Graft Preservation and Ischemia Reperfusion Injury in Liver Transplantation. Studies in Immunomodulation and Attenuation of Injury

Juan Echeverri Cifuentes

Universitat Autònoma de Barcelona

Departament de Cirurgia

Graft Preservation and Ischemia Reperfusion Injury in Liver Transplantation. Studies in Immunomodulation and Attenuation of Injury

DOCTORAL THESIS presented by Juan Echeverri Cifuentes to opt to the grade of Doctor

Programa de Doctorat en Cirurgia i Ciències Morfològiques de la Universitat Autònoma de Barcelona, Barcelona, Spain

Doctoral Thesis directed by Ramón Charco Torra

Co-directors. Markus Selzner and Gonzalo Sapisochin

Tutor. Manuel Armengol Carrasco

D. RAMON CHARCO TORRA, Jefe del Servicio de Cirugía HBP y Trasplantes del Hospital Universitario Vall d'Hebron de Barcelona

HACE CONSTAR

Que la **TESIS DOCTORAL** titulada **"Graft Preservation and Ischemia Reperfusion Injury in Liver Transplantation. Studies in Immunomodulation and Attenuation of Injury"** presentada por **Juan Echeverri Cifuentes** para optar al grado de Doctor se ha realizado bajo su dirección, y al considerarla concluida, autoriza su presentación para ser juzgada por el Tribunal correspondiente.

Y para que conste los efectos firma la presente.

Barcelona, Junio de 2021.

Dr. Ramón Charco Torra

Director de Tesis

ACKNOWLEDGEMENT

To Dr. Ramon Charco Torra

My deepest gratitude for his mentorship and guidance during the elaboration of this doctoral thesis. My initial experiences during surgical residency paved the way for me to pursue a career in organ transplantation. I am deeply grateful to him and all the members of the liver transplant team at Hospital Universitari Vall D ´Hebron.

To Dr. Gonzalo Sapisochin

For his unconditional support and companionship in many aspects of my life. My deepest appreciation, gratitude, and admiration.

To Dr. Markus Selzner

I will always be grateful for giving me the opportunity to work in the ischemia reperfusion lab under his supervision. This unique exposure to basic science was paramount in my career as an academic surgeon. My 4 years of mentorship in Toronto, both in the lab and during clinical fellowship, have been the highlight of my career and the solid foundation for my constant pursue of the “savoir faire”.

To Moritz Kaths and Nicolas Goldaracena

Their teachings, support, and generosity were key for the elaboration of this scientific work. More important is what remains, and that is a lifelong friendship. My deepest gratitude to all members of the Toronto Ischemia Reperfusion Lab.

To my family Maria Elena and Bere

For their unconditional love and support throughout these years of absence while pursuing my dream to become an HPB and transplant surgeon. None of this would have been possible without their constant love and encouragement.

To Maria

The best journey companion I can ever have. None of these accomplishments would have been feasible without your love and support.

List of Abbreviations

OTL	Orthotopic Liver Transplantation
ESLD	End Stage Liver Disease
HCC	Hepatocellular Carcinoma
ChC	Cholangiocarcinoma
HCV	Hepatitis C Virus
NASH	Non-alcoholic steatohepatitis
HBV	Hepatitis B virus
PSC	Primary Sclerosing Cholangitis
PBC	Primary Biliary Cirrhosis
FHF	Fulminant Hepatic Failure
MELD	Model for End Stage Liver Disease
PNF	Primary Non-Function
DGF	Delayed Graft Function
ECD	Extended Criteria Donors
SFSS	Small for Size Syndrome
ACS	Abdominal Compartment Syndrome
PRS	Post-Reperfusion Syndrome
CNI	Calcineurin Inhibitors
MMF	Mycophenolate Mofetil
HAT	Hepatic Artery Thrombosis
NAS	Non-Anastomotic Strictures
ERCP	Endoscopic Retrograde Cholangio Pancreatography
UNOS	United Network of Organ Sharing
DCD	Donation After Circulatory Death

DBD	Donation After Brain Death
IRI	Ischemia Reperfusion Injury
WHO	World Health Organization
SCS	Static Cold Storage
WIT	Warm Ischemia Time
ROS	Reactive Oxygen Species
LSEC	Liver Sinusoidal Endothelial Cells
KC	Kupffer Cells
ATP	Adenosine Triphosphate
NO	Nitric Oxide
ET	Endothelin
TXA2	Thromboxane A2
TNF α	Tumor Necrosis Factor Alpha
IL-1	Interleukin-1
IL-12	Interleukin-12
INF γ	Interferon Gamma
DC	Dendritic Cells
HMP	Hypothermic Machine Perfusion
HOPE	Hypothermic Oxygenated Machine Perfusion
AST	Aspartate Aminotransferase
ALT	Alanine Aminotransferase
FMN	Flavin Mononucelotide
SNMP	Sub-normothermic Machine Perfusion
NRP	Normothermic Regional Perfusion
PECAM	Platelet-endothelial cell adhesion molecule

HSP70	Heat shock protein 70

TABLE OF CONTENTS

1. Introduction / Literature Review

1.1 Liver Transplantation. Overview and Important Implications

Orthotopic liver transplantation (OTL) is the only curative treatment for patients with acute or chronic liver failure [1,2,3]. Since its early beginnings in the 1960s, both the number of patients who have received an organ and those in need of a transplant have increased dramatically [4,5]. Outcomes have evolved from a 5-year survival of 50 % to greater than 70 % in most recent years [6,7]. Advances in donor selection and management, perioperative patient optimization, organ preservation, surgical techniques, and immunosuppression have resulted in 1-year survival rates of 80- 90% [8,9,10,11].

1.1.2 Indications and contraindications for liver transplantation

End stage liver disease (ESLD) causes over one million deaths per year worldwide. Therapeutic options for these patients are limited and transplantation remains the best treatment modality as it cures the complications associated with ESLD and the underlying disease in the majority of cases. The rationale to offer transplantation to a patient with chronic liver disease focuses on prolonging survival and improving quality of life [12]. Due to the scarcity of organs, the challenge remains in selecting patients who are most appropriate for transplantation in regard to the natural course of their disease and the number of complications which limit their quality of life [13]. From a practical point of view, liver transplantation is indicated in patients who have an expected survival of less than one year due to the severity of their disease or to those who have an important limitation in their quality of life due to disease complications [14]. In the case of hepatic malignancy such as patients with hepatocellular carcinoma (HCC), cholangiocarcinoma (ChC), neuroendocrine tumors or metastatic colorectal disease, the

indications and window of opportunity are much narrower and must adhere to oncological and non-futility principles in transplantation [15,16,17,18].

The main indications for liver transplantation are ever evolving **(Figure 1)** [19]. Hepatotropic viruses such as hepatitis C (HCV) which evolve to cirrhosis or HCC has been the main indication for liver transplant historically. However, the burden of HCV has been in decline with the advent of new antiviral therapies. Non-alcoholic steatohepatitis (NASH) has been increasing in recent years and positioning itself as the main indication in the western world [20,21]. Other causes comprise alcoholic liver disease, hepatitis B virus (HBV), metabolic diseases such as primary sclerosing cholangitis (PSC) or primary biliary cirrhosis (PBC), idiopathic liver failure, and fulminant hepatic failure (FHF). In recent years patients with perihilar cholangiocarcinoma, intra hepatic cholangiocarcinoma, and metastatic colorectal liver disease have been given the opportunity to be treated by the means of transplantation [22].

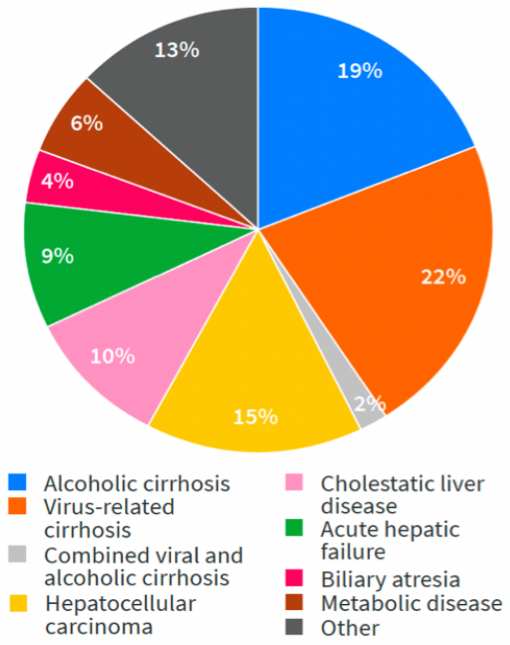

Figure 1. Main Indications for Liver Transplantation in Europe. Adapted from the Annual Report of the European Liver Transplantation Registry (ELTR).

Absolute contraindications for transplantation are few these days. In general, the different scenarios that preclude transplantation are those in which there is enough evidence to assure that the outcome of transplantation is not acceptable given the conditions of the patient and its comorbidities. As a rule, uncontrolled infection, extrahepatic malignancy, active substance abuse or non-compliance, and irreversible brain damage are universal contraindications to transplantation. Other conditions such as age of the recipient, anatomical considerations, technical limitations, or adverse psychosocial factors are deemed as relative contraindications that should be reviewed by each transplant unit and decided upon their expertise [23].

1.1.3 Timing of Transplantation

Liver transplantation has been a victim of its own success. Due to the scarcity of organs the allocation process has undergone strict scrutiny over the years. Before 2002, patients were prioritized using the Child-Turcotte-Pugh scoring system [24] **(Table 1)**, time on the waiting list, and location at time of organ offer. Although useful to some extent, the ever-increasing nature of transplant waiting lists demonstrated that the Child scoring system was not able to discriminate regarding disease severity and prioritization on the waiting list [25]. Due to this limitation, the Model for End Stage Liver Disease (MELD) was developed as a strong predictor of mortality at 3 months, shifting the allocation strategy to a "sickest first" system [26,27] (**Table 2**). Since then, patients have been prioritized using this score world-wide. There are some caveats, as for patients with HCC who have low MELD scores but are in need of an organ due to the risk of tumor progression. In the case of oncologic progression, the situation leads them to be unsuitable for transplantation. To palliate this pitfall, patients with malignancies are given exception points while waiting for an organ offer, this way they are given the

opportunity of an organ despite not being fully affected by their underlying liver function as compared to their peers. Of note, both Child and MELD scoring systems are poor predictors of outcomes and survival after transplantation. Despite these limitations, the MELD scoring system continues to be the most used tool world-wide for allocation of organs for patients with ESLD.

Table 1. Child – Turcotte – Pugh Classification

	POINTS		
	1	2	3
Encephalopathy	None	Grade 1 or 2	Grade 3 or 4
Ascites	Absent	Slight	Moderate
Bilirubin mg/dl	1 to 2	2 to 3	>3
Albumin	>3.5	2.8 to 3.5	<2.8
Prothrombin Time (seconds prolonged)	1 to 4	4 to 6	>6

Child A: 5 – 6 Points. 100% 1-year survival
Child B: 7 – 9 Points. 81% 1-year survival
Child C: 10 – 15 Points. 45% 1-year survival

Table 2. Predicted Mortality According to Model for End Stage Liver Disease (MELD) Scoring System

Score	Mortality Rate (%)	Death or Removal of List due to Illness (%)
<9	1.9	2.9
10 – 19	6	7.7
20 – 29	19.6	23.5
30 – 39	52.6	60.2
>40	71.3	79.3

1.1.4 Donor Selection and The Surgical Procedure

Adequate liver function once the organ is grafted to the recipient is of utmost importance. Different to other organs as kidney, pancreas, or in some cases heart transplantation, there is no artificial support for patients with post-transplant liver failure. Primary non-function (PNF), defined as non-life sustaining function of the graft, or delayed graft function (DGF), defined as impaired graft function which responds to support therapy, are to be avoided and kept in mind when accepting an organ offer. In PNF the only treatment is re-transplantation, taking into account the increase of morbidity and mortality in the recipient. Delayed graft function also increases morbidity and may lead to other complications associated with hemodynamic support [28,29,30].

Donor selection is important to avoid PNF or DGF [10]. Also, cold ischemia time and method of preservation previous to transplant are variables that impact the incidence of these complications. The use of extended criteria donors (ECD) defined as donation with advanced age, donation after cardiac death, prolonged ischemia time, steatotic grafts, and grafts from donors positive for HCV or HBV, is a tool to expand the donor pool [10, 31, 32, 33, 34, 35]. However, the use of these marginal grafts portends an increase in post-transplant organ dysfunction. Other techniques such as splitting of liver grafts also lead to long procurement times, over manipulation of the graft and vascular complications in the recipient which may result in poorer outcomes [37,38,39,40].

Donor and recipient are usually matched by ABO compatibility. Size matching is also important, a small graft transplanted into a large recipient could suffer of small for size syndrome (SFSS) and develop either PNF or DGF [41]. Small for size syndrome is defined as prolonged cholestasis, coagulopathy, and ascites in the absence of ischemia

in the first week post-transplant by a liver graft that is unable to meet the metabolic demands of the recipient [42, 43]. Smaller organs are easily adaptable to a big recipient with the risk of SFSS. A large liver adapted to a small recipient carries technical issues of abdominal closure or abdominal compartment syndrome (ACS), which in turn lead to vascular complications as arterial or venous thrombosis [44].

Two different techniques have been described throughout the years. The classic technique comprises of en-block resection of the recipient's liver with the retro-hepatic vena cava, thus being preceded by a total cross-clamping of the vena cava and portal vein [45]. An alternative "Piggy-back" technique has been described with preservation of the vena cava. Partial clamping of the vena cava in the "Piggy-back" technique has gained acceptance over the years now that it reduces warm ischemia time, ensues hemodynamic stability during cross-clamping, preserves renal perfusion pressure, and improves post-operative renal recovery [46, 47, 48]. In some cases, splachnic flow can be maintained through a porto-caval shunt during hepatectomy. This shunt is particularly useful in patients with FHF or metabolic diseases who lack portal-systemic collateral circulation in the splanchnic bed [49, 50, 51, 52, 53]. Both the classical and "Piggy-back technique are valid from a technical point of view. The implementation of one technique over the other depends on the preference and expertise of each transplant unit, having a wide array of possibilities depending on the complexity of each particular patient. In general, the peri-operative mortality of liver transplantation nowadays ranges from 3-5% and the 1-and 5 -year survival ranges between 80-90%, and 70-80 % respectively [54].

1.1.5 Post-operative Graft Assessment and General Considerations

Several peri-operative events deserve to be mentioned. Since the very moment of reperfusion of the graft in the recipient, diverse immunological and hemodynamic events come into play.

Post-reperfusion syndrome (PRS) has been described as a decrease in systemic arterial pressure to less than 70 % of the pre reperfusion value or a mean blood pressure below 60 mmHg within 5 minutes of reperfusion and lasting at least one minute [55]. The event of PRS has been described to be present between 25-55% of patients. Depending on the severity of the event, PRS can escalate to significant bradycardia resistant to chronotropic therapy and cardiac arrest [56,57]. Although the mechanisms of PRS and bradycardia are not fully understood, marginal grafts or grafts from ECD are more prone to present this complication. Post reperfusion syndrome is associated with adverse outcomes, increased blood loss, kidney failure, higher incidence of graft loss and increased mortality [58]. All strategies which optimize graft preservation previous to transplant seem to ameliorate this alarming complication [59].

Other typical complications in the early post-operative period include bleeding with the need of transfusion of blood derived products, bowel injury, bowel obstruction, incisional hernia, deep vein thrombosis, pulmonary embolism, and infection [54].

Following liver transplantation recipients depend on lifelong immunosuppression to prevent rejection and graft loss. Most common regimes of immunosuppression include a combination of steroids, calcineurin inhibitors (CNI), and mycophenolate mofetil (MMF). Over time, as the risk of acute rejection decreases, patients are weaned off

immunosuppression in an effort to minimize the occurrence of malignancy, CNI induced kidney injury, and infection.

Biliary complications after liver transplantation have an incidence of 10 - 40%, having a significant impact on morbidity and mortality (8 -15%) [60,61,62]. These complications comprise either biliary leak or stenosis, which lead to infectious complications and graft dysfunction. Risk factors influencing their occurrence are often related to technical issues during transplant, immunological phenomena, and graft preservation previous to transplant. Bile duct strictures are most often related to bile duct ischemia during procurement or preservation, and to hepatic artery thrombosis (HAT) in the transplanted graft [63,64] These are divided into anastomotic and non-anastomotic strictures (NAS) [65]. Anastomotic strictures are more prone to be solved by endoscopic techniques such as endoscopic retrograde cholangio pancreatography (ERCP) or by surgical revision and conversion to a Roux-en-Y-hepaticojejunostomy [66,67,68]. On the other hand, NAS are more troublesome and difficult to solve due to their heterogeneous location within the bile duct. The incidence of NAS varies from 1-20%, and up to 50% of patients with this complication die or require re-transplantation [69]. The bile duct epithelium is very sensitive to ischemia, as its blood supply and oxygenation depends solely on the arterial system [60]. Hepatocytes on the other hand receive dual blood supply from the hepatic artery and portal vein. Bile duct epithelial cells subjected to long periods of cold and warm ischemic injury are more prone to develop complications. All efforts focused to minimize bile duct injury during organ preservation may decrease morbidity and mortality associated with this deleterious entity.

1.2 Historical aspects of liver transplantation and graft preservation:

Dr. Thomas Earl Starzl started developing the liver transplant model in humans around 1963 [70]. It wasn't until 1967 when his team performed the first successful liver transplant in humans at the University of Colorado and Denver Veterans Administration Hospitals. Among with his many accomplishments, he was responsible for the development of immunosuppression drugs such as Cyclosporine and Tacrolimus. Treatment of rejection along with the optimization of the surgical procedure, represented a significant advancement in transplant medicine. Dr. Starzl, then relocating at the University of Pittsburgh Medical Center, continued to move forward the field of transplantation taking it from an experimental procedure to an accepted form of treatment for patients with ESLD [71].

During the first transplant procedures under Dr. Starzl´s supervision, organ preservation during procurement was performed using extracorporeal perfusion at 15º Celsius of the abdominal organs. Perfusion was started once the donor was declared dead as by cardiac arrest (donation after cardiac death). The perfusion device consisted of a glucose-primed bubble oxygenator, a single DeBakey pump, and a heat exchanger [70]. The circuit was primed with 2 liters of 5% dextrose in water, procaine hydrochloride, penicillin, and heparin. Extracorporeal perfusion was maintained all throughout graft dissection. Once the team was ready to take out the liver, the organ was flushed with Lactate Ringers solution cooled to 15 º Celsius.

Along with the development of surgical techniques for kidney and liver transplantation, physiologists and anatomists were gaining significant knowledge on how to keep the organs viable and functioning outside the body [72,73,74]. Organ perfusion with blood was

being replaced by synthetic perfusates. Other preservation techniques rendered the use of lower temperatures to alter the organs metabolism and decrease tissue damage. The first cooling experiments were performed by Calne and colleagues, achieving kidney preservation by perfusion of cooled blood through the renal artery [75]. Later, Collins would develop an acellular solution that would mimic the intracellular electrolyte balance of the cell and move forward the field of static cold storage [76]. From that moment on, static flush cooling of organs followed by preservation on ice expanded the occurrence of multiple organ procurement. Multiple organs could be harvested from a same donor without jeopardizing the viability of one organ over the other [77].

1.3 Organ shortage

1.3.1 Donation After Brain Death vs Donation After Circulatory Death

Despite the advances in the field of liver transplantation, scarcity of organs continues to be an important limitation. Expanding criteria for marginal donors with the aim to increase the donor pool is not sufficient to fulfil the need for this life saving procedure.

According to the United Network of Organ Sharing (UNOS), during 2018, a total of 11,844 patients were added to the liver transplant waiting list in the United States [78]. Of these, 8,250 patients were transplanted. Of note, 95.7% of these recipients received an organ from a deceased donor. The remaining transplants were performed with grafts from living donors. Waitlist mortality varied geographically, reaching 37.4 per 100 waitlist-years in some regions. Despite the increase in organ donation from young donors due to the opioid epidemic in the US, 707 livers were discarded during 2018. The organ discard rate (percentage of livers recovered for transplant and not transplanted) was 8.4%. The discard rate varied among donor age groups, being higher

in older donors [78](**Figure 2**). This suggest an opportunity to increase the donor pool. Ex vivo machine perfusion arises as an objective tool for assessment and repair of these grafts that otherwise would be discarded according to current clinical practice.

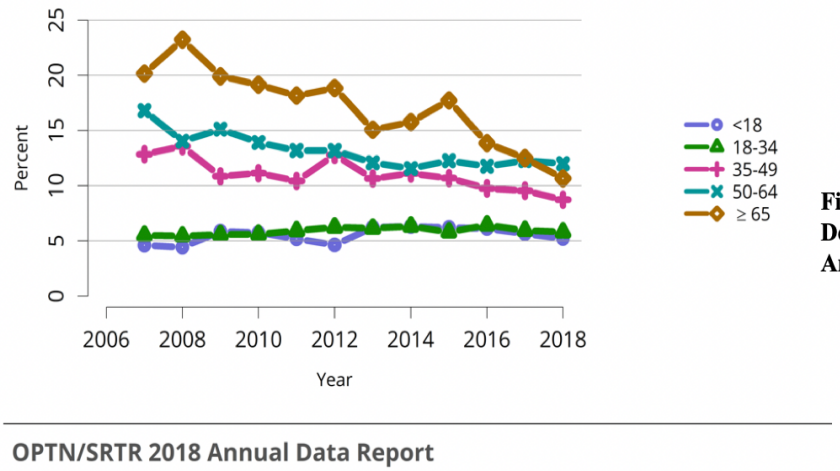

Figure 2. Organ Discard According to Donor Age. Adapted from UNOS 2018 Annual Report.

