Initial Organ Flush Temperature in Liver Preservation

by

Jordan Joseph Nostedt

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

Department of Surgery
University of Alberta

Abstract

Waitlist mortality resulting from a shortage of transplantable liver grafts has led to increased use of donation after circulatory death (DCD) grafts. However these marginal grafts are associated with higher complication rates. Normothermic *ex-situ* perfusion of DCD livers has shown encouraging results in both animal and clinical trials as a potential preservation strategy to expand safe use of DCD grafts. Following procurement, initial flush with a cold preservation solution is the standard of care. There is concern that initial cold flush followed by warming may lead to additional liver injury, however the optimal initial flush temperature has not yet been identified. We hypothesized that avoiding hypothermia during initial organ flush will lead to better quality DCD liver grafts. Following simulated DCD with 60 minutes of warm ischemic time, 24 pigs underwent liver procurement and initial organ flush for a period of 5 minutes. The current clinical standard of 4°C histidine-tryptophan-ketoglutarate (HTK) served as the control group. Livers were also flushed with HTK at 25°C and 35°C (n=4 per group). For additional comparison a normokalemic adenosine-lidocaine crystalloid solution (AD) at the same temperatures (n=4 per group) was also used. Livers then underwent 12 hours of normothermic perfusion. Adenosine triphosphate, lactate and hepatocellular injury markers were determined. In the HTK groups hepatocellular injury markers and hemodynamic parameters were lower in the 4°C group while the AD groups demonstrated the opposite pattern. There was no statistical difference in these parameters when all groups were compared. All groups demonstrated similar recovery of ATP levels and lactate clearance after warm ischemic time. These results suggest that altering the temperature of the initial flush solution alone does not provide added benefit over the current clinical standard of cold initial flush with HTK for DCD livers preserved with normothermic perfusion. However, larger powered studies to investigate the effects of alternative solutions such as AD at warmer initial flush temperatures may be warranted.

Preface

The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project name “Improving Liver Preservation in a Large Animal Model by NPM”, AUP00001036.

Chapter 2 is adapted from a manuscript that was submitted for publication March 16, 2019. Jordan Nostedt performed the literature review and was the primary author of this review Dr. Darren Freed, Dr. James Shapiro, and Dr. David Bigam also contributed to the final manuscript.

Chapter 3 is adapted from:

Nostedt, JJ., Skubleny, DT., Shapiro, AMJ., Campbell, S., Freed, DH., Bigam, DL., "Normothermic Ex Vivo Machine Perfusion for Liver Grafts Recovered from Donors after Circulatory Death: A Systematic Review and Meta-Analysis," HPB Surgery, vol. 2018, Article ID 6867986, 8 pages, 2018.

JN designed the study, reviewed abstracts/articles for inclusion, analyzed data and was the primary author of the manuscript. DTS reviewed abstracts/articles for inclusion. SC assisted with the literature search. DHF, AMJS, DLB contributed to the study design. All co-authors contributed to the final version of the manuscript.

Chapter 4 describes the main project of this thesis. It is in preparation for publication. Jordan Nostedt had a primary role in experimental design, performed all experiments, data collection,

and data analysis and was the primary author of the final manuscript. Dr. Jessica Hopkins and Dr. Mackenzie Lees provided assistance with performing experiments. Dr. Thomas Churchill assisted with data collection. Dr. Sunita Ghosh provided statistical assistance. Dr. Aducio Thiesen and Dr. Benjamin Adam provided histologic assessments. Dr. Darren Freed, Dr. James Shapiro and Dr. David Bigam provided guidance with experiment design, and data analysis. All co-authors contributed to the final version of the manuscript.

Acknowledgements

I would first like to thank my supervisor Dr. Bigam. I am grateful for your support and guidance throughout the completion of this project, but also for your ongoing mentorship. I have the highest respect for you as a clinician, researcher, and overall mentor.

I would also like to thank Dr. Freed and Dr. Shapiro for being a part of my supervisory committee. I appreciate your input and guidance for this project. Your inquisitive approach has developed from a long history of research experience and I have a learned a great deal from you both.

I would also like to acknowledge Xiuhua (Sue) Wang for your daily support in the lab. Additionally I would like to thank Dr. Jessica Hopkins and Dr. Mackenzie Lees for your assistance in the operating room without which this project would have been much more difficult, and certainly less enjoyable. Lastly, I would like to thank Tomiko McCall and Tracey Zawalusky for your help with navigating my way through the CIP program.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AD	adenosine-lidocaine crystalloid solution
ALT	alanine transaminase
ANOVA	analysis of variance
AST	aspartate transaminase
ATP	adenosine triphosphate
°C	degrees Celsius
COR	controlled oxygenated rewarming
DAMP	damage associated molecular pattern
DBD	donation after brain death
DCD	donation after circulatory death
EAD	early allograft dysfunction
EC	Euro-Collins solution
ECD	extended criteria donor
H&E	hematoxylin & eosin
HES	hydroxyethyl starch
HMGB-1	high mobility group box 1
HMP	hypothermic machine perfusion
HTK	histidine-tryptophan-ketoglutarate
IC	ischemic cholangiopathy
IL-6	interleukin 6
IGL-1	Institut George Lopez -1
IR	ischemia reperfusion
LDH	lactate dehydrogenase
MD	mean difference
MPT	mitochondrial permeability transition pore

NADPH	reduced nicotinamide adenine dinucleotide phosphate
NECMO	normothermic extracorporeal membrane oxygenation
NMP	normothermic machine perfusion
NO	nitric oxide
NRP	normothermic regional perfusion
PNF	primary non-function
PRR	pattern recognition receptor
ROS	reactive oxygen species
SCS	static cold storage
SD	standard deviation
SNMP	subnormothermic machine perfusion
SYRCLE	Systematic Review Centre for Laboratory Animal Experimentation
TLR	toll-like receptor
TNF-α	tissue necrosis factor alpha
tPA	tissue plasminogen activator
UW	University of Wisconsin solution
WIT	warm ischemic time

Chapter 1

A Brief History of Liver Preservation for Transplantation

Introduction

In the 1930s Carrel and Lindbergh were the first to describe *ex-vivo* perfusion of organs with the intent of organ preservation and study.(1) Subsequently in 1960 Lapchinsky demonstrated successful kidney re-implantation was possible after prolonged preservation using perfusion with hypothermic blood. (2) This sparked interest in the field of organ preservation with multiple studies published utilizing *ex-vivo* hypothermic perfusion to preserve kidneys for increasing periods of time.(3-5) However, in 1969 using a combination of surface cooling and Collins Solution, Collins et al. were able to successfully preserve kidneys for up to 30 hours.(6) This static cold storage (SCS) technique proved to be less expensive and technically much simpler than machine perfusion. With the subsequent development of University of Wisconsin Solution (UW) in 1987 after prolonged preservation of the canine pancreas,(7) this became the preferred method of organ preservation. Despite the later development of other solutions, UW continues to be a commonly used solution for the preservation of intra-abdominal organs and its discovery was a major stimulus for the cold preservation era in transplantation. More recently, as a result of quality organ shortages, there has been an increased utilization of extended criteria donors (ECD).(8) These marginal grafts, which do not tolerate SCS well, have led to resurgence in research looking at the use of machine perfusion for organ preservation in recent years. This chapter will review both cold storage and the current status of machine perfusion, as they relate to liver preservation, and particularly donation after circulatory death (DCD) grafts as compared to donation after brain death (DBD). The key difference between DCD and DBD grafts is that DCD livers endure a period of warm ischemic time (WIT) from the point of cardiac arrest until organ procurement. This WIT is variable depending on local regulations and logistics, however

during this period the liver suffers ischemic damage. On the other hand, DBD grafts experience minimal WIT as the liver is perfused right up until the time of procurement.

Static Cold Storage

Effects of Hypothermia

The premise of cold storage relies on depressed metabolic function. A 1.5-2 fold drop in metabolic activity is observed for each 10 degree drop in temperature, the "Q10 effect."(9) While this temperature change allows for prolonged periods of storage it also generates injury to the organ. During the process of liver procurement the inciting ischemia results in anaerobic metabolism and a resulting depletion in adenosine triphosphate (ATP).(10) With lower temperatures, although slowing metabolic rate, the cell remains metabolically active to some degree, but its ability to regenerate ATP cannot keep up to the metabolic requirements during this ischemic period and cellular energy levels are depleted.(11, 12) The sodium/potassium ATPase pump, which is responsible for maintaining the cell membrane potential(13), is impaired by the low levels of ATP and it's loss of function leads to disruption of the cell's membrane leading to accumulation of intracellular sodium, altered membrane charge and a reversal in flow of potassium and chloride ions.(14) The overall result of these membrane changes is calcium influx(15), cellular edema(14), and leukocyte adhesion leading to subsequent activation of the inflammatory cascade and further cell damage. (16) These cellular changes also prime the cell for further damage upon reperfusion.(17)

Ischemia-Reperfusion injury

Following ischemic cold storage, the reperfusion phase is an additional critical period for significant cell damage during organ transplantation. Ischemia-reperfusion (IR) injury is a multifaceted process that leads to inflammation and cell death. IR injury results both from direct oxidative stress injury mediated through the generation of reactive oxygen species (ROS), as well as the resulting inflammatory reaction leading to further cell damage and additional ROS production that creates a cycle of cellular damage during reperfusion. (18)

ROS are generated from multiple sources including Kupffer cells(19), neutrophils(20), and intracellular sources including the mitochondria(17), xanthine oxidase(21), and phagocytes in endothelial cells and hepatocytes called NADPH oxidases.(22) ROS lead to a sequence of direct oxidative stress damage to cells that includes a rise in intracellular calcium, oxidation of pyridine nucleotides, and activation of mitochondrial membrane permeability transition pores (MPT).(23) When the MPT is inhibited pharmacologically there is a protective effect with respect to IR injury highlighting the importance of this step as a catalyst for IR injury.(24) The opening of MPTs disrupt the mitochondrial cell membrane potential leading to creation of superoxide molecules, and cell death with resultant release of damage associated molecular patterns (DAMPS).(17)

DAMPS are newly described components of, or cellular metabolites of damaged cells that are recognized by pattern recognition receptors (PRR).(25) Toll like receptor 4 (TLR-4) is a PRR that has been shown to play a prominent role in IR injury.(26) High mobility group box 1 (HMGB-1) is a key DAMP released by hypoxic hepatocytes during IR injury(27) and leads to up-regulation of transcription factors responsible for the expression of cytokines, chemokines,

and cellular adhesion molecules.(25) While Kupffer cells contribute early in IR injury, release of cytokines and chemokines attracts and activates neutrophils, and increased cellular adhesion molecules allow for neutrophil extravasation, a crucial step required for neutrophils to cause further cellular injury.(28) Neutrophils are more prominent later on in the IR process and damage cells via the release of proteases and further ROS generation.(17) CD4 lymphocytes also contribute by amplifying the Kupffer cell and neutrophil response.(29)

Changes in the microvasculature also contribute to IR injury. Activation of Kupffer cells can lead to microvascular endothelial damage with resulting adhesion of neutrophils and platelets in the microcirculation leading to worsening ischemia.(30) Prostaglandin E-1 has been shown to reduce IR injury by preventing leukocyte adhesion to damaged endothelial cells.(31) Within the microcirculation, nitric oxide (NO) is produced from two sources, endothelial nitric oxide synthetase, present only in endothelial cells, and inducible nitric oxide synthetase, which can be up-regulated in endothelial cells and hepatocytes.(32) NO is a vasodilator in the sinusoidal and post sinusoidal vasculature.(18) Both adenosine, which prevents a drop in NO levels, and exogenous use of NO have been suggested to be beneficial in limiting IR injury.(33, 34) As such, the summative impact of NO during IR injury is thought to be protective.(35) Endothelin on the other hand counters the effect of NO and results in vasoconstriction.(36) During the IR process, there is increased sensitivity to endothelin leading to excessive vasoconstriction above and beyond what can be balanced out by NO leading to hypoperfusion of liver tissue. (37)

IR injury is a complex process with mechanistic details still being investigated. However, the generation of ROS, inflammatory response, immune response and microcirculatory changes discussed above all play a role in IR injury during liver transplantation. Regarding organ

preservation, interventions at these steps in the IR injury pathway aimed toward limiting IR injury remain an active area of research.

Liver Preservation Solutions

Commonly used preservation solutions include Euro-Collins (EC), UW, Histidine-tryptophan-ketoglutarate (HTK), and newer solutions that have been developed include Celsior and Institut Georges Lopez-1 (IGL-1). The compositions of these preservation solutions are outlined in **Table 1-1**. These solutions aim to reduce cellular edema, limit oxidative stress and buffer shifts in acid-base status. The key differences amongst available solutions will be highlighted here.

To prevent cellular edema UW has the large molecules raffinose, lactobionate and hydroxyethyl starch (HES) to maintain oncotic pressure. (38) To limit damage of ROS the reducing agent glutathione and xanthine oxidase inhibitor allopurinol are included.(38) To limit cellular acidosis hydrogen ions are buffered by phosphate, and adenosine is added as an energy substrate. (38)

HTK uses mannitol to prevent cellular edema, which also, alongside tryptophan and histidine function to limit ROS injury. Buffering is through histidine, and glutamate is added as an energy precursor.(39)

EC limits edema with glucose and mannitol, while mannitol also has antioxidant capacity. (39) EC contains both phosphate and bicarbonate to buffer hydrogen ions and does not contain a cellular energy substrate. (39)

Celsior is a relatively new solution that combines mannitol and lactobionate for oncotic pressure, buffers hydrogen ions with histidine and utilizes glutamate for an energy substrate, while glutathione, mannitol and histidine protect against oxidative damage.(39) IGL-1 is a newer solution with higher sodium relative to other solutions, as well as polyethylene glycol added for its microcirculatory and antioxidant properties in place of HES in UW. (40) Other additives in IGL-1 include adenosine for an energy substrate and allopurinol to limit ROS production. (40)

Preservation Solutions: Clinical Outcomes

UW is commonly used in the preservation of abdominal organs for transplant but it has the disadvantages of being difficult to flush due to high viscosity, hyperkalemic cardiac arrest due to high potassium concentration, impaired microcirculation preservation as a result of particle deposition, higher cost and a possible increased rate of ischemic type biliary complications in the setting of DCD liver transplantation.(40) HTK has seen increased use as it has lower viscosity, lower potassium and potentially improved biliary and microcirculatory preservation with lower costs. (41) However when directly compared the clinical outcomes for UW and HTK have shown mixed results.

Multiple studies show clinical equivalence between HTK and UW with respect to primary non-function (PNF), primary dysfunction and overall patient and graft survival. (42-46) A systematic review by Feng *et al*. showed no significant difference in patient or graft survival,

PNF or primary dysfunction. (47) However, in a retrospective review of the United Network for Organ Sharing database comparing HTK (n=4755) and UW (n=12673), Stewart *et al*. reported an increased risk of graft loss, particularly in DCD livers and those with more than eight hours of cold ischemic time when preserved with HTK.(48) A meta-analysis in 2013 showed no significant difference in primary graft dysfunction and patient or graft survival when comparing UW to HTK. (49) Overall no reproducible difference between UW and HTK regarding primary graft function, and overall patient and graft survival has been demonstrated.

Biliary complications are one area where HTK and UW are thought to differ.(50) Yet again, the literature is mixed in this regard. In some studies, biliary complications have been found to be more common in livers preserved with UW.(43, 46, 51) Due to the high viscosity of UW solution it causes aggregation of red blood cells, which leads to stasis, possible incomplete organ washout and lower penetration into the biliary tissues.(52) Poor flush can lead to biliary stasis, increased bile salt toxicity,(53) and a higher bile salt to phospholipid ratio which has been linked to non-anastomotic biliary strictures.(54) HTK on the other hand is much less viscous, resulting in easier and more rapid tissue penetration although at the expense of requiring more solution volume.(43) However, Meine *et al.* in a randomized prospective study showed increased biliary complications in the HTK group when compared to UW.(55) The authors note that donors from the HTK group were older and that low volumes of HTK were used which may contribute to the increased biliary complication rate.(55) A subsequent meta-analysis showed no statistically significant difference regarding biliary complications between UW and HTK.(47) Although more recently, increased biliary complications were also seen in the HTK group from a single center study comparing HTK to UW for DCD liver transplants.(56) More evidence is needed to definitively conclude the correlation between either UW or HTK and increased biliary

complications in DCD transplants, however given these graft's known susceptibility to biliary complications this is an important question to be answered.

While UW and HTK remain the two most common solutions used in liver transplantation (50), there has also been interest in Celsior. Two early prospective trials comparing UW and Celsior showed no significant difference in primary graft function or overall graft and patient survival. (57, 58) Garcia-Gil et al. confirmed this in a randomized trial with long term five-year follow up data. (59) This study also showed no difference in biliary complications between the two solutions after five-year follow up. (59)

In a randomized trial comparing IGL-1 (n=48) to UW (n=92) no significant difference in PNF, biliary strictures or ICU stay were reported and the IGL-1 was associated with lower costs. (60) More clinical studies will be required before it extends to more broad clinical use.

Machine Perfusion

Waitlist mortality remains a major concern in liver transplantation. This has led to pressure to expand the donor pool through utilization of DCD and other ECD grafts as well as living donor liver transplantation. Machine perfusion offers the possibility to salvage, repair and provide a more detailed assessment of these marginal organs prior to transplant. While machine perfusion was previously studied in the early history of transplant, the limited technologies available in the 1960s and 1970s made this approach impractical, especially after the advent of SCS.

Transplant is the only definitive therapy for end stage liver disease, and this has led to the search for novel solutions to improve organ preservation and organ quality. The total number of transplants performed in the US continued to increase in 2016, however 11,340 candidates were added to the waiting list and the organ shortage remains severe with 1399 patients dying while on the waitlist and a further 1205 removed, too sick to undergo transplant. (8, 61) This shortage has resulted in increased use of ECD, particularly DCD grafts. However, the percentage of DCD grafts recovered but not transplanted remains high.(8) (61) DCD grafts seem to be more vulnerable to cold storage and reperfusion injury with early-published data showing ischemic biliary cholangiopathy and lower patient and graft survival.(62) More recent results have improved graft and patient survival, though ischemic cholangiopathy is still a frequent complication of DCD grafts.(63) DCD grafts show early histological changes during WIT prior to organ removal and storage, including vacuolization, sinusoidal swelling and morphologic changes to biliary epithelial cells which may be the early changes leading to long term biliary complications in these grafts.(64) During WIT, cells are ischemic yet have not been cooled so these cells are working under normal metabolic rates and must rely on anaerobic metabolism.(65) This leads to increased lactate levels and acidosis prior to preservation, and depleted cellular energy precursors during cold storage.(65) This primes the cell for further damage upon reperfusion, and DCD grafts demonstrate higher rates of cell death proportional to WIT upon reperfusion relative to DBD grafts. (66)

The discard rate of liver grafts reflects the fact that the current preservation technique of SCS is not adequately preserving DCD grafts and as a result, in recent years, there has been significant interest in investigating machine perfusion techniques to reverse some of the

physiologic damage incurred by these grafts during WIT. There is a growing amount of research both in animal models and clinical studies exploring new perfusion technologies.

Assessing Liver Grafts During Machine Perfusion

Machine perfusion has been classified by temperature of perfusate. Regardless of the technique used, a common requirement is the ability to assess the quality of the organ while on the perfusion circuit. Several parameters are used across most experimental and clinical studies, while other more technically challenging techniques have been utilized in the experimental setting to further the understanding of IR injury mechanisms. A set of reliable assessment criteria to predict successful transplantation following machine perfusion is an important goal in *ex-vivo* perfusion.

Hepatocellular injury markers aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) are used clinically and thus are a natural starting point for assessing these grafts during *ex-vivo* perfusion. These enzymes are released into the perfusate as parenchymal cell damage occurs and the severity of rise correlates to the degree of IR injury seen histologically.(67) These markers are easy to measure, translate well from animal models to the clinical setting and have been used consistently across animal and clinical studies making comparisons easy to draw between studies.

Beta-galactosidase, and hyaluronic acid can be used as indicators of Kupffer cell damage, and sinusoidal endothelial damage respectively. Beta- galactosidase is an intracellular enzyme that is released when Kupffer cells are damaged in the face of IR injury.(68) Hyaluronic acid is a glycosaminoglycan that is a component of the protective covering in the sinusoids(17), increased

levels of hyaluronic acid are used in machine perfusion studies as it is a marker of sinusoidal endothelial cell damage.(69)

During ischemic periods cells experience decreased ATP levels.(11, 12) With the added insult of WIT in DCD grafts ATP levels have also been studied during *ex-vivo* perfusion. Xu *et al*. demonstrated a drop in ATP levels to 30% of baseline following 60 minutes of WIT with levels recovering to 80% after normothermic machine perfusion (NMP).(70) ATP levels have also been recovered using hypothermic machine perfusion (HMP)(71) and a gradual re-warming perfusion technique(72) however, others have suggested that ATP levels are not recovered following longer WIT.(73) ATP levels in the peri-transplant period have been shown to correlate to post transplant outcomes, particularly early allograft dysfunction.(74) The ability to recover ATP levels in DCD grafts thus is an important measure in *ex-vivo* preservation.

Histologic changes have also demonstrated consistent features of IR injury. Parenchymal cells damaged from IR injury show ischemic vacuolization, hemorrhage, necrosis, mitochondrial injury, and cellular edema on hematoxylin and eosin staining. Sinusoidal endothelial cells are also susceptible to injury from both hypothermia and IR injury.(9) Histologic liver injury scales have been developed for reporting IR changes.(75) (**Table 1-2**)

Bile production has been suggested to be a key criterion in predicting post transplant function as it was linked to lower transaminase levels and improved lactate clearance.(76) It has been routinely measured in animal and clinical perfusion studies. However, more recent studies have suggested that bile production does not correlate with graft function post transplant.(75, 77, 78) Therefore, in addition to total bile production, the quality of bile and bile duct histology has also been suggested to be important in predicting clinical outcomes. The ability to produce bile

with pH greater than 7.4 correlated with reduced risk for development of cholangiopathy.(77) LDH in the bile has been measured as well as an indicator of biliary endothelial injury.(79) This is of particular interest in DCD grafts, as they have demonstrated a propensity for ischemic type biliary complications. Biliary regeneration following damage incurred during the transplant process has been investigated by histologic assessment of the peribiliary glands(80) and Ki67 staining as a marker of biliary cell proliferation.(81)

There have been multiple different markers of liver function utilized in perfusion studies with the most common being bile production and lactate clearance. The MEGX test measures the livers ability to convert lidocaine to its metabolite monoethylglycinexylidide(82) and has been used to measure liver function during *ex-vivo* perfusion.(81, 83) Clearance of indocyanine green (83) and factor V levels (84, 85) have also been used to assess hepatic clearance and synthetic functions respectively.

Hemodynamic stability is key for monitoring machine perfusion of liver grafts. Not only is it important to ensure the system is functioning properly, changes in hepatic artery, and portal vein flow and vascular resistance are indicative of reperfusion damage and flow rates during machine perfusion have been proposed in criteria to assess viability for transplant. (75, 86)

The advancement of machine perfusion preservation for clinical liver transplantation depends on understanding of the ischemic reperfusion injury mechanism. As our knowledge in this area expands, more assays are being utilized to elucidate the details of this process including but not limited to cellular energy levels, cytokine levels and gene regulation. Many of these techniques are complex, expensive, time consuming and not readily available clinically. The use of clinically available biochemical markers of hepatocellular injury and liver function, in

addition to histological assessment will remain important not only for comparison between studies to draw meaningful conclusions, but also to be used clinically to assess marginal grafts for transplantation as this technology progresses toward more regular clinical use.

Hypothermic Perfusion

Hypothermic machine perfusion (HMP) has shown better graft survival in kidney transplant relative to SCS.(87) With the increasing liver shortage driving more frequent use of ECD, this technology has been revisited in recent years to see if benefits seen in kidney transplantation are translatable to liver grafts.

Early studies established the capacity of HMP to preserve large animal livers for up to 24 hours. (88, 89) In these studies HMP showed superiority with respect to ALT levels and bile production(88) as well as improved intravascular resistance when compared to SCS. (89) In 2007 Monbaliu *et al*. using non-oxygenated kidney preservation solution-1 perfusate found increased vascular resistance in the first 6 hours, increased AST and LDH during perfusion as well as significant increased lactate and decrease in pH. (90) The authors concluded that the addition of oxygen would be beneficial in the perfusate.(90) Vekemans *et al*. confirmed the importance of oxygen in the perfusion system after showing improved histology with the presence of an oxygenated system.(10) In addition, this study also revealed increased sinusoidal cell damage when a high flow system was used relative to sub-physiologic flow parameters. This effect was thought by the authors to be secondary to increased shear stress on the sinusoidal endothelial cells created by higher flow states during hypothermic conditions.(90)

Subsequent to these studies, the role of oxygen and flow were assessed in more detail in a DCD large animal pig model.(91) Following 60 minutes of WIT, livers were preserved either for seven hours with SCS or for 6 hours of SCS followed by 1 hour of HMP. There were HMP groups with high flow, low flow, oxygen and no oxygen. The grafts exposed to oxygenated HMP displayed decreased Kupffer cell activation, decreased leukocyte adhesion and less mitochondrial and nuclear injury.(91) Those perfused under low flow conditions showed even greater mitochondrial protection in addition to preservation of sinusoidal cells.(91) These are important steps in the IR injury cascade and highlight HMP's capacity to protect liver grafts from IR damage.

A potential advantage of machine perfusion over cold storage is the ability to assess liver viability prior to transplantation. Several studies have investigated evaluation parameters that could potentially be used to assess viability during HMP. Obara *et al*. concluded that monitoring of hepatic artery pressure drop rate during HMP could be used to assess viability after this parameter was found to correlate with LDH release from grafts at various durations of WIT.(92) Liu *et al*. using mathematical curve fitting developed a damage index score based on AST and pH values that correlated with WIT and morphological changes as another possible tool for graft assessment.(67) There has been no consensus on the ideal assessment criteria for liver graft viability during HMP and one of the criticisms of this modality is that graft metabolic rate is slowed due to hypothermia. Thus assessing graft function poses a significant challenge under these non-physiologic conditions where metabolic activities are suppressed. Conversely the ability to evaluate graft viability under more physiologic conditions where metabolic processes are active has been proposed as one of the advantages of SNMP and NMP.

Regarding biliary complications, Op den Dries *et al*. showed in a large animal porcine simulated transplant model that after 30 minutes of WIT oxygenated HMP improved hepatic arterial flow and prevented arteriolonecrosis of the peribiliary plexus in extra hepatic ducts relative to grafts preserved by SCS.(93) There were no differences between these groups with respect to biliary epithelial lining, however the improvements in peribiliary vasculature and hepatic arterial flow, were concluded by the authors to be important mechanisms in potentially preventing biliary complications that currently limit the use of DCD liver grafts.(71)

PNF and graft survival have been studied in several animal transplant models. Guarrera *et al*. concluded HMP was superior to SCS with 100% survival of HMP pigs to post operative day 5.(94) The results in DCD transplant models however have been more variable. After 30 minutes of WIT there was 75% survival in the HMP group and no pigs surviving when livers were preserved with SCS.(95) Another group compared HMP after a period of cold storage, to cold storage alone. HMP did demonstrate improved ATP levels, better lactate clearance, and improved architectural preservation after reperfusion.(96) When the post transplant observation period was extended, HMP treated animals were able to be extubated, but did not survive beyond 18 hours.(96) Fondevila *et al*. showed higher rates of sinusoidal endothelial and Kupffer cell injury relative to cold storage groups and only 20% five-day survival of HMP treated DCD graft recipients.(97)

Following these animal studies, HMP technology has made its way to the clinical realm. Guarrera *et al*. were the first group to publish results for the clinical use of HMP.(98) In a prospective cohort study they compared 20 human livers preserved with HMP to a control group of patients preserved with SCS using UW. There were no cases of PNF but one early allograft dysfunction (EAD) in the HMP group. Two HMP patients developed biliary complications.

However, the HMP group did show improved biochemical signs of hepatocellular injury and had shorter hospital stays overall leading the authors to conclude HMP is a safe option in human liver transplant.(98) Further analysis of this group of patients suggested the improved preservation of these grafts was a result of decreased IR injury.(99) This study however did not specifically evaluate DCD grafts, which is arguably the area that may potentially lead to the greatest expansion of the donor pool. In another study involving 8 clinical DCD transplants preserved by HMP there was no PNF, no biliary strictures at 6-month follow up, and no difference in ICU or hospital stay relative to DBD controls.(100) These livers did have a relatively short storage time with mean cold time of 4.6 hours, which may have contributed to the positive results.(100) In a more recent clinical series of DCD grafts, HMP demonstrated lower rates of ischemic cholangiopathy, increased survival and reduced re-transplant rate relative DCD grafts preserved with SCS.(101) A current randomized trial will provide further information regarding the use of this modality for DCD liver preservation. (NCT02584283) Another advantage of HMP relative to other machine perfusion approaches is simplicity and lower cost. Dutkowski reported lower costs in DCD HMP group relative to SCS controls (100) however; little has been reported on the costs of implementing this technology clinically.

Another clinical area where HMP has been introduced to potentially aid in expanding the donor pool is for uncontrolled DCD grafts (Maastricht category II). Maastricht category II donors are when cardiac arrest occurs suddenly, often out of hospital and then procurement occurs after unsuccessful resuscitation.(102) (**Table 1-3**) The majority of DCD grafts are from category III donors, where cardiac arrest is induced in a controlled setting by removing ventilator support.(103) However, as a result of the organ shortage for liver transplantation, there has been increased interest in developing strategies to increase utilization of category II grafts. A recent

clinical study combined normothermic regional perfusion (NRP) with HMP in uncontrolled DCD grafts.(104) In 6 uncontrolled DCD grafts that were preserved by NRP and HMP there were no cases of PNF, no ischemic cholangiopathy and 100% patient and graft survival at 6 month follow up.(104) Uncontrolled DCD liver grafts while offering a potential source to expand the donor pool do also present significant logistical challenges. The benefits of regional perfusion in these cases are becoming more apparent, and the addition of machine perfusion preservation may be beneficial for safely prolonging storage times while other logistics can be sorted out in this population.(104) This combined approach may offer a tool to utilize more uncontrolled DCD grafts, and has also been trialed with NMP which will be discussed later, however more experience in this regard is required prior to more widespread implementation.

