Preconditions for warm organ preservation
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Summary, conclusions and future perspectives
This thesis deals with new avenues to facilitate, create, or appraise techniques in organ preservation and to ultimately enable the shift from hypothermic to (sub)normothermic perfusion preservation of organs.

**Appraisal of organ transplantation and the use of large-animal models**

That prolonged, (sub)normothermic organ preservation is an unestablished method of the past, is evident from the historical development of organ preservation and transplantation. To put novel methods into perspective, Chapter 1 provides an overview over the historical development of organ preservation and highlights the current, renewed interest in warm organ preservation.

As is evident from the historical perspective, large animal models, comprising dogs and non-human primates are the cornerstones for gaining insight in novel methods of organ preservation. However, animal models have their limitations and are vulnerable to bias from a plethora of influences. To assess these concerns in current transplantation research, Chapter 2 provides an appraisal of large animal kidney transplantation models reported in literature from 2000-2008. The literature not only showed a species diversity in large animal models, but also a major lack of information on practical points concerning housing, anesthesia, and postoperative care.

While non-human primates and dogs have traditionally been used for transplantation-related research, a dissimilar renal anatomy, resilience to ischemia-reperfusion injury, high costs, and societal condemnation and protest have deemed these models less preferable. As a result, pigs are now considered more favorable with respect to renal function, anatomy, and the associated factors stated above. Female pigs of 5 months (approximately 50 kg) are found to be optimal for transplantation studies, as they combine the advantages of a manageable weight, absence of hormonal influences, mature renal anatomy, and comparable function to humans. However, dietary changes can induce a pro-inflammatory state of the gut in the short timespan of an experiment. It is therefore advisable to maintain the farmers’ diet and also to properly acclimatize the pig and avoid prolonged fasting in order to prevent a starvation response.

For anesthesia, ventilator-assisted anesthesia using oro-tracheal intubation but avoidance of volatile anesthesia is advisable to minimize interference with ischemia-reperfusion related processes. Additionally, many drugs may influence hemodynamic parameters requiring exploration of the specific side-effects. For example, propofol has a greater anti-inflammatory and kidney-protective effect than sevoflurane. The
postoperative period should be regarded as an intensive care period inasmuch as pigs' oral intake is dependent on their well-being.\textsuperscript{17} Therefore, intravenous fluids should be administered liberally and effective pain management structured to minimize influences on the pigs' physiology. Furthermore, avoidance of solitary housing, provision of a familiar surrounding (i.e. smells, sounds, and caretakers) while under non-threatening observation, should improve the outcome and comparability of transplantation studies across different research groups.

**Reconsideration of systems for machine perfusion preservation**

With current organ preservation techniques shifting from static storage to machine perfusion preservation, a novel array of devices has been developed in the last decades.\textsuperscript{18-20} All devices are based upon pulsatile perfusion despite the ongoing question regarding the advantage over non-pulsatile perfusion.\textsuperscript{21} However, recent *in vitro* and *ex vivo* studies show an advantage for pulsatile perfusion on endothelial function\textsuperscript{22}, cell survival\textsuperscript{23}, and expression of vasoprotective agents.\textsuperscript{24} Although many experimental systems use pulsatile perfusion pumps to perfuse organs, they have the drawback of employing ultrasonic flow sensors. While experimental systems may especially be capable of investigating the effect of different preservation solutions and temperatures on the organ, conventional ultrasonic sensors may be inaccurate when employed under changing conditions.\textsuperscript{25} This introduces a crucial inaccuracy as perfusate flow is used to assess an important viability parameter of the organ, namely intrarenal vascular resistance.

This potential inaccuracy is addressed in Chapter 3, together with the viscosity of different perfusates used for organ preservation. Perfusate viscosity can influence the organs’ vascular resistance\textsuperscript{26}, certainly at lower temperatures when viscosity is significantly higher. Colloid-containing solutions were 2.5-fold more viscous than non-colloidal solutions at 4 °C. The difference in viscosity between colloidal and non-colloidal solutions remained 2-fold higher at 37 °C. Across this temperature range, the ultrasonic flow sensor overestimated the flow rate with a maximum of 7.6 mL/ min as opposed to a maximum overestimation of 0.5 mL/ min using the coriolis-based, mass-flow sensor. The coriolis-based sensor provided accurate measurement of flow regardless of the solution's composition and temperature. Our redesigned perfusion system, incorporating the coriolis-based sensor, proved highly stable in maintaining perfusion settings for preservation of porcine kidneys. Furthermore, the system was able to reproduce perfusion characteristics of other experimental or commercially employed machine perfusion
Summary, conclusions, and future perspectives

The Airdrive, the recreation of a machine for organ preservation, is an oxygen-driven perfusion machine, providing the first disposable device for kidney and liver preservation. This device, as described in Chapter 4, employs pressurized oxygen to pump, oxygenate, and maintain sterility of the perfusate and organ at hypothermic conditions. Preservation using the Airdrive of three porcine livers for 20 h resulted in increased portal flow to 177 mL/min at 13-mmHg pressure in the absence of histological damage. Previously, the Airdrive successfully preserved porcine kidneys for 20 h at hypothermic conditions.\textsuperscript{27, 28} We conclude that the Airdrive is capable of preserving organs using an oxygen-driven pump providing opportunities in the clinical field.

