immunosuppressive regimen was based on induction therapy with basiliximab on day 0 and 4; and tacrolimus or cyclosporine A in addition with mycophenolate mofetil and...
steroids. For technical reasons (renal vein sampling) only patients receiving a left kidney were included. Patient and graft characteristics and ischemia times are summarized in Table 1. The postoperative course was uneventful in all patients. One-year patient and graft survival was 100%. The study protocol was approved by the local ethics committee, and informed consent was obtained from each patient.

Table 1. Transplantation and outcome characteristics in living donor (LD) and deceased donor (DD) kidney transplantation (mean ± SD).

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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>44</td>
<td>0.15</td>
</tr>
<tr>
<td><strong>Preservation fluids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HTK* (n=8)</td>
<td>UW† (n=11)</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>H1TK* (n=5)</td>
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</tr>
<tr>
<td><strong>CIT‡ (min.)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean ± SD</td>
<td>179.1 ± 18.6</td>
<td>1117.7 ± 299.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>WIT§ (min.)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean ± SD</td>
<td>34.0 ± 6.3</td>
<td>33.5 ± 6.1</td>
<td>0.85</td>
</tr>
</tbody>
</table>

* HTK: Histidine-tryptophan-ketoglutarate
† UW: University of Wisconsin
‡ CIT: cold ischemia time
§ WIT: warm ischemia time

IL-9 plasma measurements in arteriovenous samples

Arterial and renal venous blood samples were obtained during human kidney transplantation as described previously. In short, blood aliquots were sampled at 30 seconds, 3, 10 and 30 minutes after reperfusion via a catheter placed in the renal vein. Paired arterial blood samples were obtained at 0, 3, 10 and 30 minutes after reperfusion. The same method was used to obtain a control arterial and venous blood sample during donor nephrectomy prior to the induction of renal ischemia. All samples were collected in tubes containing EDTA (BD Vacutainer, Plymouth, UK) and centrifuged twice (1550 g, 20 min, 4°C) to deplete it of leukocytes and thrombocytes. Plasma was aliquotted and stored at -70°C until analysis. IL-9 was measured in a custom-made multiplex assay in accordance with the manufacturer’s instructions (X-plex, Biorad, Veenendaal, The Netherlands).
Immunohistochemical evaluation of IL-9 source in human kidney biopsies

A renal cortical biopsy was obtained before transplantation, after cold storage of a deceased donor graft. Kidney tissue was fixed in formalin for 24 hours and then embedded in paraffin for light microscopy. Longitudinal sections of 4 μm were prepared. After EDTA-retrieval, IL-9 deposition was assessed using a human IL-9 mAb in a 1:100 dilution (cat. no. 507602, Biolegend, Cambridge, UK). Staining was visualized using Nova RED (Vector Labs, Peterborough, UK). Sections were counterstained with hematoxylin (Merck, Darmstadt, Germany).

IL-9 expression in human kidney biopsies

A renal cortical biopsy was obtained before transplantation after cold storage, and a post-reperfusion biopsy was collected 45 minutes after reperfusion. Biopsies were immediately snap frozen in liquid nitrogen and stored at -70°C. Total RNA was extracted from renal tissues using RNAzol (Campro Scientific, Veenendaal, The Netherlands) and glass beads. The integrity of each RNA sample was examined by Agilent Lab-on-a-chip technology using the RNA 6000 Nano LabChip kit and a bioanalyzer 2100 (Agilent Technologies, Amstelveen, The Netherlands). RNA preparations were considered suitable for array hybridization only if samples showed intact 18S and 28S rRNA bands, and displayed no chromosomal peaks or RNA degradation products (RNA Integrity Number >8.0). Subsequent microarray analysis was performed using Illumina whole-genome gene expression BeadChips (Illumina BeadArray®, San Diego, CA, USA) according to instructions of the manufacturer at Service XS (Leiden, The Netherlands). IL-9 expression data were obtained from this array.

Animals and surgical procedures

Male BALB/c Jico mice (Charles River, L’Arbresle, France) of 8 weeks old (20-30 g) were divided into the following four groups: (1) I/R pre-treated with anti-IL-9 antibody (Ab) (n=12); (2) I/R pre-treated with control Ab (n=12); (3) sham-operated, pre-treated with anti-IL-9 Ab (n=4), and (4) sham-operated pre-treated with control Ab (n=4). Monoclonal anti-IL-9 Ab (no. 504802, clone D9302C12, BioLegend, San Diego, CA, USA) or isotype control IgG (no. 400916, clone HTH888, BioLegend, San Diego, USA) was administered at 1 and 2 days before surgery. At 2 days before surgery, 2 ug/g body weight anti-IL-9 Ab or its equivalent of isotype control Ab was administered by i.p. injection, followed by 4 ug/g body weight the day before surgery. This dosage is based on previous research in which a significant effect of anti-IL-9 treatment was observed after administration of a similar dose of the antibody. Mice were anesthetized with isoflurane. Bilateral kidney ischemia was induced by clamping the renal artery and vein for 35 minutes, followed by reperfusion. In the sham group, identical surgical procedures were used, except that clips were not applied. Kidney function was measured by plasma urea concentration (Reflotron®, Roche...
diagnostics, Almere, The Netherlands) the day before administration of antibodies (day -3),
day of surgery (day 0), and two consecutive days after surgery (day 1 and 2). Two days after
surgery, mice were killed and kidneys retrieved. Mice were maintained on standard diet and
given water ad libitum throughout the whole experimental period. The study was approved
by the veterinary authorities of our institute.

**Histological evaluation of murine kidney tissue**
The mouse kidneys were fixed in formalin for 24 hours and then embedded in paraffin for
light microscopy. Longitudinal sections of 4 μm were prepared and stained with periodic
acid-Schiff (PAS). The degree of tissue injury was scored by two blinded observers. Kidney
injury was scored semi-quantitatively using the amount of proximal tubule necrosis (score
0-3), band necrosis of the cortex and the medulla (score 0-3) and protein casts in the
tubules (score 0-3).

**Data collection and statistical analysis**
Clinical donor data were retrieved from the Eurotransplant database. Delayed graft function
was defined as the need for dialysis within the first week after transplantation. Statistical
analysis was performed using SPSS 16.0 statistical analysis software (SPSS Inc, Chicago, IL,
USA). Patient characteristics were compared by paired t-test and are expressed as mean ±
standard deviation (SD). Area under the curve (AUC) was calculated for the arterial and
venous curve of the human plasma measurements for the total of 30 minutes. The delta AUC
was calculated (venous minus arterial) and the null-hypothesis (delta AUC is 0) was tested
by a Wilcoxon signed rank test. Urea concentrations were compared using a Mann Whitney
U-test, Wilcoxon signed rank test or Kruskal-Wallis test where appropriate. Histological
score was tested by the Mann Whitney U-test. P value < 0.05 was considered significant.

**Results**

**IL-9 is released from deceased donor kidneys during reperfusion**
Living donor kidneys released no IL-9 during the first thirty minutes after reperfusion (P =
0.78, n = 8, Figure 1A). In contrast, deceased donor kidneys, which generally show more
dysfunction after transplantation, released a vast amount of IL-9 in the first thirty minutes
after reperfusion (P = 0.006, n = 16, Figure 1B). More specifically, only brain-dead donor
kidneys showed a significant release of IL-9 (P = 0.04, n = 9). However, cardiac-dead donors
showed the same trend (P = 0.07, n = 7), and therefore deceased donor data are clustered.
IL-9 release was specific for I/R, as control measurements over the non ischemic kidney in
the donor did not show a transrenal difference in IL-9 levels (P = 0.89, data not shown). This
indicates that IL-9 release is not a consequence of manipulation of the kidney during surgery or a reaction to anesthetics.

Source of human IL-9 release is mainly tubular epithelial cells

The source of IL-9 released from deceased donor kidneys could be local resident cells or circulating cells. An immunohistochemical staining of IL-9 was performed on renal biopsy tissue from a deceased donor graft, which showed that local stores of IL-9 are present in the kidney. IL-9 signals were present predominantly in renal tubular epithelial cells and also in macrophages and dendritic cells (Figure 2).
Figure 2. Typical example of IL-9 staining in deceased donor kidney biopsy collected before transplantation. IL-9 signal was positive in renal tubular epithelial cells, macrophages, and dendritic cells. Original magnification x200.

**No change in human renal IL-9 expression after reperfusion**

No differences in IL-9 RNA expression were observed between pre- and post-reperfusion biopsies in living \( (P = 0.89, \text{Figure 3A}) \) as well as deceased donor kidneys \( (P = 0.59, \text{Figure 3B}) \). In addition, similar baseline expression of IL-9 in living and deceased donor kidneys was observed \( (P = 0.57) \).

![Graph A](image1.png)  ![Graph B](image2.png)

**Figure 3. Interleukin-9 expression in human kidney biopsies.** IL-9 expression in human pre- and post-transplantation biopsies of (A) living donor kidneys; and (B) deceased donor kidneys. There was no difference in IL-9 RNA expression between pre- and post-reperfusion biopsies in living and deceased donor kidneys \( (P = 0.89 \text{ and } P = 0.59, \text{respectively}) \) nor in baseline IL-9 expression between both donor types \( (P = 0.57) \).
Anti-IL-9 treatment does not prevent functional and structural kidney I/R injury
Given the dominance of IL-9 release from deceased donor kidneys, we hypothesized that IL-9 is critical in the initiation of I/R injury. Therefore, mice were treated with anti-IL-9 Ab or isotype control Ab before undergoing renal I/R. All mice survived the end of the experiment. Baseline urea concentrations were identical in all animals (Figure 4). In both anti-IL-9 Ab and isotype control Ab treated mice that underwent renal I/R, urea concentration was significantly raised at 1 and 2 days after reperfusion as compared with baseline (all, $P = 0.002$). As expected, urea of sham-operated animals, both anti-IL-9 Ab and isotype control Ab pre-treated, did not change after surgery as compared with baseline (all, $P = NS$). Urea concentration was similar in anti-IL-9 treated and control Ab treated mice at day 1 ($P = 0.91$) and 2 ($P = 0.53$) after reperfusion (Figure 4).

Figure 4. Plasma urea levels after murine renal ischemia/reperfusion.
Plasma urea levels measured at baseline (day -3), at the day of surgery (day 0), and two consecutive days after surgery (day 1 and 2). Plasma urea levels were similar in anti-IL-9 Ab treated mice compared with isotype control Ab treated mice after I/R at day -3 ($P = 0.77$), day 0 ($P = 0.69$), day 1 ($P = 0.91$), and day 2 ($P = 0.53$). The median and interquartile range are plotted.
Structural kidney damage was moderate to severe in all kidneys that underwent I/R, whereas kidneys of sham-operated mice showed normal histology. Kidney injury was scored on several characteristics and then quantified. There was significantly more structural damage in kidneys of anti-IL-9 Ab treated mice than in the control Ab treated group after I/R. In detail, anti-IL-9 Ab treated mice showed more proximal tubule necrosis ($P = 0.02$), band necrosis of the cortex and medulla ($P = 0.01$) and protein casts in the tubules ($P = 0.005$) (Figure 5).
Figure 5. PAS-staining of murine kidney biopsies. Representative PAS-stained kidney sections harvested 2 days after surgery of (A) mice that received anti-IL-9 Ab before renal I/R; (B) mice that received control Ab before kidney I/R; (C) sham-operated mice that received anti-IL-9 or control Ab treatment before operation (no difference). Original magnification x200. Mice receiving anti-IL-9 Ab before I/R had evident protein casts and tubule necrosis, more than isotype control Ab treated mice. Sham-operated mice receiving either anti-IL-9 Ab or control Ab had no PAS-positive deposits and no structural kidney damage. (D) Semi-quantitative histological damage score for the severity of kidney damage is shown. Damage was scored (0-3) on presence of proximal tubule necrosis (PTN), band necrosis (BN) and protein casts (PC). Kidneys of all mice that underwent kidney I/R showed moderate to severe damage. There were significant differences between anti-IL-9 Ab-treated and isotype control Ab-treated groups (PTN, $P = 0.02$; BN, $P = 0.01$; PC, $P = 0.005$). Kidneys of sham-operated mice scored 0 for all measures. Median and interquartile ranges are plotted. Significant differences are indicated by an asterisk.

Discussion

In this study it is shown that IL-9 is released exclusively from human deceased donor grafts directly after reperfusion. Since deceased donor grafts are clinically more affected by I/R injury, we hypothesized that IL-9 is a mediator of renal I/R injury. In a subsequent mouse experiment, inhibition of IL-9 did not influence kidney function after I/R and even aggravated structural kidney injury.

IL-9 was released instantly after reperfusion from deceased donor grafts and its release persisted during the next thirty minutes. The substantial and immediate character of this IL-9 release suggests a pre-stored pool, since no change in IL-9 expression in kidney tissue upon reperfusion was observed. Pre-transplantation infiltrated cells are probably not involved, since baseline biopsies showed no difference between human living and deceased donor IL-9 expression, although there was a vast difference in cellular infiltrate before transplantation. Immunohistochemical staining of IL-9 showed its presence in tubular epithelium and resident macrophages and dendritic cells. These cells are likely responsible for secretion of their pre-stored IL-9.
IL-9 is considered a pro-inflammatory cytokine, although as yet little is known about its exact functions. Preclinical studies have shown that IL-9 promotes mast cell growth and plays a crucial role in the nephroprotective effects of regulatory T cells.\textsuperscript{12, 20} In addition, a potential role in the stimulation of erythropoiesis has been suggested\textsuperscript{21, 22}, as well as an antiapoptotic effect.\textsuperscript{23} The observation in our study that IL-9 was released exclusively from deceased donor kidneys, in which tissue damage is most severe, argues for an active role of IL-9 in initiating I/R injury. Therefore, the effect of IL-9 inhibition on kidney I/R injury was assessed in a mouse experiment. Remarkably, results showed aggravated structural kidney damage after I/R in anti-IL-9 Ab treated animals. When extrapolating these findings, it can be suggested that IL-9 plays a regulating role in clinical renal I/R injury or can be an initial trigger for salvaging or limiting injury.

Although no previous data are available regarding the role of IL-9 in I/R injury, the few studies on allograft rejection after transplantation are conflicting. An association between IL-9 and acute, eosinophil-driven rejection has been suggested by Poulin et al. in mismatched heart allografts.\textsuperscript{24} However, no involvement of IL-9 was observed in rejection on a longer term in both mouse islets and human kidney allografts.\textsuperscript{25}

Our arteriovenous sampling time was limited to thirty minutes after reperfusion, the time needed to complete the operative procedure. Deceased donor kidney grafts showed an extensive release of IL-9 within the studied timeframe; therefore it is highly unlikely that living donor kidney grafts will start releasing IL-9 after the studied period. Finally, one might argue that a mouse model of renal I/R injury can only approximate the human situation. Nevertheless, the effects of IL-9 inhibition could be accurately assessed in our animal experiment and a negative effect of anti-IL-9 treatment was indeed observed.

In conclusion, this study shows a substantial and exclusive release of IL-9 from deceased donor grafts in human kidney transplantation, while living donor kidneys, which generally have a more favourable clinical outcome, did not release IL-9 during early reperfusion. The source of released IL-9 was its pre-stored pool in renal tubular cells. Experimental inhibition of IL-9 in mice aggravated kidney damage after I/R injury. Altogether, these results suggest a regulating or minor role of the released IL-9 on I/R injury in human kidney transplantation.
References


Chapter 3


Donor brain death predisposes human kidney grafts to a proinflammatory reaction after transplantation


Am J Transplant. 2011;11:1064-70
Abstract

Donor brain death has profound effects on post-transplantation graft function and survival. We hypothesized that changes initiated in the donor influence the graft’s response to ischemia and reperfusion. In this study, human brain dead donor kidney grafts were compared to living and cardiac dead donor kidney grafts. Pre-transplant biopsies of brain dead donor kidneys contained notably more infiltrating T lymphocytes and macrophages. To assess whether the different donor conditions result in a different response to reperfusion, local cytokine release from the reperfused kidney was studied by measurement of paired arterial and renal venous blood samples. Reperfusion of kidneys from brain dead donors was associated with the instantaneous release of inflammatory cytokines, such as G-CSF, IL-6, IL-9, IL-16 and MCP-1. In contrast, kidneys from living and cardiac dead donors showed a more modest cytokine response with release of IL-6 and small amounts of MCP-1. In conclusion, this study shows that donor brain death initiates an inflammatory state of the graft with T lymphocyte and macrophage infiltration and massive inflammatory cytokine release upon reperfusion. These observations suggest that brain dead donors require a novel approach for donor pretreatment aimed at preventing this inflammatory response to increase graft survival.
**Introduction**

Despite better immunological matching, kidney allografts of deceased donors demonstrate inferior graft function and survival in comparison with living unrelated donor grafts. This can only partially be explained by their longer cold ischemia time.\(^1\) It is thus likely that donor-specific characteristics influence post-transplantation graft function and explain differences in clinical success rate.

Donor brain death has a significant influence on graft function and survival.\(^2\) The unphysiological state of brain death is associated with systemic pro-inflammatory changes, as illustrated by increased levels of circulating cytokines which reflect an inflammatory state in peripheral organs. Indeed, the expression of inflammatory factors in the kidney at time of donation is increased in brain dead donors.\(^3\)-\(^5\)

We have previously shown that the graft reperfusion phase is dominated by an inflammatory response, and that kidney grafts of living donors release cytokines immediately after reperfusion.\(^6\) It was thus hypothesized that the inflammatory changes associated with brain dead influence the kidney graft and result in a different response to ischemia and reperfusion. In this explorative study we first assessed whether there are differences in inflammatory cell content between brain dead and living as well as cardiac dead donor kidney grafts before transplantation. We subsequently evaluated the inflammatory response to reperfusion of the donor kidney through measurement of arteriovenous concentration differences over the transplanted organ. Cardiac dead donor grafts were included in this study in order to evaluate a potential effect of longer cold ischemia times in the deceased donors.

**Methods**

**Patient population**
Twenty-four patients undergoing renal allograft transplantation were included; 8 patients receiving a kidney from a living donor, 9 patients receiving a kidney from a brain dead donor, and 7 patients receiving a kidney from a cardiac dead donor (Maastricht category III, table 1). For technical reasons (renal vein sampling) only patients receiving a left kidney were included. The study protocol was approved by the local ethics committee, and informed consent was obtained from each patient.
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Operation and materials
Kidney transplantations were performed according to local standardized protocol. In living donors open nephrectomy was performed and Custodiol® Histidine–tryptophan–ketoglutarate solution (HTK) solution (Tramedico, Weesp, The Netherlands) was used for cold perfusion and storage of the kidney. Brain dead and cardiac dead donor kidneys were perfused and stored with either University of Wisconsin solution (UW) or HTK (table 1). The immunosuppressive regimen was based on induction therapy with basiliximab; and tacrolimus or cyclosporine A in addition with mycophenolate mofetil and steroids in all groups.

A renal cortical biopsy was obtained just before transplantation, when the graft was still on ice. Biopsy tissue was frozen and cut into 4 mm thick sections in a cryostat. Arterial and renal venous blood samples were obtained as described before in detail. In short, via an umbilical vein catheter placed in the renal vein at 30 sec., 1, 3, 5, 10, 20 and 30 min. after reperfusion (i.e., t=0) blood aliquots were sampled. For ethical reasons our study was limited to a sample collection time of 30 minutes, which was the timeframe needed to anastomose the ureter to the bladder. Paired arterial blood samples were obtained at 0, 3, 5, 10, 20 and 30 min. after reperfusion. All samples were collected in tubes containing EDTA and immediately placed on ice. Blood samples were centrifuged (1,550 g, 20 min, 4ºC) and the derived plasma was re-centrifuged (1,550 g, 20 min, 4ºC) to deplete it from leukocytes and thrombocytes. Plasma was aliquotted and stored at -70ºC until assayed.

Validation of the arteriovenous measurements
The method of arteriovenous measurements over the reperfused kidney has been validated before. Because cholesterol is not secreted, filtered or re-absorbed by the kidney, the plasma cholesterol was measured to evaluate potential blood concentrating effect of the kidney. Lactate dehydrogenase (LDH) activity was determined as measure of cell lysis. Cholesterol and LDH concentrations were measured in a certified facility, using routine laboratory assays. There was no evidence for hemoconcentration over the kidney, as cholesterol concentrations did not show an arteriovenous difference. LDH was not released into venous blood either, excluding cell lysis as source of cytokines. Therefore, all measurements are shown as uncorrected values.

Immunohistochemical analysis of biopsies
Cryostat sections were rehydrated and incubated with the primary antibody raised against CD3 (1:200, Abcam, Cambridge, MA, USA), CD68 (1:2000, DAKO, Glostrup, Denmark) or Myeloperoxidase (MPO) (1:1000, DAKO, Glostrup, Denmark). A standard streptavidin-biotin-peroxidase complex method was applied to visualize T-lymphocytes, macrophages and polymorphonuclear neutrophils (PMN) using DAB or nova red respectively as a chromogen.
Slides were counterstained with Mayer’s haematoxylin. The number of CD3 positive cells was counted as a percentage of total cells in at least 5 views at a magnification x20 by automated analysis using AxioVision software, version 4.4.1.0 (Carl Zeiss MicroImaging Inc, Gottingen, Germany). The numbers of CD68 and MPO positive cells were quantified in each specimen (magnification x20) using a calibrated grid. The number of cells over 5-7 grid areas of each kidney biopsy was counted by two independent blinded observers (kappa=0.97).

**Plasma measurements**

We first performed a pilot measurement in venous and arterial blood samples collected 30 minutes after reperfusion for both living donor, brain dead donor and cardiac dead donor kidney transplantation to identify candidate cytokines and chemokines in the human 27-plex panel for multiple cytokines (Biorad, Veenendaal, The Netherlands). The panel included interleukin (IL)-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin, basic fibroblast growth factor (b-FGF), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, interferon-inducible protein (IP)-10, monocytes chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α and -1β, platelet-derived growth factor-BB, regulated upon activation, normal T cell expressed and secreted (RANTES), tumour necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF). Next, we performed a detailed analysis of the targets above the detection threshold and showing a trend towards an arteriovenous difference. IL-1ra, IL-6, IL-8, IL-9, IL-16, G-CSF and MCP-1 were measured in a custom made multiplex assay in accordance with the manufacturer’s instructions (X-plex, Biorad, Veenendaal, The Netherlands).

**Data collection and statistical analysis**

Clinical donor data were retrieved from Eurotransplant database. Delayed graft function (DGF) was defined as the need for dialysis within 1 week after transplantation. Statistical analysis was performed using SPSS 16.0 statistical analysis software (SPSS Inc, Chicago, Ill). The number of positive cells in the immunohistochemical analysis was compared for all three donor types using ANOVA, with post-hoc Bonferroni test to assess the individual differences. Area under the curve (AUC) was calculated for the arterial and venous curve of the plasma measurements for the total of 30 minutes. The delta AUC was calculated (venous minus arterial) and the null-hypothesis (delta AUC is 0) was tested by t-test or non-parametric test in case of a non-normal distribution. Benjamini-Hochberg correction for multiple testing was applied to the cytokine measurements. Graph error bars represent the standard error of the mean (SEM). A p-value of less than 0.05 was considered significant.
Chapter 4

Results

Transplant and recipient characteristics
Recipient and donor age and gender were similar in living donor, brain dead donor and cardiac dead donor groups (table 1). As expected, warm and cold ischemia times differed between groups, with shorter cold ischemia times in living donor kidney transplantation and a donor warm ischemic period for cardiac dead donor kidney transplantation. The immunosuppressive regimen was equal in all recipients. All kidneys were still functioning at 1 year post transplantation, except for one graft from a cardiac dead donor (the recipient was not compliant with immunosuppressive medication).

Table 1: Transplantation and outcome characteristics in living donor (LD), brain dead donor (BDD) and cardiac dead donor (CDD) kidney transplantation.

<table>
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<th>BDD</th>
<th>CDD</th>
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<td>9</td>
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<td>107 (139)</td>
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ICU stay: the period the donor was admitted to intensive care unit, BD: the total duration of brain dead of the donor, WIT1: first warm ischemia time, CIT: cold ischemia time, WIT2: second warm ischemia time, DGF: delayed graft function.
*p<0.05 compared to BDD, ∇ p<0.05 compared to CDD.

T lymphocyte and macrophage infiltration in brain dead donor grafts
T lymphocytes and macrophages were found in all pre-transplant biopsies, however the amount of T lymphocytes was significantly higher in brain dead donor kidney grafts as compared to living and cardiac dead donor grafts (p<0.001, figure 1). Moreover, macrophage counts were more than twice as high in brain dead donor kidneys as compared to living donor and cardiac dead donor kidneys (p=0.009, figure 2). Neutrophils were only present in minimal amounts in grafts of all three donor types and were not significantly different between groups (data not shown).
Figure 1: CD3 positive cells in biopsies. Pre-transplantation biopsies of living donor (LD) (A), brain dead donor (BDD) (B) and cardiac dead donor (CDD) (C) were stained for CD3. Representative images are shown. Original magnifications were x20. (D) The amount of CD3 positive cells was significantly different between brain dead donor (BDD), living donor (LD) and cardiac dead donor (CDD) grafts (p<0.001). Post-hoc test showed that BDD and LD (p<0.001) and BDD and CDD (p<0.001) differed significantly, whereas there was no difference between LD and CDD grafts (p=0.181).
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Figure 2: CD68 positive cells in biopsies. Pre-transplantation biopsies of living donor (LD) (A), brain dead donor (BDD) (B) and cardiac dead donor (CDD) (C) were stained for CD68. Representative images are shown. Original magnifications were x20. (D) The amount of CD68 positive cells was significantly different between brain dead donor (BDD), living donor (LD) and cardiac dead donor (CDD) grafts (p<0.001). Post-hoc test showed that BDD and LD (p=0.001) and BDD and CDD (p=0.009) differed significantly, whereas there was no difference between LD and CDD grafts (p=1.00).

Brain dead donor grafts release many inflammatory cytokines

To assess whether infiltrating cells may influence post-transplantation graft characteristics, local cytokine release from the kidney was assessed by measuring arteriovenous differences over the kidney. To illustrate the method of measurement and analysis, the arterial and renal venous concentration of MCP-1 during the first 30 minutes of reperfusion is shown for living, brain dead and cardiac dead donor kidney transplantation (figure 3). The difference in area under the curve (AUC) of the arterial and venous curve was used as measure of total release from the kidney. First, in the explorative study, 27 different cytokines were measured in arteriovenous samples collected at 30 minutes after reperfusion. The majority of cytokines, i.e. interleukin (IL)-1β, IL-4, IL-5, IL-7, IL-10, IL-12, IL-13, eotaxin, GM-CSF, IFN-γ, IP-10, MIP-1β, PDGF-BB, RANTES, TNF-α, and VEGF were not released from the kidney. In the measurement of IL-2, IL-15, IL-17, b-FGF and MIP-1α all samples were below the detection limit of the assay, and thus interpreted as clinically non relevant.
Figure 3: Release of MCP-1 from the reperfused kidney. A: Arterial and renal venous plasma concentrations of MCP-1 during the first 30 min of reperfusion in living donor (LD) kidney transplantation showed no significant MCP-1 release from the graft (p = 0.08, n = 8); B: In cardiac dead donor (CDD) kidney transplantation there was a modest, steady MCP-1 release in the first 30 min of reperfusion (p = 0.002, n = 7); C: In brain dead donor (BDD) kidney transplantation MCP-1 was released in high amounts (p = 0.027, n = 9).

Five cytokines changed in concentration over the kidney in at least one of the donor types, and figure 4 shows their total release from the kidney in the first 30 minutes of reperfusion. Only statistically significant differences are shown. Brain dead donor kidney grafts massively released various cytokines, including G-CSF (p=0.011), IL-6 (p=0.011), IL-9 (p=0.029), IL-16 (p=0.017) and MCP-1 (p=0.012) immediately after transplantation. In contrast, living donor and cardiac dead donor kidney grafts show a very modest response with only release of IL-6 (p=0.003) and MCP-1 (p=0.002) respectively. No correlation could be established between clinical outcome variables (as shown in table 1) and the distribution or magnitude of cytokine release.
Brain dead donor kidneys show an inflammatory pattern of cytokine release. Delta AUC of venous minus arterial release of seven different cytokines for living donor (LD), cardiac dead donor (CDD) and brain dead donor (BDD) kidney transplantation. LD and CDD kidney grafts only released IL-6 (p=0.003) and MCP-1 (p=0.002) respectively. In contrast, BDD grafts released many different pro-inflammatory cytokines, such as G-CSF (p=0.011), IL-6 (p=0.011), IL-9 (p=0.029), IL-16 (p=0.017) and MCP-1 (p=0.012) in vast amounts. The box runs from the lower to upper quartile, the whiskers indicate the 5 to 95 percentile, while the horizontal line is set at the median.

Discussion

Donor brain death is considered an independent risk factor for graft function and survival. Yet it is unclear what processes are responsible for the impaired function and survival. We hypothesized that the response to ischemia and reperfusion in brain dead donor kidneys is different to that of non brain dead donor grafts. This study shows that donor brain death predisposes the kidney graft to a pro-inflammatory reaction upon reperfusion. Before transplantation, brain dead donor grafts already contain many infiltrated macrophages and T lymphocytes. Subsequently, after reperfusion brain dead donor kidneys release various pro-inflammatory cytokines. This inflammatory response is specific for brain death, as cardiac dead donor kidney grafts with equally long periods of cold ischemia do not demonstrate this pro-inflammatory cytokine release.
It is known that brain death leads to a systemic pro-inflammatory state of the donor. Circulating levels of TNF-\(\alpha\), IL-2R, IL-6 and IL-8 are many times higher in brain dead donors compared to living donors at time of organ procurement.\(^7\) Similarly it has been shown that brain death induces endothelial cell activation in organs to be recovered as indicated by increased expression of adhesion molecules such as E-selectin,\(^4\) ICAM-1 and VCAM-1 \(^8\) and increased presence of leukocytes.\(^5,9\) Whether these inflammatory changes persist after transplantation and influence the reperfusion response of the graft has been unclear. This study not only shows that kidneys of brain dead donors contain certain cellular infiltrates, i.e. T lymphocytes and macrophages before transplantation, but also that upon reperfusion many inflammatory mediators are released from the graft. This different response of brain dead donor kidneys may be explained by changes in the kidney tissue itself; however a potentially more likely reason is that these mediators are released from the infiltrated T lymphocytes and macrophages. These leukocytes of donor origin may enhance the pro-inflammatory state of the organ and may increase the immunogenicity of the organ. Many of the cytokines that are released upon reperfusion are chemo attractants and may attract recipient leukocytes to the kidney, increasing the threat of acute rejection.\(^10,11\) Indeed, animal experiments demonstrate that brain death increases the rejection rate of allogeneic transplants.\(^12,13\)

In most previously published studies where the effect of brain death after reperfusion was examined, only brain dead donor and living donor kidney transplantation were compared. However, in these studies the question remains to what extent results can be explained by the great differences in cold ischemia time, as it is known that renal inflammation correlates with duration of cold ischemia.\(^14\) In this study, cardiac dead donor kidney grafts were included in the comparison, as their period of cold ischemia is comparable to that of brain dead donor kidney grafts.

