Strategies to Improve Outcome after Transplantation of Extended Criteria Donor Livers

Westerkamp, Andrie Cornelis

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Summary, Discussion and Future Perspectives
Summary and General Discussion

The studies in this thesis have provided evidence for new strategies to improve outcome after transplantation of extended criteria donor (ECD) livers. Especially, the chapters in which the applications of machine perfusion were investigated confirm previous findings and contribute additional evidence that machine perfusion has the ability to lower preservation injury and improves organ quality of ECD livers before transplantation.

In this chapter, the results of this PhD thesis are summarized and discussed, followed by a section on future perspectives.

Chapter 1 provides a short introduction to this thesis and the aims of this thesis are discussed.

Part A: Strategies to Improve Outcome after Transplantation of Extended Criteria Donor Livers in Clinical and Animal Studies

In chapter 2 we examined whether donor livers with advanced age (≥ 65 years) can be used safely for transplantation compared to a control group that received a graft from younger donors (< 65 years). We performed this retrospective study together with the other two liver transplant centers in the Netherlands. We demonstrated that transplantation of livers from elderly donors does not result in inferior patient/graft survival rates or a higher incidence of biliary complications such as non-anastomotic biliary strictures (NAS). A plausible reason for the good outcomes after transplantation of elderly donor livers could be the short cold ischemia times obtained in this cohort. However, it should be noticed that the elderly donor livers were transplanted in recipients with minimal additional risk factors (low MELD [model for end-stage liver disease] score, no high urgency, no hepatitis C cirrhosis) and other donor related risk factors such as donation after circulatory death (DCD), split livers, or steatosis were avoided.

In chapter 3 we assessed whether short cold ischemia times during transplantation of moderate (30-60%) macrovesicular steatotic donor livers can lead to similar postoperative outcomes compared to transplantation of nonsteatotic donor livers. In 19 patients who received a moderately steatotic donor liver, similar patient and graft survival rates were observed in comparison to a control group of 95 patients that were transplanted with a nonsteatotic liver. Likewise, no differences were found in the incidences of primary non-function, retransplantation, or NAS formation. In addition, we have shown that liver function, reflected by prothrombin time and serum bilirubin was comparable between the two groups. Similarly, no differences were observed in postoperative lactate levels. However, recipients of a
moderately steatotic liver suffered from more postoperative complications such as respiratory or kidney dysfunction, which necessitated intensive care treatment.

In **chapter 4** and **5** we analyzed whether the diffuse reflectance spectroscopy (DRS) system of Philips Healthcare was able to quantify steatosis in liver tissue of humans and mice. Both studies demonstrated that the DRS can measure steatosis accurately and in-real time. A good agreement was shown between DRS analysis and pathological assessment (gold standard) of the degree of steatosis. Moreover, we showed that DRS is also able to differentiate between mild and moderate/severe steatotic livers with high sensitivity and specificity. As a result, we suggest that the DRS can be used to assist the surgical team in the assessment of the hepatic fat content during donation and/or transplantation procedures. However, it was not possible to discriminate between macrovesicular and microvesicular steatosis with the DRS system. With the DRS device it was only possible to measure the total hepatic fat content.

We explored in **chapter 6** whether transplantation of ECD livers is associated with higher need for blood transfusions during transplantation. A donor liver was classified as ECD when the donor risk index (DRI) was 1.7 or higher. ECD livers were used in 115 of the 318 primary adult liver transplant recipients (36%) of this cohort. In 95 (30%) of all recipients no red blood cell concentrate (RBC) was administered during transplantation. After uni- and multivariate analysis the following variables were found to be independently associated with postreperfusion RBC transfusion requirements: DRI ≥ 1.7, female recipient, recipient age, and no aprotinin administration. The increased blood loss after graft reperfusion in ECD livers could be related to the higher susceptibility of ischemia/reperfusion (I/R) injury in ECD livers. It has been shown that increased reperfusion injury is associated with the release of fibrinolytic proteins from the donor graft, which results in premature breakdown of hemostatic blood clots.