Organ washout and preservation

The effect of colloidal additions on viscosity and the related vascular resistance provides the basis for a long-lasting discussion in organ retrieval. The organ needs to be cleared of blood by a washout procedure prior to or just after retrieval whereas an incomplete washout impairs organ function.\textsuperscript{27} However, the agglutinative effects of colloids\textsuperscript{29} in combination with the increased viscosity of the solution (Chapter 4), potentially impair proper blood washout. Chapter 5 deals with the effect of colloids on blood and their consequences in a washout solution. Firstly, colloid-induced erythrocyte agglutination was independent of the solutions’ composition, whereas the use of Ringer’s lactate (RL) resulted in erythrocyte blebbing. A rat liver washout with \textsuperscript{99m}Tc-pertechnetate labeled blood was performed with RL, histidine-tryptophan-ketoglutarate solution (HTK), University of Wisconsin solution (UW), or Polysol (PS) at 4 or 37 °C with 15 or 100 mmHg pressure. Using RL resulted in an 81.3 \% reduction of blood retention after arterial washout at 37 °C and 100 mmHg portal pressure.

The remnant-blood fraction of 18.7 \% was intriguing as the washout effluent was macroscopically clear. A small amount of residual erythrocytes could be explained by their presence in the otherwise injury-free, histological liver sections. Therefore, dynamic gamma-scans of the rat with special focus on the duodenal region (in which the rats’ biliary system directly drains) were performed. This way, the much faster radioactive biliary excretion in the rat, in comparison to humans, was confirmed and seen as a contributory cause for the remaining 18.7 \% retention.

Not only retained erythrocytes can occlude the livers microvasculature as we observed during (sub)normothermic experiments with machine perfusion of porcine...
kidneys. Bacteria from an infected preservation additive had overgrown during kidney perfusion experiments and occluded the peritubular capillaries. As antibiotic prophylaxis was administered, the efficacy of antibiotic agents under these conditions can be questioned. Unfortunately, literature only provides information on risks of preservation solution infections under hypothermic conditions and no references on antibiotic efficacy during (sub)normothermic perfusion. Therefore, Chapter 6 is concerned with the currently applied antibiotic prophylaxis during (sub)normothermic perfusion and the efficacy of two cephalosporins under these conditions. Only 14 of the 33 recently published papers on (sub)normothermic organ preservation mention the use of antibiotic prophylaxis. The risk of bacterial growth in perfusates was proven by the overgrowth of different \textit{S. Epidermidis} and \textit{S. Aureus} strains in Polysol (PS) at 28 and 37 °C. However, not all preservation media provide a bacteria-supporting basis as PS, which is a culture medium-derived perfusate designed for (sub)normothermic organ preservation.

The efficacy of ceftriaxone and cefazolin was assessed in PS and showed that 75 \( \mu \text{g}/\text{mL} \) cefazolin had a similar effect as 1000 \( \mu \text{g}/\text{mL} \) ceftriaxone at 37 °C. Although the efficacy of cefazolin decreased at 28 °C, requiring 1000 \( \mu \text{g}/\text{mL} \) for a similar effect, the bactericidal property of cefazolin was observed even after washout and warming to 37 °C. Unfortunately, cephalosporins might induce cytochrome C uncoupling at high dosages, leading to cellular toxicity. The non-toxicity of 1000 \( \mu \text{g}/\text{mL} \) cefazolin was proven by the unaltered viability of porcine kidney endothelium cells. As a conclusion from these results, a 1000 \( \mu \text{g}/\text{mL} \) dose of cefazolin is recommended to safeguard the sterility of the perfusate.

After a proper washout and maintenance of perfusate sterility, the preservation solution must maintain or even recover the organ’s function. To this end, Chapter 7 was added to look into the most optimal condition to maintain cell viability. While RL showed to be advantageous for washout in chapter 5, the advantage is lost during human umbilical vein endothelial cell (HUVEC) preservation, as previously mentioned in literature. At 4 °C, no solution was capable of sustaining HUVEC monolayer integrity and viability after 20 hours, underscoring the detrimental effects of prolonged hypothermic preservation. At 15 and 20 °C, a sharp increase in endothelial viability was observed using the nutrient-enriched solutions UW, PS, and the endothelial cell culture medium (ECGM), showing the best results with UW. Interestingly, 20 °C, which comes down to room temperature preservation, has previously shown to yield good functional results using a similar, nutrient-enriched preservation solution. However, at temperatures above 20 °C, all commercial and experimental perfusates were unable to sustain monolayer integrity and viability. Only ECGM was capable of preserving excellent HUVEC-viability and
monolayer integrity after 20-h preservation up to 37 °C.