The period of HMP perfusion extends the period where the liver is exposed to hypothermic conditions. Not only does this promote ongoing hypothermic injury, as evidenced by increased sinusoidal endothelial cell damage and Kupffer cell activation during HMP (97), but it also maintains the liver in a state of minimal metabolic activity limiting the capacity for improving cellular function, which may be particularly important in DCD grafts that have already suffered cellular damage during the period of WIT. As such, for DCD grafts there is also interest in investigating SNMP, and NMP where the liver is perfused under more metabolically active conditions.

Subnormothermic perfusion and rewarming machine perfusion

By perfusing at warmer temperatures, hypothermic injury is avoided and the liver is metabolically active. However, the sub-physiologic temperatures do reduce the metabolic rate

enough that oxygen and metabolic substrate requirements are reduced, translating to a simplified perfusion setup relative to perfusion at normothermia. This balance has made SNMP a technology of interest, particularly in the preservation of DCD grafts.

In early large animal studies SNMP at 20°C for 6 hours resulted in lower transaminase levels, better lactate clearance and reduced ischemic vacuolization and necrosis relative to the same duration of SCS following 60 minutes of WIT.(105) Most clinical transplant situations still require a period of SCS following WIT while the organ is transported to the transplant center for machine perfusion. With previous results showing that periods of SCS following WIT are deleterious to DCD grafts(84), gradual rewarming in combination with SNMP for DCD grafts was proposed as a new strategy for these cold preserved livers and showed promising results.(72, 73, 106-108) In a porcine simulated transplant model, Minor *et al*. compared SCS, HMP, SNMP and gradual rewarming SNMP after a prolonged period of SCS.(72) Gradual rewarming reduced post-reperfusion AST and necrotic tissue scores, improved bile production, improved portal vein resistance and reduced tissue necrosis factor alpha (TNF-α) and caspase-3 levels. In addition, ATP levels were improved in the gradual rewarming group.(72) Similar findings were revealed when comparing gradual rewarming to NMP following 18 hours of SCS.(109) After the period of SCS livers were perfused for three hours with Custodial-N solution and temperature gradually increased from 8 to 20 degrees versus diluted blood at 37 degrees in the NMP group. The gradual rewarming group showed lower transaminase levels, greater bile production, improved ATP levels and mitochondrial function relative to the NMP group.(109) Mitochondria play a key role in IR injury, the improved energy profiles and reduced oxygen

consumption on reperfusion suggest mitochondrial preservation leading to reduced IR injury with gradual rewarming to sub-physiologic temperatures during preservation. (109)

Morito *et al*. compared constant oxygen concentration and flow rate to a graduated increase of flow rate and oxygen concentration to match the temperature increase. By altering oxygenation with the progressive increase in temperature these DCD grafts showed lower ALT and LDH levels, with improved hepatic artery pressures.(107) Thus suggesting benefit to matching oxygenation with the level of metabolic activity at different temperatures during rewarming machine perfusion. However, determining the optimal oxygenation strategy will require further study.

SNMP has demonstrated recovery of metabolic function and minimization of IR damage in discarded human livers following combined WIT and SCS. (110) However, this technology has not yet been studied in clinical trials to the extent of other machine perfusion strategies and thus its effect on long-term outcomes for DCD grafts, particularly biliary complications are not yet known.

Normothermic perfusion

NMP aims to recreate physiologic conditions during liver preservation. The potential benefits of such a system prevent further injury from hypothermia, allow potential regeneration of cellular energy stores and provide a possible platform to assess liver grafts for their ability to function and be safely transplanted. However, perfusing at physiologic temperatures does come with inherent risks. If there were to be a technical failure, the graft would be lost, physiologic

temperatures also increase the risk of infection and the metabolic activity of grafts during NMP necessitate a more complex perfusion system relative to HMP or SNMP.

While some have used perfusate without an oxygen carrier(111), the majority of NMP studies have been done using either whole blood, or dilute blood in the perfusate. The need for an oxygen carrier was emphasized in a study by Liu *et al*.(83) Cold stored livers were used as controls with three experimental groups compared based on perfusate used: 1: Steen solution, 2: Steen with washed RBC and 3: whole blood. Steen solution is a buffered extracellular solution containing human serum albumin for regulating osmotic pressure first used to prevent pulmonary edema during *ex-vivo* lung perfusion.(112) Groups 2 and 3 were superior to group 1 with respect to hepatocellular injury, bile production and hemodynamic stability with no significant difference noted between group 2 and 3.(83) This is the only trial directly comparing different perfusates head to head and provides supportive evidence for including an oxygen carrier during NMP.

Other regular additives in the perfusate include heparin, antibiotics, and insulin. Bile salts are added to simulate entero-hepatic recirculation, particularly in prolonged perfusions where studies show depleted bile salts.(113) After periods of 12 hours the supply of bile salts required for bile production are significantly depleted and bile production rates fall.(114) Parenteral nutrition is utilized in clinical *ex-vivo* circuits after it had been used in early studies for a prolonged 72 hour perfusions based on the premise that the liver would require nutritional substrate for such a long perfusion.(113) Since an early study by Schon *et al*. that showed less hepatocellular damage with the addition of metabolic substrates relative to control groups(115), there has not been further evidence to suggest the optimal nutrient supplementation strategy during *ex*-vivo perfusion.

Vasoactive drugs, namely prostaglandin have also been included in *ex-vivo* circuits after being shown in clinical trials to reduce IR injury when given prior to procurement.(116) Since that time Nassar *et al*. demonstrated lower transaminases, increased bile production and improved preservation of hepatic architecture when a prostacyclin analog was added to the perfusate relative to the addition of adenosine or NMP with no vasoactive additives.(117)

NMP studies have utilized dual perfusion through the hepatic artery and portal vein trying to simulate normal physiology, either utilizing pulsatile hepatic arterial flow, or continuous flow through both portal vein and hepatic artery. The perfusion system pumps are controlled to maintain physiologic pressure and flow through the hepatic artery and portal vein where as some systems utilize gravity to perfuse the portal vein.(111, 118) Contrary to HMP where higher flow rates have shown shear stress and endothelial damage(90), the vascular compliance at normothermia mimics that of physiologic conditions allowing for similar pressure and flow rates seen *in-vivo*. NMP systems utilize an oxygenator and heat exchanger to control oxygenation and temperature respectively. (**Figure 1-1**)

The results of NMP have been encouraging thus far. One of the concerns for NMP was that if there were to be a malfunction at physiologic temperatures the graft would be lost. However, NMP circuits have proven to be safe and reliable, demonstrating consistent ability to maintain target physiologic parameters.(119) Early studies showed feasibility of NMP for preserving grafts after a period of WIT(115) and even superior preservation in NMP groups relative to SCS.(120) Schon *et al*. showed that after a WIT of 60 minutes all grafts stored via SCS demonstrated PNF, whereas those preserved with NMP were all functional grafts following

porcine orthotopic liver transplant.(121) Since that study there has been significant interest in this technology as a potential vehicle for expanding the donor pool, particularly for DCD grafts. NMP has shown significantly improved hepatic arterial flow, and biliary endothelial cell regeneration in DCD grafts relative to SCS(81), and superior parenchymal and biliary preservation of DCD grafts relative to SNMP.(122) These findings are suggestive that NMP may be the optimal perfusion strategy to preserve the biliary system and protect DCD grafts from cholangiopathy, however additional short and long-term data from clinical trials will be required before definitive conclusions can be drawn.

Another important breakthrough was establishing the capacity of NMP to preserve grafts exposed to WIT for extended periods of time, 24 hours in some cases(118, 123) and even up to 72 hours(113) in large animal models. In more recent years, prolonged perfusion of discarded human grafts has also been demonstrated for greater than 24 hours (124, 125) and even 86 hours in a single case.(126) This has important implications clinically as it would remove some of the time constraints on preservation of marginal organs, simplify coordinating transplant logistical considerations, while also providing time to assess recovery of the graft and possibly provide treatments to optimize the graft.(124)

In order to move toward clinical use liver grafts would either require a portable NMP system, or have exposure to a period of SCS until they could be perfused *ex-vivo* at a transplant center. This is problematic as the marginal DCD liver grafts are very susceptible to cold ischemia following WIT. Reddy *et al*. showed that even a brief one-hour period of SCS prior to NMP significantly increased IR injury.(84) However, NMP has shown some promise in recovering DCD grafts following a period of SCS in an animal model.(111) There have also been discarded human liver studies where NMP has been successfully used to resuscitate grafts after exposure to

both WIT and SCS (76, 127) with some planned for discard livers going on to be utilized in human transplant. (77, 86) These human transplant case series have shown variable success. Mergental *et al*. in a series of 5 cases had all patients well with normal liver function at 7-month follow up.(86) More recently Watson *et al*. had 3 DCD recipients develop cholangiopathy.(77) The aforementioned strategy of gradually rewarming cold stored livers prior to NMP has shown promise, and portable perfusion systems do exist and could potentially eliminate the cold storage state in the clinical transplant sequence. More clinical studies will be needed not only to confirm the efficacy of these different strategies for DCD grafts, but also to provide an estimate of the costs of implementing such systems in our current clinical setting, as this will be another obstacle to overcome prior to this technology entering regular clinical use.

The mixed results for NMP after periods of SCS also highlight the importance of establishing reliable criteria to predict successful transplantation as this technology allows for biochemical, functional and histologic assessment prior to proceeding with transplantation. This is of particular importance when using marginal DCD grafts. As this technology advances, it will be important to have reliable, standardized criteria to select marginal organs that are safe for transplantation. This has become an area of increasing interest. Bile production has been used as a marker of liver function during many animal studies. Sutton *et al*. after trials with human discarded livers, showed grafts with bile output measured by weight, of 30 grams or more after 6 hours of perfusion also had lower markers of hepatocellular injury, better lactate clearance and improved histological scores with respect to necrosis and venous congestion.(76) Another study utilizing NMP in discarded human livers, with a large number of DCD grafts, was also able to show strong correlation between bile production and histological grading.(124) However, in one study, where discarded human livers went on to clinical transplant following preservation with

NMP, 2 out of 3 grafts that went on to develop cholangiopathy produced significant levels of bile while on the perfusion circuit suggesting that bile production alone is insufficient to predict graft viability.(77) Furthermore, multiple studies have now suggested bile production *ex*-vivo does not predict post transplant outcomes.(75, 77) Nasralla et al. in the first clinical randomized control trial comparing NMP to SCS found no correlation between bile production and post transplant liver function or ischemic cholangiopathy.(78) Given that ischemic cholangiopathy remains a problematic complication of DCD liver transplant, there is ongoing research interest in determining better predictors of risk for ischemic cholangiopathy.(80)

The ideal viability criteria would accurately predict graft function based on simple, inexpensive and easily available testing. Discarded livers were selected for transplant based on their ability to clear lactate to levels less than 2.5 mmol/L or produce bile and additionally maintain perfusate pH greater than 7.3, stable arterial flow greater than 150 ml/min and portal flows greater than 500ml/min with satisfactory gross appearance of the liver graft.(86) There were 4-discarded DCD livers meeting these criteria, which were then transplanted and at 7-month follow up all were doing well clinically with normalized liver function tests.(86) Although this is a small number of patients, they are encouraging results for NMP's utility as a tool for assessing grafts. However, no standardized set of viability criteria has yet been agreed upon and this will be crucial for widespread clinical implementation to ensure safety of patients receiving these potentially marginal grafts. Another important logistical question will be how long must these organs be perfused in order to confidently make an assessment of viability. Sutton *et al*. suggested that significant conclusions could be drawn after only 2.5 hours of NMP(76), however there is no consensus on how much time is considered reasonable to decide on the safety of a graft for transplant.

Fueled by the significant organ shortage and promising early results in large animal models, NMP progressed to phase-I clinical trials(128-130). The 3 phase-I clinical trials done up to this point have been similar in their design, comparing NMP preservation to matched control liver transplants preserved with SCS. Two studies perfused with Gelofusine (a volume expander that utilizes succinylated gelatin as an intravenous colloid) with PRBC(128, 130) and the other, Steen solution with PRBC.(129) Ravikumar *et al*. had 100% 30-day survival in the NMP group, with all NMP grafts and patients alive at 6-month follow up and there was no difference in ICU or hospital stay when NMP was compared to SCS matched controls.(128) Similar results were seen in a study by Selzner *et al*. leading these authors to conclude Steen solution NMP was as safe as SCS for liver preservation.(129) The clinical results from Bral *et al*. were also similar in that there was no PNF, and 30-day and 6-month survival were equivalent between groups, however there was a longer median ICU and overall hospital stay in the NMP group.(130) Transaminase levels in all three studies were higher in SCS groups, reaching statistical significance in only one study.(128) Transaminase levels were higher in DCD grafts relative to DBD in all three studies. This was thought to be contributory to the overall higher transaminase levels seen in the study by Bral *et al*. where the proportion of DCD grafts was higher than the other two studies.(130) There was also increased cold ischemia time due to vascular reconstruction and prolonged NMP periods reported, which both also could have contributed to the higher enzyme levels seen in this study.(130) Subsequent to these studies, and despite the optimal protocol still being a target of ongoing research, NMP has quickly reached clinical use. In the first randomized control trial comparing NMP to SCS there was a reduction in peak AST, a decrease in EAD and an increase in graft utilization. These effects were more pronounced for DCD grafts, however there was no significant difference between modalities in preventing

ischemic cholangiopathy.(78) Finding further ways to optimize NMP for DCD grafts remains a significant research focus.

Similar to HMP, another area where NMP has started to be used is in uncontrolled DCD (Maastricht category II). This is more prominent in some European countries, especially in Spain where the use of normothermic extracorporeal membrane oxygenation (NECMO) has been shown to improve outcomes from these grafts.(131) NMP has now been studied in combination with NECMO with hopes that this combined methodology may allow for more extensive use of category II donors. In a pig transplant model, the combination of NECMO with NMP showed preservation of endothelial cells, reduced inflammatory response and minimal histologic signs of IR injury with 100% 5-day survival following transplant.(132) Further clinical studies are required to confirm these results and investigate long-term outcomes, however the combination of these technologies may allow for more countries to adopt this technique and expand the donor pool through utilization of more category II donors. Similar to other machine perfusion technologies, the question of economic impact of implementing this will also need to be investigated prior to its more widespread use.

Summary and future directions

Significant shortage of quality organs remains a challenge within liver transplantation and has led to utilization of ECD. DCD grafts represent a significant potential resource to expand the donor pool, however current preservation strategies are not ideal for these marginal grafts, which have demonstrated sensitivity to SCS. As a result, machine perfusion has come to the forefront of liver transplantation research with recent and ongoing studies in hypothermic,

subnormothermic and normothermic *ex-vivo* perfusion strategies in search of the optimal preservation technique to safely expand the donor pool through the utilization of DCD, and other ECD grafts. Hypothermic and normothermic perfusion, have shown promising results in large animal models, and more recently have expanded into clinical trials with encouraging results. Normothermic perfusion with its benefit of physiologic conditions seems to be favored in DCD grafts as it offers the benefit of assessing the viability and safety of these grafts for clinical transplant. However NMP has not yet been able to demonstrate a reduction in ischemic cholangiopathy and this will remain an important area of ongoing research efforts. A set of standardized, reliable viability assessment criteria will also be crucial for utilizing this technology clinically.

Despite promising results with NMP, there still exists questions regarding the safety of this preservation technique following periods of cold storage required to get the liver to the transplant center. Portable perfusion machines now exist to potentially eliminate this step, however logistical and economic details will still need to be worked out before this technology becomes mainstream in clinical practice. An alternative strategy that has shown promising results is to gradually rewarm cold stored livers and perfuse at sub-normothermic temperatures. More clinical studies with long-term outcomes will be important in establishing this technique as a reliable modality for cold stored DCD livers.

Once the optimal preservation conditions have been established, NMP also will provide a platform to administer therapies to these damaged liver grafts. There is a growing interest in gene modification therapy(133) and some early work in trying to reverse steatosis while on the perfusion circuit.(134) This would potentially expand the donor pool to include more steatotic livers, which are becoming increasingly common with rising obesity rates. In addition to

targeting steatosis, the NMP system provides a platform for an unlimited number of pharmacological interventions to further optimize liver preservation and function prior to transplantation. This will likely be another area of future research should this technology gain wider utilization in the clinical realm.

Initial organ flush has traditionally been with a cold preservation solution (UW, HTK etc.) immediately after the organ is procured. However in grafts being preserved with NMP, this results in rapid cooling for a brief period, to then warm the organ again on the NMP circuit. This has been called in to question for DCD heart perfusions.(135) The effect of temperature of initial organ flush of DCD livers prior to NMP has not yet been studied in a porcine model, and may be another period in the transplant timeline that can be optimized to improve the preservation of DCD grafts following periods of WIT. We hypothesize that avoiding the rapid temperature shifts during initial organ flush of DCD livers will lead to improved liver graft quality. The main work of this thesis focuses on testing this hypothesis.

Table 1-1: Composition of commonly used preservation solutions

	University of Wisconsin	Histidine-tryptophan-ketoglutarate	Euro-Collins	Celsior	Institut Georges Lopez-1
Na	25	15	10	100	125
K	125	10	115	15	30
pH	7.40 (25.0° C)	7.02-7.20 (25° C)	7.30 (0° C)	7.30 (20° C)	7.40 (25° C)
Buffer	Phosphate	Histidine	Phosphate Bicarbonate	Histidine	Phosphate
Energy Source	Adenosine	Glutamic acid/glutamate		Glutamic acid/glutamate	Adenosine
Osmotic Agents	Lactobionate Raffinose Hydroxyethyl starch	Mannitol	Glucose Mannitol	Lactobionate Mannitol	Polyethylene glycol, Raffinose
Osmolality	320	310	340	242-368	320
Additional Additives	Insulin, Dexamethasone, Allopurinol, Glutathione	Tryptophan, ketoglutarate			Allopurinol

Units are mmol/L unless otherwise specified.

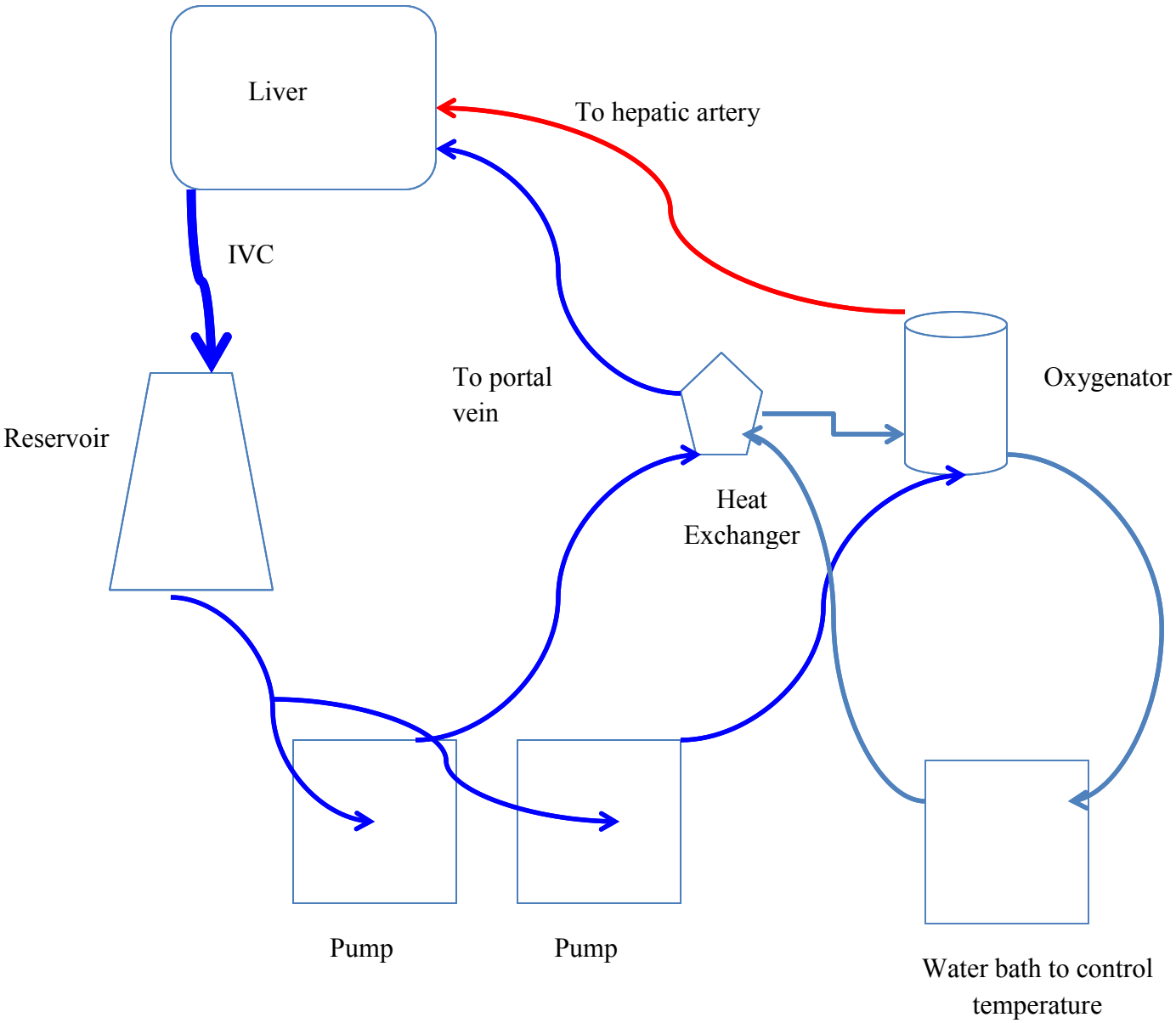
Table 1-2: Histologic scoring system for ischemia reperfusion liver injury.(75)

Score	Hemorrhage	Necrosis	Cholestasis	Sinusoidal Dilatation
0	Absent	Absent	Absent	None
1	Focal	Peri-central	Present	Mild
2	Zonal	Zone 2 + 3	__	Moderate
3	Pan-lobular	Pan-lobular	__	Severe

Table 1-3: Maastricht categories of Non Heart-Beating Donor(102)

Category	Description
I	Dead on arrival at hospital
II	Death with Unsuccessful resuscitation
III	Awaiting cardiac death
IV	Cardiac arrest while brain dead

Figure 1-1: Example of experimental NMP circuit



References

1. Carrel A, Lindbergh CA. The Culture of Whole Organs. Science. 1935;81(2112):621-3.
2. Lapchinsky AG. Recent results of experimental transplantation of preserved limbs and kidneys and possible use of this technique in clinical practice. Ann N Y Acad Sci. 1960;87:539-71.
3. Belzer FO, Ashby BS, Dunphy JE. 24-hour and 72-hour preservation of canine kidneys. Lancet. 1967;2(7515):536-8.
4. Ackermann JR, Barnard CN. Successful storage of kidneys. Br J Surg. 1966;53(6):525-32.
5. Humphries AL, Jr., Russell R, Stoddard LD, Moretz WH. Successful five-day kidney preservation. Perfusion with hypothermic, diluted plasma. Invest Urol. 1968;5(6):609-18.
6. Collins GM, Bravo-Shugarman M, Novom S, Terasaki PI. Kidney preservation for transplantation. I. Twelve-hour storage in rabbits. Transplant Proc. 1969;1(3):801-7.
7. Wahlberg JA, Love R, Landegaard L, Southard JH, Belzer FO. 72-hour preservation of the canine pancreas. Transplantation. 1987;43(1):5-8.
8. Kim WR, Lake JR, Smith JM, Schladt DP, Skeans MA, Harper AM, et al. OPTN/SRTR 2016 Annual Data Report: Liver. Am J Transplant. 2018;18 Suppl 1:172-253.
9. Clavien PA, Harvey PR, Strasberg SM. Preservation and reperfusion injuries in liver allografts. An overview and synthesis of current studies. Transplantation. 1992;53(5):957-78.
10. Vekemans K, Liu Q, Brassil J, Komuta M, Pirenne J, Monbaliu D. Influence of flow and addition of oxygen during porcine liver hypothermic machine perfusion. Transplant Proc. 2007;39(8):2647-51.
11. Harvey PR, Iu S, McKeown CM, Petrunka CN, Ilson RG, Strasberg SM. Adenine nucleotide tissue concentrations and liver allograft viability after cold preservation and warm ischemia. Transplantation. 1988;45(6):1016-20.
12. Nishida T, Koseki M, Kamiike W, Nakahara M, Nakao K, Kawashima Y, et al. Levels of purine compounds in a perfusate as a biochemical marker of ischemic injury of cold-preserved liver. Transplantation. 1987;44(1):16-21.
13. Leaf A. Maintenance of Concentration Gradients and Regulation of Cell Volume. Ann Ny Acad Sci. 1959;72(12):396-404.
14. Carini R, Autelli R, Bellomo G, Albano E. Alterations of cell volume regulation in the development of hepatocyte necrosis. Exp Cell Res. 1999;248(1):280-93.
15. Snowdowne KW, Freudenrich CC, Borle AB. The effects of anoxia on cytosolic free calcium, calcium fluxes, and cellular ATP levels in cultured kidney cells. J Biol Chem. 1985;260(21):11619-26.
16. Jaeschke H, Farhood A, Smith CW. Neutrophils contribute to ischemia/reperfusion injury in rat liver in vivo. FASEB J. 1990;4(15):3355-9.
17. van Golen RF, van Gulik TM, Heger M. Mechanistic overview of reactive species-induced degradation of the endothelial glycocalyx during hepatic ischemia/reperfusion injury. Free Radic Biol Med. 2012;52(8):1382-402.
18. Jaeschke H. Molecular mechanisms of hepatic ischemia-reperfusion injury and preconditioning. Am J Physiol Gastrointest Liver Physiol. 2003;284(1):G15-26.
19. Jaeschke H, Farhood A. Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver. Am J Physiol. 1991;260(3 Pt 1):G355-62.

20. Jaeschke H, Bautista AP, Spolarics Z, Spitzer JJ. Superoxide generation by neutrophils and Kupffer cells during in vivo reperfusion after hepatic ischemia in rats. J Leukoc Biol. 1992;52(4):377-82.
21. Fernandez L, Heredia N, Grande L, Gomez G, Rimola A, Marco A, et al. Preconditioning protects liver and lung damage in rat liver transplantation: role of xanthine/xanthine oxidase. Hepatology. 2002;36(3):562-72.
22. Li JM, Shah AM. Differential NADPH- versus NADH-dependent superoxide production by phagocyte-type endothelial cell NADPH oxidase. Cardiovasc Res. 2001;52(3):477-86.
23. Nieminen AL, Byrne AM, Herman B, Lemasters JJ. Mitochondrial permeability transition in hepatocytes induced by t-BuOOH: NAD(P)H and reactive oxygen species. Am J Physiol. 1997;272(4 Pt 1):C1286-94.
24. Nieminen AL, Saylor AK, Tesfai SA, Herman B, Lemasters JJ. Contribution of the mitochondrial permeability transition to lethal injury after exposure of hepatocytes to t-butylhydroperoxide. Biochem J. 1995;307 (Pt 1):99-106.
25. Lu L, Zhou H, Ni M, Wang X, Busuttil R, Kupiec-Weglinski J, et al. Innate Immune Regulations and Liver Ischemia-Reperfusion Injury. Transplantation. 2016;100(12):2601-10.
26. McDonald KA, Huang H, Tohme S, Loughran P, Ferrero K, Billiar T, et al. Toll-like receptor 4 (TLR4) antagonist eritoran tetrasodium attenuates liver ischemia and reperfusion injury through inhibition of high-mobility group box protein B1 (HMGB1) signaling. Mol Med. 2015;20:639-48.
27. Tsung A, Sahai R, Tanaka H, Nakao A, Fink MP, Lotze MT, et al. The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. J Exp Med. 2005;201(7):1135-43.
28. Granger DN, Kubes P. The microcirculation and inflammation: modulation of leukocyte-endothelial cell adhesion. J Leukoc Biol. 1994;55(5):662-75.
29. Zwacka RM, Zhang Y, Halldorson J, Schlossberg H, Dudus L, Engelhardt JF. CD4(+) T-lymphocytes mediate ischemia/reperfusion-induced inflammatory responses in mouse liver. J Clin Invest. 1997;100(2):279-89.
30. Shiratori Y, Kiriyama H, Fukushi Y, Nagura T, Takada H, Hai K, et al. Modulation of ischemia-reperfusion-induced hepatic injury by Kupffer cells. Dig Dis Sci. 1994;39(6):1265-72.
31. Natori S, Fujii Y, Kurosawa H, Nakano A, Shimada H. Prostaglandin E1 protects against ischemia-reperfusion injury of the liver by inhibition of neutrophil adherence to endothelial cells. Transplantation. 1997;64(11):1514-20.
32. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med. 1993;329(27):2002-12.
33. Fukazawa K, Lang JD. Role of nitric oxide in liver transplantation: Should it be routinely used? World J Hepatol. 2016;8(34):1489-96.
34. Peralta C, Hotter G, Closa D, Prats N, Xaus C, Gelpi E, et al. The protective role of adenosine in inducing nitric oxide synthesis in rat liver ischemia preconditioning is mediated by activation of adenosine A2 receptors. Hepatology. 1999;29(1):126-32.
35. Vardanian AJ, Busuttil RW, Kupiec-Weglinski JW. Molecular mediators of liver ischemia and reperfusion injury: a brief review. Mol Med. 2008;14(5-6):337-45.
36. Goto M, Takei Y, Kawano S, Nagano K, Tsuji S, Masuda E, et al. Endothelin-1 is involved in the pathogenesis of ischemia/reperfusion liver injury by hepatic microcirculatory disturbances. Hepatology. 1994;19(3):675-81.