During the beginnings of liver transplantation, grafts from donation after circulatory death (DCD) were the main donor source. Along with the development of preservation solutions, the definition of brain death by the Ad Hoc Committee of the Harvard Medical School in 1968 increased the use of grafts from donation after brain death (DBD) [79-80].

In contrast to DCD, DBD grafts are of better quality and less prone to ischemia reperfusion injury (IRI) during procurement. Oxygenated blood is perfused through the organs by the donor´s heart while the surgical team dissects the organs. Warm ischemia is minimized in DBD. Immediately after physiological blood perfusion of the organs ceases, cold preservation solution is infused to the organs [81]. In DCD, procurement can only be executed once cardiac arrest is certified. This period of time between cessation of hemodynamic support and cardiac arrest portends a critical period of warm ischemia

and poor perfusion that harms the graft. Organs from older donors, or those with fatty infiltration tolerate badly this period of warm ischemia. Discard rates of DCD are known to be higher than DBD grafts due to the lower quality and nature of procurement technique [82]. Nowadays, DBD represents the main source of organs for transplantation in the western world.

Despite being a good source, DBD is not enough to supply the demand and limit waitlist mortality. In 1990, DCD regained interest by the initiative of transplant teams in the Netherlands [83,84]. It was not until 2011 when this procurement modality was approved by the World Health Organization (WHO) [85]. Since then, this practice has been adopted by many countries world-wide. Donation after cardiac death donors are classified according to the Maastritch Classification [86] (**Table 3**). Type I, II, and V donors are uncontrolled and within the DCD classification carry a higher risk of graft dysfunction upon their use. Type III and IV donors are controlled, thus facilitating logistics during procurement and minimizing warm ischemic injury previous to transplantation.

Table 3. Maastritch Classification.

I	Dead on arrival	Uncontrolled
II	Unsuccessful resuscitation (CPR)	
III	Awaiting cardiac arrest	Controlled
IV	Cardiac arrest after brain-stem death	
V	Cardiac arrest in a hospital patient	Uncontrolled (added in 2000)

In recent years the use of DCD grafts has increased in North America and Europe demonstrating promising results. Extended criteria donors along with DCD has expanded the donor pool. However, the discard rate of DCD grafts compared to those derived from DBD continues to be high [78] (**Figure 3)**. In general, 20-30 % discard rates following recovery are reported for DCD. An important limitation of DCD grafts is the associated risk of developing post-transplant complications such as PNF and ischemic type biliary strictures. Despite these limitations and concerns, some series report that long term results in patients transplanted with DCD grafts seem to have similar patient survival compared to those receiving DBD grafts [87, 88]. Of note, in donors with a warm ischemia time (WIT) over 25 minutes, an increased risk for diminished graft survival has been seen.

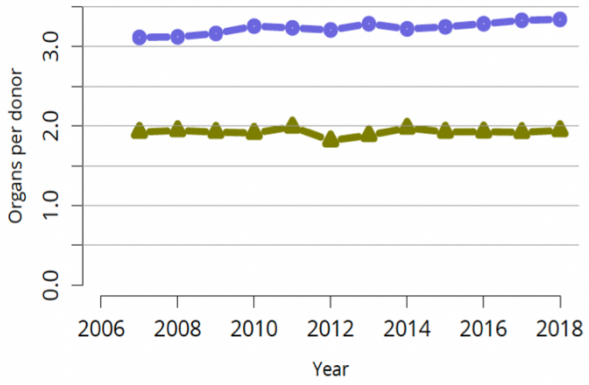

Figure 3. Organs Transplanted from Donation After Brain Death (DBD) vs Donation After Cardiac Death (DCD). Adapted from UNOS 2018 Annual Report.

1.4 Principles of Static Cold Storage:

Static cold storage (SCS) inhibits enzymatic activity, limits ATP consumption, and decreases redox potential of the cell [89]. During hypoxia transport of H^+ and e^- ceases, thus creating accumulation of reduced species and subsequent mitochondrial dysfunction. These phenomena are linked with a disbalance in ionic transport which

results in edema and alterations of water homeostasis. In turn, the cell swells and is more prone to injury and dysfunction as a whole [90,91].

The basis of preservation solutions is protection of intracellular compartments upon the onset of ischemic damage. The initial Collins solution introduced for clinical use had a high content of potassium and used glucose to create an osmotic barrier within the cell [76]. The development of solutions towards longer preservation periods was achieved by substituting glucose with mannitol or sucrose. Euro-Collins solution was widely used as an optimal preservation solution until the 1980s. Since then, many other solutions have been developed having colloids and citrate as impermeant molecules; buffers such as bicarbonate, phosphate, histidine, and lactobionate to stabilize pH and protein degradation; free radical scavengers as glutathione and tryptophan to counteract reactive oxygen species (ROS); and nutrients to support metabolic activity of the cell during cold ischemia [92] **(Table 4)**.

Table 4. Preservation Solutions for Static Cold Storage.

	Celsior	EC	HOC	HTK	IGL-1	UW	Belzer MPS
Colloids (mM)							
HES	-	-	-	-	-	0.25	0.25
PEG	-	-	-	-	0.03	-	-
Impermeants (mM)							
Citrate*	-	-	80	-	-	-	-
Gluconate	-	-	-	-	-	-	85
Glucose	-	195	-	-	-	-	10
Histidine*	30	-	-	198	-	-	-
Lactobionate*	80	-	-	-	100	100	-
Mannitol*	60	-	185	38	-	-	30
Raffinose	-	-	-	-	30	30	-
Ribose	-	-	-	-	-	-	5
Buffers (mM)							
HEPES	-	-	-	-	-	-	10
K_2HPO_4	-	15	-	-	-	-	-
KH_2PO_4	-	43	-	-	25	25	25
$NaHCO_3$	-	10	10	-	-	-	-
Electrolytes (mM)							
Calcium	0.25	-	-	0.0015	-	-	0.5
Chloride	42	15	-	32	20	20	1
Magnesium	13	-	40	4	5	5	5
Potassium	15	115	84	9	25	120	25
Sodium	100	10	84	15	120	30	100
ROS scavenger (mM)							
Allopurinol	-	-	-	-	1	1	-
Glutathione	3	-	-	-	3	3	-
Tryptophan	-	-	-	2	-	-	-
Nutrients (mM)							
Adenine	-	-	-	-	-	-	5
Adenosine	-	-	-	-	5	5	-
Glutamate	20	-	-	-	-	-	-
Ketoglutarate	-	-	-	1	-	-	-
Osmolality (mOsm)	255	406	400	310	320	320	300

1.4.1 Hepatic Ischemia Reperfusion Injury:

Ischemia reperfusion injury continues to be the most common cause of post-transplant graft dysfunction. Tissue damage during IRI is biphasic in nature. The first hit is encountered by the graft during organ procurement once perfusion with oxygenated blood ceases and cold static preservation solution is infused to the liver bed. The second hit comes when the graft is re-perfused in the recipient with warm blood [93, 94]. Both events are characterized by amplification of an inflammatory response which takes into

account hepatocytes, liver sinusoidal endothelial cells (LSEC), Kupffer cells (KC), stellate cells, lymphocytes, macrophages, and platelets [95,96].

Hypoxia during cold ischemia leads to mitochondrial dysfunction which in turn triggers abruption of oxidative phosphorylation and adenosine triphosphate (ATP) depletion. This creates an imbalance in electrolyte homeostasis, acceleration of glycolysis and lactate accumulation. During reperfusion injury (once the graft is transplanted), reintroduction of oxygen to ischemic tissues amplifies the inflammatory cascade generating ROS. Shear stress derived from blood perfusion onto the LSEC, recruits neutrophils which in turn activate immunological signaling pathways within the liver milieu [90] .

Liver sinusoidal endothelial cells are in the front line of defense against IRI. Their role is to control vascular tone, inflammation, homeostasis, and maintain toxic clearance. During cold ischemia, LSEC and KP undergo edema due to transient transmembrane transport dysfunction. Along with the dysregulation of nitric oxide (NO), endothelin (ET), and thromboxane A2 (TXA2), narrowing of the sinusoidal space leads to microcirculation dysfunction and alteration of homeostasis [97]. During reperfusion, LSEC and KC suffer a profound activation process which involves activation of tumor necrosis factor alpha (TNF α), interleukin-1 (IL-1), interleukin-12 (IL-12), and interferon γ (INF γ). These pro-inflammatory cytokines upregulate the expression of adhesion molecules and activate the recruitment of CD4+ T Lymphocytes [98,99,100].

The initial innate immune response is non-antigen specific and supports itself in the activation of macrophages, neutrophils, dendritic cells (DC), and natural killer cells.

Amplification of the immune response leads to activation of the antigen-specific pathway, which in turn leads to recruitment of antigen presenting cells, and T/B cell interactions. The bigger the response, the more prone is the recipient to develop rejection in the future [101].

Better understanding of the molecular mechanisms involved in the two phases of IRI have led to investigate immunomodulation in murine and porcine models. Unfortunately, none of these models have been translated to clinical practice. Any intervention in the donor to modulate IRI solely for its attenuation on the liver would affect other organs during procurement. These interventions are not realistic from a clinical perspective and none have been implemented with success in clinical practice. Ex vivo machine perfusion gives us the opportunity for the first time to apply these interventions without harming other grafts.

1.5 Principles of Machine Perfusion as a Preservation Technique.

The organ shortage has resulted in a high mortality on the waiting list for liver transplantation. This has triggered interest in extending the donor pool by using marginal grafts, such as those retrieved from elderly donors, DCD grafts, or livers with steatosis. Strategies as hypothermic machine perfusion (HMP), hypothermic oxygenated machine perfusion (HOPE), sub-normothermic machine perfusion, and normothermic machine perfusion have the potential to improve the outcome of liver transplantation with marginal grafts by reducing preservation injury and improving graft assessment.

Ex vivo liver perfusion was tested initially in animal models and with discarded human grafts before its application in clinical trials. In this portion of the literature review we will focus on the different types of ex vivo perfusion techniques.

1.5.1 Hypothermic Machine Perfusion (HMP)

Several groups have focused their research on HMP following the promising results of cold perfusion in kidney grafts [102]. These strategies are based on the principle of "washing out" waste products from the vascular endothelium, thus enhancing preservation time and allowing assessment previous to transplantation. None of these advantages are seen with SCS.

1.5.2 Hypothermic Anoxic Machine Perfusion

The team from Columbia University validated their perfusion device with the use of non-transplantable discarded human livers along with a porcine liver transplant model. Grafts were perfused via portal vein and hepatic artery with Vasosol solution at a temperature of 3 – 5ºC [103]. The device used was a flow-controlled modified Medtronic Portable By-Pass system. In the discard protocol the group reports no technical issues or machine failure. Once translated to the porcine liver transplant model, no differences were seen between livers subjected to HMP compared to those preserved by SCS. This proof of concept study was the basis to move forward to the first HMP clinical trial [104]. The study included 20 liver transplant recipients of standard criteria grafts undergoing HMP preservation. Cases were matched to SCS controls. The HMP group presented less post-transplant hospital stay, no vascular complications, less biliary complications, lower IRI markers and less incidence of EAD. There were no cases of PNF in either group [104].

The model was challenged with the use of ECD grafts. Published in 2015, the study demonstrated the benefits of hypothermic anoxic machine perfusion over SCS regarding

outcomes with extended criteria grafts [105]. Assessment of adhesion molecules as well as inflammatory cytokines indicated decreased markers of inflammation within grafts preserved with HMP. As seen in other studies, HMP proved to achieve better results than SCS regarding post-transplant complications and length of stay [105, 106].

1.5.3 Hypothermic Oxygenated Machine Perfusion (HOPE)

Hypothermic oscillating machine perfusion was initially described in a murine perfusion model [107,108]. Compared to SCS, rat livers subjected to cold machine perfusion were charged regarding ATP stores. This was measured by computer-aided liver perfusion devices. In subsequent animal experiments HOPE demonstrated reduction of necrosis, less release of aspartate aminotransferase (AST) and increased bile flow in DCD grafts during reperfusion [109,110, 111] .

Similar, hypothermic oxygenated machine perfusion was evaluated in a human study involving eight end stage liver disease recipients receiving DCD grafts (Maastricht category III). Livers were perfused for a period of 1-2 hours prior to transplantation. Recipients of perfused DCD grafts were matched with recipients of standard criteria grafts (brain death donors). After reperfusion, each perfused DCD graft had excellent function with low serum AST and alanine aminotransferase (ALT) levels. Post-transplant hospital-stay and costs were comparable in DCD and standard criteria graft recipients. During the 6-month follow up no patients in the study group presented bile duct strictures [112,113,114]. Seven-year results of HOPE treatment for EC grafts from DBD and DCD have been presented validating fluorometric analysis of flavin mononucleotide (FMN) as a marker of mitochondrial injury. The levels of FMN

correlate with the grade of injury and identify those livers which pose a risk for PNF or DGF [115].

1.5.4 Sub-normothermic and Normothermic Machine Perfusion

The principle of normothermic and sub-normothermic machine perfusion (SNMP) is to maintain a metabolically active graft prior to transplantation. Opposed to cold perfusion, temperatures above 33 ºC are expected to maintain metabolic activity, thus giving physicians the opportunity to objectively assess the graft and modulate IRI.

Several groups demonstrated that sub-normothermic ex vivo liver perfusion reduced liver and bile duct injury in DCD and marginal grafts in porcine liver transplant models [116, 117, 118, 119, 120, 121, 122]. This technology offers for the first time a platform with the potential to modulate the immune response, treat hepatitis C, apply stem cell therapy and deliver nanoparticles previous to transplantation [123].

The first clinical trial of Normothermic Machine Perfusion (NMP) was presented in 2016 by Ramikuvar et al [124]. Twenty grafts were perfused and subsequently transplanted. Compared to SCS controls, seven-day peak AST levels post-transplant were lower in the NMP group. Other groups subjected discarded livers to NMP and transplanted these grafts obtaining good results and function at 7 months post-transplant [125]. The first clinical trial results in North America presented by Selzner et al, demonstrated the feasibility of perfusing standard criteria grafts. Twelve perfused grafts had lower levels of AST and ALT post-transplant compared to SCS controls [126]. Ultimately, Nasralla et al presented the first randomized controlled trial comparing NMP to SCS. The authors selected differences in peak AST levels as the primary end

point. As hypothesized, grafts undergoing NMP presented lower levels of peak AST compared to those preserved on ice [127]. The organ discard rate was 50% lower in the NMP group. No differences in bile duct complications, graft survival or patient survival were seen between groups.

Despite the promising results of NMP and SNMP, perfusion devices and perfusates continue to pose a challenge regarding costs and logistics. Blood-based perfusates lead to dependence on blood products from the blood bank. Devices such as the Organox ®, Liver Assist®, and Transmedics® perfusion device use blood derived products combined with albumin-based colloids to maintain osmotic pressure.

Normothermic regional perfusion (NRP) has appeared as an initiative to overcome the logistic and economical limitations of NMP and SNMP [128,129]. Following femoral artery and vein donor cannulation after cardiac arrest, the abdominal organs are perfused in situ using the donor´s blood. Liver function is assessed for a period of 2 – 4 hours by measurement of liver enzymes and lactate clearance. In countries as Spain the law consents donor cannulation previous to treatment withdrawal. In uncontrolled DCD Maastricht type III this advantage has shortened warm ischemia times and shown promising results compared to other countries who have a more rigorous law. Normothermic regional perfusion has shown a benefit in reducing biliary complications and graft loss with the use of controlled and uncontrolled DCD grafts [130,131].

1.5.5 Combined Strategies

Combined strategies of NRP and HMP for DCD grafts have been reported as feasible [132]. Good outcomes regarding post-transplant graft function and ischemic bile duct injury have been reported. Other groups have reported an “all warm” technique by combining NRP and NMP. This technique supports an ischemia free procurement and

preservation avoiding cold flush of the liver. Of note, the procedure is complex from a technical point of view and has limitations regarding it´s application to current clinical practice [133]. To date there is no clinical trials comparing all different strategies of machine perfusion.

2. Justification and Aims

The optimal perfusion setup and temperature for ex-vivo machine preservation of liver grafts has not been defined yet. Despite numerous studies in porcine models and human clinical trials, no direct comparison has been done using different compositions of the perfusate. Adding anti-inflammatory agents plus sub-normothermic temperature could improve the protective effects of ex vivo organ perfusion.

On the other hand, the use of vasodilators during ex vivo normothermic and sub-normothermic graft preservation has not been compared and validated among different perfusion setups. Vasodilators reverse hepatic artery vasospasm caused by static cold storage and attenuate ischemia reperfusion injury. The optimal vasodilator to be used during ex vivo graft preservation with an effective and safe profile is still to be determined.

Our goal is to study the application of protective anti-inflammatory strategies with the addition of substances known to attenuate ischemia reperfusion injury.

Therefore, the **general objective** of this thesis is to provide knowledge on liver graft preservation with the use of ex-vivo machine perfusion.

Specific Aims

1. To evaluate if the addition of anti-inflammatory agents plus sub-normothermic temperature improves the protective effects of ex vivo organ perfusion on preservation injury in a porcine liver transplant model.

This aim was developed in the study “Anti-inflammatory Signaling During Ex Vivo Liver Perfusion Improves the Preservation of Pig Liver Grafts Before Transplantation” published in *Liver Transplantation* in November 2016.

2. To compare the safety and efficacy of 3 different types of vasodilators, BQ123 (endothelin 1 antagonist), Epoprostenol (prostacyclin, prostaglandin I2) and Verapamil (non-dihydropyridine calcium channel─antagonist) during normothermic ex-vivo liver machine perfusion in a porcine transplant model.

These aims were developed in the second study “Comparison of BQ123, Epoprostenol, and Verapamil as Vasodilators During Normothermic Ex Vivo Liver Machine Perfusion” published in *Transplantation* in April 2018.

3. Publications

STUDY 1.

Anti-inflammatory Signaling During Ex Vivo Liver Perfusion Improves the Preservation of Pig Liver Grafts Before Transplantation.

Authors: Nicolas Goldaracena, **Juan Echeverri**, Vinzent N. Spetzler, Johan M. Kaths, Andrew S. Barbas, Kristine S. Louis, Oyedele A. Adeyi, David R. Grant, Nazia Selzner, Markus Selzner.

Journal: Liver Transplantation. 22. November 2016. 1573-1583.

Impact factor: 4.57

STUDY 2

Comparison of BQ123, Epoprostenol, and Verapamil as Vasodilators During Normothermic Ex Vivo Machine Perfusion.

Authors: **Juan Echeverri**, Nicolas Goldaracena, Johan Moritz Kaths, Ivan Linares, Roizar Roizales, Dagmar Kollmann, Matyas Hamar, Peter Urbanellis, Sujani Ganesh, Oyedele A. Adeyi, Mahmood Tazari, Markus Selzner, Nazia Selzner.

Journal: Transplantation. 102. April 2018. 601-608.

Impact factor: 3.67

TOTAL IMPACT FACTOR OF THE THESIS: 8.24

4. Anti-inflammatory Signaling During Ex Vivo Liver Perfusion Improves the Preservation of Pig Liver Grafts Before Transplantation

Anti-inflammatory Signaling During Ex Vivo Liver Perfusion Improves the Preservation of Pig Liver Grafts Before Transplantation

Nicolas Goldaracena,[1] Juan Echeverri,[1,4] Vinzent N. Spetzler,[1] Johan M. Kaths,[1] Andrew S. Barbas,[1] Kristine S. Louis,[1] Oyedele A. Adeyi,[2] David R. Grant,[1] Nazia Selzner,[3] and Markus Selzner[1]

Departments of [1]Surgery, [2]Pathology, and [3]Medicine, Multi-Organ Transplant Program, Toronto General Hospital, University of Toronto, Toronto, Ontario, Canada; and [4]Programa de Doctorat en Cirurgia i Ciències Morfològiques, Universitat Autònoma de Barcelona, Barcelona, Spain

Normothermic ex vivo liver perfusion (NEVLP) improves graft preservation by avoiding cold ischemia injury. We investigated whether the protective effects of NEVLP can be further improved by applying strategies targeted on reducing the activation of proinflammatory cytokines during perfusion. Livers retrieved under heart-beating conditions were perfused for 4 hours. Following the preservation period, a pig liver transplantation was performed. In group 1 (n = 5), anti-inflammatory strategies (alprostadil, n-acetylcysteine, carbon monoxide, sevoflurane, and subnormothermic temperature [33°C]) were applied. This was compared with a perfused control group (group 2) where livers (n = 5) were perfused at 37°C without anti-inflammatory agents, similar to the setup used in current European clinical trials, and to a control group preserved with static cold storage (group 3). During 3-day follow-up, markers of reperfusion injury, bile duct injury, and liver function were examined. Aspartate aminotransferase (AST) levels during perfusion were significantly lower in the study versus control group at 1 hour (52 ± 6 versus 162 ± 86 U/L; $P = 0.01$), 2 hours (43 ± 5 versus 191 ± 111 U/L; $P = 0.008$), and 3 hours (24 ± 16 versus 218 ± 121 U/L; $P = 0.009$). During perfusion, group 1 versus group 2 had reduced interleukin (IL) 6, tumor necrosis factor α, and galactosidase levels and increased IL10 levels. After transplantation, group 1 had lower AST peak levels compared with group 2 and group 3 (1400 ± 653 versus 2097 ± 1071 versus 1747 ± 842 U/L; $P = 0.47$) without reaching significance. Bilirubin levels were significantly lower in group 1 versus group 2 at day 1 (3.6 ± 1.5 versus 6.60 ± 1.5 μmol/L; $P = 0.02$) and 3 (2 ± 1.1 versus 9.7 ± 7.6 μmol/L; $P = 0.01$). A trend toward decreased hyaluronic acid, as a marker of improved endothelial cell function, was observed at 1, 3, and 5 hours after reperfusion in group 1 versus group 2. Only 1 early death occurred in each group (80% survival). In conclusion, addition of anti-inflammatory strategies further improves warm perfused preservation.