Measuring the organ’s viability during preservation has been extensively explored using different biomarkers, for example NGAL, KIM-1, IL-16, and vascular resistance in the organ. While (sub)normothermic preservation could provide the opportunity to maintain normal organ function, it does require physiological support of the metabolism. As shown in chapter 7, the endothelium is vulnerable for preservation-related injury and potentially provides a valuable biomarker in the form of circulating endothelial cells (CEC). Because to date, no CEC-detection method exists for porcine blood, a method for the detection and quantification of circulating endothelial cells in porcine whole blood is described in Chapter 8. To enable translation to HUVEC, swine umbilical vein endothelial cells (SUVEC) were harvested from porcine umbilical cords and a culture method devised. Characterization of endothelial cells was only possible after application of a minimum of two, non-overlapping epitopes of anti-human endothelium antibodies. Of 11 anti-human endothelium antibodies (21 clones), only endoglin, CD105 MEM-229, and melanoma cell adhesion molecule, CD146 P1H12, showed cross-species immunoreactivity during flow-cytometric comparison of HUVEC and SUVEC. Immunofluorescent imaging of HUVEC, SUVEC, and porcine kidney endothelial cells showed a homologous distribution of the fluorescently labeled CD105 MEM229 and CD146 P1H12 on the cell membrane. Using these two antibodies after osmotic erythrolysis of whole blood, flow cytometric quantification of baseline levels of CEC in porcine whole blood yielded 673.1 ± 551.4 events/ mL in a 50 kg female pig.

Conclusions

In our efforts to unlock the full potential of (sub)normothermic organ perfusion, the underreporting of transplantation-model specific variables like acclimatization, fasting, anesthesia protocol, antibiotic prophylaxis during organ preservation, postoperative care, hinders comparison between research groups. While needing a clinically relevant, large-animal transplantation model, a 50-kg female pig will provide the best comparable model to humans. However, special attention should be paid to the design of preoperative care, anesthesia, surgical practice, and postoperative care.

When changing conditions in kidney preservation are investigated, mass-flow sensors are more reliable than ultrasonic flow sensors. The Airdrive, a new disposable, oxygen-driven pump device for machine perfusion preservation proved efficacious in preserving kidney and liver grafts.
During organ procurement, a colloidal washout solution should be avoided to reduce erythrocyte retention. A washout with Ringer’s lactate via the portal vein and hepatic artery at 37 °C and 100-mmHg pressure, will significantly reduce erythrocyte retention. However, Ringer’s lactate is not advised for prolonged storage at any temperature because of the cell damage induced. Endothelial integrity and viability is best maintained using University of Wisconsin solution at 20 °C while at higher temperatures only the endothelial cell growth medium was equally effective.

During organ preservation, the prophylactic addition of 1000 μg/ mL cefazolin maintains perfusate sterility. The risk of bacterial overgrowth is significantly increased when employing perfusates especially designed for perfusion at (sub)normothermic temperature. A high-dose cefazolin (1000 μg/ mL) prophylaxis proved non-toxic to the endothelium. Damaged endothelial cells detach and can be detected as circulating endothelial cells in porcine whole blood by flow cytometry using the anti-human endothelium antibodies CD105 MEM229 and CD146 P1H12. Osmotic erytholysis proved sufficient to reliably determine the CEC-levels in porcine whole blood.

**Future Prospects**

There is a persistent shortage of donor organs that requires novel tactics to make better use of the currently available organs. An assignment that is as difficult as it is challenging in the current times. Improving current organ procurement with donor pretreatment is promising. Not only by avoiding cardiac arrest (as is required by law for brain dead donors in the Netherlands), administration of pharmaceuticals, preconditioning by peripheral ischemia, but also by extracorporeal perfusion.

Improvements in the method of blood washout will contribute to novel developments in organ procurement. The major benefit, however, will lie in the improvement of organ preservation techniques. As evidenced by the literature and the results derived from the studies in this thesis, the era of hypothermic organ preservation is likely to come to an end. Although application of normothermia, a major research topic at many research institutes, is not likely to be developed in a widespread, clinically applicable method.

Oxygen demand of the organ will require addition of oxygen carriers. With the scarcely available packed red blood cells being in high demand, artificial oxygen carriers are key additives but are, unfortunately, far from clinical application. Therefore, room temperature preservation appears to be a relatively unexplored, but highly promising
organ preservation condition.

It is advisable that novel research into warm organ preservation is therefore changed to room temperate. This provides the benefits of warm organ preservation, including the possibility to conduct organ function tests, without the drawbacks of hypothermic organ preservation. The benefits of warm organ preservation can only be explored in full if the energized preservation systems are redesigned.

In conclusion, the paradigm shift of hypothermic to subnormothermic organ preservation is dependent on a mentality change. The current perception of organ preservation should be reexplored and constructed using the technical, pharmaceutical, and practical possibilities of the current time.