Hemostatic system activation and reperfusion injury in liver machine preservation and transplantation of extended criteria donor livers

Karangwa, Shanice

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CHAPTER 10

Summary, Discussion and Future perspectives

SUMMARY

Extended-criteria donor (ECD) livers, particularly those retrieved from donation after circulatory death (DCD) donors currently comprise more than 50% of all deceased donor organs in the Netherlands. Even though utilization of these livers has resulted in successful transplantations, these livers are frequently associated with a higher incidence of intraoperative and post-transplant complications. Development of these complications has mainly been attributed to ischemia-reperfusion (IR) injury. In this section, the main findings from the studies described in this thesis are summarized and discussed. This chapter is then concluded with a section on the future perspectives within this field of research.

PART I: ACTIVATION OF THE HEMOSTATIC SYSTEM

Chapter 1 provides the reader with an introduction to this research field, followed by an outline of the chapters within this thesis.

The specific effect of transplantation of a DCD liver on intraoperative hemostasis is not fully known. Therefore, in **Chapter 2** we aimed to investigate whether transplantation of DCD compared to DBD liver grafts is associated with an increased incidence of (severe) intraoperative blood loss and greater intraoperative transfusion requirements following graft reperfusion. For this single center retrospective cohort study, intraoperative data collected during all primary adult liver transplantations performed in the UMCG in the last 20 years were analyzed. Propensity score matching analysis was performed in which a total cohort of 218 patients were included with 109 patients in each group (DCD vs. DBD). We concluded that DCD liver recipients do not face a risk of increased post-reperfusion blood loss nor is there a difference between total blood loss between DCD and DBD liver transplantation procedures. Moreover, DCD liver recipients do not receive significantly more RBC, FFP or thrombocyte transfusions after reperfusion nor during the entire transplantation procedure. Furthermore, a subset analysis of plasma collected during 30 transplantations belonging to the matched cohort showed that transplantation of DCD livers does not increase the risk of

post-reperfusion hyper fibrinolysis as DCD and DBD liver transplant recipients possessed similar fibrinolytic profiles. Collectively, these results signify that the selective use of DCD liver grafts result in safe and successful transplant procedures without exposing patients to an increased chance of severe blood loss.

A predominant feature of reperfusion of a transplanted liver graft *in vivo* is the simultaneous activation of the coagulation and fibrinolytic systems[1]. Whether this similarly occurs during *ex situ* normothermic machine perfusion (NMP) was unknown. The objective of **Chapter 3** was to determine whether activation of the hemostatic system occurs during NMP and what impact this may have on predicting graft function and/or injury of DCD liver grafts. Twelve ECD donor livers declined for transplantation underwent 6 hours of end-ischemic NMP using a heparinized plasma-based perfusion fluid. Markers of coagulation activation (prothrombin fragment F1+2) and fibrinolysis (D-dimer, PAP complex, tPA) were measured in perfusion fluid at regular intervals and liver biopsies were examined for the presence of fibrin formation. Our results showed no increase in coagulation activation markers throughout NMP. Moreover, histological analysis did not present any evidence of new fibrin formation. Contrastingly, the fibrinolytic markers D-dimer and PAP complex levels significantly increased soon after starting NMP. D-dimer concentrations also correlated significantly with levels of injury marker ALT throughout NMP. With these results, we concluded that end-ischemic *ex situ* NMP results in the activation of fibrinolysis and not of coagulation in a heparinized system. We also showed that markers of fibrinolysis activation correlate significantly with markers of I/R injury and thus could potentially serve as markers for (severe) I/R injury and predictors of poor liver graft function.

Donor livers undergoing *ex-situ* NMP usually resume normal metabolic and synthetic functions, such as hemostatic protein production. However, the quantity and rate of (hemostatic) protein production is yet to be clearly defined. If the production supersedes the capability of the administered anti-coagulant agent, then the liver could potentially be at risk of developing intravascular thrombosis, which would hamper perfusion of the liver graft. In

Chapter 4, six donor livers declined for transplantation underwent 6 hours of end-ischemic NMP using a heparinized plasma-free perfusion fluid. Concentrations of key pro-coagulant, anti-coagulant and fibrinolytic proteins were measured in the perfusion fluid at regular intervals which were compared with a plasma-based reference solution. With net increases of greater than 50% for majority of these proteins, we demonstrated that donor livers perfused with a plasma-free perfusion fluid are capable of producing substantial amounts of hemostatic proteins during a relatively short period of NMP. As (clinical) NMP is increasingly being performed for longer periods, with the recent study by Eshmuminov et al, reporting one week long perfusion of liver grafts[2], our findings emphasize the importance for the need for the administration of adequate anticoagulant therapy during NMP of donor liver grafts.