Liver Transplantation 22 1573–1583, 2016 AASLD.
Received January 15, 2016; accepted July 21, 2016.

Warm ex vivo liver perfusion has emerged as an alternative organ preservation method for liver transplantation (LT).[1-3] Reducing cold ischemia time is an attractive strategy to minimize cold ischemia injury, which is poorly tolerated by marginal grafts. Most studies so far have focused on using warm perfused preservation as a technique to avoid cold storage. The new technique of warm perfused graft preservation, however, extends beyond changing cold storage to warm perfusion. The active metabolism during the warm perfused preservation time offers the opportunity to assess graft function, but also to provide mediators against preservation injury prior to reperfusion. Reperfusion injury in the setting of LT is characterized by the activation of a proinflammatory cascade, which includes release of interleukin (IL) 1, IL6, and tumor necrosis factor α (TNF-α). Warm perfused graft

Abbreviations: ALP, alkaline phosphatase; AST, aspartate aminotransferase; CO, carbon monoxide; DCD, donation after circulatory death; EC, endothelial cell; ELISA, enzyme-linked immunosorbent assay; H & E, hematoxylin-eosin; HA, hyaluronic acid; HBD, heart-beating donor; HPF, high-power field; HSP70, heat shock protein 70; IL, interleukin; INR, international normalized ratio; IRI, ischemia/reperfusion injury; LT, liver transplantation; NEVLP, normothermic ex vivo liver perfusion; PECAM-1, platelet-endothelial cell adhesion molecule; SCS, static cold storage; TNF-α, tumor necrosis factor α.

preservation represents a new platform to apply anti-inflammatory strategies before graft reperfusion.

Both subnormothermic and normothermic techniques have been investigated with very good results in porcine models.[1,2,4] The optimal perfusate composition and temperature have not been defined yet. The impact of applying anti-inflammatory strategies to the perfusion system is unclear. The addition of different anti-inflammatory agents during ex vivo perfusion might have additional protective effects on the graft besides avoiding cold ischemia injury. In addition, subnormothermic temperatures have strong inhibitory effects on Kupffer cells with a reduction of proinflammatory cytokine release in rodent and porcine models.[5-8]

In the present study, we evaluated if the addition of anti-inflammatory agents plus subnormothermic temperatures improves protective effects of ex vivo organ perfusion on preservation injury in a porcine transplant model.

Material and Methods

STUDY DESIGN

Using a porcine LT model, 2 different ex vivo liver perfusion setups were applied as preservation methods and compared with one another. Only livers retrieved from heart-beating donors (HBDs) were used in this study. In 1 group, before LT occurred, livers were perfused under subnormothermic conditions (33°C) using our previously described perfusion system (group 1).[2,9] In the second group (group 2), livers were perfused under normothermic conditions (37°C) as described and were used in current clinical trials.[10] A third group (group 3) of livers were preserved under static cold storage (SCS) for a period of 6 hours without ex vivo machine perfusion before transplantation and were compared with the 2 perfused liver groups in order to assess differences between standard current practice and novel perfusion setups. Besides the temperature at which the grafts were perfused, perfusates differed only in the presence or absence of anti-inflammatory strategies and the type of vasodilator. In both groups, a HBD pig liver retrieval was performed and after a short period of cold ischemia time (2 hours), liver grafts were perfused for 4 hours with either perfusion method. Following the preservation period (6 hours overall), orthotopic pig LT was performed as previously described.[11] Pigs were killed 3 days after transplantation. Five pigs were included in each group.

Address reprint requests to Markus Selzner, M.D., Department of Surgery, Multi-Organ Transplant Program, Toronto General Hospital, University of Toronto, NCSB 11C-1244, 585 University Avenue, Toronto, ON, Canada, M5G2N2. Telephone: 1-416-340-4800, ext. 5884; FAX: 1-416-340-5321; E-mail: markus.selzner@uhn.ca

View this article online at wileyonlinelibrary.com.

DOI 10.1002/lt.24603

Potential conflict of interest: Nothing to report.

ANIMALS

Male Yorkshire pigs, 30-35 kg, were used for this study. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care of Laboratory Animals" published by the National Institutes of Health. The Animal Care Committee of the Toronto General Research Institute approved all studies.

EX VIVO PERFUSION SETUP DESCRIPTION

In group 1, a subnormothermic ex vivo liver perfusion was performed as previously described by our group.[2,9] Briefly, the liver was perfused at 33°C with a continuous flow through the hepatic artery and portal vein. Perfusion through the hepatic artery was set at a pressure of 60 mm Hg resulting in a flow of approximately 400 mL/minute. The portal vein pressure was adjusted between 2 and 4 mm Hg corresponding to a flow of 900 to 1100 mL/minute. In this perfusion system, different agents were added to the perfusate due to their anti-inflammatory properties. Therefore, composition of the perfusate consisted of 1.5 L of Steen solution (XVIVO Perfusion, Goteborg, Sweden) and washed pig erythrocytes to achieve a hematocrit of 15%. Before adding them to the perfusion system, erythrocytes were passed through a leukocyte filter and washed once in sterile saline solution to avoid contamination with leukocytes or plasma. The Steen solution is a buffered extracellular-type solution containing dextran and albumin to provide an optimized colloid osmotic pressure. The perfusate contained heparin

(10,000 IU; Sandoz Canada, Quebec, QC, Canada), amino acid concentrate (Travasol 4.25%, 50 mL bolus plus 8 mL/hour; Baxter, Hamilton, ON, Canada), D50W (2-5 mL/hour; Baxter, Mississauga, ON, Canada), and insulin (125 IU/hour; NovoRapid, Novo Nordisk, Mississauga, ON, Canada). Also, cefazolin (1 g; Pharmaceutical Partners of Canada, Richmond Hill, ON, Canada) and metronidazole (500 mg; Baxter, Mississauga, ON, Canada) were added to prevent bacterial contamination. To improve flow properties by vasodilatation, a bolus of BQ123[12] at both perfusion initiation and after 2 hours (1.25 mg; AG Scientific, Kelowna, BC, Canada) were used. Also, a continuous infusion of prostaglandin E1 (500 μg/3 hours; Pfizer, Kirkland, QC, Canada) was administrated for its anti-inflammatory property as described.[13] Acetylcysteine[14] (6 g; Sandoz, Quebec, QC, Canada) was added for its radical scavenging properties. A gas composition containing 95% O_2 and 5% CO_2 was connected to the oxygenator at a sweep of 2 L/minute. Additionally, active gaseous components were added: carbon monoxide (CO; 750 ppm; Praxair, Burlington, ON, Canada) for its vasodilatative and anti-inflammatory properties[15-18] and sevoflurane (1%, Abbott, Saint-Laurent, QC, Canada) for its protective properties on endothelial cells (ECs).[19]

Our perfusion system was compared with a different perfusion setup (group 2) whose composition was based on the perfusion system used for the recently completed European randomized controlled trial using the Metra perfusion device.[1,10] To facilitate the comparison between the 2 perfusion systems, Steen solution was also used to provide physiological oncotic pressure to the group 2 perfusate. In this case, perfusion was performed at 37°C (normothermic conditions). The circuit was primed with the following components: 1.5 L of Steen solution and washed pig erythrocytes, heparin (10,000 IU), 20 mmol of sodium bicarbonate, and 9.2 mmol/L of calcium chloride. As a vasodilator, prostacycline (Flolan, GlaxoSmithKline Inc., Mississauga, ON, Canada) was administered at a continuous infusion of 4 mL/hour (8 μg/hour) as previously described.[10] Nutrition of the perfusate consisting of 96 g aminoacids and 300 g glucose were adjusted to keep a perfusate glucose level of $\geq$10 mmol/L. Regular human insulin (NovoRapid Insulin Aspart, Novo Nordisk Inc., Mississauga, ON, Canada) at 100 IU was also administered to the perfusate. The same antibiotic prophylaxis described in group 1 was used to prevent bacterial contamination in the control group perfusion. Gas composition consisted only of 95% O_2 and 5% CO_2. No anti-inflammatory additives were included in this group.

PIG LT

A model of HBD liver procurement was used. Donor pigs received 30,000 IU of heparin 5 minutes prior to organ flush. All livers were flushed with a total of 3 L of cold University of Wisconsin solution (SPS-1, Organ Recovery Systems, Itasca, IL) through both the aorta and portal vein. After being flushed, liver grafts were stored on ice (4°C) for 2 hours followed by 4 hours of ex vivo liver perfusion. At the end of the preservation time (6 hours), orthotopic pig LT was performed using an active portojugular shunt (Rotaflow centrifugal pump, Maquet, Hirlingen, Germany) as previously described.[11] In group 3, liver procurement was performed as described previously. These grafts underwent a period of SCS of 6 hours without exposure to ex vivo machine perfusion. After SCS, livers were transplanted to recipient pigs using the same technique described above.

The animals were followed up for 3 days after LT. If any animal met suffering criteria (lethargy, failure to move coordinately, metabolic or respiratory decompensation, excessive bleeding) before the intended survival, pigs were sacrificed in accordance to our animal use protocol to avoid animal suffering. At autopsy, the patency of all anastomoses was confirmed. Pigs were exsanguinated under deep isoflurane anesthesia after central liver and bile duct (each right and left bile duct) specimens were obtained.

LIVER AND EC INJURY EVALUATION

During perfusion, serum aspartate aminotransferase (AST) levels were measured hourly for 3 hours. After reperfusion, AST was measured every hour for 6 hours and daily thereafter. Total serum bilirubin and alkaline phosphatase (ALP) were also measured after transplantation on a daily basis as markers of bile duct damage. A hyaluronic acid (HA) enzyme–linked immunosorbent assay (ELISA) was used to assess the EC function during machine perfusion and at 3 hours and 5 hours after transplantation (quantitative sandwich enzyme immunoassay technique, R&D Systems, Minneapolis, MN).[20,21] During the follow-up, international normalized ratio (INR) was measured daily as a liver function marker.

TNF-α AND β GALACTOSIDASE ANALYSIS DURING PERFUSION AND AFTER TRANSPLANTATION

TNF-α is an acute phase reaction cytokine involved in the immune response to diverse forms of inflammation. β galactosidase is a glycohydrolase that predominates within lysosomes and is released during Kupffer cell activation and death. In the liver, both are key mediators of ischemia and reperfusion injury. TNF-α and β galactosidase release was measured during ex vivo machine perfusion and after transplantation in both groups and compared between one another to evaluate the inflammatory response in each group. TNF-α and β galactosidase levels were assessed at 3 and 5 hours after reperfusion.

CYTOKINE PROFILING: IL6 AND IL10

Proinflammatory and anti-inflammatory cytokines play important roles in hepatic ischemia/reperfusion injury (IRI). IL6 promotes injury by altering the expression of other cytokines and influencing neutrophil recruitment. In contrast, IL10 is a potent anti-inflammatory cytokine that protects against IRI. Cytokine levels were determined by ELISA in group 1 (33°C) and group 2 (37°C) both during ex vivo machine perfusion and after reperfusion in recipient pigs.

HISTOLOGICAL EVALUATION

At 2 hours after reperfusion, core liver biopsies were performed and stained with cleaved caspase 3 antibody immunohistochemistry (Cell Signaling Technology, Danvers, MA) as a marker of apoptosis.[22] Histological slides were evaluated at 20× objective magnification. Staining positive cells were counted and averaged for 10 random high-power fields (HPFs).

Sinusoidal EC integrity was assessed by CD31 immunohistochemistry (PECAM, Santa Cruz Biotechnology, Dallas, TX) from core biopsies obtained 2 hours after reperfusion.[23] Integrity of the sinusoidal cell lining was evaluated and scored by a blinded investigator. Also, at the end of survival, liver parenchyma and bile duct specimens were obtained to assess necrosis by hematoxylin-eosin (H & E) staining. All histological specimens were stored in 10% formalin for 24 hours and then transferred to 70% ethanol until paraffin embedding. Sections of 5-μm thickness were cut and processed according to protocols of the Pathology Research Program, University Health Network, Toronto, Ontario, Canada.

STATISTICAL ANALYSIS

The data were analyzed with the SPSS 22 statistical package (IBM, Chicago, IL). Mann-Whitney U test was used for the comparison of continuous variables, whereas a chi-square test was applied for categorical outcome. The 1-way analysis of variance was used for the comparison of variables between 3 groups. The results are presented as mean ± standard deviation and were considered significant at the level of $P \leq 0.05$.

Results

HEPATOCYTE INJURY AND EC FUNCTION DURING NORMOTHERMIC EX VIVO LIVER PERFUSION

During ex vivo liver perfusion, AST levels in group 1 (33°C) remained at close to normal levels comparable to sham-operated pigs. In contrast, AST levels in group 2 (37°C) showed a progressive increase during ex vivo perfusion. AST levels at 1 hour ($P = 0.01$), 2 hours ($P = 0.008$), and 3 hours ($P = 0.009$) were significantly lower in group 1 (33°C) versus group 2 (37°C; Fig. 1).

We also assessed EC function by measuring HA clearance. HA is removed by ECs from the circulation and therefore increased HA levels correspond to

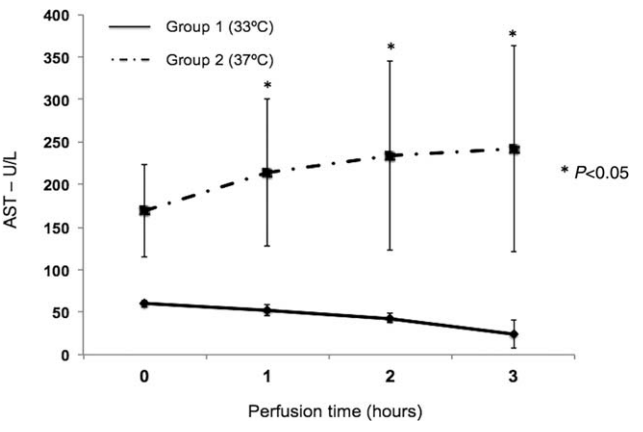

FIG. 1. AST values during ex vivo machine perfusion.

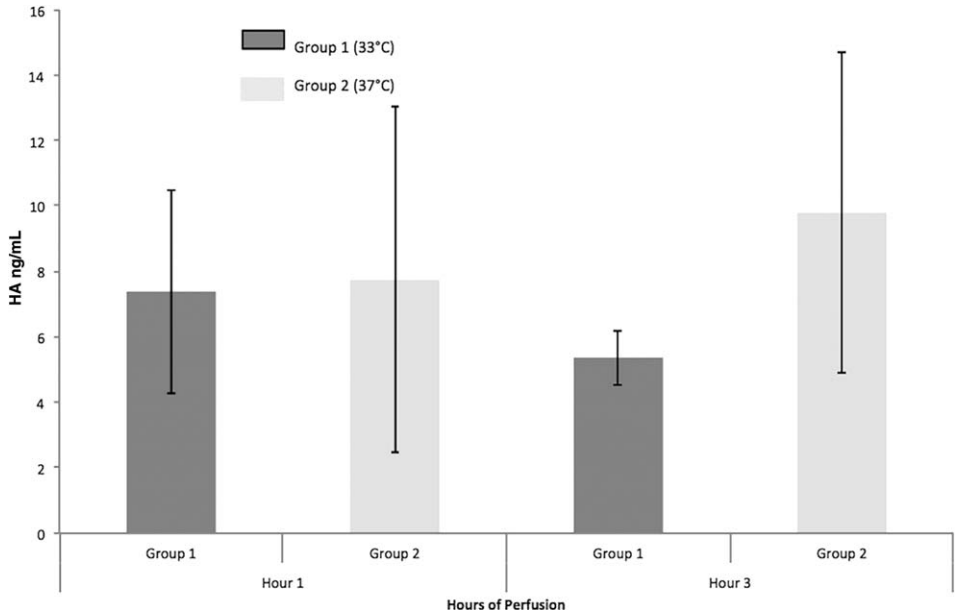

FIG. 2. HA concentration during machine perfusion.

decreased EC function. HA levels during perfusion were lower in group 1 (33°C) during the first and third hour of perfusion compared with group 2 (37°C; hour 1, 7.4 ± 3.1 versus 7.7 ± 5.3 ng/mL; $P = 0.91$; hour 3, 5.4 ± 0.8 versus 9.8 ± 4.9 ng/mL; $P = 0.08$, respectively; Fig. 2).

INFLAMMATORY SIGNALING DURING PERFUSION AND AFTER TRANSPLANTATION AS A MARKER OF INFLAMMATORY RESPONSE

TNF-α release as a marker of inflammatory response and β galactosidase as a specific marker of Kupffer cell activation were analyzed during ex vivo machine perfusion and after transplantation. After 3 hours of perfusion, TNF-α levels were significantly lower in group 1 versus group 2 (102.8 ± 101.3 versus 394.1 ± 154.5 pg/mL; $P = 0.01$). Similarly, 3 hours (186 ± 67 versus 349 ± 155 pg/mL; $P = 0.06$) and 5 hours (148 ± 94 versus 181 ± 49 pg/mL; $P = 0.49$) after transplantation, TNF-α levels were lower in group 1 (33°C) versus group 2 (37°C) without reaching statistical significance (Fig. 3). β galactosidase levels were lower in group 1 (33°C) at 1 (100.3 ± 35 versus 122.9 ± 59 U/mL; $P = 0.54$) and 3 hours (93.5 ± 54.6 versus 170.2 ± 49.5 U/mL; $P = 0.08$) of perfusion compared with group 2 (37°C). On the postoperative period, levels were higher in group 1 (33°C) compared with group 2 (37°C; hour 3, 166.4 ± 17 versus 127.2 ± 9.6 U/mL; $P = 0.004$; hour 5, 178.4 ± 8.4 versus 126.4 ± 7.8 U/mL; $P < 0.001$, respectively; Fig. 3).

Proinflammatory IL6 expression was measured during perfusion and after transplantation in the recipient. IL6 levels were significantly lower in group 1 (33°C) at the third hour of perfusion compared with group 2 (37°C; 73.8 ± 34.6 versus 1124.5 ± 355.4 pg/mL; $P = 0.01$, respectively; Fig. 4). Posttransplant levels of IL6 were lower in group 1 (33°C) versus group 2 (37°C) during the third and fifth hour after reperfusion (third hour, 1156.3 ± 269.8 versus 3582 ± 1604.7 pg/mL; $P = 0.02$; fifth hour, 925 ± 851 versus 1425.7 ± 994 pg/mL; $P = 0.43$) respectively (Fig. 4).

Although not significant, anti-inflammatory IL10 expression was higher during the first hour of perfusion in group 1 (33°C) versus group 2 (37°C; Fig. 4). Postreperfusion expression of IL10 was higher in group 1 (33°C) both during the third and fifth hour (third hour, 18.8 ± 1.48 versus 14.5 ± 2.7 pg/mL; $P = 0.07$: fifth hour, 16 ± 5 versus 11.6 ± 1.5 pg/mL; $P = 0.22$; Fig. 4).

HEPATOCYTE AND EC INJURY AND FUNCTION AFTER TRANSPLANTATION

AST levels were assessed from the early postreperfusion period until the day of euthanasia. Group 1

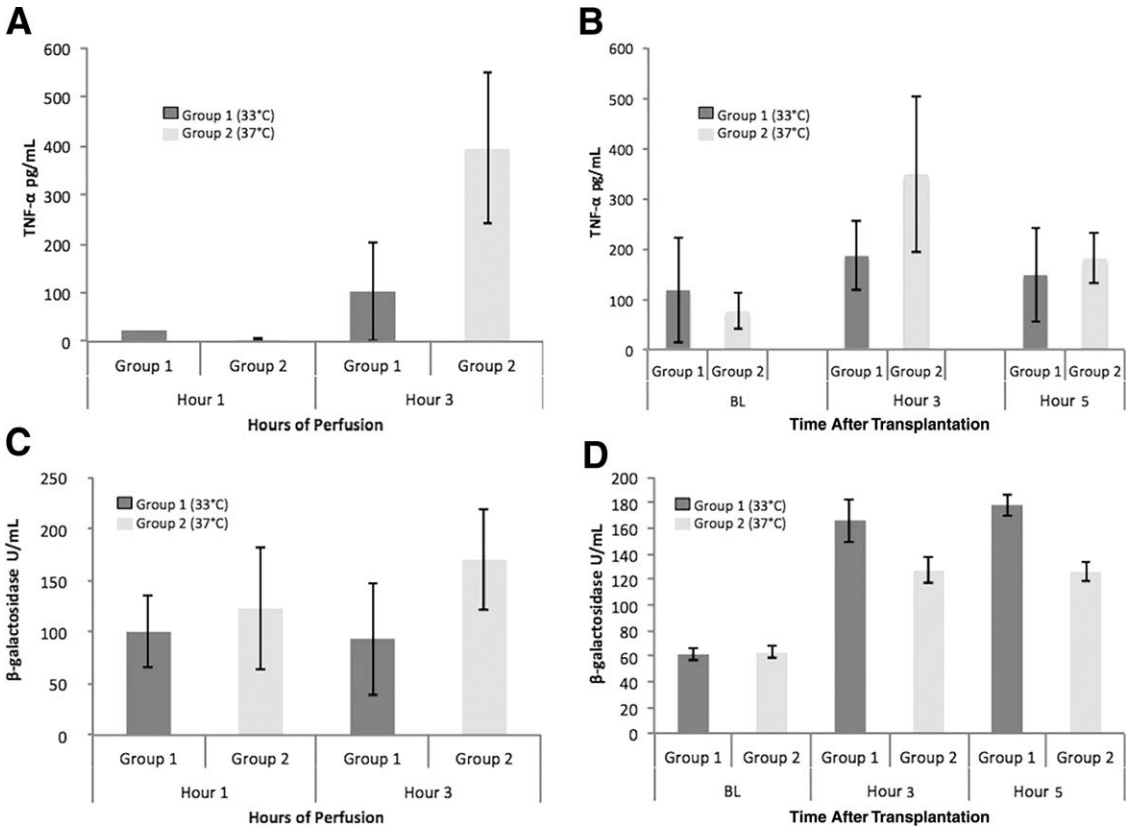

FIG. 3. TNF-α and β galactosidase concentration during machine perfusion and after pig LT. (A) TNF-α levels during machine perfusion. (B) TNF-α levels after pig LT. (C) β galactosidase levels during machine perfusion. (C) β galactosidase levels after pig LT.