Brain dead donor kidney grafts release the inflammatory cytokines G-CSF, IL-6, IL-9, IL-16 and MCP-1 immediately after reperfusion. Living donor and cardiac dead donor grafts only released IL-6 and MCP-1. IL-6 is released locally from both LD and BDD kidney grafts. The role of IL-6 in acute kidney injury is not straightforward, and in the context of I/R IL-6 can have protective effects as well.\(^6\) Of the cytokines that are selectively released in vast amounts from brain dead donor grafts, such as IL-16 and MCP-1, the effects are more clear-cut. These are considered pro-inflammatory cytokines that are harmful and can contribute to kidney ischemia/reperfusion (I/R) injury.\(^15,16\) However, cytokines can not be classified as exclusively pro- or anti-inflammatory and their actions are unpredictable because they generally depend on the presence of responsive cells, the combination of cytokines and timing of release. Therefore, when converging all these results towards a therapeutical
consequence, it is clear that instead of aiming to inhibit cytokines after reperfusion, a more straightforward approach would be to avoid the pro-inflammatory response after reperfusion. Pre-treating the brain dead organ donor to prevent changes that cause the pro-inflammatory state in the graft may constitute a promising modality to increase graft function and survival. Donor pretreatment with steroids has been shown to significantly decrease tissue expression and serum concentrations of pro-inflammatory cytokines.\textsuperscript{17,18} Moreover, preclinical data indicate that donor pretreatment with steroids increases kidney graft survival after transplantation.\textsuperscript{19} In clinical liver transplantation, donor pretreatment with steroids decreased the incidence of acute rejection.\textsuperscript{18} Other anti-inflammatory acting agents may hold promise as well. Statins are known to lower cytokine responses and may therefore be an interesting, safe option for future donor pretreatment.\textsuperscript{20}

Since the goal of this study was to detect differences in the nature of the inflammatory response in living and brain dead donor grafts, instead of correlating findings to clinical outcome; small patient numbers were sufficient. Indeed, we were able to detect differences in patterns of cytokines released from the kidney. By comparing arterial and venous curves, the net release from the kidney was assessed and systemic influences or release of cytokines from other sites than the kidney did not influence measurements.

In conclusion, this study shows that local inflammatory cell infiltration in brain dead donor kidney grafts influences their response to ischemia and reperfusion. Transplanted kidneys from brain dead donors release many pro-inflammatory cytokines immediately after reperfusion. This cytokine profile is specific for brain death and is not related to ischemia time since both cardiac dead and living donor kidney grafts showed a minimal response. Therefore, it is suggested that brain dead donors require a more specialized, targeted approach for donor pretreatment and graft preservation aimed at reducing the inflammatory reaction and thereby improving graft outcome.

**Acknowledgements**
The Netherlands Organization for Health Research and Development project 92003525 (D.K.V.)
Inflammatory profile of kidneys of brain dead donors

References


Acute but transient release of terminal complement complex after reperfusion in clinical kidney transplantation


These authors contributed equally to this article

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Abstract

**Background.** Ischemia/reperfusion injury has a major impact on kidney graft function and survival. Animal studies have suggested a role for complement activation in mediating I/R injury, however results are not unambiguous. Whether complement activation is involved in clinical I/R injury in humans is still unclear.

**Methods.** In the present study, we assessed the formation and release of C5b-9 during early reperfusion in clinical kidney transplantation in both living, brain dead and cardiac dead donor kidney transplantation. By arteriovenous measurements and histological studies, local terminal complement activation in the reperfused kidney was assessed.

**Results.** There was no release of sC5b-9 from living donor kidneys, nor was there a release of C5a. In contrast, instantly after reperfusion, there was a significant but transient venous release of soluble C5b-9 from the reperfused kidney graft in brain dead and cardiac dead donor kidney transplantation. This short-term activation of the terminal complement cascade in deceased donor kidney transplantation was not reflected by renal tissue deposition of C5b-9 in biopsies taken 45 minutes after reperfusion.

**Conclusions.** This systematic study in human kidney transplantation shows an acute but non-sustained sC5b-9 release upon reperfusion in deceased donor kidney transplantation. This instantaneous, intravascular terminal complement activation may be induced by intravascular cellular debris and hypoxic or injured endothelium.
Introduction

Ischemia/reperfusion (I/R) injury is an inevitable consequence of organ transplantation and a major determinant of patient and graft survival. Current therapy is supportive and there are no specific therapeutical options yet. The pathophysiology of I/R injury is complex and incompletely understood. The innate immune system has been suggested to play an important role in potentiating an injurious reaction upon reperfusion since it is prone to recognize not only pathogens but also ‘damaged self’.

The complement system is one of the fastest responding basal defense mechanisms of the innate immune system. Activation of either the classical, alternative, or Mannan-binding lectin pathway ultimately leads to the formation of C5b-9, otherwise known as the terminal complement complex or membrane attack complex (MAC). Release of soluble (s)C5-9 has been described in a variety of renal disorders, such as lupus nephritis, Henoch-Schönlein Pupura and aHUS, and has been shown to be a sensitive marker in assessing disease activity. Renal I/R affects the endothelial as well as the epithelial compartment and might activate the complement cascade leading to deposition of C5b-9 or release of non-lytic sC5b-9.

Animal studies of renal I/R injury generally show that complement inhibition reduces post-reperfusion damage. Zhou et al more specifically demonstrated the involvement of terminal complement complex C5b-9. However, in recent rat experiments by our group, inhibition of complement activation did not reduce kidney damage and only 24 hours after reperfusion the first signs of complement activation were observed. Moreover, Mannan-binding lectin itself appears to exert cytotoxic effects on the tubular epithelium early after reperfusion, far before first complement deposition was observed. These recent findings raise questions about the contribution of complement activation as initiator of I/R injury.

Despite the extensive number of animal experiments, studies on the involvement of complement in human I/R injury are scarce. Studies in the human heart have suggested a role for complement activation in I/R induced tissue damage. However, the diverse studies on experimental anti-complement therapy in human myocardial I/R injury did not lead to major improvements yet.

To address the recent contradictory findings in animals and the lack of evidence for involvement of complement in human I/R injury, we investigated the role of complement activation in the initiation of clinical renal I/R injury. I/R induced complement activation may take place in both the tubular and vascular compartment. Therefore, we systematically measured terminal complement activation during early reperfusion in human
kidney transplantation in both the tubular compartment by immunohistochemistry and the intravascular compartment by selective arteriovenous measurements over the transplanted kidney.

**Materials and Methods**

**Patient population**

Twenty-four patients undergoing renal allograft transplantation were included for arteriovenous sampling: 8 patients receiving a kidney from a living donor, 9 patients receiving a kidney from a brain dead donor and 7 patients receiving a kidney from a cardiac dead donor (patient and transplantation characteristics are in table 1), as previously described.\textsuperscript{24} Brain dead and cardiac dead donors together were referred to as deceased donors. For technical reasons (renal vein sampling) only patients receiving a left kidney were included. In another 33 patients (13 living donor, 10 brain dead donor, and 10 cardiac dead donor kidney recipients) renal biopsies were collected. The study protocol was approved by the local ethics committee, and informed consent was obtained from each patient.

**Table 1:** Transplantation and outcome characteristics of patients undergoing arteriovenous measurements.

<table>
<thead>
<tr>
<th></th>
<th>LD</th>
<th>BDD</th>
<th>CDD</th>
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</thead>
<tbody>
<tr>
<td>N</td>
<td>8</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
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<td>43.9(10.6)</td>
<td>54.1(17.1)</td>
<td>52.7(15.3)</td>
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<td>Donor gender (M:F %)</td>
<td>75:25%</td>
<td>44:56%</td>
<td>43:57%</td>
</tr>
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<td>Duration ICU stay in h. (SD)</td>
<td>N/A</td>
<td>126 (211)</td>
<td>107 (139)</td>
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<td>Duration of BD in h. (SD)</td>
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<td>14.7(9.7)</td>
<td>N/A</td>
</tr>
<tr>
<td>Preservation fluid</td>
<td>HTK (n=8)</td>
<td>UW (n=9)</td>
<td>UW(n=2), HTK (n=5)</td>
</tr>
<tr>
<td>WIT1 in min. (SD)</td>
<td>N/A</td>
<td>N/A</td>
<td>23.1(7.7)</td>
</tr>
<tr>
<td>CIT in h. (SD)</td>
<td>3.0(0.3) * V</td>
<td>19.7(6.2)</td>
<td>17.3(2.6)</td>
</tr>
<tr>
<td>WIT2 in min. (SD)</td>
<td>34.0(6.3)</td>
<td>33.0(6.1)</td>
<td>34.1(6.4)</td>
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<td>Recipient age: mean (SD)</td>
<td>41.1(10.5)</td>
<td>55.1(13.5)</td>
<td>54.0(11.2)</td>
</tr>
<tr>
<td>Recipient gender (M:F %)</td>
<td>38:62%</td>
<td>44:56%</td>
<td>71:29%</td>
</tr>
<tr>
<td>Preemptive transplantation (n)</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Creatinine clearance day 30 in ml/min (SD)</td>
<td>73.3 (20.5) * V</td>
<td>49.3(15.3) V</td>
<td>27.1(10.3)</td>
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<td>DGF (%)</td>
<td>0%</td>
<td>56%</td>
<td>86%</td>
</tr>
<tr>
<td>DGF: dialysis after transplantation in days (SD)</td>
<td>0 (0) * V</td>
<td>7.0 (5.3) V</td>
<td>17.2 (7.2)</td>
</tr>
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</table>

Groups undergoing living donor (LD), brain dead donor (BDD) and cardiac dead donor (CDD) kidney transplantation were compared. Intensive care unit (ICU) stay: the period the donor was admitted to intensive care unit, BD: the total duration of brain dead of the donor, WIT1: first warm ischemia time, CIT: cold ischemia time, WIT2: second warm ischemia time, DGF: delayed graft function. *p<0.05 compared to BDD, V p<0.05 compared to CDD.
Operation and materials

Kidney transplantations were performed according to local standardized protocol. In living donors minimally invasive nephrectomy was performed and Custodiol® Histidine-tryptophan-ketoglutarate solution (HTK) solution (Tramedico, Weesp, The Netherlands) was used for cold storage of the kidney. Brain dead and cardiac dead donor kidneys were perfused and stored with either University of Wisconsin solution (UW) or HTK. The immunosuppressive regimen was based on tacrolimus or cyclosporine A in addition with mycophenolate mofetil and steroids in all groups.

Arterial and renal venous blood samples were obtained as described before in detail.25 A schematic drawing of the arteriovenous sampling method is shown in figure 1. In short, via a small catheter placed in the renal vein blood aliquots were sampled at 0, 3, 10 and 30 min. after reperfusion. Paired arterial blood samples were obtained at 0, 3, 10 and 30 min. after reperfusion. All samples were collected in tubes containing EDTA and immediately placed on ice. Blood samples were centrifuged (1,550 g, 20 min, 4ºC) and the derived plasma was re-centrifuged (1,550 g, 20 min, 4ºC) to deplete it from leukocytes and thrombocytes. Material was aliquotted and stored at -70ºC until assayed.

Figure 1. Schematic representation of the arteriovenous sampling method over the reperfused kidney by simultaneous blood collection from the renal artery and vein. Illustration by Manon Zuurmond© (www.manonproject.com)

In another 33 patients (13 living donor, 10 brain dead donor, and 10 cardiac dead donor kidney recipients) renal biopsies were collected (table 2). A renal cortical biopsy was obtained after cold storage, and a second biopsy of the same kidney was collected 45 minutes after reperfusion.
Table 2: Transplantation and outcome characteristics of patients from which renal biopsies were studied.

<table>
<thead>
<tr>
<th></th>
<th>LD</th>
<th>BDD</th>
<th>CDD</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>13</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Donor age: mean (SD)</td>
<td>45 (12)</td>
<td>43 (13)</td>
<td>54 (15)</td>
</tr>
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<td>Donor gender (M:F %)</td>
<td>77:23%</td>
<td>20:80%</td>
<td>70:30%</td>
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<td>Duration ICU stay in h. (SD)</td>
<td>N/A</td>
<td>90 (121)</td>
<td>86 (100)</td>
</tr>
<tr>
<td>Duration of BD in h. (SD)</td>
<td>N/A</td>
<td>12 (9)</td>
<td>N/A</td>
</tr>
<tr>
<td>Preservation fluid</td>
<td>UW (n=13)</td>
<td>UW (n=10)</td>
<td>UW (n=10)</td>
</tr>
<tr>
<td>WIT1 in min. (SD)</td>
<td>N/A</td>
<td>N/A</td>
<td>19 (7)</td>
</tr>
<tr>
<td>CIT in h. (SD)</td>
<td>132 (40) *</td>
<td>1131 (369)</td>
<td>1093 (273)</td>
</tr>
<tr>
<td>WIT2 in min. (SD)</td>
<td>38 (7)</td>
<td>43 (7)</td>
<td>41 (17)</td>
</tr>
<tr>
<td>Recipient age: mean (SD)</td>
<td>42 (16)</td>
<td>55 (14)</td>
<td>53 (12)</td>
</tr>
<tr>
<td>Recipient gender (M:F %)</td>
<td>46:54%</td>
<td>40:60%</td>
<td>100:0%</td>
</tr>
<tr>
<td>Creatinine clearance day 30 in ml/min (SD)</td>
<td>65 (15)</td>
<td>54 (15)</td>
<td>50 (22)</td>
</tr>
<tr>
<td>DGF (%)</td>
<td>8%</td>
<td>20%</td>
<td>100%</td>
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Groups undergoing living donor (LD), brain dead donor (BDD) and cardiac dead donor (CDD) kidney transplantation were compared.

Intensive care unit (ICU) stay: the period the donor was admitted to intensive care unit, BD: the total duration of brain dead of the donor, WIT1: first warm ischemia time, CIT: cold ischemia time, WIT2: second warm ischemia time, DGF: delayed graft function.

*p<0.05 compared to BDD, V p<0.05 compared to CDD.

sC5b-9 and C5a plasma measurements
sC5b-9 and C5a levels were assessed by sandwich ELISA. In short, 96-well ELISA plates (Nunc Bioscience, Belgium) were coated with a monoclonal antibody (mAb) to a neo-epitope on C5b-9 (aE11; Hycult Biotechnology, Uden, Netherlands) or C5a (mAb 2952; Hycult Biotechnology). Plasma was incubated in the coated wells and bound sC5b-9 or C5a was detected with a biotin-labeled mAb to C6 (9C4; in-house made) or C5a (mAb 561; Hycult Biotechnology) respectively, followed by detection with streptavidin-poly-horseradish peroxidase (Sanquin, Amsterdam, The Netherlands). Enzyme activity was detected using 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (Sigma Chemical Co., St. Louis, MO). The optical density was measured at 415 nm using a microplate reader (Model 680; Biorad, Philadelphia, USA). The detection limits for C5a and C5b-9 were 1.95 ng/ml and 0.01 U/ml, respectively.

Immunohistochemistry
Sections (4 µm) of paraffin embedded, formaldehyde fixed biopsies were deparaffinized and treated with 0.1% protease (type XXIV pronase, Sigma) for antigen retrieval. Endogenous peroxidase activity was blocked with 0.1% H2O2 and 0.1% NaN3. Consequently, C5b-9 deposition was assessed using a mAb to a neoepitope on C5b-9 (aE11, Hycult Biotechnology)
followed by anti-mouse peroxidase-conjugated EnVision™ (DAKO, Glostrum, Germany). The staining was visualized using Nova RED (Vector Labs, Peterborough, United Kingdom). Sections were counterstained with hematoxylin (Merck, Darmstadt, Germany). As a positive control, a renal biopsy of a patient with acute graft rejection was used.

Data collection and statistical analysis
Clinical donor data were retrieved from Eurotransplant. Outcome measures were creatinine clearance at 30 days after transplantation, presence and duration of delayed graft function (DGF) and patient and graft survival. DGF was defined as need for dialysis within 7 days post-transplantation. Statistical analysis was performed using SPSS 16.0 statistical analysis software (SPSS Inc, Chicago, Ill). Wilcoxon signed ranks test was used for paired non-parametric data, the Mann-Whitney test for unrelated non-parametric data, i.e. comparison of different donor types. Graph points represent the median and error bars represent the interquartile range. A p-value of less than 0.05 was considered significant.

Results
Donor and transplant characteristics
Recipient and donor age and gender were similar in living donor, brain dead donor and cardiac dead donor groups (Table 1). As expected, warm and cold ischemia times differed between the groups, with shorter cold ischemia times in living donor kidney transplantation. The immunosuppressive regimen did not differ between groups. A significantly higher rate of delayed graft function (DGF) was observed in brain dead donor and cardiac dead donor as compared to living donor kidney transplantation. Brain dead donor and cardiac dead donor transplantation were equal in occurrence, but not in duration of DGF. All kidneys were still functioning at 1 year post-transplantation, except for one kidney from a cardiac dead donor (the recipient was not compliant with immunosuppressive medication).

Early release of soluble complement complex C5b-9 from the kidney into the circulation
Activation of the terminal complement cascade during reperfusion was assessed by measuring the release of soluble (s)C5b-9 complex from the kidney by arteriovenous measurements (Figure 1). Immediately at reperfusion there was an acute but transient release of sC5b-9 from deceased donor kidneys, which was not observed from living donor grafts (LD p=0.46, BDD p=0.011, CDD p=0.028; Figure 2). There was no release of sC5b-9 at later timepoints, at 3 (LD p=0.31, BDD p=0.77, CDD p=0.06), 10 (LD p=0.48, BDD p=0.68, CDD p=0.08) or 30 minutes (LD p=0.12, BDD p=0.78, CDD p=0.74) after reperfusion. Soluble C5a, as an alternative sign of complement activation, was measured in arteriovenous samples in living
donor kidneys. In accordance with sC5b-9 measurements, there was no difference in arterial and renal venous C5a levels (p=1.00 at 5 minutes, p=0.29 at 30 minutes after reperfusion, data not shown). Differences in the net release of sC5b-9 from the kidney for the total of 30 minutes were assessed by comparing arterial and venous area under the curve. For living (p=0.87) and brain dead donor grafts (p=0.26) no net release was observed from the kidney. Cardiac dead donor kidneys, however, showed a significant release of C5b-9 from the kidney for the total first half hour after reperfusion (p=0.018). Baseline values of sC5b-9 were not different between groups (p=NS).

Figure 2. sC5b-9 concentration in arterial and venous blood samples in living donor (LD), brain dead donor (BDD) and cardiac dead donor (CDD) kidney transplantations in the first 30 minutes of reperfusion. There was a significant release of sC5b-9 within seconds after reperfusion (first time point) from BDD kidney grafts (n=9, p=0.011) and from CDD grafts (n=7, p=0.028), but not from LD grafts (n=8, p=0.46). At later time points there was no significant difference anymore. Graphs show median and interquartile range.

No increase in local, tissue-bound complement complex C5b-9 after reperfusion
The acute but transient release of soluble C5b-9 into the circulation might be accompanied by local C5b-9 deposition in the kidney, and local deposition could contribute to the absence of circulating C5b-9. Therefore, presence and localization of C5b-9 in pre- and post-reperfusion kidney biopsies was assessed. Whereas the renal biopsy of acute rejection tissue showed extensive C5b-9 positivity, staining for C5b-9 revealed no vascular or tubular depositions of C5b-9 before or after reperfusion in any of the three donor types (Figure 3).
Figure 3. Representative sections showing distribution of C5b-9 staining in a pre- and post-transplantation biopsies of living donor (LD), brain dead donor (BDD) and cardiac dead donor (CDD) kidneys. There were no C5b-9 depositions in any of the biopsies (LD n=13, BDD n=10, CDD n=10). In contrast, the positive control biopsy of a kidney graft with acute rejection showed massive peritubular and tubular C5b-9 depositions. Original magnification, x200.

Discussion

I/R injury is one of the main causes of delayed graft function in transplantation. Studies in mice have suggested a predominant role for complement activation in renal I/R injury. However, in our recent study we show that not complement activation, but rather direct cytotoxic effects of circulation derived Mannan-binding lectin initiate tissue injury in rat renal I/R experiments. Studies on timing and role of complement activation in human renal I/R injury are scarce and inconclusive. Therefore, we set out to assess the role of terminal complement activation in the initiation of renal I/R injury in humans. Our data show that there is acute, non-sustained terminal complement activation upon reperfusion in deceased donor kidney transplantation.

We concentrated on measurement of sC5b-9 as it is the common end-point of both the classical, alternative and Mannan-binding lectin pathway of the complement cascade. Moreover, in mice it is suggested that specifically C5b-9 is essential in the induction of tubular damage in renal I/R injury. By measuring arteriovenous differences over the reperfused organ, we were able to obtain very specific data on local venous release of sC5b-9 from the human kidney. In a previous study involving living donor kidney transplantations only, we found no release of C5b-9, but rather an early and vast release of interleukin-6 from the kidney. In the current study the group is expanded with kidneys from brain dead and cardiac dead donors which are more severely affected by I/R. We show that from these deceased donor kidney grafts sC5b-9 is indeed released directly after reperfusion, indicative
Chapter 5.1

of intravascular terminal complement activation. Since C5b-9 is released transiently, directly after reperfusion, this may result from a wash out effect. The complement system may be triggered upon encounter with intravascular cellular debris accumulated during the cold ischemic period or by encounter with hypoxic or injured endothelium. Studies in other human organs, such as the heart confirm complement activation in I/R injury, although these observations may be influenced by complement activating effects of the cardiopulmonary bypass machine.

Besides the intravascular sC5b-9 formation, C5b-9 could be formed locally in the tissue without any release into the circulation. To assess this tubular activation, tissue content and distribution of C5b-9 was assessed in kidney biopsies collected before and after reperfusion. There was no deposition of C5b-9 in the kidney after reperfusion in both living and deceased donor kidney transplantation. This is confirmed by a study of Haas et al. where in post transplantation biopsies no complement depositions as consequence of reperfusion were detected either. In contrast, renal tissue of a patient with acute graft rejection showed extensive C5b-9 deposition in the tubular compartment.

Finally, the possibility remained that the complement cascade is activated in living donor kidneys as well, without leading to terminal complement activation. Therefore, release of C5a from the reperfused kidney was assessed because C5a is more upstream in the complement cascade than the terminal complex C5b-9 is. In agreement with C5b-9 measurements, there was no C5a release from living donor kidneys. This excludes complement activation after reperfusion in living donor kidneys and also excludes early involvement of C5a, which has also been ascribed a harmful role in I/R injury.

A limitation of our study was the fact that the sampling time was restricted to maximally 30 minutes following reperfusion. Although complement activation in this study was only observed instantly after reperfusion, mouse experiments show membrane attack complex elements C6 and C9 later on, at 12 and 24 hours after reperfusion, respectively. Furthermore, one may consider the sample size as a limitation. However, as the goal of this study was to assess the basic pathophysiological role of complement activation in I/R injury, instead of correlating findings to clinical outcomes, small patient numbers were sufficient. Finally, only cortical biopsies could be collected, as deeper puncture holds a high risk of bleeding and calycal injury and was considered unsafe.

In summary, this systematic study in human kidney transplantation shows acute, non-sustained intravascular terminal complement activation during early reperfusion of deceased donor kidney grafts, likely to be initiated by contact with intravascular cellular debris and injured
or hypoxic endothelium. These results indicate that terminal complement activation may play a role in early renal I/R injury in deceased donor kidney grafts.

**Acknowledgements**

We thank The Netherlands Organization for Health Research and Development project 92003525 (D.K. de Vries) and the Dutch Kidney Foundation project NSN C03-6014 (P. van der Pol) for the financial support. We thank Kim Zuidwijk, Nicole Schlagwein and Karin Koekkoek for technical assistance. Marc Seelen and Henri Leuvenink are thanked for providing patient material.
References


Complement activation in renal I/R injury

5.1


5.2

Pitfalls in urinary complement measurements


Transpl Immunol 2012;27:55-8
Abstract

Local activation of the complement system has been associated with ischemia/reperfusion injury following kidney transplantation and tubular injury under proteinuric conditions. The soluble terminal complement complex sC5b-9 is a stable end-product of the complement cascade, and as such a promising urinary biomarker. In the early post-transplant period we found high urinary levels of sC5b-9, significantly correlating with the degree of proteinuria, suggesting activation of filtered complement components at the tubular epithelial surface of the kidney. However, when mimicking proteinuria in vitro by exposing serum (or blood) to urine (both negative for sC5b-9), we found extensive generation of sC5b-9 in urine. This process was inhibited by EDTA, confirming activation of the complement system. In conclusion, although sC5b-9 is an attractive urinary biomarker, one should be aware of the risk of extra-renal complement activation independent of a renal contribution. This may be of special interest when measuring urinary sC5b-9 following kidney transplantation in which procedure-related (microscopic) hematuria and proteinuria are common.
Introduction

Complement activation at the tubular epithelial surface of the kidney, which lacks several important complement regulators (CD46, CD55), is considered to be a mediator of tubular injury in the proteinuric condition. In proteinuria, complement proteins, which normally are retained in circulation, are able to pass the glomerular filter barrier, end up in the tubular lumen and are activated by the unprotected epithelial surface of the tubuli. To this end, the detection of soluble (s)C5b-9 in urine is widely considered as a clinical indicator of tubular complement activation.

The complement system, a set of circulating proteins of the innate immune system that forms a biochemical cascade, is activated by the binding of complement recognition molecules (e.g. C1q, MBL or properdin) to their respective target (e.g. pathogens or apoptotic cells). Three activation pathways have been recognized, namely the classical, lectin and alternative pathway, which all converge at the level of complement component C3. Subsequent downstream activation of the complement cascade leads to formation of the lytic terminal complement complex C5b-9, which is able to damage and lyse target cells. Recently, it was shown that complement activation and deposition of C5b-9 on tubular epithelial cells is mediated by binding of properdin, the initiator of the alternative pathway of complement.

Increased glomerular permeability to large plasma proteins (proteinuria) is common in the early period following renal transplantation, with a prevalence of 15% to 30% at 1 year post-transplantation. Activation of filtered or locally produced complement components is likely to be involved in tubulotoxicity of proteinuria. Complement activation products indeed are detectable in the urine of patients with different proteinuric renal diseases and are believed to be one of the possible candidates mediates tubular injury in the proteinuric condition. However, in the days after transplantation, not only glomerular damage may be responsible for proteinuria, but also a procedure-related (microscopic) hematuria.

In the present study we confirm the relation of urinary sC5b-9 and proteinuria in a renal transplantation cohort and we investigated the possibility whether in proteinuric urine sC5b-9 can be generated independent of a renal contribution.

Material and methods

Patient population
Twenty-four patients undergoing a renal allograft transplantation receiving a kidney from a deceased cardiac death donors in the period between August 2005 and September 2006
were recruited (Table 1). The study protocol was approved by the local ethics committee, and informed consent was obtained from each patient.

### Table 1: Donor, recipient and graft characteristics

<table>
<thead>
<tr>
<th></th>
<th>All (n=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor age (years)</td>
<td>47 ± 16</td>
</tr>
<tr>
<td>Donor gender (% female)</td>
<td>58 %</td>
</tr>
<tr>
<td>Recipient age (years)</td>
<td>52 ± 14</td>
</tr>
<tr>
<td>Diuresis preTx (number of patients)</td>
<td>17</td>
</tr>
<tr>
<td>Cold ischemia time (hours)</td>
<td>17,5 ± 5,0</td>
</tr>
<tr>
<td>Warm ischemia time 1 (min)</td>
<td>20,3 ± 6,6</td>
</tr>
<tr>
<td>Warm ischemia time 2 (min)</td>
<td>29,0 ± 7,2</td>
</tr>
<tr>
<td>Delayed graft function (number of patients)*</td>
<td>17</td>
</tr>
<tr>
<td>One-year patient survival (%)</td>
<td>91,7%</td>
</tr>
<tr>
<td>One-year graft survival (%)</td>
<td>88,5%</td>
</tr>
</tbody>
</table>

* DGF was defined as cases in which creatinine level increased, remained unchanged, or decreased by less than 10% per day immediately after transplantation during 3 consecutive days within the first week.

### Operation and materials

Donor kidney transplantations were performed according to the local protocol. From allograft recipients, urine was collected at consecutive days after transplantation. Urine samples were centrifuged at 2500g at 4°Celsius for 10 minutes, aliquotted and stored at −80°C for later complement measurements.

### Urine measurements

Soluble C5b-9 was measured by ELISA using an antibody to a neoeptipe on C5b-9 (AE11). 

Total protein was measured by a colorimetric method.

### Urinary complement activation

Whole blood or serum from 7 healthy volunteers was 1:4 or serially diluted in their corresponding urine or PBS, and incubated for 60 minutes at 37°C (mimicking the hematuric or proteinuric condition, respectively). Additionally, the process of complement activation was prevented by adding 10mM EDTA. To investigate any involvement of remaining renal cells or cellular debris, urine from 4 healthy volunteers was filtered with a 0.2 µm filter (GE Healthcare, Little Chalfont, UK). Subsequently, corresponding sera were 1:4 diluted in the filtered and unfiltered urines and incubated for 60 minutes at 37°C. After incubation, in all samples further complement activation was blocked by adding 10mM EDTA. Samples were then immediately processed for sC5b-9 measurement as described.
Statistics
Correlation analysis between variables was performed by linear regression and the significance of differences was calculated by a Mann-Whitney test using GraphPad Prism software. Differences with P<0.05 were considered significant.

Results
In a cohort of deceased cardiac dead donors (Table 1), high levels of urinary sC5b-9 were detected at day 2 after transplantation, which decreased slowly after 10 and 42 days (Fig 1A). Although urinary output was still variable at day 2, almost all patients showed normal diuresis at day 10. Most patients suffered from proteinuria (Fig 1B), and the degree of proteinuria strongly correlated with the urinary sC5b-9 levels (Fig 1C).

![Figure 1](image)

Figure 1. Urine from recipients of a renal allograft were collected at day 2, 10 and 42 after transplantation and assessed for sC5b-9 (A) and proteinuria (B). Levels of sC5b-9 at day 2, 10 and 42 (C) were correlated to the measured proteinuria.