**Part B: Machine Perfusion as a Strategy to Improve Hepatobiliary Viability of Extended Criteria Donor Livers**

**Chapter 7** provides a general overview of biliary complications after liver transplantation. In particular we focussed on the role of machine perfusion as a potential technique to lower biliary complications after liver transplantation.

In a recent study performed by our group, it has been shown that injury to the peribiliary glands during static cold storage (SCS) is associated with the development of NAS after transplantation (1). Therefore, we aimed in **chapter 8** to study whether the peribiliary glands are also injured in the late stages of severe NAS. Another aim of this study is to hypothesize new starting points for protection of the bile duct epithelium with machine perfusion. To study whether the
peribiliary glands are injured in late stages of severe NAS, we analyzed large bile duct biopsies of explants of patients retransplanted for severe NAS. Large bile duct biopsies of explants from patients without concomitant biliary pathology were chosen as controls. Our study demonstrated that the peribiliary glands are significantly more injured in patients retransplanted for severe NAS compared to patients retransplanted without concomitant biliary pathology. In addition, also significantly more biliary epithelial injury was observed in large bile duct biopsies of patients retransplanted for severe NAS compared to control patients. Therefore, our study supports our earlier hypothesis that injury to the peribiliary glands during SCS increases the risk for the development of NAS after transplantation. Furthermore, we postulate that these ongoing injuries to the peribiliary glands and consequently insufficient regeneration of the biliary epithelium are important contributing factors in the pathogenesis of NAS. Additionally, in another recent study, it has been shown that it is possible to isolate stem cells from the peribiliary glands. Moreover, the same study showed that therapy with these stem cells in two patients with liver cirrhosis, improved the functionality of their cirrhotic liver (2). Besides, it has been suggested that normothermic machine perfusion ([NMP] perfusion at 37°C) can function as vehicle for the delivery of stem cells (3-5). Hence, the combination of NMP and stem cell therapy may be an interesting approach to reduce bile duct injury after transplantation of ECD livers.

In chapter 9 we investigated whether 2 hours of end-ischemic machine perfusion was able to improve liver viability of human ECD livers after regular SCS. During machine perfusion, the inflow temperature was set at 12°C (hypothermic conditions) and the perfusion fluid was actively oxygenated. The liver was perfused through both the portal vein and hepatic artery. After 2 hours of oxygenated hypothermic machine perfusion (HMP), livers were perfused using NMP at 37°C for 6 hours, to evaluate hepatobiliary viability. NMP was performed with a perfusion solution enriched with an oxygen carrier (RBCs) and nutrients. Livers with only SCS preservation and NMP served as control group. Our study demonstrated that 2 hours of HMP significantly increased hepatic ATP content as well as liver functionality, as seen by a better cumulative bile production and improved composition of bile (more bicarbonate secretion) compared to livers with only SCS preservation. However, HMP treatment did not lower markers of hepatobiliary injury such as transaminases, lactate dehydrogenase (LDH), biliary LDH, and biliary gamma-glutamyl transferase (gamma-GT) after 6 hours of ex situ viability testing using NMP. A possible explanation for the similar level of hepatobiliary injury markers can be the almost equivalent degree of pre-existing injury in the two groups. Immediately after SCS, in the majority of ECD livers, a substantial degree of parenchymal ischemic necrosis was observed. This substantial degree of injury can be explained by the combination of suboptimal graft quality, substantial donor warm ischemia time, and the subsequent period of SCS (median 7-9 hours). Likewise, after 6 hours of NMP, the patterns of ischemic necrosis were comparable and no increase in injury was observed between the group with HMP and only SCS preservation.
The study in chapter 10 assessed the optimal end-ischemic machine perfusion fluid temperature for protection of the bile ducts. We performed this study with DCD rat livers. Three temperature conditions were compared: hypothermic (8°C), subnormothermic (20°C), and gradual rewarming, or so-called controlled oxygenated rewarming (8-20°C). In the control group no end-ischemic machine perfusion was performed. After 1 hour of oxygenated end-ischemic MP, the livers were 2 hours reperfused ex situ at 37 degrees for hepatobiliary viability assessment. Independently of the machine perfusion temperature, end-ischemic machine perfusion improved markers of biliary function, such as bile and bicarbonate production, and reduced markers of biliary injury as well as biliary histological injury. In addition, end-ischemic machine perfusion reduced hepatocyte injury markers (transaminases and LDH) and improved mitochondrial function, all irrespective of the perfusion temperature.

