Ischemia targeted therapies during critical periods of organ preservation

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Chapter 1

General introduction

Organ shortage

In 2021, 10.269 patients were on the active waiting list in the Eurotransplant region for a kidney transplantation.[1] In the US, the current list is even bigger with 100.791 patients waiting for a kidney.[2] In the Netherlands, 883 patients are currently on the active waiting list.[3] With an equal number of patients being added to the waiting list compared to those leaving it, the number of patients waiting for a donor kidney is stalled. The long waiting time for a post mortal donor kidney in the Netherlands (average 2.4 years, median 1.8 years), led to the death of 6.2% of the patients in 2020. In addition, 13.2% of the patients were removed from the waiting list due to deterioration in health status precluding them from transplantation.[4] This emphasizes the urgency for more kidneys to become available for transplantation. Over the years the traditional donor pool of Deceased Brain Death (DBD) donors has already expanded by the use of "expanded criteria" donor (ECD) kidneys and circulatory death donor (DCD) kidneys.[5] However, the number of available kidneys is still insufficient despite the use of these donor types. In addition, high donor age and ischemic injury makes these donor organs suboptimal for transplantation. ECD kidneys have a higher risk of graft loss (Hazard ratio (HR) 1.35; 95% confidence interval (CI), 1.28-1.42)[6] and warm ischemia in DCD donors is an important risk factor for patient and graft survival.[7-9] Graft loss results in the need for re-transplantation or return to dialysis, increasing the pressure on the waiting list. Therefore, diminishing or preventing injury from the onset of ischemia could be an important strategy to increase the quality of kidneys offered for transplantation. Preventing ischemic injury could be achieved by improving organ preservation in periods of ischemia. In addition, organ quality assessment techniques could provide a better insight if the ECD or DCD donor kidneys are suitable for transplantation.

Ischemic injury

Ischemia, which starts with the cessation of blood flow through an organ,[10] results in a lack of oxygen and nutrient supply to support physiologic metabolic processes. A total ischemic time of 82 minutes was reported in 1954 during the first successful kidney transplantation.[11] Despite all improvements, ischemia is still unavoidable and can be divided into two different categories: warm and cold ischemia. Warm ischemia (WI) occurs after cessation of the blood flow during different phases of transplantation, such as extraction surgery, in (sub)normothermic temperatures. In contrast, cold ischemia (CI) occurs during organ preservation at temperatures around 4 °C.

The length of ischemia depends on the type of donor, which are subdivided into living donors and deceased donors. Deceased donor organs can be retrieved from either DBD or DCD patients. Kidney transplantation using kidneys from living donors results in excellent outcomes in regard to graft survival and post-transplant function.[12] The retrieval time is short, which results in warm ischemic times (WIT) of only 2-5 minutes before the organ is flushed and cooled effectively.[10] In DBD donors, a few minutes of non-circulatory WI might occur, similar to living donation procedures.10 This is not the case for DCD donors, in which total WIT is comprised of different phases (Fig. 1). There is non-circulatory WIT directly after asystole or a cardiac arrest until the start of the vascular flush. In a large Eurotransplant cohort the median WIT was 17 minutes in controlled DCD donors.[7] Prior to the non-circulatory WIT, functional WIT occurs when the mean arterial pressure is below 50 mmHg or when arterial saturation is <80% in controlled DCD donors. In uncontrolled DCD donors, the time from stop resuscitation until the start of the vascular flush resembles the functional WIT (Fig. 1).[10]