37. Yokoyama Y, Baveja R, Sonin N, Nakanishi K, Zhang JX, Clemens MG. Altered endothelin receptor subtype expression in hepatic injury after ischemia/reperfusion. Shock. 2000;13(1):72-8.
38. Southard JH, van Gulik TM, Ametani MS, Vreugdenhil PK, Lindell SL, Pienaar BL, et al. Important components of the UW solution. Transplantation. 1990;49(2):251-7.
39. Latchana N, Peck JR, Whitson BA, Henry ML, Elkhammas EA, Black SM. Preservation solutions used during abdominal transplantation: Current status and outcomes. World J Transplant. 2015;5(4):154-64.
40. Parsons RF, Guarrera JV. Preservation solutions for static cold storage of abdominal allografts: which is best? Curr Opin Organ Transplant. 2014;19(2):100-7.
41. Ringe B, Braun F, Moritz M, Zeldin G, Soriano H, Meyers W. Safety and efficacy of living donor liver preservation with HTK solution. Transplant Proc. 2005;37(1):316-9.
42. Testa G, Malago M, Nadalin S, Treptow B, Paul A, Frilling A, et al. Histidine-tryptophan-ketoglutarate versus University of Wisconsin solution in living donor liver transplantation: results of a prospective study. Liver Transpl. 2003;9(8):822-6.
43. Mangus RS, Fridell JA, Vianna RM, Milgrom MA, Chestovich P, Chihara RK, et al. Comparison of histidine-tryptophan-ketoglutarate solution and University of Wisconsin solution in extended criteria liver donors. Liver Transpl. 2008;14(3):365-73.
44. Mangus RS, Tector AJ, Agarwal A, Vianna R, Murdock P, Fridell JA. Comparison of histidine-tryptophan-ketoglutarate solution (HTK) and University of Wisconsin solution (UW) in adult liver transplantation. Liver Transpl. 2006;12(2):226-30.
45. Erhard J, Lange R, Scherer R, Kox WJ, Bretschneider HJ, Gebhard MM, et al. Comparison of histidine-tryptophan-ketoglutarate (HTK) solution versus University of Wisconsin (UW) solution for organ preservation in human liver transplantation. A prospective, randomized study. Transpl Int. 1994;7(3):177-81.
46. Canelo R, Hakim NS, Ringe B. Experience with hystidine tryptophan ketoglutarate versus University Wisconsin preservation solutions in transplantation. Int Surg. 2003;88(3):145-51.
47. Feng L, Zhao N, Yao X, Sun X, Du L, Diao X, et al. Histidine-tryptophan-ketoglutarate solution vs. University of Wisconsin solution for liver transplantation: a systematic review. Liver Transpl. 2007;13(8):1125-36.
48. Stewart ZA, Cameron AM, Singer AL, Montgomery RA, Segev DL. Histidine-Tryptophan-Ketoglutarate (HTK) is associated with reduced graft survival in deceased donor livers, especially those donated after cardiac death. Am J Transplant. 2009;9(2):286-93.
49. Lema Zuluaga GL, Serna Agudelo RE, Zuleta Tobon JJ. Preservation solutions for liver transplantation in adults: celsior versus custodiol: a systematic review and meta-analysis with an indirect comparison of randomized trials. Transplant Proc. 2013;45(1):25-32.
50. Voigt MR, DeLario GT. Perspectives on abdominal organ preservation solutions: a comparative literature review. Prog Transplant. 2013;23(4):383-91.
51. Heidenhain C, Pratschke J, Puhl G, Neumann U, Pascher A, Veltzke-Schlieker W, et al. Incidence of and risk factors for ischemic-type biliary lesions following orthotopic liver transplantation. Transpl Int. 2010;23(1):14-22.
52. Morariu AM, Vd Plaats A, W VO, NA TH, Leuvenink HG, Graaff R, et al. Hyperaggregating effect of hydroxyethyl starch components and University of Wisconsin solution on human red blood cells: a risk of impaired graft perfusion in organ procurement? Transplantation. 2003;76(1):37-43.

53. Hertl M, Harvey PR, Swanson PE, West DD, Howard TK, Shenoy S, et al. Evidence of preservation injury to bile ducts by bile salts in the pig and its prevention by infusions of hydrophilic bile salts. Hepatology. 1995;21(4):1130-7.
54. Buis CI, Geuken E, Visser DS, Kuipers F, Haagsma EB, Verkade HJ, et al. Altered bile composition after liver transplantation is associated with the development of nonanastomotic biliary strictures. J Hepatol. 2009;50(1):69-79.
55. Meine MH, Zanotelli ML, Neumann J, Kiss G, de Jesus Grezzana T, Leipnitz I, et al. Randomized clinical assay for hepatic grafts preservation with University of Wisconsin or histidine-tryptophan-ketoglutarate solutions in liver transplantation. Transplant Proc. 2006;38(6):1872-5.
56. Gulsen MT, Girotra M, Cengiz-Seval G, Price J, Singh VK, Segev DL, et al. HTK preservative solution is associated with increased biliary complications among patients receiving DCD liver transplants: a single center experience. Ann Transplant. 2013;18:69-75.
57. Cavallari A, Cillo U, Nardo B, Filipponi F, Gringeri E, Montalti R, et al. A multicenter pilot prospective study comparing Celsior and University of Wisconsin preserving solutions for use in liver transplantation. Liver Transpl. 2003;9(8):814-21.
58. Pedotti P, Cardillo M, Rigotti P, Gerunda G, Merenda R, Cillo U, et al. A comparative prospective study of two available solutions for kidney and liver preservation. Transplantation. 2004;77(10):1540-5.
59. Garcia-Gil FA, Serrano MT, Fuentes-Broto L, Arenas J, Garcia JJ, Guemes A, et al. Celsior versus University of Wisconsin preserving solutions for liver transplantation: postreperfusion syndrome and outcome of a 5-year prospective randomized controlled study. World J Surg. 2011;35(7):1598-607.
60. Dondero F, Paugam-Burtz C, Danjou F, Stocco J, Durand F, Belghiti J. A randomized study comparing IGL-1 to the University of Wisconsin preservation solution in liver transplantation. Ann Transplant. 2010;15(4):7-14.
61. Kim WR, Lake JR, Smith JM, Skeans MA, Schladt DP, Edwards EB, et al. OPTN/SRTR 2015 Annual Data Report: Liver. Am J Transplant. 2017;17 Suppl 1:174-251.
62. Foley DP, Fernandez LA, Leverson G, Anderson M, Mezrich J, Sollinger HW, et al. Biliary complications after liver transplantation from donation after cardiac death donors: an analysis of risk factors and long-term outcomes from a single center. Ann Surg. 2011;253(4):817-25.
63. Coffey JC, Wanis KN, Monbaliu D, Gilbo N, Selzner M, Vachharajani N, et al. The influence of functional warm ischemia time on DCD liver transplant recipients' outcomes. Clin Transplant. 2017;31(10).
64. Rhee JY, Alroy J, Freeman RB. Characterization of the withdrawal phase in a porcine donation after the cardiac death model. Am J Transplant. 2011;11(6):1169-75.
65. Perera MT, Richards DA, Silva MA, Ahmed N, Neil DA, Murphy N, et al. Comparison of energy metabolism in liver grafts from donors after circulatory death and donors after brain death during cold storage and reperfusion. Br J Surg. 2014;101(7):775-83.
66. Xu J, Sayed BA, Casas-Ferreira AM, Srinivasan P, Heaton N, Rela M, et al. The Impact of Ischemia/Reperfusion Injury on Liver Allografts from Deceased after Cardiac Death versus Deceased after Brain Death Donors. PLoS One. 2016;11(2):e0148815.
67. Liu Q, Vekemans K, Iania L, Komuta M, Parkkinen J, Heedfeld V, et al. Assessing warm ischemic injury of pig livers at hypothermic machine perfusion. J Surg Res. 2014;186(1):379-89.

68. McGuire JB, James TJ, Imber CJ, St Peter SD, Friend PJ, Taylor RP. Optimisation of an enzymatic method for beta-galactosidase. Clin Chim Acta. 2002;326(1-2):123-9.
69. Adams DH, Wang L, Neuberger JM. Serum hyaluronic acid following liver transplantation: evidence of hepatic endothelial damage. Transplant Proc. 1989;21(1 Pt 2):2274.
70. Xu H, Berendsen T, Kim K, Soto-GutiÃ©rrez A, Bertheium F, Yarmush ML, et al. Excorporeal normothermic machine perfusion resuscitates pig DCD livers with extended warm ischemia. Journal of Surgical Research. 2012;173(2):e83-e8.
71. den Dries So, Sutton ME, Karimian N, de Boer MT, Wiersema-Buist J, Gouw ASH, et al. Hypothermic Oxygenated Machine Perfusion Prevents Arteriolonecrosis of the Peribiliary Plexus in Pig Livers Donated after Circulatory Death. PLoS One. 2014;9(2):e88521.
72. Minor T, Efferz P, Fox M, Wohlschlaeger J, Luer B. Controlled oxygenated rewarming of cold stored liver grafts by thermally graduated machine perfusion prior to reperfusion. Am J Transplant. 2013;13(6):1450-60.
73. Furukori M, Matsuno N, Meng LT, Shonaka T, Nishikawa Y, Imai K, et al. Subnormothermic Machine Perfusion Preservation With Rewarming for Donation After Cardiac Death Liver Grafts in Pigs. Transplant Proc. 2016;48(4):1239-43.
74. Bruinsma BG, Avruch JH, Sridharan GV, Weeder PD, Jacobs ML, Crisalli K, et al. Peritransplant Energy Changes and Their Correlation to Outcome After Human Liver Transplantation. Transplantation. 2017;101(7):1637-44.
75. Brockmann J, Reddy S, Coussios C, Pigott D, Guirriero D, Hughes D, et al. Normothermic perfusion: a new paradigm for organ preservation. Ann Surg. 2009;250(1):1-6.
76. Sutton ME, op den Dries S, Karimian N, Weeder PD, de Boer MT, Wiersema-Buist J, et al. Criteria for viability assessment of discarded human donor livers during ex vivo normothermic machine perfusion. PLoS One. 2014;9(11):e110642.
77. Watson CJE, Kosmoliaptsis V, Randle LV, Gimson AE, Brais R, Klinck JR, et al. Normothermic Perfusion in the Assessment and Preservation of Declined Livers Before Transplantation: Hyperoxia and Vasoplegia-Important Lessons From the First 12 Cases. Transplantation. 2017;101(5):1084-98.
78. Nasralla D, Coussios CC, Mergental H, Akhtar MZ, Butler AJ, Ceresa CDL, et al. A randomized trial of normothermic preservation in liver transplantation. Nature. 2018.
79. Vajdova K, Smrekova R, Kukan M, Lutterova M, Wsolova L. Bile analysis as a tool for assessing integrity of biliary epithelial cells after cold ischemia--reperfusion of rat livers. Cryobiology. 2000;41(2):145-52.
80. op den Dries S, Westerkamp AC, Karimian N, Gouw AS, Bruinsma BG, Markmann JF, et al. Injury to peribiliary glands and vascular plexus before liver transplantation predicts formation of non-anastomotic biliary strictures. J Hepatol. 2014;60(6):1172-9.
81. Liu Q, Nassar A, Farias K, Buccini L, Baldwin W, Mangino M, et al. Sanguineous normothermic machine perfusion improves hemodynamics and biliary epithelial regeneration in donation after cardiac death porcine livers. Liver Transpl. 2014;20(8):987-99.
82. Oellerich M, Armstrong VW. The MEGX test: a tool for the real-time assessment of hepatic function. Ther Drug Monit. 2001;23(2):81-92.
83. Liu Q, Nassar A, Farias K, Buccini L, Mangino MJ, Baldwin W, et al. Comparing Normothermic Machine Perfusion Preservation With Different Perfusates on Porcine Livers From Donors After Circulatory Death. Am J Transplant. 2016;16(3):794-807.

84. Reddy S, Greenwood J, Maniakin N, Bhattacharjya S, Zilvetti M, Brockmann J, et al. Non-heart-beating donor porcine livers: the adverse effect of cooling. Liver Transpl. 2005;11(1):35-8.
85. Reddy SP, Bhattacharjya S, Maniakin N, Greenwood J, Guerreiro D, Hughes D, et al. Preservation of porcine non-heart-beating donor livers by sequential cold storage and warm perfusion. Transplantation. 2004;77(9 SUPPL):1328-32.
86. Mergental H, Perera MT, Laing RW, Muiesan P, Isaac JR, Smith A, et al. Transplantation of Declined Liver Allografts Following Normothermic Ex-Situ Evaluation. Am J Transplant. 2016;16(11):3235-45.
87. Moers C, Pirenne J, Paul A, Ploeg RJ, Machine Preservation Trial Study G. Machine perfusion or cold storage in deceased-donor kidney transplantation. N Engl J Med. 2012;366(8):770-1.
88. Jain S, Lee CY, Baicu S, Duncan H, Xu H, Jones JW, Jr., et al. Hepatic function in hypothermically stored porcine livers: comparison of hypothermic machine perfusion vs cold storage. Transplant Proc. 2005;37(1):340-1.
89. Bessems M, Doorschodt BM, Dinant S, de Graaf W, van Gulik TM. Machine perfusion preservation of the pig liver using a new preservation solution, polysol. Transplant Proc. 2006;38(5):1238-42.
90. Monbaliu D, Vekemans K, De Vos R, Brassil J, Heedfeld V, Qiang L, et al. Hemodynamic, biochemical, and morphological characteristics during preservation of normal porcine livers by hypothermic machine perfusion. Transplant Proc. 2007;39(8):2652-8.
91. Schlegel A, de Rougemont O, Graf R, Clavien P-A, Dutkowski P. Protective mechanisms of end-ischemic cold machine perfusion in DCD liver grafts. Journal of hepatology. 2013;58(2):278-86.
92. Obara H, Matsuno N, Enosawa S, Shigeta T, Huai-Che H, Hirano T, et al. Pretransplant screening and evaluation of liver graft viability using machine perfusion preservation in porcine transplantation. Transplant Proc. 2012;44(4):959-61.
93. Op DD, Sutton ME, Karimian N, De Boer MT, Wiersema-Buist J, Gouw ASH, et al. Hypothermic oxygenated machine perfusion prevents arteriolonecrosis of the peribiliary plexus in pig livers donated after circulatory death. PLoS ONE. 2014;9(2).
94. Guarrera JV, Estevez J, Boykin J, Boyce R, Rashid J, Sun S, et al. Hypothermic machine perfusion of liver grafts for transplantation: technical development in human discard and miniature swine models. Transplant Proc. 2005;37(1):323-5.
95. Shigeta T, Matsuno N, Obara H, Mizunuma H, Kanazawa H, Tanaka H, et al. Functional recovery of donation after cardiac death liver graft by continuous machine perfusion preservation in pigs. Transplantation proceedings. 2012;44(4):946-7.
96. de Rougemont O, Breitenstein S, Leskosek B, Weber A, Graf R, Clavien PA, et al. One hour hypothermic oxygenated perfusion (HOPE) protects nonviable liver allografts donated after cardiac death. Ann Surg. 2009;250(5):674-83.
97. Fondevila C, Hessheimer AJ, Maathuis MH, Munoz J, Taura P, Calatayud D, et al. Hypothermic oxygenated machine perfusion in porcine donation after circulatory determination of death liver transplant. Transplantation. 2012;94(1):22-9.
98. Guarrera JV, Henry SD, Samstein B, Odeh-Ramadan R, Kinkhabwala M, Goldstein MJ, et al. Hypothermic machine preservation in human liver transplantation: the first clinical series. Am J Transplant. 2010;10(2):372-81.

99. Henry SD, Guarrera JV. Protective effects of hypothermic ex vivo perfusion on ischemia/reperfusion injury and transplant outcomes. Transplant Rev (Orlando). 2012;26(2):163-75.
100. Dutkowski P, Schlegel A, de Oliveira M, Mullhaupt B, Neff F, Clavien PA. HOPE for human liver grafts obtained from donors after cardiac death. J Hepatol. 2014;60(4):765-72.
101. Dutkowski P, Polak WG, Muiesan P, Schlegel A, Verhoeven CJ, Scalera I, et al. First comparison of hypothermic oxygenated perfusion versus static cold storage of human donation after cardiac death liver transplants. Annals of Surgery. 2015;262(5):764-71.
102. Kootstra G, Daemen JH, Oomen AP. Categories of non-heart-beating donors. Transplant Proc. 1995;27(5):2893-4.
103. Foley DP, Fernandez LA, Leverson G, Chin LT, Krieger N, Cooper JT, et al. Donation after cardiac death: the University of Wisconsin experience with liver transplantation. Ann Surg. 2005;242(5):724-31.
104. De Carlis R, Di Sandro S, Lauterio A, Ferla F, Dell'Acqua A, Zanierato M, et al. Successful donation after cardiac death liver transplants with prolonged warm ischemia time using normothermic regional perfusion. Liver Transplantation. 2017;23(2):166-73.
105. Gringeri E, Bonsignore P, Bassi D, D'Amico FE, Mescoli C, Polacco M, et al. Subnormothermic machine perfusion for non-heart-beating donor liver grafts preservation in a Swine model: a new strategy to increase the donor pool? Transplant Proc. 2012;44(7):2026-8.
106. Obara H, Matsuno N, Shigeta T, Hirano T, Enosawa S, Mizunuma H. Temperature controlled machine perfusion system for liver. Transplant Proc. 2013;45(5):1690-2.
107. Morito N, Obara H, Matsuno N, Enosawa S, Watanabe K, Furukori M, et al. Regulated Oxygenation of Rewarming Machine Perfusion for Porcine Donation After Cardiac Death Liver Transplantation. Transplantation proceedings. 2016;48(4):1244-6.
108. Matsuno N, Obara H, Watanabe R, Iwata S, Kono S, Fujiyama M, et al. Rewarming preservation by organ perfusion system for donation after cardiac death liver grafts in pigs. Transplantation proceedings. 2014;46(4):1095-8.
109. Hoyer DP, Paul A, Luer S, Reis H, Efferz P, Minor T. End-ischemic reconditioning of liver allografts: Controlling the rewarming. Liver Transpl. 2016;22(9):1223-30.
110. Bruinsma BG, Yeh H, Ozer S, Martins PN, Farmer A, Wu W, et al. Subnormothermic machine perfusion for ex vivo preservation and recovery of the human liver for transplantation. Am J Transplant. 2014;14(6):1400-9.
111. Boehnert MU, Yeung JC, Bazerbachi F, Knaak JM, Selzner N, McGilvray ID, et al. Normothermic acellular ex vivo liver perfusion reduces liver and bile duct injury of pig livers retrieved after cardiac death. Am J Transplant. 2013;13(6):1441-9.
112. Steen S, Liao Q, Wierup PN, Bolys R, Pierre L, Sjoberg T. Transplantation of lungs from non-heart-beating donors after functional assessment ex vivo. Ann Thorac Surg. 2003;76(1):244-52; discussion 52.
113. Butler AJ, Rees MA, Wight DG, Casey ND, Alexander G, White DJ, et al. Successful extracorporeal porcine liver perfusion for 72 hr. Transplantation. 2002;73(8):1212-8.
114. Imber CJ, St Peter SD, de Cenarruzabeitia IL, Lemonde H, Rees M, Butler A, et al. Optimisation of bile production during normothermic preservation of porcine livers. Am J Transplant. 2002;2(7):593-9.
115. Schon MR, Hunt CJ, Pegg DE, Wight DG. The possibility of resuscitating livers after warm ischemic injury. Transplantation. 1993;56(1):24-31.

116. Klein M, Geoghegan J, Wangemann R, Bockler D, Schmidt K, Scheele J. Preconditioning of donor livers with prostaglandin I2 before retrieval decreases hepatocellular ischemia-reperfusion injury. Transplantation. 1999;67(8):1128-32.
117. Nassar A, Liu Q, Farias K, D'Amico G, Buccini L, Urcuyo D, et al. Role of vasodilation during normothermic machine perfusion of DCD porcine livers. International Journal of Artificial Organs. 2014;37(2):165-72.
118. St Peter SD, Imber CJ, Lopez I, Hughes D, Friend PJ. Extended preservation of non-heart-beating donor livers with normothermic machine perfusion. British Journal of Surgery. 2002;89(5):609-16.
119. Nassar A, Liu Q, Farias K, D'Amico G, Tom C, Grady P, et al. Ex vivo normothermic machine perfusion is safe, simple, and reliable: Results from a large animal model. Surgical Innovation. 2015;22(1):61-9.
120. Hellinger A, Fiegen R, Lange R, Rauen U, Schmidt U, Hirche H, et al. Preservation of pig liver allografts after warm ischemia: normothermic perfusion versus cold storage. Langenbecks Arch Chir. 1997;382(4):175-84.
121. Schon MR, Kollmar O, Wolf S, Schrem H, Matthes M, Akkoc N, et al. Liver transplantation after organ preservation with normothermic extracorporeal perfusion. Ann Surg. 2001;233(1):114-23.
122. Nassar A, Liu Q, Farias K, Buccini L, Baldwin W, Bennett A, et al. Impact of Temperature on Porcine Liver Machine Perfusion From Donors After Cardiac Death. Artif Organs. 2016;40(10):999-1008.
123. Imber CJ, St Peter SD, Lopez de Cenarruzabeitia I, Pigott D, James T, Taylor R, et al. Advantages of normothermic perfusion over cold storage in liver preservation. Transplantation. 2002;73(5):701-9.
124. Vogel T, Brockmann JG, Quaglia A, Morovat A, Jassem W, Heaton ND, et al. The 24-hour normothermic machine perfusion of discarded human liver grafts. Liver Transplantation. 2017;23(2):207-20.
125. Bellomo R, Marino B, Starkey G, Fink M, Wang BZ, Eastwood GM, et al. Extended normothermic extracorporeal perfusion of isolated human liver after warm ischaemia: a preliminary report. Critical care and resuscitation : journal of the Australasian Academy of Critical Care Medicine. 2014;16(3):197-201.
126. Liu Q, Nassar A, Buccini L, Grady P, Soliman B, Hassan A, et al. Ex situ 86 hours liver perfusion: Pushing the boundary of organ preservation. Liver Transpl. 2018.
127. op den Dries S, Karimian N, Sutton ME, Westerkamp AC, Nijsten MW, Gouw AS, et al. Ex vivo normothermic machine perfusion and viability testing of discarded human donor livers. Am J Transplant. 2013;13(5):1327-35.
128. Ravikumar R, Jassem W, Mergental H, Heaton N, Mirza D, Perera MT, et al. Liver Transplantation After Ex Vivo Normothermic Machine Preservation: A Phase 1 (First-in-Man) Clinical Trial. Am J Transplant. 2016;16(6):1779-87.
129. Selzner M, Goldaracena N, Echeverri J, Kaths JM, Linares I, Selzner N, et al. Normothermic ex vivo liver perfusion using steen solution as perfusate for human liver transplantation: First North American results. Liver Transpl. 2016;22(11):1501-8.
130. Bral M, Gala-Lopez B, Bigam D, Kneteman N, Malcolm A, Livingstone S, et al. Preliminary Single-Center Canadian Experience of Human Normothermic Ex Vivo Liver Perfusion: Results of a Clinical Trial. American Journal of Transplantation. 2017;17(4):1071-80.

131. Fondevila C, Hessheimer AJ, Ruiz A, Calatayud D, Ferrer J, Charco R, et al. Liver transplant using donors after unexpected cardiac death: novel preservation protocol and acceptance criteria. American Journal of Transplantation. 2007;7(7):1849-55.
132. Fondevila C, Hessheimer AJ, Maathuis M-HJ, Munoz J, Taura P, Calatayud D, et al. Superior preservation of DCD livers with continuous normothermic perfusion. Annals of Surgery. 2011;254(6):1000-7.
133. Morales-Ruiz M, Fondevila C, Munoz-Luque J, Tugues S, Rodriguez-Laiz G, Cejudo-Martin P, et al. Gene transduction of an active mutant of akt exerts cytoprotection and reduces graft injury after liver transplantation. Am J Transplant. 2007;7(4):769-78.
134. Nagrath D, Xu H, Tanimura Y, Zuo R, Berthiaume F, Avila M, et al. Metabolic preconditioning of donor organs: defatting fatty livers by normothermic perfusion ex vivo. Metab Eng. 2009;11(4-5):274-83.
135. White CW, Ambrose E, Muller A, Li Y, Le H, Thliveris J, et al. Avoidance of Profound Hypothermia During Initial Reperfusion Improves the Functional Recovery of Hearts Donated After Circulatory Death. Am J Transplant. 2016;16(3):773-82.

Chapter 2

Addressing Organ Shortages: Progress in Donation after Circulatory Death for Liver Transplant

Submitted for publication: March 16, 2019

Abstract

Reducing waitlist mortality in liver transplantation remains a significant challenge due to organ shortages. In efforts to expand the donor pool there has been a trend toward increased use of donation after circulatory death (DCD) liver grafts. However these marginal grafts are prone to higher complication rates, particularly biliary complications. In addition many procured DCD livers are then deemed unsuitable for transplant and do not end up going on to be transplanted. Despite these limitations, DCD grafts represent an important resource to address current organ shortages and as such there are significant research efforts directed toward improving the utilization and outcomes for transplantation of these grafts. This article reviews the current progress in DCD liver transplantation.

Liver transplant remains the definitive treatment for end stage liver disease. During 2017, 182 Canadians either died on the waitlist or became too sick to undergo the operation. (136) A recent *Canadian Medical Association Journal* article by Shemie highlighted the current trend toward increasing utilization of donation after circulatory death (DCD) grafts to address organ shortages. (137) In 2017 53 Canadians underwent liver transplant with DCD grafts, the highest number to date. (136) DCD grafts differ from the more common donation after brain death (DBD) donors in that before procurement, livers are exposed to a period of warm ischemic time (WIT) after cardiopulmonary arrest where the organ is no longer perfused. This contrasts to DBD donors where perfusion of the organ persists right up until the moment of procurement in the operating room.

As a result, higher complication rates persist. (103) Although DCD liver transplants have increased in recent years, in 2017 15% of the DCD liver grafts procured in Canada were subsequently discarded before transplantation.(136) The high discard rate and higher complication rates induced by WIT suggests that the current preservation strategy of static cold storage (SCS), is not well tolerated by these injured grafts. We present a brief overview of DCD liver transplant outcomes and review progress made in improving DCD outcomes (donor selection, use of thrombolytic medications, and machine perfusion preservation strategies).

We searched PubMed for the Medical Subject Heading (MESH) terms “donation after circulatory death” and “liver transplant”. This resulted in 211 articles of which the best matched were reviewed. Relevant articles from the references listed in select articles were also reviewed. When possible we discussed the highest level of evidence in randomized controlled trials although this level of evidence was limited and therefore observational data was also reviewed.

Current outcomes in DCD liver transplantation

A 2011 meta-analysis which included 489 DCD and 4455 DBD recipients revealed a 1.6 times increase in 1-year mortality and 2.1 times increased risk of 1-year graft failure for DCD grafts when compared to DBD.(138) More recently however in a meta-analysis inclusive of over 12000 patients, there was no difference found in patient or graft survival when comparing DCD and DBD liver transplants.(139)

The same improvement has not been observed regarding ischemic cholangiopathy (IC). IC is defined as the occurrence of multiple intra-hepatic strictures in the absence of hepatic artery thrombosis or stenosis. (140) IC is 2.5 times more likely in DCD liver grafts relative to DBD.(139) While hepatocytes receive dual blood supply from both the portal vein and hepatic artery, biliary cells receive only arterial blood.(141) Both DBD and DCD livers demonstrate loss of intra-luminal biliary epithelium as a result of procurement and preservation however only those with significant damage to the deep peribiliary plexus, the site of progenitor cells for regeneration of the biliary ducts show an increased risk of IC.(80) Thus it has been hypothesized that IC develops from an impaired ability to regenerate biliary cells after revascularization secondary to peribiliary vascular plexus injury in DCD grafts.(80)

IC leads to higher rates of graft failure, longer hospitalizations, increased biliary procedures, re-transplantation and overall increased health care costs.(142) Improving IC rates in DCD transplants remains a significant challenge for ongoing research efforts.

Donor factors impacting DCD liver transplant outcomes

Donor Age

Advanced donor age has been identified consistently as a risk factor for complications in DCD liver transplantation.(143) Increased donor age above 50 has been shown to predict risk for IC (144) and graft failure.(143) However when other risk factors were minimized, Schlegel et al. showed no difference in biliary or overall complications in DCD liver grafts between cohorts above or below 60 years. (145) This led the authors to conclude that older DCD donors may provide viable grafts when other risk factors are optimized.

Donor Obesity

Rising obesity rates have resulted in increasing numbers of steatotic donors.(146) DCD liver grafts from obese donors have shown poor tolerance of SCS evidenced by worse transplant outcomes.(146) Elevated body mass index (BMI) has been associated with increased IC as well as lower graft and patient survival.(143-145, 147) Liver steatosis leads to lower ATP levels, microcirculatory dysfunction, increased inflammation and reactive oxygen species production with ultimately more severe ischemia reperfusion injury. (146) Improving outcomes of this expanding subset of donor livers has become an area of keen research interest for machine perfusion.

Warm Ischemic Time

For DCD procurement the WIT cut off in many centers is 30 minutes. Both graft failure and IC have been linked to prolonged WIT.(63, 143, 147) Tun-Abraham et al. evaluated their

single center series dividing their cohort into early: 2006-2011, and late: 2011-2016. The late group showed reduced times from incision to arterial cannulation and organ flush.(148) This was accompanied by a statistically significant reduction in IC for the late group.(148) These improvements were attributed to a learning curve, suggesting procurements done by DCD experienced staff could improve outcomes.(148) In addition a meta-analysis looking at DCD liver transplant outcomes between those who had life support withdrawn in the intensive care unit compared with the operating room. Both graft survival and rate of IC were improved by withdrawal of life support in the operating room.(149) This varies by institution however a shift in policies to allow withdrawal of life support in the operating room may contribute to improved outcomes.

