Ischemia targeted therapies during critical periods of organ preservation

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DOI:
10.33612/diss.658835672

Chapter 9

General discussion

Improvement of kidney transplantation

Ischemic tissue damage resulting from the surgical extraction procedure remains an important clinical problem in kidney transplantation. This thesis aims to gain insight and find potential solutions to mitigate the impact of ischemic injury. In addition to quantification of the impact of the duration of ischemia, also the role of temperature during this ischemic period is investigated. Furthermore, the effect of temperature on mitochondrial function and reactive oxygen species (ROS) production were determined and new strategies to decrease ischemic injury were evaluated. These strategies include the use of hydrogen sulphide (H_2S), oxygen and glucose during the flush-out at the start of extraction procedure. Lastly, a new way of visualizing renal cortical microperfusion (RCM) was studied, thereby improving the assessment of damage resulting from ischemia and getting better insight into organ quality.

Extraction time

First, research was performed to identify the optimal moment to apply protective therapies to counteract ischemic injury during organ retrieval, transport and transplantation. For donation after brain death (DBD), the first option would be to supply therapeutics during the ICU phase. This, however, brings ethical and logistical concerns. Also, interventions in the donor will not only affect the kidneys, but all organs. During donation after circulatory death (DCD) it is not possible in most countries to intervene before the surgery starts due to legislation and logistics. Therefore, the first more realistic moment to start a therapy to treat the transplantable organs would be during the abdominal aortic flush-out in both deceased donation types. The aortic flush is also the start of the cold-ischemia time and is often referred to as the start of organ protection. However, it is important to realize that the organ is still in situ and has not reached the required low temperature for organ protection.

Previous research showed a negative impact of prolonged extraction time on liver transplantation outcome.[1] In smaller cohorts, the impact of extraction time of kidney transplants was studied, showing a higher risk of delayed graft function (DGF) with increasing extraction times.[2] Animal studies showed that an increase in extraction time resulted in an increase in kidney temperature.[3] This increase causes warm ischemic injury instead of the presumed cold ischemic injury.[4] **Chapter 2** of this thesis shows that prolonged postmortem kidney extraction time is associated with a higher rate of graft loss in Dutch donors. The effect on graft loss was not found in American donors, for which prolonged extraction time was only associated with increased odds of developing DGF.

The results from the American donors might however be biased due to the large number of missing extraction times in the U.S. donor database (UNOS). Nonetheless, the presented research combined with previous data demonstrates the impact of prolonged extraction time on transplantation outcomes. These findings emphasize the need to minimize extraction time as much as possible or otherwise to reduce the ischemic injury that occurs during extraction surgery. The harmful impact of prolonged extraction time on the transplant is presumably due to rise of temperature during extraction period. It is well known that warm ischemia is injurious to grafts, leading to worse kidney transplantation outcomes[5,6] and should therefore be counteracted.

Renal temperature, metabolism and ROS

Mitochondria play an important role in ischemia and ischemia reperfusion injury. During ischemia succinate accumulates which is re-oxidized when oxygen is introduced during reperfusion.[7] Re-oxidation of succinate results in a reverse electron transport in complex I of the mitochondria which in turn causes the production of ROS.[7] The function of the mitochondria in producing ATP is highly affected by temperature,[8] but less is known on their temperature dependent role during ischemia. Therefore, we studied the differences in metabolism and ROS production between 4 °C and 37 °C in **Chapter 3**. Oxygen consumption was highly affected by the reduction in temperature, whereas ROS production was only mildly decreased. The observed discrepancy in lowered metabolism and relative limited decreased in ROS production could either be the result of impaired ROS scavenging capacity in a cold environment or a relatively high ROS production by complexes of the mitochondria. Complex I and III of the electron transport chain (ETC) are known to be the primary source of mitochondrial ROS production and could have been reduced less in activity compared to the ATP producing complexes IV/V.[9] In addition, one of the defence mechanisms of the mitochondria to reduce ROS levels, i.e. MnSOD levels, were decreased during hypothermia compared to normothermia.[10] Regardless of the cause of the relative minor drop in ROS production, these results emphasize that simply forcing hypothermia onto an organ is not without risk. Although warm ischemia is more detrimental on transplantation outcome than cold ischemia,[5,6] cold temperatures might not be sufficient in preserving transplantable organs. Although organs can be preserved for hours instead of minutes during cold preservation, their quality is reduced equivalently on the time spend in the cold.5 Simply forcing hypothermia onto an organ during the extraction period might not be the key to a successful preservation. In addition, in current clinical practice it is not possible to cool down the organ during extraction to the intended 4 °C.[3] Especially in prolonged extraction surgery this temperature is not reached in the current clinical setting due