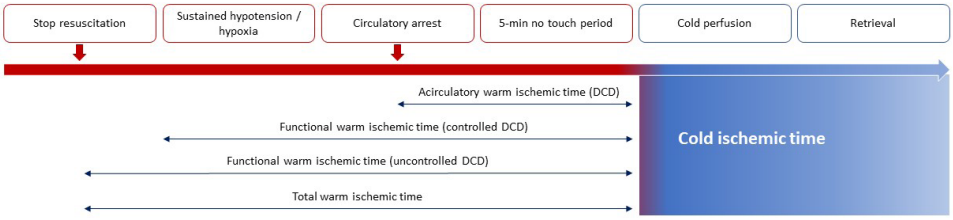

Figure 1 - overview of ischemic times

When the functional and non-circulatory WITs are combined, especially in uncontrolled DCD donors, warm ischemia is considerably longer in DCD than in living donation or DBD. Ischemia has a profound impact on kidney transplantation outcome as defined by graft loss and patient survival.[7,8,13-15] Each additional hour of cold ischemia increases the risk of graft failure.[13] The negative impact of warm ischemia on kidney transplantation outcome occurs faster than cold ischemia. Warm ischemia longer than 30 minutes is associated with an adjusted relative hazard of 1.13 [95% CI 1.04-1.23] for death or graft failure compared with patient with warm ischemia of 10-20 minutes.[8]

After the first warm-ischemic time in deceased donors, organs are cooled via an abdominal aortic flush and are cooled topically with slushed ice. Next, they are preserved via static cold storage, surrounded with cold preservation fluid and ice, or on hypothermic machine perfusion. The rationale behind this is to reduce the metabolic rate by introducing hypothermia.

Ischemia reperfusion injury

Active metabolic processes under physiological conditions can either occur in the presence of oxygen (aerobic) or in absence of oxygen (anaerobic). Without oxygen, anaerobic glycolysis results in a nett production of 2 adenosine triphosphate (ATP) and the formation of lactate.[16] ATP is an organic compound that provides energy for many different cell functions. Aerobic metabolism will result in 2 ATP after completion of the Krebs cycle and 34 ATP in the subsequent oxidation of hydrogen atoms in the mitochondria.[16] Mitochondria oxidize substrates via the electron transport chain (ETC), by creating an electrochemical gradient through the coupled transfer of electrons to oxygen, the ETC creates a proton flux that is used to drive the ATP synthesis.[17] A schematic overview of the ETC can be seen in Fig. 2. The ETC is built up of 5 different complexes, and the proton flux is used by the terminal complex V to power the ATP production.[17] Oxygen is essential in the coupled transfer of electrons, during ischemia the ETC becomes uncoupled which results in succinate accumulation.[18] During reperfusion oxygen is re-introduced, resulting in re-oxidisation of the accumulated succinate.[18] This re-oxidation results in a reverse electron transport at complex I, subsequently producing reactive oxygen species (ROS). The overproduction of ROS results in ischemia/reperfusion injury (IRI) after the organ is transplanted.[19] Thus, mitochondrial dysfunction plays a key role in IRI, leading to a depletion of ATP and the production of ROS.[18] Mitochondrial ROS production can induce cell death (apoptosis and necrosis) and immune responses.[20] It is well known that a burst of ROS is a dominant injurious effector during reperfusion of an organ.[21] The burst of ROS activates injurious pathways through carbonylation of proteins or lipid peroxidation, inducing cellular injury.[22] In addition, dysfunction of the mitochondria results in opening of the mitochondrial permeability transition pores (mPTP) and release of damage associated molecular patterns (DAMPs), activating apoptosis and regulated necrosis.[22] In addition to the production of ROS and mitochondrial dysfunction during reperfusion, IRI induces several pathophysiological pathways including endothelial dysfunction and activation of the innate and adaptive immune system.[22,23] IRI also causes a sterile inflammation which results in the activation of the complement system and a cytokine burst. Taken together, reducing IRI is one of the major goals in improving organ quality for transplantation.