To assess whether this observed complement activation may be an extra-renal phenomenon, the proteinuric condition was mimicked in vitro. Seven urine samples from healthy volunteers, all free of sC5b-9, were incubated with a small amount of freshly drawn blood from the corresponding individuals, which strikingly resulted in extensive generation of sC5b-9 in urine (Fig 2A). In contrast, levels of sC5b-9 remained low when the same amount of blood was added to PBS instead of urine, indicating that complement can be activated in urine ex vivo, independent of any renal contribution. To assess whether this effect was due to the presence of blood cells (e.g. in hematuric conditions), similar dilutions of human serum in urine were tested. This also resulted in an extensive generation of sC5b-9 (Fig 2B), ruling out any effect of blood cells on the observed complement activation.
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The active process of complement activation in the urine samples *in vitro* was confirmed by the complete abolishment of complement activation when EDTA was added before incubation (Fig 2C). Since EDTA chelates calcium and magnesium needed for complement activation, measured urinary sC5b-9 must be formed by active complement activation *in vitro* in urine.

![Figure 2](image)

**Figure 2.** Blood (A) or serum (B, C) from seven healthy volunteers was 1:4 diluted in the corresponding urines or PBS and incubated for 60 minutes at 37°C followed by assessment of sC5b-9. Additionally, 10mM EDTA was added to the serum/urine to block complement activation (C). **P < 0.01; *** P<0.001.

The urinary protein content in the transplantation patients varied from 0 to 10 mg/ml (Fig 1C). In an additional experiment, this range was approached by serially diluting serum (normal serum protein content is 60-80 mg/ml) 4 to 32 times (Fig 3A). Even when serum was 32 times diluted in urine (reflecting a urinary protein content of 1.8-2.5 mg/ml), significant levels of sC5b-9 could be detected. This indicates that also in less severe proteinuric conditions, sC5b-9 can be generated without any renal contribution.

Urine, even from healthy subjects, often contains viable or apoptotic renal cells and cellular debris, as turnover or injury to epithelial cells lining the urinary tract results in shedding of these cells into urine which potentially could lead to activation of complement proteins present in proteinuric urines. To investigate such an involvement, the urine was filtered with a 0.2 µm filter to remove cells and cellular particles which possibly remained after centrifugation. Next, the corresponding serum was added to the filtered or unfiltered urine to allow sC5b-9 generation (Fig 3B). Removal of remaining cells and cellular debris reduced the sC5b-9 generation in the urine by half, suggesting that sC5b-9 generation in proteinuric urine is partially caused by cellular debris and apoptotic epithelial cells. The remaining complement activation observed after filtration, may be explained by high urinary levels of ammonia or low urinary pH, which have been shown to favor urinary complement activation.
Figure 3. Serum from a healthy volunteer was serially diluted in the corresponding urine and incubated for 60 minutes at 37°C followed by assessment of sC5b-9. Represented data are true sC5b-9 concentrations, uncorrected for dilution. sC5b-9 levels in the diluted serum were compared to undiluted serum. * P < 0.05; ** P < 0.01; *** P<0.001 (A).

Urine from 4 healthy volunteers was filtered with a 0.2 µm filter to remove any remaining cells or cellular debris. Subsequently, corresponding sera were 1:4 diluted in the filtered and unfiltered urine for 60 minutes after which sC5b-9 generation was assessed. ** P < 0.01 (B).

Discussion

After renal transplantation, proteinuria is a common event, with a prevalence of 15% to 30% at 1 year after transplantation. Here, we show that after renal transplantation, urinary sC5b-9 can be detected in the majority of renal allograft recipients and significantly correlates with the degree of proteinuria. This would classically be interpreted as activation of complement by the tubular epithelial surface. However, in this study we show that, independent of a renal contribution, presence of blood or serum constituents in urine form healthy subject could lead to complement activation and generation of sC5b-9 in vitro. This implies that following transplantation, proteinuria and procedure-related (microscopic) hematuria may cause urinary complement activation resulting in high urinary sC5b-9 levels. Centrifugation or filtration following collection of patient urines would not prevent this, since urinary complement activation most probably occurs in the urinary tract.

We would like to conclude that urinary sC5b-9 measurement is troubled by extra-renal or even ex vivo complement activation in case of hematuric or proteinuric conditions, rendering the implications and clinical relevance of measured urinary sC5b-9 rather unpredictable.
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Acknowledgements
This work was supported by the Dutch Kidney Foundation project NSN C03-6014 (P. van der Pol) and the Netherlands Organization for Health Research and Development project 92003525 (D.K. de Vries).
References


Inhibition of platelet activation in human clinical renal ischemia/reperfusion


Submitted for publication
Abstract

Apart from their primary function in haemostasis, platelets serve as modulators of inflammation. Although in preclinical studies activated platelets have been implicated in the inflammatory response after reperfusion, their role in clinical renal ischemia/reperfusion (I/R) injury is unknown. In this study, the role of platelets in living, brain dead and cardiac dead donor kidney transplantation was assessed by our unique method of arteriovenous measurements over the reperfused kidney. Markers of platelet activation and degranulation, i.e. β-thromboglobulin, soluble glycoprotein Ib and platelet derived growth factor did not show an arteriovenous concentration difference over the reperfused kidney. The solitary RANTES release from brain dead donor kidneys presumably reflects leukocytic release. Since no overt platelet activation was observed, more subtle changes in excitability of platelets were measured. Remarkably, platelets in renal venous blood were less easily and less intensely excitable than platelets in arterial blood. In conclusion, results of this study unequivocally deny platelet activation in early reperfusion in both living and deceased donor kidney transplantation. Platelet excitability was even inhibited in the reperfused kidney, suggesting platelets do not initiate the inflammatory response of renal I/R injury.
Introduction

Ischemia/reperfusion (I/R) is an important clinical problem and an inevitable component of organ transplantation. I/R injury is characterized by inflammation, but the mechanism that initiates this inflammatory reaction is unknown. At present no effective therapy is available. A role of platelets in the etiology of I/R injury has been suggested but is not fully sustained or understood. Upon activation, platelets release granules containing growth factors, cytokines, chemokines and leukotrienes into the circulation by exocytosis. As such, platelets serve as inflammatory mediators that can initiate recruitment and activation of leukocytes and thereby significantly aggravate tissue injury.

Increasing experimental evidence suggests that platelet activation orchestrates an inflammatory response upon reperfusion. However, clinical studies on the mechanism and timing of platelet activation in I/R injury are scarce. In myocardial infarction, platelets mediate thrombotic occlusion and increase damage by causing micro vascular occlusions and no-reflow phenomenon. Administration of anti-platelet agents is routine therapy in acute coronary syndrome. Whether platelet activation, apart from its role in infarction and thrombosis, is involved in the initiation of inflammation in clinical I/R injury in humans is not clear yet.

In this study, kidney transplantation was used as model of human I/R injury. Since peripheral blood measurements are influenced by many systemical influences, and factors of interest are diluted in the total intravascular volume, we instead applied selective arteriovenous measurements over the reperfused graft to specifically assess local release of mediators. The role of platelet activation and excitability was systematically investigated using these arteriovenous measurements over the reperfused kidney. We show that there is no platelet activation in both living, brain dead and cardiac dead donor kidney transplantation in the immediate period following reperfusion. In fact, platelet excitability is even repressed in the reperfused kidney, indicating that regulatory mechanisms prevent post-reperfusion platelet activation and subsequent thrombus formation. It is therefore unlikely that platelets are an initiator of the inflammatory response leading to I/R injury.

Patients and Methods

Patient population
Thirty-four patients undergoing renal allograft transplantation were included for arteriovenous sampling. Of these, 8 patients received a kidney from a living donor, 9...
patients received a kidney from a brain dead donor, and 7 patients received a kidney from a cardiac dead donor. Brain dead and cardiac dead donors together are referred to as deceased donors. Ten other patients that received a kidney from a living donor were included for direct arteriovenous measurements of platelet excitability, and biopsy collection for immunohistochemical analysis. Patient characteristics are shown in Table 1. For technical reasons (renal vein sampling), only patients receiving a left kidney were included. The study protocol was approved by the local ethics committee, and informed consent was obtained from each patient.

Table 1: Transplantation and outcome characteristics in living donor (LD), brain dead donor (BDD) and cardiac dead donor (CDD) kidney transplantation.

<table>
<thead>
<tr>
<th></th>
<th>LD PEA, IHC</th>
<th>LD</th>
<th>BDD</th>
<th>CDD</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>8</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>Donor age: mean (SD)</td>
<td>53.1(10.0)</td>
<td>43.9(10.6)</td>
<td>54.1(17.1)</td>
<td>52.7(15.3)</td>
</tr>
<tr>
<td>Donor gender (M:F %)</td>
<td>30:70%</td>
<td>75:25%</td>
<td>44:56%</td>
<td>43:57%</td>
</tr>
<tr>
<td>Preservation fluid</td>
<td>HTK (n=10)</td>
<td>HTK (n=8)</td>
<td>UW (n=9)</td>
<td>UW(n=2)</td>
</tr>
<tr>
<td>WIT1 in min. (SD)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>23.1(7.7)</td>
</tr>
<tr>
<td>CIT in h. (SD)</td>
<td>30.9(8.1)</td>
<td>34.0(6.3)</td>
<td>33.0(6.1)</td>
<td>34.1(6.4)</td>
</tr>
<tr>
<td>Recipient age: mean (SD)</td>
<td>47.8(16.1)</td>
<td>41.1(10.5)</td>
<td>55.1(13.5)</td>
<td>54.0(11.2)</td>
</tr>
<tr>
<td>Recipient gender (M:F %)</td>
<td>70:30%</td>
<td>38:62%</td>
<td>44:56%</td>
<td>71:29%</td>
</tr>
<tr>
<td>Creatinine clearance day 30 (ml/min)</td>
<td>49.8(6.1)</td>
<td>73.3 (20.5)</td>
<td>49.3(15.3)</td>
<td>27.1(10.3)</td>
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<tr>
<td>DGF (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DGF: dialysis after transplantation in days (SD)</td>
<td>0 (0) *</td>
<td>0 (0) *</td>
<td>7.0 (5.3)</td>
<td>17.2 (7.2)</td>
</tr>
</tbody>
</table>

LD procedures that were exclusively sampled for platelet excitability assay (PEA) and biopsy collection (IHC) are presented in a separate column. WIT1: first warm ischemia time, CIT: cold ischemia time, WIT2: second warm ischemia time, DGF: delayed graft function. * P <.05 compared to BDD, ∇ P <.05 compared to CDD.

Operation and materials
Kidney transplantations were performed according to local standardized protocol. In living donors minimally invasive nephrectomy was performed. For cold perfusion and storage of the kidney, Custodiol<sup>®</sup> Histidine-tryptophan-ketoglutarate solution (HTK) solution (Tramedico, Weesp, The Netherlands) was used. Deceased donor kidneys were perfused and stored with University of Wisconsin solution (UW) or HTK (Table 1). The immunosuppressive regimen was based on induction therapy with an interleukin-2 receptor blocker and maintenance treatment with tacrolimus or cyclosporine A, in addition to mycophenolate mofetil and steroids in all groups. During surgery, no heparin or other drugs influencing platelet
aggregation were administered. Donors, not recipients, were routinely administered a daily prophylactic dose of low molecular weight heparin. Arterial and renal venous blood samples were obtained as described before in detail. In short, via a small catheter placed in the renal vein, 10 ml blood aliquots were sampled at 30 seconds, 1, 3, 5, 10, 20 and 30 minutes after reperfusion (i.e., t=0). Paired arterial blood samples were obtained at 0, 3, 5, 10, 20 and 30 minutes after reperfusion (Figure 1). All samples were centrifuged (1,550 g, 20 min, 4°C) and the derived plasma was subsequently centrifuged to deplete it from leukocytes and platelets (1,550 g, 20 min, 4°C). Plasma was aliquotted and stored at -70°C until assayed. A renal cortical biopsy was obtained after cold storage, and a second biopsy of the same kidney was collected 45 minutes after reperfusion.

Figure 1. Schematic representation of the arteriovenous sampling method over the reperfused kidney by simultaneous blood collection from the renal artery and vein. Illustration by Manon Zuurmond© (www.manonproject.com).

**Plasma measurements**

Platelet activation was assessed by measuring the local renal release of platelet granule contents into the circulation. Platelets contain three main types of secretory granules: the α-granules (the most prominent population in size and number), the dense granules and the lysosomal granules. Beta-thromboglobulin (β-TG) is abundant in α-granules and is a specific marker for platelet activation. Levels of β-TG and soluble GPIb (sGPIb) were determined in plasma using semi-automated ELISA on a TECAN Freedom EVO 150. Each antigen was measured on a separate Nunc maxisorb 384 well ELISA plate (Thermofischer Scientific, Roskilde, Denmark). The capture antibodies, MAB393 (1 µg/mL) and mouse anti-GP1b (clone 6.30; 0.9 µg/mL) were coated on two different plates for 2 hours. Unbound antibodies were
removed by washing five times using phosphate-buffered saline (PBS)/0.5% Tween. Plasma samples were diluted 1/80 for NAP-2 and 1/25 for sGPIb measurements and added to the plate with the corresponding capture antibody. Each plate contained four calibration curves consisting of standard serum sample with known β-TG and sGPIb concentrations. Dilutions were made in PBS/1% BSA and incubated for 2 hours on the capture antibodies. Unbound antigens were removed by washing five times with PBS/0.5% Tween. The detection antibodies BAF393 (50 ng/mL) and biotinylated M1852 (0.25 µg/mL, Sanquin Amsterdam) were added to the corresponding plates. Unbound detection antibody was removed by three wash steps with PBS/0.5% Tween, after which streptavidin/horseradish peroxidase (HRP) was added for 2 hours to bind the biotin on the detection antibody. Bound antigen was quantified using Supersignal West Pico Chemiluminescent Substrate (#34080, Thermoscientific, Rockford USA) on a Spectramax Luminescence Microplate Reader (Molecular Devices, Sunnyvale, USA). The chemokine ‘Regulated on Activation, Normal T Cell Expressed and Secreted’ (RANTES) and platelet-derived growth factor (PDGF)-BB can be released from platelet α-granules upon activation. Both RANTES and PDGF-BB were measured in a custom made multiplex assay in accordance with the manufacturer’s instructions (X-plex, Biorad, Veenendaal, the Netherlands).

Platelet excitability assay (PEA)

In the absence of platelet activation, more subtle changes in excitability of platelets were explored, using a renewed, bedside applicable assay for platelet excitability. For this purpose, arterial and renal venous samples from another 10 patients undergoing living donor kidney transplantation were collected in citrate coated tubes at 5 and 30 minutes after reperfusion. Whole blood was added to tubes containing either platelet agonist adenosine diphosphate (ADP), collagen-related peptide (C-RP) or thrombin related activatable peptide (TRAP) in eight increasing concentrations. Fluorescent anti-P-selectin and anti-GP1b antibody were co-incubated for subsequent flow cytometry analysis. Final concentrations in the tubes were 0.01, 0.03, 0.12, 0.49, 1.95, 7.81, 31.25, 125.00 μM ADP; 0.2, 0.6, 2.4, 9.8, 39.1, 156.3, 625 and 2500 ng/ml C-RP; and 0.04, 0.15, 0.61, 2.44, 9.77, 39.06, 156.25, 625.00 μM TRAP. After 20 minutes of incubation, the reaction was stopped by adding 0.5% formaldehyde in heparin buffered saline. FACS-Calibur flow cytometer adjusted in a standard configuration (Cytomics FC 500 flow cytometer, Beckman and Coulter, Krefeld, Germany) was used to assess the activation state and excitability of platelets. Samples were analyzed on GP1b positivity (all platelets) and P-selectin positivity (activated platelets). A typical example of the flow cytometry result of a low and high percentage of activated platelets is shown in Figure 2A and 2B. Analysis involved assessment of the maximum percentage of activated platelets (% P-selectin positive) and their intensity of activation (mean fluorescence index; MFI). The percentage activated platelets increased with concentration of the agonist in a sigmoid shaped curve (Figure 2C).
Figure 2. Typical examples of flow cytometry results after stimulation with low (A) and high (B) concentration agonist, i.e. thrombin related activatable peptide (TRAP). The corresponding P-selectin positive percentage of platelets was 0.1% and 96.6%, respectively. The eight results of a stimulation series are depicted in sigmoid shaped curves where arterial □ and renal venous ■ percentages of P-selectin positive platelets can be compared (C).

Platelet staining
Pre-transplant and reperfusion (45 min. post reperfusion) cortical biopsies were collected from living donor kidneys. Biopsies were snap frozen and cryostat sections (5 µm) were fixed in acetone for 10 min. Endogenous peroxidase activity was blocked with 0.1% H$_2$O$_2$. Slides were incubated with primary antibody biotin labeled CD41 antibody (ab30434, Abcam, Cambridge, United Kingdom), followed by ABC complex (Vectastain, Vector laboratories, Peterborough, United Kingdom). The staining was visualized using DAB (DAKO, Glostrup, Denmark). Sections were counterstained with hematoxylin (Merck, Darmstadt, Germany).

Analysis
Clinical donor data were retrieved from Eurotransplant Foundation (Leiden, The Netherlands). Delayed graft function (DGF) was defined as the need for dialysis within one week after transplantation. Statistical analysis was performed using SPSS 16.0 statistical analysis software (SPSS Inc, Chicago, Ill). The area under the curve (AUC) was calculated for the arterial and venous curve of the plasma measurements for the total of 30 minutes. The delta AUC was calculated (venous minus arterial) and the null-hypothesis (delta AUC is 0) was tested by t-test or non-parametric test in case of a non-normal distribution. Wilcoxon test was applied to test for differences between arterial and venous outcome per agonist and per time-point in the analysis of the PEA. Graph error bars represent the standard error of the mean (SEM). A $P$-value of less than 0.05 was considered significant.
Chapter 6

Results

Donor and transplant characteristics
Recipient and donor age and gender were similar in all three donor groups (Table 1). As expected, the warm and cold ischemia duration differed between the groups, with a shorter duration of cold ischemia in living donor kidney transplantation. The immunosuppressive regimen was similar in the three groups. A significantly higher rate of DGF was observed in brain dead and cardiac dead donor groups as compared to living donor kidney transplantation. Although incidence of DGF was similar in brain dead donor and cardiac dead donor kidney transplantations, DGF was more prolonged in the cardiac dead donor group. With the exception of one kidney from a cardiac dead donor in which the recipient was not compliant with immunosuppressive medication, all kidneys were still functioning at one year after transplantation. There were no complications because of local or deep venous thrombosis or any (re-)bleeding problems.

No platelet activation in the reperfused kidney
Platelet activation leading to degranulation was assessed by comparing plasma concentrations of granule contents, i.e. RANTES, PDGF-BB, β-TG and GP1b between arterial and renal venous samples, i.e. net change over the kidney. RANTES was not released from the kidneys of living or cardiac dead donors (P=0.11, P=0.34 respectively). Kidneys of brain dead donors however, showed a significant RANTES release during the first 30 minutes of reperfusion (P=0.03, Figure 3). PDGF-BB was not released from living, brain dead, or cardiac dead donor kidneys (P=0.28, P=0.51, P=0.14, respectively). Similarly, β-TG and GP1b were not released from the kidney in any of the three donor groups (P=0.68, 0.72, 0.17 for β-TG, and P=0.08, 0.64, 0.51 for GP1b in LD, BDD and CDD respectively).

Inhibition of platelet excitability in the reperfused kidney
Although direct arteriovenous measurements over the kidney did not indicate platelet activation and degranulation in the reperfused kidney, more subtle changes in platelet excitability may be induced when platelets pass through the graft. Maximum platelet excitability was measured by an established platelet activation assay, now optimized for bedside use. Before stimulating the samples, there was no arteriovenous difference in percentage of spontaneously P-selectin positive platelets at 5 minutes (arterial 0.24%, venous 0.22%; P=0.36) or 30 minutes after reperfusion (arterial 0.25%, venous 0.23%; P=0.21). After in vitro stimulation of arterial or renal venous blood with platelet activation agonists ADP, C-RP and TRAP, the maximum total percentage of activated platelets and the mean fluorescence index (MFI) of activated platelets were measured. There was a significant decrease in maximum percentage activated platelets over the kidney for both
C-RP (P=0.01) and TRAP (P=0.005) at 5 minutes after reperfusion. At 30 minutes after reperfusion this venous decrease in activation was not significant anymore (Figure 4). The MFI of activated platelets was significantly lower in renal venous blood compared to arterial blood at 5 minutes after reperfusion for both C-RP (P=0.047) and TRAP (P=0.009). After 30 minutes, only ADP activated platelets showed a significant decrease in MFI (P=0.013, Figure 5). Overall results of the platelet excitability assay indicate that platelets are less easily and less intensely excitable after they passed the reperfused kidney.

**Figure 3.** Arterial □ and venous ■ concentrations of (A) RANTES, (B) PDGF, (C) β-TG and (D) GP1b are shown for the first 30 minutes of reperfusion of brain dead donor kidney grafts. RANTES was significantly released (P=0.03), however other platelet degranulation markers were not released from the kidney. Graph error bars represent the standard error of the mean (SEM).
Figure 4. Maximum percentage activated platelets after stimulation with agonists adenosine diphosphate (ADP), collagen-related peptide (C-RP) or thrombin related activatable peptide (TRAP). Five minutes after reperfusion there was a significant decrease in maximum percentage activated platelets over the kidney for both C-RP (P=0.013) and TRAP (P=0.005). After 30 minutes of reperfusion changes were not statistically different anymore. Graph error bars represent the standard error of the mean (SEM).

Figure 5. Maximum intensity of activated platelets after stimulation with agonists adenosine diphosphate (ADP), collagen-related peptide (C-RP) or thrombin related activatable peptide (TRAP). Five minutes after reperfusion there was a significant decrease in maximum intensity over the kidney for both C-RP (P=0.047) and TRAP (P=0.009). After 30 minutes of reperfusion only ADP activated platelets showed a significant decrease in intensity (P=0.013). Graph error bars represent the standard error of the mean (SEM).

No platelet retention in the kidney
The difference between arterial and venous platelet excitability could be explained by retention of a subpopulation of (activated) platelets in the kidney. To evaluate platelet retention, arterial and renal venous platelet concentrations were measured 5 and 30 minutes after reperfusion. There was no difference between arterial and renal venous concentration of platelets at 5 minutes after reperfusion (mean arterial 148.6 x 10^9/l, renal venous 145.1 x 10^9/l, P=0.08) and at 30 minutes after reperfusion (mean arterial 147.6 x 10^9/l, renal venous 145.2 x 10^9/l, P=0.29), and therefore retention of platelets in the kidney is unlikely. To
substantiate the lack of platelet infiltration in the reperfused kidney, immunohistochemical analysis of renal biopsies collected before and 45 minutes after reperfusion was executed as well. CD41 staining showed occasional clusters of platelets, mostly in glomeruli (Figure 6). Pre- and post-reperfusion patterns were highly similar, and thereby substantial platelet retention after reperfusion could be excluded.

![Figure 6](image.png)

**Discussion**

Platelets have been suggested to be involved in experimental I/R injury. However, their potential role in human I/R injury and the opportunities of anti-platelet agents in clinical I/R injury are unclear. In this study, we show there is no platelet activation in early I/R injury in human kidney transplantation. Moreover, platelet activation is even repressed in renal venous blood, suggesting a platelet inhibitory effect of the reperfused kidney.

It is well-established that platelets, aside from their thrombotic role, are contributors to the inflammatory response through release of cytokines, chemokines and growth factors from their granules.15 Platelets have been suggested to be involved in the inflammatory response of I/R injury in various organs. In mouse gut, platelets roll and adhere to post-reperfusion endothelium in a P-selectin dependent mechanism.16 Furthermore, I/R injury of the liver results in platelet adhesion to the sinusoidal endothelium.17-21 The first activated platelets are present in mouse myocardial tissue within two minutes after reperfusion,22 and then accumulate in the infarcted myocardium. In the mouse renal vascular bed, platelet accumulation has been shown early after reperfusion as well.24 However, the role of platelet activation in the initiation of human clinical I/R injury in general is unclear. In kidney biopsies potential infiltration of P-selectin positive platelets after reperfusion was not consistent.25 The many intervention studies using anti-platelet agents in myocardial...
infarction show beneficial effects, however it is difficult to discern the effects of diminished (microvascular) thrombosis from potential anti-inflammatory effects.

In this study, platelet activation upon reperfusion was examined in living and deceased donor kidney transplantation. Since deceased donor kidneys have more clinical organ damage, platelet activation was expected to be more pronounced in these kidneys. In the arteriovenous measurements, renal release of various platelet degranulation products into the circulation was assessed. Among all of the measured factors, only RANTES was found to be minimally released from the reperfused kidney of brain dead donors. Since none of the other platelet degranulation markers were released from the reperfused kidney, it is more likely that RANTES is released from another source. Increased T-lymphocyte and macrophage content is found in kidneys from brain dead donor grafts already before transplantation, and both cell types can be a source of RANTES. Moreover, RANTES is constitutively released during the first 30 minutes of reperfusion, while a peak after reperfusion could be expected should the source of this RANTES be degranulating platelets. Since none of the other platelet activation markers were released in any of the groups, platelet granule release upon reperfusion is probably minimal and as such there is no evidence for platelet activation upon reperfusion.

Although platelet activation leading to degranulation was not observed in the reperfused kidney, more delicate changes in the excitability of platelets could potentially be sufficient to change platelet homeostasis. We used a novel and sensitive bedside test to determine platelet excitability. Results showed that platelet excitability decreased when blood passed through the reperfused graft. This inhibitory effect on platelets passing through the reperfused kidney may be explained very well by an endothelial regulatory release of short-acting inhibitors of platelet activation, such as nitric oxide and prostacyclin. Release of these factors unfortunately is difficult to confirm due to their short half-life. The difference is probably not mediated by adherence of platelets in the kidney, since platelet concentration was equal in arterial and venous blood, and immunohistochemical analysis did not reveal renal platelet deposits after reperfusion. Moreover, one would then expect a measurable release of platelet activation markers as well.

A limitation of our study was the fact that the sampling time was restricted to a maximum of 30 minutes following reperfusion. Although platelet activation is considered to be a short-term process, thought to occur during the first contact between recipient blood and the graft, we cannot exclude that platelet activation commences after this time period. Hence, apart from their potential role at later time points, platelets do not appear to be involved in the primary initiation of I/R injury. One may consider the sample size a limitation, but as
the goal of this study was to assess the basic pathophysiological role of platelet activation in I/R injury, instead of correlating findings to clinical outcomes, small patient numbers were sufficient.

In conclusion, we unequivocally show absence of platelet activation in early reperfusion injury in both living and deceased donor kidney transplantation. In fact, platelets display inhibited excitability upon passing the reperfused kidney. This indicates that platelets do not initiate the inflammatory response of I/R injury and other factors are more important in regulating the immune response.

Acknowledgements

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References


Renal ischemia/reperfusion induces release of angiopoietin-2 from human grafts of living and deceased donors


Abstract

Background: Recent insights suggest that endothelial cell (EC) activation plays a major role in renal ischemia/reperfusion (I/R) injury. Interactions between ECs and pericytes via signaling molecules, including angiopoietins, are involved in maintenance of the vascular integrity. Experimental data have shown that enhancement of Angiopoietin (Ang)-1 signaling might be beneficial in renal I/R injury. However, little is known about the role of angiopoietins in human renal I/R injury.

Methods: In this study, EC activation and changes in angiopoietins are assessed in human living and deceased donor kidney transplantation. Local release of angiopoietins was measured by unique, dynamic arteriovenous measurements over the reperfused kidney.

Results: Renal I/R is associated with acute EC activation shown by a vast Ang-2 release from both living and deceased donors shortly after reperfusion. Its counterpart Ang-1 was not released. Histological analysis of kidney biopsies showed EC loss after reperfusion. Baseline protein and mRNA Ang-1 expression was significantly reduced in deceased compared to living donors and declined further after reperfusion.

Conclusions: Human renal I/R injury induces EC activation after reperfusion reflected by Ang-2 release from the kidney. Interventions aimed at maintenance of vascular integrity by modulating angiopoietin signaling may be promising in human clinical kidney transplantation.
Introduction

Ischemia/reperfusion (I/R) injury is an inevitable consequence of organ transplantation and a major determinant of patient and graft survival. The pathophysiology of renal I/R injury is complex and incompletely understood. Although the role of tubular cell injury in post-transplantation graft dysfunction is widely acknowledged, microvascular endothelial cell (EC) damage is considered increasingly important. ECs line the lumen of all blood vessels within the kidney graft and in this unique position they form the interface between the recipient blood and the allograft tissue. ECs are very susceptible to damage, including I/R injury. The repetitive insults during transplantation may induce loss of the microvasculature, on the long term resulting in impaired delivery of oxygen and nutrients to renal tubular epithelial cells, chronic ischemia and cell death.

The molecular mechanisms that lead to microvascular graft injury are largely unknown. Endothelial homeostasis is regulated by the angiopoietin system and pericytes. Pericytes are the basic supportive cells of the endothelium, connected to the vessel’s basement membrane. Angiopoietin-1 (Ang-1) and -2 (Ang-2) are both ligands for the TIE2 receptor, but have opposite effects. Ang-1 is produced by pericytes and is responsible for suppressing vascular leakage, maintaining EC survival and inhibiting vascular inflammation. Ang-2 acts as an antagonist of Ang-1 and thereby destabilizes quiescent endothelium. Ang-2 promotes pericyte loss, leading to loosening contacts between ECs and pericytes, subsequent vessel destabilization and abnormal microvascular remodeling. ECs store Ang-2 in Weibel–Palade bodies from where it can be released quickly following stimulation, and Ang-2 expression can be upregulated manifold following endothelial activation. A few experimental studies have focused on the functional role of angiopoietins in endothelial damage in the kidney. The relation between a disbalance in angiopoietins and EC loss was demonstrated in a mouse model of anti-glomerular basement membrane glomerulonephritis, where glomerular capillary loss was associated with reduced Ang-1 and increased Ang-2 expression. Similarly, Ang-1 expression decreased after renal I/R injury in mice. Moreover, Ang-1 overexpression even significantly improved renal function and renal tissue blood flow after renal I/R in mice and decreases inflammatory cells and renal interstitial fibrosis.