Cold Ischemic Time

Cold ischemic time has been identified as a significant risk factor for poor outcomes in DCD liver transplantation.(51, 150) In order to reduce risk of IC and graft failure, cold ischemic time is suggested to be limited to less than 10 hours.(150) Where as other authors suggest even more strict time limits of less than 9 hours of total ischemic time.(144)

Thrombolytic medications during DCD transplantation

The addition of thrombolytic medications has received recent research interest. Tissue plasminogen activator (tPA) was injected via the hepatic artery on the back table based on the hypothesis that micro-thrombi from stasis during WIT led to damaged peribiliary vasculature and

ultimately contributed to IC.(151) In this initial study 22 DCD transplants were carried out with the tPA protocol, 9% developed IC, lower than previously reported, however excessive bleeding was encountered in 14 of the 22 recipients.(151) Subsequent studies modified the tPA protocol and injected the drug via the hepatic artery after venous reperfusion in the recipient.(152, 153) A description of the approaches for tPA administration in DCD liver transplant has been described elsewhere.(154) The more recent studies using tPA showed improved 1 and 3 year patient and graft survival and a significant reduction in IC relative to controls.(152, 153) These studies did not show the same increased transfusion requirement previously described by Hashimoto et al.(151) A detailed meta-analysis that included 249 patients in the tPA group and 178 patients not receiving tPA showed a significant reduction in IC and re-transplant rates for the tPA group without increased transfusion requirements leading the authors to conclude that tPA provides an advantage for preventing IC.(154) Further supporting the use of tPA protocols for DCD liver grafts is the recent demonstration of cost savings with this intervention.(155)

Progress in *ex-situ* machine perfusion

The higher incidence of biliary complications and graft failure with DCD grafts has been attributed to the damage incurred during WIT and subsequent SCS. Machine perfusion has made significant gains as an alternative preservation strategy. Machine perfusion is classified by temperature of the perfusate: hypothermic machine perfusion (HMP), sub-normothermic machine perfusion (SNMP), and normothermic machine perfusion (NMP).

Hypothermic Machine perfusion

Providing oxygen in the perfusate, at sub-physiologic flow rates and temperatures allows for improved mitochondrial and endothelial protection.(91) In a large animal model, DCD livers undergoing HMP showed lower alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels(156), and improved preservation of the biliary microvasculature relative to SCS.(157) There were also significant improvements in ATP levels, bile production, bile composition, and lactate levels relative to those transplanted after SCS.(158, 159) A clinical series compared 50 DCD livers treated with HMP to 50 DCD SCS livers and 50 DBD controls. There was a significantly lower rate of IC relative to the DCD SCS group with increased survival and reduced re-transplant rate at 1 year.(101) At 5 year follow up graft survival was 94% compared to 78% in DCD grafts without HMP.(160)

A criticism of this modality is that it remains difficult to assess liver function during perfusion, as metabolic activity is minimized under hypothermic conditions. Thus defining criteria of organs suitable for transplantation will be an important goal if this modality is to be utilized more widely in clinical practice. HMP has shown benefit following periods of SCS, which in the Canadian population where organs are being transported long distances, would allow for implementation without significant alteration of current procurement strategies. An ongoing randomized trial (NCT02584283) will shed further light on the potential of this technology.

Subnormothermic machine perfusion and controlled oxygenated rewarming

In animal studies controlled oxygenated rewarming (COR) with gradual rewarming followed by perfusing at subnormothermic temperatures (20-21°C) was superior to HMP and SCS with improved serum enzyme levels, portal vein resistance, bile production and histological injury scores.(72) It also demonstrated improved ATP recovery relative to NMP or SCS. (109) The first clinical series involved 6 patients that underwent COR for 90 minutes following SCS and were compared to a historic cohort. COR had a statistically significant reduction in peak AST and at 6 month follow up all patients had normal liver function tests.(161) However this series did not include any DCD grafts. There is limited data regarding SNMP or COR for DCD liver grafts. In a series of perfusions consisting of 5 discarded DCD human livers, SNMP demonstrated increased ATP levels and clearance of lactate during 3 hours of SNMP.(110) More evidence is required in order to make any conclusions regarding the utilization of SNMP/COR for DCD liver grafts.

Normothermic machine perfusion

NMP aims to recreate physiological conditions to recover and assess liver grafts prior to transplantation. Large animal models have demonstrated lower transaminase levels, improved lactate clearance and better histologic preservation of hepatocytes relative to SCS.(81, 111) In addition, NMP has shown improved preservation of biliary endothelial regeneration capacity, which is hypothesized to prevent the development of IC.(81) It is also being investigated as a modality to “de-fat” livers from obese donors.(146) However, consensus on the optimal NMP perfusate composition, circuit set up, and viability criteria have not yet been reached.(162)

Nonetheless, results of animal studies have led to multiple phase 1 clinical trials and more recently the first clinical randomized control trial comparing NMP to SCS.(78) One hundred-seventy livers (63 DCD) and 164 livers (64 DCD) were randomized to NMP and SCS respectively. There was a statistically significant reduction in peak AST and early allograft dysfunction in the NMP group relative to SCS.(78) An important finding highlighted by the authors was an increase in organ utilization.(78) Fifteen percent of DCD grafts procured never went on to be transplanted in Canada in 2017(136), thus if NMP could increase the number of these grafts that are successfully transplanted, further progress could possibly be made to reduce waitlist mortality. However, biliary complications after transplantation remain a significant problem in DCD grafts and Nasralla et al. demonstrated no statistically significant difference in IC between NMP and SCS preserved DCD liver grafts.(78) Reducing IC in DCD grafts remains a major research target for NMP, but it remains unclear whether any interventional strategies will ultimately mitigate the risk of IC.

Conclusions

DCD liver transplantation has resulted in an increase in transplants being performed in Canada however discard rates are significant and higher rates of IC persist. Donor selection remains critical, while thrombolytic protocols have shown early benefits for graft survival and IC. Machine perfusion has promise in both increasing utilization and improving outcomes however clinical data is still emerging and ongoing research aims to optimize machine perfusion protocols, establish reliable viability criteria and demonstrate consistent long-term outcomes prior to widespread clinical implementation.

References

51. Heidenhain C, Pratschke J, Puhl G, Neumann U, Pascher A, Veltzke-Schlieker W, et al. Incidence of and risk factors for ischemic-type biliary lesions following orthotopic liver transplantation. Transpl Int. 2010;23(1):14-22.
63. Coffey JC, Wanis KN, Monbaliu D, Gilbo N, Selzner M, Vachharajani N, et al. The influence of functional warm ischemia time on DCD liver transplant recipients' outcomes. Clin Transplant. 2017;31(10).
72. Minor T, Efferz P, Fox M, Wohlschlaeger J, Luer B. Controlled oxygenated rewarming of cold stored liver grafts by thermally graduated machine perfusion prior to reperfusion. Am J Transplant. 2013;13(6):1450-60.
78. Nasralla D, Coussios CC, Mergental H, Akhtar MZ, Butler AJ, Ceresa CDL, et al. A randomized trial of normothermic preservation in liver transplantation. Nature. 2018.
80. op den Dries S, Westerkamp AC, Karimian N, Gouw AS, Bruinsma BG, Markmann JF, et al. Injury to peribiliary glands and vascular plexus before liver transplantation predicts formation of non-anastomotic biliary strictures. J Hepatol. 2014;60(6):1172-9.
81. Liu Q, Nassar A, Farias K, Buccini L, Baldwin W, Mangino M, et al. Sanguineous normothermic machine perfusion improves hemodynamics and biliary epithelial regeneration in donation after cardiac death porcine livers. Liver Transpl. 2014;20(8):987-99.
91. Schlegel A, de Rougemont O, Graf R, Clavien P-A, Dutkowski P. Protective mechanisms of end-ischemic cold machine perfusion in DCD liver grafts. Journal of hepatology. 2013;58(2):278-86.
101. Dutkowski P, Polak WG, Muiesan P, Schlegel A, Verhoeven CJ, Scalera I, et al. First comparison of hypothermic oxygenated perfusion versus static cold storage of human donation after cardiac death liver transplants. Annals of Surgery. 2015;262(5):764-71.
103. Foley DP, Fernandez LA, Leverson G, Chin LT, Krieger N, Cooper JT, et al. Donation after cardiac death: the University of Wisconsin experience with liver transplantation. Ann Surg. 2005;242(5):724-31.
109. Hoyer DP, Paul A, Luer S, Reis H, Efferz P, Minor T. End-ischemic reconditioning of liver allografts: Controlling the rewarming. Liver Transpl. 2016;22(9):1223-30.
110. Bruinsma BG, Yeh H, Ozer S, Martins PN, Farmer A, Wu W, et al. Subnormothermic machine perfusion for ex vivo preservation and recovery of the human liver for transplantation. Am J Transplant. 2014;14(6):1400-9.
111. Boehnert MU, Yeung JC, Bazerbachi F, Knaak JM, Selzner N, McGilvray ID, et al. Normothermic acellular ex vivo liver perfusion reduces liver and bile duct injury of pig livers retrieved after cardiac death. Am J Transplant. 2013;13(6):1441-9.
136. Information CIfH. Canadian Organ Replacement Register, 2017 Cumulative Report 2018 [Available from: https://www.cihi.ca/en/e-statistics-on-organ-transplants-waiting-lists-and-donors.
137. Shemie SD. Trends in deceased organ donation in Canada. CMAJ. 2017;189(38):E1204-E5.
138. Jay CL, Lyuksemburg V, Ladner DP, Wang E, Caicedo JC, Holl JL, et al. Ischemic cholangiopathy after controlled donation after cardiac death liver transplantation: a meta-analysis. Ann Surg. 2011;253(2):259-64.

139. Tang JX, Na N, Li JJ, Fan L, Weng RH, Jiang N. Outcomes of Controlled Donation After Cardiac Death Compared With Donation After Brain Death in Liver Transplantation: A Systematic Review and Meta-analysis. Transplant Proc. 2018;50(1):33-41.
140. Mourad MM, Algarni A, Liossis C, Bramhall SR. Aetiology and risk factors of ischaemic cholangiopathy after liver transplantation. World J Gastroenterol. 2014;20(20):6159-69.
141. Adkins RB, Jr., Chapman WC, Reddy VS. Embryology, anatomy, and surgical applications of the extrahepatic biliary system. Surg Clin North Am. 2000;80(1):363-79.
142. Skaro AI, Jay CL, Baker TB, Wang E, Pasricha S, Lyuksemburg V, et al. The impact of ischemic cholangiopathy in liver transplantation using donors after cardiac death: the untold story. Surgery. 2009;146(4):543-52; discussion 52-3.
143. Mathur AK, Heimbach J, Steffick DE, Sonnenday CJ, Goodrich NP, Merion RM. Donation after cardiac death liver transplantation: predictors of outcome. Am J Transplant. 2010;10(11):2512-9.
144. Chan EY, Olson LC, Kisthard JA, Perkins JD, Bakthavatsalam R, Halldorson JB, et al. Ischemic cholangiopathy following liver transplantation from donation after cardiac death donors. Liver Transpl. 2008;14(5):604-10.
145. Schlegel A, Scalera I, Perera M, Kalisvaart M, Mergental H, Mirza DF, et al. Impact of donor age in donation after circulatory death liver transplantation: Is the cutoff "60" still of relevance? Liver Transpl. 2018;24(3):352-62.
146. Boteon YL, Boteon A, Attard J, Mergental H, Mirza DF, Bhogal RH, et al. Ex situ machine perfusion as a tool to recondition steatotic donor livers: Troublesome features of fatty livers and the role of defatting therapies. A systematic review. Am J Transplant. 2018;18(10):2384-99.
147. Kalisvaart M, de Haan JE, Polak WG, Metselaar HJ, Wijnhoven BPL, JNM IJ, et al. Comparison of Postoperative Outcomes Between Donation After Circulatory Death and Donation After Brain Death Liver Transplantation Using the Comprehensive Complication Index. Ann Surg. 2017;266(5):772-8.
148. Tun-Abraham ME, Wanis K, Garcia-Ochoa C, Sela N, Sharma H, Al Hasan I, et al. Can we reduce ischemic cholangiopathy rates in donation after cardiac death liver transplantation after 10 years of practice? Canadian single-centre experience. Can J Surg. 2018;62(1):8.
149. Cao Y, Shahrestani S, Chew HC, Crawford M, Macdonald PS, Laurence J, et al. Donation After Circulatory Death for Liver Transplantation: A Meta-Analysis on the Location of Life Support Withdrawal Affecting Outcomes. Transplantation. 2016;100(7):1513-24.
150. Lee KW, Simpkins CE, Montgomery RA, Locke JE, Segev DL, Maley WR. Factors affecting graft survival after liver transplantation from donation after cardiac death donors. Transplantation. 2006;82(12):1683-8.
151. Hashimoto K, Eghtesad B, Gunasekaran G, Fujiki M, Uso TD, Quintini C, et al. Use of tissue plasminogen activator in liver transplantation from donation after cardiac death donors. Am J Transplant. 2010;10(12):2665-72.
152. Seal JB, Bohorquez H, Reichman T, Kressel A, Ghanekar A, Cohen A, et al. Thrombolytic protocol minimizes ischemic-type biliary complications in liver transplantation from donation after circulatory death donors. Liver Transpl. 2015;21(3):321-8.
153. Bohorquez H, Seal JB, Cohen AJ, Kressel A, Bugeaud E, Bruce DS, et al. Safety and Outcomes in 100 Consecutive Donation After Circulatory Death Liver Transplants Using a Protocol That Includes Thrombolytic Therapy. Am J Transplant. 2017;17(8):2155-64.

154. Jayant K, Reccia I, Virdis F, Shapiro AMJ. Systematic Review and Meta-Analysis on the Impact of Thrombolytic Therapy in Liver Transplantation Following Donation after Circulatory Death. J Clin Med. 2018;7(11).
155. Jones JM, Bhutiani N, Wei D, Goldstein L, Jones CM, Cannon RM. A literature-based cost analysis of tissue plasminogen activator for prevention of biliary stricture in donation after circulatory death liver transplantation. Am J Surg. 2018;216(5):959-62.
156. Li P, Liu YF, Yang L. Advantages of dual hypothermic oxygenated machine perfusion over simple cold storage in the preservation of liver from porcine donors after cardiac death. Clinical transplantation. 2015;29(9):820-8.
157. Op den Dries S, Sutton ME, Karimian N, de Boer MT, Wiersema-Buist J, Gouw AS, et al. Hypothermic oxygenated machine perfusion prevents arteriolonecrosis of the peribiliary plexus in pig livers donated after circulatory death. PLoS One. 2014;9(2):e88521.
158. Westerkamp AC, Karimian N, Matton AP, Mahboub P, van Rijn R, Wiersema-Buist J, et al. Oxygenated Hypothermic Machine Perfusion After Static Cold Storage Improves Hepatobiliary Function of Extended Criteria Donor Livers. Transplantation. 2016;100(4):825-35.
159. van Rijn R, Karimian N, Matton APM, Burlage LC, Westerkamp AC, van den Berg AP, et al. Dual hypothermic oxygenated machine perfusion in liver transplants donated after circulatory death. Br J Surg. 2017;104(7):907-17.
160. Schlegel A, Muller X, Kalisvaart M, Muellhaupt B, Perera M, Isaac JR, et al. Outcomes of DCD liver transplantation using organs treated by hypothermic oxygenated perfusion before implantation. J Hepatol. 2019;70(1):50-7.
161. Hoyer DP, Mathe Z, Gallinat A, Canbay AC, Treckmann JW, Rauen U, et al. Controlled Oxygenated Rewarming of Cold Stored Livers Prior to Transplantation: First Clinical Application of a New Concept. Transplantation. 2016;100(1):147-52.
162. Nostedt JJ, Skubleny DT, Shapiro AMJ, Campbell S, Freed DH, Bigam DL. Normothermic Ex Vivo Machine Perfusion for Liver Grafts Recovered from Donors after Circulatory Death: A Systematic Review and Meta-Analysis2018. 8 p.

Chapter 3

Normothermic *Ex-vivo* Machine Perfusion for Liver Grafts Recovered from Donors after Circulatory Death: A Systematic Review and Meta-Analysis

Adapted from:

Nostedt, JJ., Skubleny, DT., Shapiro, AMJ., Campbell, S., Freed, DH., Bigam, DL., "Normothermic Ex Vivo Machine Perfusion for Liver Grafts Recovered from Donors after Circulatory Death: A Systematic Review and Meta-Analysis," HPB Surgery, vol. 2018, Article ID 6867986, 8 pages, 2018.

Abstract

As a result of donation after circulatory death liver grafts' poor tolerance of cold storage, there has been increasing research interest in normothermic machine perfusion. This study aims to systematically review the current literature comparing normothermic perfusion to cold storage in donation after circulatory death liver grafts and complete a meta-analysis of published large animal and human studies. A total of nine porcine studies comparing cold storage to normothermic machine perfusion for donation after circulatory death grafts were included for analysis. There was a significant reduction in AST; mean difference -2291 U/L, CI= (-3019, -1563); p=<0.00001 and ALT; mean difference -175 U/L, CI= (-266, -85); p=0.0001, for normothermic perfusion relative to static cold storage, with moderate (I^2=61%) and high (I^2=96%) heterogeneity respectively. Total bile production was also significantly higher, mean difference =174 ml, CI= (155, 193) p<0.00001. Further research focusing on standardization, performance of this technology following periods of cold storage, economic implications, and clinical trial data focused on donation after circulatory death grafts will be helpful to advance this technology toward routine clinical utilization for these grafts.

Introduction

Liver transplant remains the only definitive therapy for end stage liver disease. However the shortage of quality organs remains significant in the United States with 1673 patients dying while on the waitlist and a further 1227 removed, too sick to undergo transplant during 2015. (61) Due to organ shortage, there has been a rise in the use of extended criteria donors (ECD). These donors include those with significant steatosis, advanced age and donation after circulatory death (DCD) liver grafts.(163)

DCD grafts represent an important source of organs to expand the donor pool. The number of DCD grafts used continues to increase however there is also a rise in the percentage of DCD grafts recovered but not transplanted.(61) This is a result of these grafts' poor tolerance of static cold storage (SCS)(84), the current standard for organ preservation. DCD grafts are more prone to reperfusion injury and susceptible to ischemic biliary cholangiopathy. As a result, outcomes of DCD transplants have traditionally been marginal showing lower long-term patient and graft survival and increased biliary complications.(62) More recent results show improved graft and patient survival, though ischemic cholangiopathy is still a frequent complication of DCD grafts.(63)

Ex-vivo perfusion is now being studied as a method of increasing use of DCD grafts. Studies using hypothermic and subnormothermic perfusion have shown promising results both in large animal (10, 71, 105, 164), and clinical studies(98), however in marginal grafts such as those from DCD, normothermic machine perfusion (NMP) showed superior graft function and preservation of biliary epithelium in animal

models.(111, 122) In addition to organ preservation, NMP offers the advantage of being able to assess graft viability during perfusion under physiologic conditions where the graft is metabolically active. It also provides opportunity to deliver and monitor response to therapies in order to resuscitate marginal grafts prior to transplantation. These added benefits have led to growing research interest in NMP for DCD grafts in an effort to expand the organ donor pool. NMP for DCD grafts has been studied primarily in large animal studies where resource allocation only allows for small study subject numbers, and study design is critical to advance this complex technology. Although not used often in animal research, systematic reviews can have an important role for the development of future studies.(165) To our knowledge this is the first systematic review of NMP for DCD liver grafts with a meta-analysis of published data.

The aim of this paper is to systematically review the current literature comparing NMP to SCS in DCD liver grafts in large animal (pig) and human studies. The secondary aim is to complete a meta-analysis of NMP vs. SCS livers in published DCD porcine liver perfusions.

Methods

Search Strategy

Searches were conducted in Ovid MEDLINE, OVID EMBASE, EBSCO CINAHL, WOS, SCOPUS, Proquest Dissertations and Theses, PROSPERO by an expert librarian (SC) June, 2017 and updated in July, 2017. Searches employed both controlled vocabularies (eg: MeSH,. EMTREE, etc.) and key words such as: (DCD livers) and (ex-

vivo perfusion or normothermic perfusion) Search strategies were adapted for each database. Search strategies are available in the supporting information (S1). No limits were applied.

All full text, porcine and human trials comparing NMP to SCS for the preservation of DCD livers were included for analysis. Studies that did not include DCD livers, and those that focused only on hypothermic or sub-normothermic machine perfusion were excluded.

Selection of Studies

Titles and abstracts from the primary search were reviewed independently by two authors (JN, DS) for studies that met inclusion criteria. When this was not clear from the titles and abstracts, full text articles were reviewed to determine inclusion.

Outcome measures

Primary outcomes in *ex*-vivo perfusion studies included assessment of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels as markers of hepatocellular damage, as well as bile production and lactate clearance as markers of liver function. Secondary outcomes were histological preservation, and hemodynamic stability indicated by hepatic arterial flow. Primary outcomes in orthotopic pig liver transplant studies included post-transplant peak AST, bile production and graft-survival. Secondary outcomes included histologic preservation. Where there was missing data for

quantitative analysis, this information was requested via email from the publication corresponding authors. We received two responses, but no further data for inclusion. Where possible, this data was estimated from published figures using Adobe Acrobat Reader DC software.

Assessment of Bias

Articles were assessed by two authors (JN, DS) using the Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE) risk of bias assessment tool.(166)

Statistical Analysis

A trained statistician performed statistical analysis. Outcomes assessed in the meta-analysis included AST, ALT, total bile production and hepatic artery flow for perfusion studies, as well as peak AST for transplant studies. They are all continuous variables expressed as mean ± standard deviation (SD). The mean difference (MD) was used as a summary measure of efficacy between groups treated by NMP and SCS. When no SD was provided, a pooled SD was estimated as previously described.(167) Meta-analysis was performed using RevMan 5.3 software. Heterogeneity of studies was assessed and the following cut-offs were applied, low (>25%), moderate (>50%), and high (>75%) as described by Higgins et al.(168)

Results

Search Results

Three hundred and eighty-six titles were identified through our primary search, with 228 remaining for screening after the removal of duplicates. Of these, 201 titles were excluded for the following reasons: published abstract with no complete full text article, comparison of hypothermic or subnormothermic perfusion without NMP and studies without DCD grafts. Nine articles that directly compared cold storage to NMP for DCD grafts were included for analysis (**Figure 3-1**). Six articles were perfusion studies(81, 118, 119, 122, 169, 170), and two pig-transplant models(121, 132). One article published results of both a perfusion model, and pig transplant model(111). There are no clinical trials directly comparing SCS to NMP specifically for DCD livers and as such the studies included for analysis were limited to porcine experimental studies. The results of included studies are summarized in **Table 3-1** and **Table 3-2**.

Pig liver *ex-vivo* perfusion studies

Pooled data showed a significant reduction in AST at the end of the simulated transplant phase in the NMP group relative to SCS. MD= -2291 U/L, CI= (-3019, -1563); p=<0.00001. A similar trend was seen in ALT MD= -175 U/L, CI= (-266, -85); p=0.0001. However the heterogeneity was moderate (I^2=61%) and high (I^2=90%) respectively for these two variables. (**Figure 3-2**)

Total bile production following the simulated transplant phase was significantly higher in the NMP group, MD=174 ml, CI= (155, 193) p<0.00001. There was low heterogeneity (I^2=45%). (**Figure 3-2**)

There was insufficient data available to perform meta-analysis for lactate clearance.

Limited data was available for hepatic arterial flow. The NMP group did demonstrate higher flows, although this did not reach statistical significance (p=0.09). (**Figure 3-2**)

Different histological scoring systems were used by different centers, thus were not suitable for meta-analysis. All perfusion studies showed less necrosis and improved architectural preservation in the NMP group relative to SCS.(81, 83, 111, 118, 119, 122, 169) Similarly, NMP demonstrated improved preservation of the biliary epithelium and peribiliary plexus (81, 83, 122)

Pig liver orthotopic transplant Studies

Post transplant peak AST was lower in the NMP group MD= -1019, CI= (-1276, -762) p<0.00001. There was a high level of heterogeneity (I^2=78%). (**Figure 3-3**) There was insufficient data available to compare bile production. Graft survival also was not assessed in the meta-analysis, as the recovery period in each of these studies were different (**Table 3-2**). Boehnert et al. reported no difference in bile production in the eight hours following transplant after perfusing with acellular solution.(111) Schon et al. showed all grafts transplanted after 60 minutes WIT and SCS suffered primary graft non-function.(121) In an uncontrolled DCD transplant model where normothermic extracorporeal membrane oxygenation was combined with either NMP or SCS, there was

100% five-day survival in the NMP group relative to 83% survival in the SCS group.(132)

NMP groups demonstrated less necrosis, sinusoidal swelling and improved overall architectural preservation relative to SCS groups.(121, 132) One pig transplant model did not report histologic data.(171)

Risk of Bias Assessment

The allocation process of animals was unclear in several studies (111, 118, 121, 132) however no other significant sources of bias within the included studies were identified.

Discussion

The results of this review and meta-analysis must be interpreted with caution, as heterogeneity was high within the perfusion studies limiting the strength of conclusions that can be drawn. Experimental design for the included perfusion studies varied in several fundamental parameters. Major differences included surgical model, duration of preservation and reperfusion, and *ex-vivo* circuit design.

Pigs used as liver donors were 30-40kg and included Landrace(118, 169) and Yorkshire(81, 83, 111, 119, 122), with male pigs used only by Boehnert et al.(172) and gender unspecified in one study.(118) The DCD model also varied between studies with

the majority inducing cardiac arrest with potassium chloride injection(81, 83, 118, 119, 122, 169), while one study induced cardiac arrest via exsanguination.(111)

Boehnert et al. was the only perfusion study to compare SCS to NMP following a period of SCS.(111) The WIT in all included perfusion studies was 60 minutes except Banan et al.(169) who compared SCS after 40 minutes WIT to NMP following 20, 40 and 60 minutes of WIT. Following WIT livers were flushed with histidine-tryptophan-ketoglutarate (81, 83, 119, 122, 169), University of Wisconsin (111) or Euro Collins (118) cold preservation solutions. Livers were flushed *in-situ* (111, 118, 119, 122, 169) or *ex-situ* (81, 83) with dual perfusion through the hepatic artery and portal vein (81, 83, 119, 122, 169) or single arterial flush.(118) One study did not specify if dual vessel flush was used.(111)

NMP was then carried out for either 6 (169), 8(111), 10(81, 83, 119, 122), or 24 (118) hours. Simulated transplant with whole blood reperfusion was for either 2 (169), 12 (111) or 24 (81, 83, 118, 119, 122) hours. Which is of important note as transaminase levels were reported at the end of the reperfusion stage.

One study included a dialysis circuit as part of their perfusion set up.(169) Flow was driven by either dual centrifugal pumps(169), the combination of a centrifugal pump and roller pump(81, 83, 119, 122) or a centrifugal pump to perfuse the hepatic artery and the portal vein perfused by gravity.(111, 118) With regards to perfusate used: three studies used whole blood (81, 118, 122), two dilute whole blood(119, 169), and one used acellular perfusate.(111) The study by Liu et al. is the only to directly compare different perfusates (83), using Steen solution, Steen solution with washed red blood cells, and

whole blood compared to SCS. Hepatocellular injury and liver function were significantly better in the Steen solution with red blood cells, and whole blood groups relative to both SCS or Steen solution alone. There was no significant difference between the whole blood or Steen solution with washed red blood cells.(83) Within the included studies there was not enough available data to perform subgroup analysis based on type of perfusate used. However, the results with acellular perfusion(111) to our knowledge have not been replicated and more studies are still needed to determine the optimal NMP perfusate composition for DCD livers, however the results from Liu et al(83) suggest the need for an oxygen carrier.

In porcine liver transplant models there was also significant study heterogeneity. The post transplant observation period ranged from eight hours to seven days. Fondevila et al.(132) compared NMP to SCS following a period of normothermic extracorporeal machine oxygenation, which was significantly different from the other included transplant studies. Schon et al. compared SCS to NMP with no period of SCS and all grafts that were exposed to 4 hours of cold storage following 60 minutes WIT suffered primary non function.(121) This is in keeping with previous data suggesting that even brief periods of cold storage can impact positive effects of NMP.(84) The study by Boehnert et al. however, compared SCS alone to a period of SCS followed by NMP and reported less hepatocellular injury in the NMP group(111), but data from these grafts were only reported for eight hours post transplant and longer-term survival of the grafts was not assessed. In discarded human liver studies, NMP has shown the ability to recover function of damaged livers even after extensive periods of cold storage.(124) Further research to address NMP's ability to safely recover and transplant DCD grafts following

periods of cold storage is needed. Devices available for NMP were reviewed by Ravikumar et al. (173) and portable perfusion devices are now available to try and eliminate cold storage time in the transplant sequence for these marginal organs. Whether NMP can successfully recover DCD grafts after periods of cold storage remains an important question that will impact the clinical implementation of *ex-vivo* NMP. The economic impact of these systems has not yet been studied and will also remain a factor in clinical implementation of NMP for DCD grafts. The use of gradual rewarming has shown promise for this population of liver grafts (72, 106, 174, 175) and may play an important role moving forward in utilizing machine perfusion after periods of SCS.

NMP has shown capacity to recover function in discarded DCD human liver studies (76, 124, 125, 127, 176), and has been used to recover these grafts for clinical transplant.(77, 86) NMP has also been studied as a method to assess which marginal DCD grafts are safely transplantable. A set of viability criteria has been proposed by Mergental et al. (86) Establishing a standardized set of criteria will be an important goal for clinical implementation of NMP for DCD grafts.

There are phase-I clinical trials comparing NMP to SCS (128-130), however these studies have only limited numbers of DCD and otherwise marginal grafts. To date no randomized control trials have been published comparing NMP to SCS specifically in DCD grafts. Results of a multicenter European randomized control trial (ISRCTN39731134) comparing NMP to SCS, once published, may be pivotal for this technology moving forward into clinical practice.

Limitations

There was a large amount of heterogeneity amongst the small number of studies as outlined above. These significant differences in experimental design limit the strength of conclusions that could be drawn from meta-analysis. Furthermore, multiple data points included for meta-analysis were estimated from published figures which may differ slightly from the measured values.

Conclusion

Meta-analysis of published porcine perfusion studies demonstrates NMP is superior to SCS regarding the preservation of liver architecture and function in DCD grafts. Given significant differences between studies, these results are to be taken with caution. Further study is still required in order to optimize and standardize perfusate composition and to evaluate NMP's role in preservation following periods of cold storage. Clinical studies involving more DCD grafts will help bring this technology closer to clinical implementation. Economic factors need to be considered in subsequent studies to ensure feasibility within current healthcare systems.