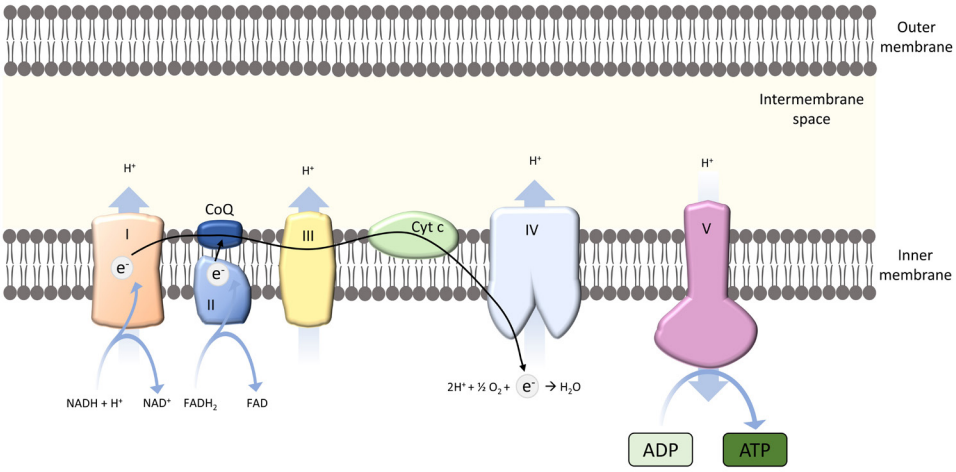

Figure 2 - schematic overview of the electron transport chain

Abbreviations: CoQ = Coenzyme Q, Cyt c = Cytochrome c.

Impact of temperature on kidney transplantation

Although cold organs can sustain ischemia longer than warm organs, IRI also occurs after cold storage. Metabolism during cold storage is not fully reduced to 0%, but remains at 5-10%, thereby impacting transplantation outcome.[13,15] Indeed, the addition of oxygen to support the remaining metabolism during hypothermic machine perfusion (HMP) increased ATP levels in an experimental setup.[24] Also, oxygenated HMP decreased serum creatinine after kidney transplantation in pigs[25] and reduced post transplantation complications in human transplantation.[26] The mitochondria play a vital role in IRI and are highly affected in their function by temperature. Decreasing the temperature of normothermic animals show a 1.5-2 fold decrease in activity for every 10 °C decrease in temperature.[27] The metabolic rate is suppressed by 12-13-fold when temperature is reduced from 37 °C to 0 °C.27 Although it is clear that the metabolic rate slows down with lower temperatures, it is unclear how it affects mitochondrial ROS formation. Since cold ischemia results in IRI, ROS production might still be present in cold temperatures. Decreasing the metabolic rate during different phases of transplantation, without forcing hypothermia upon the organ, might limit the adverse effects of cold ischemia. On the other hand, decreasing the metabolic rate in phases of transplantation in which the objected cold temperature is not reached could also limit the adverse effects of warm ischemia. One important time-point during the whole process of transplantation in which temperature increases above the intended temperature, is during prolonged extraction surgery times.[28] Extraction time is

defined as the start of the vascular flush until the extraction of the organ.[10] Improving organ preservation by enhancing the flush of the organ during the extraction time could have a vast impact on renal quality.

In this thesis we propose two ways of improving the flush-out.
First, by artificially inducing a hypometabolic state, which might limit the adverse effects of the forced hypothermia or decrease metabolism when hypothermia is not reached. This can be achieved by using hydrogen sulphide, a gasotransmitter that has been researched extensively for its ability of inducing a hibernation like state in small animals.
Secondly, by supporting the remaining metabolism through the use of essential oxygen and nutrients in order to support ongoing cellular respiration.

Enhancing the flush-out

Hypometabolism

Gasotransmitters such as nitric oxide, carbon monoxide and hydrogen sulphide (H_2S), are endogenous gaseous mediators that gained expanding interest over the past decades.[29,30] They play an essential role in regulating mitochondrial function and ROS production and are therefore a potential therapeutic agent during ischemia.[31] Hydrogen sulphide (H_2S) is one of the three gasotransmitters with a potent beneficial effects on ischemic injury. Experiments in small mammals showed protective effects of H_2S as therapeutic compound in preventing ischemic injury after kidney IRI.[32] H_2S can induce hypometabolism when supplied in high concentrations through reverse inhibition of the mitochondrial ETC, specifically complex IV (cytochrome c oxidase).[33] Hypometabolism, artificially induced by H_2S, could provide a suitable alternative for hypothermia. This could reduce IRI that still occurs after cold ischemia. It might also be used in situations where the targeted cold temperature is not reached, reducing metabolism if hypothermia fails. Unfortunately, hypometabolism was not reached in larger mammals when supplied systemically.[34,35] Still, H_2S might induce a hypometabolic state in isolated perfused kidneys due to its relatively small size compared to a complete animal.[36] Next to the hypometabolic effect of H_2S, the gasotransmitter shows promising results in decreasing ROS damage,[37,38] inflammatory,[39] and apoptosis.[40] Even without full hypometabolism, H_2S reduced ischemic injury in mice.[41] Therefore, adding H_2S to the aortic flush might decrease ischemic injury during procurement surgery via hypometabolism or via the anti-inflammatory and anti-oxidant capacities of H_2S.