Although these experimental data suggest a functional role of angiopoietins in renal I/R injury, their involvement in human renal I/R injury had not been investigated yet. In addition, their involvement in living and deceased donor kidney transplantation had not been compared. In our recent studies on renal I/R, we exploited selective arteriovenous measurements over the kidney as a reliable method to study inflammatory processes after reperfusion. In this study, local renal angiopoietin expression and release during renal I/R is systematically assessed in human living and deceased donor kidney transplantation.
Chapter 7

Materials and methods

Patient population
Eighteen patients undergoing renal allograft transplantation were included for arteriovenous sampling, 6 living donor (LD), 6 brain dead donor (BDD), and 6 cardiac dead donor (CDD) kidney recipients. BDD and CDD together are referred to as deceased donors (DD). Twenty-three other patients receiving a kidney from a LD (n=10), a BDD (n=7) or a CDD (n=6) were included for mRNA isolation from renal biopsies. Immunohistochemical analyses were performed on biopsies of 10 LD, BDD and CDD kidney graft recipients. Patient characteristics are shown in Table 1. All kidneys were still functioning at 1 year after transplantation, except for one patient in the arteriovenous sampling cohort who received a kidney from a CDD (the recipient was not compliant with immunosuppressive medication) and one patient in the PCR cohort, that received a BDD kidney transplant and died 6 months after transplantation (because of veno-occlusive disease ultimately causing hepatic failure). For technical reasons (renal vein sampling) only patients receiving a left kidney were included in the arteriovenous group. The reason for including different cohorts was to minimize patient burden by collecting one type of material per patient, i.e. either blood or a single renal biopsy. The study protocol was approved by the local ethics committee, and informed consent was obtained from each patient.

Operation and materials
Kidney transplantations were performed according to local standardized protocol. In LD minimally invasive nephrectomy was performed. For cold perfusion and storage of the kidney, Custodiol® Histidine-tryptophan-ketoglutarate solution (HTK) solution (Tramedico, Weesp, The Netherlands) was used. DD kidneys were perfused and stored with University of Wisconsin solution (UW) or HTK. The immunosuppressive regimen was based on induction therapy with an interleukin-2 receptor blocker (basiliximab, day 0 and day 4) and maintenance treatment with tacrolimus or cyclosporine A, in addition to mycophenolate mofetil and steroids in all groups.

Arterial and renal venous blood samples were obtained as described before in detail 21. In short, via a small catheter placed in the renal vein, blood aliquots were sampled at 3, and 30 minutes after reperfusion. Paired arterial blood samples were obtained simultaneously. All samples were collected in tubes containing EDTA and immediately placed on ice. Blood samples were centrifuged (1,550 g, 20 min, 4°C) and the derived plasma was subsequently centrifuged to deplete it from leukocytes and platelets (1,550 g, 20 min, 4°C). Plasma was aliquotted and stored at -70°C until assayed.
Table 1: Transplantation and outcome characteristics of the three different patient cohorts that were included. In each cohort material was collected from living donor (LD), brain dead donor (BDD) and cardiac dead donor (CDD) grafts during kidney transplantation.

<table>
<thead>
<tr>
<th>N</th>
<th>LD</th>
<th>BDD</th>
<th>CDD</th>
<th>LD</th>
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<th>CDD</th>
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<tr>
<td>Donor age: mean (SD)</td>
<td>42(11)</td>
<td>51(21)</td>
<td>52(16)</td>
<td>57(10)</td>
<td>48(16)</td>
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<td>N/A</td>
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<td>N/A</td>
<td>18(5)</td>
<td>N/A</td>
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<td>0/10</td>
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<td>7/10</td>
<td>0/10</td>
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<td>DGF duration in days (SD)</td>
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<td>Acute rejection n (%)</td>
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<td>2(20%)</td>
<td>1(10%)</td>
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<td>0(0%)</td>
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AV: cohort of patients included for arteriovenous sampling over the kidney.
IHC: cohort of patients in which renal biopsies were used for immunohistochemical analysis. PCR: cohort of patients from which renal biopsies were used for PCR analysis.
WIT1: first warm ischemia time.
CIT: cold ischemia time.
WIT2: second warm ischemia time.
DGF: delayed graft function.
DGF duration: dialysis after transplantation in days (SD).
CrCl: Creatinine clearance.

Immunohistochemical analysis
A renal cortical biopsy was obtained after cold storage from LD, BDD and CDD kidney graft recipients. For logistical reasons, only in the LD group a second biopsy 45 minutes after reperfusion was collected as well. Immunohistochemical analyses were performed on 4 µm cryostat kidney tissue sections fixed in acetone. Slides were washed and incubated with peroxidase blocking solution and blocked with PBS containing 5% normal human serum (NHS) and 1% BSA. Then slides were incubated with the following primary antibodies: Ang-1 mouse monoclonal IgG, 1:800 (R&D systems), Ang-2 mouse monoclonal IgG, 1:100 (Novus Biologicals), ECs (CD34) mouse polyclonal IgG, 1:3200 (Dako), vWf, rabbit polyclonal, 1:2500 (Dako). Next, the following peroxidase-conjugated secondary antibodies were applied: goat anti-rabbit IgG, 1:200 (Dako); goat anti-mouse IgG, 1:200 (Dako); or rabbit anti-goat IgG, 1:200 (Dako), followed by sequential fluorescein isothiocyanate (FITC, amplification reagent) and anti-fluorescein-HRP. Staining was completed by incubation with 3,3’ diaminobenzidine tetrahydrochloride (DAB)/ hydrogen peroxide and counterstained with Mayer’s Hematoxylin Solution (Merck, Darmstadt, Germany). At 100 × magnification, 10 microscopic fields of each kidney section were quantified using computerized image analysis (ImageJ).
Plasma measurements of endothelial cell activation

Activation of the endothelium was assessed by measuring the local release of endothelial activation markers from the kidney. Release of Ang-1 and Ang-2 from the kidney was assessed by ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s protocol. The paired arteriovenous samples were also analyzed on more classical markers of endothelial activation; von Willebrand factor (vWF) and vWF propeptide. The propeptide has a shorter circulating half-time (2–3 h), compared with vWF itself (>12 h), and may therefore be a more sensitive marker of acute endothelial activation. Plasma levels of vWF and vWF propeptide were determined using a semi-automated ELISA. Plates were coated overnight with coating buffer, consisting of sodium carbonate, sodium bicarbonate and NaN3. Commercial antibody duo sets, optimized for ELISA, were used (rabbit anti-human vWF and peroxidise-conjugated rabbit anti-human vWF, A00082 and P0226, Dako, Glostrup, Denmark). vWF propeptide, rabbit-anti-propeptide, and rabbit-anti-propeptide-biotine were used for the analysis of vWF propeptide as described previously.25

mRNA expression in human kidney biopsies

A renal cortical biopsy was obtained after cold storage from LD, BDD and CDD kidneys, and a second biopsy was collected 45 minutes after reperfusion from LD kidneys. Biopsies were immediately snap frozen in liquid nitrogen and stored at -70°C. Total RNA was extracted from renal tissues using RNAzol (Campro Scientific, Veenendaal, The Netherlands) and glass beads.26 The integrity of each RNA sample was examined by Agilent Lab-on-a-chip technology using the RNA 6000 Nano LabChip kit and a bioanalyzer 2100 (Agilent Technologies, Amstelveen, The Netherlands). RNA preparations were considered suitable for further processing if samples showed intact 18S and 28S rRNA bands, and displayed no chromosomal peaks or RNA degradation products (RNA Integrity Number >8.0).27 cDNA was synthesized from 1 µg total RNA, using an oligo dT primer, RNase-OUT, M-MLV reverse transcriptase, 0.1 M-DTT and buffers in a volume of 20 µL (all purchased from Invitrogen, Breda, The Netherlands). Quantitative real-time PCR was performed in duplicate by using iQ SYBR Green Supermix on iCycler Real-Time Detection System (BioRad). The amplification reaction volume was 20 µL, consisting of 10 µL iQ SYBR Green PCR mastermix, 1 µL primers, 1 µL cDNA, and 8 µL water. Data were analyzed using Gene Expression Analysis for iCycler Real-Time PCR Detection System (BioRad). Expression of each gene was normalized against mRNA expression of the housekeeping gene GAPDH. The primer sequences are shown in Table 2.
Ang-2 release and endothelial injury

Table 2: Primer sequences used for quantitative real-time polymerase chain reaction.

<table>
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<tr>
<th>Gene</th>
<th>Forward primer 5’-&gt;3’</th>
<th>Reverse primer 5’-&gt;3’</th>
<th>Supplier</th>
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<td>Ang-1</td>
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<td>CATCTGCACAGTCTCATAATGGT</td>
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<tr>
<td>Ang-2</td>
<td>TGGGATTGTGAAACCCTTCA</td>
<td>GTAAGCTCATTGCCCTTCCC</td>
<td>Biolegio</td>
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<tr>
<td>Tie-2</td>
<td>GTATGGACTCTTTAGCCGCTT</td>
<td>TTCGCCCATTCCTGGTCAC</td>
<td>Biolegio</td>
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</table>

Analysis

Clinical donor data were retrieved from Eurotransplant Foundation (Leiden, The Netherlands). Delayed graft function (DGF) was defined as the need for dialysis within one week after transplantation. Acute rejection was defined as biopsy proven rejection within 60 days after transplantation. Statistical analysis was performed using SPSS 17.0 statistical analysis software (SPSS Inc, Chicago, Ill). Paired, non-parametric test (Wilcoxon) was applied to assess differences between arterial and venous concentration, and between pre- and post-reperfusion samples. Graph error bars represent the standard error of the mean (SEM), unless otherwise stated. A P-value of less than 0.05 was considered significant.

Results

Integrity of the renal microvasculature is disturbed after reperfusion

To assess endothelial structure and viability, staining for ECs was quantified in both living and deceased donor kidney tissue. Both CD34 and vWF staining showed a characteristic positive pattern of peritubular and glomerular ECs. Pre-reperfusion biopsies did not show a difference between living and deceased donor kidneys in either CD34 (p=0.76) or vWF (p=0.07; Figure 1). Next, pre- and post-reperfusion biopsies of LD kidneys were compared. There was a trend towards a decrease in CD34 positive cells after reperfusion (p=0.08, data not shown), whereas vWF staining of CD34 positive cells showed a significant decrease in expression after reperfusion (p<0.001; Figure 1).
Figure 1: *vWf staining decreased after reperfusion*. Staining for vWf showed a characteristic positive endothelial pattern. Typical example of vWf staining in A a pre-reperfusion biopsy, B a post-reperfusion biopsy of a LD kidney. C Quantification of vWf staining showed no differences in intensity between groups before transplantation (p=0.07). D After reperfusion, vWf signal showed a vast decrease (p<0.005) in LD kidneys. The boxes run from the lower to upper quartile, the whiskers indicate the 5 to 95 percentile, while the horizontal line is set at the median.

**Ang-2 is released from the reperfused kidney**

Arteriovenous differences for Ang-1 and Ang-2 were assessed over the kidney during reperfusion. Ang-1 levels did not change significantly over the reperfused kidney in LD, BDD and CDD kidney transplantation, making it unlikely that anti-inflammatory Ang-1 is released from the kidney after reperfusion (Figure 2A-C). In contrast Ang-2, a marker for endothelial activation, was released from the kidney both early and late after reperfusion (Figure 2D-F). Ang-2 release was statistically significant in LD kidneys early after reperfusion (3 min., p=0.005; 30 min. p=0.064), in BDD kidneys only late after reperfusion (3 min. p=0.15; 30 min., p=0.036) and in CDD kidneys both early and late after reperfusion (3 min., p<0.001; 30 min., p=0.017). In the total group of deceased donor kidney transplantations, both early and late Ang-2 release was statistically significant (3 min., p=0.024; 30 min., p=0.001). The Ang-1/Ang-2 ratio was consistently higher in the arterial compared to renal venous samples at both time points for all donor types, although this trend was statistically not significant.
(data not shown). There was no early or late release of vWf or its more dynamic propeptide (Figure 3).

![Figure 2: Ang-2 release from the kidney during reperfusion.](image)

**Figure 2:** Ang-2 is released from the kidney during reperfusion. Ang-1 was not released from the kidney either 3 or 30 minutes after reperfusion in A LD (3 min. p=0.18; 30 min. p=0.59); B BDD (3 min. p=0.29, 30 min. p=1.0); C CDD (3 min. p=0.47; 30 min. p=1.0). Ang-2 was significantly released from the kidney in D LD kidneys early after reperfusion (3 min. p=0.005; 30 min. p=0.064), E in BDD kidneys only later after reperfusion (3 min. p=0.15; 30 min. p=0.036) and F in CDD kidneys both early and late after reperfusion (3 min. p<0.001; 30 min. p=0.017). N=6 in each group. Bars represent mean and error bars represent SEM.
Figure 3: No vWF or vWF propeptide release from the reperfused kidney. vWF was not released from the reperfused graft both early (3 min.) and later (30 min.) after reperfusion in kidney transplantation with A LD (p=0.89, p=0.78), B BDD (p=0.89, p=0.33) and C CDD (p=0.40, p=0.18) respectively. The shorter lived and more dynamic vWF propeptide, was not released either from the kidney in D LD (p=0.78, p=0.62), E BDD (p=0.19, p=0.89) and F CDD (p=0.35, p=0.74) kidney transplantation. Bars represent mean and error bars represent SEM.

Ang-1 protein and mRNA expression are significantly higher in LD kidneys and are reduced after reperfusion

Protein and mRNA expression of angiopoietsins was assessed in the three donor groups. Ang-1 protein was expressed in glomeruli and capillary walls (Figure 4A, B). The protein expression was already reduced before transplantation in CDD kidneys (p=0.003), not BDD kidneys (p=0.17) compared to LD kidneys (Figure 4C, D). Consistently, Ang-1 mRNA expression before transplantation was significantly higher in LD kidneys compared to CDD kidneys (p=0.006) (Figure 4E). In LD kidney biopsies collected before and 45 minutes after reperfusion, there was a significant reduction in both Ang-1 mRNA (p=0.007) and protein expression after reperfusion (p=0.001; Figure 4D, F).

Ang-2 protein and RNA expression levels are similar between LD and deceased donors

Ang-2 immunostaining of kidney sections revealed expression in interstitial vessels and negative staining in the glomeruli (Figure 5A, B). There were no differences in Ang-2 protein and mRNA expression between LD, BDD and CDD kidneys (p=0.69, p=0.73, respectively; Figure 5C, E). Quantification of Ang-2 staining showed a decrease of protein expression
after reperfusion in LD kidneys (p=0.006, Figure 5D). However, mRNA expression of Ang-2 did not change after reperfusion in LD kidney transplantation (p=0.72, Figure 5F). The mRNA expression of the angiopoietin receptor Tie-2 was similar between the different donor types (p=0.48) and not upregulated after reperfusion in LD grafts (p=0.32) (data not shown).

**Figure 4:** Ang-1 protein and mRNA expression decreased after reperfusion. Ang-1 staining was positive in glomeruli and peritubular capillaries. Typical example of Ang-1 staining in A a pre-reperfusion biopsy and B a post reperfusion biopsy of a LD kidney. C Quantification of Ang-1 in pre-transplantation kidney biopsies showed a significantly decrease in CDD (p=0.003) compared to LD grafts. D Ang-1 in LD kidney biopsies taken before (pre) and after (post) reperfusion showed a significant decrease in Ang-1 protein expression (p=0.001). The boxes run from the lower to upper quartile, the whiskers indicate the 5 to 95 percentile, while the horizontal line is set at the median. In kidney biopsies mRNA expression of Ang-1 was assessed. E pre-transplantation Ang-1 mRNA expression was significantly reduced in CDD kidneys compared to LD kidneys (p=0.006). F when comparing biopsies collected before (pre) and after (post) reperfusion, in LD kidneys there was a significant reduction in Ang-1 mRNA expression after reperfusion (p=0.007)
Figure 5: Ang-2 protein and mRNA levels are similar between living and deceased donors.
Ang-2 staining was positive in interstititial vessels and peritubular capillaries. Typical example of Ang-2 staining in A a pre-reperfusion biopsy and B a post-reperfusion biopsy of a LD kidney. C There were no differences in Ang-2 protein expression between groups before transplantation (p=0.69). D After reperfusion, there was a decrease in Ang-2 protein expression (p=0.006), consistent with degranulation of ECs and loss of Ang-2. The boxes run from the lower to upper quartile, the whiskers indicate the 5 to 95 percentile, while the horizontal line is set at the median. mRNA expression of Ang-2 was assessed in kidney biopsies. E Ang-2 expression was similar before reperfusion in all groups (p=0.73). F In LD kidney biopsies collected before (pre) and after (post) reperfusion, Ang-2 expression did not change (p=0.721).
Discussion

Previous studies, primarily exploiting animal models, have revealed that EC activation plays a major role in I/R injury and that the angiopoietin balance may be critical in maintaining vascular integrity. Enhancement of Ang-1 signaling has been demonstrated to be beneficial in renal I/R injury. However, thus far, no study has reported on the role of angiopoietins in human renal I/R injury or local release of angiopoietins from the kidney. In this study, the local response of the reperfused kidney was assessed by arteriovenous concentration differences of angiopoietins during the first 30 minutes after reperfusion in living and deceased donor kidney transplantation. Results demonstrate that renal I/R is associated with acute EC activation shown by a vast Ang-2 release from both living and deceased donor kidneys shortly after reperfusion.

In the present study, a reduction in CD34 and vWF protein expression was observed in tissue biopsies after reperfusion, consistent with either EC death or endothelial injury that induces loss of their characteristic epitopes. These findings are consistent with experimental models of acute ischemic renal endothelial injury after reperfusion and recently observed in humans as well. EC activation represents a switch from a quiescent phenotype toward one that is involved in the host defense response. This process will result in expression of chemokines, cytokines, angiogenic factors and adhesion molecules designed to interact with leukocytes and platelets and target inflammation. In our previous study in human living donor kidney transplantation no evidence for EC activation was found by absence of local release of ICAM-1, P-selectin and vWF. However, the angiopoietins and endothelial/pericyte interactions were not studied.

Angiopoietins have been shown to play a critical role in the maintenance of the microvasculature. This study focused on the release of these factors from both living and deceased donor kidneys. Results show that Ang-1 was not released from the kidney during reperfusion in any of the three groups, consistent with the theory that Ang-1 is constitutively expressed and dynamic changes in Tie-2 signaling are mediated via Ang-2. Consistent with previous reports, Ang-1 protein was found in glomeruli and capillary walls. Interestingly, we show that Ang-1 mRNA and protein expression decreased after reperfusion in human renal I/R injury. In contrast, in rodents Ang-1 expression has been shown to increase after chemical or ischemic kidney injury. However, these observations in experimental animals were done days after the injury, in contrast to the immediate changes observed in our patients. The loss of Ang-1 signal may indicate loss of vital pericytes, since pericytes are the main Ang-1 producing cells. However, decreased Ang-1 protein in renal tissue may also be related to the decrease in Ang-2 signal. Since Ang-1 and Ang-2 share a 60% amino acid
identity, the storage and release of Ang-1 may have the same mechanism as that of Ang-2.\textsuperscript{38} Loss of Ang-1 signaling appears important in many disease processes, and enhancement of Ang-1 signaling was beneficial in sepsis and renal I/R injury.\textsuperscript{28,39}

High Ang-2 levels have repeatedly been shown in pathological conditions, such as in diabetes mellitus, renal failure and in myocardial I/R injury.\textsuperscript{40-42} Ang-2 triggers an inflammatory response by inducing permeability of the vascular lining,\textsuperscript{43} possibly mediated by the Ang-2 induced loss of pericytes.\textsuperscript{44} Results of this study show a vast Ang-2 release from both living and deceased donor kidneys shortly after reperfusion. This indicates injury to ECs, which can release Ang-2 from Weibel-Palade bodies upon activation.\textsuperscript{45} Since expression of Ang-2 did not change upon reperfusion, the arteriovenous difference may reflect Ang-2 release from Weibel-Palade bodies upon stress. This was confirmed by decreased endothelial Ang-2 tissue biopsy staining after reperfusion, consistent with exocytosis of Ang-2 containing granulae. In contrast to Ang-1, there was no expression of Ang-2 in the glomeruli, and positive signal was mainly seen on peritubular capillaries.\textsuperscript{35} The Ang-2 release from the kidney may induce continuing endothelial damage, ultimately leading to pericyte dropout, loosening contacts between endothelial and perivascular cells, with subsequent vessel destabilization and fibrosis.

In our arteriovenous measurements directly over the reperfused graft no release of vWF itself or its propeptide was observed, while Ang-2, another constituent of Weibel-Palade bodies was released. Possible explanations include that Ang-2 may be more easily detectable by arteriovenous measurements due to a shorter half-life, or that Ang-2 release from Weibel-Palade bodies is regulated independent from vWF release.\textsuperscript{46} Moreover, when vWF multimers are secreted from stimulated ECs they may remain anchored to the endothelial surface.\textsuperscript{47} This early retention of vWF to endothelium may provide a further explanation for the lack of a detectable release of vWF into the renal vein.

A limitation of our study was the fact that sampling time was restricted to maximally 45 minutes following reperfusion. Although endothelial damage is considered to be initiated during cold ischemia and expected to be vast around reperfusion, we cannot exclude continuing damage after 45 minutes. Another limitation is that we had to include different patient cohorts for arteriovenous measurements and collection of biopsies. Since for ethical reasons from every patient only one biopsy could be obtained, either DNA isolation or immunohistochemistry was done. Although one cohort for all sampling may have been preferable, our sampling is not expected to have influenced the conclusions from this study, since no correlations with patient characteristics are made. The aim of this study was to explore the endothelium-pericyte interactions in the pathophysiology of renal I/R injury. In
future studies correlations between patient characteristics and angiopoietin release will be made.

In conclusion, human renal I/R injury induces EC activation after reperfusion which leads to Ang-2 release from the kidney in both living and deceased donor kidney transplantation. This is accompanied by loss of ECs and diminished Ang-1 protein and mRNA expression. Moreover, compared to living donors expression of Ang-1 was significantly reduced in deceased donors. Interventions aimed at maintenance of vascular integrity by modulation of angiopoietin signaling may provide new therapeutical insights for I/R injury in human clinical kidney transplantation.

Acknowledgements
We thank The Netherlands Organization for Health Research and Development for the financial support: project AGIKO 92003525 (D.K. de Vries) and Veni grant (M.E.J. Reinders). Ellen Lievers is thanked for technical assistance.
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Changes in adenosine generating enzymes
CD39 and CD73 upon reperfusion in clinical kidney transplantation


In preparation
Abstract

Ischemia reperfusion (I/R) injury is an inevitable consequence of organ transplantation. Its pathophysiology is complex and treatment is primarily supportive. Recent animal experiments suggest that extracellular adenosine may be a critical mediator in protection from renal I/R injury. However, information on adenosine production in human kidney transplantation is lacking. In this study changes in protein and mRNA expression of adenosine generating enzymes CD39 and CD73 in transplanted human kidneys are assessed. Results show that more CD39 protein is present in living compared to deceased donor kidneys before transplantation. CD39 mRNA expression was not different between groups and did not change upon reperfusion. However, CD73 mRNA expression was significantly downregulated after reperfusion, while its tissue protein expression did not change. Altogether, results show that living donor kidneys may be protected by higher pre-transplantation CD39 expression. Yet, this effect may be counteracted by the decrease in CD73 expression after reperfusion. Further studies will need to focus on the consequences of these enzyme changes for renal adenosine generation and kidney graft injury.
Introduction

Ischemia reperfusion (I/R) injury is an inevitable consequence of organ transplantation, and a major determinant of patient and graft survival.\(^1\)\(^3\) The pathophysiology of I/R injury is complex and incompletely understood, and effective treatment is currently lacking. Recent animal experiments suggest that adenosine may be a critical mediator in protection from renal I/R injury.\(^4\)\(^5\)

Adenosine is a nucleoside, acting as a signaling molecule when present in the extracellular space. Extracellular adenosine can be generated by metabolization of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) to adenosine through an enzymatic reaction initiated by ectonucleoside-triphosphate-diphosphohydrolase-1 (CD39) and completed by the enzyme ecto-5′-nucleotidase (CD73) (Figure 1). Hypoxia and ischemia are known to induce CD39 and CD73.\(^6\)\(^7\) Animals genetically modified by CD39 or CD73 deletion experience lower adenosine levels during ischemia and a vast increase in I/R induced inflammation and subsequent tissue damage in many organs.\(^6\)\(^9\) Consequently, CD39 overexpression has beneficial effects in mouse renal I/R injury.\(^10\)

Altogether, animal models provide clear evidence for the protective role of CD39 and CD73 activity in renal I/R injury. In this study, we report on changes in adenosine generation by CD39 and CD73 in human clinical renal I/R injury.

![Figure 1: Schematic representation of extracellular adenosine generation from ADP and ATP by the enzymes ectonucleoside-triphosphate-diphosphohydrolase-1 (CD39) and ecto-5′-nucleotidase (CD73).](image-url)
Materials and methods

Patient population
Twenty-three patients receiving a kidney from a living donor (n=10), a brain dead donor (n=7) or a cardiac dead donor (n=6) were included for mRNA isolation from renal biopsies and subsequent expression analysis. From a different cohort of 10 living, 10 brain dead donor and 10 cardiac dead donor kidney graft recipients, renal biopsies were collected and processed for immunohistochemical analysis. Patient characteristics are shown in Supplemental Table 1. All kidneys were still functioning at 1 year after transplantation, but one patient in the mRNA cohort, that received a BDD kidney transplant died 6 months after transplantation (because of veno-occlusive disease ultimately causing hepatic failure). The study protocol was approved by the local ethics committee, and informed consent was obtained from each patient.

Table 1: Transplantation and outcome characteristics of the three different patient cohorts that were included. In each cohort material was collected from living donor (LD), brain dead donor (BDD) and cardiac dead donor (CDD) grafts during kidney transplantation.

<table>
<thead>
<tr>
<th></th>
<th>LD</th>
<th>IHC</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BDD</td>
<td>CDD</td>
<td>BDD</td>
</tr>
<tr>
<td>N</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Donor age: mean (SD)</td>
<td>57(10)</td>
<td>48(16)</td>
<td>41(10)</td>
</tr>
<tr>
<td>Donor gender (M:F)</td>
<td>5:5</td>
<td>5:5</td>
<td>6:4</td>
</tr>
<tr>
<td>WIT1 in min. (SD)</td>
<td>N/A</td>
<td>N/A</td>
<td>18(5)</td>
</tr>
<tr>
<td>CIT in h. (SD)</td>
<td>2.8(0.5)</td>
<td>18.6(9.6)</td>
<td>17.8(3.4)</td>
</tr>
<tr>
<td>WIT2 in min. (SD)</td>
<td>29(8)</td>
<td>28(4)</td>
<td>28(8)</td>
</tr>
<tr>
<td>Recipient age: mean (SD)</td>
<td>54(16)</td>
<td>51(13)</td>
<td>45(15)</td>
</tr>
<tr>
<td>Recipient gender (M:F)</td>
<td>4:6</td>
<td>7:3</td>
<td>7:3</td>
</tr>
<tr>
<td>CrCl day 30 (ml/min)</td>
<td>45(11)</td>
<td>63(30)</td>
<td>48(21)</td>
</tr>
<tr>
<td>DGF</td>
<td>0/10</td>
<td>3/10</td>
<td>7/10</td>
</tr>
<tr>
<td>DGF duration in days (SD)</td>
<td>0</td>
<td>5(2)</td>
<td>13(6)</td>
</tr>
</tbody>
</table>

IHC: cohort of patients in which renal biopsies were used for immunohistochemical analysis.
PCR: cohort of patients from which renal biopsies were used for PCR analysis.
WIT1: first warm ischemia time.
CIT: cold ischemia time.
WIT2: second warm ischemia time.
DGF: delayed graft function.
DGF duration: dialysis after transplantation in days (SD).
CrCl: Creatinine clearance.

Operation and materials
Kidney transplantations were performed according to local standardized protocol. In living donors minimally invasive nephrectomy was performed. For cold perfusion and storage of the
kidney, Custodiol® Histidine–tryptophan–ketoglutarate solution (HTK) solution (Tramedico, Weesp, The Netherlands) was used. Deceased donor kidneys were perfused and stored with University of Wisconsin solution (UW) or HTK. The immunosuppressive regimen was based on induction therapy with an interleukin-2 receptor blocker (basiliximab, day 0 and day 4) and maintenance treatment with tacrolimus or cyclosporine A, in addition to mycophenolate mofetil and steroids in all groups.

**Immunohistochemical analysis**

To assess presence of CD39 and CD73, a renal cortical biopsy was obtained after cold storage from living, brain dead and cardiac dead donor kidney grafts. Only in the LD group, a second cortical biopsy was collected as well at 45 minutes after reperfusion. Immunohistochemical analyses were performed on 4 μm cryostat kidney tissue sections. Frozen sections were fixed in acetone for 10 min. Slides were washed and incubated with peroxidase blocking solution for 30 minutes and blocked with PBS containing 5% normal human serum (NHS) and 1% BSA for 45 minutes at room temperature. Then slides were incubated overnight at room temperature with the following primary antibodies: CD39 mouse monoclonal IgG1, 1:50 (eBioscience, Vienna, Austria); CD73 mouse monoclonal IgG1, 1:3200 (Hycult Biotechnology, Uden, Netherlands). Next, a peroxidase-conjugated goat anti-mouse IgG (1:200) secondary antibody was applied (DAKO, Glostrum, Germany), followed by sequential fluorescein isothiocyanate (FITC, amplification reagent, NENTM Life Science Products, Boston, MA, USA) and anti-fluorescein-HRP (DAKO, Glostrum, Germany). Staining was completed by incubation with 3,3' diaminobenzidine tetrahydrochloride (DAB)/ hydrogen peroxide (Sigma, St Louis, MO, USA) and counterstained with Mayer’s Hematoxylin Solution (Merck, Darmstadt, Germany). At 100× magnification, 10 microscopic fields of each kidney section were quantified using computerized image analysis (ImageJ).

**mRNA expression in human kidney biopsies**

A renal cortical biopsy was obtained after cold storage, before transplantation, from living, brain dead and cardiac dead donor kidneys. A second, post-reperfusion biopsy was collected 45 minutes after reperfusion from living donor kidneys only. Biopsies were immediately snap frozen in liquid nitrogen and stored at -70°C. Total RNA was extracted from renal tissues using RNAzol (Campro Scientific, Veenendaal, The Netherlands) and glass beads. The integrity of each RNA sample was examined by Agilent Lab-on-a-chip technology using the RNA 6000 Nano LabChip kit and a bioanalyzer 2100 (Agilent Technologies, Amstelveen, The Netherlands). RNA preparations were considered suitable for further processing if samples showed intact 18S and 28S rRNA bands, and displayed no chromosomal peaks or RNA degradation products (RNA Integrity Number >8.0). cDNA was synthesized from 1 μg total RNA, using an oligo dT primer, RNase-OUT, M-MLV reverse transcriptase, 0.1 M-DTT
and buffers in a volume of 20 µL (all purchased from Invitrogen, Breda, The Netherlands). Quantitative real-time PCR was performed in duplicate by using iQ SYBR Green Supermix on iCycler Real-Time Detection System (BioRad). The amplification reaction volume was 20 µL, consisting of 10 µL iQ SYBR Green PCR mastermix, 1 µL primers, 1 µL cDNA, and 8 µL water. Data were analyzed using Gene Expression Analysis for iCycler Real-Time PCR Detection System (BioRad). Expression of each gene was normalized against mRNA expression of the housekeeping gene GAPDH. The primer sequences are shown in Supplemental Table 2.