Chapter 5 summarizes published literature on hemostatic management employed during machine perfusion. Our group is currently the only group thoroughly investigating hemostasis in donor livers during *ex situ* machine perfusion. Given the increasing implementation of (clinical) NMP, the objective of chapter 5 was to report on current anticoagulant agents used and the variation in dosing and administration of these agents during liver machine perfusion. Furthermore, we discussed the possibilities of using different anticoagulant agents during the various phases of NMP and the utilization the synthesis of liver-derived coagulation factors as potential viability markers during ex-situ NMP.

PART II: REPERFUSION INJURY

Non-anastomotic biliary strictures (NAS) are a major complication after liver transplantation and are known to critically affect patients' long-term survival and often result in an increased rate of re-transplantation. NAS occurring early after transplantation is largely associated with an ischemia-related pathogenesis and DCD liver grafts, with the additional ischemia they typically undergo, are particularly more susceptible to developing NAS. The biliary tree is principally perfused by the hepatic artery, therefore in **Chapter 6** we hypothesized that shorter arterialization time (time to hepatic artery anastomosis during implantation and thus

shorter ischemia to the biliary tree) would result in a lower incidence of NAS in DCD liver grafts. In this multi-center retrospective study, a total of 289 DCD-III liver transplantations were analyzed. Median arterialization time was 33 (IQR 25 - 48) minutes and NAS was diagnosed in 26% of the total cohort. Multivariate cox proportional-hazards regression analysis showed that arterialization time was not an independent risk factor for the development of NAS. Therefore, we could conclude that if not extensively prolonged, arterialization time is not associated with development of NAS and transplant surgeons do not necessarily need to perform the arterial anastomosis first as opposed to the standard portal vein anastomosis solely to prevent the development of NAS.

Chapter 7 is a systematic literature review cataloging the differences and variation in techniques and methodology of donor liver machine perfusion reported in current published literature. This review demonstrated that different terms are frequently used to denote the same modality of machine perfusion. We also noted that the temperature ranges reported for particular types of machine perfusion are highly variable and that reporting of some crucial aspects of the methodology of perfusion are lacking. This review proposed a standardization of nomenclature and provided guidelines on how methodology can be reported so as to facilitate comparison as well as clinical implementation of liver MP procedures for future studies.

Chapter 8 focuses on hypothermic machine perfusion (HMP). This review principally focuses on the role HMP plays in mitigating IR injury. This protective effect is discussed in depth whilst presenting promising results from all the HMP clinical studies performed so far. Furthermore, as result of recently published data by Muller et al[3] showing that increasing levels of mitochondria-derived flavin mononucleotide (FMN) measured in the perfusate during HMP (as a result of mitochondrial IR injury) was predictive of poor graft function post-transplantation, the new hot topic; viability assessment of donor livers during HMP is discussed. Lastly, we describe the injury to the biliary tree throughout the processes of liver

donation, preservation and transplantation as well as outline the protective effect HMP has on the biliary tree.

Chapter 9 is a pilot study investigating the safety and efficacy of cytokine adsorption during NMP of DCD donor livers. Six porcine livers randomly assigned to two groups (intervention vs. control) underwent 3 hours of NMP with or without addition of a cytokine adsorber to the NMP circuit. The CytoSorb cytokine adsorber is a non-selective adsorber that is designed to remove both pro- and anti- inflammatory cytokines depending on the concentration of the these cytokines in any given solution. During NMP, no decrease in cytokine levels in the perfusion solution of the intervention group was seen. Moreover, similar levels of cytokines were measured in the pre- and post-adsorber perfusion samples at all time-points during NMP. These findings suggest that the CytoSorb cytokine adsorber was not efficient in removing inflammatory mediators during NMP. Short ischemia times may have limited the extent of injury incurred by these livers, thereby preventing measurable/observable efficacy of the CytoSorb adsorber from occurring. Nevertheless, addition of the CytoSorb adsorber did not lead to any complications or damage of the graft during and after perfusion. Moreover, graft function in the intervention group was comparable to controls. Therefore, from this preliminary, proof-of-concept study, we concluded that cytokine adsorption during NMP of donor livers is safe and feasible. However, the efficacy remains to be determined in future studies.