Oxygen and glucose

Enhancing the standard flush-out solution could possibly diminishing ischemic injury directly from the onset. Since glucose and oxygen play such an important role in the production

of ATP to sustain normal cell function, addition of both during the flush-out of the kidneys might decrease ischemic injury upon reperfusion. In this way, cellular metabolism might be sustained during the course of extraction surgery, even with increasing temperatures. Theoretically, this could reduce accumulation of succinate, since less ischemia is forced upon the mitochondria.[18] This would result in a lower degree of ROS formation during reperfusion,[18] following better organ quality after prolonged extraction times.

Perfusion monitoring

In addition to minimizing ischemic injury that occurs during the course of transplantation using H_2S, oxygen and glucose, visualizing perfusion of an organ during critical moments of transplantation could identify ischemic injury. By visualizing ischemia, quick and proper therapy can be supplied to reduce the injury. Another important hurdle to overcome in kidney transplantation is that not all organs are accepted for transplantation due to the lack of confidence that they will prevail as suitable donor organs due to missing insight in the quality of the organ.[42-44] Eight percent of donor kidneys that were retrieved from deceased donors were not transplanted in the Netherlands in 2020.[4] It is likely that some of these organs might have been suitable for transplantation. The potential of kidneys could even be higher, since in some cases patients are exempted from donating organs. The underlying reason for decline is the lack of suitable quality assessment tools. Clinical decision-making is currently based on a wide variety of factors, such as donor age, cause of death, and ischemic times. However, a complete objective measurement or standard is currently not available. Organ quality assessment, provided by an objective perfusion measurement tool, could result in better transplantation outcomes due to ischemia visualization and correct organ selection for transplantation.

Normothermic machine perfusion (NMP) is a perfusion technology that can provide organs with an oxygenized blood-based solution, mostly performed around 37 °C. NMP is a widely used method in an experimental setup to assess renal function and organ quality and test different therapeutic possibilities.[45] For kidney transplantation it is currently not clinically applied as a standard treatment. The first clinical trial in the Netherlands on NMP of kidneys started very recently.[46] This trial investigates if NMP can be applied safely in kidneys that are accepted for donation, from both DBD and DCD donors. NMP might be useful in quality assessment of the kidney, especially in combination with imaging techniques. An overview of the NMP setup we used in our experiments is displayed in Fig. 2. The setup consists of a perfusion pump, connected to a laptop with perfusion software, oxygenator, organ chamber, flow and pressure sensor, an infusion pump and water bath for temperature regulation.

Imaging techniques revealed promising results in visualization the graft and might be indicative of organ quality. Magnetic Resonance Imaging (MRI) can provide a very detailed image and offer a large base regarding different imaging options.[47] Downsides include the costs and impracticality of an MRI scanner. Laser speckle contrast imaging (LSCI) could provide a more simple and cheaper solution. LSCI is a non-contact, full-field imaging technique of the microperfusion of an organ. LSCI is based on the principle that the backscattered light from a tissue that is illuminated with the coherent laser light forms a random interference pattern at the detector, the so-called speckle pattern.[48] Speckle images can be blurred when red blood cells cause fluctuations in the speckle pattern; this blurring can then be related to the current blood flow.[48] An overview of the LSCI setup used for our experiments is displayed in Fig. 3. It consists of a camera, a laser with a diffuser, both connected to a laptop with imaging software.