### Table 2: Primer sequences used for quantitative real-time polymerase chain reaction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5’-&gt;3’</th>
<th>Reverse primer 5’-&gt;3’</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD39</td>
<td>AGCAGCTGAAATATGCTGGC</td>
<td>GAGACAGTATCTGCGAAGTCC</td>
<td>Biolegio</td>
</tr>
<tr>
<td>CD73</td>
<td>GCAGACATTACAAATGGAGG</td>
<td>CATCCGTGTGTCTCAGGTTG</td>
<td>Biolegio</td>
</tr>
</tbody>
</table>

### Analysis

Clinical donor data were retrieved from Eurotransplant Foundation (Leiden, The Netherlands). Delayed graft function (DGF) was defined as the need for dialysis within one week after transplantation. Statistical analysis was performed using SPSS 17.0 statistical analysis software (SPSS Inc, Chicago, Ill). Graph error bars represent the standard error of the mean (SEM), unless otherwise stated. A P-value of less than 0.05 was considered significant.

### Results

**CD39**

CD39 staining was positive in peritubular capillaries and in glomeruli. Already before transplantation, living donor kidneys had a significantly higher CD39 tissue expression as compared to both brain dead (p=0.004), and cardiac dead (p<0.0001) donor kidneys (Figure 2). After reperfusion, CD39 tissue expression did not change in living donor kidneys (p=0.16). CD39 mRNA expression was similar in grafts of all three donor types before transplantation (p=0.27). Upon reperfusion CD39 mRNA expression did not change (p=0.49).

**CD73**

CD73 signal was positive in endothelial cells and to a lesser extent in tubular epithelial cells. Before transplantation, CD73 protein and mRNA expression was equal in renal biopsies of the three different donor types (p=0.52, p=0.13, respectively, Figure 3). After reperfusion, CD73 tissue expression did not change (p=0.20). However, CD73 mRNA expression was significantly reduced after reperfusion in living donor (P=0.002) kidneys.
Figure 2: CD39 protein levels are higher in living than deceased donor kidneys. CD39 staining was positive in peritubular capillaries and in glomeruli. Typical example of CD39 staining in A a pre-reperfusion biopsy and B a post-reperfusion biopsy of a LD kidney. C Before transplantation, CD39 tissue expression was significantly higher in LD kidneys as compared to BDD (p=0.004) or CDD kidneys (p<0.0001). D Upon reperfusion, there was no change in CD39 protein expression in living donor kidneys (p=0.16). The boxes run from the lower to upper quartile, the whiskers indicate the 5 to 95 percentile, while the horizontal line is set at the median. mRNA expression of CD39 was assessed in kidney biopsies. E CD39 mRNA expression was similar before reperfusion in all groups (p=0.27). Similar to protein expression, CD39 mRNA expression did not change upon reperfusion in LD, BDD and CDD kidneys (p=0.87, p=0.57, p=0.20, respectively).
Figure 3: CD73 mRNA levels decrease after reperfusion.
CD73 staining was positive in endothelial and tubular epithelial cells. Typical example of CD73 staining in A a pre-reperfusion biopsy and B a post-reperfusion biopsy of a LD kidney. C Before transplantation, CD73 tissue expression was similar in kidneys of all three donor types (p=0.52). D 45 minutes after reperfusion, CD73 protein expression had not changed in living donor kidneys (p=0.20). The boxes run from the lower to upper quartile, the whiskers indicate the 5 to 95 percentile, while the horizontal line is set at the median. mRNA expression of CD39 was assessed in kidney biopsies. E CD73 mRNA expression was similar before reperfusion in kidneys of all three donor groups (p=0.13). While CD73 mRNA expression was massively down regulated after reperfusion (p=0.002) in LD kidneys, BDD kidneys showed no significant changes in expression although mean CD73 showed a trend towards decrease after reperfusion. In CDD kidneys the CD73 decrease did reach statistical significance. (P=0.043)
Discussion

I/R injury is a compulsory component of kidney transplantation, and its pathophysiology is still unclear. Although animal experiments have suggested protection from renal I/R injury by extracellular adenosine, data on adenosine generation in human renal I/R have been missing. In this study we show changes in adenosine generation by the enzymes CD39 and CD73 in transplanted human kidneys.

CD39 mediates the first step in the extracellular hydrolysis of ATP and ADP towards adenosine. Animal experiments show critical involvement of CD39 in renal I/R injury.\textsuperscript{10,13} In human renal I/R injury, we found no changes in CD39 expression after reperfusion in kidney transplantation. However, before transplantation CD39 tissue expression was significantly higher in living compared to deceased donor kidneys. This may suggest a protective effect of CD39 in living donor kidneys, and a potential beneficial effect of CD39 over expression in humans as well.

CD73 is the enzyme that generates adenosine from AMP in the extracellular space. In contrast to CD39, results on the exact role of CD73 in renal I/R injury are conflicting. Although its essential role in ischemic preconditioning has been shown,\textsuperscript{14} others report on a protective effect of CD73 deletion mediated by AMP accumulation or by nonenzymatic functions of CD73.\textsuperscript{15-17} Here, we show a vast decrease in CD73 mRNA expression after reperfusion in both living and deceased donor kidneys. Since these are the first human data describing expression of these enzymes in renal I/R injury, we can only relate our results to previous experimental studies. These quite unambiguously show the in vitro upregulation of CD73 after hypoxia \textsuperscript{6} and an increase in CD73 activity after ischemic preconditioning in mice.\textsuperscript{14}

Although in this study CD39 and CD73 are central, when extrapolating results, it may be suggested that extracellular adenosine are involved in human renal I/R injury. In the transplantation setting, it is of interest that adenosine has been added to UW preservation solution. Although this adenosine appears critical in the beneficial effects of UW solution,\textsuperscript{18} the effect of enhanced adenosine generation is probably many times larger, since the in vivo effect of adenosine lasts only minutes.\textsuperscript{19}

In conclusion, we show that expression of adenosine generating enzymes CD39 and CD73 is different between living and deceased donor kidneys and changes after reperfusion. Further studies are needed to assess the consequences of these enzyme changes for renal adenosine generation and kidney graft injury.
Chapter 8

References


Oxidative damage in clinical ischemia/reperfusion injury: a reappraisal


*These authors contributed equally to this article

Abstract

Background: Ischemia/reperfusion (I/R) injury is a common clinical problem. Although the pathophysiological mechanisms underlying I/R injury are unclear, oxidative damage is considered a key factor in the initiation of I/R injury. Findings from preclinical studies consistently show that quenching reactive oxygen and nitrogen species (RONS), thus limiting oxidative damage, alleviates I/R injury. Results from clinical intervention studies on the other hand are largely inconclusive. In this study, we systematically evaluated the release of established biomarkers of oxidative and nitrosative damage during planned I/R of kidney and heart in a wide range of clinical conditions.

Results: Sequential arteriovenous concentration differences allowed specific measurements over the reperfused organ in time. None of the biomarkers of oxidative and nitrosative damage (that is, malondialdehyde, 15(S)-8-iso-prostaglandin F2α, nitrite, nitrate and nitrotyrosine) were released upon reperfusion. Cumulative urinary measurements confirmed plasma findings. As of these negative findings, we tested for oxidative stress during I/R and found activation of the nuclear factor erythroid 2-related factor 2, the master regulator of oxidative stress signaling.

Innovation: This comprehensive, clinical study evaluates the role of RONS in I/R injury in two different human organs (kidney and heart). Results show oxidative stress, but do not provide evidence for oxidative damage during early reperfusion, thereby challenging the prevailing paradigm on RONS-mediated I/R injury.

Conclusions: Findings from this study suggest that the contribution of oxidative damage to human I/R may be less than commonly thought and propose a re-evaluation of the mechanism of I/R.
Introduction

Ischemia/reperfusion injury (I/R) is the paradoxical increase of tissue damage upon reperfusion of ischemic tissue. I/R is considered a major contributor to tissue damage in multiple clinical situations such as myocardial infarction, stroke and organ transplantation. The pathophysiology of I/R injury is complex and incompletely understood, and effective treatment is currently lacking.

Reactive oxygen and nitrogen species (RONS) are considered key initiators of I/R injury. Ischemia-related metabolic adaptations and dysregulated mitochondrial homeostasis are thought to result in substantial RONS release upon reintroduction of oxygen. This RONS overload can overwhelm the endogenous antioxidant system, resulting in oxidative damage. This may trigger secondary processes such as a pro-inflammatory response.\(^1\)\(^-\)\(^4\)

It has long been supposed that antioxidant therapy mitigates I/R injury. The validity of this concept has been proven in numerous animal studies, which all clearly demonstrate that antioxidant therapy ameliorates I/R injury.\(^5\)\(^-\)\(^7\) Despite these findings, studies in humans consistently fail to show any clinically relevant effect.\(^5\),\(^8\)\(^-\)\(^11\) The basis for this discrepancy between human and animal studies is still unclear, yet it may suggest that the contribution of RONS to I/R injury in humans may be less than commonly thought.

In this study, a double approach was used to evaluate the role of RONS in clinical I/R injury. First, release of established biomarkers of oxidative damage from the human kidney and heart was assessed during planned I/R. By cannulation of the efferent vein, that is, the renal vein during kidney transplantation and the coronary sinus during cardiac valve surgery, sensitive and organ-specific measurements of net changes in oxidative damage biomarkers over the organ were conducted. Next, as none of the biomarkers were released during the reperfusion phase, we tested for presence of oxidative stress during early reperfusion, which did show activation of nuclear factor erythroid 2-related factor 2 (Nrf2), a critical transcription factor in oxidative stress signaling. All together, results from these studies, comprising different organs, clinical settings and durations of ischemia, are highly consistent and do indicate oxidative stress, but no RONS-related damage during the acute reperfusion phase in humans, suggesting that the endogenous antioxidant system is able to cope with I/R related oxidative stress.
Methods

Patients and surgical procedures
This prospective observational study involved patients undergoing renal I/R during kidney transplantation with living donor (LD) and deceased donor (DD) grafts, and patients undergoing myocardial I/R (that is, during cardiac valve surgery) with and without preexisting heart failure. The study protocols were approved by the local Ethics Committee, and written informed consent was obtained from each patient.

Kidney transplantation
Twenty-four patients undergoing renal allograft transplantation were included: eight patients receiving a kidney from a LD and 16 patients receiving a kidney from a DD (nine brain-dead donors and seven cardiac-dead donors). LD and DD kidney transplantations were separately analyzed and compared because of large differences in ischemia times, pre-transplantation graft handling, and clinical outcome between these groups. Within groups variability of patient and graft characteristics was small for both donor types, rendering the comparison more reliable. LD kidney transplantations allowed for measurements in urine produced by the graft directly after reperfusion, providing a more cumulative measure than the arteriovenous measurements.

Kidney transplantations were performed according to the local standardized protocol. For technical reasons (renal vein sampling), only patients receiving a left kidney were included. In LDs a minimally invasive open nephrectomy was performed. The immunosuppressive regimen was based on induction therapy with basiliximab followed by maintenance therapy with tacrolimus or cyclosporine A in addition to mycophenolate mofetil and steroids.

Cardiac valve surgery
Myocardial I/R was studied in patients undergoing cardiac valve surgery with aortic cross-clamping (that is, cessation of blood flow in the coronary arteries). Twenty-four patients scheduled for elective mitral valve annuloplasty with use of cardiopulmonary bypass were included: 12 patients with preexisting heart failure and 12 patients without heart failure. Heart failure was defined as an inadequate pump function with an echocardiographically estimated ejection fraction biplane below 35%\(^\text{12}\) and the presence of one or more clinical symptoms of heart failure (New York Heart Association classification).\(^\text{13}\) Exclusion criteria were perioperative corticosteroid therapy, minimal invasive surgical procedures, emergency cardiac operations and previous cardiac surgery.
Cardiac surgery was performed according to the local standardized protocol. All surgical procedures were performed via a midline sternotomy under normothermic cardiopulmonary bypass (Jostra Maquet, Maquet, Hirrlingen, Germany) with intermittent potassium-enriched antegrade warm-blood cardioplegia. This solution was administered every 15 to 20 minutes throughout the entire ischemic period. The cardiopulmonary bypass system was coated with a heparin softline coating. No antioxidants or other preservational or therapeutical substances were added.

During cardioplegia-induced cardiac arrest (myocardial ischemia), valve surgery was executed. Eventually, the aortic cross-clamp was removed to restore the blood flow through the heart (start of reperfusion).

**Arteriovenous measurements**
Arterial and venous blood samples were collected directly over the reperfused organ, that is, from the afferent and efferent blood vessel. Comparison of multiple biomarkers in these samples allowed for accurate and specific assessment of locally ongoing processes in the reperfusion phase. This method was used to determine the release of biomarkers of oxidative damage in both kidney and heart I/R.

**Kidney transplantation**
Arterial and renal venous blood samples were obtained as described before. Via an umbilical vein catheter placed in the renal vein, blood aliquots were sampled at 30 seconds, 3, 10 and 30 minutes after reperfusion. Paired arterial blood samples were simultaneously obtained via the iliac artery at 0, 3, 10 and 30 minutes after reperfusion (Figure 1A). The endpoint of sampling was reached 30 minutes after reperfusion by closing the abdominal wall. The initial urine produced by the graft in the first 10 minutes after reperfusion was collected directly from the graft’s ureter. Control urine was collected from the urethral catheter of LDs during the nephrectomy procedure before kidney ischemia was induced. All samples were collected in pre-cooled tubes containing ethylenediaminetetraacetic acid (EDTA) (BD Vacutainer, Plymouth, UK) and placed on melting ice immediately. Blood and urine samples were centrifuged (1.550 g, 20 min, 4°C) within one hour after collection and the derived plasma or supernatant was re-centrifuged (1.550 g, 20 min, 4°C) to deplete it from remaining leukocytes and platelets. Material was aliquoted and stored at -70°C until analysis.

**Cardiac valve surgery**
A 5 French indwelling jugular vein catheter (PICC, Arrow International Inc., REF PS-01651, PA, USA) was inserted in the right atrium and placed in the coronary sinus during
cardiac surgery (Figure 1B). As all patients underwent mitral valve surgery using a vertical transseptal incision, the coronary sinus could easily be cannulated during the surgical procedure. An arterial catheter was routinely placed in the radial artery. Arterial and paired myocardial venous blood (coronary sinus) samples were obtained at 0, 15, 30 and 60 minutes after reperfusion as described recently. All samples were collected in pre-cooled tubes containing EDTA (BD Vacutainer, Plymouth, UK) and placed on melting ice immediately. Blood samples were centrifuged (1,550 g, 10 min, 4°C) within one hour after collection and the derived plasma was re-centrifuged (10,000 g, 4 min, 4°C) to obtain leukocyte and platelet poor plasma. Aliquots were stored at -70°C until analysis.

Figure 1. Schematic representation of the arteriovenous measurements of the kidney and heart. The figure shows the position of the arterial and renal venous catheter in the kidney (A), and the coronary sinus catheter in the heart (B).

Biopsy collection
A pre-transplantation renal cortical biopsy was obtained just before transplantation, when the graft was still on ice. A paired needle biopsy of the same kidney was collected 45 minutes after reperfusion. Myocardial biopsies were collected at start of cardioplegia-induced cardiac arrest and a paired, second biopsy was obtained at the end of the ischemic period, just before reperfusion of the heart. Because of technical reasons, a post-reperfusion biopsy could not be collected since most of the included heart failure patients were hemodynamically instable in the early reperfusion phase. Lifting the heart, to take a post reperfusion biopsy, was considered unsafe. All biopsies were snap-frozen in liquid nitrogen and stored at -70°C until analysis.
Laboratory plasma measurements
To validate the method of arteriovenous measurements and to assess whether there is oxygen consumption by the reperfused organ, the oxygen saturation level was assessed in arterial as well as venous blood samples of a LD by means of a routinely used validated blood gas analyzer. The extent of RONS-mediated damage was evaluated by measuring different well-established biomarkers of oxidative and nitrosative damage by state-of-the-art stable isotope dilution gas chromatography-mass spectrometry (GC-MS) and gas chromatography-tandem mass spectrometry (GC-MS/MS) methods. Malondialdehyde (MDA) and 15(S)-8-iso-PGF2α are widely recognized biomarkers of lipid peroxidation.\(^{16}\) In humans, nitrite and nitrate are reliable indicators of nitric oxide synthesis and frequently measured breakdown products of RONS.\(^{16-18}\)

MDA and total 15(S)-8-iso-prostaglandin F2α (15(S)-8-iso-PGF2α), that is, free and esterified 15(S)-8-iso-PGF2α, were assessed with an extensively validated GC-MS/MS method as described previously.\(^{19, 20}\) In parallel, the thiobarbituric acid-reactive substances (TBARS) method was used to detect MDA in plasma as well as in urine samples.\(^{21}\) Free 15(S)-8-iso-PGF2α in urine was measured by GC-MS/MS.\(^{20}\) Nitrite and nitrate were measured simultaneously by GC-MS in plasma and urine aliquots as described elsewhere in detail.\(^{19}\)\(^{22}\) The prostaglandin E\(_2\) (PGE\(_2\)) concentration in urine was measured by GC-MS/MS after immunoaffinity column chromatography extraction.\(^{20}\) Urinary parameters were corrected for creatinine excretion.\(^{23}\) Study samples were analyzed alongside quality control (QC) samples as described previously.\(^{19, 22, 23}\) The MDA plasma concentration in the QC samples was determined to be 57.3 ± 7.8 nmol/L (mean ± standard deviation (SD), n = 25) corresponding to an imprecision (relative SD) of 13.7 %. The total 15(S)-8-iso-PGF2α plasma concentration in the QC samples was determined to be 157 ± 6.4 pmol/L (mean ± SD, n = 10) corresponding to an imprecision of 4.1%. In the QC samples for plasma nitrite and nitrate (n = 30), accuracy and imprecision were 90% to 112% and below 6%, respectively. 3-Nitrotyrosine in proteins, a biomarker of nitrosative damage, was measured by ELISA in accordance with the manufacturer's instructions (Biotech nitrotyrosine EIA, Oxis, Portland, Oregon, USA).\(^{16}\)

Nrf2/ARE pathway
Nrf2 activation was assessed in whole-cell lysate. Snap-frozen renal biopsies were homogenized and whole-cell extracts were prepared according to the manufacturers’ instructions (Active Motif, Carlsbad, CA, USA). Next, activated Nrf2 in cell lysates was quantified by DNA-binding ELISA according to the manufacturers’ instructions (TransAM Nrf2, Active Motif, Carlsbad, CA, USA). Total RNA was extracted from renal or myocardial tissues as described earlier, using glyceraldehyde phosphate dehydrogenase (GAPDH) as internal control.\(^{24}\) For expression profiling, the integrity of each RNA sample
was examined by Agilent Lab-on-a-chip technology using the RNA 6000 Nano LabChip kit and a bioanalyzer 2100 (Agilent Technologies, Amstelveen, The Netherlands). RNA preparations were considered suitable for array hybridization only if samples showed intact 18S and 28S rRNA bands, and displayed no chromosomal peaks or RNA degradation products (RNA Integrity Number >8.0). Subsequent microarray analysis was performed using Illumina whole-genome gene expression BeadChips (Illumina BeadArray®, San Diego, CA, USA) according to instructions of the manufacturer at Service XS (Leiden, The Netherlands). Selected probes from the canonical Nrf2 pathway were used as an input for pathway analysis through the Ingenuity Pathway Analysis suite (http://www.ingenuity.com). Hierarchical clustering and heatmap visualization were carried out using Hierarchical Clustering Viewer and Heatmap Viewer modules within the GenePattern analysis suite.

**Histological analyses**

In kidney biopsies collected before and after reperfusion, presence of various antioxidant enzymes and RONS generation by xanthine oxidase (XO) was assessed. Antioxidant enzymes superoxide dismutase (SOD) and peroxidases (catalase and glutathione peroxidase) were measured by histochemical methods as described previously in detail. The XO activity was measured in renal tissue, based on the generation of superoxide anions from hypoxanthine, as described earlier. Specificity of the assay was demonstrated by inhibition of XO by allopurinol. Rat liver tissue was included as positive control.

**Statistical analysis**

Statistical analysis was performed with the Statistical Package for the Social Sciences 16.0 (SPSS Inc., Chicago, IL, USA). All data in the text and tables are expressed as mean ± SD. Patient characteristics were compared by paired t-test. The area under the curve (AUC) was calculated for the arterial and venous curve. AUC’s and urinary measurements were compared by using the Wilcoxon signed rank test. Differential expression of probes was assessed using unpaired moderated t-test (LIMMA) through the Remote Analysis Computation for gene Expression data (RACE) suite at http://race.unil.ch. A P value less than 0.05 was considered significant. Graph error bars indicate the standard error of the mean (SEM).

**Results**

**Patient characteristics and outcome**

Patient characteristics for kidney transplantation and cardiac valve surgery are summarized in Tables 1 and 2, respectively. One-year patient and graft survival was 100% for the patients who underwent kidney transplantation. One-year survival was also 100% for all patients who underwent cardiac valve surgery.
### Table 1. Transplantation and outcome characteristics in living donor and deceased donor kidney transplantations

<table>
<thead>
<tr>
<th></th>
<th>LD</th>
<th>DD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Recipient age (years) mean ± SD</td>
<td>41.1 ± 10.5</td>
<td>54.6 ± 12.2</td>
<td>0.02</td>
</tr>
<tr>
<td>Recipient gender (% male)</td>
<td>38 (n = 3)</td>
<td>56 (n = 9)</td>
<td>0.39</td>
</tr>
<tr>
<td><strong>Cause of renal failure</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADPKD</td>
<td>2 (25%)</td>
<td>4 (25%)</td>
<td></td>
</tr>
<tr>
<td>Renal fibrosis</td>
<td>2 (25%)</td>
<td>1 (6%)</td>
<td></td>
</tr>
<tr>
<td>MPG</td>
<td>1 (13%)</td>
<td>1 (6%)</td>
<td></td>
</tr>
<tr>
<td>IgA nephropathy</td>
<td>1 (13%)</td>
<td>1 (6%)</td>
<td></td>
</tr>
<tr>
<td>BM nephropathy</td>
<td>0</td>
<td>2 (13%)</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>0</td>
<td>2 (13%)</td>
<td></td>
</tr>
<tr>
<td>FSG</td>
<td>0</td>
<td>1 (6%)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>2 (25%)</td>
<td>4 (25%)</td>
<td></td>
</tr>
<tr>
<td>Donor age (years) mean ± SD</td>
<td>43.9 ± 10.6</td>
<td>53.5 ± 16.1</td>
<td>0.14</td>
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<tr>
<td>Donor gender (% male)</td>
<td>75 (n = 6)</td>
<td>44 (n = 7)</td>
<td>0.15</td>
</tr>
<tr>
<td><strong>Cause of death donor</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>BDD: trauma</td>
<td>3 (19%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BDD: SAH</td>
<td>2 (13%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BDD: hypoxic</td>
<td>3 (19%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDD: traumatic brain injury</td>
<td>2 (13%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDD: intracranial hemorrhage</td>
<td>4 (25%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDD: SAH</td>
<td>2 (13%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Preservation fluids</strong></td>
<td></td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>HTK (n = 8)</td>
<td></td>
<td>UW (n = 11)</td>
<td></td>
</tr>
<tr>
<td>CIT (min) mean ± SD</td>
<td>179.1 ± 18.6</td>
<td>1117.7 ± 299.1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>WIT (min) mean ± SD</td>
<td>34.0 ± 6.3</td>
<td>33.5 ± 6.1</td>
<td>0.85</td>
</tr>
</tbody>
</table>

**Abbreviations:** ADPKD, Autosomal Dominant Polycystic Kidney Disease; BDD, Brain-Dead Donor; BM, Basement Membrane; CDD, Cardiac-Dead Donor; CIT, Cold Ischemia Time; DD, Deceased Donor; FSG, Focal Segmental Glomerulosclerosis; HTK, Histidine–Tryptophan–Ketoglutarate; LD, Living Donor; MPG, Membranous Glomerulonephritis; SAH, Subarachnoid Hemorrhage; UW, University of Wisconsin; WIT, Warm Ischemia Time.

### Table 2. Characteristics of patients with and without preexisting heart failure undergoing cardiac surgery

<table>
<thead>
<tr>
<th></th>
<th>Heart failure</th>
<th>No heart failure</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>12</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>75 (n = 9)</td>
<td>42 (n = 5)</td>
<td>0.11</td>
</tr>
<tr>
<td>Age (years) Mean ± SD</td>
<td>66.75 ± 8.91</td>
<td>62.68 ± 11.65</td>
<td>0.20</td>
</tr>
<tr>
<td>Ischemia time (min) Mean ± SD</td>
<td>128.25 ± 50.66</td>
<td>127.00 ± 32.62</td>
<td>0.89</td>
</tr>
<tr>
<td>Duration ICU stay (h)' Mean ± SD</td>
<td>62.50 ± 40.75</td>
<td>31.58 ± 23.90</td>
<td>0.02</td>
</tr>
</tbody>
</table>

**Abbreviation:** ICU, Intensive Care Unit.
Validation of arteriovenous measurements
The methodology of the arteriovenous sampling is schematically shown in Figure 1. The principle of the method is illustrated by arterial and venous oxygen saturation levels measured during early reperfusion, showing an effective and consistent consumption of oxygen (Figure 2).

Figure 2. A typical example of arteriovenous oxygen saturation differences during reperfusion of the kidney.
Arterial and renal venous oxygen saturation was measured during the first 30 minutes of reperfusion. The consistently lower venous oxygen saturation level indicates the uptake of oxygen by the reperfused kidney, demonstrating its immediate metabolic activity.

Biomarkers of oxidative damage
Renal I/R is not associated with oxidative damage
MDA and 15(S)-8-iso-PGF2α were measured as established biomarkers of RONS-mediated damage. Arteriovenous measurements of MDA did not indicate its release from the kidney during the first 30 minutes of reperfusion of LD kidneys by either GC-MS ($P = 0.17$; Figure 3) or TBARS method ($P = 0.06$). Urinary MDA levels (TBARS method) were also assessed in the first urine produced immediately after reperfusion (LDs only) as an aggregate measure of total MDA formation in the kidney. The MDA levels in this first urine collected after reperfusion were similar to the control urine, that is, urine produced by the kidney before its removal from the donor ($P = 0.40$).
Figure 3. Arterial and venous plasma concentrations of MDA.
(A) Arterial and renal venous plasma MDA concentrations during the first 30 minutes of reperfusion in living donor kidney transplantation as determined by GC-MS/MS showed no MDA release \((p = 0.17)\); (B) No MDA release was observed when determined by TBARS method either \((p = 0.06)\); (C) Arterial and myocardial venous plasma MDA concentrations during the first 60 minutes of reperfusion in patients with preexisting heart failure showed no release from the myocardium \((p = 0.05)\). In fact, venous MDA levels tended to be lower than the arterial levels in patients with preexisting heart failure; (D) In patients without preexisting heart failure MDA was not released either \((p = 0.43)\).

Minimal release was observed for 15(S)-8-iso-PGF\(_2\alpha\) from reperfused LD kidney grafts \((p = 0.03)\). However, clinically more vulnerable DD kidneys did not show any 15(S)-8-iso-PGF\(_2\alpha\) release upon reperfusion \((p = 0.16; \text{Figure 4})\). Arteriovenous measurements over the non-ischemic kidney in LDs, that is, before donor nephrectomy, revealed no release of 15(S)-8-iso-PGF\(_2\alpha\) \((p = 0.69)\), demonstrating that its post-reperfusion release is specific for I/R. Urinary 15(S)-8-iso-PGF\(_2\alpha\) levels were not influenced by reperfusion \((p = 0.35; \text{Table 3})\). Urinary PGE\(_2\) was measured as marker of cyclooxygenase-2 (COX-2) activity. PGE\(_2\) levels in the first urine collected after reperfusion were higher than those of the control urine, that is, urine produced by the kidney prior to its removal from the donor \((p = 0.02; \text{Figure 5})\). This shows that in contrast to the absence of apparent oxidative damage, oxidative stress appears to be increased after renal reperfusion.
Figure 4. Arterial and venous plasma concentrations of 15(S)-8-iso-prostaglandin F2α.
(A) Arterial and renal venous plasma 15(S)-8-iso-PGF2α concentrations during the first 30 minutes of reperfusion in living donor (LD) kidney transplantation showed a small, but significant release from the kidney ($P = 0.03$); (B) In deceased donor (DD) kidney transplantation, 15(S)-8-iso-PGF2α was not released from the reperfused kidney during the first 30 minutes of reperfusion ($P = 0.16$); (C) Arterial and myocardial venous plasma 15(S)-8-iso-PGF2α concentrations during the first 60 minutes of reperfusion in patients with preexisting heart failure showed a small, but significant release from the myocardium ($P = 0.02$); (D) In patients without preexisting heart failure, 15(S)-8-iso-PGF2α was not released from the reperfused heart during the first 60 minutes of reperfusion ($P = 0.18$).
Table 3. AUC venous minus AUC arterial (delta AUC) of the measured plasma biomarkers for oxidative and nitrosative damage during the early reperfusion phase.

<table>
<thead>
<tr>
<th></th>
<th>Kidney transplantation</th>
<th>Cardiac valve surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LD</td>
<td>DD</td>
</tr>
<tr>
<td>Oxidative damage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malondialdehyde</td>
<td>138.2</td>
<td>Not measured</td>
</tr>
<tr>
<td>(nmol/L×min)</td>
<td>(0.17)</td>
<td></td>
</tr>
<tr>
<td>15(S)-8-iso-PGF2α</td>
<td>356</td>
<td>-1306</td>
</tr>
<tr>
<td>(pmol/L×min)</td>
<td>(0.03)</td>
<td></td>
</tr>
<tr>
<td>Nitrotyrosine</td>
<td>-113</td>
<td>Not measured</td>
</tr>
<tr>
<td>(nmol/L×min)</td>
<td>(0.93)</td>
<td></td>
</tr>
</tbody>
</table>

* Delta AUC: AUC venous minus AUC arterial (P value) of the measured plasma markers during the early reperfusion phase. A positive number indicates a release from the reperfused organ, whereas a negative number indicates an uptake of the biomarker.

† Results in urine indicate P values of the difference of the pre- and post reperfusion measure.

‡ Significant venous release (P < 0.05).

Abbreviation: AUC, area under the curve.