Altogether, the studies summarized above have helped provide further understanding of the hemostatic system and reperfusion injury during machine perfusion and transplantation of ECD/DCD donor livers. **The most important findings of this thesis are:**

1. Liver transplantation using DCD donor livers, compared to DBD donor livers, does not lead to higher intraoperative blood loss after reperfusion of the DCD liver graft or greater post-reperfusion transfusion requirements.

2. Activation of fibrinolysis, and not of coagulation occurs during end-ischemic *ex situ* normothermic machine perfusion of human donor livers.
3. Donor livers are capable of producing substantial quantities of hemostatic proteins during a mere six hours of *ex situ* normothermic machine perfusion with a plasma-free perfusion fluid.
4. The time between portal and arterial reperfusion in DCD-LT is not a significant risk factor for developing NAS, patient death and graft failure. Prolonged cold ischemia time and advanced donor age, however, do increase the risk of the development of NAS in DCD-LT.
5. The increased experimental and clinical application of machine perfusion of donor livers called for the need for the standardization of nomenclature and methodology reporting.
6. Cytokine adsorption during end-ischemic *ex situ* normothermic machine perfusion of DCD livers is safe and feasible. However, effective removal of cytokines during NMP remains to be determined. Future studies with longer, yet clinically relevant ischemia and perfusion times are necessary to determine the efficacy of cytokine adsorption during NMP and whether it results in improved graft quality and function.

DISCUSSION AND FUTURE PERSPECTIVES

The studies described in this thesis have provided insightful answers to the questions asked at the beginning of this trajectory, which I believe have contributed notably to the field of DCD liver transplantation. Needless to say, with these answers, new unanswered questions arose and we realise that new challenges remain to be tackled. In this part of the chapter, I'd like to further discuss the findings from the studies performed in this thesis, address the new unanswered questions and challenges, as well as discuss possible directions and opportunities for future research.

Hemostasis during liver transplantation using DCD liver grafts

DCD liver transplantation is typically associated with a higher incidence of complications such as early allograft dysfunction and non-anastomotic biliary strictures. Although these complications are of utmost importance, they are not the only complications to be concerned about. Patients undergoing liver transplantation are typically at risk for severe blood loss and high blood-product transfusion rates, particularly during reperfusion of the implanted graft[4-6]. The findings from **Chapter 2** however**,** showed that DCD transplantations in fact, have similar rates of blood loss and transfusion requirements in comparison to DBD transplantations. Our results therefore reiterate the safety of the utilization of DCD livers for transplantation. Moreover, liver transplant anesthesiologists need not be afraid of a potential increased risk of haemodynamic instability specifically because a liver is derived from a DCD donor. These findings are especially relevant for countries like Germany and Hungary (within the Eurotransplant zone) in which only DBD liver grafts are currently used for transplantation. This may encourage such countries to consider the implementation of DCD liver transplantation which would potentially expand the current donor organ pool.

Nonetheless, our results partly dispute the findings and conclusions drawn by recent studies performed mostly in North America. These studies showed profound hyper-fibrinolysis, increased blood loss, higher transfusion requirements and a higher incidence of post-

reperfusion hemodynamic instability[7-9] in DCD liver transplantations as compared to DBD liver transplantations. Contrasting findings from a recent study by Kalisvaart et al showed transfusion requirements and the development of post-operative vascular complications to be comparable between DCD and DBD liver recipients[10]. These contradicting findings bring to light the difficulty in reaching a generally accepted consensus on the specific effect of DCD transplantation on intraoperative hemostasis. The criteria deeming a DCD liver suitable for transplantation is highly variable and in many instances, transplant center-specific. Moreover, the retrospective nature of these studies, the variation in recipient demographics, variable cold ischemia times owing to greater travel distances and varied anesthesiological hemostatic protocols hamper the ability to widely extrapolate these results. Therefore, prospective, larger cohort and preferably multi-center and/or multi-national studies are essential in order to investigate this further.