(33°C) had a trend toward lower AST peak levels once compared with group 2 (37°C) and group 3 (SCS) without reaching statistical significance (group 1 versus group 2 versus group 3, 1400 ± 653 versus 2097 ± 1071 versus 1747 ± 841.6 U/L, respectively; $P = 0.5$; Fig. 5). Liver necrosis was determined at the end of the survival period by H & E staining. Minimal necrosis (<5%) was present in all groups without evidence of significant ischemia injury.

Liver tissue was stained for cleaved caspase 3 at 2 hours after reperfusion as a marker of apoptosis. Cleaved caspase 3 staining was significantly lower in group 1 (33°C) and group 2 (37°C) when compared with group 3 (SCS; 21 ± 6 versus 36.2 ± 15 versus 89.2 ± 19.1 positive cells/HPF; $P < 0.001$), respectively (Fig. 6). The positively stained cells were predominantly sinusoidal ECs and not hepatocytes.

As previously described,[23] EC viability was assessed by CD31 immunohistochemistry. All slides were scored for integrity of the sinusoidal EC lining and evaluated by a blinded pathologist (Table 1). All liver grafts in group 1 (33°C) had an intact sinusoidal EC lining and minimal EC injury. In contrast, group 2 (37°C) grafts showed disruption of the EC lining ($P = 0.01$). Grafts subjected to SCS without machine perfusion had a severe disruption of the EC lining and architecture compared with perfused grafts (Fig. 7; Table 2).

Posttransplant EC function was assessed by measuring HA clearance. Three and 5 hours after reperfusion, HA levels were lower in group 1 (33°C) versus group 2 (37°C) recipients without reaching significance (third hour after reperfusion, 224 ± 4.2 versus 267.8 ± 29.5 ng/mL; $P = 0.15$; fifth hour after reperfusion, 222.6 ± 16.1 versus 304.8 ± 57 ng/mL; $P = 0.14$, respectively).

Animal survival was considered permanent 3 days after pig LT. There was no statistical difference between all groups regarding survival. One pig in each perfused group died during the follow-up. In both cases, pigs were killed on day 2. In group 1 (33°C), the pig was killed because of ventilatory and renal failure

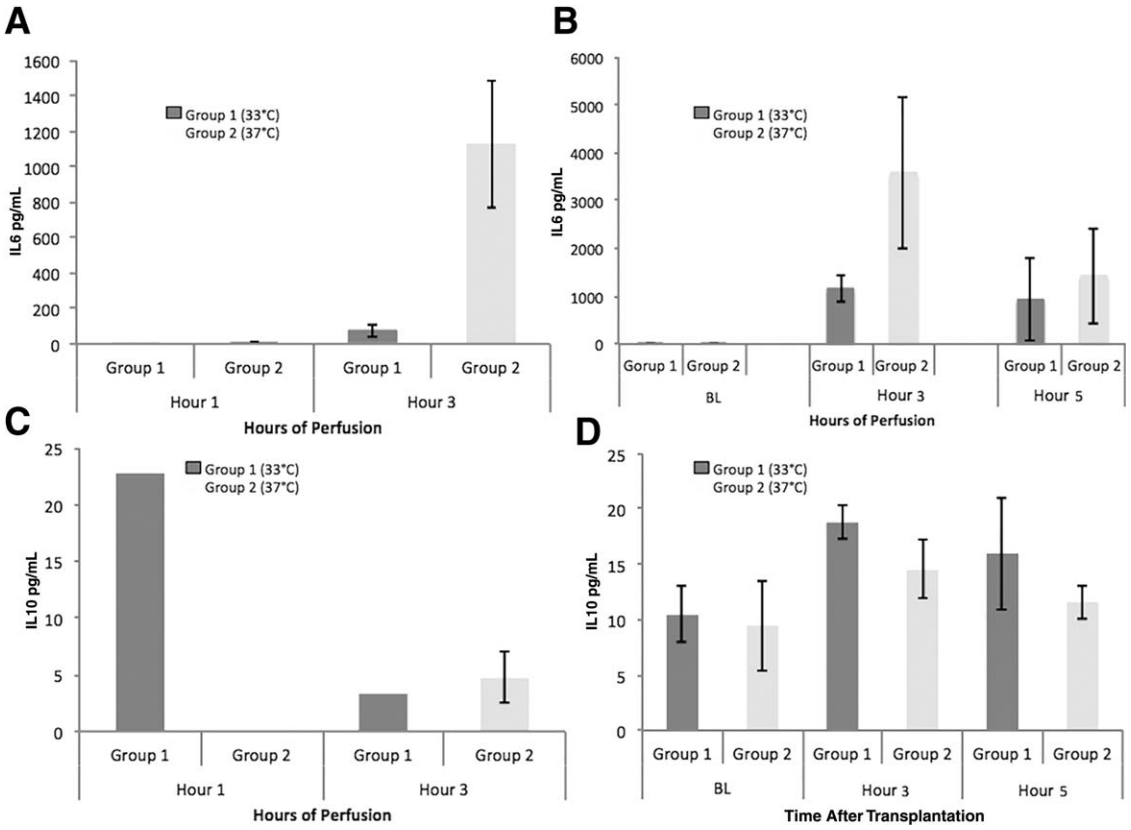

FIG. 4. Expression of IL6 and IL10 during machine perfusion and after pig LT. (A) Expression of IL6 during machine perfusion. (B) Expression of IL6 after pig LT. (C) Expression of IL10 during machine perfusion. (D) Expression of IL10 after pig LT.

due to an abdominal compartment syndrome that occurred following a severe bowel distention. One animal in group 2 (37°C) was killed on day 2 due to a severe rectal prolapse that caused severe pain and bleeding. All pigs included in group 3 (SCS) survived until the third postoperative day (Fig. 8).

INR levels were higher in group 2 (37°C) compared with group 1 (33°C) and group 3 (SCS) until the third postoperative day, as a marker of graft function. No statistical significant differences were seen in this regard (Fig. 9).

BILE DUCT INJURY AND FUNCTION

Bile duct injury and function was assessed by serum ALP and total bilirubin levels. Recipient pigs from group 1 (33°C) had lower ALP levels from postoperative day 1 until the end of follow-up without reaching statistical significance (Fig. 10). Regarding total bilirubin levels, group 1 (33°C) had significantly lower levels compared with group 2 (37°C) at days 1 (3.6 ± 1.5 versus 6.60 ± 1.5 μmol/L; $P = 0.02$) and 3 (2 ± 1.1 versus 9.7 ± 7.6 μmol/L; $P = 0.01$; Fig. 11).

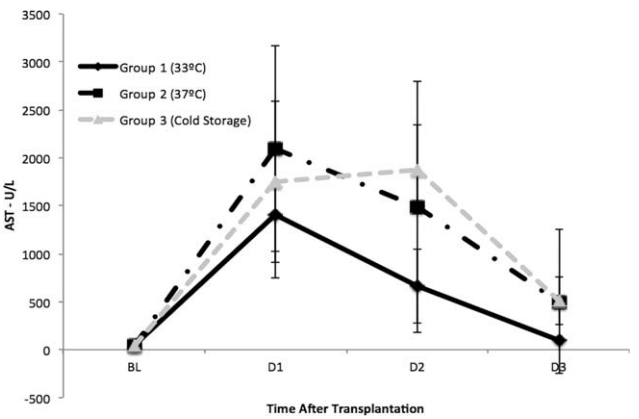

FIG. 5. AST values after pig LT. Group 1 (33°C) versus group 2 (37°C) versus group 3 (SCS).

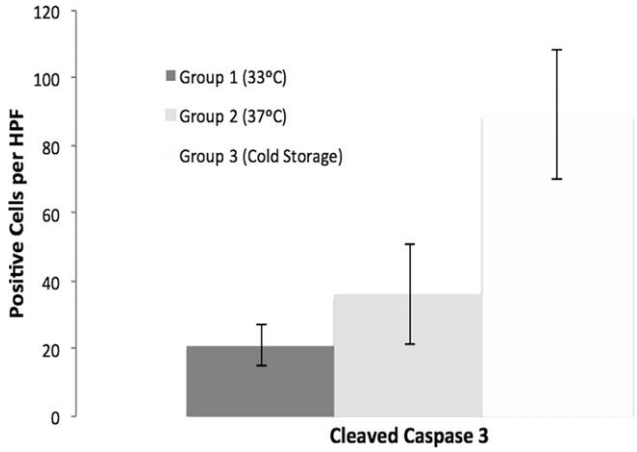

FIG. 6. Cleaved caspase 3 staining in biopsies 2 hours after pig LT. Group 1 (33°C) versus group 2 (37°C) versus group 3 (SCS; $P = 0.001$).

TABLE 1. EC Lining Integrity Score

CD31 Score	Pattern
0	No staining
1	Scattered staining without obvious architecture
2	Reduced staining throughout lobule but intact architecture
3	Reduced staining in zone 3 only
4	Physiological cell lining

Discussion

In this study, we evaluated the addition of anti-inflammatory strategies to the ex vivo liver perfusion system. We demonstrated that providing anti-inflammatory signaling during ex vivo liver perfusion further improves the outcome after transplantation.

TABLE 2. CD31 Scores 2 Hours After Reperfusion and Percentages of Recipient Pigs of Group 1 (33°C) Versus Group 2 (37°C) Versus Group 3 (SCS)

CD31 Score	0	1	2	3	4
Group 1	0%	0%	0%	50%	50%
Group 2	0%	0%	40%	40%	20%
Group 3	0%	75%	25%	0%	0%

In the past, we[2,24,25] and others[1,26,27] have demonstrated that warm perfusion (subnormothermic or normothermic) on its own improves liver grafts during the preservation period. In 2014, we demonstrated in a donation after circulatory death (DCD) pig LT model that warm perfusion improves the preservation of ECs and reduces bile duct injury.[2] Likewise, others have shown that normothermic perfusion provides a superior preservation of DCD liver grafts improving survival after transplantation.[1,26] It has been demonstrated that normothermic ex vivo liver perfusion (NEVLP) is a safe procedure, and normothermic machine preservation of liver grafts is currently being applied in clinical studies in both Europe and Canada.[3,4,25]

In the current study, we added several anti-inflammatory agents to the perfusion system and a reduction of temperature with the objective to further reduce injury during the preservation period. Different agents were added to act at different levels. Prostaglandin E1, a prostacyclin analogue with vasodilator, antiplatelet, fibrinolytic, and several other anti-inflammatory properties were continuously administered to our perfusion system.[28] In 2007, Hafez et al.[29] proved in a canine model of ischemia and

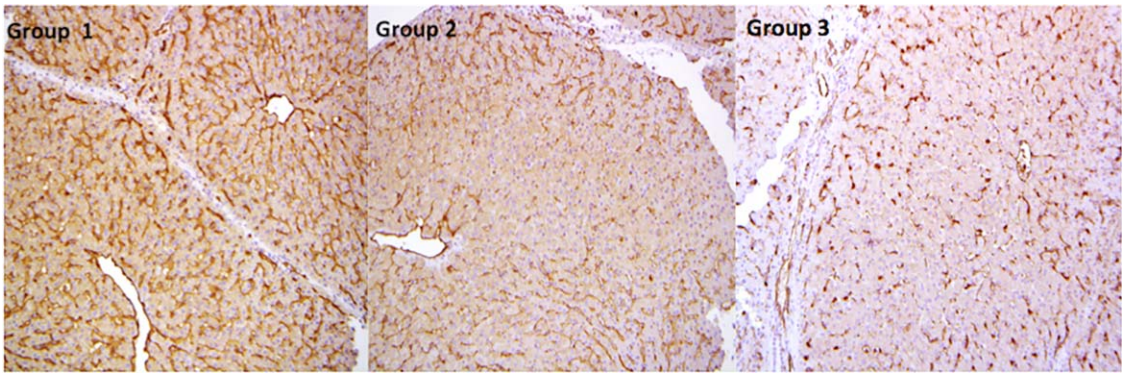

FIG. 7. CD31 staining 2 hours after reperfusion in group 1 (33°C) versus group 2 (37°C) versus group 3 (SCS). 10× objective magnification.

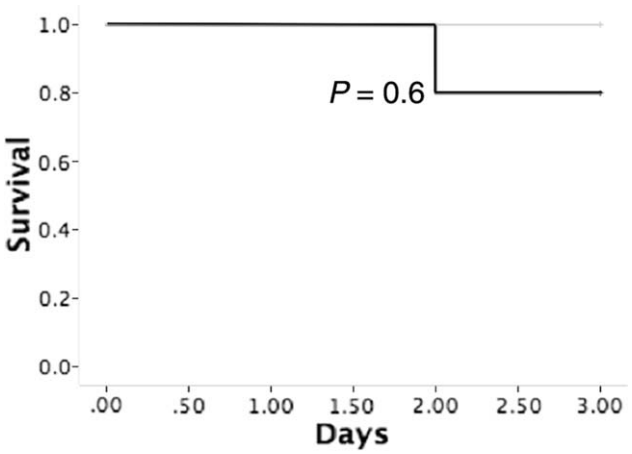

FIG. 8. Recipient survival after LT.

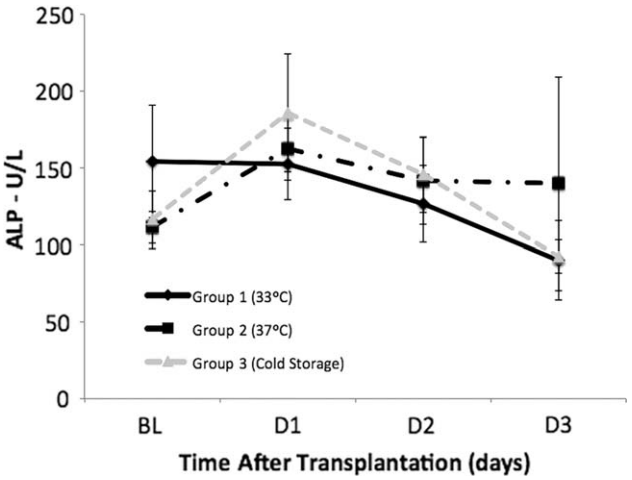

FIG. 10. ALP values after pig LT. Group 1 (33°C) versus group 2 (37°C) versus group 3 (SCS).

reperfusion that the administration of prostaglandin E1 through the portal vein provides a significant reduction in IRI. The authors found a significant reduction in the levels of transaminases and TNF-α. In addition, they demonstrated a down-regulation of the adhesion molecules: intercellular cell adhesion molecule 1, platelet-endothelial cell adhesion molecule (PECAM-1), P-selectin, and E-selectin. Others have shown that prostaglandin E1 reduces hepatocyte injury by increasing the production of the heat shock protein 70 (HSP70), which protects cells against various forms of stress.[30] In addition, in our study we also provided acetylcysteine to the perfusate due to its free radicals scavenging properties.[14] In this manner, liver cells would be protected from reactive oxygen species produced during ischemia and reperfusion. Moreover, our perfusion system was treated with active gaseous components that induce an anti-inflammatory effect on the liver parenchyma. On one hand, sevoflurane was administered due to its protective properties on EC. It is well described that IRI does not only affect the liver parenchyma but also directly targets the vascular endothelium.[2] The endothelial glycocalyx, a protective layer on the luminal endothelial surface, provides an

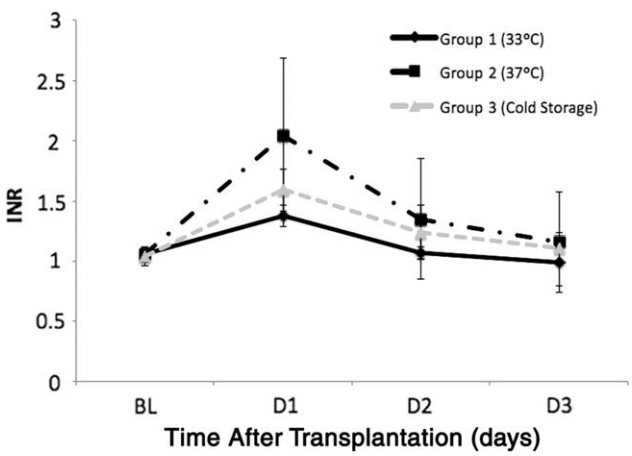

FIG. 9. INR levels after pig LT. Group 1 (33°C) versus group 2 (37°C) versus group 3 (SCS).

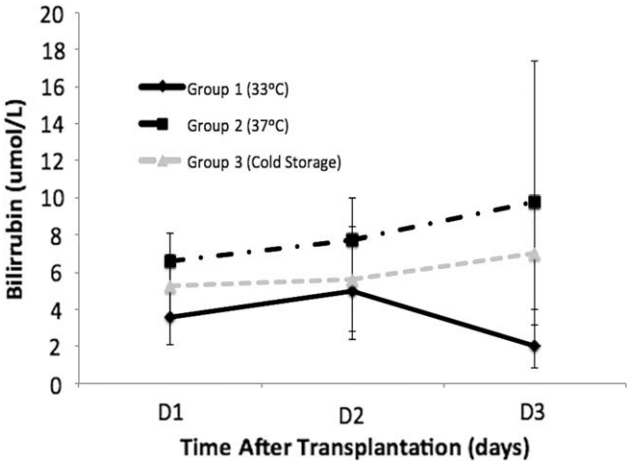

FIG. 11. Total bilirubin levels after pig LT. Group 1 (33°C) versus group 2 (37°C) versus group 3 (SCS). Group 1 (33°C) had significantly lower levels of total bilirubin at day 1 (P = 0.02) and day 3 (P = 0.01) compared with group 2 (37°C).

important barrier function that induces an anticoagulant environment and regulates leukocyte-endothelial interaction.[31] Sevoflurane has been demonstrated to protect and better preserve the endothelial glycocalyx in ischemia/reperfusion models.[19] Furthermore, in our study group (group 1), CO was added to the perfusion solution with the objective to improve vasodilatation and reduce inflammation. In 2008, Tomiyama et al.[16] reported a study in which they proved that administration of CO before and after rat LT provides a reduction of IRI by inducing a down-regulation of graft Kupffer cells. Moreover, Lee et al.[17] showed also in a rat LT model, that CO increases the expression of HSP70 in liver grafts, especially in Kupffer cells, resulting in a reduction of IRI. Subnormothermic temperatures have been found to have strong anti-inflammatory properties similar to the additives described above. In rodent and porcine models, it has been demonstrated that temperatures of 33°C-34°C reduce Kupffer cell activation and suppress release of IL1, IL6, and TNF-α.[5-8] In preliminary studies, we found that the combination of different strategies is most effective to block the activation of proinflammatory pathways during ex vivo perfusion.

Therefore, the strategy of our perfusion setup was to combine the benefits of perfusing livers at close to normal temperatures of 33°C with the benefits of adding several anti-inflammatory strategies. By doing this, we were able to provide an active metabolic scenario wherein livers could be treated with different agents during the preservation period. We believe that perfusing livers at subnormothermic temperatures instead of normothermic provides enough active metabolism so as to apply these protective strategies. Temperatures of 37°C (normothermic conditions) might not be necessary to reach adequate metabolic requirements to improve organ preservation.

This is the first study in which liver grafts are treated with anti-inflammatory approaches during ex vivo liver perfusion. However, this study has several limitations. First, no detailed mechanisms of the molecular effects of these anti-inflammatory agents were explored. Second, no DCD model was used in this model, and therefore, severe injury was not inflicted to the liver grafts to analyze the degree of recovery these agents might achieve. On the other hand, we believe that using a model of HBD organs instead of DCD grafts provides a more homogeneous study group. While performing the analysis of the effects of this novel approach, confounding factors derived from varying degrees of injury (DCD model) are avoided. In addition, following LT with HBD grafts, animals recover quickly with minimal secondary organ injury. This allows a more detailed evaluation of liver function over several days.