Table 3-1: Summary of Pig Liver Perfusion Study Results

Perfusion studies	Perfusate	Preservation Time (hr)	Simulated transplant phase (hr)	WIT (min)	N-NMP	N- SCS	AST (U/L) NMP	AST (U/L) SCS	ALT (U/L) NMP	ALT (U/L) SCS	NMP total bile(ml)	SCS total bile(ml)	NMP HA flow (ml/min)	SCS HA flow (ml/min)
Boehnert et al. 2013	Steen	4 SCS+8 NMP vs. 12 SCS	12	60	6	6	_	_	*69±21	*308±45	_	_	340±85	180±35
Liu et al. 2014	whole blood	10	24	60	5	5	*309	*3163±1545	*25	*186±98	219±42.5	11.6±16.3	23±7 ml/min/ 100g liver	13±3ml/ min/100 g
Banan et al. 2015	saline + whole blood	6	2	40	3	3	610±121	1942 ± 641	63±10	109±10	_	_	*504	*480±60
Nassar et al. 2015	acellular solutions+ whole blood	10	24	60	15	5	1029±230	3150±691	46±8	184±43	181±18	12±7	94±7ml/ min/100 g	57±14ml /min/10 0g
Liu et al. 2016	Steen+RBC	10	24	60	5	5	*931±793	3151±1547	*40	185±97	174±30	12±16	_	_
Nassar et al. 2016	whole blood	10	24	60	5	5	277±69	3150±1546	22±2	185±97	219±43	12±16	_	_
St. Peter et al. 2002	whole blood	24	24	60	4	4	259	3810	*66±20	*398±74	_	_	1400 ml/min	440ml/m in

*Denotes values estimated from published figures where raw data not available for analysis. –Denotes data not available for meta-analysis
AST/ALT values are taken at the end of the simulated transplant reperfusion phase. HA flows are ml/min unless units otherwise specified

Table 3-2: Summary of Pig Orthotopic Liver Transplant Studies

Pig Transplant Studies	Preservation Time (hr)	Duration of post transplant monitoring	NMP n=	SCS n=	NMP peak AST (U/L)	SCS Peak AST (U/L)
Schon et al. 2001	4	7 days	6	6	603±141	1570±171
Fondevila et al. 2011	4	5 days	6	6	692±77	1500±269
Boehnert et al. 2013	12	8 hours	6	6	524±187	1809±205

Figure 3-1: Study selection

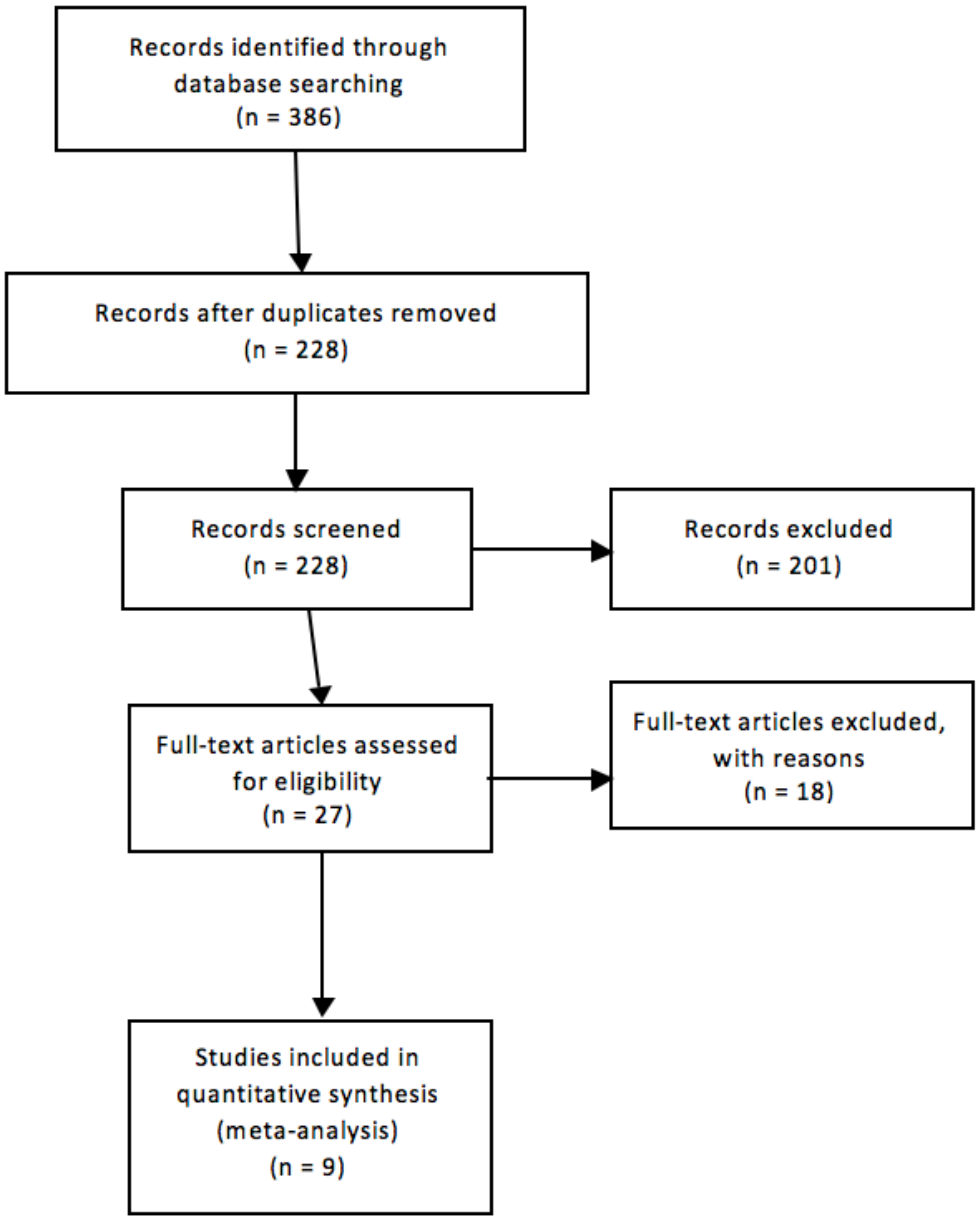
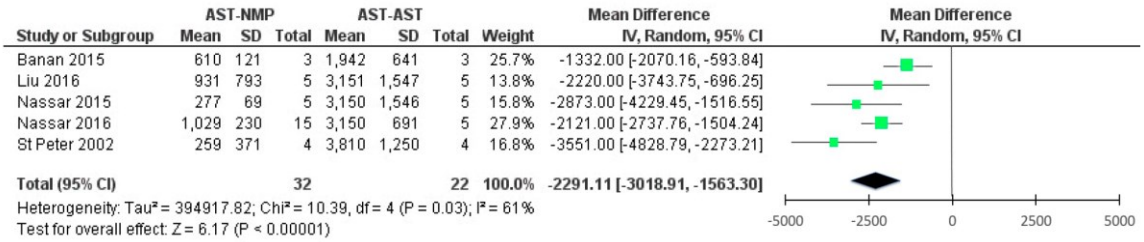
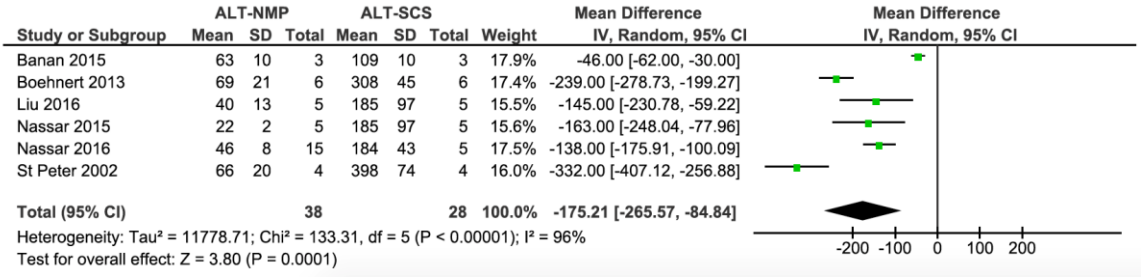
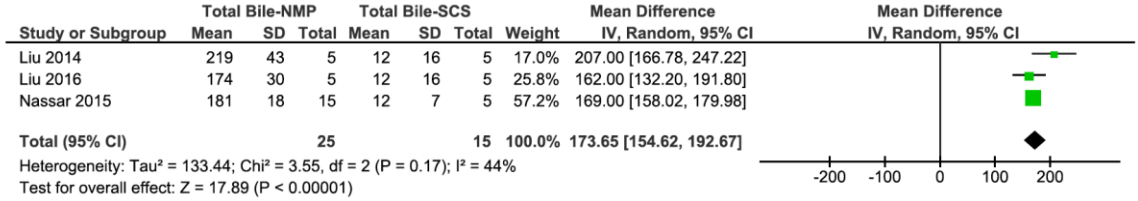


Figure 3-2: Pooled AST, ALT, and bile production results from porcine liver perfusion studies







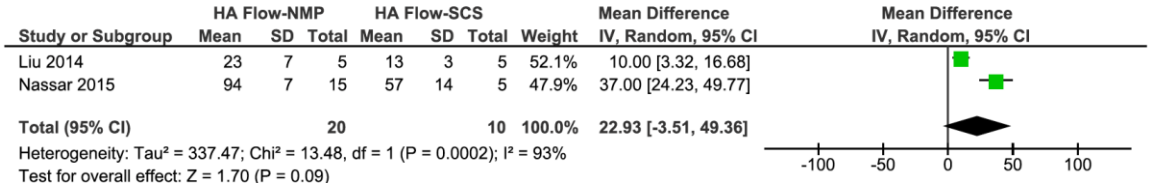
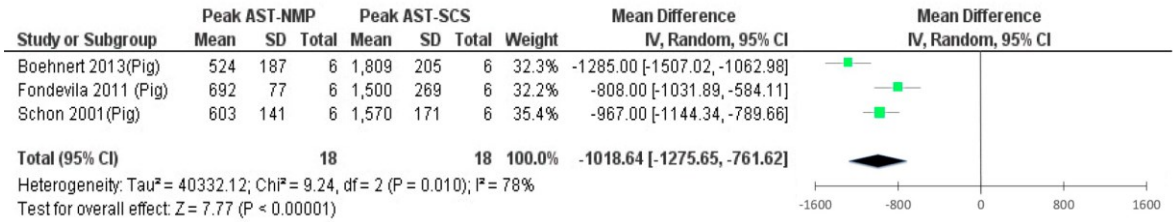


Figure 3-3: Pooled peak AST results from porcine transplant studies



S1: Description of literature search strategy

Database: Ovid MEDLINE(R) Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R) Daily and Ovid
MEDLINE(R) <1946 to Present>
Search Strategy:
---

1 liver transplantation after organ preservation.m_titl. (1)
2 superior preservation of DCD livers.m_titl. (1)
3 criteria for viability assessment of discarded.m_titl. (1)
4 non heart beating donor porcine livers.m_titl. (1)
5 Sanguineous normothermic machine perfusion improves hemodynamics.m_titl. (1)
6 (normothermic machine perfusion and viability testing).m_titl. (1)
7 First human liver transplantation using a marginal allograft.m_titl. (2)
8 ". Impact of Temperature on Porcine Liver Machine Perfusion".m_titl. (1)
9 or/1-8 (9)
10 exp Liver Transplantation/ or exp Liver/ (462813)
11 (liver or livers).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms] (1012778)
12 hepatic.mp. (282677)
13 10 or 11 or 12 (1078000)
14 ((donat* adj2 circulat* death) or (donat* adj2 cardiac* death)).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms] (1178)
15 ("non heart beat*" or NHBD).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms] (1272)
16 dcd.mp. (2013)
17 ((discarded or marginal or declined or rejected) adj5 liver*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms] (1048)
18 14 or 15 or 16 or 17 (4764)
19 (exvivo or "ex-vivo" or exsitu or "ex-situ" or excorporeal or extracorporeal or

"extra corporeal" or cold storage).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms] (109551)
20 ("normo therm*" or normotherm* or warm perfusion*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms] (7886)
21 13 and 18 and 19 and 20 (78)
22 exp Rats/ or (rat or rats).mp. (1679155)
23 21 not 22 (60)

Database: Embase <1974 to 2017 July 20>
Search Strategy:
---
1 exp liver transplantation/ or exp liver/ (671770)
2 (liver or livers).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer,
drug manufacturer, device trade name, keyword, floating subheading word] (1382551)
3 hepatic.mp. (359985)
4 1 or 2 or 3 (1453549)
5 ((donat* adj2 circulat* death) or (donat* adj2 cardiac* death)).mp. [mp=title, abstract, heading word, drug trade
name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word]
(2430)
6 ("non heart beat*" or NHBD).mp. [mp=title, abstract, heading word, drug trade name, original title, device
manufacturer, drug manufacturer, device trade name, keyword, floating subheading word] (1768)
7 dcd.mp. (4169)
8 ((discarded or marginal or declined or rejected) adj5 liver*).mp. [mp=title, abstract, heading word, drug trade
name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word]
(1564)
9 5 or 6 or 7 or 8 (8072)
10 (exvivo or "ex-vivo" or exsitu or "ex-situ" or excorporeal or extracorporeal or

"extra corporeal" or cold
storage).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer,
device trade name, keyword, floating subheading word] (162280)
11 ("normo therm*" or normotherm* or warm perfusion* or NMP or "37 adj degree*").mp. [mp=title, abstract, heading
word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating
subheading word] (11768)
12 4 and 9 and 10 and 11 (192)
13 exp rat/ or (rat or rats).mp. (1875464)
14 12 not 13 (159)
15 remove duplicates from 14 (156)

References

10. Vekemans K, Liu Q, Brassil J, Komuta M, Pirenne J, Monbaliu D. Influence of flow and addition of oxygen during porcine liver hypothermic machine perfusion. Transplant Proc. 2007;39(8):2647-51.
61. Kim WR, Lake JR, Smith JM, Skeans MA, Schladt DP, Edwards EB, et al. OPTN/SRTR 2015 Annual Data Report: Liver. Am J Transplant. 2017;17 Suppl 1:174-251.
62. Foley DP, Fernandez LA, Leverson G, Anderson M, Mezrich J, Sollinger HW, et al. Biliary complications after liver transplantation from donation after cardiac death donors: an analysis of risk factors and long-term outcomes from a single center. Ann Surg. 2011;253(4):817-25.
63. Coffey JC, Wanis KN, Monbaliu D, Gilbo N, Selzner M, Vachharajani N, et al. The influence of functional warm ischemia time on DCD liver transplant recipients' outcomes. Clin Transplant. 2017;31(10).
71. den Dries So, Sutton ME, Karimian N, de Boer MT, Wiersema-Buist J, Gouw ASH, et al. Hypothermic Oxygenated Machine Perfusion Prevents Arteriolonecrosis of the Peribiliary Plexus in Pig Livers Donated after Circulatory Death. PLoS One. 2014;9(2):e88521.
72. Minor T, Efferz P, Fox M, Wohlschlaeger J, Luer B. Controlled oxygenated rewarming of cold stored liver grafts by thermally graduated machine perfusion prior to reperfusion. Am J Transplant. 2013;13(6):1450-60.
76. Sutton ME, op den Dries S, Karimian N, Weeder PD, de Boer MT, Wiersema-Buist J, et al. Criteria for viability assessment of discarded human donor livers during ex vivo normothermic machine perfusion. PLoS One. 2014;9(11):e110642.
77. Watson CJE, Kosmoliaptsis V, Randle LV, Gimson AE, Brais R, Klinck JR, et al. Normothermic Perfusion in the Assessment and Preservation of Declined Livers Before Transplantation: Hyperoxia and Vasoplegia-Important Lessons From the First 12 Cases. Transplantation. 2017;101(5):1084-98.
81. Liu Q, Nassar A, Farias K, Buccini L, Baldwin W, Mangino M, et al. Sanguineous normothermic machine perfusion improves hemodynamics and biliary epithelial regeneration in donation after cardiac death porcine livers. Liver Transpl. 2014;20(8):987-99.
83. Liu Q, Nassar A, Farias K, Buccini L, Mangino MJ, Baldwin W, et al. Comparing Normothermic Machine Perfusion Preservation With Different Perfusates on Porcine Livers From Donors After Circulatory Death. Am J Transplant. 2016;16(3):794-807.
84. Reddy S, Greenwood J, Maniakin N, Bhattacharjya S, Zilvetti M, Brockmann J, et al. Non-heart-beating donor porcine livers: the adverse effect of cooling. Liver Transpl. 2005;11(1):35-8.
86. Mergental H, Perera MT, Laing RW, Muiesan P, Isaac JR, Smith A, et al. Transplantation of Declined Liver Allografts Following Normothermic Ex-Situ Evaluation. Am J Transplant. 2016;16(11):3235-45.
98. Guarrera JV, Henry SD, Samstein B, Odeh-Ramadan R, Kinkhabwala M, Goldstein MJ, et al. Hypothermic machine preservation in human liver transplantation: the first clinical series. Am J Transplant. 2010;10(2):372-81.

105. Gringeri E, Bonsignore P, Bassi D, D'Amico FE, Mescoli C, Polacco M, et al. Subnormothermic machine perfusion for non-heart-beating donor liver grafts preservation in a Swine model: a new strategy to increase the donor pool? Transplant Proc. 2012;44(7):2026-8.
106. Obara H, Matsuno N, Shigeta T, Hirano T, Enosawa S, Mizunuma H. Temperature controlled machine perfusion system for liver. Transplant Proc. 2013;45(5):1690-2.
111. Boehnert MU, Yeung JC, Bazerbachi F, Knaak JM, Selzner N, McGilvray ID, et al. Normothermic acellular ex vivo liver perfusion reduces liver and bile duct injury of pig livers retrieved after cardiac death. Am J Transplant. 2013;13(6):1441-9.
118. St Peter SD, Imber CJ, Lopez I, Hughes D, Friend PJ. Extended preservation of non-heart-beating donor livers with normothermic machine perfusion. British Journal of Surgery. 2002;89(5):609-16.
119. Nassar A, Liu Q, Farias K, D'Amico G, Tom C, Grady P, et al. Ex vivo normothermic machine perfusion is safe, simple, and reliable: Results from a large animal model. Surgical Innovation. 2015;22(1):61-9.
121. Schon MR, Kollmar O, Wolf S, Schrem H, Matthes M, Akkoc N, et al. Liver transplantation after organ preservation with normothermic extracorporeal perfusion. Ann Surg. 2001;233(1):114-23.
122. Nassar A, Liu Q, Farias K, Buccini L, Baldwin W, Bennett A, et al. Impact of Temperature on Porcine Liver Machine Perfusion From Donors After Cardiac Death. Artif Organs. 2016;40(10):999-1008.
124. Vogel T, Brockmann JG, Quaglia A, Morovat A, Jassem W, Heaton ND, et al. The 24-hour normothermic machine perfusion of discarded human liver grafts. Liver Transplantation. 2017;23(2):207-20.
125. Bellomo R, Marino B, Starkey G, Fink M, Wang BZ, Eastwood GM, et al. Extended normothermic extracorporeal perfusion of isolated human liver after warm ischaemia: a preliminary report. Critical care and resuscitation : journal of the Australasian Academy of Critical Care Medicine. 2014;16(3):197-201.
127. op den Dries S, Karimian N, Sutton ME, Westerkamp AC, Nijsten MW, Gouw AS, et al. Ex vivo normothermic machine perfusion and viability testing of discarded human donor livers. Am J Transplant. 2013;13(5):1327-35.
128. Ravikumar R, Jassem W, Mergental H, Heaton N, Mirza D, Perera MT, et al. Liver Transplantation After Ex Vivo Normothermic Machine Preservation: A Phase 1 (First-in-Man) Clinical Trial. Am J Transplant. 2016;16(6):1779-87.
129. Selzner M, Goldaracena N, Echeverri J, Kaths JM, Linares I, Selzner N, et al. Normothermic ex vivo liver perfusion using steen solution as perfusate for human liver transplantation: First North American results. Liver Transpl. 2016;22(11):1501-8.
130. Bral M, Gala-Lopez B, Bigam D, Kneteman N, Malcolm A, Livingstone S, et al. Preliminary Single-Center Canadian Experience of Human Normothermic Ex Vivo Liver Perfusion: Results of a Clinical Trial. American Journal of Transplantation. 2017;17(4):1071-80.
132. Fondevila C, Hessheimer AJ, Maathuis M-HJ, Munoz J, Taura P, Calatayud D, et al. Superior preservation of DCD livers with continuous normothermic perfusion. Annals of Surgery. 2011;254(6):1000-7.

163. Busuttil RW, Tanaka K. The utility of marginal donors in liver transplantation. Liver Transpl. 2003;9(7):651-63.
164. Knaak JM, Spetzler VN, Goldaracena N, Boehnert MU, Bazerbachi F, Louis KS, et al. Subnormothermic ex vivo liver perfusion reduces endothelial cell and bile duct injury after donation after cardiac death pig liver transplantation. Liver Transpl. 2014;20(11):1296-305.
165. de Vries RB, Wever KE, Avey MT, Stephens ML, Sena ES, Leenaars M. The usefulness of systematic reviews of animal experiments for the design of preclinical and clinical studies. ILAR J. 2014;55(3):427-37.
166. Hooijmans CR, Rovers MM, de Vries RB, Leenaars M, Ritskes-Hoitinga M, Langendam MW. SYRCLE's risk of bias tool for animal studies. BMC Med Res Methodol. 2014;14:43.
167. Furukawa TA, Barbui C, Cipriani A, Brambilla P, Watanabe N. Imputing missing standard deviations in meta-analyses can provide accurate results. J Clin Epidemiol. 2006;59(1):7-10.
168. Higgins JP, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. BMJ. 2003;327(7414):557-60.
169. Banan B, Chung H, Xiao Z, Tarabishy Y, Jia J, Manning P, et al. Normothermic extracorporeal liver perfusion for donation after cardiac death (DCD) livers. Surgery (United States). 2015;158(6):1642-50.
170. Liu Q, Nassar A, Farias K, Buccini L, Mangino MJ, Baldwin W, et al. Comparing Normothermic Machine Perfusion Preservation With Different Perfusates on Porcine Livers From Donors After Circulatory Death. American Journal of Transplantation. 2016;16(3):794-807.
171. Boehnert MU, Yeung J, Knaak J-M, Selzner N, Bazerbachi F, McGilvray I, et al. Acellular Normothermic Ex Vivo Liver Perfusion (Nevlp) Decreases Hepatocyte and Bile Duct Injury in Livers Retrieved After Cardiac Death. Hepatology. 2011;54(Suppl. 1):386A.
172. Boehnert MU, Yeung JC, Knaak JM, Selzner N, Selzner M. Normothermic acellular ex vivo liver perfusion (NEVLP) reduces liver and bile duct in DCD liver grafts. American Journal of Transplantation. 2013;13(12):3290.
173. Ravikumar R, Leuvenink H, Friend PJ. Normothermic liver preservation: a new paradigm? Transpl Int. 2015;28(6):690-9.
174. Shigeta T, Matsuno N, Obara H, Kanazawa H, Tanaka H, Fukuda A, et al. Impact of rewarming preservation by continuous machine perfusion: Improved post-transplant recovery in pigs. Transplantation proceedings. 2013;45(5):1684-9.
175. Banan B, Xiao Z, Watson R, Xu M, Jia J, Upadhya GA, et al. Novel strategy to decrease reperfusion injuries and improve function of cold-preserved livers using normothermic ex vivo liver perfusion machine. Liver Transpl. 2016;22(3):333-43.
176. Banan B, Watson R, Xu M, Lin Y, Chapman W. Development of a normothermic extracorporeal liver perfusion system toward improving viability and function of human extended criteria donor livers. Liver Transplantation. 2016;22(7):979-93.

Chapter 4

Avoiding Initial Hypothermia Does Not Affect Liver Graft Quality in a Porcine Donation After Circulatory Death Model of Normothermic Perfusion

This chapter is in preparation to submit for publication.

Abstract

Background:

Normothermic machine perfusion (NMP) of liver grafts donated after circulatory death (DCD) has shown promise in large animal and clinical trials. Following procurement, initial flush with a cold preservation solution is the standard of care. There is concern that initial cooling followed by warming may exacerbate liver injury, and the optimal initial flush temperature has yet to be identified. We hypothesize that avoidance of the initial cold flush will yield better quality liver grafts.

Methods:

Twenty-four anaesthetized pigs were withdrawn from mechanical ventilation. After 60 minutes of warm ischemia to simulate a DCD procurement, livers were flushed with histidine-tryptophan-ketoglutarate (HTK) at 4°C, 25°C or 35°C (n=4 per group). For comparison, an adenosine-lidocaine crystalloid solution (AD), shown to have benefit at warm temperatures in heart perfusions, was also used (n=4 per group). During 12 hours of NMP, adenosine triphosphate (ATP), lactate, transaminase levels, and histological injury were determined. Bile production and hemodynamics were monitored continuously.

Results:

ATP levels recovered substantially following 1-hour of NMP reaching pre-ischemic levels by the end of NMP with no difference between groups. There was no difference in peak aspartate

aminotransferase (AST) or in lactate dehydrogenase (LDH). Portal vein resistance was lowest in the 4°C group reaching significance after 2 hours (0.13 CI -0.01,0.277, p=0.025). Lactate levels recovered promptly with no difference between groups. Comparison to AD groups showed no statistical difference in the abovementioned parameters.

Conclusions:

Avoidance of the initial cold flush failed to demonstrate added benefit over standard 4°C HTK in this DCD model of liver perfusion.

Introduction

Liver transplant remains the only definitive therapy for end stage liver disease. However growing waitlist mortality has necessitated increased use of extended criteria (8) and donation after circulatory death (DCD) donors to address the shortage of transplantable livers.(163) Higher complication rates have been associated with use of DCD livers, most notably ischemic cholangiopathy.(63) The poor tolerance of prolonged static cold storage (SCS) for DCD liver grafts has led to recent growing interest in normothermic machine perfusion (NMP) as an alternative preservation strategy. Relative to SCS, NMP has shown improved preservation of DCD liver grafts in animal models(81, 83, 111, 121, 122) and promising results more recently in a large UK-European adequately powered randomized clinical trial.(78) As a result, this technology is progressing rapidly into the clinical realm. However the optimal perfusion strategy still remains a key focus for ongoing research. The initial liver flush before establishment of NMP represents an opportunity to further potentially optimize recovery and preservation of DCD liver grafts. The current clinical standard is to flush livers flushed with a cold (4°C) preservation solution at the time of procurement and then store in cold preservation solution on ice until implantation. This practice of cold initial flush has continued in clinical trials where grafts are being preserved with NMP. Rapid cooling of organs before rewarming during NMP preservation has recently been found to be detrimental in experimental cardiac preservation, (135) but has not been explored previously to our knowledge in DCD liver grafts.

The aim of this study is to determine if avoidance of hypothermia at the time of initial organ flush could improve liver graft quality for DCD liver grafts preserved using NMP.

Methods

Twelve DCD pigs were block randomized based on initial flush protocol. Histidine-tryptophan-ketoglutarate (HTK) solution (Servator H, Global Transplant Solutions, Ontario Canada), the standard clinical preservation solution, was used at 4°C (control), 25°C or 35°C (n=4 per group). For comparison, using an additional 12 pigs, an adenosine-lidocaine crystalloid solution (AD) shown to have benefit at warmer temperatures in heart perfusions(135) was used at similar temperatures (n=4 per group). Solution compositions are shown in **Table 4-1**. All animals received humane care in compliance with the National Institute of Health's Guide for the Care of Laboratory Animals. The Institutional Animal Care Committee approved the experimental protocol.

Liver procurement

Pigs were sedated with intramuscular injection of ketamine (20mg/kg) (Bimeda, Ontario, Canada) and anesthesia induced with inhaled isoflurane (Fresenius Kabi Canada Ltd, Ontario, Canada) with subsequent orotracheal intubation performed. General anesthesia was maintained with 2-3% isoflurane. A neck cut-down was performed for placement of an arterial pressure line in the left carotid artery for continuous pressure monitoring. A midline laparotomy was performed and a 10-gauge angiocath placed in the infra-renal vena cava for venous access. Hemodilution using 1 liter of Ringer's Lactate was performed to allow for later extraction of maximal blood to prime the NMP circuit. The porta hepatis was dissected to isolate the common bile duct, portal vein and common hepatic artery. A baseline liver parenchyma biopsy was obtained and then 500 units/kg of sodium heparin (Fresenius Kabi, Ontario, Canada) was

administered. Approximately 800 ml of whole blood was extracted via the vena cava line and processed with a cell saver (Sorin Xtra, Germany). The washed red blood cells were used to prime the NMP circuit. Inspired oxygen was decreased to room air and the isoflurane increased to 5% to deepen anesthesia and then mechanical ventilation discontinued. Circulatory death was declared when a pulse on the arterial line waveform was no longer present and loss of cardiorespiratory activity confirmed with auscultation. The abdomen was closed using towel clips with intra-abdominal temperature measured throughout the warm ischemia period. A second liver biopsy was taken immediately following warm ischemia. The previously dissected vascular and biliary structures were divided and the liver taken to the back table for cannulation of the hepatic artery (RMI 8 French PED-A-008, Edwards Life Sciences, California, United States) and portal vein (Intersept Tubing Connector, Medtronic, Minnesota, United States).

The warm ischemic time (WIT) was 60 minutes starting from the point where mean arterial pressure dropped below 50mmHg or oxygen saturation below 70% and extended to the onset of initial perfusion.

Initial Organ Flush

The liver was flushed on the back table with dual perfusion through the hepatic artery and portal vein for 5 minutes. Temperature and composition of the flush solution was as per the groups described above. Temperature of the initial flush solution was controlled via a circuit that included a heat exchanger (Trillium Myotherm XP, Medtronic, Minnesota, United States), controlled water bath circulator (Poly Science PD07R-20-A11B, Illinois, United States), a centrifugal pump (BPX-80 Bi-Medicus, Medtronic, Minnesota, United States) and Affinity

Fusion cardiotomy/venous reservoir (Medtronic, Minnesota, United States). During the 5-minute initial flush, temperature of the liver parenchyma was continually monitored. After the initial perfusion phase, livers were transferred to the primed NMP circuit and perfused continuously for 12 hours.

Normothermic Perfusion Circuit

Perfusate consisted of Krebs-Henseleit based solution with albumin (glucose, sodium chloride, potassium chloride, calcium chloride, magnesium chloride, sodium bicarbonate, sodium phosphate and 8% bovine serum albumin) and autologous red blood cells in a 3 to 1 ratio. The circuit was primed with 3.375g Piperacillin-tazobactam (Sandoz, Quebec, Canada), 500mg methylprednisolone (Pfizer Canada, Quebec, Canada), and 10,000 units of sodium heparin (Fresenius Kabi, Ontario, Canada). Sodium bicarbonate 8.4% (Hospira, Quebec, Canada) was titrated to maintain pH of ≥7.30. Insulin was infused at 2 units/hour (NovoRapid, Novo Nordisk, Ontario, Canada). Glucose (Sigma Aldrich, Missouri, United States) was added as required based on hourly blood gas glucose readings titrating to 4-10mmol/L. Hepatic temperature was maintained at 37°C. Hepatic artery pressure was maintained at 60mmHg and the portal vein was perfused at a constant weight adjusted flow.