Normothermic machine perfusion of donor livers and the optimization of anticoagulant management

Anticoagulant management during NMP has thus far, solely consisted of the use of (unfractionated) heparin. **Chapter 3** illustrated that a singular bolus dose of 20,000 IU of heparin is capable of inhibiting coagulation activation and subsequent thrombin formation for at least six hours of NMP. However, NMP is increasingly being performed for extended durations with studies reporting perfusion periods of 15+, 24 hour, and even week long perfusions[2,11,12]. Therefore, **Chapter 4** opens the discussion about appropriate and adequate anticoagulation during MP. Collectively, these findings have shown that in order to ensure maximum protection of the liver graft from coagulation activation due to *de novo* hemostatic protein production, it is essential that anticoagulant dosing and administration during *ex situ* NMP is adequate and effective. It is worth further investigating the exact rate of hemostatic protein production during these extended periods of NMP in order to assess whether regular-interval or continuous anticoagulant administration is more protective than bolus dosing and perhaps eventually develop a standardized guideline on optimal anticoagulant

management during NMP of donor livers. Moreover, future studies are required to investigate the potential use of alternative anticoagulants such as direct thrombin inhibitors that do not rely on the presence of other proteins to function (such as in the case of heparin and antithrombin) or perhaps those that do not specifically require metabolic activation by the liver to function. These alternative anticoagulant agents may prove to be more efficient and/or more protective than heparin. For instance, in poorly functioning livers that are yet to be improved during NMP or in cases in which perfusate antithrombin levels in the perfusate are (still) low which would prohibit the anticoagulant effect of heparin.

Altogether, these studies have provided pioneering insight into hemostasis during normothermic machine perfusion of donor livers. However, as machine perfusion continues to advance, more knowledge is required in this area of research to optimize anticoagulant management during dynamic preservation of donor organs.

Utilization of hemostatic proteins for viability assessment of donor livers during normothermic machine perfusion

As machine perfusion steadily makes its transition from bench to bedside, the search for valid and reliable markers for graft viability and injury during machine perfusion is actively on-going. A consensus on universally validated viability criteria for livers undergoing *ex situ* NMP is yet to be established. Currently, majority of studies on liver NMP use common clinical biochemical markers such as transaminase and lactate levels, pH, electrolyte, glucose and bilirubin levels as viability markers of organ injury and function. In **Chapter 3**, we show that D-dimer too, could be used as a marker of injury during NMP. Prior to this study, D-dimer had not been considered as a potential injury marker, however, similar to the above-mentioned injury and functional markers, D-dimer is widely used clinically, hence, is easily interpretable. Additionally, the measurement of D-dimer in perfusate is quick allowing for real-time evaluation of graft injury prior to transplantation. These factors deem D-dimer a suitable injury marker.

Studies have shown that poorly-flushed livers during organ procurement tend to result in marginal quality and thus face a greater risk of developing intra- and post- operative complications[13,14]. The utilization of D-dimer as an injury marker during NMP may possibly provide insight into the patency of the (micro) vasculature. The hypotensive-hypoxic phase during donor demise triggers coagulation activation and consequent (micro) clot formation. Given that a high release of D-dimer upon NMP signifies fibrinolysis of these pre-existing clots, this could imply suboptimal flushing of the donor liver resulting in residual clots in the (micro) vasculature. These micro (clots), as has been suggested in previous studies, could hinder adequate perfusion and perpetuate injury to the bile ducts during reperfusion[15,16]. A potential area for future research would be to investigate this further in a larger cohort of clinical donor livers in order to assess whether D-dimer is indeed a reliable and sensitive injury marker and whether or not a threshold concentration of D-dimer can be determined, which could be used to predict of poor graft function.

Besides the widely used clinical biochemical markers, coagulation factors have also been directly (by measuring coagulation factor concentrations in the perfusate), or indirectly (by measuring INR) used as viability markers to demonstrate synthetic function of donor livers during NMP[17,18]. The utilization of liver-derived hemostatic proteins as viability markers is promising as it provides insight into the overall synthetic capacity of the liver. However, measurement of these proteins is currently limited by the lack of rapid and sensitive assays that are capable of generating results real-time during NMP. This is necessary in order to determine whether a donor liver is fit for transplantation prior to the procedure itself. Unfortunately, current methods/assays such as ELISAs only allow for this to be performed retrospectively. Therefore, future research calls for the development of quicker assays. Furthermore, alternative ways to measure these proteins in the perfusate without being affected/limited by the presence of heparin should be explored. If this can be achieved, hemostatic proteins could potentially be universally implemented as reliable viability markers of hepatocellular synthetic function.