A detailed and objective measurement of the perfusion of the organ could indicate ischemic injury, organ quality and provide vital information regarding acceptance of the organ for donation. LSCI could be used during two different phases of kidney transplantation. Firstly, LSCI could provide a fast and objective measurement of the reperfusion of the graft during reperfusion in the recipient. Which is an important time-point in transplantation regarding warm ischemic injury.[14] Secondly, during NMP, to assess the quality of the organ and it might provide information on if the graft should be accepted for transplantation.

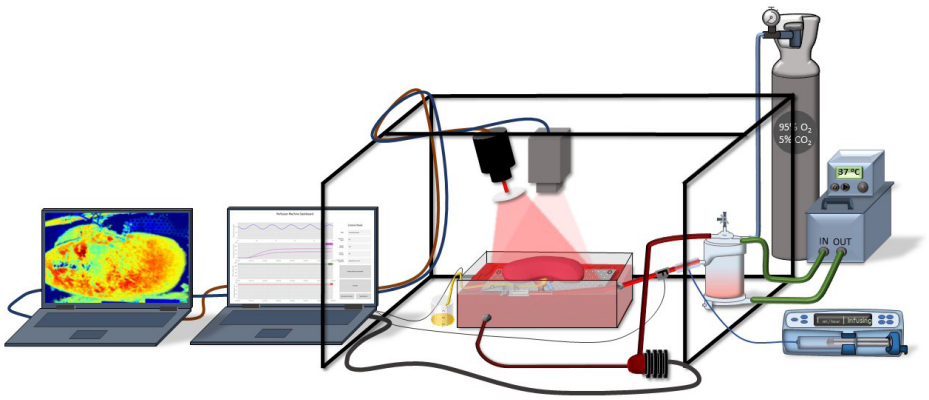

Figure 3 - Normothermic machine perfusion setup, including laser speckle contrast imaging

Aims of this Thesis

The research described in this thesis aims to develop mechanisms that reduce ischemic injury and to explore suitable techniques for perfusion monitoring during NMP

We hypothesize that an increase in temperature during extraction surgery induces significant ischemic injury. The higher metabolic rate during extraction could be lowered by artificially inducing hypometabolism with H_2S. Alternatively, the remaining metabolic rate could be supported with sufficient amounts of oxygen and nutrients. Furthermore, we study perfusion monitoring during transplantation in order to provide valuable information regarding organ quality.

Chapter 2 describes the impact of prolonged extraction time on kidney transplantation outcome. Temperature increase during prolonged extraction surgery might induce a significant amount of injury that impacts graft function or even patient survival.
Chapter 3 researched the impact of temperature on mitochondrial function, respiration and ROS production. This study was performed in isolated mitochondria and perfused kidneys, which were subjected to different temperatures. The goal was to identify possible therapeutic substances that could reduce injury as a result of ischemia. **Chapter 4** reviewed three gasotransmitters on their role in mitochondrial function in health and disease. We also provide an overview of their possible therapeutic properties. Subsequently, **Chapter 5** tested one of these gasotransmitters, i.e. H_2S, on its ability of inducing a hypometabolic state during NMP. Hypometabolism which is artificially induced by H_2S could have beneficial effects when supplied in periods of ischemia. Next, H_2S was tested as a therapeutic enhancement during the flush-out and subsequent cold storage as described in **Chapter 6**. The aim was to reduce ischemic injury and inflammation due to the H2S enrichment of the flush. This was tested in both DBD and non-DBD porcine kidneys, to evaluate the effect of H_2S on different donor types. **Chapter 7** researched another possible improvement of the flush-out of kidneys. The flush-out fluid was supplemented with oxygen and glucose and the kidneys were tested on mitochondrial and renal function during the flush-out and subsequent NMP. Next, we used our NMP model in **Chapter 8** to assess and validate LSCI. The aim was to provide insight in the applicability of LSCI in visualising the renal cortical microperfusion. In addition, in **Chapter 7**, LSCI was tested during NMP to assess its ability of renal graft quality assessment. To conclude this thesis, **Chapter 9** discusses the data obtained in this thesis and provides future perspectives.

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