Ischemia/reperfusion injury : a metabolic meltdown

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Part III

Summary

Future Perspectives

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SUMMARY AND FUTURE PERSPECTIVES

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SUMMARY

Kidney transplantation is considered one of the greatest medical achievements of the last century. Although developments have resulted in significant improvements in both patient and graft survival rates, ischemia/reperfusion (I/R) injury continues to negatively impact the success rate of kidney transplantations. Despite numerous experimental studies indicating promising results in the prevention and treatment of I/R injury, to date no clinical interventions are available. This highlights the importance of unravelling the pathophysiological mechanisms causing I/R injury in humans.

This thesis explores the pathophysiology of I/R injury. *Chapter 1* outlines the objective of the thesis and describes why kidney transplantation is a predictable, accessible and clinically relevant model for studying I/R injury in humans. In addition, the latest research regarding the pathophysiology of I/R injury is reviewed. Previous studies by de Vries et al. demonstrate that the culprit mechanisms commonly assumed (such as thrombocyte, endothelial, neutrophil and complement activation) are not critical mechanisms in the acute phase of reperfusion injury.[1–3] In addition, the lack of effective antioxidant therapies and absence of radical oxygen species (ROS)-mediated-damage biomarkers suggest that the role of ROS in initiating clinical I/R injury may be lesser and perhaps not as critical as generally thought.[4]

In *Chapter 2* the presumed key role for ROS in I/R injury is further examined. The hypoxanthine-xanthine oxidase axis is assessed as a potential source of ROS. The demonstration of low and stable xanthine oxidase (XO) activity in ischemic and reperfused human kidneys indicated that the enzyme xanthine oxidase is not a source of ROS in human I/R injury. The absence of end products of xanthine oxidase reactions (i.e. uric acid and oxypurinol) further validates this finding. In humans, allantoin is the stable end product of the antioxidant activity of uric acid. The fact that no allantoin was released from the reperfused grafts, not even from DGF kidneys, fundamentally challenges the notion that I/R injury in human kidneys is driven by (XO produced) ROS.

In situ enzymology did show substantial XO activity in ischemic rat kidneys, contrasting the absence of activity in human biopsies. This interspecies difference explains why preclinical studies targeting the XO system did protect against I/R injury - while there was no effect in human studies. *Chapter 3* describes differences in I/R injury between species, indicating a translational gap between experimental models (e.g. rodents) and the clinical situation.[5,6] A widely acknowledged

letter in Nature stated a new, promising explanation for the mechanism of I/R injury: "ischemic accumulation of succinate drives reperfusion injury through mitochondrial ROS".[7] This theory was deducted from studies using mice and results described in Chapter 3 indicate that succinate-driven reactive oxygen formation does not occur in human kidney transplantation. This profound difference between mice and humans could be attributed to mitochondrial susceptibility to I/R. Human mitochondria were far more vulnerable than mice mitochondria, with human mitochondria having impaired capacity to oxidize succinate after exposure to brief ischemia. Thus succinate driven I/R injury appears to be a murine phenomenon and consequently addresses the difficulty of translating findings in mice to humans with regards to I/R injury research.

In the absence of evidence for involvement of the canonical pathways in clinical I/R injury, new approaches were used to unravel underlying mechanisms. Unbiased transcriptomic and metabolomic analyses were performed in the setting of clinical kidney transplantation. DGF development was used as the readout for I/R injury. *Chapter 4* describes measurements of arteriovenous(AV) concentration differences over the reperfused graft. These AV measurements show persistent lactate release in the acute phase of reperfusion in grafts that later developed DGF. Conversely control grafts did not show persistent lactate release and renal acidosis after reperfusion, indicating immediate reestablishment of aerobic respiration in control grafts. Biopsies taken at the end of the ischemic period and 45 minutes after reperfusion validated the results of the plasma measurements. Metabolomic analysis of these biopsies shows an almost immediate increase of tissue glucose/lactate rate in grafts with adequate functional recovery, on the contrary DGF grafts showed persistently low glucose/lactate rates. These observations implicate defective mitochondrial oxidative phosphorylation (OXPHOS) in DGF grafts. Transmission electron microscopy confirmed mitochondrial damage, with imaging showing mitochondrial morphology recovery in grafts that functioned adequately after reperfusion and deterioration in DGF grafts. These findings imply that DGF development is preceded by a profound post-reperfusion metabolic deficit that results from severe mitochondrial damage.