Our study does not allow us to conclude which of the different anti-inflammatory strategies contributed to the beneficial effects on organ injury and function after transplantation. All mediators had protective effects in rodent models, but a detailed analysis in a pig model would be difficult to achieve, as well as costly. Similarly, most cold preservation solutions contain multiple mediators and the role of each is often based on theoretical considerations rather than data. The future of warm ex vivo liver perfusion will require us to identify the optimal perfusion conditions that allow us to block multiple reperfusion injury pathways prior to the activation of the reperfusion injury cascade. It is likely that the reperfusion injury cascade is redundant and that multiple mediators and pathways have to be addressed to have a significant impact on preservation injury and graft function.

In summary, our study demonstrates that warm ex vivo liver perfusion can be further improved by refining perfusate mediators and perfusion temperature. The protective effects of avoiding cold ischemia injury can be further improved if we capitalize on the new option to modify proinflammatory signaling and block multiple pathways prior to the initiation of the reperfusion injury cascade.

REFERENCES

1) 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;250:1–6.
2) 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;20:1296–1305.
3) 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;13:1327–1335.
4) 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;14:1400–1409.
5) Fröhlich M, Hildebrand F, Weuster M, Mommsen P, Mohr J, Witte I, et al. Induced hypothermia reduces the hepatic inflammatory response in a swine multiple trauma model. J Trauma Acute Care Surg 2014;76:1425–1432.
6) Todani M, Fujita M, Tsuruta R, Nakahara T, Yagi T, Oshima C, et al. Moderate hypothermia suppressed excessive generation of superoxide anion radical and inflammatory reactions in blood

and liver in heatstroke: laboratory study in rats. Free Radic Res 2010;44:462–472.
7) Kuboki S, Okaya T, Schuster R, Blanchard J, Denenberg A, Wong HR, Lentsch AB. Hepatocyte NF-kappaB activation is hepatoprotective during ischemia-reperfusion injury and is augmented by ischemic hypothermia. Am J Physiol Gastrointest Liver Physiol 2007;292:G201–G207.
8) Behrends M, Hirose R, Serkova NJ, Coatney JL, Bedolli M, Yardi J, et al. Mild hypothermia reduces the inflammatory response and hepatic ischemia/reperfusion injury in rats. Liver Int 2006;26:734–741.
9) Knaak JM, Spetzler VN, Goldaracena N, Louis KS, Selzner N, Selzner M. Technique of subnormothermic ex vivo liver perfusion for the storage, assessment, and repair of marginal liver grafts. J Vis Exp 2014;90:e51419–e51419.
10) St Peter SD, Imber CJ, Lopez I, Hughes D, Friend PJ. Extended preservation of non-heart-beating donor livers with normothermic machine perfusion. Br J Surg 2002;89:609–616.
11) Spetzler VN, Goldaracena N, Knaak JM, Louis KS, Selzner N, Selzner M. Technique of porcine liver procurement and orthotopic transplantation using an active porto-caval shunt. J Vis Exp 2015;99:e52055.
12) Zhang B, Calmus Y, Wen L, Sogni P, Lotersztajn S, Houssin D, Weill B. Endothelin-1 induces liver vasoconstriction through both ETA and ETB receptors. J Hepatol 1997;26:1104–1110.
13) Nakai T, Tanimura H, Hirokawa F, Tamaki T. Altered hepatic hemodynamics and improved liver function following intrahepatic vascular infusion of prostaglandin E1. J Gastroenterol 1998;33:362–367.
14) Winbladh A, Björnsson B, Trulsson L, Bojmar L, Sundqvist T, Gullstrand P, Sandström P. N-acetyl cysteine improves glycogenesis after segmental liver ischemia and reperfusion injury in pigs. Scand J Gastroenterol 2012;47:225–236.
15) Vollmar B, Menger MD. The hepatic microcirculation: mechanistic contributions and therapeutic targets in liver injury and repair. Physiol Rev 2009;89:1269–1339.
16) Tomiyama K, Ikeda A, Ueki S, Nakao A, Stolz DB, Koike Y, et al. Inhibition of Kupffer cell-mediated early proinflammatory response with carbon monoxide in transplant-induced hepatic ischemia/reperfusion injury in rats. Hepatology 2008;48:1608–1620.
17) Lee LY, Kaizu T, Toyokawa H, Zhang M, Ross M, Stolz DB, et al. Carbon monoxide induces hypothermia tolerance in Kupffer cells and attenuates liver ischemia/reperfusion injury in rats. Liver Transpl 2011;17:1457–1466.
18) Spetzler VN, Goldaracena N, Kaths JM, Marquez M, Selzner N, Cattral MS, et al. High preoperative bilirubin values protect against reperfusion injury after live donor liver transplantation. Transpl Int 2015;28:1317–1325.
19) Annecke T, Rehm M, Bruegger D, Kubitz JC, Kemming GI, Stoeckelhuber M, et al. Ischemia-reperfusion-induced unmeasured anion generation and glycocalyx shedding: sevoflurane versus propofol anesthesia. J Invest Surg 2012;25:162–168.
20) Deaciuc IV, Bagby GJ, Lang CH, Spitzer JJ. Hyaluronic acid uptake by the isolated, perfused rat liver: an index of hepatic sinusoidal endothelial cell function. Hepatology 1993;17:266–272.
21) Soejima Y, Yanaga K, Wakiyama S, Nishizaki T, Yoshizumi T, Sugimachi K. Serum hyaluronic acid as a reliable parameter of allograft viability in porcine liver transplantation. Hepatogastroenterology 1996;43:590–595.
22) Gown AM, Willingham MC. Improved detection of apoptotic cells in archival paraffin sections: immunohistochemistry using antibodies to cleaved caspase 3. J Histochem Cytochem 2002;50:449–454.
23) Pusztaszeri MP, Seelentag W, Bosman FT. Immunohistochemical expression of endothelial markers CD31, CD34, von Willebrand factor, and Fli-1 in normal human tissues. J Histochem Cytochem 2006;54:385–395.
24) 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;13:1441–1449.
25) Spetzler VN, Goldaracena N, Echiverri J, Kaths JM, Louis KS, Adeyi OA, et al. Subnormothermic ex vivo liver perfusion is a safe alternative to cold static storage for preserving standard criteria grafts. Liver Transpl 2016;22:111–119.
26) Fondevila C, Hessheimer AJ, Maathuis MH, Muñoz J, Taurá P, Calatayud D, et al. Superior preservation of DCD livers with continuous normothermic perfusion. Ann Surg 2011;254:1000–1007.
27) 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. Surg Innov 2015;22:61–69.
28) Hossain MA, Izuishi K, Maeta H. Effect of short-term administration of prostaglandin E1 on viability after ischemia/reperfusion injury with extended hepatectomy in cirrhotic rat liver. World J Surg 2003;27:1155–1160.
29) Hafez T, Moussa M, Nesim I, Baligh N, Davidson B, Abdul-Hadi A. The effect of intraportal prostaglandin E1 on adhesion molecule expression, inflammatory modulator function, and histology in canine hepatic ischemia/reperfusion injury. J Surg Res 2007;138:88–99.
30) Togo S, Chen H, Takahashi T, Kubota T, Matsuo K, Morioka D, et al. Prostaglandin E1 improves survival rate after 95% hepatectomy in rats. J Surg Res 2008;146:66–72.
31) 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;52:1382–1402.

5. Comparison of BQ123, Epoprostenol, and Verapamil as Vasodilators During Normothermic Ex Vivo Liver Machine Perfusion

Comparison of BQ123, Epoprostenol, and Verapamil as Vasodilators During Normothermic Ex Vivo Liver Machine Perfusion

Juan Echeverri, MD,[1,2] Nicolas Goldaracena, MD,[1] Johan Moritz Kaths, MD,[1] Ivan Linares, MD,[1] Roizar Roizales, MD,[1] Dagmar Kollmann, MD,[1] Matyas Hamar, MD,[1] Peter Urbanellis, MD,[1] Sujani Ganesh, MD,[1] Oyedele A. Adeyi, MD,[3] Mahmood Tazari, PhD,[1] Markus Selzner, MD,[1] and Nazia Selzner, MD[1]

Background. The optimal vasodilator to avoid hepatic artery vasospasm during normothermic ex vivo liver perfusion (NEVLP) is yet to be determined. We compared safety and efficacy of BQ123 (endothelin1 antagonist), epoprostenol (prostacyclin analogue), and verapamil (calcium channel antagonist). **Methods.** Livers from porcine heart beating donors were perfused for 3 hours and transplanted into recipient pigs. Four groups were compared: group 1, livers perfused with a dose of 1.25 mg of BQ123 at baseline and at 2 hours of perfusion; group 2, epoprostenol at a continuous infusion of 4 mg/h; group 3, verapamil 2.5 mg at baseline and at 2 hours of perfusion; group 4, no vasodilator used during ex vivo perfusion. Liver injury and function were assessed during perfusion, and daily posttransplantation until postoperative day (POD) 3. All groups were compared with a cold storage group for postoperative graft function. **Results.** Hepatic artery flow during NEVLP was significantly higher in BQ123 compared with verapamil, epoprostenol, and no vasodilator-treated livers. Aspartate aminotransferase levels were significantly lower with BQ123 and verapamil compared with epoprostenol and control group during perfusion. Peak aspartate aminotransferase levels were lower in pigs receiving BQ123 and verapamil perfused grafts compared with epoprostenol and control group. International Normalized Ratio, alkaline phosphatase, and total bilirubin levels were lower in the BQ123 and verapamil groups compared to epoprostenol group. Cold storage group had increased markers of ischemia reperfusion injury and slower graft function recovery compared to machine perfused grafts. **Conclusion.** The use of BQ123, epoprostenol, and verapamil during NEVLP is safe. Livers perfused with BQ123 and verapamil have higher hepatic artery flow and reduced hepatocyte injury during perfusion compared with epoprostenol. Hepatic artery flow is significantly reduced in the absence of vasodilators during NEVLP.

(*Transplantation* 2018;102: 601–608)

Received 12 April 2017. Revision received 11 October 2017.

Accepted 14 October 2017.

[1] *Multi Organ Transplant Program, Toronto General Hospital, Toronto, ON, Canada.*

[2] *Programa de Doctorat en Cirurgia i Ciències Morfològiques de la Universitat Autònoma de Barcelona, Barcelona, Spain.*

[3] *Department of Pathology, Toronto General Hospital, University of Toronto, Toronto, ON, Canada.*

The authors declare no conflicts of interest.

J.E. participated in the conception and design of the study, completion of experiments, acquisition of data, data analysis and interpretation, drafted the article, final approval for publication, and agreement to be accountable for all aspects of the work. N.G. participated in the completion of experiments, acquisition of data, data analysis and interpretation, revised the article critically for important intellectual content, final approval for publication, and agreement to be accountable for all aspects of the work. J.M.K. participated in the completion of experiments, acquisition of data, data analysis and interpretation, revised the article critically for important intellectual content, final approval for publication, and agreement to be accountable for all aspects of the work. I.L. participated in the completion of experiments, acquisition of data, data analysis and interpretation final approval for publication, and agreement to be accountable for all aspects of the work. R.R. participated in the completion of experiments, acquisition of data, data analysis and interpretation final approval for publication, and agreement to be accountable for all aspects of the work. D.K. participated in the completion of experiments, acquisition of data, data analysis and interpretation final approval for publication, and agreement to be accountable for all aspects of the work. M.H. participated in the acquisition of data, data analysis and interpretation final approval for publication, and agreement to be accountable for all aspects of the work. P.U. participated in the Analysis and interpretation of data, revised manuscript critically for important intellectual content, final approval for publication, and agreement to be accountable for all aspects of the work. S.G. participated in the acquisition of data, analysis and interpretation of data, revised the article critically for important intellectual content, final approval for publication, and agreement to be accountable for all aspects of the work. O.A. participated in the analysis and interpretation of data, revised manuscript critically for important intellectual content, final approval for publication, and agreement to be accountable for all aspects of the work. M.T. participated in the statistical analysis and interpretation of data, revised manuscript critically, final approval for publication, and agreement to be accountable for all aspects of the work. M.S. participated in the conception and design of the study, completion of experiments, acquisition of data, data analysis and interpretation, drafted the article, final approval for publication, and agreement to be accountable for all aspects of the work. N.S. participated in the conception and design of the study, acquisition of data, data analysis and interpretation, drafted the article, final approval for publication, and agreement to be accountable for all aspects of the work.

Correspondence: Nazia Selzner, MD, PhD, Toronto General Hospital, NCSB 11C-1244, 585 University Avenue, Toronto, ON, Canada M5G2N2. (nazia.selzner@uhn).

ISSN: 0041-1337/18/10204-0601

DOI: 10.1097/TP.0000000000002021

Ex vivo liver perfusion has become an alternative strategy to cold static storage for the preservation of liver grafts.[1-5] The possibility of graft assessment and repair during normothermic ex vivo liver perfusion (NEVLP) has the potential to increase the pool of marginal livers that are currently declined for transplantation, thus increasing the number of donor grafts available.[6,7] In the past decade, research has mainly focused on the feasibility of preserving standard criteria and marginal grafts at normothermic and subnormothermic environments.

One of the main challenges encountered during NELVP is maintaining grafts with an active metabolism and within physiological parameters. This is mandatory for an optimal graft assessment and repair before transplantation. One key component is the use of vasodilators during perfusion to reverse hepatic artery vasospasm caused by static cold storage and manipulation. These agents are known to attenuate ischemia reperfusion injury in the graft.[8-11] Human clinical trials using normothermic ex vivo machine perfusion before transplantation are currently underway across Europe and North America. The optimal vasodilator used during perfusion is yet to be defined.[12,13]

We compared the safety and efficacy of 3 different types of vasodilator agents, BQ123 (endothelin 1 antagonist), epoprostenol (prostacyclin, prostaglandin I2), and verapamil (nondihydropyridine calcium channel antagonist) during NELVP in a porcine transplant model. A control group with no vasodilator during NEVLP was used to evaluate the benefit of all 3 vasodilators. All 4 machine perfusion groups were compared with cold storage control group regarding postoperative graft function.

MATERIALS AND METHODS

Study Design

Three different vasodilators, BQ123 (endothelin 1 antagonist), epoprostenol (prostacyclin, prostaglandin I2), and verapamil (nondihydropyridine calcium channel antagonist) were used to perfuse livers retrieved from heart-beating donors (HBD). A fourth group with no vasodilator during NEVLP was used as a control group. Finally, all machine perfused grafts were compared with a cold storage control group (n = 5). In all 4 machine perfused groups (n = 5 in each group), livers were stored on ice (4°C) for a period of 2 hours after retrieval, while proceeding to back table dissection, insertion of cannulas and spinning of blood used for perfusion. After 2 hours of cold storage, grafts were perfused for 3 hours with normothermic (37°C) ex vivo liver perfusion machine and were transplanted into recipient pigs as previously described.[3,14] Ex vivo perfusion setups for all 3 groups were based on our protocol currently used in human clinical trials.[12] Perfusate setup only differed in the type of vasodilator used: BQ123, epoprostenol, verapamil, and control group with no vasodilator. In the cold storage control group, livers were stored on ice for 5 hours and then transplanted into recipient pigs. After transplantation, all pigs were followed for 3 days until surgical euthanasia.

TABLE 1.

ECs integrity score

CD 31 score	Pattern
0	No staining
1	Scattered staining without obvious architecture
2	Reduced staining throughout lobule but intact architecture
3	Reduced staining in zone 3 only
4	Physiological cell lining

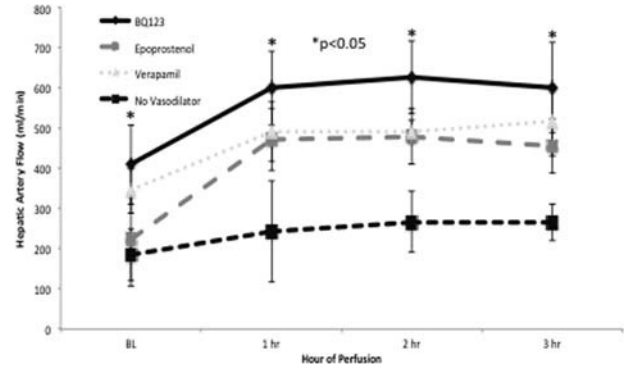

FIGURE 1. Hepatic artery flow during ex vivo machine perfusion using BQ123 vs epoprostenol vs verapamil vs no vasodilator.

Animals

Thirty to 35 kg male Yorkshire pigs were used for this study. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care of Laboratory Animals" published by the National Institutes of Health. All studies were approved by the Animal Care Committee of the Toronto General Hospital.

Normothermic Ex Vivo Perfusion

NELVP was performed for 3 hours before transplantation using the same set up for all 4 groups. Briefly, our perfusion device is propelled by a centrifugal pump set to reach pressures and flows near physiologic levels through the portal vein and hepatic artery. Our NEVLP setup is pressure controlled. Perfusion through the hepatic artery was set to achieve pressures around 60 mm Hg and flows up to 400 mL/h. Portal vein pressure was set to 2 to 4 mm Hg corresponding to a flow of 900 to 1200 mL/h. Perfusate was composed of 1.5 L of Steen Solution (XVIVO Perfusion, Goteborg, Sweden) mixed with washed pig erythrocytes to acquire an hematocrit of 15%. As part of our protocol, erythrocytes were passed through leukocyte filters and washed with sterile saline to avoid contamination with leukocytes or plasma before added to the perfusate. Steen solution is a buffered extracellular-type solution, which is dextran and albumin based. This stabilizes the perfusate with an adequate colloid osmotic pressure, mimicking a near to physiologic environment. To prevent clothing and thrombus formation, the perfusate contained heparin (10 000 iU, Sandoz Canada, Quebec, QC, Canada). Nutrition and metabolic homeostasis was maintained by administering amino acid concentrate (travasol, 4.25%; 50 mL; bolus plus, 8 mL/h; Baxter, Hamilton, ON, Canada), D50W (2-5 mL/h; Baxter, Mississauga, ON, Canada), and Insulin (125 iU/h, NovoRapid, Novo Nordisk, Mississauga, ON, Canada). Prophylactic antibiotics Cefazolin (1 g; Pharmaceutical Partners of Canada, Richmond Hill, ON, Canada) and Metronidazole (500 mg; Baxter), against bacterial contamination was administered during the priming of the circuit. Also, a continuous infusion

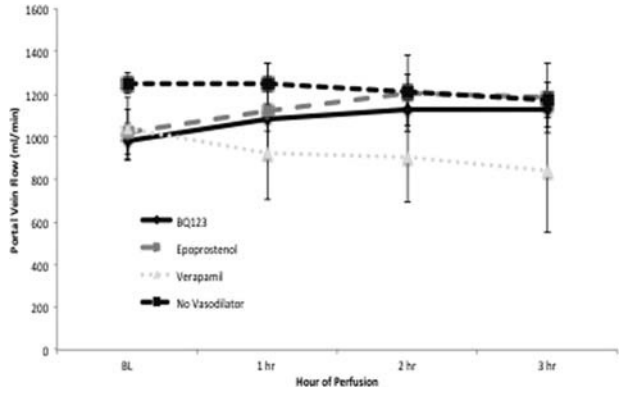

FIGURE 2. Portal vein flows during ex vivo machine perfusion using BQ123 vs epoprostenol vs verapamil vs no vasodilator.

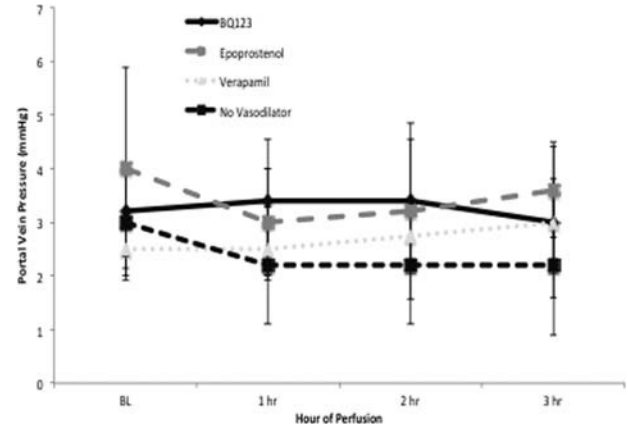

FIGURE 4. Portal vein pressures during ex vivo machine perfusion using BQ123 vs epoprostenol vs verapamil vs no vasodilator.

of Prostaglandin E1 (500 μg/3 hours; Pfizer, Kirkland, QC, Canada) was administrated for its anti-inflammatory properties. Other components added to the perfusate were 20 mmol of sodium bicarbonate, 9.2 mmol/L of calcium chloride, and 2% taurocholic acid (Sigma-Aldrich, St Louis, MO) infused at 7 mL/h as a precursor for bile production. Gases were administered to the perfusate through a gas exchanger membrane with a composition of 95% O_2 and 5% CO_2.

The type of vasodilator agents utilized during NELVP was the only difference between the study groups. Livers perfused with BQ123 (AG Scientific, Kelowna, BC, Canada) received an initial bolus of 1.25 mg at baseline, and a second injection at 2 hours of perfusion to achieve a hepatic artery flow around 400 mL/h. In the epoprostenol group (Flolan; GlaxoSmithKline Inc, Mississauga, ON, Canada) livers were perfused at a continuous infusion of 4 mL/h (8 μg/h) as used in human clinical trials.[12,13] Verapamil (nondihydropyridine calcium channel antagonist, Sandoz Canada Inc, QC, Canada) group received 2.5 mg of verapamil at baseline and at the second hour of perfusion. Finally, a control group of machine perfusion without the use of vasodilator was used for comparison. Ex vivo perfusion set up only differed on the lack of vasodilator when compared to the other groups.