In chapter 11 we examined whether pharmacological pre- or postconditioning of rat donor livers using metformin, was able to improve hepatobiliary function and reduce preservation injury as assessed during 3 hours of NMP. Metformin was administered 12 and 2 hours before the hepatectomy via oral gavage (preconditioning), or was added to the perfusion fluid in two different concentrations (300 mg/L and 30 mg/L) (reconditioning). Rats who were pretreated with only saline in combination with NMP without metformin administration served as control group. Three hours of NMP was used as method to assess hepatobiliary viability. The study demonstrated that metformin, as preconditioning agent, significantly improved ATP production, markers for hepatobiliary function (total bile production, biliary bilirubin and bicarbonate), and significantly lowered levels of lactate and glucose during NMP. On the other hand, metformin preconditioning lowered total bile salt secretion and expression of the FXR target genes CYP7A1 and BSEP. Furthermore, metformin preconditioning did not reduce markers for hepatobiliary injury such as AST, ALT, LDH, caspase-3 activity, TBARS or biliary gamma-GT and LDH. In addition, reconditioning with metformin did not improve hepatobiliary function or reduce hepatobiliary injury markers during NMP. In contrast, metformin added in a high concentration to the perfusion solution (300 mg/L) had adverse effects as it significantly increased levels of glucose and lactate, decreased bile production and reduced glycogen content during 3 hours of NMP. Contrary to expectations, in this study, metformin preconditioning or reconditioning was not able to significantly reduce preservation injury. A possible explanation for this may be that the reference group (heart beating model) did not show major injury after 4 hours of SCS. Therefore, it still remains unknown whether metformin preconditioning is also able to lower preservation injury in a model with more severe injury at baseline (e.g. donation after circulatory death model).
Future Perspectives

The current strategy to optimize the use of ECD livers is to find a balance between additional donor related risk factors and recipient factors. Although no strict guidelines are available for the allocation of ECD livers, a couple of models are useful that can help with balancing of risks between the recipient and the donor. These models are able to predict recipient patient/graft survival when ECD livers are used for transplantation. Examples of such models are the donor risk index (DRI) (6) or DRI adjusted for the Eurotransplant registry (ET-DRI) (7), donor age combined with model for end-stage liver disease (D-MELD) score (8), and balance of risk (BAR) score (9), all using donor related risk factors whether or not combined with recipient risk factors. Although these models are applicable for risk assessment, usage of these models will not lead to a major increase of ECD livers that are necessary to reduce waiting list mortality (10). In addition, the common perception is that availability of suitable donor organs is getting worse due to the increased prevalence of diabetes type II and obesity in the general population. As a consequence, other strategies are needed to improve postoperative outcomes of transplantation of ECD livers.

A possible way to reduce preservation injury compared to the current preservation method SCS is the application of machine perfusion as preservation method. In the last years, substantial evidence, mainly in animal experiments and studies using (discarded) human donor livers, have shown that machine perfusion is a very promising method to lower preservation injury and improve function of ECD livers (11-19). In two clinical studies, Guarrera et al. and Dutkowskji et al. have reported that human recipients of an end-ischemic hypothermic machine perfused liver showed less early allograft dysfunction, fewer biliary complications, and shorter length of hospital stay compared to recipients with a liver, which was only preserved with SCS (11,12,19). Although these reports included a relatively small numbers of recipients, the results are very encouraging and support the initiation of randomized clinical trials with hypothermic machine perfusion (HMP). For subnormothermic machine perfusion (SNP) and NMP the first (proof-of-principle) clinical studies have yet to be published.