Figure 5. Prostaglandin E₂ excretion in urine.
Creatinine-corrected levels of prostaglandin E₂ (PGE₂) in the first urine collected after reperfusion (post) were higher than those of control urine (pre), that is, urine produced by the kidney before its removal from the donor (P = 0.02).
Myocardial I/R is not associated with oxidative damage

Results of myocardial I/R resembled those of the kidney. There was no myocardial MDA release during the first hour after reperfusion in both patients with and without heart failure ($P = 0.05$ and $P = 0.43$, respectively; Table 3, Figure 3). In fact, venous MDA levels tended to be lower than arterial levels in patients with preexisting heart failure. 15(S)-8-iso-PGF2α showed a small local release early after reperfusion in patients with preexisting heart failure ($P = 0.02$; Figure 3). In contrast, arteriovenous measurements of 15(S)-8-iso-PGF2α in patients without preexisting heart failure did not indicate a local release during the first hour after reperfusion ($P = 0.18$; Table 3).

**Figure 6.** Arterial and venous plasma concentrations of nitrite.

(A) Arterial and renal venous plasma nitrite concentrations during the first 30 minutes of reperfusion in living donor kidney transplantation showed no release from the kidney ($p = 0.50$); (B) In deceased donor kidney transplantation nitrite was not released either during the first 30 minutes of reperfusion ($p = 0.87$); (C) Arterial and myocardial venous plasma nitrite concentrations during the first 60 minutes of reperfusion in patients with preexisting heart failure showed no myocardial release. However, a myocardial uptake was observed ($p = 0.002$); (D) Similarly, in patients without preexisting heart failure a myocardial nitrite uptake instead of a release was observed ($p = 0.03$).
Biomarkers of nitrosative damage

No evidence for nitrosative damage in human kidney transplantation

Nitrite concentrations were equal in renal arterial and venous blood samples (LDs $P = 0.50$, DDs $P = 0.87$; Figure 6). Likewise, nitrate was not released from the reperfused graft either (LDs $P = 0.50$, DDs $P = 0.98$; Figure 7). These findings were confirmed by the additional measurement of plasma nitrotyrosine in LD kidney grafts, indicating no such release ($P = 0.93$, Figure 8). The integral urinary measurement of nitrite and nitrate remained similar after reperfusion in LD kidney transplantations ($P = 0.06$, $P = 0.69$ respectively; Table 3).

![Figure 7. Arterial and venous plasma concentrations of nitrate.](image)

(A) Arterial and renal venous plasma nitrate concentrations during the first 30 minutes of reperfusion in living donor kidney transplantation showed no release from the kidney ($P = 0.50$); (B) In deceased donor kidney transplantation nitrate was not released either during the first 30 minutes of reperfusion ($P = 0.98$); (C) Arterial and myocardial venous plasma nitrate concentrations during the first 60 minutes of reperfusion in patients with preexisting heart failure showed no myocardial release ($P = 0.53$); (D) Similarly, in patients without preexisting heart failure no myocardial nitrate release was observed ($P = 0.81$).
Figure 8. Arterial and venous plasma concentrations of nitrotyrosine. These was no release of nitrotyrosine from living donor kidney grafts during early reperfusion ($p = 0.93$).

**No evidence for nitrosative damage after reperfusion of the myocardium**

Arterial nitrite concentrations were higher than venous concentrations in both patient groups during the early reperfusion phase ($P = 0.002$ preexisting heart failure, $P = 0.03$ no preexisting heart failure; Figure 6), excluding myocardial nitrite release. Nitrate did not change over the reperfused myocardium in both patient groups ($P = 0.53$ preexisting heart failure, $P = 0.81$ no preexisting heart failure; Table 3, Figure 7).

**Oxidative stress**

**Kidney**

Since the above-mentioned results indicated no extracellular evidence for RONS-mediated damage, we tested for the occurrence of oxidative stress during I/R by assessing activation of Nrf2, the master regulator of the antioxidant stress response. Activation of Nrf2 was quantified, as well as the upregulation of Nrf2 responsive genes, heme oxygenase-1 (HMOX-1), NAD(P)H quinone oxidoreductase (NQO1), and glutathione S-transferase A2 (GSTA2) in pre- and post biopsies collected from both kidney and heart (Figure 9). Using a DNA binding ELISA for activated Nrf2, we found clear activation of this transcription factor upon reperfusion ($P = 0.01$; Figure 10), while Nrf2 gene expression increased as well in both LD and DD kidney grafts ($P = 0.03$, $P = 0.04$ respectively; Figure 11). Pathway-based analysis of Nrf2 downstream genes (Ingenuity Pathway Analysis suite, Figure 12) showed highly significant ($P = 7.06 \times 10^{-12}$) upregulation of the canonical antioxidant responsive element (ARE) pathway in kidneys from LDs.
Environmental stress exerts oxidative stress on cells through production of reactive oxygen and nitrogen species (RONS). The oxidative stress response is largely coordinated by a transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2). Under homeostatic conditions, there is only an inactive minimal basal level of Nrf2-directed gene expression under tight control of protein Keap 1. RONS lead to the activation of mitogen-activated protein kinases (such as MAPK, ERK, p38), protein kinase C (PKC), phosphatidylinositol 3 kinase (PI3K), and others, which phosphorylate Keap1 and Nrf2. The Keap1-Nrf2 complex is then disrupted and the transcriptionally active Nrf2 is translocated to the nucleus where it binds to antioxidant responsive elements (AREs) in association with Maf proteins. This results in an increase of the transcriptional expression of Nrf2-inducible genes such as those encoding heme oxygenase-1 (HMOX1), NAD(P)H quinone oxidoreductase (NQO1), and glutathione S-transferase A2 (GSTA2). Together, these proteins scavenge RONS and limit oxidative damage.

Figure 10. Nuclear factor erythroid 2-related factor 2 (Nrf2) activation upon renal reperfusion.
Nuclear factor erythroid 2-related factor 2 (Nrf2) in renal tissue was significantly converted to its activated form after reperfusion ($P = 0.01$) during living donor (LD) kidney transplantation.
Figure 11. Changes in renal expression of nuclear factor erythroid 2-related factor 2 gene. Renal expression of nuclear factor erythroid 2-related factor 2 (Nrf2) gene in biopsies collected before (pre) and 45 minutes after (post) reperfusion during living donor (LD) and deceased donor (DD) kidney transplantation. Y-axis shows the relative change in gene expression as calculated by log(1/2\(^{dCt}\)), where the dCt is the Ct of studied gene minus the Ct of GAPDH, and ddCt is dCt of post-biopsy minus dCt of pre-biopsy. A positive number indicates an increase in expression after reperfusion, whereas a negative number indicates a downregulation after reperfusion. Expression of the transcription factor Nrf2 gene NFE2L2 was significantly upregulated after reperfusion in both LD (P = 0.03) and DD (P = 0.04) kidney grafts.

Conversion of xanthine reductase to XO and accumulation of hypoxanthine during ischemia has been proposed as a major source of oxygen radicals during I/R injury. Histochemical analysis of kidney biopsies collected before and after reperfusion did not indicate the XO activity in renal tissue, whereas ample XO activity and abundant hypoxanthine were observed in the positive control (rat liver, Figure 13).
Figure 12. Nrf2 downstream genes.
Pathway-based analysis of Nrf2 downstream genes showed highly significant upregulation of the canonical antioxidant responsive element (ARE) pathway in kidneys from living donors ($p = 7.06 \times 10^{-12}$).
Figure 13. Xanthine oxidase activity.
Histochemical analysis showing minimal xanthine oxidase activity in pre and post perfusion kidney biopsies. Liver biopsies showing xanthine oxidase activity in the presence (left) and absence of hypoxanthine (middle) are included as a positive control. Staining in the absence of hypoxanthine implies endogenous hypoxanthine. Specificity of the analysis is shown by including the xanthine oxidase inhibitor allopurinol (right).

Antioxidant enzyme activities
SOD and peroxidase activities (catalase and glutathione peroxidase) were evaluated by histochemical analyses of kidney biopsies taken before and 45 minutes after reperfusion. Results of this analysis show abundant and similar antioxidant activities before and after reperfusion (Figure 14).
Figure 14. Endogenous antioxidant enzymes.
Histochemical analysis showing the endogenous antioxidant enzyme systems superoxide dismutase, and peroxidases (catalase and glutathione peroxidase) are active in pre and post perfusion kidney biopsies.

Discussion

Oxidative damage is long considered a key factor in the initiation of I/R injury. Although animal studies repeatedly showed the involvement of free radicals in I/R injury, studies in humans are scarce, and results of clinical intervention studies using antioxidants are inconclusive. Our study shows minimal release of markers of oxidative and nitrosative damage, and did not indicate upregulation of the classical oxidative response genes in both heart and kidney I/R. Results of our study are highly consistent and suggest that the role of oxidative and nitrosative damage in the initiation of clinical I/R injury may be less than commonly thought.

RONS have long been held responsible for the initiation and propagation of I/R injury in many different tissue types, including the kidney, liver, and heart. Initial studies date back to 20 years ago when an increase in lipid peroxidation was shown after experimental
reperfusion of rat kidneys. Since then, further animal studies have supported a role for oxidative damage in I/R. In contrast, studies in humans are scarce and findings are inconclusive. In a clinical study of Grech et al, it was confirmed that RONS are formed after I/R. By using direct spin trapping methods, they demonstrated an immediate free radical production during the first four hours after myocardial reperfusion. However, whether RONS actually lead to tissue damage was not studied. Further studies on oxidative damage in I/R injury in humans show conflicting results. Zahmatkesh et al. showed that after kidney transplantation circulating lipid peroxidation biomarkers rise in peripheral blood. Whether this can be attributed to oxidative damage in the kidney itself or in any other part of the body can not be distinguished by peripheral measurements. Some studies involving myocardial I/R used more specific methods, that is, arteriovenous measurements over the reperfused heart, after percutaneous transluminal coronary angioplasty (PTCA). F2-isoprostanes were released from the reperfused myocardium after PTCA. However, results on MDA release were contradictory, showing no myocardial release after PTCA and low extent MDA release during coronary artery bypass grafting.

Although available antioxidant intervention studies are limited and results are inconclusive, antioxidant treatment appears a potential therapy to study in humans. Somehow, only few trials using antioxidants in human I/R injury are known, and it seems reasonable that a certain amount of publication bias has been introduced. El-Hamamsy et al. showed that administration of the antioxidant N-acetylcysteine around human coronary artery bypass surgery did not lead to improvement in clinical endpoints or decreased release of biochemical markers. Administration of tirilazad mesylate, a nonglucocorticoid 21-aminosteroid inhibiting lipid peroxidation, to patients with acute ischemic stroke even increased the end-point of death instead of having beneficial effects. In kidney I/R only two clinical trials have been published using antioxidants in renal transplantation. Pollak et al. administered SOD intravenously before and one hour after reperfusion, but no beneficial effects were observed with respect to early graft function. One year later, Land et al. administered a single, higher intravenous dose of SOD, immediately before reperfusion. The results demonstrated a significant reduction in first acute rejection. However, up to now, none of both observations have been confirmed in other studies or have led to application in daily practice.

Thus, despite encouraging results of antioxidant I/R therapy in animals, published clinical studies lack an appreciable, clinically relevant effect thus far. Let alone minor differences in clinical set up, there appears to be a mechanistic disparity in the role of oxidative damage and endogenous antioxidant capacity in I/R injury between animals and humans. An explanation could be derived from studies on ageing that have shown that organisms
with large mass-specific metabolic rate, such as mice, have a relative high RONS production and a weak capacity to maintain homeostasis compared with humans. This difference in homeostatic capacity may explain differences in the role of oxidative damage between species as well. Thus, it can be suggested that endogenous antioxidant systems in humans are sufficiently equipped to handle the excess RONS load during clinical reperfusion, thus preventing damage. A finding that is supported by abundant SOD and peroxidases activities before and after I/R.

Whether oxidative damage is indeed prevented in human I/R injury was assessed in this study. RONS have a transient nature and are difficult to assess in vitro and in vivo. Consequently, we measured more stable oxidation products caused by RONS to determine the extent of oxidative damage in I/R injury. By combining multiple biomarkers, a footprint of oxidative and nitrosative damage could be reconstructed. Arteriovenous concentration differences of various established oxidative and nitrosative damage biomarkers were sequentially measured during reperfusion in kidney transplantation. To exclude organ- and procedure-specific findings, kidney results were compared with a different clinical setting, that is, myocardial I/R.

Arteriovenous measurements did not indicate release of oxidative or nitrosative damage biomarkers upon reperfusion of both the kidney and heart with the sole exception of a minimal and transient release of 15(S)-8-iso-PGF2α in a selection of patients. Although the arteriovenous measurements provide important information on local processes in the reperfused organ, sensitivity may be restricted by dilution of released biomarkers because of high flow rates. Therefore, accumulation of oxidative damage markers was measured in urine produced by the transplanted kidney (LD) during the first minutes of reperfusion. Urinary measurements were in accordance to plasma findings, showing no release of oxidative or nitrosative damage biomarkers, including 15(S)-8-iso-PGF2α.

In the absence of other markers, it can be questioned whether venous 15(S)-8-iso-PGF2α release observed in subgroups of patients in this study reflects oxidative damage or alternatively reflects activation of inflammatory pathways. Since all other markers refute oxidative damage, it is highly unlikely that 15(S)-8-iso-PGF2α is the sole reflection of oxidative damage. Altogether, the multiple biomarkers of oxidative and nitrosative damage showed no consistent release upon reperfusion, denying RONS-mediated tissue damage.

Absence of venous release of oxidative and nitrosative damage markers may suggest that oxidative and nitrosative stress during I/R is less pronounced than commonly thought or may even be absent. We evaluated activation of Nrf2, the master regulator in cellular oxidative
defense pathways, in a DNA-binding ELISA that exclusively measures the activated form of this transcription factor. Moreover, the activation of the conical downstream pathway of Nrf2 was evaluated through pathway analysis. Clear increases in Nrf2 activation and its downstream pathway show that oxidative stress does occur during I/R.

Evaluation of the XO activity, an often referred source of oxygen radicals in I/R injury, did not indicate XO activity in human kidney, although abundant XO was found in rat liver. These observations are in line with previous reports showing abundant XO activities in rodent tissue but not in human tissue.46 XO therefore appears not to be a prominent source of RONS generation in human tissue.

Limitations

This is a clinical study, and as such, procedures and timing were dictated by the operative procedures. We did include two clinically distinct situations of I/R to identify universal mechanisms of free radical-mediated damage after reperfusion. In human kidney transplantation, I/R included extremes in both short and long cold ischemia duration, the use of antioxidant preservation fluids, corticosteroids, and other immunosuppressive drugs. In cardiac surgery, I/R involved a normothermic ischemic period, and no antioxidants, corticosteroids, or other immunosuppressive drugs were administered. Throughout this whole spectrum of clinical conditions, independent of antioxidant use or immunosuppression, results unequivocally showed no dominant role of oxidative and nitrosative damage in human I/R injury.

Sampling in our study was restricted to less than an hour post-reperfusion. This time window should be adequate as it is generally assumed that I/R injury is an acute process, initiated directly after reperfusion.18 This notion is supported by spin trap experiments indicating free radical formation within 15 minutes after reperfusion.37 Yet, we can not exclude that I/R injury is less acute than commonly thought and that oxidative damage occurs after an hour of reperfusion.

A second limitation of the study is that it largely relies on repetitive measurement of arteriovenous concentration differences. Although this methodology has the advantage of specificity, and has been proven effective in elaborating the inflammatory response after I/R, the high flow rates over the organs may compromise the sensitivity. To compensate for this limitation, we considered an evaluation of the first urine produced after I/R and measurement of the classical redox response genes relevant.
In conclusion, the findings from this study do not indicate a dominant role for RONS-induced damage in the pathophysiology of early I/R injury. The production of RONS during I/R is explicitly not questioned by our findings. In fact, Nrf2 activation and the higher PGE₂ levels in post-transplantation urine indicate increased COX activity after reperfusion, suggesting that oxidative stress does occur during early reperfusion. Yet, the generated RONS apparently do not induce tissue damage in human I/R. These observations suggest an efficient antioxidant system, providing an explanation for the limited efficacy of antioxidant therapy in human I/R. Altogether, this study challenges the prevailing paradigm of prominent involvement of oxidative damage in the initiation of human I/R injury.

Innovation
I/R injury is a common clinical problem, complicating myocardial infarction, cardiovascular surgery, and organ transplantation. As such, it is of great importance to unravel the exact pathophysiological mechanisms leading to I/R injury. This comprehensive clinical study systematically assessed the putative role of oxidative stress in human I/R injury in two diverse clinical settings. Findings for all biomarkers studied were highly consistent and did not indicate release of markers of oxidative damage from the reperfused organs nor did we observe upregulation of redox-response genes. As such, this study questions the involvement of RONS in the pathophysiology of I/R injury in humans.

Acknowledgements
We thank Lars Verschuren for his help with the pathway analysis. Manon Zuurmond and Gerrit Kracht are gratefully acknowledged for providing, respectively, the image in Figure 1 and Figure 4. Frank-Mathias Gutzki is thanked for performing GC-MS and GC-MS/MS analyses. Professor Anton K. Raap is gratefully acknowledged for critical reading of the manuscript.
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45. Demetrius L. Of mice and men. When it comes to studying ageing and the means to slow it down, mice are not just small humans. EMBO Rep 2005;6:39-44.


Exploring human renal ischemia/reperfusion injury:
a gene array-based bioinformatics approach


In preparation
Abstract

Ischemia/reperfusion (I/R) injury impairs short and long term graft function and survival. Its pathophysiology is still largely unresolved. Proposed mechanisms underlying I/R injury include oxidative damage, complement activation, and inflammation. While targeting of these mechanisms effectively alleviated I/R injury in preclinical models, results from clinical interventions remained disappointing. Therefore, in this study a whole genome expression array was conducted, enabling the comparison of expression profiles in kidney biopsies taken before and after reperfusion. Ingenuity pathway analysis was used to assess the effect of reperfusion, mapping genes in functional categories and canonical pathways. Results show that the majority of metabolism related pathways were exclusively upregulated after reperfusion in grafts of living donors, while they remained unchanged or were downregulated in deceased donor grafts. In conclusion, results from this systematic study show there is no upregulation of metabolism related genes after reperfusion in kidneys of deceased donors. Therefore it has been hypothesized that I/R injury is instigated by metabolic exhaustion, incapacitating the kidney in resuming homeostasis or repair upon reperfusion.
Introduction

Kidney transplantation is the only curative treatment for end-stage renal disease. A persistent donor shortage has led to extension of donor criteria, and use of organs with a less favourable profile. These kidneys are more likely to experience delayed graft function (DGF), which is characterized by a slow functional recovery and impaired long term graft survival. The mechanisms underlying delayed graft function are thought to involve both ischemic damage as well as ischemia/reperfusion (I/R) injury.\(^1\) Although graft procurement methods aimed at reducing ischemic damage have greatly improved in the last decade, currently there is no established therapy that ameliorates I/R injury. This sharply contrasts with an abundance of preclinical studies showing that pharmaceutical interventions reduce I/R injury.\(^2\)\(^-\)\(^7\) This contrast between preclinical and clinical studies points towards poor translatability of preclinical models and an incomplete understanding of clinical (renal) I/R injury.

In a series of studies performed during human kidney transplantation we systematically evaluated factors that have been generally mentioned in the pathophysiology of I/R injury. These studies did not identify oxidative damage, neutrophil or thrombocyte activation as major triggers for I/R injury; whereas indications were found that the emergent post-reperfusion inflammatory response is protective.\(^8\)\(^-\)\(^11\) As such, these observations suggest that human renal I/R injury is (in part) driven by factors beyond those commonly implicated in the process. From this perspective, we considered an unbiased evaluation of the processes involved in the early phase of reperfusion through gene-expression profiling relevant. A comparison between living, brain dead and cardiac dead donor grafts was specifically chosen as kidneys from living donors exhibit outstanding short and long term function and survival, and as such can be considered the primary comparator.

Results of this analysis show that while early reperfusion of kidneys from living donors is characterized by the broad upregulation of metabolism-related pathways, and the pronounced upregulation of the NRF-2 redox-response pathway; kidneys from deceased donors are largely unresponsive and only exhibit a weak upregulation of the redox response pathway. The comprehensive upregulation of metabolic pathways in living donors kidneys and the generic inertness of cadaveric donors imply that metabolic impotence is a driving force of I/R injury.
Materials and Methods

Patient population
Twenty-five patients undergoing renal allograft transplantation were included; of these, ten patients received a kidney from a living donor (LD), 8 from a brain dead donor (BDD) and 7 of a cardiac dead donor (CDD). Kidney transplantations were performed according to the local standardized protocol. In living donors open nephrectomy was performed and Custodiol Histidine-tryptophan-ketoglutarate solution (Tramedico, Weesp, The Netherlands) was used for cold perfusion and storage of the kidney. Brain dead and cardiac dead donor kidneys were perfused and stored with either University of Wisconsin solution or Custodiol Histidine-tryptophan-ketoglutarate solution. The immunosuppressive regimen was based on induction therapy with basiliximab on day 0 and day 4; and tacrolimus or cyclosporine A in addition with mycophenolate mofetil and steroids. Postoperative course was uneventful in all patients. One-year patient and graft survival was 100%. The study protocol was approved by the local ethics committee, and informed consent was obtained from each patient.

Renal Transcriptome Analysis
A renal cortical biopsy was obtained before transplantation at the end of the cold storage phase, and a post-reperfusion biopsy was collected 45 minutes after reperfusion. Biopsies were immediately snap frozen in liquid nitrogen and stored at -70ºC. Total RNA was extracted from renal tissues using RNAzol (Campro Scientific, Veenendaal, The Netherlands) and glass beads. The integrity of each RNA sample was examined by Agilent Lab-on-a-chip technology using the RNA 6000 Nano LabChip kit and a bioanalyzer 2100 (Agilent Technologies, Amstelveen, The Netherlands). RNA preparations were considered suitable for array hybridization only if samples showed intact 18S and 28S rRNA bands, and displayed no chromosomal peaks or RNA degradation products (RNA Integrity Number >8.0). Subsequent microarray analysis was performed using Illumina whole-genome gene expression BeadChips (Illumina BeadArray®, San Diego, USA) according to instructions of the manufacturer at Service XS (Leiden, The Netherlands).

Results were analyzed by biostatistic methods using average replicate values for each of the groups of samples. Log2 ratios were computed and one value per gene was calculated for the average expression of probes with same Entrez ID, resulting in 15093 unique gene profiles. ToxProfiler was used to compute pathway/geneset scores using KEGG pathways. Ingenuity pathway analysis was used for a functional analysis of the data.
Results

Three different groups were compared in this study: living, brain dead and cardiac dead donor kidneys. Living donor kidney transplantations served as primary comparator, while deceased donor kidneys from brain dead and cardiac dead donors were studied separately. Clinical donor and graft characteristics are provided in table 1.

<table>
<thead>
<tr>
<th>Table 1: Transplantation and outcome characteristics.</th>
<th>LD</th>
<th>BDD</th>
<th>CDD</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>10</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Donor age: mean (SD)</td>
<td>52(8)</td>
<td>47(22)</td>
<td>45(18)</td>
</tr>
<tr>
<td>Donor gender (M:F)</td>
<td>3:7</td>
<td>4:4</td>
<td>4:3</td>
</tr>
<tr>
<td>CIT in h. (SD)</td>
<td>3.6(0.5)</td>
<td>17.2(10)</td>
<td>15.1(4)</td>
</tr>
<tr>
<td>Recipient age: mean (SD)</td>
<td>51(16)</td>
<td>56(16)</td>
<td>58(7)</td>
</tr>
<tr>
<td>Recipient gender (M:F)</td>
<td>7:3</td>
<td>3:5</td>
<td>4:3</td>
</tr>
<tr>
<td>DGF</td>
<td>0/10</td>
<td>3/8</td>
<td>6/7</td>
</tr>
</tbody>
</table>

LD: Living donor.
BDD: brain dead donor.
CDD: cardiac dead donor.

Principle component analysis was performed on the pre and post reperfusion biopsy samples. This analysis revealed a clear linear separation between the pre and post reperfusion samples, as well as a separation between the different donor types, thus indicating clear biological differences.

After determining the quantitative gene expression upon reperfusion, the expressed genes were analyzed in clusters or pathways. We first mapped genes in pre-fitted KEGG pathways. This approach revealed a comprehensive upregulation of multiple metabolic pathways upon reperfusion of living donor kidneys. Such an upregulation was not observed in kidney of deceased donors, in fact we only observed upregulation of immunity related pathways such as asthma, cellular antigens and complement in kidneys of brain dead donors, whereas cardiac dead donor kidneys appeared largely unresponsive.

We further explored the array data through the ingenuity database. This pathway based platform identified an unequivocal upregulation of the NRF-2 response pathway as the dominant response in early reperfusion, both in kidneys of living and deceased donors. The ingenuity-based analysis further confirmed conclusions from the KEGG-based pathway analysis. These analyses all point towards a comprehensive upregulation of metabolic pathways in living donor kidneys, whereas deceased donor kidneys were largely unresponsive.
Figure 1: KEGG pathway analysis demonstrates the rapid upregulation of metabolism-related pathways after reperfusion in living donor (LD) kidney transplantation, while in brain dead donor (BDD) and cardiac dead donor (CDD) kidneys expression did not change. Blue color indicates upregulation at 45 minutes after reperfusion compared to before reperfusion. Red color indicates downregulation after reperfusion.
Discussion

Ischemia reperfusion is the inevitable consequence of kidney transplantation. Despite decades of research its pathophysiology has not been unraveled yet. Although many experimental strategies proved successful in preclinical studies, none of them made an appreciable impact on clinical I/R injury. This contrast points to a poor translatability of preclinical models, and an incomplete understanding of I/R injury in humans. We systematically evaluated mechanisms implied in human I/R in clinical kidney transplantation. Findings from this series of studies were highly consistent and did not identify oxidative damage, neutrophil or thrombocyte activation as major triggers for I/R injury.\(^8\)\(^-\)\(^11\) There was an immediate release of IL-6 and IL-9 upon reperfusion, yet evaluation of these inflammatory mediators in an animal model of renal I/R injury suggested a protective or modulating role for these factors. Overall, these observations challenge the current paradigms of I/R injury.

In an effort to find new clues of processes involved in human renal I/R injury, we performed an evaluation of the changes in gene expression during the early phase of reperfusion. We used living donor kidney transplantation as the comparator. Results from the array analysis were evaluated by two bio-informatics platforms: KEGG pathways and the ingenuity database, a functional analysis. Results from the ingenuity based analysis showed that the early reperfusion phase was dominated by upregulation of the NRF-2 pathway: a quickly responsive tissue protective pathway that is activated by redox-stress. Both the ingenuity data base and KEGG pathways also indicated a broad upregulation of metabolism related pathways in living donor kidneys. Kidneys from brain dead and in particular cardiac dead donors exhibited a highly mitigated response with moderate activation of the NRF-2 pathway. Brain dead donor kidneys showed an activation of immune related pathways (KEGG: asthma, rejection) after reperfusion instead. An effect that may reflect activation of infiltrating lymphocytes which are less abundant in living and cardiac dead donor kidneys compared to brain dead donor kidneys.\(^9\)

Results from living donor kidneys showed a clear response to the redox stress during early reperfusion but also a comprehensive upregulation of metabolism related pathways. Deceased donor kidneys on the other hand only showed suppressed upregulation of the NRF-2 pathway and no upregulation of metabolic pathways. This provides a hypothesis in which an adequate response to ischemia and reperfusion in living donor kidneys is characterized by upregulation of a tissue protective network and restoration of metabolic pathways. The suppressed NRF-2 response and absent upregulation of other gene pathways in deceased donor kidneys may reflect a metabolic inadequacy with insufficient energy supply to sustain adequate gene transcription. Such a scenario may well explain the apparent paradox of I/R
injury, in which metabolic failure persists despite adequate oxygen supply. We therefore hypothesize that preservation of an adequate metabolic reserve may ameliorate I/R injury in kidney transplantation and other conditions associated with ischemia reperfusion.
References


11

Future perspectives
The ultimate aim of all studies on the pathophysiology of I/R injury would be to gain insight into the processes which directly cause I/R injury in patients. When the sequence of events and mechanisms involved in I/R injury become more clear, a specific, more targeted therapy may be within reach. However, current treatment is still supportive and simultaneous with mechanistic studies, others focus on experimental therapies that alleviate I/R injury.

The first moment of intervention is already prior to transplantation. Before the actual transplantation the graft has already been exposed to various noxious events, including potential donor brain death and cold preservation. These non-immunological factors such as donor health and the duration of the ischemic period probably have substantial impact on short and long term graft function. Consequently, interventions in the donor aimed at minimizing pre-transplantation graft injury, may potentially have large effects in preventing acute and long term graft dysfunction. In chapter 11.1 the successes of donor pretreatment are discussed.

Next, during transplantation, I/R injury may be limited by modulation of the inflammatory response. Various anti-inflammatory drugs have been tested in animal experiments and human trials. Unfortunately they generally had more side-effects than wanted effects on I/R injury. From this perspective, mesenchymal stromal cells (MSCs) are under extensive investigation, since MSCs are able to exert immune regulatory and reparative effects. These versatile cells have been shown to migrate to sites of injury and to enhance repair by paracrine mechanisms instead of by differentiating and replacing the injured cells. In chapter 11.2 various preclinical studies and the first clinical study are discussed demonstrating the beneficial effects of MSC’s.
11.1

Donor pretreatment in clinical kidney transplantation: a critical appraisal


Accepted for publication in Clinical Transplantation
Abstract

Kidney transplantation represents one of the medical achievements of the 20th century. Its continued success, however, is limited by the increasing shortage of donor grafts. As a result more kidney grafts from marginal donors are being considered for transplantation, with concomitantly more initial graft injury and limited organ and patient survival. This has led to an increased need for interventions aiming to optimize and preserve graft quality. Interventions within the donor may protect against ischemia/reperfusion injury and therefore donor pretreatment is a promising strategy to increase graft function and survival. During the last decade, diverse donor pretreatment interventions have been explored in animal studies. Moreover, the first human trials concerning donor pretreatment in kidney transplantation have provided encouraging results. Unfortunately, it remains difficult to determine how and where to intervene in the multifactorial and complex processes that affect the donor kidney. Moreover, ethical matters play a critical role in donor interventions, and pretreatment should principally not have any potentially unfavorable effects on other organs to be transplanted or on the living donor. This review provides an overview of promising therapeutical strategies for donor pretreatment in kidney transplantation and discusses the clinical trials that have been conducted thus far.
Introduction

Kidney transplantation is the preferred treatment of patients with end-stage renal disease.\textsuperscript{1-3} The previous decade has been characterized by a steady increase in the number of kidney transplantations. This increase largely reflects improved medical therapy for renal failure. Besides the fact that more patients are being considered eligible for kidney transplantation, improved patient survival rate after transplantation led to the emergence of patients requiring re-transplantation, because of progressive loss of graft function in the long run.\textsuperscript{4-6} The augmented demands for kidney transplantation resulted in organ shortage and as a consequence, waiting lists for kidney transplantation are ever increasing. As of June 2012 more than 99,000 American citizens were on the waiting list for a kidney transplantation.\textsuperscript{7-9} Kidney grafts can be derived from living or deceased donors. For the deceased donors, more kidneys are recovered by donation after brain death than from donation after cardiac death. The current organ shortage, however, necessitates expansion of the donor pool by the increased use of marginal donor kidneys. Since these marginal donor kidneys have worse long term outcome, it has been proposed that these may benefit most from pre-transplantation interventions that preserve or even improve graft quality.\textsuperscript{10}

Before and during the process of transplantation the graft is exposed to various noxious events, including donor brain death, cold preservation and ischemia/reperfusion (I/R) injury; potentially all contributing to the functional deterioration of the graft. The importance of these harmful mechanisms is illustrated by the superior results of living donor transplantation. Despite generally more accepted HLA mismatches, living donor transplantation is associated with minimal delayed graft function and improved long term outcomes. This observation suggests that non-HLA-specific factors such as donor health and duration of the ischemic period before transplantation have substantial impact on short and long term graft function.\textsuperscript{11-13}

Consequently, interventions in the donor, aimed at minimizing pre-transplantation graft injury, may potentially have large effects in preventing acute and long term graft dysfunction. This review will focus on the prevention of harmful processes that initiate graft damage in the donor. Various intervention strategies for donor pretreatment that have been tested in clinical kidney transplantation or in animal experiments involving kidney transplantation will be discussed.