Pig Liver Transplantation

All recipients were transplanted with a graft retrieved under heart beating conditions (HBD). Donor pigs received 30 000 IU of heparin 5 minutes before cross clamping. All livers were flushed through the aorta and portal vein with a total volume of 3 L of University of Wisconsin solution (SPS-1, Organ Recovery Systems, Itasca, IL, USA). Perfused grafts were cold stored at 4°C, while proceeding to back table preparation completing a total of 2 hours of cold static storage time. Livers were then perfused at 37°C for 3 hours and transplanted into their respective recipient pigs using a venovenous portojugular bypass shunt technique (Rotaflow centrifugal pump; Maquet, Harlingen, Germany) as described previously.[14] Liver core biopsies were taken before abdominal wall closure at 2 hours postreperfusion.

Recipient signs of distress or suffering were assessed during the 3-day survival period posttransplantation according to our institution animal use protocol. If signs of suffering were noted (lethargy, excessive bleeding, uncoordinated movements and/or severe acid base disturbances), animals were euthanized under deep anesthesia. At the time of euthanasia, the well being of the graft and patency of vascular anastomoses was assessed. Once the specimen was exsanguinated, we proceeded to perform a total hepatectomy. For doing so, we first dissected all vascular anastomoses (upper vena cava, lower vena cava, portal vein and hepatic artery) dividing the vessel from distal to proximal. The occurrence of thrombus formation, intimal dissection, and marginal flow was assessed thoroughly. Pigs were euthanized under deep isoflurane anesthesia. Samples for pathology analysis from the liver parenchyma and bile duct were taken and stored on 10% formalin.

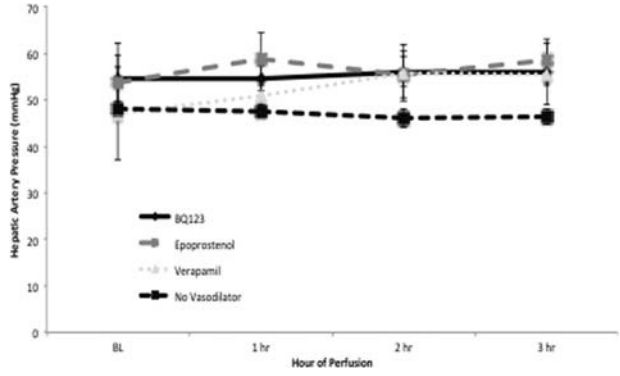

FIGURE 3. Hepatic artery pressures during ex vivo machine perfusion using BQ123 vs epoprostenol vs verapamil vs no vasodilator.

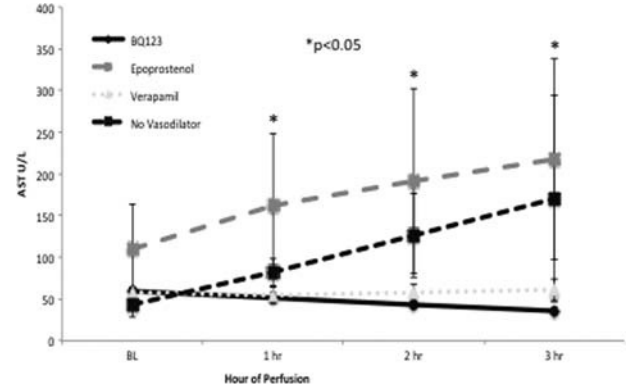

FIGURE 5. AST values during ex vivo machine perfusion using BQ123 vs epoprostenol vs verapamil vs no vasodilator.

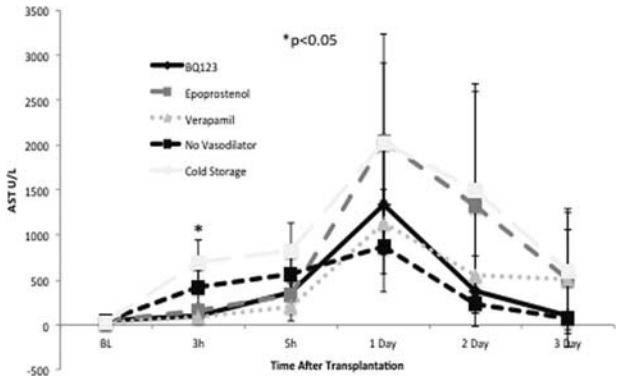

FIGURE 6. Posttransplant and postoperative AST levels using bq123 vs epoprostenol vs verapamil vs no vasodilator vs cold storage.

Assessment of Liver Injury

Levels of aspartate aminotransferase (AST) were measured hourly during ex vivo machine perfusion. Similarly, serum AST levels were measured 3 and 5 hours after reperfusion in the recipient pig and then on a daily basis until euthanasia. Total serum bilirubin and alkaline phosphatase were measured daily as a marker of bile duct injury. International Normalized Ratio (INR) was measured on a daily basis to assess liver function.

TNF and Galactosidase Analysis During Perfusion

Tumor Necrosis Factor-α and β Galactosidase release was measured during ex-vivo machine perfusion in all groups to evaluate the inflammatory response mediated by each vasodilator. Both TNF-α and β Galactosidase are important acute phase cytokines involved in the inflammatory response. Their levels are directly proportional to inflammation and are mediators of ischemia reperfusion injury in the graft. β Galactosidase is a glycohydrolase that predominates within lysosomes acting as a specific marker of Kupffer cell activation.

Cytokine Analysis During Perfusion

Interleukin 6 and IL-10 are pro and anti-inflammatory mediators, respectively. They both mediate cytokine expression and amplification of the inflammatory response. Levels of IL-6 and IL-10 were determined by enzyme-linked immunosorbent assay analysis during perfusion.

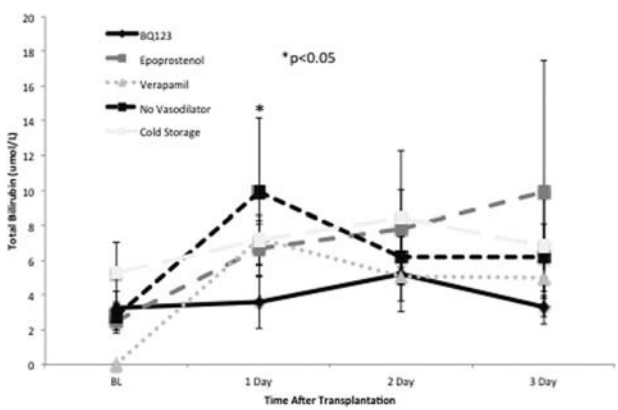

FIGURE 7. Total bilirubin levels in the posttransplant period in recipients of grafts perfused using BQ123 vs epoprostenol vs verapamil vs no vasodilator vs cold storage.

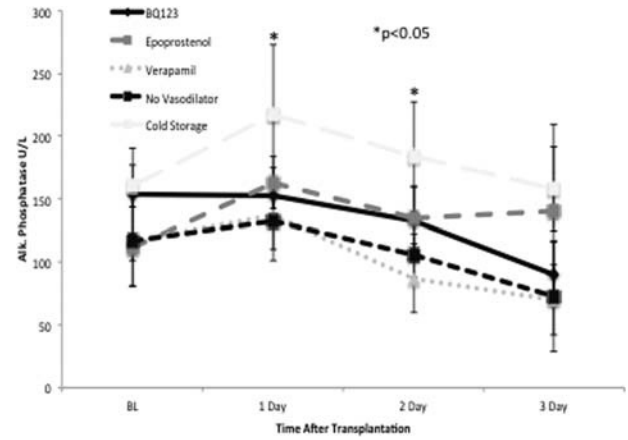

FIGURE 8. Alkaline phosphatase levels in the posttransplant period in recipients of grafts perfused using BQ123 vs epoprostenol vs verapamil vs no vasodilator vs cold storage.

Histological Evaluation

All biopsies taken at 2 hours postreperfusion and at the time of euthanasia were stored in 10% formalin for 24 hours and then transferred to 70% ethanol. After paraffin embedding, sections of 5 μm were cut and processed.

Postreperfusion biopsies were assessed for apoptosis by cleaved caspase-3 and Tunel antibody immunohistochemistry staining (Cell Signaling Technology, Danvers, MA).[15] Slides were evaluated at 20× objective magnification. Stained positive cells were counted and averaged for 10 random high power fields.

Liver parenchyma and bile duct necrosis was assessed by hematoxylin-eosin (H&E) staining. Two hours postreperfusion biopsies were assessed for sinusoidal endothelial cell (EC) integrity by CD31 immunohistochemistry (PECAM; Santa Cruz Biotechnology, Dallas, TX).[16] Slides were evaluated and graded by a blinded pathologist according to a scoring system for assessment of EC lining integrity (Table 1).

Statistical Analysis

Data was analyzed with the SAS 9.4 (SAS Institute Inc, Cary, NC) package. For hemodynamic parameters on ex vivo liver perfusion and follow-up parameters posttransplant, the data were measured equally spaced over different time points for all groups. The statistical methodology was based on

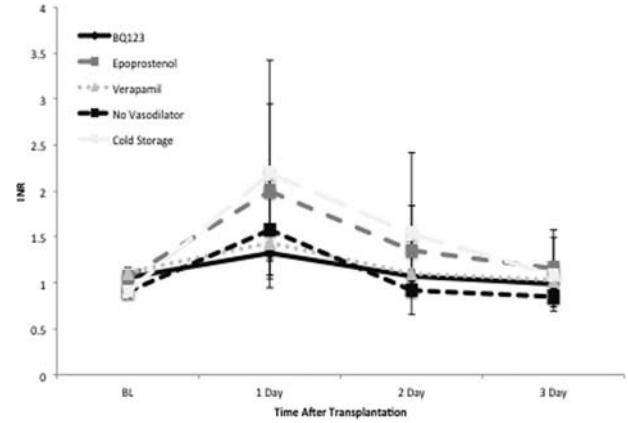

FIGURE 9. INR levels in the posttransplant period in recipients of grafts perfused using BQ123 vs epoprostenol vs verapamil vs no vasodilator vs cold storage.

TABLE 2.

CD 31 score for BQ123, epoprostenol, verapamil, and control group samples 2 hours postreperfusion

CD 31 score	0	1	2	3	4
BQ123	0%	0%	0%	50%	50%
Epoprostenol	0%	0%	40%	40%	20%
Verapamil	0%	33%	33%	0%	33%
No vasodilator (control)	0%	40%	40%	20%	0%

generalized linear mixed models with correlation variability, which arises from repeated observation over time period in each subject. The covariance matrix structure was tested with different structure and the fitted one to the statistical model was Unstructured Correlations (UNR). Due to differences among treatments we computed Least Squares Means and determined simulation method for adjusting the *P* value with correlated association test. The data is presented as mean ± SD and was considered significant at the level of *P* value of 0.05 or less.

RESULTS

Hemodynamic Parameters During Normothermic Ex Vivo Machine Perfusion in Grafts Perfused With BQ123, Epoprostenol, Verapamil or Without Vasodilator

Hepatic artery (HA) flow was higher during the perfusion in BQ123 and verapamil groups compared with epoprostenol and control (without vasodilator). At baseline, HA flow was significantly different between all 4 groups (600±92 mL/h vs 490 ±75 mL/h vs 471±77 mL/h vs 242±126 mL/h; respectively *P* = 0.01). At the second hour of perfusion, BQ123 presented higher HA flow compared to verapamil, epoprostenol and control group (626±91 mL/h vs 492±29 mL/h vs 471±77 mL/h vs 266±76 mL/h; *P* = 0.005) (Figure 1). Similar differences were noted at 3 hours of perfusion (600 ±113 mL/h vs 517 ±86 mL/h vs 454 ±68 mL/h vs 264 ±45 mL/h; *P* = 0.005, respectively). Hepatic artery flow was statistically higher when comparing all vasodilator groups to the no vasodilator group at hour 1, hour 2, and hour 3 of perfusion (Figure 1). No difference was observed in portal vein (PV) flow between all 4 groups (Figure 2). Similarly, no significant differences in HA and PV pressure was seen throughout the perfusion in all groups (Figures 3 and 4).

Ischemia Reperfusion Injury During NEVLP and After Transplantation in Grafts Perfused With BQ123 vs Epoprostenol vs Verapamil vs No Vasodilator vs Cold Storage

Aspartate amino transferase (AST) levels in the perfusate were significantly lower at hours 1, 2, and 3 of perfusion with

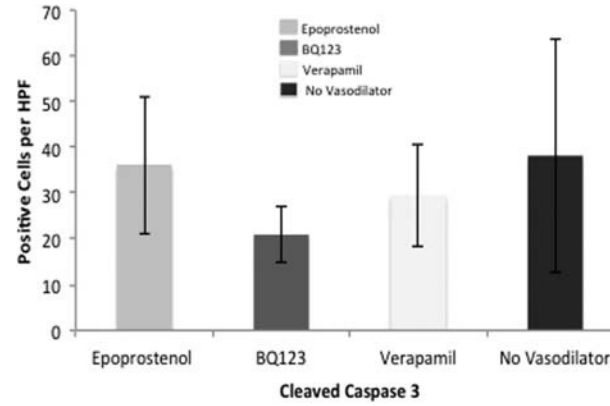

FIGURE 11. Cleaved caspase 3 staining 2 hours posttransplant.

BQ123 and verapamil compared to epoprostenol and no vasodilator control group (hour 1: 52 ± 6.2 U/L vs 54.5 ± 10 U/L vs 162 ± 86 U/L vs 82 ± 16 U/L; *P* = 0.01, hour 2: 43.2 ± 5.2 U/L vs 57.2 ± 10 U/L vs 191 ± 110 U/L vs 126 ± 50 U/L; *P* = 0.01, hour 3: 35.6 ± 4.6 U/L vs 61.2 ±13 U/L vs 218 ± 120 U/L vs 170 ± 124 U/L, *P* = 0.01), respectively (Figure 5). Similarly postliver transplantation, serum AST levels were significantly lower at the third hour in pigs receiving grafts perfused with BQ123 and verapamil compared to epoprostenol and control groups (107 ± 36 U/L vs 89 ± 24 U/L vs 157 ± 61 U/L vs 427 ±192 U/L, respectively; *P* = 0.01). Serum AST levels remained lower in the BQ123 and verapamil groups compared to the epoprostenol and control groups throughout the postoperative period until animals were sacrificed at day 3 after transplantation (Figure 6). Cold storage control group had higher levels of serum AST throughout all the postoperative period compared with the machine perfused groups (Figure 6).

H&E staining was performed at the end of survival period (day 3 posttransplantation). Minimal necrosis (<5%) was encountered in all groups without evidence of severe ischemic injury.

Bile Duct Injury and Graft Function After Transplantation in Grafts Perfused With BQ123 vs Epoprostenol vs Verapamil

Total bilirubin, alkaline phosphatase and INR were lower but not statistically different in the postoperative period in the BQ123 and verapamil groups compared to the epoprostenol group, indicating perhaps a better recovery of hepatocyte function (Figures 7-9). Total bilirubin levels were significantly higher on the first POD in the no-vasodilator control group compared to the vasodilator groups (Figure 7). Cold storage grafts had higher levels of serum

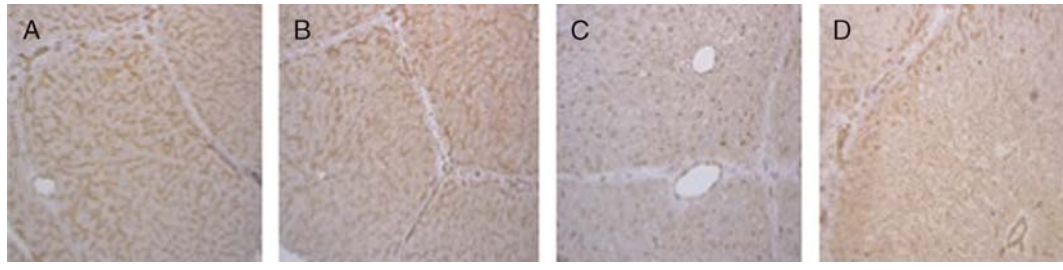

FIGURE 10. CD 31 staining 2 hours posttransplant in (A) grafts perfused with BQ123, (B) grafts perfused with epoprostenol, (C) grafts perfused with verapamil, (D) grafts with no vasodilator.

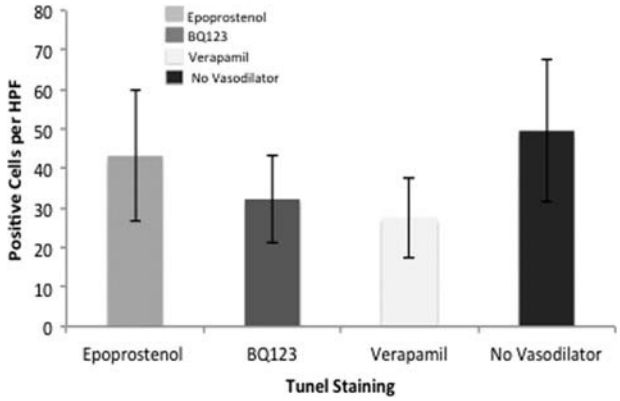

FIGURE 12. Tunel Staining 2 hours posttransplant.

alkaline phosphatase and INR until end of follow-up compared to the machine perfused grafts (Figures 8 and 9). H&E staining of the liver sections did not show bile duct necrosis between the 3 vasodilators groups or the 2 control groups.

EC Injury and Histological Evaluation

EC viability was assessed 2 hours postreperfusion by CD 31 immunostaining. Liver grafts perfused with BQ123 and epoprostenol had an intact architecture in all specimens. Verapamil perfused grafts presented an intact architecture in 66% of specimens evaluated (Table 2) (Figure 10). Apoptosis was measured by cleaved caspase 3 and Tunel staining 2 hours postreperfusion. Although not significant, a lower number of ECs inducing apoptosis was seen 2 hours postreperfusion in the BQ123 and verapamil groups compared to epoprostenol and the no-vasodilator groups (Figures 11-13).

Inflammatory Response During Perfusion

TNFα levels were significantly lower in the BQ123 group compared with the epoprostenol and verapamil group during the third hour of perfusion (102.8± 101 pg/mL vs 394 ± 154 pg/mL vs 429.7 ± 45 pg/mL, $P = 0.01$) respectively (Figure 14). Beta galactosidase levels, as a marker of Kupffer cell activation were lower during perfusion in the BQ123 and verapamil group (Figure 14).

IL-6 levels during machine perfusion were lower in livers perfused with BQ123 and verapamil compared to epoprostenol (73.8 ± 34.6 pg/mL vs 156.6 ± 2 pg/mL vs 1124.4 ± 355 pg/mL, $P = 0.01$) respectively (Figure 15). Although not significant, levels of IL-10 were higher in the BQ123 and verapamil group during the first hour of perfusion compared with epoprostenol (Figure 15).

Animal Survival After Transplantation

No statistical differences were seen between groups regarding survival until the end of the study period. All pigs survived until the day of euthanasia in the BQ123, epoprostenol, and control groups. One pig in the verapamil group had to be euthanized shortly after transplantation due to a technical malfunction in the veno-venous bypass that resulted in a severe metabolic acidosis and multiorgan dysfunction after reperfusion and skin closure. Vessel patency assessment in all specimens did not reveal any signs of thrombosis or intimal dissection.

DISCUSSION

We compared the safety and efficacy of 3 different vasodilators; BQ123 (endothelin 1 antagonist), epoprostenol (prostacyclin, prostaglandin I2), and verapamil (nondihydropyridine calcium channel antagonist) during NEVLP and the outcome post-pig liver transplantation. All 3 vasodilators proved to be safe to use before transplantation. BQ123 and verapamil seemed to achieve better results regarding HA flow and preservation injury during ex vivo perfusion, as well as a better postoperative graft recovery once compared to epoprostenol. A control group without the use of vasodilator during NEVLP revealed lower hepatic artery flows throughout the 3 hours of perfusion. AST values during perfusion were higher in the epoprostenol group compared to BQ123 and verapamil. All 3 vasodilators were compared to a 5 hours cold storage control group, showing higher degree of reperfusion injury and lower graft recovery in the grafts subjected to cold storage.

Normothermic and Sub normothermic ex vivo perfusion systems have been recently validated in animal models as an alternative strategy for the preservation of standard criteria grafts and for the assessment and recovery of extended criteria grafts.[6] Human clinical trials assessing safety and feasibility of ex vivo liver perfusion are underway, and no consensus has yet been established as to the use of optimal vasodilators for organ preservation and posttransplant outcomes.[12,13]

Preservation solutions are rich in potassium, which in turn promote hepatic artery vasospasm once the organ is reperfused. During organ recovery and before perfusion of liver grafts, the organs are flushed with preservation solution, which may cause hepatic artery spasm at the initial phase of perfusion, compromising flow and adequate delivery of nutrients to the liver. Biliary arterial blood supply is of upmost importance during retrieval of marginal grafts such as donation after circulatory death (DCD) livers. Unfortunately, our study was performed in a heart beating liver transplant model. All study and control groups showed no bile duct necrosis at moment of euthanasia. Despite having lower

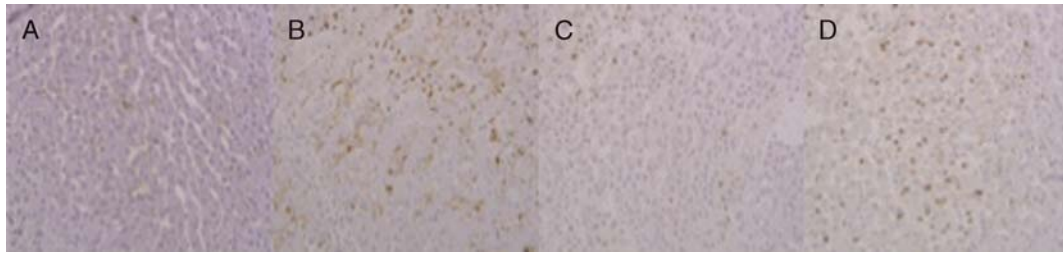

FIGURE 13. Tunel staining 2 hours posttransplant in (A) grafts perfused with BQ123, (B) grafts perfused with epoprostenol, (C) grafts perfused with verapamil, (D) grafts with no vasodilator.