Despite recent substantial improvements in machine perfusion of the liver, several basic parameters of this technology are still subject to ongoing (pre)clinical research, including temperature of the perfusion fluid, timing of perfusion, pressure, single or dual perfusion, nature of perfusion solution, oxygenation of perfusate, and suitable biomarkers for hepatobiliary injury in the perfusate. In the next section of this chapter, these parameters will be discussed and directions for further research will be given.
**Timing and Duration of Perfusion**

Machine perfusion can be performed at different time points between organ procurement and transplantation. Machine perfusion can be carried out in the donor (normothermic regional perfusion) (20), immediately after procurement, during transport, or at the end of transport (so called end-ischemic machine perfusion) (21). Depending on the perfusion temperature, some time intervals are not beneficial such as a short period of NMP at the end of SCS (end-ischemic NMP) (22-24). Because machine perfusion during the transport phase is a logistic challenge, most experience has been gained with a short period (1-2 hours) of end-ischemic MP in the transplantation hospital under hypothermic, subnormothermic or controlled rewarming conditions. However, a combination of machine perfusion during different phases between organ procurement and transplantation will synergistically act to make the effects of single periods of machine perfusion more powerful (21).

**Temperature of the Perfusion Fluid**

As mentioned previously, machine perfusion can be performed under different temperature conditions such as HMP (4-12°C), SNP (20-30°C) and NMP (37°C). In addition, gradual warming-up of the perfusion solution (controlled oxygenated rewarming; COR) from 8 to 20 degrees Celsius has also shown to be a feasible temperature strategy (25-27). In our study, performed with DCD rat livers, as described in chapter 10, we found no differences between the three temperature conditions HMP, COR, and SNP. However, in a previous study using a porcine model, Minor et al. indicated that COR might be the best perfusion temperature for the preservation of liver grafts after living donation (25). A possible explanation for the dissimilarity in the results between the study of Minor et al. and our study, could be next to the various donation models (DCD versus living donation), also the difference in specific liver mass. A porcine liver has a higher weight and therefore the influence of slowly rewarming might be more intensive than in a rat liver, which has a relatively small liver mass. In addition, in the field of ex vivo lung perfusion, it is known that gradual rewarming of the lung after SCS provides the best results (28,29). Clinical trials with COR are therefore strongly recommended. Interestingly, Minor and coworkers already initiated such a study; six ECD livers were recently transplanted after COR. All recipients showed a good liver function with a median peak aspartate aminotransferase (AST) level within the first 7 days of 564 (305-1525) U/L (D. Hoyer et al. presented at World Transplant Congress, Philadelphia 2015). The final outcomes of this study are awaited with great interest.

With respect to NMP, a short period of NMP prior to reperfusion has shown no beneficial effects compared to only SCS preservation. During a short period of NMP, the full cascade of I/R injury will be activated without a conditioning or protective effect of NMP (22-24). The general hypothesis is that NMP is only beneficial if it is performed during the entire preservation period or for a couple of hours after
SCS to washout the effects of preservation injury (5). The results of a clinical study using NMP during the entire period of preservation are expected soon (study performed under supervision of Professor Peter Friend, Controlled-Trials.com ID: ISRCTN14355416, OrganOx, Oxford, UK). In addition, another advantage of NMP is that liver viability can be assessed prior to transplantation (4,30). We used this application of NMP in experiments described in this thesis. A disadvantage of NMP is that an interruption of the perfusion or a failure of the perfusion system may cause substantial tissue injury like an “ex situ DCD” procedure. Moreover, the available machines for NMP do not have a built-in cold storage back-up system to prevent tissue injury when the perfusion is interrupted.

**Pressure of Perfusion**

During perfusion it is necessary to find a balance between optimal tissue perfusion and shear stress induced endothelial injury. A low pressure can lead to incomplete perfusion, while a high pressure can cause injury to the endothelial cells. It is well known that high portal pressure disrupts endothelial sinusoidal cell integrity (31). For HMP and NMP, studies have given directions to the optimal perfusion pressure (4,32). However, the ideal pressure for SNP has to be determined. So far, experiments with SNP are only performed under flow-controlled conditions (no pressure control used) or the pressures during these SNP experiments were similar to hypothermic conditions (13,14,18,25,33). In our experiment described in chapter 10, we perfused rat livers under subnormothermic conditions with a pressure of 40 mmHg on the arterial and 4 mmHg on the portal side. However, it has yet to be established whether this pressure is the most optimal.