The experimental circuit was composed of a Dideco D764 cardiotomy reservoir (Dideco Inc. Italy) two BPX-80 Bi-Medicus centrifugal pumps (Medtronic, Minnesota, United States) for the hepatic artery and portal vein respectively, water bath circulator (Lab companion CW-05G, Jeio Tech, Korea) heat exchanger (Trillium Myotherm XP, Medtronic, Minnesota, United States) and Dideco D903 Avant Oxygenator (Dideco Inc. Italy). The vena cava flowed freely with

venous outflow draining back to the reservoir. The bile duct was cannulated and bile production continuously monitored.

Cellular Energy Levels

Biopsies were taken prior to the onset of WIT, following WIT, and at 1, 2, 4, 8 and 12 hours of NMP. Tissue samples were immediately frozen using Wollenberger clamps and liquid nitrogen then stored at -80°C. The frozen tissue was homogenized using 6% perchloric acid. Fifty microliters of homogenate was extracted and transferred to 0.15 M sodium hydroxide for measurement of protein content using the Lowry method as previously described.(177) The remaining homogenate supernatant was neutralized and ATP levels determined enzymatically.(178) Values were calculated in millimoles of ATP per gram of protein.

Measurement of Hepatocellular injury and Inflammation

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) were measured in the perfusate every 2 hours during NMP in our clinical laboratory using an enzymatic rate method on a Beckman Coulter DxC 800 analyzer (Beckman Coulter Canada, Ontario Canada). Inflammatory markers included tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6) levels in the perfusate, which were determined using porcine TNF-alpha and IL-6 ELISA kits (R&D Systems, Minnesota, United States). To further assess parenchymal injury a biopsy was taken at the end of dissection (pre-ischemia), and again at the end of 12 hours NMP. Samples were preserved in 10% formalin for hematoxylin and eosin

(H&E) staining. H&E specimens were assessed by a blinded pathologist using light microscopy to assign injury score based on necrosis, hemorrhage, cholestasis and sinusoidal dilatation (**Table 4-2**).(75)

Liver Function

Bile production was monitored continuously throughout perfusion. Tissue lactate was determined enzymatically.(178)

Hemodynamics and Endothelial Injury

Pressure, flow and vascular resistance were continuously monitored for both the hepatic artery and portal vein.

Hyaluronic acid, a marker of sinusoidal endothelial cell injury(179), was measured using an ELISA kit (R&D Systems, Minnesota, United States). A random subset of biopsies taken after 12 hours of NMP (3 per group) was examined using electron microscopy for endothelial injury. Biopsies were fixed with a 2.5% glutaraldehyde/2% paraformaldehyde solution and then 1% osmium tetroxide. Samples were subsequently dehydrated and embedded in Spurr's Resin. Two blocks from each sample (n=6 per group) were sectioned and stained with uranyl acetate and lead citrate and photographed with a Philips Morgagni 268 transmission electron microscope (North American NanoPort, Hillsboro, Oregon, United States). A pathologist blinded to the experimental group assignment assessed maximum sinusoidal diameter, endothelial cell thickness at the nucleus and endothelial cell thickness at the thinnest

point to determine endothelial cell edema. All measurements were taken perpendicular to the basement membrane.

Statistical Analysis

Graphs display data as mean values with standard error. ANOVA with Tukey Test for multiple comparisons was used to compare temperature groups for each solution. To compare all 6 groups, repeated measures ANOVA was used to include assessment of changes over time. Ordinal scale data was analyzed with the Kruskal-Wallis test. Statistical significance was defined as a p value of <0.05. Analysis was performed using SPSS (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.).

Results

Cellular Energy Levels

ATP levels as a fraction of pre-ischemia values are shown for the duration of the perfusion in **Figure 4-1**. Perturbations in ATP level were observed in all groups and found to be significant over time ($P<0.001$). There was a significant decrease following 60 minutes of WIT with partial recovery after 1 hour of perfusion followed by more gradual recovery to pre-ischemic levels, or higher by 8 hours of NMP. AD groups followed a similar pattern. There was no significant difference at any time point between temperature groups for any of the study solutions. When all groups were compared there was also no significant difference between groups ($p=0.88$).

Hepatocellular Injury and Inflammation

Hepatocellular enzyme levels in the perfusate are shown in **Figure 4-2**. In the HTK groups, the mean peak AST was lowest in the 4°C group although there was no statistically significant difference between groups (p=0.51). Peak ALT and LDH levels showed a similar pattern with no significant difference between groups for either peak ALT (p=0.76) or peak LDH (p=0.14). Though LDH was significantly lower at 4 (p=0.04), 8 (p=0.03) and 10 hours (p=0.03) in the 4°C group relative to the 25°C, it did not reach significance relative to 35°C. For AD, the 4°C group showed the highest perfusate enzyme levels but no statistically significant difference between groups for peak AST (p=0.53), ALT (p=0.50) or LDH (p=0.74). When all groups were compared there was no statistically significant difference in peak AST (p=0.63), ALT (p=0.80) or LDH (p=0.31).

Histologic injury scores after 12 hours of NMP were lowest in the 4°C initial flush groups for both solutions. The HTK4°C and AD4°C groups scored 4.75 and 5 respectively on the 10 point injury scale (**Table 4-2**), where as the highest scores were seen in the HTK35°C and AD35°C groups at 6 and 6.25 respectively. This difference did not reach statistical significance (p=0.78).

TNF-α levels were similar across temperatures in the HTK groups. For AD, the 4°C group had significantly lower levels of TNF-α in the first 2 hours of perfusion compared to

both 25°C (p=0.004) and 35°C (p=0.002). When all groups were compared there was a significant difference between groups (p<0.001) with warm AD groups showing the highest levels, while IL-6 also showed elevated but non-significant levels in the warm AD groups (p=0.08).

Liver Function

Tissue lactate levels changed significantly over time in all groups (p<0.001) with a significant rise following 60 minutes WIT (**Figure 4-3**). Lactate was cleared rapidly in the first 2 hours of perfusion for all groups and remained low for the duration of perfusion in all of the HTK and AD groups with no differences between temperatures for either solution. There was also no statistically significant difference when all groups were compared (p=0.95).

The cold initial flush groups HTK4°C and AD4°C had the highest mean bile production of 117 grams (95% CI 0, 234) and 171 grams (95% CI 63, 279) over the 12 hours of NMP respectively, however there was no statistically significant difference between groups with respect to volume of bile produced (p=0.67) (data not shown).

Hemodynamics and Endothelial Injury

Hepatic artery pressure was maintained at 60 mmHg and the arterial flow showed a similar pattern across all 6 groups with an initial increase over the first 2 hours of perfusion. Flows then decreased to reach steady flows by hour 6 for the remainder of the perfusion (**Figure 4-4**). The 4°C group showed initially higher flows for HTK but similar steady state flows to

the warm groups were reached by 6 hours. Statistically there was no significant difference noted between temperature groups at any time point. The AD4°C group had lower flows throughout the perfusion relative to the AD25°C and AD35°C groups though this difference did not reach statistical significance at any time point. When all 6 groups were compared there was no statistically significant difference between groups (P=0.60).

Portal vein flow was kept constant. Portal vein resistance decreased significantly over the first 2 hours of perfusion ($p<0.001$) and remained stable for the remainder of the 12-hour perfusion (**Figure 4-4**). For HTK, portal vein resistance was lowest in the 4°C group, reaching statistical significance at 2 hours of perfusion relative to 25°C ($p=0.03$) but not statistically significant relative to 35°C ($p=0.21$). For AD, the 25°C group had the lowest portal vein resistance throughout the perfusion though this did not reach statistical significance at any time point. When all groups were compared there was no statistical difference between groups ($p=0.26$).

Electron microscopy demonstrated a significant difference in sinusoidal dilatation between the HTK groups (**Figure 4-5**) with the largest diameter seen in the 35°C group (7.60µm 95% CI 5.70, 9.5, $p=0.04$). There was no difference in endothelial cell thickness ($p=0.40$) (**Table 4-3)**. In the AD groups there was no significant difference in sinusoid diameter ($p=0.28$) though endothelial cell thickness trended higher in the 4°C group ($p=0.09$).

There was no significant difference in hyaluronic acid levels in the perfusate between groups at any time point (data not shown).

Discussion

DCD liver grafts represent a large potential resource for addressing the organ shortage currently facing liver transplantation. The results of recent clinical trials have expedited delivery of NMP technology in clinical liver transplantation. However optimal NMP protocols, especially for DCD grafts remain an ongoing target for research. Warm ischemia followed by immediate cooling endured by DCD grafts has been shown to be detrimental and leads to worse transplant outcomes.(103) Avoidance of initial cooling before establishment of NMP has not been investigated previously in DCD liver grafts to our knowledge, but has been shown to be beneficial in experimental cardiac preservation.(135) Our study in a large animal model suggests that altering the HTK flush temperature alone is insufficient to improve DCD liver graft quality. However warm initial flush with alternative solution compositions may indeed warrant further investigation.

After withdrawal of life support, DCD livers are exposed to a period of WIT where cells quickly shift to anaerobic metabolism. However at normothermic temperatures ATP consumption rapidly outpaces ATP production leading to depletion of cellular ATP stores.(65) This was confirmed in our DCD model with low ATP levels following 60 minutes of WIT, and is consistent with previous studies.(70) Maintenance of normal ion gradients across the cell membrane is vital for cellular function and is dependent upon the membrane sodium-potassium ATPase which is impaired by these low ATP levels.(14) Altered ionic flows ultimately lead to calcium influx, cellular edema and overall damage of cells priming them for further injury upon reperfusion.(14, 17) ATP levels in the peri-transplant period have been correlated with clinical transplant outcomes, with early allograft dysfunction.(74) Rapidly cooling the liver during initial

flush reduces metabolic demands of the organ for subsequent SCS in accordance with the Q10 effect where each 10°C reduction in temperature is accompanied by a 1.5-2.5 fold drop in metabolic activity.(9) During SCS the reduced metabolic rate slows down ATP depletion but levels continue to decline during SCS resulting in severely depleted levels at the time of reperfusion.(180)

NMP has been shown in animal models to replenish cellular ATP levels in DCD liver grafts following WIT to levels nearly 80% of pre-ischemic values by 4 hours of perfusion.(70) Initial flush represents an important transition phase in the timeline of DCD liver grafts as they move from warm ischemia, to re-oxygenation on the NMP circuit. Reddy *et al.* found that even a brief one-hour period of SCS before NMP negated some of the positive effects of this modality.(84) SCS is eliminated in the setting where organs can be placed directly on the NMP circuit at the donor site as observed in clinical case reports. (78, 128-130) In this setting the period of initial flush is very short and the resulting negative effects of hypothermia may outweigh the benefit of a reduced metabolic rate for such a short period.(135) We hypothesized that rapid cooling during initial flush would impair the ability of the organ to recover cellular energy levels relative to liver grafts that were not exposed to hypothermic conditions. However, all of the groups in our study demonstrated significant recovery of ATP levels within the first hour of NMP, with ongoing recovery to pre-ischemic values by the end of the NMP period with no clear difference between temperatures for either HTK or AD. Additionally, comparing HTK and AD groups did not demonstrate a difference in ATP levels despite the presence of adenosine in AD, which has previously shown benefit for ATP levels in liver ischemia reperfusion injury.(181) The ATP results from this study confirm nearly total cellular ATP depletion following 60 minutes of WIT but also further validate previous studies (70) demonstrating NMP

is capable of restoring ATP levels prior to transplantation. ATP levels were not significantly different between groups suggesting warm initial flush strategies with either HTK or AD provide no additional benefit over the current practice of cold HTK with respect to recovering cellular ATP levels for DCD grafts being preserved with NMP.

During WIT the depletion in ATP levels and the resultant disruption of membrane ionic gradients lead to hepatocyte damage that can be quantified by the measurement of transaminase release in the perfusate with transaminase levels during perfusion correlating with post transplant levels.(77) Kupffer cells in the liver clear transaminases from the circulation after they are released from hepatocytes.(182) A continuous rise in transaminase levels during NMP has been shown to predict poor graft viability.(183) Therefore the enzyme pattern during NMP gives an indication of the degree of hepatocellular damage, but also the recovery of the liver graft. Altering the initial flush strategy in our study did not significantly change the peak transaminase levels or the overall perfusate transaminase pattern over time with all groups demonstrating steady or declining transaminase levels by approximately 4 hours of perfusion. Enzyme levels in the HTK experiments appeared to be lower in the 4°C group, particularly LDH, while the 4°C initial flush seemed to have a pattern of higher enzyme levels in the AD groups. Reactive oxygen species (ROS) play a significant role in ischemic reperfusion injury. The AD solution contains reduced glutathione, absent from HTK; it plays a role in clearing ROS and has shown to be protective from ischemia reperfusion injury.(181) At cold temperatures during the transition to NMP where the tissue is first re-exposed to oxygen, glutathione peroxidase, which uses glutathione as a substrate to clear ROS, will function at a reduced capacity and thus, the benefit of this additive may be limited. That being said, we were underpowered to detect a significant differences when all groups were compared, and

comparison of HTK4°C, AD25°C and AD35°C with larger numbers may be warranted to further assess the degree of hepatocellular injury with these novel strategies relative to the current clinical standard.

Of note, the warm AD groups did demonstrate higher TNF-α levels, which is suggestive of a higher degree of kupffer cell activation.(184) This would potentially place these grafts at risk for more significant ischemia reperfusion injury following reperfusion, however in the absence of a transplant phase this cannot be confirmed by this study.

Recovery of hepatocellular function was not different between the initial flush strategies tested. Lactate levels have consistently been used as a marker of liver function during *ex-situ* perfusion and have been included as a key component of proposed clinical viability criteria for liver grafts preserved with NMP.(185) During WIT reliance on anaerobic metabolism causes lactate levels to increase significantly as seen in our DCD model. Once NMP is initiated, rapid lactate clearance to within the normal range has been suggested to correlate with recovery of liver function and predict favorable transplant outcomes even in marginal grafts.(185) Our DCD model led to significant lactate accumulation following WIT followed by rapid clearance to low levels after only 1-2 hours of NMP. This pattern was consistently observed regardless of the initial flush strategy used, suggesting that altering the temperature of the brief initial flush does not impact recovery of hepatic function during NMP.

Bile production during NMP was initially thought to be an important predictor of graft viability however more recent data has emphasized the variability of *ex-situ* bile production.(183) Although still included in proposed viability criteria(185) low or absent bile production does not seem to reliably predict graft outcomes.(78, 185) The variability within

groups in our study was significant making it difficult to draw any meaningful conclusions with respect to the effect of initial flush strategy on bile production.

Proposed graft viability criteria also include flows greater than 150 ml/min for the hepatic artery, and 500ml/min for the portal vein during NMP with these values correlating to improved graft outcomes.(185) Portal vein flow was controlled in our study and thus the portal vein resistance, also suggested to be an important marker of subsequent post transplant viability(75), was monitored. Portal vein resistance was lowest in the control group, suggesting no added benefit of warm flush on portal resistance. Regarding arterial flow, all groups maintained hepatic artery flows higher than the abovementioned threshold. When looking at the AD groups, although not statistically significant there was a pattern of higher flows in the warm AD groups where as the same could not be said for HTK. Additional studies with a larger sample size may further characterize these patterns and would be important given hepatic artery flow has been hypothesized to be an important factor in ischemic cholangiopathy pathophysiology.(151) Ischemic cholangiopathy is the most unique and troubling complication of DCD grafts. It often presents in a delayed fashion up to six months after transplant and thus assessing whether changes in flow could contribute to reducing ischemic cholangiopathy would require a far more complex longitudinal transplant model with a more focused assessment of biliary injury.

Our study has several limitations. The primary operator in our experiments was not blinded to the conditions of each experiment due to logistical reasons regarding the set up of each experiment. Additionally, the conclusions drawn from this study are limited to the preservation phase, as we did not include a simulated transplant, or orthotopic transplantation phase. This limits our ability to determine whether or not altering the initial flush strategy would make a difference following transplantation. Furthermore, our experimental perfusion circuit was

created in our lab as described above. This perfusion setup differs in some ways from other experimental circuits and clinical set ups and thus may further limit the generalizability of these results. Additionally, DCD liver transplantation has commonly been limited by ischemic biliary cholangiopathy. As mentioned above, this complication tends to occur months after transplantation and is thus difficult to study in a large animal model. In a recent study where immediate cooling following WIT in DCD liver grafts was avoided by the use of normothermic regional perfusion Watson *et al*. were able to show a significant reduction in ischemic cholangiopathy.(186) Thus the impact of avoiding immediate hypothermia during initial flush on the bile ducts of DCD liver grafts preserved with NMP may warrant further investigation. There were also some potential differences in hepatocellular injury markers and hemodynamic parameters that we were underpowered to show in our large animal model.

Conclusion

This study suggests that initial flush at warmer temperatures provides no added benefit relative to the current standard of care of initial cold flush with HTK or alternate standard preservation solutions. Although temperature alone may not significantly change graft quality, solutions such as AD at warmer temperatures, may warrant subsequent investigation in larger powered models in efforts to further optimize NMP for DCD liver grafts.

Acknowledgements

The authors would like to thank Global Transplant Solutions (Ontario, Canada) for providing the HTK solutions for these experiments.

Table 4-1: Composition of initial flush solutions

	HTK (mmol/L)	**AD** (mmol/L)
Sodium chloride	15	123
Potassium chloride	9	5.9
Magnesium chloride	4	13
Histidine hydrochloride	18	
Histidine	180	
Tryptophan	2	
Mannitol	30	120
Calcium chloride	0.015	0.22
Potassium hydrogen 2-oxopentadioate	1	
Sodium bicarbonate		20
Sodium phosphate		1.2
Insulin		10 unit/L
Pyruvate		1
Reduced glutathione		3
Adenosine		0.4
Lidocaine		0.05
Glucose		10

Table 4-2: Hepatocellular histologic injury scoring system(75)

Score	Hemorrhage	Necrosis	Cholestasis	Sinusoidal Dilatation
0	Absent	Absent	Absent	None
1	Focal	Peri-central	Present	Mild
2	Zonal	Zone 2+3	-	Moderate
3	Pan-lobular	Pan-lobular	-	Severe

Table 4-3: Electron microscopy assessment of sinusoidal endothelial cells

Maximum sinusoidal diameter, endothelial cell thickness at the nucleus and endothelial cell thickness at the thinnest point were measured to assess endothelial cell edema. All measurements were taken perpendicular to the basement membrane.

	thickest/nucleus measurement (µm)	**thinnest point measurement (µm)**	**sinusoidal max diameter (µm)**
HTK4°C	2.92	0.14	5.36
HTK25°C	3.30	0.14	6.23
HTK35°C	3.22	0.15	7.60
P Value	0.41	0.90	0.04*

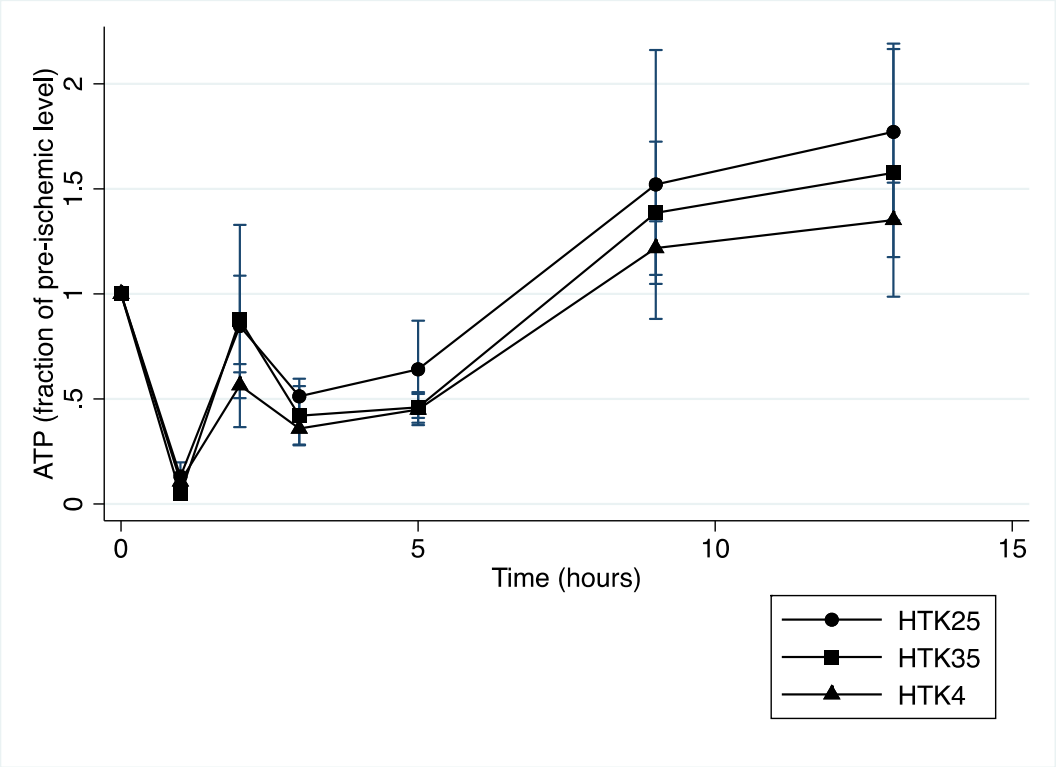
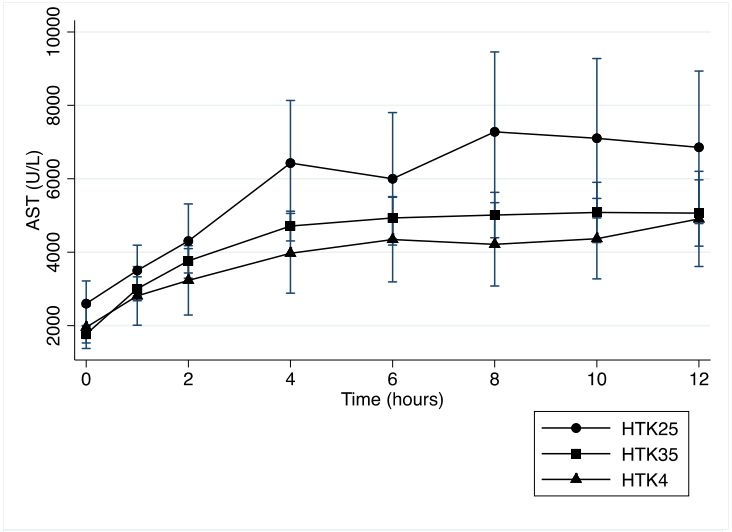
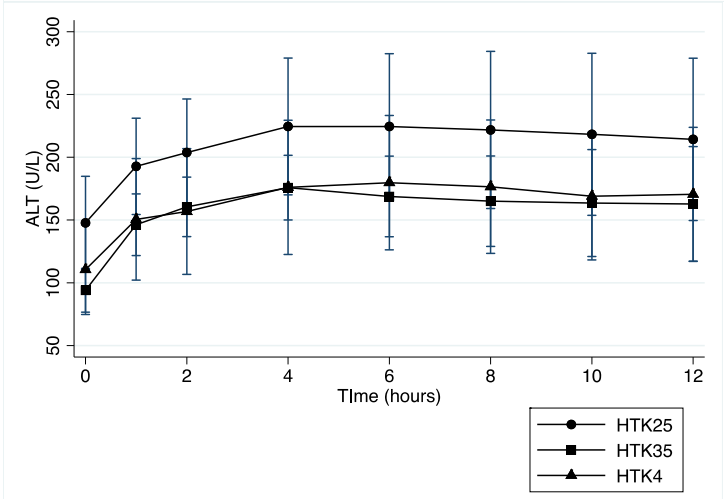


Figure 4-1: Tissue ATP during NMP

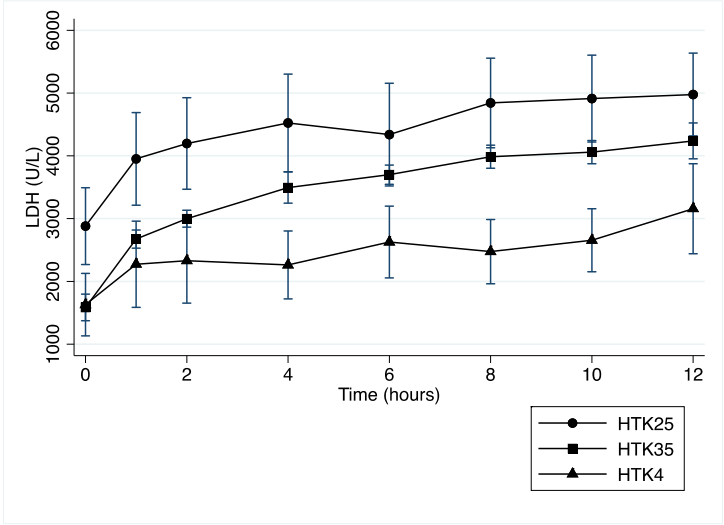
ATP levels during NMP presented as a fraction of the pre-ischemia levels. The first 2 time points represent pre-ischemia and post 60 minutes WIT respectively.



A)



B)



C)

Figure 4-2: Hepatocellular injury markers during NMP

A) AST, B) ALT and C) LDH levels during NMP.

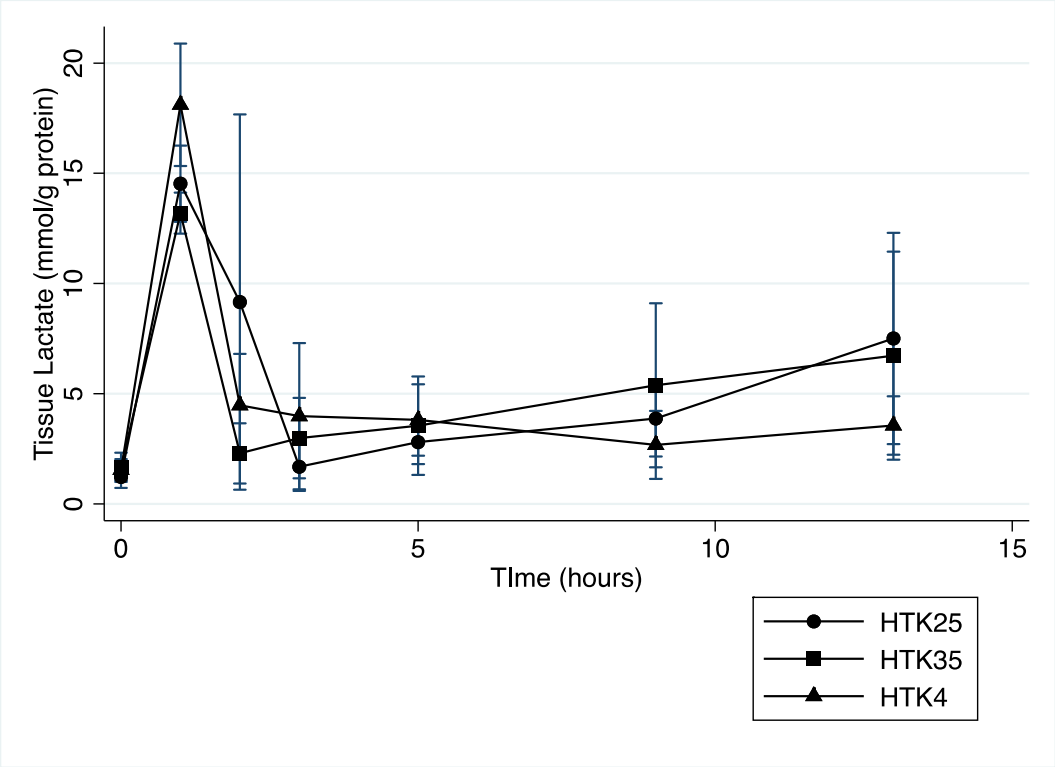
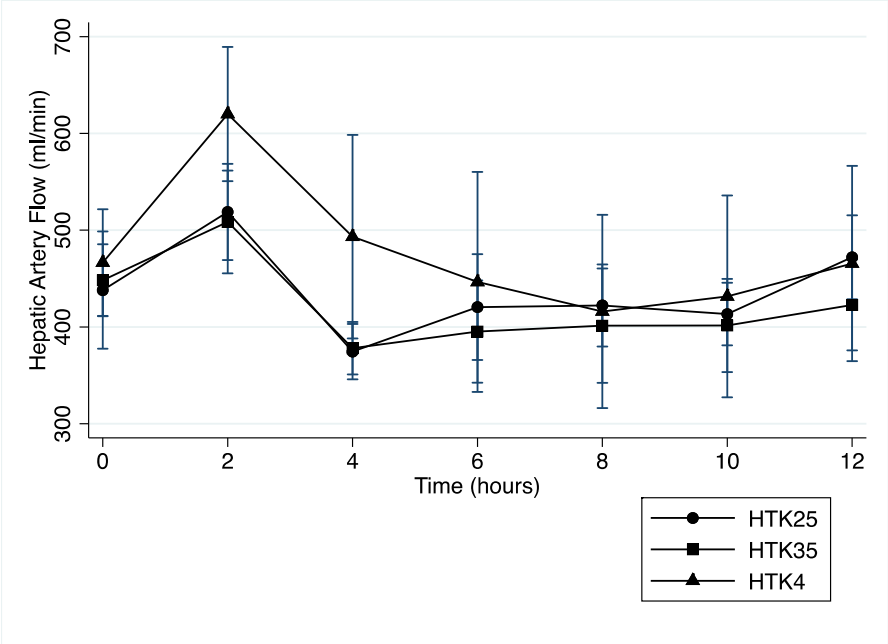


Figure 4-3: Tissue lactate during NMP

Lactate levels measured enzymatically from tissue biopsies during NMP preservation.