The results of Chapter 4 suggest that mitochondrial dysfunction and sequential energetic deficits are key drivers of I/R injury. It was realized (and concluded following the cohort study in Chapter 6) that almost all kidney grafts suffering from DGF start recovering within a few days to sometimes weeks after transplantation. As recovery is energy-dependent, this raises the question which metabolic pathways would still function in DGF grafts. To address this question, *Chapter 5* compares metabolic adaptation to ischemia and reperfusion in DGF

versus control grafts.

In summary, the differences in metabolic adaptation between DGF and control grafts were identified in 5 functional clusters: (I) metabolic collapse (power outage); (II) β-oxidation; (III) glycolysis/glutamine oxidation and autophagy; (IV) Krebs cycle (entry) defects, and (V) phospholipolysis/cell damage. This metabolome-broad approach shows that DGF grafts are hallmarked by energetic exhaustion ('metabolic meltdown'). Unlike control grafts, kidneys that developed DGF showed a decrease in phosphocreatine levels and continuous production of hypoxanthine and xanthine after reperfusion. This indicates ATP/GTP catabolism and a failure of ATP production in grafts that later develop DGF. This apparent metabolic deficit appears despite abundant activation of β-oxidation, glycolysis/glutamine oxidation and possibly autophagy. The accumulation of the Krebs-cycle entry-products acetylcarnitine and pyruvate point to Krebs cycle (entry) defect(s) in DGF grafts and release of these metabolites indicates that these carbon flows exceed the capacity of the Krebs cycle. Release of the Krebs cycle intermediate α-ketoglutarate, in combination with the absence of succinate recovery implies graded defects of oxoglutarate dehydrogenase activity in DGF grafts.

Findings in chapter 5 extend conclusions from experimental studies and our earlier reports. It shows that incident DGF not only associates with mitochondrial dysfunction, but that damage extends beyond the membrane bound respiratory complexes and also involves complexes located in the mitochondrial cytosol. The fully preserved β-oxidation in DGF grafts implies that these effects are specific, and not merely reflect gross mitochondrial damage. Subsequent to this mitochondrial dysfunction, the ATP deficit leads to inability to maintain homeostasis, which results in a state of on-going tissue damage after reperfusion. This is reflected by the continuous release of phospholipids and uracil, which mark membrane and cell damage respectively.

Since DGF resembles a state of energetic crisis, attempts to mitigate DGF should focus on sustaining a minimal level of metabolic competence. In order to identify candidate targets, the post-reperfusion metabolome of renal I/R injury was established. It is hypothesized that ATP could be provided both by enhancing glycolysis (via inhibiting glutaminolysis) and the catabolism of inosine. This is discussed in the 'Future Perspectives' section of this thesis.

To further explore factors determining the short- and long-term outcomes of kidney transplantation, graft function and survival were analysed in *Chapter 6*. Analysis of the Netherlands Organ Transplant Registry (NOTR) indicates a

50% higher incidence of primary non-function (PNF), and an almost tripled incidence of delayed graft function (DGF) in kidneys donated after cardiac death (DCD) (n=2891), compared to kidneys donated after brain death (DBD) (n=4084). The higher incidence of DGF in DCD compared to DBD grafts results in reservations regarding the use of DCD grafts in kidney transplantation. However, after excluding the grafts with primary non-function (7,9% of all DCD and 4.5% of all DBD grafts) 10-year graft survival was similar for both donortypes. Further evaluation shows that duration of cold ischemia longer than 24 h disproportionally mitigates graft survival of DCD grafts ($P<0.001$).

It was shown that incident DGF negatively impacts graft survival in DBD grafts, while it does not so in DCD grafts. It was realized that this differential impact of DGF on DBD grafts might reflect biological differences between the graft-types. Indeed, functional recovery curves show an exponential "catch-up" in DCD grafts that fully compensates for the initial graft loss, thus resulting in similar long-term graft survival for both donortypes. Hence, the current negative conception regarding the survival rate of DCD donors might need revision.

Following conclusions in Chapter 3, 4 and 5, *Chapter 7* proceeds on the role of mitochondria. No biomarkers currently exist to predict functional graft recovery during DGF. With mitochondrial dysfunction underlying DGF, it was hypothesized that mitochondrial regeneration precedes functional recovery. Therefore mitochondrial regeneration was expected to be a potent biomarker for functional recovery. To validate this hypothesis, graft biopsies taken during the period of DGF were examined (n=30). Mitochondrial SOD and YAP-1 (activation of Hippo signalling) qualified as histological markers of upcoming functional graft recovery. It came to our attention that these markers may also hold the key to potential therapeutic targets.