Processes in brain death

To date, the majority of deceased kidney grafts are derived from donation after brain death. Brain dead donor kidneys unfortunately have a worse graft and patient survival rate
as compared to living donor kidneys. Brain death leads to dysregulation of the autonomic nerve system, inducing many pathophysiological processes in the human body. Brain death is usually provoked by a period of increased intracranial pressure exceeding the mean arterial pressure and thereby blocking brain perfusion. The physiological responses to this increased pressure and brain damage can have effects on multiple organ systems. The most prevalent derangements are cardiovascular. With increasing intracranial pressure, a compensatory arterial hypertension is induced, sometimes with bradycardia. The catecholamine storm sets in next, with sympathetic stimulation, vasoconstriction, raised systemic vascular resistance, and tachycardia. After the catecholamine storm, there is a loss of sympathetic tone and peripheral vasodilatation. Next, brain death results in severe hemodynamic instability and the resulting hypotension, if untreated, leads to hypoperfusion of all organs. This phase is well-known for the damage it can inflict in organs to be transplanted. Other common clinical problems associated with donor brain death may include diabetes insipidus, disseminated intravascular coagulation, arrhythmias and pulmonary edema. Injury to the hypothalamus and the pituitary gland causes disturbances of hormonal homeostasis and thermoregulation.

On the microvascular level, brain death is associated with the induction of adhesion molecule expression and endothelial cell activation. The hemodynamic, neurogenic, hormonal and microvascular disturbances lead to a generalized inflammatory response in the donor. This is characterized by the release of cytokines into the circulation which can trigger an inflammatory response in all organs with tissue infiltration by granulocytes, monocytes and lymphocytes. All these physiological derangements should be limited as far as possible to maintain optimal graft condition before donation. Donor management is the primary approach to do so.

**Donor management**

After the diagnosis of brain death, there is a change from curative patient care to optimizing organ function for subsequent transplantation. This donor management is the active care of the donor from the time of diagnosis of brain death until procurement of organs and involves correction of the widespread physiological changes that occur during brain death. Early recognition of the potential organ donor and aggressive correction of the non-physiological state, even before consent to organ donation, are crucial to optimize post-transplantation graft function.

In order to standardize management, donor goals have been developed. These aim to maintain physiology close to normal values and were based on measurements performed routinely in patients in the intensive care unit. They include objectives to maintain body temperature, ensure adequate oxygenation, circulating volume, cardiovascular stability,
and adequate urine output. Indeed, in a prospective study, the application of a standardized donor management protocol increased the number of retrieved and transplanted organs per donor substantially.\textsuperscript{18}

One part of donor management is providing cardiovascular support. This support principally includes stabilization of hemodynamics in the donor. Treatment of hypertension associated with the catecholamine storm may significantly increase graft availability for transplantation.\textsuperscript{19} In the consecutive hypotensive period, the first priority is to maintain an adequate intravascular volume. Fluid therapy should, however, be carefully titrated. Recent studies recommend restrictive fluid management, since this restriction increases the number of transplantable lungs without influencing kidney graft function or survival after transplantation.\textsuperscript{20, 21}

Hormone replacement can aid by correcting the loss of pituitary function after brain death, as a method of stabilizing the donor. Posterior pituitary function is lost very commonly, leading to diabetes insipidus with associated fluid and electrolyte changes. Anterior pituitary function may be preserved or only partially affected. Most hormone replacement therapies use a combination of methylprednisolone, vasopressin and thyroid hormone. The fraction of donors that received replacement therapy with these three hormones had an increase in the number of procured organs by 22.5\%, as demonstrated by a retrospective study.\textsuperscript{22}

Aggressive donor management increases the number of organs available for transplantation and has minimized loss of potential donors due to cardiovascular collapse in the process of brain death. Most studies, however, deny a major effect on graft quality and survival of donor management. Grafts may therefore benefit from additional interventions that more specifically prevent organ damage before procurement. This donor pretreatment aiming to further maximize organ quality is an evolving field that constitutes the next step in optimizing kidney graft survival.

**Donor pretreatment**

Donor pretreatment is the active treatment of the donor in order to improve organ quality before and after transplantation. It distinguishes itself from donor management by the fact that donor management concentrates on stabilizing the donor to normal physiological ranges, while pretreatment aims to inhibit potentially harmful processes. Many therapies used as donor pretreatment have been investigated for their ability to reduce or prevent renal I/R injury in animal experiments. Few interventions have been studied in clinical trials, which are summarized in Table 1. Here we discuss the most frequently applied and most promising approaches to donor pretreatment.
Table 1: Human clinical trials of donor pretreatment and result on outcome in kidney transplantation.

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Design</th>
<th>n</th>
<th>Main result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine</td>
<td>Single blind RCT</td>
<td>264 brain dead donors</td>
<td>Dopamine pretreatment decreased the incidence of dialysis post-transplantation. No change in acute rejection or patient or graft survival after 3 years.</td>
<td>Schnuelle et al., 2009 42</td>
</tr>
<tr>
<td>Steroids</td>
<td>Double blind RCT</td>
<td>306 brain dead donors</td>
<td>Donor pretreatment with corticosteroids did not reduce the incidence or duration of DGF.</td>
<td>Kainz et al., 2010 60</td>
</tr>
<tr>
<td>PUVA</td>
<td>Non-randomized</td>
<td>59 deceased donors</td>
<td>PUVA pretreated grafts had a significantly lower number of rejection episodes, other outcome parameters were not different.</td>
<td>Oesterwitz et al., 1987 70</td>
</tr>
<tr>
<td>Hyperoxia</td>
<td>Double blind RCT</td>
<td>60 living donors</td>
<td>Donor oxygen pre-treatment the day before transplantation improved kidney function at 10 days after transplantation.</td>
<td>Montazeri et al., 2011 79</td>
</tr>
</tbody>
</table>

RCT: randomized controlled trial
DGF: delayed graft function
PUVA: psoralen plus ultraviolet A

Ischemic preconditioning

Over the past decades several studies were performed, exposing an organ to brief periods of ischemia to protect against subsequent periods of ischemia and reperfusion. This phenomenon of ischemic preconditioning has been first described in 1986 in the heart.23 Many animal studies, mainly in rats, have reported beneficial effects of donor renal ischemic preconditioning before kidney transplantation since then.24-27 Our present understanding of the molecular mechanisms causing these effects is still largely incomplete. Experimental studies have shown that the protective effects of renal ischemic preconditioning are mediated by adenosine28, nitric oxide 29, 30 and subsequent activation of signal networks involving protein kinases and transcription factors. More complex mechanisms have been proposed recently as well, including cellular actions of regulatory T cells and endothelial progenitor cells.31, 32 It is generally acknowledged that the mechanism of ischemic preconditioning may differ between species and organs and it still remains controversial whether ischemic preconditioning is beneficial in large animals as well. Studies on kidney transplantation in dogs and renal I/R injury in pigs in fact both failed to confirm beneficial effects of renal ischemic preconditioning. Although the first description of ischemic preconditioning dates from almost thirty years ago, the technique has not yet been successfully translated into the clinical setting.33, 34
More recent studies demonstrated that remote ischemic preconditioning confers protection to I/R injury by preceding ischemia and reperfusion of another organ or tissue. In animal experiments, the donor kidney can be protected after transplantation by remote ischemic preconditioning of the hindlimb. Remote ischemic preconditioning suggests the involvement of humoral mediators and consequently protection is both dialyzable, transferable, and receptor-mediated. Remote ischemic preconditioning has the advantage that it is more easily applicable in clinical transplantation than ischemic preconditioning of the graft itself. At present, clinical studies in which remote ischemic preconditioning of the lower limb is explored to improve outcome of kidney transplantation have been initiated and patients are being recruited (www.clinicaltrials.gov).

**Catecholamines**

Before transplantation, the graft is exposed to harmful periods of warm and cold ischemia. Dopamine is capable of protecting endothelial cells from damage during cold preservation by inducing protective enzymes, such as heme oxygenase-1 (HO-1). Dopamine could therefore be a good donor pretreatment candidate, rendering the kidney graft more resistant to I/R injury. In a rat allogeneic kidney transplantation model it was shown that donor dopamine pretreatment diminished histological damage, monocyte infiltration and cytokine expression in the kidney graft. Moreover, both short- and long-term graft function significantly improved. Other catecholamines, like dobutamine and norepinefrin, did not influence post transplantation kidney function.

This preclinical experimental evidence resulted in a clinical trial of donor dopamine pretreatment. Almost 300 brain dead donors were randomized to receive low dose dopamine pretreatment or placebo. Donors had to be stable on low dose noradrenalin and dopamine was continuously infused at a standard rate. The main outcome measure, need for dialysis during the first week after transplantation, was significantly reduced in recipients of a dopamine pretreated graft. Dopamine pretreatment however, did not affect graft or patient survival. Donors in the dopamine administered group showed a significant but clinically not relevant increase in systolic blood pressure. In addition, effects of dopamine pretreatment were more pronounced with increasing cold ischemia time, supporting the hypothesis that the beneficial effects of dopamine are mediated by its protective effects on the endothelium. Dopamine pretreatment may therefore even have the largest effects in marginal donors.

**Heme oxygenase-1**

The ischemic period before transplantation can induce oxidative stress, which on its turn induces the release of the cytoprotective enzyme HO-1. Both HO-1 and carbon monoxide (CO),
a product of HO-1 metabolism, are potential candidates for donor pretreatment. Induced expression of HO-1 in rat kidney donors led to decreased cell infiltration, downregulation of inflammatory genes and diminished histological signs of chronic rejection, resulting in increased graft function and survival after transplantation.\textsuperscript{43}

The effects of HO-1 may be mediated by its downstream CO production, since induction of CO in the donor improved graft function similarly to HO-1, and both HO-1 and CO were able to diminish donor immunogenicity.\textsuperscript{44-47} Cellular effects however, may also be involved in the protective effect of HO-1. HO-1 induction in the donor decreased early post transplant alloreactivity, donor-derived dendritic cells, and T-cell reactivity in the recipient.\textsuperscript{48} Moreover, donor pre-treatment with HO-1 has been shown to improve microcirculation after transplantation.\textsuperscript{49} Both HO-1 and CO appear promising opportunities for donor pretreatment. Whether their application is feasible and beneficial in clinical practice still remains uncertain.

\textbf{Anti-complement therapy}

The inflammatory storm in the process of brain death may induce complement activation in the donor, thereby causing damage in the kidney graft before transplantation.\textsuperscript{50} Inhibition of complement activation in the donor could therefore be beneficial for the kidney graft. The complement system is part of the innate immune system and it can be activated through the classical, alternative and lectin pathway. Soluble complement receptor 1 (sCR1) acts as an inhibitor of the common part of all three complement pathways. Pretreatment of donor rats with sCR1 around induction of brain death prevented the increase in circulating C3d and significantly improved renal function immediately after transplantation.\textsuperscript{51} These first encouraging results should be confirmed in further animal experiments applying other complement inhibitors, before translation to clinical application can be made.

\textbf{Erythropoietin}

Erythropoietin (EPO) was originally identified for its role in erythropoiesis, but is now known for its anti-apoptotic and cytoprotective effects as well. These protective effects are mediated by different receptors and mechanisms than the ones regulating the hematopoietic effects and may defend the kidney from I/R injury, potentially even when administered to the donor. This is particularly interesting considering the conceivably unfavorable side effects of systemic EPO treatment to the recipient.

Two very recent animal studies addressed donor pretreatment with EPO.\textsuperscript{52, 53} In a rat model, brain dead donors were pretreated with EPO or carbamylated EPO (cEPO), which lacks the hematopoietic effects of EPO. Although kidneys were not actually transplanted, short term graft function was analyzed in an isolated perfused kidney set-up. Both EPO and
Donor pretreatment

cEPO diminished the influx of polymorphonuclear cells into the kidney and grafts showed a normalization of creatinine clearance in this isolated perfused kidney model. In a study with larger animals, involving porcine kidney transplantation, cardiac dead donors were pretreated with EPO. In as little as 4 hours after reperfusion, renal injury and inflammation decreased and renal function improved in the EPO pretreated group.

**Immunosuppressive and anti-inflammatory agents**

The period of donor brain death is well known for causing a systemic, nonspecific inflammatory reaction, and organs from brain dead donors are influenced by this inflammatory state. Moreover, the ongoing inflammatory reaction after transplantation is responsible for reperfusion induced tissue damage. When translating findings of animal experiments into human therapies, the general immunosuppressive effects of corticosteroids could be suitable to suppress the inflammatory response in brain dead donors. Methylprednisolone is frequently administered in donor management as part of hormone replacement therapy, but may also be administrated at a higher dose than normally given for replacement therapy, to decrease the inflammatory response. Limited recent data from a large randomized, blind, placebo-controlled trial on donor steroid pretreatment are available. The results show that expression of pro-inflammatory genes in brain dead donors normalized after steroid pretreatment. Although the incidence and duration of delayed graft function did not change with steroid pretreatment, the follow-up period was fairly short and information on longer term graft function is not available as yet. Nevertheless, reducing the pro-inflammatory storm after brain death remains a promising approach, as illustrated by the improved kidney graft survival rate in rats after donor pretreatment with JNK signal transduction inhibition.

Preconditioning of rat donors with calcineurin inhibitors cyclosporine A or tacrolimus decreased structural damage and resulted in improved graft function after kidney transplantation. Pretreatment with tacrolimus combined with rapamycin even improved outcome synergistically. Not all studies could confirm these results, potentially explained by gross differences in experimental set-up and dosing of immunosuppressives between studies.

The basic mechanism behind immunosuppressive donor pretreatment remains unknown. The responsible mechanism appears not to be, as expected, an additional inhibition of the alloimmune response. An effect on renal I/R injury is more likely, since the protective effects have been observed in syngeneic kidney transplant models and appear related to acute renal stress.
**Other pretreatment therapies**

Photosensitizer + UVA (PUVA) treatment was applied in a series of studies nearly 30 years ago. Donor rats were pretreated with a photosensitizer and during hypothermic storage the kidney was irradiated with UVA. PUVA pretreatment improved graft survival in rats, with a dose-response relationship of the irradiation period. The positive effect is attributed to decreased graft immunogenicity. To validate these results, a clinical study was performed in 1986 showing that PUVA pretreated grafts had a significantly lower number of rejection episodes, although all other outcome parameters were not different. Altogether, PUVA pretreatment showed some successes but since then results have never been validated or reproduced by others. Multiple rodent studies report on donor pretreatment with various substances with antioxidant capacities. Among others N-acetylcysteine (NAC), melatonin, danshen, and taurine were applied in animal kidney donors. Most studies showed improvements in biochemical parameters or secondary endpoints only, although some also demonstrated increased survival rates. Nevertheless, unconfirmed results of these single studies on various antioxidants will probably not have a great impact on clinical donor pretreatment in the next few years, unless confirmed or applied in clinical studies.

Miscellaneous studies using diverse donor pretreatment strategies have been published the last few years with varying results. Vagus nerve stimulation was applied as donor pretreatment in brain dead donors for its potential anti-inflammatory effects. Vagus nerve stimulation decreased the expression of pro-inflammatory genes, decreased TNF-α production, diminished monocyte infiltration and more importantly, improved post-transplantation graft function. Another study showed that donor statin pretreatment increased graft function and reduced renal inflammation in a rat kidney transplantation model. Others showed that ICAM-1 inhibition in rat kidney donors improved graft survival, although effects were even larger when ICAM-1 was inhibited in the recipient or during preservation. Disappointing results in animal studies have also been reported; glutamine donor pretreatment did not affect the post-transplantation renal function in rats.

Finally, in humans a remarkable randomised clinical trial showed that hyperoxic donor pretreatment resulted in improved urine production and creatinine clearance after transplantation. Previous animal experiments involving renal I/R without transplantation showed identical results. It may be speculated that hyperoxia induces oxidative stress in the donor, which enhances endogenous antioxidant mechanisms of the kidney. Another explanation is that hyperoxia leads to an improved oxygen reserve capacity of the kidney that protects the energy metabolism during the ischemic period. Finally, there have been reports on successes of hyperthermic donor preconditioning in rodent experiments. Two studies of the same group described beneficial effects of donor hyperthermia on kidney
function and graft survival after transplantation. Hyperthermia induced renal expression of heat shock proteins was held responsible for the beneficial effects.\textsuperscript{82, 83}

**Future perspectives**

Although the principle of donor pretreatment is not new, the clinical trend to use more marginal donors for transplantation only recently necessitated the search for new ways to optimize donor organ quality. Much preclinical research on donor pretreatment has been done, with promising results. The first human trials have recently shown protective effects of donor pretreatment with dopamine and corticosteroids, and these are likely to be of great influence in the years to come. Despite all the promising results from preclinical studies, clinical trials studying donor pretreatment are scarce, especially studies aiming specifically at the effects in marginal donor grafts. It has been suggested that the great amount of groups of interests involved in transplantation, the difficult ethical debate concerning informed consent of deceased donors and the effect of pretreatment on other organs considered for transplantation hampers the translation into the clinical setting.\textsuperscript{9, 84}

In the near future we expect results from some ongoing clinical trials, studying the effects of glucose, ischemic preconditioning, HO-1 induction and dopamine. Donor pretreatment targeting the immune system or oxidative stress responses remain interesting topics, but still have to prove themselves in the clinical setting.

In this review we focused on kidney transplantation. Research on transplantation of other organs will also provide new targets for pretreatment of kidney donors. Animal experiments with 17β-Estradiol as donor pretreatment, for example, showed improved outcome after transplantation of several different organs.\textsuperscript{85} Donor pretreatment with metformin improved acute and chronic rejection in cardiac transplantation in mice.\textsuperscript{86} Ultimately, it is expected that a combination of agents will be used as tailored donor pretreatment, and timing may turn out to be crucial in the success of donor pretreatment.

**Ethical considerations**

In trying to translate donor pretreatment strategies to the clinical setting, ethical issues may be raised, particularly involving deceased donors. Most issues are related to the fact that manipulations will not benefit the donor directly in any way. Living donors provide the simplest situation, where the donor is fully aware of the donation procedure and can provide detailed consent, with prolonged time for consideration and reflection. It is obvious that donor pretreatment may never harm the living donor. The situation is more difficult for deceased donors. The act of joining an organ donor register or carrying a donor card is considered as consent to organ donation, but it is not clear whether such consent would extend to invasive pretreatment strategies, and when they could be started. There is a
need to establish whether pretreatment could be seen as no different from other medical interventions, such as the administration of heparin to donors. One can argue that consent to organ donation suggests consent to all techniques required to allow optimal quality of grafts. Pretreatment may represent part of the normal process of organ donation in the near future, and therefore should not require further consent or lack of objection beyond that associated with organ donation itself.

The timing of pretreatment may be crucial, particularly with regard to agents that require a significant length of time prior to donation, to provide the beneficial effect. Thus, there may be requirements to commence administration of the agent before lack of objection is obtained. Although this might be an unusual principle, this type of approach has already been approved by ethical committees and has been used in clinical trials on organ donors. Deceased donors are often multiple organ donors. Intervention on behalf of one organ may be harmful to other potentially transplantable organs or at least affect them differentially. Donor pretreatment should be carefully tested for the effects on other potential grafts. With progression of the clinical applications of pretreatment, potential new ethical issues will arise and boundaries have to be continuously redefined.

**Conclusion**

Nowadays we are facing a scarcity of donor organs, hence it is absolutely necessary to search for therapeutical options to render more marginal donor organs suitable for transplantation and to improve graft quality. During and before the process of transplantation the graft is exposed to various noxious events, which will lead to functional deterioration. Prevention of injury already in the donor could facilitate transplantation of more marginal donor grafts and provide better outcomes. Donor management is the first step to prevent derangements in the donor and has been much improved by standardization. Further, more specific improvement of graft condition is the aim of donor pretreatment. Donor pretreatment by various strategies, including ischemic preconditioning, HO-1 induction, anti-inflammatory and anti-complement interventions, erythropoietin and catecholamines has been successful in animal experiments. Although many of these promising results in animals have yet to be confirmed in human kidney transplantation, the first pretreatment strategies have already shown encouraging beneficial effects in clinical studies.
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11.2

Mesenchymal stromal cells in treatment of renal ischemia/reperfusion injury

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Introduction

Ischemia/reperfusion (I/R) injury is the exacerbation of tissue damage upon reestablishment of circulation after a period of ischemia. I/R injury is considered a major contributor to tissue damage in multiple clinical situations such as myocardial infarction, stroke and organ transplantation. In many clinical settings, the duration of ischemia is beyond control, and preventive and therapeutical measures are required to reduce the extent of I/R injury. Unfortunately, current treatment is primarily supportive. The pathophysiology of I/R injury is multifactorial and only partially understood. However, the general local reaction to reperfusion is thought to involve an inflammatory response that leads to tissue damage. In the quest for new therapeutical options for renal I/R injury, stem cells have come into play. With their multipotent immune modulating properties they hold promise to lead to improvement in the repair phase after I/R injury is evident.

Renal repair

In recent years, it has become clear that not only fibrotic repair but also restoration of damaged kidney tissue can occur. This has been best established for acute kidney injury, where resident tubular epithelial cells that survive a given damage dedifferentiate and subsequently re-enter the cell cycle and replace the necrotic tubular epithelium. In this process they take up an immature mesenchymal phenotype and re-express transcription factors that are involved in fetal nephrogenesis. More recently, glomerular epithelial repair, involving both local resident cells as well as multipotent progenitor cells has been described.1

Dedifferentiated cells outside the injured kidney may also migrate to the site of injury within the kidney. Kidney biopsies in male recipients of a female donor kidney with acute tubular necrosis showed presence of the male Y chromosome in renal tubular cells. No Y chromosome staining was seen in patients without acute tubular necrosis. This provides evidence that extra-renal cells may participate in renal regeneration.2,3

The call for better treatment strategies for I/R injury has directed research toward more encompassing cellular-based therapies, particularly aimed at the use of stem cells. The multi-factorial pathophysiology of I/R injury makes a pharmacological agent that has a single mechanistic target less likely to be therapeutically effective. In contrast, stem cells are versatile, and able to target a whole cascade of repair mechanisms simultaneously and successively, thereby improving organ protection and repair.
Mesenchymal stromal cells

Of all bone marrow-derived cells, mesenchymal stromal cells (MSCs) hold special promise in attenuating kidney injury, since nephrons are largely of mesenchymal origin and stromal cells are of crucial importance for signalling, leading to differentiation of both nephrons and collecting ducts. MSCs are characterized by three main criteria; 1) The ability to differentiate into osteoblasts, adipocytes and chondroblasts in vitro, 2) the expression of surface makers CD73, CD90 and CD105, and 3) plastic adherence in culture.

MSCs have the ability to secrete numerous growth factors and cytokines that collectively stimulate mitogenesis, inhibit apoptosis and modulate immune responses. MSCs can alter cytokine secretion profiles of naïve and effector T cells, DCs and natural killer cells to induce a more anti-inflammatory or tolerant phenotype. It is proposed that in an inflammatory microenvironment, MSCs promote a Th1 to Th2 shift. Furthermore, MSCs have been reported to induce T-cell division arrest, to inhibit differentiation and maturation of DCs and to decrease production of inflammatory cytokines by various immune cell populations. These immune modulating effects could be achieved both with autologous and allogeneic MSCs.

The mechanism of MSC-induced kidney repair has been addressed in numerous studies. There is growing evidence that the process of transdifferentiation is rare and it probably does not have any relevance to renal repair in vivo. The primary means of these cells most likely involve paracrine and endocrine effects; including mitogenic, anti-apoptotic, anti-inflammatory, antifibrotic and angiogenic influences (Figure 1). The factors that mediate the paracrine effects are obviously of great interest. Several factors, such as IDO, VEGF, HGF and IGF-1 have been mentioned because they are abundant in MSC-conditioned medium.

Chapter 11.2
MSC’s in the treatment of I/R injury

Figure 1: MSCs diminish damage and induce repair. Schematic illustration of the paracrine effects of MSCs on their environment. While stimulating repair by mitogenic and angiogenic effects, MSCs inhibit ongoing inflammation, apoptosis and later fibrosis of injured tissue.

An important aspect of the effect of MSCs is their ability to home to areas of injury or inflammation. Both animal and human studies have provided evidence that stem cells from hematopoietic tissues are able to engraft in nephrons as cells with tubular phenotype. Exogenously administered MSCs can engraft into various injured structures in the kidney. Recently, studies have shed light on the exact factors that facilitate homing of MSCs. Amid them, CD44 and hyaluronic acid interactions were shown to be crucial in recruiting exogenous MSCs to injured renal tissue and to enhance renal regeneration. Others have shown that stromal-derived factor-1 (SDF-1) and CXCR4 interactions may play a role in tubular homing.

Sources of MSCs

While initially isolated from the bone marrow (bm), MSCs have now been identified within most tissues and are thought to represent a perivascular cell population involved in normal tissue homeostasis. Indeed, MSCs have been isolated from adipose tissue, umbilical cord (uc) blood, placenta and different organs. Recently MSCs have also been isolated from the human and mouse kidney. In mice these cells were extensively compared to bm cells. Transcriptome and immunophenotype analysis of the renal MSC-like populations supported strong congruence with bmMSCs. Interestingly, despite this molecular congruence, distinct identities were found within the renal MSC-like population, suggesting organ-specific functions. In addition, it is suggested that the adult mouse kidney contains interstitial mesenchymal cell progenitors that are able to provide paracrine support for surrounding vessels and tubular epithelial cells. Future studies are needed to elucidate whether regeneration and functional repair of damaged kidney epithelium and endothelium can be enhanced via the resident renal stem cells. In the meantime, bmMSCs are the best
characterized population and currently more than 200 clinical trials are ongoing using bmMSCs (www.clinicaltrials.gov).

**MSCs ameliorate renal ischemia/reperfusion injury in vivo**

Although MSCs most probably do not replace damaged cells, evidence on beneficial effects of MSCs in renal I/R injury is accumulating in animal experiments. Intravenous injection of bm derived lineage-negative pluripotent cells after experimental renal I/R significantly attenuated the creatinine rise. Although peripherally administered purified MSCs were quickly present in peritubular capillaries and glomeruli after reperfusion, and functional and histological damage was significantly attenuated by MSC therapy. Even when administered 24 hours after I/R injury, MSCs still were able to ameliorate damage.

Different studies have reported beneficial effects of human MSCs on acute repair in the kidney. The therapeutic potential of human bmMSCs was studied in immunodeficient NOD-SCID mice. Infused bmMSCs reduced renal cell apoptosis and increased proliferation after cisplatin-induced acute renal failure. bmMSCs also preserved the integrity of the tubular epithelium and peritubular vessels, and prolonged survival. In search for new sources of MSCs for renal repair, human ucMSCs were shown to ameliorate both renal dysfunction and tubular cell injury, and prolong survival in cisplatin-induced acute kidney injury. Furthermore, IGF-1 gene-silenced MSCs were limited in their protective effects. These findings indicate that MSCs exert beneficial effects on tubular cell repair in acute kidney injury by producing the mitogenic and pro-survival factor IGF-1.

**Clinical applications of MSCs in renal disease**

There are only limited clinical data concerning MSC therapy in renal disease. The first phase I trial of autologous MSCs in kidney injury enrolls cardiac surgery patients with preexisting renal risk factors and therefore at high risk for developing acute kidney injury. In addition, the first safety and feasibility data of autologous MSC administration in the week after kidney transplantation were recently published. Although data are limited to two patients, MSC infusion appeared feasible and restricted memory T cell expansion while enlarging Treg population. However, both patients showed a transient increase in serum creatinine levels within two weeks after cell infusion. In one patient a renal biopsy was performed, which demonstrated a focal inflammatory infiltrate in the renal interstitium, consisting mainly of granulocytes with very few lymphocytes. It was suggested that the various soluble factors produced by MSCs also include proinflammatory mediators, which eventually may have contributed to the intragraft recruitment of granulocytes, and deterioration of renal function. This may suggest that timing of infusion is of particular importance. Indeed, other studies have shown that the therapeutic properties of MSCs may largely depend on
the timing of their infusion. Importance of timing is probably related to the necessity for the appropriate micro-environment to allow MSCs to acquire their anti-inflammatory properties. In our clinical trial we investigate safety and feasibility of autologous bmMSC treatment in patients with subclinical rejection and/or IF/TA in the renal biopsy at 4 weeks or 6 months after renal transplantation (Clinical trials NCT00734396). Hereby we expect to provide additional information about the importance of timing in the transplant setting.

**Autologous versus allogeneic MSCs**

Until now, most studies have focused on the use of autologous cells, since allogeneic cell transplantation has been reported to be connected with allograft rejection and possibly sensitization. However, the use of autologous MSCs also has disadvantages. The cells need weeks of culture and it is still unclear whether autologous MSCs are affected by the renal disease. A few studies have reported influence of renal failure on MSC behavior. In mice, functional incompetence of MSCs was reported under uremic conditions. In human MSCs it was shown that uremic serum induced an osteoblast-like phenotype in MSCs accompanied by matrix remodeling and calcification.