A)



B)

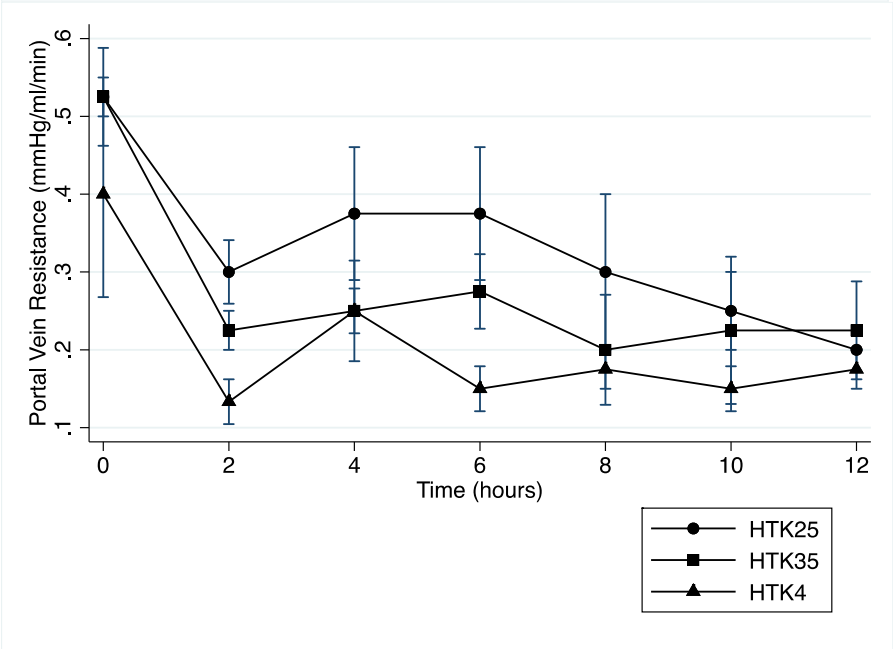
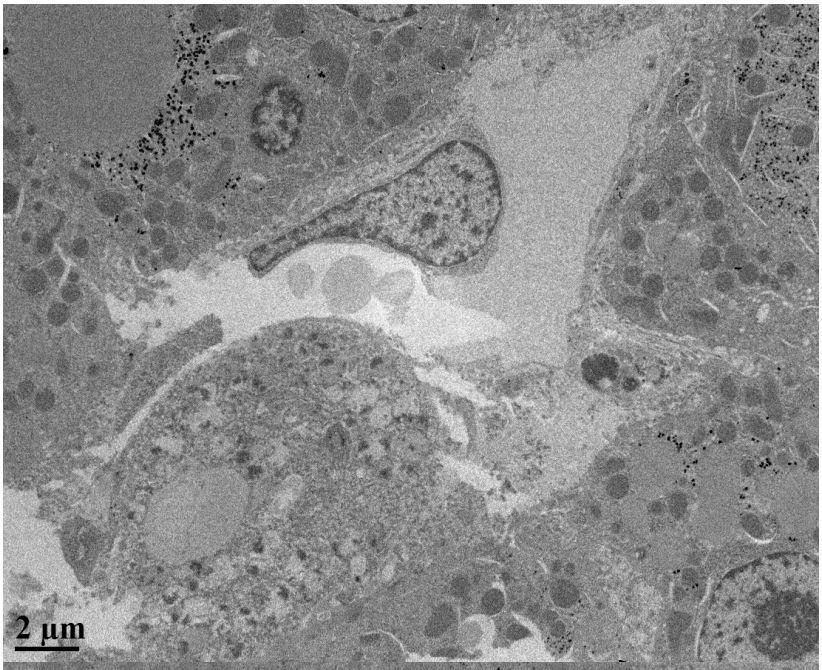


Figure 4-4: Hemodynamic parameters during NMP

A) Hepatic artery flow during NMP B) Portal vein resistance (PVR) during NMP

A)



B)

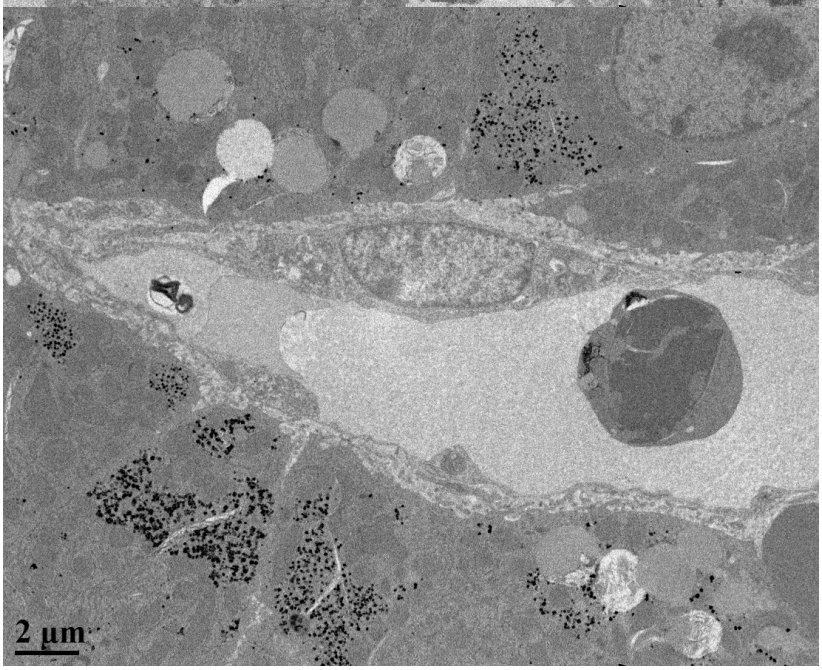


Figure 4-5: Electron micrographs of endothelial cells

Smaller sinusoidal luminal diameter was seen in HTK4°C groups (A), compared with HTK35°C groups (B).

References

8. Kim WR, Lake JR, Smith JM, Schladt DP, Skeans MA, Harper AM, et al. OPTN/SRTR 2016 Annual Data Report: Liver. Am J Transplant. 2018;18 Suppl 1:172-253.
9. Clavien PA, Harvey PR, Strasberg SM. Preservation and reperfusion injuries in liver allografts. An overview and synthesis of current studies. Transplantation. 1992;53(5):957-78.
14. Carini R, Autelli R, Bellomo G, Albano E. Alterations of cell volume regulation in the development of hepatocyte necrosis. Exp Cell Res. 1999;248(1):280-93.
17. van Golen RF, van Gulik TM, Heger M. Mechanistic overview of reactive species-induced degradation of the endothelial glycocalyx during hepatic ischemia/reperfusion injury. Free Radic Biol Med. 2012;52(8):1382-402.
63. Coffey JC, Wanis KN, Monbaliu D, Gilbo N, Selzner M, Vachharajani N, et al. The influence of functional warm ischemia time on DCD liver transplant recipients' outcomes. Clin Transplant. 2017;31(10).
65. Perera MT, Richards DA, Silva MA, Ahmed N, Neil DA, Murphy N, et al. Comparison of energy metabolism in liver grafts from donors after circulatory death and donors after brain death during cold storage and reperfusion. Br J Surg. 2014;101(7):775-83.
70. Xu H, Berendsen T, Kim K, Soto-GutiÃ©rrez A, Bertheium F, Yarmush ML, et al. Excorporeal normothermic machine perfusion resuscitates pig DCD livers with extended warm ischemia. Journal of Surgical Research. 2012;173(2):e83-e8.
74. Bruinsma BG, Avruch JH, Sridharan GV, Weeder PD, Jacobs ML, Crisalli K, et al. Peritransplant Energy Changes and Their Correlation to Outcome After Human Liver Transplantation. Transplantation. 2017;101(7):1637-44.
75. Brockmann J, Reddy S, Coussios C, Pigott D, Guirriero D, Hughes D, et al. Normothermic perfusion: a new paradigm for organ preservation. Ann Surg. 2009;250(1):1-6.
77. Watson CJE, Kosmoliaptsis V, Randle LV, Gimson AE, Brais R, Klinck JR, et al. Normothermic Perfusion in the Assessment and Preservation of Declined Livers Before Transplantation: Hyperoxia and Vasoplegia-Important Lessons From the First 12 Cases. Transplantation. 2017;101(5):1084-98.
78. Nasralla D, Coussios CC, Mergental H, Akhtar MZ, Butler AJ, Ceresa CDL, et al. A randomized trial of normothermic preservation in liver transplantation. Nature. 2018.
81. Liu Q, Nassar A, Farias K, Buccini L, Baldwin W, Mangino M, et al. Sanguineous normothermic machine perfusion improves hemodynamics and biliary epithelial regeneration in donation after cardiac death porcine livers. Liver Transpl. 2014;20(8):987-99.
83. Liu Q, Nassar A, Farias K, Buccini L, Mangino MJ, Baldwin W, et al. Comparing Normothermic Machine Perfusion Preservation With Different Perfusates on Porcine Livers From Donors After Circulatory Death. Am J Transplant. 2016;16(3):794-807.
84. Reddy S, Greenwood J, Maniakin N, Bhattacharjya S, Zilvetti M, Brockmann J, et al. Non-heart-beating donor porcine livers: the adverse effect of cooling. Liver Transpl. 2005;11(1):35-8.
103. Foley DP, Fernandez LA, Leverson G, Chin LT, Krieger N, Cooper JT, et al. Donation after cardiac death: the University of Wisconsin experience with liver transplantation. Ann Surg. 2005;242(5):724-31.
111. Boehnert MU, Yeung JC, Bazerbachi F, Knaak JM, Selzner N, McGilvray ID, et al. Normothermic acellular ex vivo liver perfusion reduces liver and bile duct injury of pig livers retrieved after cardiac death. Am J Transplant. 2013;13(6):1441-9.

121. Schon MR, Kollmar O, Wolf S, Schrem H, Matthes M, Akkoc N, et al. Liver transplantation after organ preservation with normothermic extracorporeal perfusion. Ann Surg. 2001;233(1):114-23.
122. Nassar A, Liu Q, Farias K, Buccini L, Baldwin W, Bennett A, et al. Impact of Temperature on Porcine Liver Machine Perfusion From Donors After Cardiac Death. Artif Organs. 2016;40(10):999-1008.
128. Ravikumar R, Jassem W, Mergental H, Heaton N, Mirza D, Perera MT, et al. Liver Transplantation After Ex Vivo Normothermic Machine Preservation: A Phase 1 (First-in-Man) Clinical Trial. Am J Transplant. 2016;16(6):1779-87.
129. Selzner M, Goldaracena N, Echeverri J, Kaths JM, Linares I, Selzner N, et al. Normothermic ex vivo liver perfusion using steen solution as perfusate for human liver transplantation: First North American results. Liver Transpl. 2016;22(11):1501-8.
130. Bral M, Gala-Lopez B, Bigam D, Kneteman N, Malcolm A, Livingstone S, et al. Preliminary Single-Center Canadian Experience of Human Normothermic Ex Vivo Liver Perfusion: Results of a Clinical Trial. American Journal of Transplantation. 2017;17(4):1071-80.
135. White CW, Ambrose E, Muller A, Li Y, Le H, Thliveris J, et al. Avoidance of Profound Hypothermia During Initial Reperfusion Improves the Functional Recovery of Hearts Donated After Circulatory Death. Am J Transplant. 2016;16(3):773-82.
151. Hashimoto K, Eghtesad B, Gunasekaran G, Fujiki M, Uso TD, Quintini C, et al. Use of tissue plasminogen activator in liver transplantation from donation after cardiac death donors. Am J Transplant. 2010;10(12):2665-72.
163. Busuttil RW, Tanaka K. The utility of marginal donors in liver transplantation. Liver Transpl. 2003;9(7):651-63.
177. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193(1):265-75.
178. Passonneau JV, Lowry, Oliver H. . Enzymatid Analysis A Practical Guide. Totowa, New Jersey: The Humana Press Inc. ; 1993.
179. Reinders ME, van Wagensveld BA, van Gulik TM, Frederiks WM, Chamuleau RA, Endert E, et al. Hyaluronic acid uptake in the assessment of sinusoidal endothelial cell damage after cold storage and normothermic reperfusion of rat livers. Transpl Int. 1996;9(5):446-53.
180. Berendsen TA, Izamis ML, Xu H, Liu Q, Hertl M, Berthiaume F, et al. Hepatocyte viability and adenosine triphosphate content decrease linearly over time during conventional cold storage of rat liver grafts. Transplant Proc. 2011;43(5):1484-8.
181. Janssen H, Janssen PH, Broelsch CE. Celsior solution compared with University of Wisconsin solution (UW) and histidine-tryptophan-ketoglutarate solution (HTK) in the protection of human hepatocytes against ischemia-reperfusion injury. Transpl Int. 2003;16(7):515-22.
182. Radi ZA, Koza-Taylor PH, Bell RR, Obert LA, Runnels HA, Beebe JS, et al. Increased serum enzyme levels associated with kupffer cell reduction with no signs of hepatic or skeletal muscle injury. Am J Pathol. 2011;179(1):240-7.
183. Watson CJE, Jochmans I. From "Gut Feeling" to Objectivity: Machine Preservation of the Liver as a Tool to Assess Organ Viability. Curr Transplant Rep. 2018;5(1):72-81.
184. Lu TF, Yang TH, Zhong CP, Shen C, Lin WW, Gu GX, et al. Dual Effect of Hepatic Macrophages on Liver Ischemia and Reperfusion Injury during Liver Transplantation. Immune Netw. 2018;18(3):e24.

185. Mergental H, Stephenson BTF, Laing RW, Kirkham AJ, Neil DAH, Wallace LL, et al. Development of Clinical Criteria for Functional Assessment to Predict Primary Nonfunction of High-Risk Livers Using Normothermic Machine Perfusion. Liver Transpl. 2018;24(10):1453-69.
186. Watson CJE, Hunt F, Messer S, Currie I, Large S, Sutherland A, et al. In situ normothermic perfusion of livers in controlled circulatory death donation may prevent ischemic cholangiopathy and improve graft survival. Am J Transplant. 2018.

Chapter 5

Initial Organ Flush Temperature in Liver Preservation: Summary & Future Directions

Resulting from a shortage of quality liver grafts, waitlist mortality remains a significant issue in liver transplantation. In response, increasing use of extended criteria donors, particularly donation after circulatory death (DCD) livers, has followed. Higher complication rates and the risk of ischemic cholangiopathy has driven renewed interest in *ex-situ* machine perfusion as an alternative to static cold storage for preserving these injured grafts. Encouraging results in animal and clinical trials have quickly moved normothermic machine perfusion (NMP) into clinical use, however the optimal perfusion protocol to increase DCD graft utilization and reduce complications remains an area of ongoing research. Currently the clinical standard is an initial organ flush with a cold preservation solution immediately following procurement. In NMP, this process of rapidly cooling the organ only to warm it up again at the beginning of perfusion has been questioned in the setting of DCD heart perfusions. We explored the possibility that avoiding hypothermia at the time of initial organ flush for DCD liver grafts being preserved with NMP would lead to improved liver graft quality.

Using a large animal model we simulated DCD with 60 minutes of warm ischemia. As detailed in Chapter 4, altering the temperature of initial HTK flush did not show any difference in ATP recovery or lactate clearance. The 4°C group demonstrated lower portal vein resistance and LDH levels. Taken together these results suggest that altering the temperature alone does not provide added benefit over the current clinical standard of cold HTK initial organ flush. For additional comparison another solution was tested. When an adenosine-lidocaine crystalloid solution (AD) was used there was again no difference in ATP recovery or lactate clearance however, in contrast to HTK there was a trend toward improved hemodynamics and lower hepatocellular injury markers in the warm AD flush groups. No statistically significant difference was noted between the different solutions though we were underpowered in this

preliminary large animal study. Further investigation of solutions such as AD, which has shown promise at warmer temperatures in heart perfusion, may be warranted. A comparison looking only at the cold HTK and warm AD groups with larger numbers may provide further insight into potential hemodynamic and hepatocellular injury differences between this novel solution and the current clinical standard. Although animal models are limited in their ability to study ischemic cholangiopathy due to the often-delayed presentation by weeks to months after transplant, this complication remains a unique and troubling one for DCD liver transplants. Therefore in addition to assessing overall injury and graft function, which remain critical for successful DCD transplant outcomes, future studies looking at the initial flush strategy could also include specific investigation of the effects on the initial health of the bile ducts. Any additional benefits in biliary preservation will be important in hopes of moving to an optimal machine perfusion strategy to help improve successful DCD graft utilization and limit complication rates.

References

1. Carrel A, Lindbergh CA. The Culture of Whole Organs. Science. 1935;81(2112):621-3.
2. Lapchinsky AG. Recent results of experimental transplantation of preserved limbs and kidneys and possible use of this technique in clinical practice. Ann N Y Acad Sci. 1960;87:539-71.
3. Belzer FO, Ashby BS, Dunphy JE. 24-hour and 72-hour preservation of canine kidneys. Lancet. 1967;2(7515):536-8.
4. Ackermann JR, Barnard CN. Successful storage of kidneys. Br J Surg. 1966;53(6):525-32.
5. Humphries AL, Jr., Russell R, Stoddard LD, Moretz WH. Successful five-day kidney preservation. Perfusion with hypothermic, diluted plasma. Invest Urol. 1968;5(6):609-18.
6. Collins GM, Bravo-Shugarman M, Novom S, Terasaki PI. Kidney preservation for transplantation. I. Twelve-hour storage in rabbits. Transplant Proc. 1969;1(3):801-7.
7. Wahlberg JA, Love R, Landegaard L, Southard JH, Belzer FO. 72-hour preservation of the canine pancreas. Transplantation. 1987;43(1):5-8.
8. Kim WR, Lake JR, Smith JM, Schladt DP, Skeans MA, Harper AM, et al. OPTN/SRTR 2016 Annual Data Report: Liver. Am J Transplant. 2018;18 Suppl 1:172-253.
9. Clavien PA, Harvey PR, Strasberg SM. Preservation and reperfusion injuries in liver allografts. An overview and synthesis of current studies. Transplantation. 1992;53(5):957-78.
10. Vekemans K, Liu Q, Brassil J, Komuta M, Pirenne J, Monbaliu D. Influence of flow and addition of oxygen during porcine liver hypothermic machine perfusion. Transplant Proc. 2007;39(8):2647-51.
11. Harvey PR, Iu S, McKeown CM, Petrunka CN, Ilson RG, Strasberg SM. Adenine nucleotide tissue concentrations and liver allograft viability after cold preservation and warm ischemia. Transplantation. 1988;45(6):1016-20.
12. Nishida T, Koseki M, Kamiike W, Nakahara M, Nakao K, Kawashima Y, et al. Levels of purine compounds in a perfusate as a biochemical marker of ischemic injury of cold-preserved liver. Transplantation. 1987;44(1):16-21.
13. Leaf A. Maintenance of Concentration Gradients and Regulation of Cell Volume. Ann Ny Acad Sci. 1959;72(12):396-404.
14. Carini R, Autelli R, Bellomo G, Albano E. Alterations of cell volume regulation in the development of hepatocyte necrosis. Exp Cell Res. 1999;248(1):280-93.
15. Snowdowne KW, Freudenrich CC, Borle AB. The effects of anoxia on cytosolic free calcium, calcium fluxes, and cellular ATP levels in cultured kidney cells. J Biol Chem. 1985;260(21):11619-26.
16. Jaeschke H, Farhood A, Smith CW. Neutrophils contribute to ischemia/reperfusion injury in rat liver in vivo. FASEB J. 1990;4(15):3355-9.
17. van Golen RF, van Gulik TM, Heger M. Mechanistic overview of reactive species-induced degradation of the endothelial glycocalyx during hepatic ischemia/reperfusion injury. Free Radic Biol Med. 2012;52(8):1382-402.
18. Jaeschke H. Molecular mechanisms of hepatic ischemia-reperfusion injury and preconditioning. Am J Physiol Gastrointest Liver Physiol. 2003;284(1):G15-26.
19. Jaeschke H, Farhood A. Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver. Am J Physiol. 1991;260(3 Pt 1):G355-62.

20. Jaeschke H, Bautista AP, Spolarics Z, Spitzer JJ. Superoxide generation by neutrophils and Kupffer cells during in vivo reperfusion after hepatic ischemia in rats. J Leukoc Biol. 1992;52(4):377-82.
21. Fernandez L, Heredia N, Grande L, Gomez G, Rimola A, Marco A, et al. Preconditioning protects liver and lung damage in rat liver transplantation: role of xanthine/xanthine oxidase. Hepatology. 2002;36(3):562-72.
22. Li JM, Shah AM. Differential NADPH- versus NADH-dependent superoxide production by phagocyte-type endothelial cell NADPH oxidase. Cardiovasc Res. 2001;52(3):477-86.
23. Nieminen AL, Byrne AM, Herman B, Lemasters JJ. Mitochondrial permeability transition in hepatocytes induced by t-BuOOH: NAD(P)H and reactive oxygen species. Am J Physiol. 1997;272(4 Pt 1):C1286-94.
24. Nieminen AL, Saylor AK, Tesfai SA, Herman B, Lemasters JJ. Contribution of the mitochondrial permeability transition to lethal injury after exposure of hepatocytes to t-butylhydroperoxide. Biochem J. 1995;307 (Pt 1):99-106.
25. Lu L, Zhou H, Ni M, Wang X, Busuttil R, Kupiec-Weglinski J, et al. Innate Immune Regulations and Liver Ischemia-Reperfusion Injury. Transplantation. 2016;100(12):2601-10.
26. McDonald KA, Huang H, Tohme S, Loughran P, Ferrero K, Billiar T, et al. Toll-like receptor 4 (TLR4) antagonist eritoran tetrasodium attenuates liver ischemia and reperfusion injury through inhibition of high-mobility group box protein B1 (HMGB1) signaling. Mol Med. 2015;20:639-48.
27. Tsung A, Sahai R, Tanaka H, Nakao A, Fink MP, Lotze MT, et al. The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. J Exp Med. 2005;201(7):1135-43.
28. Granger DN, Kubes P. The microcirculation and inflammation: modulation of leukocyte-endothelial cell adhesion. J Leukoc Biol. 1994;55(5):662-75.
29. Zwacka RM, Zhang Y, Halldorson J, Schlossberg H, Dudus L, Engelhardt JF. CD4(+) T-lymphocytes mediate ischemia/reperfusion-induced inflammatory responses in mouse liver. J Clin Invest. 1997;100(2):279-89.
30. Shiratori Y, Kiriyama H, Fukushi Y, Nagura T, Takada H, Hai K, et al. Modulation of ischemia-reperfusion-induced hepatic injury by Kupffer cells. Dig Dis Sci. 1994;39(6):1265-72.
31. Natori S, Fujii Y, Kurosawa H, Nakano A, Shimada H. Prostaglandin E1 protects against ischemia-reperfusion injury of the liver by inhibition of neutrophil adherence to endothelial cells. Transplantation. 1997;64(11):1514-20.
32. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med. 1993;329(27):2002-12.
33. Fukazawa K, Lang JD. Role of nitric oxide in liver transplantation: Should it be routinely used? World J Hepatol. 2016;8(34):1489-96.
34. Peralta C, Hotter G, Closa D, Prats N, Xaus C, Gelpi E, et al. The protective role of adenosine in inducing nitric oxide synthesis in rat liver ischemia preconditioning is mediated by activation of adenosine A2 receptors. Hepatology. 1999;29(1):126-32.
35. Vardanian AJ, Busuttil RW, Kupiec-Weglinski JW. Molecular mediators of liver ischemia and reperfusion injury: a brief review. Mol Med. 2008;14(5-6):337-45.
36. Goto M, Takei Y, Kawano S, Nagano K, Tsuji S, Masuda E, et al. Endothelin-1 is involved in the pathogenesis of ischemia/reperfusion liver injury by hepatic microcirculatory disturbances. Hepatology. 1994;19(3):675-81.

37. Yokoyama Y, Baveja R, Sonin N, Nakanishi K, Zhang JX, Clemens MG. Altered endothelin receptor subtype expression in hepatic injury after ischemia/reperfusion. Shock. 2000;13(1):72-8.
38. Southard JH, van Gulik TM, Ametani MS, Vreugdenhil PK, Lindell SL, Pienaar BL, et al. Important components of the UW solution. Transplantation. 1990;49(2):251-7.
39. Latchana N, Peck JR, Whitson BA, Henry ML, Elkhammas EA, Black SM. Preservation solutions used during abdominal transplantation: Current status and outcomes. World J Transplant. 2015;5(4):154-64.
40. Parsons RF, Guarrera JV. Preservation solutions for static cold storage of abdominal allografts: which is best? Curr Opin Organ Transplant. 2014;19(2):100-7.
41. Ringe B, Braun F, Moritz M, Zeldin G, Soriano H, Meyers W. Safety and efficacy of living donor liver preservation with HTK solution. Transplant Proc. 2005;37(1):316-9.
42. Testa G, Malago M, Nadalin S, Treptow B, Paul A, Frilling A, et al. Histidine-tryptophan-ketoglutarate versus University of Wisconsin solution in living donor liver transplantation: results of a prospective study. Liver Transpl. 2003;9(8):822-6.
43. Mangus RS, Fridell JA, Vianna RM, Milgrom MA, Chestovich P, Chihara RK, et al. Comparison of histidine-tryptophan-ketoglutarate solution and University of Wisconsin solution in extended criteria liver donors. Liver Transpl. 2008;14(3):365-73.
44. Mangus RS, Tector AJ, Agarwal A, Vianna R, Murdock P, Fridell JA. Comparison of histidine-tryptophan-ketoglutarate solution (HTK) and University of Wisconsin solution (UW) in adult liver transplantation. Liver Transpl. 2006;12(2):226-30.
45. Erhard J, Lange R, Scherer R, Kox WJ, Bretschneider HJ, Gebhard MM, et al. Comparison of histidine-tryptophan-ketoglutarate (HTK) solution versus University of Wisconsin (UW) solution for organ preservation in human liver transplantation. A prospective, randomized study. Transpl Int. 1994;7(3):177-81.
46. Canelo R, Hakim NS, Ringe B. Experience with hystidine tryptophan ketoglutarate versus University Wisconsin preservation solutions in transplantation. Int Surg. 2003;88(3):145-51.
47. Feng L, Zhao N, Yao X, Sun X, Du L, Diao X, et al. Histidine-tryptophan-ketoglutarate solution vs. University of Wisconsin solution for liver transplantation: a systematic review. Liver Transpl. 2007;13(8):1125-36.
48. Stewart ZA, Cameron AM, Singer AL, Montgomery RA, Segev DL. Histidine-Tryptophan-Ketoglutarate (HTK) is associated with reduced graft survival in deceased donor livers, especially those donated after cardiac death. Am J Transplant. 2009;9(2):286-93.
49. Lema Zuluaga GL, Serna Agudelo RE, Zuleta Tobon JJ. Preservation solutions for liver transplantation in adults: celsior versus custodiol: a systematic review and meta-analysis with an indirect comparison of randomized trials. Transplant Proc. 2013;45(1):25-32.
50. Voigt MR, DeLario GT. Perspectives on abdominal organ preservation solutions: a comparative literature review. Prog Transplant. 2013;23(4):383-91.
51. Heidenhain C, Pratschke J, Puhl G, Neumann U, Pascher A, Veltzke-Schlieker W, et al. Incidence of and risk factors for ischemic-type biliary lesions following orthotopic liver transplantation. Transpl Int. 2010;23(1):14-22.
52. Morariu AM, Vd Plaats A, W VO, NA TH, Leuvenink HG, Graaff R, et al. Hyperaggregating effect of hydroxyethyl starch components and University of Wisconsin solution on human red blood cells: a risk of impaired graft perfusion in organ procurement? Transplantation. 2003;76(1):37-43.

53. Hertl M, Harvey PR, Swanson PE, West DD, Howard TK, Shenoy S, et al. Evidence of preservation injury to bile ducts by bile salts in the pig and its prevention by infusions of hydrophilic bile salts. Hepatology. 1995;21(4):1130-7.
54. Buis CI, Geuken E, Visser DS, Kuipers F, Haagsma EB, Verkade HJ, et al. Altered bile composition after liver transplantation is associated with the development of nonanastomotic biliary strictures. J Hepatol. 2009;50(1):69-79.
55. Meine MH, Zanotelli ML, Neumann J, Kiss G, de Jesus Grezzana T, Leipnitz I, et al. Randomized clinical assay for hepatic grafts preservation with University of Wisconsin or histidine-tryptophan-ketoglutarate solutions in liver transplantation. Transplant Proc. 2006;38(6):1872-5.
56. Gulsen MT, Girotra M, Cengiz-Seval G, Price J, Singh VK, Segev DL, et al. HTK preservative solution is associated with increased biliary complications among patients receiving DCD liver transplants: a single center experience. Ann Transplant. 2013;18:69-75.
57. Cavallari A, Cillo U, Nardo B, Filipponi F, Gringeri E, Montalti R, et al. A multicenter pilot prospective study comparing Celsior and University of Wisconsin preserving solutions for use in liver transplantation. Liver Transpl. 2003;9(8):814-21.
58. Pedotti P, Cardillo M, Rigotti P, Gerunda G, Merenda R, Cillo U, et al. A comparative prospective study of two available solutions for kidney and liver preservation. Transplantation. 2004;77(10):1540-5.
59. Garcia-Gil FA, Serrano MT, Fuentes-Broto L, Arenas J, Garcia JJ, Guemes A, et al. Celsior versus University of Wisconsin preserving solutions for liver transplantation: postreperfusion syndrome and outcome of a 5-year prospective randomized controlled study. World J Surg. 2011;35(7):1598-607.
60. Dondero F, Paugam-Burtz C, Danjou F, Stocco J, Durand F, Belghiti J. A randomized study comparing IGL-1 to the University of Wisconsin preservation solution in liver transplantation. Ann Transplant. 2010;15(4):7-14.
61. Kim WR, Lake JR, Smith JM, Skeans MA, Schladt DP, Edwards EB, et al. OPTN/SRTR 2015 Annual Data Report: Liver. Am J Transplant. 2017;17 Suppl 1:174-251.
62. Foley DP, Fernandez LA, Leverson G, Anderson M, Mezrich J, Sollinger HW, et al. Biliary complications after liver transplantation from donation after cardiac death donors: an analysis of risk factors and long-term outcomes from a single center. Ann Surg. 2011;253(4):817-25.
63. Coffey JC, Wanis KN, Monbaliu D, Gilbo N, Selzner M, Vachharajani N, et al. The influence of functional warm ischemia time on DCD liver transplant recipients' outcomes. Clin Transplant. 2017;31(10).
64. Rhee JY, Alroy J, Freeman RB. Characterization of the withdrawal phase in a porcine donation after the cardiac death model. Am J Transplant. 2011;11(6):1169-75.
65. Perera MT, Richards DA, Silva MA, Ahmed N, Neil DA, Murphy N, et al. Comparison of energy metabolism in liver grafts from donors after circulatory death and donors after brain death during cold storage and reperfusion. Br J Surg. 2014;101(7):775-83.
66. Xu J, Sayed BA, Casas-Ferreira AM, Srinivasan P, Heaton N, Rela M, et al. The Impact of Ischemia/Reperfusion Injury on Liver Allografts from Deceased after Cardiac Death versus Deceased after Brain Death Donors. PLoS One. 2016;11(2):e0148815.
67. Liu Q, Vekemans K, Iania L, Komuta M, Parkkinen J, Heedfeld V, et al. Assessing warm ischemic injury of pig livers at hypothermic machine perfusion. J Surg Res. 2014;186(1):379-89.