to a limited flush-out and removal of the ice during extraction of the liver. Therefore, organ preservation at subnormothermic temperatures might aid a solution. These temperatures, mostly around 21 °C, have been tested during organ preservation, but not to a great extent. Subnormothermic preservation is mostly tested during machine perfusion, reducing acute tubular necrosis compared to static cold storage (SCS).[11] Unfortunately, subnormothemic machine perfusion was not compared to static storage at 22 degrees Celsius and SCS was not compared to hypothermic machine perfusion (HMP). The missing data indicate that the perfusion of the organ might be responsible for the lower ischemic injury rather than the different temperature. The same shortcomings also account for other studies, in which perfusion setups are used instead of static preservations.[12] Another factor that limits the interpretability of the results is the supplementation with therapeutic substances during subnormothermic perfusion.[13] If higher temperatures are used during preservation, sufficient metabolic support during this phase could decrease the impact of ischemia. Another option would be to decrease the metabolic rate of the kidney during the flush-out of the kidneys, without inducing the injurious effects of forced hypothermia. Therefore, the following chapters focussed on improving preservation at the start of the extraction. **Chapter 5 & 6** researched the concept of artificially inducing a hypometabolic state and **chapter 7** on supporting the remaining metabolism during the flush-out to reduce ischemic injury.

H_2S and hypometabolism

First presented by Blackstone et al., H_2S was described as a potent substance to induce a hibernation-like state in small mammals.[14] By artificially inducing a suspended animation-like state in a non-hibernating species, research focussing on H_2S became increasingly prevalent. Hibernating animals can sustain life and resume normal organ function after long periods of low blood flow, low temperature, low cardiac output, and low metabolism during the hibernation season.[15,16] During the donation and transplantation procedure, periods of low blood flow and low metabolism occur which makes the induction of suspended hibernation an interesting potential therapy. In contrast to hibernators, kidney transplants do not overcome periods of ischemia without any organ injury since both warm and cold ischemic time are directly related to transplantation outcomes.[5,6] Numerous research groups presented research on decreasing ischemic injury by treatment with H_2S, primarily studied in the heart and kidneys. In kidneys, the initial studies were performed in small mammals such as mice. Treatment with H_2S prior to an ischemic event showed promising results in mice by decreasing metabolism and ischemic organ damage and improving survival.[17] These results were confirmed by others.[18,19] Translation between this pre-clinical small mammal

experimental work to actual human transplantation is an extensive journey. The first successful report in larger mammals was performed on miniature swine, which was published in 2017. Sekijima et al. showed a decrease in cytokine release in a model of warm ischemia in miniature swine.[20] These results were less evident than those in the mice and rat experiments, without any impact on the survival of the animals. In addition, other large mammal studies performed on sheep[21] and pigs[22] did not show a hypometabolic effect from the addition of H_2S, as was shown in mice.[14] The prevention of ischemic injury by H_2S is, however, shown to be partly independent of hypometabolism.[23]

In **chapter 5** we explored whether it was possible to induce a hypometabolic state in an isolated human-sized porcine kidney since induction of hypometabolism in full-size pigs and sheep has not been successful. Small mammals endure a more significant beneficial effect regarding hypothermic effects of hypometabolism due to H_2S treatment compared to larger animals due to a higher body surface to mass ratio.[24] Therefore, by using isolated porcine kidneys, we bypassed the previous mentioned issue and tested the effect of H_2S using normothermic machine perfusion (NMP) to support physiological metabolic rates. Recapitulatory, it was possible to induce a hypometabolic state using H_2S based on oxygen consumption of the kidney and the mitochondrial membrane potential (MMP) of isolated mitochondria. Oxygen consumption promptly decreased after infusion of H_2S which could imply that the mitochondria became inactive by blockade of cytochrome c oxidase (complex IV), consequently consuming less oxygen indicating hypometabolism. However, these results could be biased since H_2S can bind competitively with hemoglobin.[25] Due to this competition, the capacity of hemoglobin to bind O_2 could be decreased leading to a relative increase in dissolved O_2 levels in the venous perfusate. Since oxygen consumption was calculated with the dissolved O_2 instead of the bound O_2, this could have distorted the results. Nonetheless, it was possible to reduce the MMP, indicating a decrease in the metabolic rate of the mitochondria by infusion of H_2S. The decreased MMP advocates the first hypothesis, that O_2 consumption is decreased by blockade of complex IV. The effects were observed without harming the kidneys, preserving normal function of the kidney on the pump. Therefore, we continued the research on H_2S in decreasing ischemic injury in the following experiments.