To further explore mitochondrial targets in clinical I/R injury, *Chapter 8* studies the role of mitochondrial aldehyde dehydrogenase (ALDH) enzymes. ALDHs are enzymes catabolizing toxic aldehydes, which originate from lipid peroxidation in I/R injury. Epidemiological studies show that deactivating point mutations in the ALDH2 gene lead to increased damage following myocardial I/R injury.[8,9] Results of Chapter 8 show that ischemia and reperfusion in control grafts is marked by enrichment of (Ingenuity) pathways involving ALDH genes - this enrichment is not found in grafts that later develop DGF. Additionally, in the acute phase of reperfusion, significantly higher ALDH activity is found in controls compared to DGF grafts. Based on these results, decreased ALDH enzymatic activity can potentially be used as a biomarker to predict DGF. Above that, mitochondrial

ALDH enzymes are seen as a promising target and improving ALDH activity may potentially decrease the incidence of DGF.

Chapter 9 investigates an alternative intervention that may be effective in limiting I/R injury. The results of the 'POSITIVE' study are described: Pre-Operative STatin InterVEntion and cardioprotection for mitral valve surgery. During mitral valve surgery, ischemia of the myocardium is induced by cardiopulmonary bypass (CPB) and its reperfusion sets in when the CPB clamps are released. Results of this exploratory trial show that pre-treatment with statins at least two weeks prior to on-pump cardiac valve surgery reduces myocardial damage without affecting the inflammatory response. Further studies are needed to explore whether donor pre-treatment with simvastatin will be effective in mitigating renal I/R damage.

Chapter 10 summarizes all experimental and clinical studies that have been performed on donor pre-treatment in kidney transplantation. Several strategies showing promising results in animal studies are described (e.g. ischemic preconditioning, HO-1 induction, anti-inflammatory interventions, anti-complement interventions, epo and catecholamines) - however a clear lack of clinical studies to support these results remains (ischemic preconditioning[10,11]; epo[12]; cyclosporine[13,14]; melatonine[15]). While recognizing the ethical issues regarding including deceased donors as a research subject when studying donor pre-treatment, it is anticipated that the insightful gains that could be realized outweigh these concerns and thus enable us to overcome this moral dilemma.

FUTURE PERSPECTIVES

This thesis investigates the mechanisms underlying I/R injury in human kidneys, responsible for the development of DGF. The main conclusion (Chapters 4 and 5) is that mitochondrial dysfunction is the key driver. This is supported by a recent scientific statement published by the American Heart Association, which states that mitochondria play a critical role in cardiovascular pathologies like I/R injury.[16]

The insight that DGF grafts are in a state of deep energetic crisis (Chapter 5) indicates that that an ATP-dependent intervention given after reperfusion will not be effective. This finding sheds a whole new light on the field of I/R injury research. It is postulated that future attempts to mitigate DGF should focus on sustaining a minimal level of metabolic competence. This can be accomplished in two ways:

(I) by therapeutics targeting ATP-production pathways that are still functional
(II) through preventive strategies protecting mitochondria during I/R.[17]

I. THERAPEUTICS: TARGETING INTACT ATP-PRODUCING PATHWAYS

To identify intact ATP-producing pathways to deal with the deficit described above, the post-reperfusion metabolome of renal I/R injury (i.e. DGF grafts) was established. The metabolome of DGF grafts can be described as follows: continuous lactate, alanine and asparagine release in reperfused DGF grafts signals persistent post-reperfusion aerobic glycolysis. This process also involves extensive glutaminolysis indicated by the glutamine uptake and glutamate release into the circulation. Recovery of post-reperfusion hydroxybutyrate levels indicate intact β-oxidation in DGF grafts. Interestingly, data for lipid oxidation showed a low, albeit persistent, post-reperfusion release of short-chain carnitines (≤C6) from DGF grafts. This indicates peroxisomal β-oxidation in DGF grafts, since this is limited to large and medium chain lipids.
Despite these intact pathways providing Krebs cycle entry products (glycolysis, glutaminolysis, β-oxidation), DGF grafts do not produce sufficient ATP due to impaired oxidative phosphorylation as result of Krebs cycle defect(s). From the pathways described above, aerobic glycolysis is the only pathway that yields ATP (2*ATP).