Mitigation of ischemia-reperfusion injury and improvement of organ quality through machine perfusion

The occurrence of post-transplant graft dysfunction and the development of complications such as coagulopathy and NAS, particularly in extended-criteria donor (ECD) livers, is mainly attributed to by ischemia-reperfusion (IR) injury[19]. Despite the inferior outcomes associated with the utilization of ECD livers, transplantation of these organs has increasingly become inevitable to encumber the shortfall of suitable donor organs. Transplantation of ECD liver grafts has thus become a major driving force to discover and implement strategies to mitigate IR injury in an effort to improve ECD graft quality. In the pre-machine perfusion (MP) era, methods to mitigate of IR injury were mainly implemented in the procurement and static preservation phases; for instance by minimizing warm and cold ischemic periods through the reduction of donor hepatectomy times and instigating cold flush of the donor organ as soon as possible. Moreover, modifications of preservation fluids were made such as the addition of N-acetylhistidine, amino acids, and iron chelators to HTK preservation solution in an effort to inhibit hypoxic injury and oxidative stress in liver grafts with microvesicular steatosis[20].

Machine perfusion however, has brought forth the opportunity to further advance strategies in which IR injury could be alleviated. The various modalities of MP provide strategies to minimize IR injury. A major advantage of MP is the possibility to provide interventions and administer pharmacological agents to the circuit. In the various MP modalities, several groups have explored ways to further minimize or circumvent IR injury during MP, for instance; administration of vasodilator or thrombolytic therapy, cytokine and complement inhibitors as well as the addition of stem-cells to stimulate cellular regeneration or the administration of pharmacological agents to modulate lipid metabolism in steatotic grafts[21]. However, this particular field in MP research currently remains underexplored and published literature remains scarce. In **Chapter 9,** we explore the mitigation of IR injury in a DCD model through cytokine adsorption. As previous studies show, the activation of Kupffer cells

upon the reestablishment of blood flow during reperfusion results in the release of pro-inflammatory cytokines, activation of neutrophils and adhesion molecules on the sinusoidal endothelium. This results in the perpetuation of the inflammatory response resulting in a downstream tissue damage and initiate of cell death programs[19,22]. Our preliminary findings exhibited that cytokine adsorption is feasible and safe to implement during machine preservation of donor organs. However, we were not (yet) able to determine the efficacy. More studies are necessary in the near future to further investigate the efficacy of cytokine filtration and whether this minimizes graft injury and improves graft function.

Extending donor liver preservation beyond the current limit: The future

As machine perfusion continues to be increasingly applied, the “ice box” or static cold storage may eventually become a preservation modality of the past. Machine perfusion has proven to provide vast opportunities to not only minimize IR injury and improve graft function, but also allows for the preservation of organs for longer periods of time that would not have been possible in the past. Several studies have reported successfully preserving livers for 24+ hours, and more recently, even up to a week[2,11]. This opportunity to “buy time” in organ preservation offers possibilities of further repairing and modifying donor livers through means that require more than just a few hours to achieve desirable results. Such possibilities include; the administration of pharmaceutical agents[23], perhaps in doses that would otherwise be toxic for other systemic organs and even exploring the incorporation of stem cells into the circuit to stimulate the regenerative capacity of the liver during NMP[24]. As we enter an era of technological advancement in the preservation of donor organs, I envision a future in which extended machine perfusion will go beyond optimal preservation and improving graft function of suboptimal donor livers, but also enable: (1) the de-fatting of severely steatotic livers; a problem which continues to grow in the western world, (2) the regeneration of viable liver tissue in for instance, split or reduced livers to overcome size mismatches during transplantations and thus minimize rejections of suitable livers for size mismatch reasons and (3) in cases where a patient is too sick to be transplanted at the

moment a door organ becomes available, allow for the preservation of the donor liver until the recipient is stable/healthy enough to be transplanted or otherwise preserved long enough to permit logistical arrangements of a transplant surgical team. In so doing, extended machine preservation stands the chance to minimize the number of discarded donor organs, and vastly increase the current pool of transplantable donor organs thus helping alleviate the ever-growing disparity of donor organs.

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