H_2S-enriched flush-out

In **chapter 6**, the effect of an H_2S-enriched flush-out was investigated in both brain dead and non-brain dead donors using a pig model. H_2S treatment decreased inflammatory cytokines in the DBD kidneys without affecting renal function or injury markers.

The results presented in **chapter 6** are in accordance with in vitro studies that suggest an anti-inflammatory role of H_2S by attenuation of the nuclear-factor-kappa B (NF-kB) pathway and activation of the Keap1/NRF2 signaling pathway.[26] Additionally, these findings are in line with experimental research on H_2S-enriched University of Wisconsin (UW) fluid in rat heart transplantation model and H_2S treatment of urinary-derived sepsis. These studies showed a downregulation of pro-inflammatory markers and upregulation of anti-inflammatory markers due to H_2S treatment.[27,28] Moreover, selective Na_2S administration decreased inflammatory cytokines after reperfusion in a miniature swine experiment.[20] Although a decrease in cytokines is potentially interesting in the perspective of decreasing organ injury, solitary a decrease in cytokines is marginal proof to make a translation of H_2S into a clinical setting since merely an excessive response of cytokines results in direct tissue damage.[29]

In our experiments, reperfusion was performed with NMP. All kidneys demonstrated an increase in cytokines during NMP, independent of donation type. On the contrary, renal function during NMP was different between the brain dead donors and non-brain dead donors. However, the difference in renal function between the two donation types was not accompanied with a difference in the levels of cytokines. Therefore, cytokine release is not responsible for the observed worse renal function in DBD donors. Concluding, despite a decrease in cytokine levels, H_2S treatment did not result in a decrease of injury or improved renal function.

NMP is a suitable technique to test the reaction of a kidney on reperfusion and to test early renal function.[30] Nonetheless, NMP does not allow to research long-term outcomes and in vivo function. Long-term effects of the H_2S-flush are, therefore, not tested in the studied setup and timeframe. A diminished release in cytokines during reperfusion could result in reduced long-term fibrosis since extracellular matrix accumulation is partly activated by cytokines. In the current experiment, kidneys were flushed with H_2S after extraction in order not to affect other organs that were used in other experiments with the aim to minimize animal use. Due to this experimental setup, the therapy was supplied after initiation of ischemia which limits the potential effect since H_2S is less effective on counteracting the negative effects of ischemia if supplied after the insult.[31] Lastly, our model tested the effect of H_2S in DBD and non-DBD kidneys, but not in DCD kidneys. Especially in DCD kidneys the negative effects of a prolonged extraction surgery play a role.[1,2] Since ischemic times are longer in DCD procedures, H_2S therapy might be more beneficial in this donation category.

We showed in **chapter 3** that a decrease in temperature induces a relative lower decrease in ROS production compared to the induced lowering of metabolism. Taken the previous mentioned into consideration, addition of H_2S might function in reducing the metabolism if organs are stored subnormothermic instead of the forced hypometabolism by hypothermically preserving the kidneys at 4 degrees Celsius. This is especially interesting if the low temperatures are not reached during extraction surgery. In our experiments we used NaHS, a solid compound which releases H_2S in a gaseous form when it is dissolved in liquid. This requires constant administration to the solution if a longer effect of H_2S is desirable. Others tested slow releasing hydrogen sulphide donors such as 10-oxo-10-[4-(3-thioxo-3H-1,2-dithiol-5-yl)phenoxy]decyl] triphenyl-phosphonium (AP39) on reducing ischemic injury during subnormothermic preservation.[13,32,33] These slow releasing donors do not require constant supplementation to the preservation solution. Although interesting results are presented such as lower apoptotic injury[32] or lower tissue necrosis,[13] none of the research assessed renal function, measured by fractional sodium excretion or creatinine clearance, or performed a kidney transplantation. In addition, enrichment with AP39 showed a reduction in acute tubular necrosis values compared to the non-enriched 21 °C group, but the results were comparable with SCS. Research is also performed on other slow releasing H2S molecules, including phosphinodithioate (GYY4137), diallyl trisulfide (DATS) conjugated to a mesoporous silica nanoparticles (MSN) carrier (DATS-MSN). Compared to NaHS, DATS-MSN showed superior anti-apoptotic, anti-oxidant and anti-inflammatory abilities.[34] Theoretically, these slow releasing H_2S donors could be more suitable for prolonged preservation compared to gaseous H_2S or NaHS, but large mammal transplantation studies are currently not available.