**Single or Dual Perfusion**

It is known that under physiological conditions, perfusion through the hepatic artery is necessary to perfuse the microcirculation (peribiliary vascular plexus) around the bile ducts (34). In our experiments, in particular during HMP, we perfused through the portal vein and hepatic artery. However, Dutkowksi and coworkers who have extensive expertise in perfusion under hypothermic conditions, perfuse the liver only through the portal vein (12,15,35). Nonetheless, more research is required to determine whether only portal vein perfusion is sufficient enough to adequately preserve the biliary epithelium.

**Nature of Perfusion Solution**

Depending on the perfusion temperature and the corresponding degree of liver metabolism, it is necessary to adjust the ingredients of the perfusion solution. The available machine perfusion solutions are mainly based on a colloid with electrolytes to mimic the normal physiological osmolarity. However, amino acids or glucose are absent in these solutions. During SNP, the liver has a higher degree of metabolic activity compared to HMP. Consequently, it is questionable if the currently available perfusion solutions contain sufficient amounts of
nutrients to fulfill the metabolic demand during SNP. Experimental studies with a perfusion fluid based on William’s Medium E (hepatocyte growth medium) and with or without an artificial oxygen carrier have shown its feasibility during SNP (18,33). Therefore, future studies are needed to examine the ideal perfusion fluid for SNP. In addition, because during NMP normal physiological conditions are mimicked, it is definitely necessary to add nutrients and an oxygen carrier to the perfusion fluid. In our NMP experiments, we used human RBCs as oxygen carrier. However, given the expectation that the frequency of NMP will rise in the upcoming years, the request for human RBCs will consequently also increase. RBCs are not unlimitedly available; therefore the expectation is that studies on the use of artificial oxygen carriers such as hemoglobin-base oxygen carriers (HBOCs) added to NMP perfusion fluids, will appear in the next years. Furthermore, NMP opens new opportunities to repair preservation injury by adding pharmacological compounds or even mesenchymal stem cells (3-5). Recently, an animal study has shown that a cocktail of defatting agents lowered the amount of steatosis during NMP (36). However, these results have to be confirmed in clinical studies with severe steatotic (discarded) donor livers.

**Active Oxygenation of the Perfusate**

For HMP with human donor livers it is not known if oxygenation of the perfusion fluid is beneficial. In two clinical series, Guarrera *et al.* (11,19) did not actively oxygenate the perfusion solution, while Dutkowski *et al.* used 100% oxygen (12). Only in animal studies it has been shown that perfusion with 100% oxygen during HMP improves the functional recovery after ischemia, compared to perfusion with 20% or 0% oxygen (37,38). Advocates of perfusing with oxygen emphasize that oxygen is needed for improvement of the mitochondrial function before reperfusion and that perfusion without oxygen, only can be seen as flush out of injury markers (39). In contrast, proponents of perfusion without oxygen claim that oxygenation increases oxidative stress during reperfusion (39). However, the effects of HMP whether or not with actively dissolved oxygen in the perfusion fluid has to be explored with (discarded) human donor livers.

**Biomarkers for Biliary Injury**

During SNP and especially NMP, it is possible to assess the viability of the bile ducts by analyzing biomarkers in perfusate and bile (4,18). The current markers for biliary injury are changes in bile composition such as low glucose concentrations (< 1.0 mmol/L), or release of injury markers like gamma-GT or LDH into bile (40). In addition, also histological analysis of large bile duct biopsies can be used to predict the development of biliary injury, in particular NAS (1). Recently, Verhoeven and colleagues have reported that specific microRNA profiles in the preservation solution during SCS are associated with the development of NAS (41). It is therefore very interesting to study if these profiles of microRNAs are also measurable during machine perfusion and whether these profiles correlate with more established markers of hepatobiliary injury and/or histological assessment of the biliary tract.
References