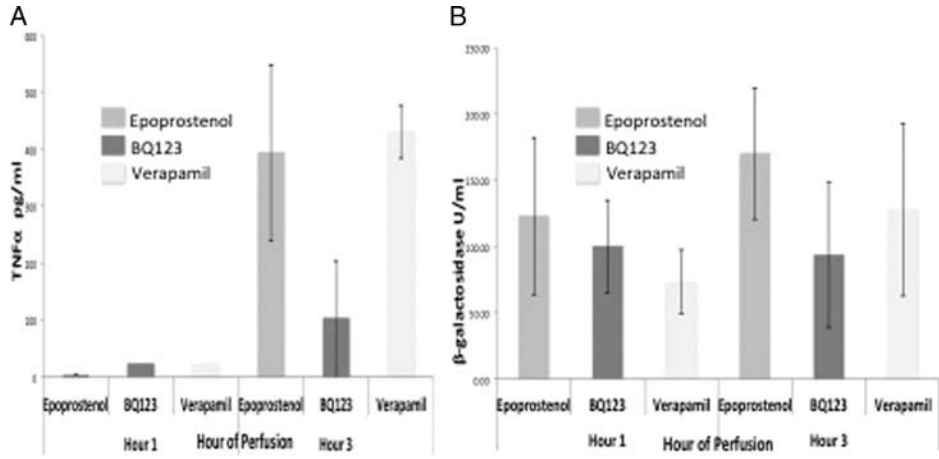

FIGURE 14. TNFα and β Galactosidase levels during ex vivo machine perfusion using BQ123 vs epoprostenol vs verapamil.

posttransplant levels of total bilirubin and alkaline phosphatase in the BQ123 and verapamil groups compared to epoprostenol, we cannot conclude that these specific vasodilator treatments prevent ischemic type biliary strictures. This should be further studied and proven in a DCD pig liver transplant model with a more specific assessment of postoperative bile duct epithelium quality. Components, such as bile salts, biliary epithelium oxygenation, and postoperative arterial flow measured by Doppler assessment until POD 3, should be included.

All 3 vasodilators assessed in this study have anti-inflammatory properties. BQ123 is a potent Endothelin 1 (ET-1) inhibitor. Its effect decreases vascular resistance, reduces cholestasis, and limits the release of eicosanoids from Kuppfer cells.[17,18] Verapamil is known to be a smooth muscle relaxant and cytoprotective agent. During ischemia, cytosolic levels of calcium rise, activating a protease capable of converting xanthine dehydrogenase to xanthine oxidase, which is a main source of superoxide radicals.[19] Calcium potentiates the damaging effects of oxygen-derived free radicals, causing an uncoupling of the mitochondrial electron transport chain due to impairment of N-acetaldehyde dehydrogenase coenzyme. Also, calcium activates phospholipases A1, A2, C and proteases.[20-22] Verapamil acts as an anti-inflammatory agent by inhibiting the activation of these cascades. Finally, epoprostenol (prostacyclin, PGI2), is a potent vasodilator that inhibits platelet aggregation, leukocyte activation, chemotaxis and production of precursors of superoxide species.[11,23] Its safety and efficacy has been proven in previous animal studies and is the mainstay of vasodilator therapy in ongoing clinical trials involving NEVLP before transplantation.[13,24,25] Dosing for epoprostenol was based on current clinical trials using the Organox/Metra device. Drug selection and dosing for verapamil and BQ123 was based on previous pilot studies performed at our laboratory which have shown that bolus doses are safer and more efficient than continuous infusion dosing.[12,13]

Interestingly, there was a decrease in proinflammatory signaling during NEVLP in livers perfused with BQ123 compared to verapamil and epoprostenol. This effect has been shown in previous studies.[26] TNF- α and β Galactosidase levels were lower during perfusion. Levels of IL-6 were lower during perfusion in BQ123 and verapamil-treated livers compared to epoprostenol. Anti-inflammatory IL-10 levels were higher in BQ123 and verapamil grafts during the first hour of perfusion compared to epoprostenol. This effect may be due to a decrease in depletion of cytokine stores during perfusion that in the end could facilitate release of inflammatory components after reperfusion in the recipient. Of note, epoprostenol has known anti-platelet activity which should be considered in current clinical trials with whole blood and colloid solutions with no anti aggregation properties. Epoprostenol may have beneficial effects in preventing prothrombotic events.

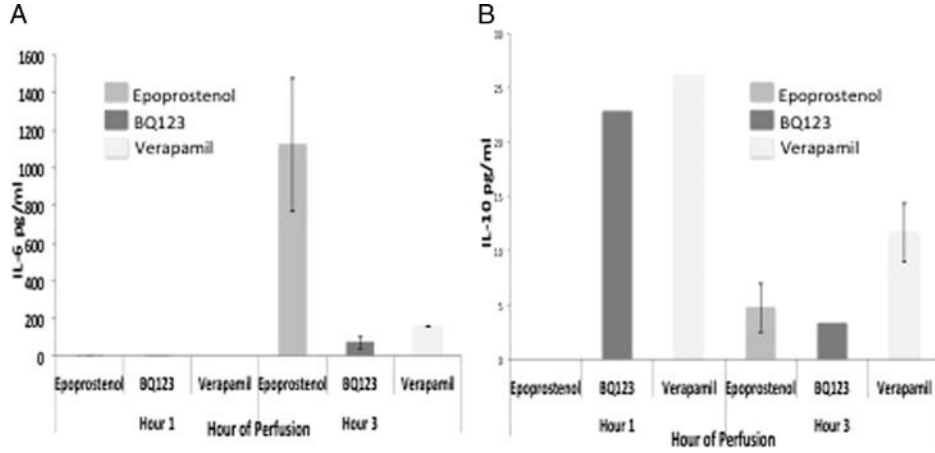

FIGURE 15. IL-6 and IL-10 levels during ex vivo machine perfusion using BQ123 vs epoprostenol vs verapamil.

Our study has several limitations. First, we used a model of HBD to assess safety of the vasodilators included in the perfusate. A more severe liver injury during organ retrieval might have provided more information about the efficacy of the vasodilators and differences on bile duct injury among groups. Performance of the vasodilators in marginal grafts remains unclear. HBD grafts, as used in the control group with no vasodilator, have little preservation injury. This may mask the risks of not using a vasodilator when comparing it with the other groups.

We conclude that the use of BQ123, epoprostenol, and verapamil during ex vivo liver machine perfusion is safe. Livers perfused with BQ123 and verapamil have higher HA flow and reduced ECs and hepatocyte injury during ex vivo liver perfusion and in the posttransplant period compared to epoprostenol. Optimizing hepatic artery flow during NEVLP could have beneficial effects of hepatocyte injury after transplantation. The prevention of bile duct injury and ischemic cholangiopathy by vasodilator treatment during NEVLP is still to be evaluated in a DCD pig liver transplant model.

REFERENCES

1. Boehnert MU, Yeung JC, Bazerbachi F, 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;13:1441–1449.
2. Knaak JM, Spetzler VN, Goldaracena N, 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;20:1296–305.
3. Spetzler VN, Goldaracena N, Echiverri J, et al. Subnormothermic ex vivo liver perfusion is a safe alternative to cold static storage for preserving standard criteria grafts. *Liver Transpl*. 2016;22:111–119.
4. Op den Dries S, Karimian N, Westerkamp AC, et al. Normothermic machine perfusion reduces bile duct injury and improves biliary epithelial function in rat donor livers. *Liver Transpl*. 2016;22:994–1005.
5. Banan B, Chung H, Xiao Z, et al. Normothermic extracorporeal liver perfusion for donation after cardiac death (DCD) livers. *Surgery*. 2015;158:1642–1650.
6. Nassar A, Liu Q, Farias K, et al. Ex vivo normothermic machine perfusion is safe, simple, and reliable: results from a large animal model. *Surg Innov*. 2015;22:61–69.
7. Vogel T, Brockmann JG, Friend PJ. Ex-vivo normothermic liver perfusion: an update. *Curr Opin Organ Transplant*. 2010;15:167–172.
8. Liang J, Yamaguchi Y, Matsumura F, et al. Calcium-channel blocker attenuates Kupffer cell production of cytokine-induced neutrophil chemoattractant following ischemia-reperfusion in rat liver. *Dig Dis Sci*. 2000;45:201–209.
9. Uchida M, Takemoto Y, Nagasue N, et al. Effect of verapamil on hepatic reperfusion injury after prolonged ischemia in pigs. *J Hepatol*. 1994;21:217–223.
10. Morioka D, Kubota T, Sekido H, et al. Prostaglandin E1 improved the function of transplanted fatty liver in a rat reduced-size-liver transplantation model under conditions of permissible cold preservation. *Liver Transpl*. 2003;9:79–86.
11. Nakai T, Tanimura H, Hirokawa F, et al. Altered hepatic hemodynamics and improved liver function following intrahepatic vascular infusion of prostaglandin E1. *J Gastroenterol*. 1998;33:362–367.
12. Selzner M, Goldaracena N, Echeverri J, et al. Normothermic ex vivo liver perfusion using steen solution as perfusate for human liver transplantation—first North American results. *Liver Transpl*. 2016;22:1501–1508.
13. Ravikumar R, Jassem W, Mergental H, et al. Liver transplantation after ex vivo normothermic machine preservation: a phase 1 (first-in-man) clinical trial. *Am J Transplant*. 2016;16:1779–1787.
14. Spetzler VN, Goldaracena N, Knaak JM, et al. Technique of porcine liver procurement and orthotopic transplantation using an active porto-caval shunt. *J Vis Exp*. 2015:e52055.
15. Gown AM, Willingham MC. Improved detection of apoptotic cells in archival paraffin sections: immunohistochemistry using antibodies to cleaved caspase 3. *J Histochem Cytochem*. 2002;50:449–454.
16. Pusztaszeri MP, Seelentag W, Bosman FT. Immunohistochemical expression of endothelial markers CD31, CD34, von Willebrand factor, and Fli-1 in normal human tissues. *J Histochem Cytochem*. 2006;54:385–395.
17. Zhang B, Calmus Y, Wen L, et al. Endothelin-1 induces liver vasoconstriction through both ETA and ETB receptors. *J Hepatol*. 1997;26:1104–1110.
18. Palmes D, Budny TB, Stratmann U, et al. Endothelin-A receptor antagonist reduces microcirculatory disturbances and transplant dysfunction after partial liver transplantation. *Liver Transpl*. 2003;9:929–939.
19. Drews G, Spiegel HU, Hermsdorf T, et al. Cytoprotective effects of a stable prostacyclin analog and a calcium channel blocker: a study on isolated rat hepatocytes. *Transplant Proc*. 1995;27:2799.
20. Nauta RJ, Tsimoyiannis E, Uribe M, et al. The role of calcium ions and calcium channel entry blockers in experimental ischemia-reperfusion-induced liver injury. *Ann Surg*. 1991;213:137–142.
21. Ishii K, Suita S, Sumimoto H. Effect of verapamil on conversion of xanthine dehydrogenase to oxidase in ischemic rat liver. *Res Exp Med (Berl)*. 1990;190:389–399.
22. Karwinski W, Garcia R, Helton WS. Protective effects of the calcium channel blocker verapamil on hepatic function following warm ischemia. *J Surg Res*. 1996;64:150–155.
23. Hossain MA, Izuishi K, Maeta H. Effect of short-term administration of prostaglandin E1 on viability after ischemia/reperfusion injury with extended hepatectomy in cirrhotic rat liver. *World J Surg*. 2003;27:1155–1160.
24. St Peter SD, Imber CJ, Lopez I, et al. Extended preservation of non-heart-beating donor livers with normothermic machine perfusion. *Br J Surg*. 2002;89:609–616.
25. Nassar A, Liu Q, Farias K, et al. Role of vasodilation during normothermic machine perfusion of DCD porcine livers. *Int J Artif Organs*. 2014;37:165–172.
26. Goldaracena N, Echeverri J, Spetzler VN, et al. Anti-inflammatory signaling during ex vivo liver perfusion improves the preservation of pig liver grafts before transplantation. *Liver Transpl*. 2016;22:1573–1583.

6. Discussion

In recent years, normothermic and sub-normothermic ex vivo machine perfusion has been validated as an alternative strategy for graft preservation. Avoiding prolonged cold static storage reduces ischemia reperfusion injury, more so in marginal and extended criteria grafts which tolerate poorly cold preservation.

This work addresses important aspects of this novel graft preservation technique. So far, most studies have focused on the validation of principles of machine perfusion in animal models with good outcomes compared to static cold storage. Despite recent advances and translating these findings to the clinical arena, there is no direct comparison of different perfusates, additives, vasodilators and temperatures used during perfusion. To date, the optimal perfusate composition and temperature have not been defined yet.

The first phase of our work focuses on the comparison of the standard normothermic technique used in the first randomized controlled trial vs an anti-inflammatory sub-normothermic setup. The study entitled "Anti-inflammatory signaling during ex vivo liver perfusion improves the preservation of pig liver grafts before transplantation" aimed to evaluate if the addition of anti-inflammatory agents plus sub-normothermic temperatures improved the protective effects of ex vivo liver prefusion on preservation injury in a porcine liver transplant model.

To do so, we added anti-inflammatory agents to the perfusion system and reduced the temperature during machine perfusion prior to transplantation. By use of controlled oxygenated rewarming we were able to start perfusion at temperatures of 28º Celsius and gradually increase until reaching peak temperatures of 33-34º Celsius. It has been

demonstrated in rodent and porcine models that sub-normothermia reduces Kupffer cell activation and downregulates the expression of pro-inflammatory cytokines such as IL-1, IL-6, and TNF α. By perfusing livers at sub-normothermic temperatures we can limit the amplification of the inflammatory response and still maintain an active metabolism wherein grafts can be treated with different agents during the preservation period.

Apart from modifying the perfusion temperature, different agents were added to the perfusate to act at different levels. Prostaglandin E1 (prostacyclin analogue) is known to have vasodilator, anti-platelet, and fibrinolytic properties. Previous studies in animal models have demonstrated that portal vein infusion of prostaglandin E1 reduces IRI by downregulating the expression of adhesion molecules such as platelet-endothelial cell adhesion molecule (PECAM), P-selectin, and E-selectin. Other studies have shown the increase of heat shock protein 70 (HSP70) with the use of prostaglandin E1. This protein is known to reduce hepatocyte injury and protect against various forms of oxidative stress.

Our sub-normothermic study group was treated with gaseous agents known to attenuate IRI. Sevoflurane, an inhaled anesthetic routinely used in surgical procedures, protects the vascular endothelium by targeting the endothelial glycocalyx. This endothelial glycocalyx acts as a luminal surface that provides a protective layer by inducing anticoagulation and regulating leukocyte-endothelial interactions. Sevoflurane seems to protect and better preserve the endothelial glycocalyx. Carbon monoxide (CO) is another gaseous agent used in our perfusion protocol with the aim of improving vasodilation and reducing inflammation. Rodent animal models have demonstrated the

increase of HSP70 and the downregulation of Kupffer cells by the use of CO previous to rat liver transplant. Finally, N-acetylcystein was administered as a ROS scavenger.

In this first study we demonstrated that ex vivo machine perfusion can act as a platform to preserve and improve grafts previous to transplantation by refining perfusate additives and temperature. Several limitations must be highlighted. First, our model was not challenged with a warm ischemia insult as in a DCD pig liver transplant protocol. Grafts in our study were not subjected to severe injury as to evaluate the degree of recovery that these anti-inflammatory agents might achieve. Secondly, we did not perform a detailed molecular analysis of each component. We were not able to accurately confirm which of all the different components contributed in a larger scale to attenuate IRI. Many pathways are modulated with the anti-inflammatory setup. Doing a study evaluating each and every component would be very cost-effective and time consuming using a pig liver transplant model. This limitation has led us to our second study, where we evaluate different vasodilators used in normothermic machine perfusion. Ideally, all components should be independently evaluated. We chose to investigate the role of vasodilation, as it is a corner stone during ex vivo machine perfusion and a component which is included in all normothermic and sub-normothermic perfusion devices used nowadays.

In the second phase of our project we evaluated different vasodilators with the study entitled “Comparison of BQ123, Epoprostenol, and Verapamil as vasodilators during normothermic ex vivo liver machine perfusion”. Vasodilators are used to reverse hepatic artery vasospasm caused by SCS and guarantee an adequate perfusion through the hepatic artery. These agents are known to attenuate IRI. Maintaining an active

metabolism during ex vivo machine perfusion is key for an optimal graft assessment and repair previous to transplantation.

We compared the performance of BQ123, Epoprostenol, and Verapamil in a pig liver transplant model using DBD grafts. All 3 perfusion groups were compared to a non-vasodilator group in order to evaluate the benefit of vasodilation. All 4 machine perfusion groups were compared to a SCS control group to evaluate post-transplant graft function. To date no consensus has been reached as to which is the best vasodilator to be used during ex vivo liver perfusion.

With the exception of in situ normothermic regional perfusion and "ischemia free" techniques as described by Fondevila et al and He et al, respectively, donor procurement protocols requiere a cold preservation flush before machine perfusion. Preservation solutions are rich in potassium, this may cause hepatic artery vasospasm in the initial phases of ex vivo perfusion. Vasodilators counteract arterial vasospasm achieving an adequate oxygen supply to the peribiliary glands. This effect may protect the graft from ischemic cholangiopathy, more so in extended criteria and DCD grafts.

All 3 vasodilators proved to be safe and not harm DBD grafts. BQ123 and verapamil perfused grafts reached higher hepatic artery flows and had less IRI during perfusion and better post-transplant graft recovery compared to Epoprostenol. Interestingly, Epoprostenol was the vasodilator used in the first ever randomized controlled trial performed worldwide. Other vasodilators which may have a better performance are yet to be validated in the clinical setting. As expected, the no-vasodilator group had lower hepatic artery flows during perfusion. Interestingly, this did not affect significantly post-

transplant function when compared to the vasodilator groups. Perfusion proved to diminish IRI and improve graft recovery when compared to SCS.

Vasodilators used during perfusion have anti-inflammatory properties. BQ123 is an endothelin-1 antagonist that reduces cholestasis and limits the release of eicosanoids from Kupffer cells. Verapamil acts as a smooth muscle relaxant with calcium channel antagonism. Calcium is known to be a cofactor in the conversion of xanthine dehydrogenase to xanthine oxidase, which is a main source of ROS. Calcium also activates phospholipases and causes uncoupling of the mitochondrial electron transport chain by impairment of N-acetaldehyde dehydrogenase coenzyme. Finally, Epoprostenol inhibits platelet aggregation, leukocyte activation and production of precursors of ROS. It is important to highlight that of all 3 vasodilators, Epoprostenol has been validated by far as the safest component in multiple animal models. Normothermic and sub-normothermic perfusates always include whole blood for its oxygen carrying capacities. The antiplatelet effects of Epoprostenol for the prevention of thrombotic events seem to favor its use in the clinical setting.

Our model included DBD grafts to assess the safety of all 3 vasodilators. One of our aims was to validate as a proof of concept that good quality livers would not be harmed. This may be interpreted as a shortcoming. The use of a DCD model may have given us more information about the efficacy of each vasodilator and its effect on the prevention on bile duct ischemic injury. Although post-transplant graft function was enhanced in the BQ123 and Verapamil groups when compared to Epoprostenol and no-vasodilator group, no mayor differences were seen upon H&E staining at time of euthanasia. Minimal necrosis (<5%) was seen in all groups. Heart beating donor grafts, as used in

all groups including the no-vasodilator control group, have little preservation injury. This may mask the risks of not using a vasodilator when comparing it with other groups.

7. Conclusions

1. Sub-normothermic ex vivo liver perfusion may be further refined by adding anti-inflammatory agents with specific pathway signaling, thus leading to improved attenuation of ischemia reperfusion injury when compared with standard normothermic perfusion used in current clinical trials.

2. The use of BQ123, Verapamil, and Epoprostenol as vasodilators during normothermic ex vivo liver perfusion is safe and does not harm standard criteria grafts.

3. Livers perfused with BQ123 and Verapamil have higher hepatic artery flow and reduced endothelial cell and hepatocyte injury during ex vivo perfusion and in the post-transplant period compared to Epoprostenol.

8. Personal Views and Future Perspectives

The constant imbalance of supply and demand of organs has motivated the academic community to focus on rescuing grafts that historically were deemed non-transplantable. Machine perfusion has revolutionized the field of transplantation in recent years. This technology may decrease organ shortage and serve as a platform to assess objectively, minimize IRI, and modify grafts previous to transplantation. Unfortunately, changes in paradigms take time. We need to responsibly evaluate all the existing data and take the best decisions maintaining patient safety while moving the field forward.