O_2 and glucose-enriched flush-out

In addition to testing the reduction of metabolism during the flush-out, we investigated the impact of supporting the remaining metabolism during this period. The experimental group was supported by addition of O_2 and was switched in flush-out fluid from UW cold storage solution to UW machine perfusion (MP) solution, which contains glucose. Taken a well-known physiological phenomenon into consideration; without oxygen, anaerobic glycolysis will lead to the formation of lactate and results in less ATP production compared to aerobic metabolism.[35] During ischemia, the lack of ATP leads to failure of Na+-K+-ATPase activity resulting in intracellular Na+ accumulation and increased levels of Ca^2+. Ca^2+ overload causes production of ROS during ischemia and opening of the mPTP after reperfusion, resulting in apoptosis and cell death.[36,37] The availability of glucose in the UW-MP solution and oxygen could support aerobic metabolism,

decreasing injury upon reperfusion. We showed that ATP levels were significantly higher and ADP:ATP ratios were significantly lower in the UW-MP and oxygen supplied group after the flush-out. However, mitochondrial activity, measured by MMP and mitochondrial respiration, was not altered by the presence of glucose and oxygen during or after the flush-out. Therefore, we hypothesised that mitochondria from the kidneys without O_2 and glucose possibly produced less ATP but more ROS since mitochondrial activity and respiration were similar to the high ATP producing mitochondria from the O_2 and glucose enriched group. However, the proposed higher ROS levels were not confirmed by ROS-related measurements. Re-oxidation of succinate at complex 1 of the ETC is thought to be the main site of ROS production.[7] On the contrary, we did not find an increased complex 1 activity in the control group. Another hypothesis is that increased glycolysis, due to the added glucose, could have led to higher ATP levels independent of mitochondrial respiration. Contradictory, we found a lower lactate to pyruvate ratio in the enriched flush-out group, indicating more aerobic than anaerobic metabolism. The enhanced energy status in the O_2 and glucose enriched group did not result in improved renal function or a decrease in injury during reperfusion. HMP increased the energy status in all groups, possibly diminishing the effects of the oxygenated flush-out compared to the control group.

As a translational step towards clinical implementation **chapter 7** also presents a feasible and easy way of supporting organ preservation during prolonged extraction surgery. By clamping the abdominal aorta cranially to the renal arteries and caudally of the superior mesenteric artery the flush of the kidneys could be prolonged. The metabolic rise of the kidney due to the temperature increases during extraction surgery could be supported, especially if oxygen and other metabolic substances are added.

Although potentially feasible, a solitary oxygenated flush-out until the organ is procured and put on transportation is probably not sufficient to completely reduce IRI. Livers flushed with oxygen enriched UW fluid before preservation via SCS showed higher ATP values but without any impact on hepatobiliary function or reduction in injury after reperfusion.[38] On the other hand, early graft function was improved using oxygenation during HMP, but not when oxygenation was only applied as an end-ischemic strategy.[39] This emphasised the need for continuous oxygenation, also during extraction surgery.