68. McGuire JB, James TJ, Imber CJ, St Peter SD, Friend PJ, Taylor RP. Optimisation of an enzymatic method for beta-galactosidase. Clin Chim Acta. 2002;326(1-2):123-9.
69. Adams DH, Wang L, Neuberger JM. Serum hyaluronic acid following liver transplantation: evidence of hepatic endothelial damage. Transplant Proc. 1989;21(1 Pt 2):2274.
70. Xu H, Berendsen T, Kim K, Soto-GutiÃ©rrez A, Bertheium F, Yarmush ML, et al. Excorporeal normothermic machine perfusion resuscitates pig DCD livers with extended warm ischemia. Journal of Surgical Research. 2012;173(2):e83-e8.
71. den Dries So, Sutton ME, Karimian N, de Boer MT, Wiersema-Buist J, Gouw ASH, et al. Hypothermic Oxygenated Machine Perfusion Prevents Arteriolonecrosis of the Peribiliary Plexus in Pig Livers Donated after Circulatory Death. PLoS One. 2014;9(2):e88521.
72. Minor T, Efferz P, Fox M, Wohlschlaeger J, Luer B. Controlled oxygenated rewarming of cold stored liver grafts by thermally graduated machine perfusion prior to reperfusion. Am J Transplant. 2013;13(6):1450-60.
73. Furukori M, Matsuno N, Meng LT, Shonaka T, Nishikawa Y, Imai K, et al. Subnormothermic Machine Perfusion Preservation With Rewarming for Donation After Cardiac Death Liver Grafts in Pigs. Transplant Proc. 2016;48(4):1239-43.
74. Bruinsma BG, Avruch JH, Sridharan GV, Weeder PD, Jacobs ML, Crisalli K, et al. Peritransplant Energy Changes and Their Correlation to Outcome After Human Liver Transplantation. Transplantation. 2017;101(7):1637-44.
75. Brockmann J, Reddy S, Coussios C, Pigott D, Guirriero D, Hughes D, et al. Normothermic perfusion: a new paradigm for organ preservation. Ann Surg. 2009;250(1):1-6.
76. Sutton ME, op den Dries S, Karimian N, Weeder PD, de Boer MT, Wiersema-Buist J, et al. Criteria for viability assessment of discarded human donor livers during ex vivo normothermic machine perfusion. PLoS One. 2014;9(11):e110642.
77. Watson CJE, Kosmoliaptsis V, Randle LV, Gimson AE, Brais R, Klinck JR, et al. Normothermic Perfusion in the Assessment and Preservation of Declined Livers Before Transplantation: Hyperoxia and Vasoplegia-Important Lessons From the First 12 Cases. Transplantation. 2017;101(5):1084-98.
78. Nasralla D, Coussios CC, Mergental H, Akhtar MZ, Butler AJ, Ceresa CDL, et al. A randomized trial of normothermic preservation in liver transplantation. Nature. 2018.
79. Vajdova K, Smrekova R, Kukan M, Lutterova M, Wsolova L. Bile analysis as a tool for assessing integrity of biliary epithelial cells after cold ischemia--reperfusion of rat livers. Cryobiology. 2000;41(2):145-52.
80. op den Dries S, Westerkamp AC, Karimian N, Gouw AS, Bruinsma BG, Markmann JF, et al. Injury to peribiliary glands and vascular plexus before liver transplantation predicts formation of non-anastomotic biliary strictures. J Hepatol. 2014;60(6):1172-9.
81. Liu Q, Nassar A, Farias K, Buccini L, Baldwin W, Mangino M, et al. Sanguineous normothermic machine perfusion improves hemodynamics and biliary epithelial regeneration in donation after cardiac death porcine livers. Liver Transpl. 2014;20(8):987-99.
82. Oellerich M, Armstrong VW. The MEGX test: a tool for the real-time assessment of hepatic function. Ther Drug Monit. 2001;23(2):81-92.
83. Liu Q, Nassar A, Farias K, Buccini L, Mangino MJ, Baldwin W, et al. Comparing Normothermic Machine Perfusion Preservation With Different Perfusates on Porcine Livers From Donors After Circulatory Death. Am J Transplant. 2016;16(3):794-807.

84. Reddy S, Greenwood J, Maniakin N, Bhattacharjya S, Zilvetti M, Brockmann J, et al. Non-heart-beating donor porcine livers: the adverse effect of cooling. Liver Transpl. 2005;11(1):35-8.
85. Reddy SP, Bhattacharjya S, Maniakin N, Greenwood J, Guerreiro D, Hughes D, et al. Preservation of porcine non-heart-beating donor livers by sequential cold storage and warm perfusion. Transplantation. 2004;77(9 SUPPL):1328-32.
86. Mergental H, Perera MT, Laing RW, Muiesan P, Isaac JR, Smith A, et al. Transplantation of Declined Liver Allografts Following Normothermic Ex-Situ Evaluation. Am J Transplant. 2016;16(11):3235-45.
87. Moers C, Pirenne J, Paul A, Ploeg RJ, Machine Preservation Trial Study G. Machine perfusion or cold storage in deceased-donor kidney transplantation. N Engl J Med. 2012;366(8):770-1.
88. Jain S, Lee CY, Baicu S, Duncan H, Xu H, Jones JW, Jr., et al. Hepatic function in hypothermically stored porcine livers: comparison of hypothermic machine perfusion vs cold storage. Transplant Proc. 2005;37(1):340-1.
89. Bessems M, Doorschodt BM, Dinant S, de Graaf W, van Gulik TM. Machine perfusion preservation of the pig liver using a new preservation solution, polysol. Transplant Proc. 2006;38(5):1238-42.
90. Monbaliu D, Vekemans K, De Vos R, Brassil J, Heedfeld V, Qiang L, et al. Hemodynamic, biochemical, and morphological characteristics during preservation of normal porcine livers by hypothermic machine perfusion. Transplant Proc. 2007;39(8):2652-8.
91. Schlegel A, de Rougemont O, Graf R, Clavien P-A, Dutkowski P. Protective mechanisms of end-ischemic cold machine perfusion in DCD liver grafts. Journal of hepatology. 2013;58(2):278-86.
92. Obara H, Matsuno N, Enosawa S, Shigeta T, Huai-Che H, Hirano T, et al. Pretransplant screening and evaluation of liver graft viability using machine perfusion preservation in porcine transplantation. Transplant Proc. 2012;44(4):959-61.
93. Op DD, Sutton ME, Karimian N, De Boer MT, Wiersema-Buist J, Gouw ASH, et al. Hypothermic oxygenated machine perfusion prevents arteriolonecrosis of the peribiliary plexus in pig livers donated after circulatory death. PLoS ONE. 2014;9(2).
94. Guarrera JV, Estevez J, Boykin J, Boyce R, Rashid J, Sun S, et al. Hypothermic machine perfusion of liver grafts for transplantation: technical development in human discard and miniature swine models. Transplant Proc. 2005;37(1):323-5.
95. Shigeta T, Matsuno N, Obara H, Mizunuma H, Kanazawa H, Tanaka H, et al. Functional recovery of donation after cardiac death liver graft by continuous machine perfusion preservation in pigs. Transplantation proceedings. 2012;44(4):946-7.
96. de Rougemont O, Breitenstein S, Leskosek B, Weber A, Graf R, Clavien PA, et al. One hour hypothermic oxygenated perfusion (HOPE) protects nonviable liver allografts donated after cardiac death. Ann Surg. 2009;250(5):674-83.
97. Fondevila C, Hessheimer AJ, Maathuis MH, Munoz J, Taura P, Calatayud D, et al. Hypothermic oxygenated machine perfusion in porcine donation after circulatory determination of death liver transplant. Transplantation. 2012;94(1):22-9.
98. Guarrera JV, Henry SD, Samstein B, Odeh-Ramadan R, Kinkhabwala M, Goldstein MJ, et al. Hypothermic machine preservation in human liver transplantation: the first clinical series. Am J Transplant. 2010;10(2):372-81.

99. Henry SD, Guarrera JV. Protective effects of hypothermic ex vivo perfusion on ischemia/reperfusion injury and transplant outcomes. Transplant Rev (Orlando). 2012;26(2):163-75.
100. Dutkowski P, Schlegel A, de Oliveira M, Mullhaupt B, Neff F, Clavien PA. HOPE for human liver grafts obtained from donors after cardiac death. J Hepatol. 2014;60(4):765-72.
101. Dutkowski P, Polak WG, Muiesan P, Schlegel A, Verhoeven CJ, Scalera I, et al. First comparison of hypothermic oxygenated perfusion versus static cold storage of human donation after cardiac death liver transplants. Annals of Surgery. 2015;262(5):764-71.
102. Kootstra G, Daemen JH, Oomen AP. Categories of non-heart-beating donors. Transplant Proc. 1995;27(5):2893-4.
103. Foley DP, Fernandez LA, Leverson G, Chin LT, Krieger N, Cooper JT, et al. Donation after cardiac death: the University of Wisconsin experience with liver transplantation. Ann Surg. 2005;242(5):724-31.
104. De Carlis R, Di Sandro S, Lauterio A, Ferla F, Dell'Acqua A, Zanierato M, et al. Successful donation after cardiac death liver transplants with prolonged warm ischemia time using normothermic regional perfusion. Liver Transplantation. 2017;23(2):166-73.
105. Gringeri E, Bonsignore P, Bassi D, D'Amico FE, Mescoli C, Polacco M, et al. Subnormothermic machine perfusion for non-heart-beating donor liver grafts preservation in a Swine model: a new strategy to increase the donor pool? Transplant Proc. 2012;44(7):2026-8.
106. Obara H, Matsuno N, Shigeta T, Hirano T, Enosawa S, Mizunuma H. Temperature controlled machine perfusion system for liver. Transplant Proc. 2013;45(5):1690-2.
107. Morito N, Obara H, Matsuno N, Enosawa S, Watanabe K, Furukori M, et al. Regulated Oxygenation of Rewarming Machine Perfusion for Porcine Donation After Cardiac Death Liver Transplantation. Transplantation proceedings. 2016;48(4):1244-6.
108. Matsuno N, Obara H, Watanabe R, Iwata S, Kono S, Fujiyama M, et al. Rewarming preservation by organ perfusion system for donation after cardiac death liver grafts in pigs. Transplantation proceedings. 2014;46(4):1095-8.
109. Hoyer DP, Paul A, Luer S, Reis H, Efferz P, Minor T. End-ischemic reconditioning of liver allografts: Controlling the rewarming. Liver Transpl. 2016;22(9):1223-30.
110. Bruinsma BG, Yeh H, Ozer S, Martins PN, Farmer A, Wu W, et al. Subnormothermic machine perfusion for ex vivo preservation and recovery of the human liver for transplantation. Am J Transplant. 2014;14(6):1400-9.
111. Boehnert MU, Yeung JC, Bazerbachi F, Knaak JM, Selzner N, McGilvray ID, et al. Normothermic acellular ex vivo liver perfusion reduces liver and bile duct injury of pig livers retrieved after cardiac death. Am J Transplant. 2013;13(6):1441-9.
112. Steen S, Liao Q, Wierup PN, Bolys R, Pierre L, Sjoberg T. Transplantation of lungs from non-heart-beating donors after functional assessment ex vivo. Ann Thorac Surg. 2003;76(1):244-52; discussion 52.
113. Butler AJ, Rees MA, Wight DG, Casey ND, Alexander G, White DJ, et al. Successful extracorporeal porcine liver perfusion for 72 hr. Transplantation. 2002;73(8):1212-8.
114. Imber CJ, St Peter SD, de Cenarruzabeitia IL, Lemonde H, Rees M, Butler A, et al. Optimisation of bile production during normothermic preservation of porcine livers. Am J Transplant. 2002;2(7):593-9.
115. Schon MR, Hunt CJ, Pegg DE, Wight DG. The possibility of resuscitating livers after warm ischemic injury. Transplantation. 1993;56(1):24-31.

116. Klein M, Geoghegan J, Wangemann R, Bockler D, Schmidt K, Scheele J. Preconditioning of donor livers with prostaglandin I2 before retrieval decreases hepatocellular ischemia-reperfusion injury. Transplantation. 1999;67(8):1128-32.
117. Nassar A, Liu Q, Farias K, D'Amico G, Buccini L, Urcuyo D, et al. Role of vasodilation during normothermic machine perfusion of DCD porcine livers. International Journal of Artificial Organs. 2014;37(2):165-72.
118. St Peter SD, Imber CJ, Lopez I, Hughes D, Friend PJ. Extended preservation of non-heart-beating donor livers with normothermic machine perfusion. British Journal of Surgery. 2002;89(5):609-16.
119. Nassar A, Liu Q, Farias K, D'Amico G, Tom C, Grady P, et al. Ex vivo normothermic machine perfusion is safe, simple, and reliable: Results from a large animal model. Surgical Innovation. 2015;22(1):61-9.
120. Hellinger A, Fiegen R, Lange R, Rauen U, Schmidt U, Hirche H, et al. Preservation of pig liver allografts after warm ischemia: normothermic perfusion versus cold storage. Langenbecks Arch Chir. 1997;382(4):175-84.
121. Schon MR, Kollmar O, Wolf S, Schrem H, Matthes M, Akkoc N, et al. Liver transplantation after organ preservation with normothermic extracorporeal perfusion. Ann Surg. 2001;233(1):114-23.
122. Nassar A, Liu Q, Farias K, Buccini L, Baldwin W, Bennett A, et al. Impact of Temperature on Porcine Liver Machine Perfusion From Donors After Cardiac Death. Artif Organs. 2016;40(10):999-1008.
123. Imber CJ, St Peter SD, Lopez de Cenarruzabeitia I, Pigott D, James T, Taylor R, et al. Advantages of normothermic perfusion over cold storage in liver preservation. Transplantation. 2002;73(5):701-9.
124. Vogel T, Brockmann JG, Quaglia A, Morovat A, Jassem W, Heaton ND, et al. The 24-hour normothermic machine perfusion of discarded human liver grafts. Liver Transplantation. 2017;23(2):207-20.
125. Bellomo R, Marino B, Starkey G, Fink M, Wang BZ, Eastwood GM, et al. Extended normothermic extracorporeal perfusion of isolated human liver after warm ischaemia: a preliminary report. Critical care and resuscitation : journal of the Australasian Academy of Critical Care Medicine. 2014;16(3):197-201.
126. Liu Q, Nassar A, Buccini L, Grady P, Soliman B, Hassan A, et al. Ex situ 86 hours liver perfusion: Pushing the boundary of organ preservation. Liver Transpl. 2018.
127. op den Dries S, Karimian N, Sutton ME, Westerkamp AC, Nijsten MW, Gouw AS, et al. Ex vivo normothermic machine perfusion and viability testing of discarded human donor livers. Am J Transplant. 2013;13(5):1327-35.
128. Ravikumar R, Jassem W, Mergental H, Heaton N, Mirza D, Perera MT, et al. Liver Transplantation After Ex Vivo Normothermic Machine Preservation: A Phase 1 (First-in-Man) Clinical Trial. Am J Transplant. 2016;16(6):1779-87.
129. Selzner M, Goldaracena N, Echeverri J, Kaths JM, Linares I, Selzner N, et al. Normothermic ex vivo liver perfusion using steen solution as perfusate for human liver transplantation: First North American results. Liver Transpl. 2016;22(11):1501-8.
130. Bral M, Gala-Lopez B, Bigam D, Kneteman N, Malcolm A, Livingstone S, et al. Preliminary Single-Center Canadian Experience of Human Normothermic Ex Vivo Liver Perfusion: Results of a Clinical Trial. American Journal of Transplantation. 2017;17(4):1071-80.

131. Fondevila C, Hessheimer AJ, Ruiz A, Calatayud D, Ferrer J, Charco R, et al. Liver transplant using donors after unexpected cardiac death: novel preservation protocol and acceptance criteria. American Journal of Transplantation. 2007;7(7):1849-55.
132. Fondevila C, Hessheimer AJ, Maathuis M-HJ, Munoz J, Taura P, Calatayud D, et al. Superior preservation of DCD livers with continuous normothermic perfusion. Annals of Surgery. 2011;254(6):1000-7.
133. Morales-Ruiz M, Fondevila C, Munoz-Luque J, Tugues S, Rodriguez-Laiz G, Cejudo-Martin P, et al. Gene transduction of an active mutant of akt exerts cytoprotection and reduces graft injury after liver transplantation. Am J Transplant. 2007;7(4):769-78.
134. Nagrath D, Xu H, Tanimura Y, Zuo R, Berthiaume F, Avila M, et al. Metabolic preconditioning of donor organs: defatting fatty livers by normothermic perfusion ex vivo. Metab Eng. 2009;11(4-5):274-83.
135. White CW, Ambrose E, Muller A, Li Y, Le H, Thliveris J, et al. Avoidance of Profound Hypothermia During Initial Reperfusion Improves the Functional Recovery of Hearts Donated After Circulatory Death. Am J Transplant. 2016;16(3):773-82.
136. Information CIfH. Canadian Organ Replacement Register, 2017 Cumulative Report 2018 [Available from: https://www.cihi.ca/en/e-statistics-on-organ-transplants-waiting-lists-and-donors.
137. Shemie SD. Trends in deceased organ donation in Canada. CMAJ. 2017;189(38):E1204-E5.
138. Jay CL, Lyuksemburg V, Ladner DP, Wang E, Caicedo JC, Holl JL, et al. Ischemic cholangiopathy after controlled donation after cardiac death liver transplantation: a meta-analysis. Ann Surg. 2011;253(2):259-64.
139. Tang JX, Na N, Li JJ, Fan L, Weng RH, Jiang N. Outcomes of Controlled Donation After Cardiac Death Compared With Donation After Brain Death in Liver Transplantation: A Systematic Review and Meta-analysis. Transplant Proc. 2018;50(1):33-41.
140. Mourad MM, Algarni A, Liossis C, Bramhall SR. Aetiology and risk factors of ischaemic cholangiopathy after liver transplantation. World J Gastroenterol. 2014;20(20):6159-69.
141. Adkins RB, Jr., Chapman WC, Reddy VS. Embryology, anatomy, and surgical applications of the extrahepatic biliary system. Surg Clin North Am. 2000;80(1):363-79.
142. Skaro AI, Jay CL, Baker TB, Wang E, Pasricha S, Lyuksemburg V, et al. The impact of ischemic cholangiopathy in liver transplantation using donors after cardiac death: the untold story. Surgery. 2009;146(4):543-52; discussion 52-3.
143. Mathur AK, Heimbach J, Steffick DE, Sonnenday CJ, Goodrich NP, Merion RM. Donation after cardiac death liver transplantation: predictors of outcome. Am J Transplant. 2010;10(11):2512-9.
144. Chan EY, Olson LC, Kisthard JA, Perkins JD, Bakthavatsalam R, Halldorson JB, et al. Ischemic cholangiopathy following liver transplantation from donation after cardiac death donors. Liver Transpl. 2008;14(5):604-10.
145. Schlegel A, Scalera I, Perera M, Kalisvaart M, Mergental H, Mirza DF, et al. Impact of donor age in donation after circulatory death liver transplantation: Is the cutoff "60" still of relevance? Liver Transpl. 2018;24(3):352-62.
146. Boteon YL, Boteon A, Attard J, Mergental H, Mirza DF, Bhogal RH, et al. Ex situ machine perfusion as a tool to recondition steatotic donor livers: Troublesome features of fatty livers and the role of defatting therapies. A systematic review. Am J Transplant. 2018;18(10):2384-99.

147. Kalisvaart M, de Haan JE, Polak WG, Metselaar HJ, Wijnhoven BPL, JNM IJ, et al. Comparison of Postoperative Outcomes Between Donation After Circulatory Death and Donation After Brain Death Liver Transplantation Using the Comprehensive Complication Index. Ann Surg. 2017;266(5):772-8.
148. Tun-Abraham ME, Wanis K, Garcia-Ochoa C, Sela N, Sharma H, Al Hasan I, et al. Can we reduce ischemic cholangiopathy rates in donation after cardiac death liver transplantation after 10 years of practice? Canadian single-centre experience. Can J Surg. 2018;62(1):8.
149. Cao Y, Shahrestani S, Chew HC, Crawford M, Macdonald PS, Laurence J, et al. Donation After Circulatory Death for Liver Transplantation: A Meta-Analysis on the Location of Life Support Withdrawal Affecting Outcomes. Transplantation. 2016;100(7):1513-24.
150. Lee KW, Simpkins CE, Montgomery RA, Locke JE, Segev DL, Maley WR. Factors affecting graft survival after liver transplantation from donation after cardiac death donors. Transplantation. 2006;82(12):1683-8.
151. Hashimoto K, Eghtesad B, Gunasekaran G, Fujiki M, Uso TD, Quintini C, et al. Use of tissue plasminogen activator in liver transplantation from donation after cardiac death donors. Am J Transplant. 2010;10(12):2665-72.
152. Seal JB, Bohorquez H, Reichman T, Kressel A, Ghanekar A, Cohen A, et al. Thrombolytic protocol minimizes ischemic-type biliary complications in liver transplantation from donation after circulatory death donors. Liver Transpl. 2015;21(3):321-8.
153. Bohorquez H, Seal JB, Cohen AJ, Kressel A, Bugeaud E, Bruce DS, et al. Safety and Outcomes in 100 Consecutive Donation After Circulatory Death Liver Transplants Using a Protocol That Includes Thrombolytic Therapy. Am J Transplant. 2017;17(8):2155-64.
154. Jayant K, Reccia I, Virdis F, Shapiro AMJ. Systematic Review and Meta-Analysis on the Impact of Thrombolytic Therapy in Liver Transplantation Following Donation after Circulatory Death. J Clin Med. 2018;7(11).
155. Jones JM, Bhutiani N, Wei D, Goldstein L, Jones CM, Cannon RM. A literature-based cost analysis of tissue plasminogen activator for prevention of biliary stricture in donation after circulatory death liver transplantation. Am J Surg. 2018;216(5):959-62.
156. Li P, Liu YF, Yang L. Advantages of dual hypothermic oxygenated machine perfusion over simple cold storage in the preservation of liver from porcine donors after cardiac death. Clinical transplantation. 2015;29(9):820-8.
157. Op den Dries S, Sutton ME, Karimian N, de Boer MT, Wiersema-Buist J, Gouw AS, et al. Hypothermic oxygenated machine perfusion prevents arteriolonecrosis of the peribiliary plexus in pig livers donated after circulatory death. PLoS One. 2014;9(2):e88521.
158. Westerkamp AC, Karimian N, Matton AP, Mahboub P, van Rijn R, Wiersema-Buist J, et al. Oxygenated Hypothermic Machine Perfusion After Static Cold Storage Improves Hepatobiliary Function of Extended Criteria Donor Livers. Transplantation. 2016;100(4):825-35.
159. van Rijn R, Karimian N, Matton APM, Burlage LC, Westerkamp AC, van den Berg AP, et al. Dual hypothermic oxygenated machine perfusion in liver transplants donated after circulatory death. Br J Surg. 2017;104(7):907-17.
160. Schlegel A, Muller X, Kalisvaart M, Muellhaupt B, Perera M, Isaac JR, et al. Outcomes of DCD liver transplantation using organs treated by hypothermic oxygenated perfusion before implantation. J Hepatol. 2019;70(1):50-7.
161. Hoyer DP, Mathe Z, Gallinat A, Canbay AC, Treckmann JW, Rauen U, et al. Controlled Oxygenated Rewarming of Cold Stored Livers Prior to Transplantation: First Clinical Application of a New Concept. Transplantation. 2016;100(1):147-52.

162. Nostedt JJ, Skubleny DT, Shapiro AMJ, Campbell S, Freed DH, Bigam DL. Normothermic Ex Vivo Machine Perfusion for Liver Grafts Recovered from Donors after Circulatory Death: A Systematic Review and Meta-Analysis2018. 8 p.
163. Busuttil RW, Tanaka K. The utility of marginal donors in liver transplantation. Liver Transpl. 2003;9(7):651-63.
164. Knaak JM, Spetzler VN, Goldaracena N, Boehnert MU, Bazerbachi F, Louis KS, et al. Subnormothermic ex vivo liver perfusion reduces endothelial cell and bile duct injury after donation after cardiac death pig liver transplantation. Liver Transpl. 2014;20(11):1296-305.
165. de Vries RB, Wever KE, Avey MT, Stephens ML, Sena ES, Leenaars M. The usefulness of systematic reviews of animal experiments for the design of preclinical and clinical studies. ILAR J. 2014;55(3):427-37.
166. Hooijmans CR, Rovers MM, de Vries RB, Leenaars M, Ritskes-Hoitinga M, Langendam MW. SYRCLE's risk of bias tool for animal studies. BMC Med Res Methodol. 2014;14:43.
167. Furukawa TA, Barbui C, Cipriani A, Brambilla P, Watanabe N. Imputing missing standard deviations in meta-analyses can provide accurate results. J Clin Epidemiol. 2006;59(1):7-10.
168. Higgins JP, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. BMJ. 2003;327(7414):557-60.
169. Banan B, Chung H, Xiao Z, Tarabishy Y, Jia J, Manning P, et al. Normothermic extracorporeal liver perfusion for donation after cardiac death (DCD) livers. Surgery (United States). 2015;158(6):1642-50.
170. Liu Q, Nassar A, Farias K, Buccini L, Mangino MJ, Baldwin W, et al. Comparing Normothermic Machine Perfusion Preservation With Different Perfusates on Porcine Livers From Donors After Circulatory Death. American Journal of Transplantation. 2016;16(3):794-807.
171. Boehnert MU, Yeung J, Knaak J-M, Selzner N, Bazerbachi F, McGilvray I, et al. Acellular Normothermic Ex Vivo Liver Perfusion (Nevlp) Decreases Hepatocyte and Bile Duct Injury in Livers Retrieved After Cardiac Death. Hepatology. 2011;54(Suppl. 1):386A.
172. Boehnert MU, Yeung JC, Knaak JM, Selzner N, Selzner M. Normothermic acellular ex vivo liver perfusion (NEVLP) reduces liver and bile duct in DCD liver grafts. American Journal of Transplantation. 2013;13(12):3290.
173. Ravikumar R, Leuvenink H, Friend PJ. Normothermic liver preservation: a new paradigm? Transpl Int. 2015;28(6):690-9.
174. Shigeta T, Matsuno N, Obara H, Kanazawa H, Tanaka H, Fukuda A, et al. Impact of rewarming preservation by continuous machine perfusion: Improved post-transplant recovery in pigs. Transplantation proceedings. 2013;45(5):1684-9.
175. Banan B, Xiao Z, Watson R, Xu M, Jia J, Upadhya GA, et al. Novel strategy to decrease reperfusion injuries and improve function of cold-preserved livers using normothermic ex vivo liver perfusion machine. Liver Transpl. 2016;22(3):333-43.
176. Banan B, Watson R, Xu M, Lin Y, Chapman W. Development of a normothermic extracorporeal liver perfusion system toward improving viability and function of human extended criteria donor livers. Liver Transplantation. 2016;22(7):979-93.
177. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193(1):265-75.
178. Passonneau JV, Lowry, Oliver H. . Enzymatid Analysis A Practical Guide. Totowa, New Jersey: The Humana Press Inc. ; 1993.

179. Reinders ME, van Wagensveld BA, van Gulik TM, Frederiks WM, Chamuleau RA, Endert E, et al. Hyaluronic acid uptake in the assessment of sinusoidal endothelial cell damage after cold storage and normothermic reperfusion of rat livers. Transpl Int. 1996;9(5):446-53.
180. Berendsen TA, Izamis ML, Xu H, Liu Q, Hertl M, Berthiaume F, et al. Hepatocyte viability and adenosine triphosphate content decrease linearly over time during conventional cold storage of rat liver grafts. Transplant Proc. 2011;43(5):1484-8.
181. Janssen H, Janssen PH, Broelsch CE. Celsior solution compared with University of Wisconsin solution (UW) and histidine-tryptophan-ketoglutarate solution (HTK) in the protection of human hepatocytes against ischemia-reperfusion injury. Transpl Int. 2003;16(7):515-22.
182. Radi ZA, Koza-Taylor PH, Bell RR, Obert LA, Runnels HA, Beebe JS, et al. Increased serum enzyme levels associated with kupffer cell reduction with no signs of hepatic or skeletal muscle injury. Am J Pathol. 2011;179(1):240-7.
183. Watson CJE, Jochmans I. From "Gut Feeling" to Objectivity: Machine Preservation of the Liver as a Tool to Assess Organ Viability. Curr Transplant Rep. 2018;5(1):72-81.
184. Lu TF, Yang TH, Zhong CP, Shen C, Lin WW, Gu GX, et al. Dual Effect of Hepatic Macrophages on Liver Ischemia and Reperfusion Injury during Liver Transplantation. Immune Netw. 2018;18(3):e24.
185. Mergental H, Stephenson BTF, Laing RW, Kirkham AJ, Neil DAH, Wallace LL, et al. Development of Clinical Criteria for Functional Assessment to Predict Primary Nonfunction of High-Risk Livers Using Normothermic Machine Perfusion. Liver Transpl. 2018;24(10):1453-69.
186. Watson CJE, Hunt F, Messer S, Currie I, Large S, Sutherland A, et al. In situ normothermic perfusion of livers in controlled circulatory death donation may prevent ischemic cholangiopathy and improve graft survival. Am J Transplant. 2018.