**MSC number, route of administration, and interaction with immunosuppressives**

Alongside the cell source, the number of MSCs and the timing of administration are critical. In clinical trials to date, most investigators have used doses of $0.4 \times 10^6$ to $10 \times 10^6$/kg body weight. However, no clear correlations have been made between cell dose and clinical effect. Dose escalation studies to monitor safety and efficacy are one of the major objectives for future studies of MSCs.

In most human trials, MSCs have been administered intravenously. Another possible successful route of administration includes intra-arterial or intrarenal infusion. An advantage of these routes may be the direct administration at the place of injury, whereas disadvantages include the complexity and possible side effects such as obstruction of capillaries. To date, there are no reports of these treatment modalities in humans.

Current immunosuppressive drugs cannot be withheld from patients receiving MSC treatment after renal transplantation. Therefore, it is of importance that an optimal concurrent immunosuppressive regimen is chosen in which drugs have no negative impact on MSC function and vice versa. So far, this interaction has mainly been assessed by *in vitro* studies and future studies are needed to elucidate their interaction with concurrent immunosuppression *in vivo* in order to facilitate successful translation to the clinic.
Chapter 11.2

Possible hurdles of MSC treatment
Although cell therapy with MSCs holds enormous promise for the treatment of many diseases, unwanted side effects of MSC infusions must be assessed with the greatest care. Experimental studies have demonstrated maldifferentiation after injecting MSC directly into damaged tissue. In addition, MSCs may adopt an unwanted, myofibroblast-like phenotype after administration. Another important concern is that MSCs may differentiate into neoplastic cells or may cause promotion of tumor cell growth, although an increased risk of tumor formation has never been confirmed in humans. Currently, more than 2000 patients have been treated with allogeneic or autologous MSCs worldwide for a variety of diseases and so far no major side effects have been reported. However, still little is known about long-term side effects.

Summary
The pathophysiology of I/R injury is complex and characterized by inflammation, leading to tissue injury and graft dysfunction. Given the current shortage of donor organs and usage of marginal donor kidneys for transplantation, novel treatment options to minimize renal I/R injury are urgently needed. Recent developments in stem cell research and derived clinical stem cell therapies have given reason to believe that such cell based treatments will become generally available in the near future. Although substantial additional time for the maturation of these therapies for routine clinical use is needed, the first steps of MSC based therapeutic strategies in the treatment of I/R injury have been taken.
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Summary

Kidney transplantation represents one of the most striking medical achievements of the 20th century. Its clinical success, however is limited by ischemia/reperfusion (I/R) injury. Renal I/R injury can lead to fibrosis and both acute and chronic graft dysfunction. Because of the increasing shortage of donors, more kidney grafts from marginal donors are being considered for transplantation, with concomitantly more initial graft injury and limited organ and patient survival. This has led to an increased need for interventions aiming to minimize I/R injury in kidney transplantation.

In chapter 1, the pathophysiology of renal I/R injury is discussed. The exact sequence of events leading to graft injury is complex and incompletely understood. Mainly based on animal experiments, inflammation is considered an important event in the development of tissue injury and graft dysfunction in renal I/R injury. Many individual factors, such as cytokines, complement and platelets have been suggested to be involved in the inflammatory response. Although some intervention studies in animals have shown promising results, results of studies in humans have generally been disappointing. Although this contrast may reflect species-related pharmacokinetic differences or differences in timing of the intervention, the poor translatability could also imply fundamental differences between experimental animals and humans in the pathophysiologic processes involved in I/R injury.

The research presented in this thesis aimed to assess which processes and factors are involved in I/R injury in human clinical kidney transplantation, in order to obtain new insights for therapeutic modalities. To be able to study local processes during I/R of the kidney, paired arteriovenous blood samples were collected during early reperfusion. In chapter 2, the first results of measurements in these arteriovenous samples are described. Interleukin (IL)-6 was released in large amounts from the graft in human living donor kidney transplantation, suggesting its potential role in the pathophysiology of I/R injury. However, in the consecutive intervention study utilizing a model of bilateral renal ischemia and reperfusion in mice, anti-IL-6 treatment did not protect from renal I/R injury. Moreover, kidney damage even increased in the intervention group. Another cytokine of interest was IL-9, which is exclusively released during reperfusion from deceased donor kidneys. Chapter 3 describes that, similar to IL-6, inhibition of IL-9 during renal I/R injury in mice did not result in improvement of renal function, but instead worsened renal injury. These studies indicate that renal I/R injury apparently is not easily modifiable by inhibiting a single factor only. The complex interplay between cytokines possibly includes the ability of other cytokines to replace the effects of the inhibited factor.
By measuring the release of multiple cytokines at once, we tried to gain insight in how the network of cytokines changes upon reperfusion in living, brain dead and cardiac dead donor kidney transplantation. In chapter 4 it is shown that brain dead donor kidneys display a massive inflammatory cytokine release upon reperfusion, while kidneys from living and cardiac dead donors showed a more modest cytokine response with release of IL-6 and small amounts of monocyte chemoattractant protein-1 (MCP-1). These released cytokines may originate from infiltrated T lymphocytes and macrophages. Already before transplantation, these cells were far more prevalent in brain dead donor kidneys than in living and cardiac dead donor kidneys. These observations suggest that brain dead donor kidneys have a pro-inflammatory tendency, that induces the release of many cytokines upon reperfusion. Brain dead donor kidneys may therefore require a different approach in the prevention of I/R injury, aimed at inhibiting this inflammatory response.

While cytokines are signaling molecules, the complement system has both signaling and lytic functions within the innate immune system. Activation of either the classical, alternative, or mannan-binding lectin complement pathway ultimately leads to the formation of the membrane attack complex, C5b-9. Animal studies of renal I/R injury generally show that complement inhibition reduces post-reperfusion damage. In chapter 5.1 we assessed whether the complement cascade is activated during clinical renal I/R injury. Arteriovenous measurements showed no release of sC5b-9 from living donor kidneys, but a transient venous release of sC5b-9 from the reperfused graft in brain dead and cardiac dead donor kidney transplantation. This activation of the terminal complement cascade was not reflected by renal tissue deposition of C5b-9 in biopsies taken 45 minutes after reperfusion. These results indicate there is instantaneous, intravascular terminal complement activation upon reperfusion, that might be induced by intravascular cellular debris and hypoxic or injured endothelium. Besides measurements in blood and biopsy tissue, urinary sC5b-9 would also be an attractive biomarker to study. However in chapter 5.2 we show that urinary sC5b-9 can be formed extra-renally in case of procedure-related (microscopic) hematuria and proteinuria. Therefore, sC5b-9 in urine is not a reliable reflection of complement activation after kidney transplantation.

Platelets also have an important role in the innate immune response, and can serve as mediators of the innate immune system, apart from their primary function in haemostasis. Although in preclinical studies activated platelets have been implicated in the inflammatory response after reperfusion, their role in clinical human I/R injury is unknown. In chapter 6 we used our method of arteriovenous measurements over the reperfused kidney to assess release of markers of platelet activation and degranulation. No evidence for platelet activation and degranulation in the reperfused kidney was found. Next, we focused on
measurement of more subtle changes in excitability of platelets. In the novel, bedside platelet excitability assay, platelets were stimulated and their response measured. Remarkably, platelets in renal venous blood were less easily and less intensely excitable than platelets in arterial blood. Since platelets were not trapped in the kidney, this may reflect true inhibition of platelet excitability upon passing the injured kidney, therefore suggesting platelets probably do not initiate the inflammatory response of I/R injury.

The most important step preceding platelet activation would be endothelial damage. Interactions between endothelial cells and pericytes, the main supportive cells of the endothelium, are important in maintenance of vascular integrity. Angiopoietins are the signaling molecules facilitating these interactions. Experimental data have shown that enhancement of Angiopoietin (Ang)-1 signaling may be beneficial in renal I/R injury. Little is known however, about the behavior of angiopoietins in human renal I/R injury. In chapter 7, endothelial cell activation and changes in angiopoietins were assessed in human living and deceased donor kidney transplantation. Local release of angiopoietins was measured by arteriovenous measurements over the reperfused kidney. Results showed acute endothelial cell activation by a vast Ang-2 release from both living and deceased donor grafts shortly after reperfusion. Its counterpart Ang-1 was not released. Histological analysis of kidney biopsies showed endothelial cell loss after reperfusion. Baseline Ang-1 protein and mRNA expression was significantly reduced in deceased compared to living donors and declined further after reperfusion. These results showed that human renal I/R injury induces endothelial cell activation after reperfusion reflected by Ang-2 release from the kidney. Interventions aimed at maintenance of vascular integrity by modulating angiopoietin signaling may be promising in human clinical kidney transplantation.

Another way by which endothelial cells may be involved in the pathophysiology of renal I/R injury is by changes in expression of the adenosine generating enzymes CD39 and CD73. Recent animal experiments suggest that extracellular adenosine may be a critical mediator in protection from renal I/R injury. Information on adenosine production in human kidney transplantation however, is lacking. In chapter 8 changes in protein and mRNA expression of adenosine generating enzymes CD39 and CD73 in transplanted human kidneys were assessed. Results showed that CD39 protein is overrepresented in living compared to deceased donor kidneys before transplantation. However, CD39 mRNA expression was not different between groups and did not change upon reperfusion. CD73 mRNA expression was significantly downregulated after reperfusion, while its tissue protein expression did not change. Results showed that living donor kidneys may be protected by higher pre-transplantation CD39 expression, although this effect may be counteracted by the decrease in CD73 expression after reperfusion. Further studies will need to focus on the consequences of these enzyme
changes for renal adenosine generation and kidney graft injury. The results from chapter 6, 7, and 8 together demonstrate that there is endothelial injury upon reperfusion, although this apparently does not lead to platelet activation.

Oxidative damage has been considered a key factor in the initiation of I/R injury for decades now. Although findings from preclinical studies show that quenching reactive oxygen and nitrogen species (RONS) alleviates I/R injury, results from clinical intervention studies in humans are largely inconclusive. In chapter 9 we systematically evaluated release of established biomarkers of oxidative and nitrosative damage during reperfusion of the transplanted kidney. Interestingly, none of the measured biomarkers of oxidative and nitrosative damage (i.e. malondialdehyde, 15(S)-8-iso-prostaglandin F$_{2\alpha}$, nitrite, nitrate and nitrotyrosine) were released upon reperfusion. Cumulative urinary measurements confirmed plasma findings. The production of RONS during I/R is explicitly not questioned by these findings. Indeed, we found increased urinary PGE$_2$ after reperfusion and activation of Nrf2, the master regulator of oxidative stress signaling. Together this illustrates that there is an increase in oxidative stress during early reperfusion. Yet, the generated RONS apparently do not induce tissue damage in human I/R. These observations suggest a very efficient antioxidant system, providing an explanation for the limited efficacy of antioxidant therapy in human I/R injury. Overall, results of this study challenge the prevailing paradigm of prominent involvement of oxidative damage in the initiation of human I/R injury.

Altogether, the studies presented in this thesis suggest that inflammation is the prevailing process in the early reperfusion period in human kidney transplantation. When assessing the mechanisms potentially underlying I/R injury however, it remained unclear what initiates I/R injury and what causes the differences in outcome between living and deceased donor kidneys. In contrast to the approach in the previous chapters, in chapter 10 we examined the genome-wide expression patterns of donor kidneys prior to and 45 minutes after reperfusion. The comparison of gene expression between living and deceased donor kidneys showed an explicit difference in metabolism related genes. Living donor kidneys regained the expression of genes associated with metabolism shortly after reperfusion, while these genes all remained downregulated after reperfusion in deceased donor kidneys. These fundamental differences provide a totally new viewpoint, indicating that failure to restart metabolism after reperfusion may lead to enhanced I/R injury in deceased donor kidney grafts. This hypothesis is strengthened by the abundant release of lactate from deceased donors, which is released in many times higher concentrations from deceased than living donor kidneys. More extensive studies to assess the mechanism and implications of the failure to restart metabolism after transplantation are required.
Therapeutical modalities and current progress in prevention of I/R injury are discussed in chapter 11. The first intervention possibility is prior to transplantation. Before the actual transplantation, the graft is already exposed to various noxious events, including potential donor brain death and cold preservation. These non-immunological factors such as donor health and the duration of the ischemic period probably have substantial impact on short and long term graft function. Consequently, interventions in the donor, aimed at minimizing pre-transplantation graft injury, may potentially have large effects in preventing acute and long term graft dysfunction. In chapter 11.1 the various therapeutical interventions that have been tested in donors are reviewed. As suggested by the results in chapter 4, anti-inflammatory interventions in the donor may prevent consequent I/R injury after transplantation. Donor pretreatment by various other strategies, including ischemic preconditioning, HO-1 induction, anti-complement interventions, erythropoietin and catecholamines has been successful in animal experiments. Although many of these promising results in animals have yet to be confirmed in human kidney transplantation, the first clinical studies have already shown encouraging results. Next, during transplantation, I/R injury may be limited by modulation of the inflammatory response. In this perspective, mesenchymal stromal cells (MSCs) are under extensive investigation, since MSCs are able to exert immune regulatory and reparative effects. These versatile cells have been shown to migrate to sites of injury and to enhance repair by paracrine mechanisms instead of by differentiating and replacing the injured cells. In chapter 11.2 various preclinical studies are discussed that demonstrate the beneficial effects of MSCs in ameliorating renal injury and accelerating tissue repair. Moreover, the first phase I studies of MSCs in human renal I/R injury and kidney transplantation have been started, and results are awaited soon. Preliminary results from clinical studies and recent developments in stem cell research have given reason to believe that MSC-based treatments will become generally available in the near future.

In conclusion, the studies in this thesis describe the systematical search for factors involved in the pathophysiology of human renal I/R injury. Many of the processes assumed to be involved in renal I/R injury based on animal studies could not be confirmed in our clinical study in humans. However, we found new evidence of complement activation and endothelial cell activation in human renal I/R injury. Moreover, there were large differences between deceased and living donor kidneys; brain dead donor kidneys have a unique pro-inflammatory cytokine release after reperfusion. Finally it appears that both brain dead and cardiac dead donor kidneys are not able to upregulate their metabolism-related genes upon reperfusion as living donor kidneys do, indicating that failure to restart metabolism may be a factor expanding I/R injury. All of these findings contribute to the understanding of renal I/R injury in humans and instigate the further search for therapeutical modalities to limit renal I/R injury.
Samenvatting in het Nederlands

Niertransplantatie is een van de meest opvallende medische prestaties van de 20e eeuw. Helaas wordt het klinisch succes ervan nog altijd beperkt door ischemie/reperfusie (I/R) schade. I/R-schade veroorzaakt acute en chronische transplantaatdisfunctie en kan leiden tot fibrose en het falen van de getransplanteerde nier. Door het toenemende tekort aan donoren worden steeds vaker organen van marginale donoren gebruikt voor transplantatie, waarbij in de regel meer schade optreedt en de overleving van het transplantaat slechter is. Dit creëert een behoefte aan interventies gericht op het verminderen van I/R-schade in de getransplanteerde nier.

In hoofdstuk 1 wordt de pathofysiologie van I/R-schade van de nier besproken. De exacte volgorde van processen die leiden tot schade aan de getransplanteerde nier is complex en niet volledig bekend. Inflammatie wordt als cruciaal beschouwd in de ontwikkeling van weefselbeschadiging en transplantaatdisfunctie door nier I/R-schade. Van vele individuele factoren, zoals cytokines, complement en bloedplaatjes wordt gesuggereerd dat zij betrokken zijn bij de inflammatoire reactie. Helaas zijn de resultaten van de uitgevoerde onderzoeken bij mensen over het algemeen teleurstellend, hoewel sommige interventiestudies bij dieren veelbelovende resultaten hebben laten zien. Hoewel verschillen in de pharmacokinetiek of verschillen in timing van de interventie dit contrast tussen uitkomsten bij mens en proefdier zouden kunnen veroorzaken, impliceert de beperkte interspecies translatie tussen soorten dat er fundamentele verschillen zijn tussen proefdieren en mensen in de pathofysiologie van I/R-schade.

De studies beschreven in dit proefschrift zijn gericht op het ontrafelen van de processen en factoren die betrokken zijn bij I/R-schade bij klinische niertransplantaties, zodat er nieuwe inzichten kunnen ontstaan om doelgerichte therapiën te ontwikkelen. Om lokale processen te bestuderen tijdens reperfusie van de nier, werden arterio-veneus gepaarde bloedmonsters afgenomen tijdens de reperfusiefase. In hoofdstuk 2 worden de eerste resultaten van de metingen in deze arterio-veneuze monsters beschreven. Bij levende donor niertransplantatie kwam interleukine (IL)-6 in grote hoeveelheden vrij uit het transplantaat. Dit suggereert dat het een rol speelt in de pathofysiologie van I/R-schade. Uit de daarop volgende interventiestudie van bilaterale renale ischemie en reperfusie in muizen, bleek echter dat de anti-IL-6 behandeling niet tegen nier I/R-schade beschermt. De schade aan de nier was zelfs toegenomen in de interventiegroep. Een ander cytokine van belang was IL-9, dat uitsluitend vrijkwam uit overleden donor nieren na reperfusie. Hoofdstuk 3 beschrijft dat, net als bij IL-6, remming van IL-9 in nier I/R-schade bij muizen niet resulteerde in verbetering van nierfunctie, maar juist in een toename van de schade aan de nier. Deze...
studies geven aan dat nier I/R-schade bij niertransplantatie blijkbaar niet gemakkelijk te beïnvloeden is door het remmen van een enkele factor alleen. De complexe wisselwerking tussen cytokines onderling maakt het waarschijnlijk mogelijk dat andere cytokines de effecten van de geremde factor overnemen.

De suggestie dat cytokines of andere inflammatoire mediatoren betrokken zijn, wordt bevestigd in een volgende studie waarbij de arterioveneuze afgifte van verschillende cytokines systematisch vergeleken is tussen levende, hersendode en hartdode donorniertransplantaties. Door het vergelijken van de afgifte van meerdere cytokines, is getrokt inzicht te krijgen in de manier waarop het cytokinenetwerk verandert na reperfusie in deze verschillende groepen. In hoofdstuk 4 wordt aangetoond dat nieren van hersendode donoren een massale inflammatoire reactie doormaken, waarbij meerdere cytokines vrijkomen na reperfusie. Getransplanteerde nieren van levende en hartdode donoren toonden een meer bescheiden cytokinerespons met afgifte van IL-6 en kleine hoeveelheden monocyte chemoattractant protein-1 (MCP-1). Deze vrijgekomen cytokines kunnen afkomstig zijn van de geïnfiltreerde T-lymfocyt en macrofagen die al vóór de transplantatie in nieren van hersendode donoren in veel grotere aantallen aanwezig waren dan in nieren van levende en hartdode donoren. De bevindingen in dit hoofdstuk suggereren dat nieren van hersendode donoren een pro-inflammatoire tendens hebben, geïnduceerd door het vrijkomen van de vele cytokines na reperfusie. Daarom vereisen hersendode donoren mogelijk een aparte aanpak bij de preventie van I/R-schade, specifiek gericht op het remmen van deze inflammatoire reactie.

Cytokines zijn signaleringsmoleculen, het complementsysteem heeft echter zowel signalerings- als lytische functies binnen het aangeboren immuunsysteem. Activering van de klassieke, alternatieve of mannan-bindende lectineroute leidt uiteindelijk tot de vorming van het functionele complex C5b-9. Dierexperimenten laten over het algemeen zien dat complementremming de post-reperfusie nierschade vermindert. Mogelijk zijn deze resultaten te translateren van de muis naar de mens. Derhalve hebben we in hoofdstuk 5.1 eerst onderzocht of de complementscascade wordt geactiveerd tijdens klinische nier I/R-schade. In de arterioveneuze metingen kwam sC5b-9 niet vrij uit nieren van levende donoren, maar bij de hersendode en hartdode donor niertransplantaties kwam sC5b-9 wel kortdurend vrij uit het gereperfundeerde orgaan. Deze activering van de terminale complementscascade leidde niet tot de afzetting van C5b-9 in nierbiopsieën 45 minuten na reperfusie. Deze resultaten wijzen op acute, intravasculaire terminale complementactivatie na reperfusie, die mogelijk geïnduceerd wordt door intravasculaire celresten, of contact met hypoxisch of beschadigd endotheel.

Naast de metingen in bloed en weefsel, zou sC5b-9 in urine ook een aantrekkelijke biomarker kunnen zijn. Helaas blijkt dit niet mogelijk, omdat sC5b-9 ook extrarenaal kan worden
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gevormd in het geval van (operatie-gerelateerde) hematurie en proteïnurie. In hoofdstuk 5.2 concluderen we dat de meting van sC5b-9 in urine niet betrouwbaar is als marker van complementactivatie na niertransplantatie.

Bloedplaatjes maken tevens onderdeel uit van het aangeboren afweersysteem. Naast hun primaire functie in hemostase, kunnen bloedplaatjes dienen als mediatoren van het aangeboren immuunsysteem. Hoewel in preklinische studies aangetoond is dat geactiveerde bloedplaatjes betrokken zijn bij de inflammatie na reperfusie, is hun rol bij humane klinische I/R-schade onbekend. In hoofdstuk 6 hebben we opnieuw gebruik gemaakt van arterioveneuze metingen over de gereperfundeerde nier, nu om het vrijkomen van markers van bloedplaatjesactivatie en -degranulatie te bepalen. Resultaten toonden geen bloedplaatjesactivatie of -degranulatie in de nier na reperfusie. Daarom werd vervolgens onderzocht of er meer subtiele veranderingen zijn in de activeerbaarheid van bloedplaatjes. In de nieuwe bloedplaatjesactiveerbaarheidsassay werden bloedplaatjes gestimuleerd in toenemende mate en hun reactie daarop gemeten. Het is opmerkelijk dat bloedplaatjes in het veneuze bloed uit de nier minder makkelijk en minder intens geactiveerd raakten dan bloedplaatjes in het arteriële bloed. Omdat de bloedplaatjes niet achterbleven in de nier, lijkt dit te berusten op een remming van de activeerbaarheid van de bloedplaatjes bij het passeren van de gereperfundeerde nier. Al met al suggereerden de bevindingen dat bloedplaatjes waarschijnlijk niet de initiator zijn van de inflammatoire reactie bij I/R-schade van de nier.

Endotheelschade wordt gezien als de belangrijkste stap voorafgaand aan activering van bloedplaatjes. Erg belangrijk bij het voorkomen van deze schade zijn de interacties tussen endotheelcellen en pericyten, de belangrijkste ondersteunende cellen van het endotheel. Deze interacties worden gefaciliteerd door signaalmoleculen, de angiopoietines. Uit dierexperimenten blijkt dat verhoging van angiopoietine (Ang)-1 signalering beschermend werkt bij nier I/R-schade. Er is echter weinig bekend over de rol van angiopoietines bij humane nier I/R-schade. In hoofdstuk 7 werden endotheelactivatie en de veranderingen in angiopoietines onderzocht en vergeleken bij niertransplantaties met levende en overleden donor. Het lokaal vrijkomen van angiopoietines werd bepaald in arterioveneuze metingen over de gereperfundeerde nier. De resultaten toonden acute activatie van het endotheel, blijvend tot het fors vrijkomen van Ang-2 uit de nieren van zowel levende als overleden donoren. De tegenhanger van Ang-2, Ang-1, kwam niet vrij uit de nier. Histologische analyse van nierbiopten toonde verlies van endotheelcellen na reperfusie. De eiwit- en mRNA-expressie van Ang-1 was significant minder in biopten van nieren van overleden donoren vergeleken met levende donoren. De Ang-1 expressie daalde verder na reperfusie. Samen tonen deze resultaten aan dat bij nier I/R-schade in de mens endotheelcellen beschadigd
raken, volgend uit de Ang-2 afgifte uit de nier na reperfusie. Interventies gericht op behoud van vasculaire integriteit door het beïnvloeden van de angioptiinesignalering zijn veelbelovend voor humane klinische niertransplantatie.

Een andere manier waarop endotheelcellen betrokken kunnen zijn bij de pathofysiologie van nier I/R-schade, is door hun expressie van de adenosinegenererende enzymen CD39 en CD73. Recente dierexperimenten suggereren dat extracellulaire adenosine een belangrijke rol speelt in de bescherming tegen I/R-schade. Er is echter nog weinig bekend over de rol van extracellulair adenosine bij niertransplantaties in de mens. In hoofdstuk 8 werd de eiwit- en mRNA-expressie van adenosinegenererende enzymen CD39 en CD73 bepaald in getransplanteerde menselijke nieren. De resultaten toonden aan dat vóór transplantatie het CD39-eiwit meer aanwezig was in nieren van levende dan overleden donoren. De mRNA-expressie van CD39 was niet verschillend tussen de groepen en veranderde ook niet na reperfusie. De mRNA-expressie van CD73 was significant minder na reperfusie, terwijl de eiwitexpressie niet veranderde. De resultaten suggereren dat nieren van levende donoren worden beschermd door hogere pre-transplantatie CD39-expressie, hoewel dit effect kan worden tegengegaan door de afname van CD73-expressie na reperfusie. Verdere studies zullen zich moeten richten op de gevolgen van deze enzymveranderingen op de adenosinegeneratie in de nier en het uiteindelijke letsel aan de nier. Samenvattend tonen de resultaten van hoofdstuk 6, 7 en 8 aan dat er endotheelschade is na reperfusie van de nier, hoewel dit niet leidt tot bloedplaatjesactivatie.

geheel genomen gaan de resultaten van deze studie in tegen het heersende paradigma van prominente betrokkenheid van oxidatieve schade in de initiatie van I/R-schade in de mens.

Concluderend suggereren de studies in dit proefschrift dat inflammatie een belangrijk proces is in de reperfusieperiode van humane niertransplantatie. Echter, het blijft moeilijk in te schatten welke processen belangrijk zijn bij het veroorzaken van I/R-schade en wat de verschillen tussen nieren van levende en overleden donoren veroorzaakt. In tegenstelling tot de aanpak in de voorgaande hoofdstukken, is in hoofdstuk 10 gepoogd nieuwe aanknopingspunten te vinden door de expressiepatronen van het totale genoom te bepalen in nierbiopten, genomen voor en 45 minuten na reperfusie. De vergelijking van genexpressiepatronen tussen nieren van levende en overleden donoren toonde een expliciet verschil in metabolisme-gerelateerde genen. Terwijl bij nieren van levende donoren de expressie van genen geassocieerd met metabolisme kort na reperfusie herstelde, bleven deze genen in nieren van overleden donoren alle ge-downreguleerd na reperfusie. Deze fundamentele verschillen impliceren een totaal nieuwe hypothese, namelijk dat het onvermogen om het metabolisme te herstarten na reperfusie kan leiden tot toegenomen I/R-schade bij niertransplantaties met nieren van overleden donoren. Deze hypothese wordt versterkt door de resultaten van lactaatmetingen, waarbij lactaat na reperfusie in vele malen hogere concentraties vrijkwam uit nieren van overleden donoren dan van levende donoren. Deze bevindingen zullen echter moeten worden bevestigd in uitgebreidere studies naar het mechanisme en de implicaties van het falen van het herstarten van het celmetabolisme.

bij dierproeven. Alhoewel menig van deze veelbelovende resultaten bij dieren nog moeten worden bevestigd bij de mens, hebben de eerste klinische studies bij niertransplantatie al bemoedigende resultaten laten zien.

Ten tweede kan tijdens transplantatie de I/R-schade beperkt worden door modulatie van de inflammatoire reactie. Vanuit dit perspectief zijn mesenchymale stromale cellen (MSC’s) uitgebreid onderzocht, omdat MSC’s in staat zijn om het immuunsysteem te beïnvloeden en herstellende effecten uit te oefenen. Van deze veelzijdige cellen is aangetoond dat ze migreren naar plaatsen van schade en daar herstel stimuleren door hun paracriene effecten en niet door differentiatie en vervanging van de beschadigde cellen. In hoofdstuk 11.2 worden verschillende preclinische studies besproken die de gunstige effecten van MSC’s in het verminderen van schade aan de nieren en het versnellen van het weefselherstel tonen. Bovendien zijn de eerste fase 1-studies van MSC’s ter vermindering van I/R-schade bij klinische niertransplantatie gestart. De definitieve resultaten worden binnenkort verwacht, echter de voorlopige resultaten van klinische studies en recente ontwikkelingen in het onderzoek naar stamcellen geven reden te geloven dat behandelingen met MSC’s toegepast zullen gaan worden in de nabije toekomst.

De studies in dit proefschrift beschrijven de systematische zoektocht naar factoren die betrokken zijn bij de pathofysiologie van I/R-schade van de menselijke nier. Veel processen die op basis van dierstudies verondersteld waren betrokken te zijn bij deze I/R-schade konden niet worden bevestigd in onze klinische studies van niertransplantatie. We vonden echter nieuw bewijs voor de betrokkenheid van complement- en endotheelcelactivatie bij nier I/R-schade in de mens. Bovendien waren er grote verschillen tussen transplantaties met nieren van levende en overleden donoren. Hierbij bleek dat nieren van hersendode donoren een unieke combinatie van pro-inflammatoire cytokines afgeven na reperfusie. Een andere belangrijke bevinding is dat nieren van zowel hersendode als hartdode donoren nieren niet in staat lijken te zijn de metabole motor opnieuw op te starten na reperfusie. Al deze bevindingen dragen bij aan een beter begrip van I/R-schade van de nier en stimuleren de verdere zoektocht naar therapeutische modaliteiten om de effecten van I/R-schade te beperken.
**List of publications**


Curriculum Vitae

The author of this thesis was born on January 23, 1982. She grew up in Zoetermeer and graduated cum laude from secondary school (Atheneum) in 2000. In the same year she started to study Biomedical Sciences at Leiden University. After two years, she decided to combine this study with the study of Medicine. In 2006 she completed Biomedical Sciences cum laude with a graduate research project at the department of Transplantation Surgery (Dr. A.F.M. Schaapherder). The research project on ischemia/reperfusion injury in kidney transplantation made her enthusiastic to continue on the subject during her clinical rotations. In 2008 she obtained her medical degree cum laude. That same year she was selected for an AGIKO scholarship by ZonMw.

Appointed by this scholarship she continued an expanded the research on I/R injury from 2008-2010. She engaged in successful research collaborations with groups from the Nephrology department. During her research years and thereafter, she presented her findings on many international meetings and was awarded multiple grants and scholarships. Based on the results described in this thesis, additional studies on the role of metabolism in I/R injury have been started, in which she is still involved.

From January 2010 the author started surgical residency at the Leiden University Medical Center (LUMC) under supervision of prof dr J.F. Hamming, and since July 2012 she continued her training in the Haga teaching hospital, The Hague, under supervision of dr. J.W.S. Merkus and later dr. J.J. Wever. She lives with her husband Paul and their son Brem in Wassenaar.
Dankwoord

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