Laser Speckle Contrast Imaging

Next to preventing ischemic injury, we assessed a new technique to visualize ischemia and indicating graft quality during NMP. Near-surface perfusion monitoring has shown to

be a possible prognostic value for reduced creatinine clearance,[40] early postoperative graft function and delayed graft function.[41-43] Although promising, these techniques are not yet fully developed or cannot be used without drawbacks. Operator dependency, a small field-of-view and contact imaging are some of the hurdles. In **chapter 8**, we tested the real-time visualization method laser speckle contrast imaging (LSCI) on its ability of visualizing the RCM. We showed a high correlation between LSCI and RCM measured by sidestream dark-field imaging. Local ischemia was visualized real-time and could be quantified in arbitrary units. The combination of a non-contact, non-invasive, real-time and non-contrast using measurement tool makes it theoretically ideal for the visualization of the RCM during different phases of transplantation. LSCI can be used during reperfusion of the organ since prolonged anastomosis time has in impact on transplantation outcome.[44] Easy and quick visualization of the RCM could help the surgeon visualizing ischemic areas and vascular obstructions and adapt the operation accordingly. The largest added value compared to conventional total renal blood flow measurements is the possibility in detecting local perfusion deficits. The largest downfall in using LSCI are movement artefacts.[45] The heartbeat or respiration of the donor can move the kidney, which can falsely be interpreted as blood flow if not corrected. The use of fiducial markers can be a solution but limit the non-contact value of LSCI.46 Recently, a multi-spectral motion artifact correction is studied with promising results regarding non-contact motion artifact correction.[47] This correction model limits the previous mentioned issue and makes LSCI more appealing for clinical use.

LSCI was also used during reperfusion of the kidneys on NMP in **chapter 7**. Here we tried to evaluate the response in reperfusion characteristics in kidneys suffering from different gradients of ischemic injury after extraction of the kidneys. The group with the most ischemic injury showed a non-significant slower reperfusion with LSCI. Since renal function, mitochondrial activity and injury markers were comparable between all groups, the induced injury might not have been sufficient to measure a difference in RCM. Although LSCI is a promising tool in visualising perfusion deficits during different phases of organ transplantation, it is not yet clear if a difference in renal quality or ischemic injury can be visualized with LSCI.

Future perspectives

The use of H_2S to preserve kidneys did, so far, not make it into a clinical transplantation setting. The presented results in using H_2S seem promising regarding the ability of inducing a hypometabolic state in an ex vivo organ, but are limited in regards to preventing injury or preserving renal function. Most research is performed on short-

term models which do not take post-transplantation survival and organ quality into consideration. Thus, a large animal study with long follow up is needed in which H_2S should be supplemented prior to the ischemic insult and the current transplantation procedure should be mimicked as much as possible. Slow releasing alternatives such as GYY4137 might be more suitable and should be taken into consideration. Although, the downside of using these forms is the longer or non-existing half-live and also the lack of large mammal transplantation experiments. These larger animal studies should unveil if H_2S or an alternative slow releasing donor could be promising for usage in a clinical setting.

The evidence for the use of oxygen during prolonged organ extraction is not sufficient for a definitive answer. However, the relatively simple clinical implication and safe use combined with continues cold regional perfusion (CCRP) make it an interesting concept in deceased donors. A better metabolic support during ischemia might be more effective in preserving organ quality than simply forcing hypothermia on the organ. Future research should focus on better insight in the metabolic needs of kidneys during different phases of transplantation. Consequently, the correct amount and type of nutrients can be supplied together with oxygen. Sufficient metabolic support should be continued throughout the entire transplantation procedure, from the start of ischemia during extraction surgery, during transportation and until reperfusion in the donor.

LSCI is a promising tool in visualising perfusion deficits during transplantation surgery. The impact of using LSCI after reperfusion in the donor needs to be tested. Therefore, clinical studies should be performed to test if using LSCI provides valuable information perioperative and if the decision of the surgeon is changed. LSCI could easily be used during surgery by installing a camera and laser in a dark plastic dome that can be placed on top of the kidney directly after finalizing the anastomosis and removal of the vasculature clamp. For now, it is too early to say if LSCI could provide information regarding quality assessment of the kidney during NMP. We performed a small pilot study and there is no additional data on the use of LSCI and kidneys on NMP.

Conclusion

Combining these results; in the future, the preservation of the kidneys could be performed under continuous oxygenation with the use of CCRP and sufficient metabolic support. Quality assessment might improve with the use of LSCI and intraoperative ischemia can be visualised, assisting the surgeon in intraoperative decision making and aiding sufficient organ quality assessment. Thereby, more organs could be transplanted and better-quality assessment might result in less discarded organs and better transplant outcome.

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