Throughout the process, it was noticed that both glutaminolysis and peroxisomal lipid oxidation could limit the ATP production of aerobic glycolysis. Glutaminolysis and lipid oxidation produce H+, leading to acidification of the

graft and causing the pH-sensitive enzyme lactate dehydrogenase (LDH) to be ineffective. In this manor, glutaminolysis and peroxisomal lipid oxidation can potentially restrict the ATP production normally generated by LDH. LDH is essential for aerobic glycolysis since it breaks down pyruvate into lactate. Therefore, it is hypothesized that inhibiting these pathways will allow for the production of more ATP on a net basis due to the removal of H+ production (acidification) which hampers the functioning of LDH. Thereby, peroxisomal lipid oxidation is accompanied by stoichiometric hydrogen peroxide production, which may perpetuate I/R injury. This is another reason why inhibiting this pathway could be a potential target for therapy.

Another hypothetical pathway that could increase ATP production is through the breakdown of inosine to hypoxanthine, which yields 8*ATP.[18] This pathway is effective in erythrocytes: aerobic glycolic cells devoid of mitochondria. Since mitochondria are dysfunctional in DGF grafts and inosine is lost in post-reperfusion biopsies, supplying inosine to the reperfused graft could potentially be an effective option to produce ATP. Along these lines, inosine is superior to glucose in preserved cellular ATP content of eukaryotic cells during hypoxia[19] and ameliorates tissue damage in experimental ischemia/reperfusion models.[20,21]

II. PREVENTIVE STRATEGIES; PRESERVING MITOCHONDRIA

In addition to maximizing the ATP production in DGF kidneys, a higher level of effectiveness will be achieved by also focusing on preventive strategies. Since mitochondrial damage underlies DGF, preventive strategies should focus on preserving mitochondria.[17] Mitochondrial preservation and recovery[22–25] are currently considered to be valid targets for intervention[26,27] and results from clinical studies are anxiously awaited. This thesis describes two other approaches to preserve mitochondria. Chapter 4 describes the protective effect of the cardiolipin-binding peptide SS-31 (Bendavia) during I/R. Chapter 8 hypothesizes about the protective role of detoxifying mitochondrial ALDH enzymes.

The organ transplantation process is exceptionally suitable for preventive strategies[28] as there are several points of intervention before I/R.

Donor pre-treatment is one option. The timeframe of donor pre-treatment encompasses a highly controlled situation in which the donor is monitored at the ICU until the moment the organs are procured for donation. During this time mitochondria could be successfully targeted prior to the induction of I/R injury. However, performing clinical studies with potential donors brings an extra level

of complexity and is accompanied by ethical difficulties (as is discussed in Chapter 10).
Another option for implementing a preventive strategy is during machine perfusion.[28,29] The time between donation and transplantation (i.e. when the ischemic donor graft is perfused on a pump) could provide an ideal opportunity to target mitochondria.[17]

CLINICAL APPLICATIONS

One of the most important conclusions of this thesis is that DGF is caused by metabolic incompetence due to mitochondrial dysfunction. To identify the clinical variables associated with DGF and transplantation outcome, a retrospective cohort study was performed (Chapter 6). DCD donortypes are associated with increased incidence of DGF and PNF, indicating superior short-term outcome of DBD grafts. Results of functional graft recovery imply a difference in biology between DBD versus DCD grafts - indicating a superior recovery potential of DCD grafts. This superior recovery potential of DCD grafts results in the most important clinical conclusion, namely that after restriction of duration of cold ischemia time to 24 h or less, 10-year graft survival is similar in DBD and DCD grafts. Currently reservations exist regarding the use of DCD grafts in kidney transplantation.[30–32] In fact, only 10 out of 27 European countries presently accept DCD donor grafts in their kidney transplantation programs [33,34], while only 10% of all deceased donor kidney transplantations in the USA are DCD donor grafts.[35] The results of this thesis suggest that hesitancy towards the use of DCD grafts might not be justified, if cold ischemia duration does not exceed 24 h. This could help alleviate current difficulties caused by donor shortages and reduce the 3,5 years average waiting time for a kidney transplant in the Netherlands (NTS data).

FUTURE RESEARCH

Research/study design

Several reports recently stated that the majority of I/R injury research had missed its purpose as they comprised of non-translatable animal studies.[36–38] Statements from the NIH[39], FDA[40] and several other (transplantation) expert institutions[6,37] confirm the translational gap between preclinical research and I/R injury in humans, disapproving non-translatable studies.
By understanding and investigating the mechanisms of I/R injury in humans, this thesis indicates that human mitochondria are more vulnerable to I/R than rodent mitochondria – this could explain the significant translational gap between experimental models and clinical I/R.[5] Based on the results of Chapter 3, it is hypothesized that pigs are a better model than rodents as their mitochondrial vulnerability to I/R is more similar to humans. Additionally, pig's metabolic

adaptation to I/R shows similarities to humans as it is characterized by the release of betaine, methionine and serine.[41] It is recommended that future studies be performed on the translation of porcine to human I/R injury.

Another recommendation (Chapter 5) is that metabolomic studies should not only rely on tissue data as this provides a static view of the actual situation and is therefore (potentially) biased. Tissue metabolomics should be combined with plasma metabolomics which will provide a more dynamic and just point-of-view of metabolic processes.

Study focus / questions to be answered
In the near future research has to localize the mitochondrial defects of grafts that later develop DGF. Labelled Krebs-cycle intermediates can be used to identify which step of the Krebs cycle is dysfunctional. When the defect is known, targeted preventive or therapeutic measures can be developed to prevent DGF or enhance the regeneration of grafts that developed DGF.
Another challenge will be unravelling epidemiological questions and possibly connecting these to graft biology. Results of Chapter 6 show that incident DGF impairs graft survival of DBD grafts, but not of DCD grafts. Dynamical studies show superior recovery potential of DCD grafts and this difference in resilience between the two donortypes needs to be investigated. Interestingly, in both donortypes increased donor age was an independent risk factor for inferior graft function. The question rises whether there is a connection between (mitochondrial) senescence (aging) and increased vulnerability to I/R or decreased resilience/regeneration capacity. Creating the bigger picture where clinical (patient/epidemiological) data is combined with cell biology will be the pursuit of future research.[42,43]

LIMITATIONS
For this thesis, human kidney transplantation was chosen to study I/R injury. It provides a situation of planned, complete organ ischemia and reperfusion and therefore is preferable for studying I/R injury than clinical stroke or myocardial infarction. However, this model does have some limitations. Due to limited size of the patient groups it was not possible to address specific points such as different donor and recipient characteristics – however larger samples sizes would not expect to change the conclusions, but only result in smaller confidence intervals. Furthermore the effects of prolonged cold ischemia, as found in the transplantation setting, are not directly translatable to other clinical situations like myocardial infarction and stroke since these are caused by so-called warm ischemia. Another concern is that the mechanism of I/R injury as found in the

kidney, may not directly be translatable to other organs, since metabolic profiles, preference substrates and mitochondrial respiratory may differ. In fact it is known that differences in vulnerability for I/R injury exists across various organs.

SUMMARY

This thesis provides insights into the mechanisms of renal ischemia/reperfusion injury based on human kidney transplantation (i.e. DGF). A severe energetic crisis differentiates DGF kidneys from adequately functioning controls. Although intact β-oxidation, aerobic glycolysis and glutaminolysis provide Krebs Cycle intermediates, these intermediates are not able to enter the mitochondrial Krebs cycle. Hence, dysfunctional mitochondria disable efficient ATP production leading to the metabolic incompetence that causes DGF. This finding sheds a whole new light on I/R injury and explains why ATP-dependent therapeutics remain ineffective.

A major difference in the vulnerability of mitochondria to ischemia and reperfusion between rodents and humans was found. This could explain the current differences in effectiveness of therapies in the experimental versus the clinical setting and highlight the translational gap. Big cohort studies as described in Chapter *6* give insights in donor, recipient and transplant-procedure variables and challenge the reluctance towards the use of DCD donor kidneys. Superior recovery potential of DCD compared to DBD grafts was established and future studies need to deepen the knowledge on these biological differences. This thesis shows that recovery of the mitochondrial pool and activation of the Hippo pathway precede functional recovery. The downstream end-products of these pathways (MnSOD, Yap-1) can be used as markers to predict functional recovery. New preventive strategies could limit I/R injury by preserving mitochondria (such as hypothetical treatments with the peptide SS-31 or activation of the mitochondrial enzyme aldehyde dehydrogenase).

The big challenge will be the identification of pathways and targets that effectively preserve mitochondrial function and prevent the energetic crisis underlying DGF. Large translational studies should be combined with clinical and cell-biological data to understand the link between I/R injury and long-term outcomes. This will overcome the detrimental effects of I/R injury on graft function and survival - thereby increasing the success rate of kidney transplantation.

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