Two main currents comprise machine perfusion nowadays. On one end sub-normothermic and normothermic perfusion strategies maintain grafts metabolically active and minimize cold ischemia by using blood derived products as oxygen carrying agents with the aim of securing physiological homeostasis. These perfusion setups are complex and leave a small margin for error during manipulation while on pump. Any problem encountered with the circuit during perfusion leads to unwanted warm ischemia and may lead to discard of an organ that could have been transplantable by using standard preservation techniques. On the other hand, hypothermic strategies (HMP and HOPE) used after cold storage and shortly before graft implantation do not rely on blood products as oxygen carriers and are comprised of simpler perfusion setups. This makes their use more appealing and cost-effective. However, at hypothermia grafts are not fully metabolically active. One of the future perspectives of ex vivo machine perfusion is to influence inflammatory pathways and modify grafts previous to transplantation by means of genetic therapy, nanoparticle delivery or defatting. Intuitively, organs with no active metabolism while on pump are less likely to be modified with this technology. As we can see, no perfusion strategy is better than the other. Each one has its strengths and weaknesses. The opportunity to objectively

evaluate and compare both methods still awaits. The implementation of a worldwide consortium where patients can be randomized to either strategy is an attractive initiative.

Static cold storage achieves excellent results (>90% 1-year graft survival) for standard criteria grafts. Currently, it seems that perfusion techniques have a limited benefit in low risk grafts. The only randomized controlled trial to date compared normothermic ex vivo perfusion with SCS using mostly good quality grafts. Despite showing a change in decision making and a decrease in organ discard rate with the assessment during ex vivo perfusion, the study was powered to evaluate differences in AST and ALT levels. In clinical practice other perfusion parameters such as lactate clearance and bile production seem to be better predictors of graft function. Transaminase levels during perfusion are still non-specific and correlate badly with post-transplant outcomes, more so with the risk of ischemic cholangiopathy which is the Achilles heel of transplanting grafts with prolonged warm and cold ischemia times.

Objective assessment and the measurement of reliable parameters during perfusion that can predict outcomes are still lacking. Not only should randomized controlled trials focus on comparison of different technologies, but also seek to define objective parameters to predict post-transplant graft function, more so with the use of extended criteria grafts. The academic community has taken a big step proving the concept of graft machine perfusion, now we await convincing data evaluating cost effectiveness directed towards relevant endpoints among different perfusion techniques. Future research in organ perfusion should also focus on graft immunomodulation, target-cell

therapy through nanomedicine and subsequently oncologic modulation in unison with the expanding of criteria for transplant oncology.

9. References

1. Ayloo S, Pentakota SR, Molinari M. Trends of characteristics of donors and recipients of deceased donor liver transplantation in the United States: 1990 to 2013. World J Transplant. 2018. September 10; 8 (5): 167 – 177.

2. Charlton MR, Burns JM, Pedersen RA, Watt KD, Heimbach JK, Dierkhising RA. Frequency and outcomes of liver transplantation for nonalcoholic steatohepatitis in the United States. Gastroenterology 2011; 141: 1249-1253.

3. Byass P. The global burden of liver disease: a challenge for methods and for public health. BMC Med. 2014.

4. Jain A, Reyes J, Kashyap R, Dodson SF, Demetris AJ, Ruppert K et al. Long-term survival after liver transplantation in 4,000 consecutive patients at a single center. Ann Surg 2000; 232: 490-500.

5. Kim WR, Lake JR, Smith JM, Skeans MA, Schladt DP, Edwards EB et al. OPTN/SRTR 2013 Annual Data Report: liver. Am J Transplant 2015; 15 Suppl 2: 1-28.

6. Wong MCS, Huang J. The growing burden of liver cirrhosis: implications for preventive measures. Hepatology International 2018. 12: 201 – 203.

7. Merion RM, Schaubel DE, Dykstra DM, Freeman RB, Port FK, Wolfe RA. The survival benefit of liver transplantation. Am J Transplant 2005;5:307-313.

8. Fritsche L, Dragun D, Neumayer HH, Budde K. Impact of cyclosporine on the development of immunosuppressive therapy. Transplant Proc 2004; 36: 130S-134S.

9. Chen T, Terrault NA. Perspectives on treating hepatitis C infection in the liver transplantation setting. Curr Opin Organ Transplant 2016; 21: 111-119.

10. Feng S, Goodrich NP, Bragg-Gresham JL, Dykstra DM, Punch JD, DebRoy MA, et al. Characteristics associated with liver graft failure: the concept of a donor risk index. Am J Transplant 2006; 6:783-790.

11. Watt KD, Pedersen RA, Kremers WK, Heimbach JK, Charlton MR. Evolution of causes and risk factors for mortality post-liver transplant: results of the NIDDK long-term follow-up study. Am J Transplant 2010; 10: 1420–27.

12. Calne RY. Transplantation of the liver. Ann Surg 1978;188:129–38.

13. Mathur AK, Talwalkar J. Quality measurement and improvement in liver transplantation. J Hepatol 2018;68:1300–10.

14. Jacob M, Copley LP, Lewsey JD, Gimson A, Rela M, van der Meulen JH; UK and Ireland Liver Transplant Audit. Functional status of patients before liver transplantation as a predictor of posttransplant mortality. Transplantation 2005; 80: 52-57.

15. Llovet JM, Bruix J. Novel advancements in the management of hepatocellular carcinoma in 2008. J Hepatol 2008; 48.

16. Sapisochin G, de Lope CR, Gastaca M, de Urbina JO, López Andujar R, Palacios F, et al. Intrahepatic cholangiocarcinoma or mixed hepatocellular cholangiocarcinoma in patients undergoing liver transplantation: a Spanish matched cohort multicenter study. Ann Surg 2014; 259: 944-952.

17. Sapisochin G, Fidelman N, Roberts JP, Yao FY. Mixed hepatocellular cholangiocarcinoma and intrahepatic cholangiocarcinoma in patients undergoing transplantation for hepatocellular carcinoma. Liver Transpl 2011; 17: 934-942.

18. Bruix J, Han KH, Gores G, Llovet JM, Mazzaferro V. Liver Cancer: Approaching a personalized care. J Hepatol. 2015 April. 62 (10): S144-S156.

19. Adam R, Karam V, Vailliez V, O Grady JG, Mirza D, Cherqui D et al. 2018 Annual report of the European liver transplant registry (ELTR) – 50-year evolution of liver transplantation. Transp Int 2018. Dec 31 (12): 1293-1317.

20. Wong RJ, Aguilar M, Cheung R, Perumpail RB, Harrison SA, Younossi ZM, et al. Nonalcoholic steatohepatitis is the second leading etiology of liver disease among adults awaiting liver transplantation in the United States. Gastroenterology 2015;148:547–555.

21. Younossi ZM, Marchesini G, Pinto-Cortez H, Petta S. Epidemiology of non-alcoholic fatty liver disease and nonalcoholic steatohepatitis: Implications for liver transplantation. Transplantation. 2019; 103: 22–27.

22. Dueland S, Syversveen T, Solheim JM, Solber S, Grut H, Bjornbeth BA et al. Survival following liver transplantation for patients with nonresectable liver-only colorectal metastases. Ann Surg 2020;271: 212-8.

23. Jarnagin, Belghiti, Buchler, Chapman, D´Angelica, De Matteo et al. Blumgart´s surgery of thhe liver, biliary tract, and pancreas. 5th edition. Philadelphia. Elsevier Saunders. 2012.

24. Maggi U, Rossi G, Colledan M, Fassati LR, Gridelli B, Reggiani P et al. Child Pugh score and liver transplantation. Transplant Proc 1993. April. 25(2). 1769-70.

25. Peng Y, Qi X, Guo X. Child-Pugh versus MELD score for the assessment of prognosis in liver cirrhosis: a systematic review and meta-analysis of observational studies. Medicine (Baltimore). 2016;95(8): e2877.

26. Martin AP, Bartels M, Hauss J, Fangmann J. Overview of the MELD score and the UNOS adult liver allocation system. Transplant Proc. 2007;39(10):3169– 74.

27. Asrani SK, Kamath PS. Model for end-stage liver disease score and MELD exceptions: 15 years later. Hepatol Int. 2015;9(3):346–54.

28. Deschênes M, Belle SH, Krom RA. Early allograft dysfunction after liver transplantation: a definition and predictors of outcome. National Institute of Diabetes and Digestive and Kidney Diseases Liver Transplantation Database. Transplantation. 1998;66:302–310.

29. Hudcova J, Scopa C, Rashid J. Effect of early allograft dysfunction on outcomes following liver transplantation. Clin Transplant. 2017;31.

30. Jochmans I, Fieuws S, Monbaliu D, Pirenne J. Model for early allograft function outperforms early allograft dysfunction as a predictor of transplant survival. Transplantation. 2017 Aug;101(8):e258-e264.

31. Tariciotti L, Rocha C, Perera MT, Gunson BK, Bramhall SR, Isaac J, et al. Is it time to extend liver acceptance criteria for controlled donors after cardiac death? Transplantation 2011;92: 1140-1146.

32. Braat AE, Blok JJ, Putter H, Adam R, Burroughs AK, Rahmel AO, et al.; for European Liver and Intestine Transplant Association (ELITA) and Eurotransplant Liver Intestine Advisory Committee (ELIAC). The Eurotransplant donor risk index in liver transplantation: ET-DRI. Am J Transplant 2012;12:2789- 2796.

33. Dubbeld J, Hoekstra H, Farid W, Ringers J, Porte RJ, Metselaar HJ, et al. Similar liver transplantation survival with selected cardiac death donors and brain death donors. Br J Surg 2010;97: 744-753.

34. Detry O, Donckier V, Lucidi V, Ysebaert D, Chapelle T, Lerut J, et al. Liver transplantation from donation after cardiac death donors: initial Belgian experience 2003-2007. Transpl Int 2010; 23:611-618.

35. Detry O, Deroover A, Meurisse N, Hans MF, Delwaide J, Lauwick S, et al. Donor age as a risk factor in donation after circulatory death liver transplantation in a controlled withdrawal protocol programme. Br J Surg 2014;101:784-792.

36. Sepulveda A, Scatton O, Tranchart H, Gouya H, Perdigao F, Stenard F et al. Split liver transplantation using extended right grafts: The natural history of segment 4 and its impact on early postoperative outcomes. Liver Transplantation 18:413-422,2012.

37. Emond JC, Freeman RB Jr, Renz JF, Yersiz H, Rogiers X, Busuttil RW. Optimizing the use of donated cadaver livers: analysis and policy development to increase the application of split-liver transplantation. Liver Transpl 2002;8:863-872.

38. Rogiers X, Malago M, Gawad K, Jauch KW, Olausson M, Knoefel WT, et al. In situ splitting of cadaveric livers. The ultimate expansion of a limited donor pool. Ann Surg 1996;224:331-339.

39. Broering DC, Topp S, Schaefer U, Fischer L, Gundlach M, Sterneck M, et al. Split liver transplantation and risk to the adult recipient: analysis using matched pairs. J Am Coll Surg 2002;195:648-657.

40. Noujaim HM, Gunson B, Mayer DA, Mirza DF, Buckels JA, Candinas D, et al. Worth continuing doing ex situ liver graft splitting? A single-center analysis. Am J Transplant 2003;3:318-323.

41. Dahm F, Georgiev P, Clavien PA. Small-for-size syndrome after partial liver transplantation: definition, mechanisms of disease and clinical implications. Am J Transplant 2005;5:2605-2610.

42. Demetris AJ, Kelly DM, Eghtesad B, Fontes P, Wallis Marsh J, Tom K, et al. Pathophysiologic observations and histopathologic recognition of the portal hyperperfusion or small-for-size syndrome. Am J Surg Pathol 2006; 30:986-993.

43. Fondevila C, Hessheimer AJ, Taurá P, Sánchez O, Catalayud D, De Riva N et al. Portal hhyperperfusion: Mechanism of injury and stimulus for regeneration in porcine small-for-size transplantation. Liver Transpl 16: 364-374, 2010.

44. Goldaracena N, Echeverri J, Kehar M, De Angelis M, Jones N, Ling S et al. Pediatric living donor liver transplantation with large-for-size lateral segment grafts. Am J Transplant. 2020 Feb20; (2) 504 – 512.

45. Penn I, Halgrimson CG, Starzl TE. Liver transplantation in man. Ann N Y Acad Sci. 1970 July; 170: 251-258.

46. Belghiti J, Panis Y, Sauvanet A, Gayet B, Fékété F. A new technique of side to side caval anastomosis during orthotopic hepatic transplantation without inferior vena caval occlusion. Surg Gynecol Obstet. 1992 Sep; 175 (3) 270-2.

47. Cherqui D, Lauzet J, Rotman N, Duvoux C, Dhumeaux D, Julien M, et al. Orthotopic liver transplantation with preservation of the caval and portal flows. Technique and results in 62 cases. Transplantation 1994;58:793-796.

48. Tzakis A, Todo S, Starzl T. Orthotopic liver transplantation with preservation of the inferior vena cava. Ann Surg 1989; 210:649-652.

49. Belghiti J, Noun R, Sauvanet A. Temporary portocaval anastomosis with preservation of caval flow during orthotopic liver transplantation. Am J Surg 1995;169:277-279.

50. Tzakis A, Reyes J, Nour B, Marino I, Todo S, Starzl T. Temporary end to side portacaval shunt in orthotopic hepatic transplantation in humans. Surg Gynecol Obstet 1993;176:180-182.

51. Hesse U, Berrevoet F, Troisi R, Mortier E, Pattyn P, de Hemptinne B. Liver transplantation by preservation of the caval flow with temporary porto-caval shunt or veno-venous bypass. Transplant Proc 1997;29:3609-3610.

52. Margarit C, de Cenarruzabeitia I, Lazaro J, Cancino J, Rojas L, Escartin A, et al. Portacaval shunt and inferior vena cava preservation in orthotopic liver transplantation. Transplant Proc 2005;37:3896-3898.

53 Davila D, Bartlett A, Heaton N. Temporary portocaval shunt in orthotopic liver transplantation: Need for a standardized approach? Liver Transpl 2008. 14; 1414-1419.

54. Killenberg PG, Clavien PA. Medical care of the liver transplant patient. 3rd edition. Massachusetts. Blackwell Publishing. 2006.

55. Aggarwal S. Postreperfusion syndrome: cardiovascular collapse following hepatic reperfusion during liver transplantation. Transplant Proc 1987;19:54-5.

56. Bezinover D, Kadry Z, McCullough P, McQuillan PM, Uemura T, Welker K, et al. Release of cytokines and hemodynamic instability during the reperfusion of a liver graft. Liver Transplant 2011;17:324-30.

57. Siniscalchi A, Gamberini L, Laici C, Bardi T, Ercolani G, Lorenzini L, et al. Post reperfusion syndrome during liver transplantation: From pathophysiology to therapy and preventive strategies. World J Gastroenterol 2016;22:1551.

58. Hilmi I, Horton CN, Planinsic RM, Sakai T, Nicolau-Raducu R, Damian D, et al. The impact of postreperfusion syndrome on short-term patient and liver allograft outcome in patients undergoing orthotopic liver transplantation. Liver Transplant 2008;14:504-8.

59. Fukazawa K, Nishida S, Hibi T, Pretto Jr EA. Crystalloid flush with backward unclamping may decrease post-reperfusion cardiac arrest and improve short-term graft function when compared to portal blood flush with forward unclamping during liver transplantation. Clin Transplant 2013;27:492-502.

60. Op Den Dries S, Sutton ME, Lisman T, Porte RJ. Protection of bile ducts in liver transplantation: Looking beyond ischemia. Transplantation. 2011; 92(4): 373-379.

61. Buck DG, Zajko AB. Biliary complications after orthotopic liver transplantation. Tech Vasc Interv Radiol 2008; 11: 51.

62. Verdonk RC, Buis CI, Porte RJ, et al. Biliary complications after liver transplantation: A review. Scand J Gastroenterol Suppl 2006; 243: 89.

63. Jagannath S, Kalloo AN. Biliary complications after liver transplantation. Curr Treat Options Gastroenterol 2002; 5: 101.

64. Verdonk RC, Buis CI, Porte RJ, et al. Anastomotic biliary strictures after liver transplantation: Causes and consequences. Liver Transpl 2006; 12: 726.

65. Sanchez-Urdazpal L, Gores GJ, Ward EM, et al. Ischemic-type biliary complications after orthotopic liver transplantation. Hepatology 1992; 16: 49.

66. Morelli J, Mulcahy HE, Willner IR, et al. Endoscopic treatment of postliver transplantation biliary leaks with stent placement across the leak site. Gastrointest Endosc 2001; 54: 471.

67. Zoepf T, Maldonado-Lopez EJ, Hilgard P, et al. Balloon dilatation vs. balloon dilatation plus bile duct endoprostheses for treatment of anastomotic biliary strictures after liver transplantation. Liver Transpl 2006; 12: 88.

68. Graziadei IW, Schwaighofer H, Koch R, et al. Long-term outcome of endoscopic treatment of biliary strictures after liver transplantation. Liver Transpl 2006; 12: 718.

69. Buis CI, Verdonk RC, van der Jagt EJ, et al. Nonanastomotic biliary strictures after liver transplantation, part 1: Radiological features and risk factors for early vs. late presentation. Liver Transpl 2007; 13: 708.

70. Starzl TE, Marchioro T, Von Kaulla K, Hermann G, Brittain R, Waddell W. Homotransplantation of the liver in humans. Surg Gynecol Obstet 1963;117:659.

71. Starzl TE, Fung JJ. Themes of liver transplantation. Hepatology. 2010;51:1869-84.

72. Humpries AL, Russell R, Gregory J, Carter RH, Moretz WH: Hypothermic perfusion of the canine kidney for 48 hours followed by reimplantation.Am Surg 1964;30:748–752.

73. Humphries A L, Russell R, Stoddard L, Moretz WH: Successful five-day kidney preservation. Perfusion with hypothermic, diluted plasma. Invest Urol 1968;5:609–618.

74. Schloerb PR, Waldorf R, Welsh JS: The protective effect of kidney hypothermia on total renal ischemia. Surg Gynecol Obstet 1959;109:561–565.

75. Calne RY, Pegg DE, Pryse-Davies J, Brown F. Renal preservation by ice-cooling; an experimental study relating to kidney transplantation from cadavers. Br Med J 1963; 2, 651-655.

76. Collins GM, Bravo-Shugarman M, Terasaki PI. Kidney preservation for transportation. Initial perfusion and 30 hours' ice storage. Lancet 1969;2:1219–1222.

77. Starzl TE, Hakala TR, Shaw BW Jr, Hardesty RL, Rosenthal TJ, Griffith BP, Iwatsuki S, Bahnson HT: A flexible procedure for multiple cadaveric organ procurement. Surg Gynecol Obstet 1984;158:223–230.

78. Israni AK, Zaun D, Hadley N, Rosendale JD, Schaffhausen C, McKinney W et al. OPTN/SRTR 2018 annual data report: deceased organ donation. Am J Transp. Supplement. 2018.

79. Diagnosis of brain death. Statement issued by the honorary secretary of the Conference of Medical Royal Colleges and their Faculties in the United Kingdom on 11 October 1976. Br Med J 2, 1187–1188 (1976).

80. Dalle Ave, A. L. & Bernat, J. L. Using the brain criterion in organ donation after the circulatory determination of death. J Crit Care (2016).

81. Kauffman, H. M., Bennett, L. E., McBride, M. A. & Ellison, M. D. The expanded donor.Transplantation Reviews 11, 165–190 (1997).

82. Monbaliu D, Pirenne J, Talbot D. Liver transplantation using donation after cardiac death donors. J of Hepatol 2012. 56; 474-485.

83. Kootstra G. Statement on non-heart-beating donor programs. Transplant Proc 1995;27:2965.

84. Kootstra G, Daemen JH, Oomen AP. Categories of non-heart-beating donors. Transplant Proc 1995;27:2893–2894.

85. WHO, Transplantation Society (TTS)Organización Nacional de Transplantes (ONT). Third WHO Global Consultation on Organ Donation and Transplantation: striving to achieve self sufficiency, March 23–25, 2010, Madrid.

86. Daemen, J. W., Kootstra, G., Wijnen, R. M., Yin, M. & Heineman, E. Nonheart-beating donors: the Maastricht experience. Clin Transpl 303–316 (1994).

87. Jay C, Ladner D, Wang E, Lyuksemburg V, Kang R, Chang Y, et al. A comprehensive risk assessment of mortality following donation after cardiac death liver transplant – an analysis of the national registry. J Hepatol 2011;55:808–813.

88. Grewal HP, Willingham DL, Nguyen J, Hewitt WR, Taner BC, Cornell D, et al. Liver transplantation using controlled donation after cardiac death donors: an analysis of a large single-center experience. Liver Transpl 2009;15: 1028–1035.

89. Montalvo-Jave EE, Escalante-Tattersfield T, Ortega-Salgado JA, Piña E, Geller DA. Factors in the pathophysiology of the liver ischemia-reperfusion injury. J Surg Res 2008;147:153–159.

90. Peralta C, Jimenez-Castro MB, Gracia-Sanchho J. Hepatic ischemia and reperfusion injury: effects on the liver sinusoidal millieu. J of Hepatol. 2013. 59; 1094-1106.

91. Serracino-Inglott F, Habib NA, Mathie RT. Hepatic ischemia-reperfusion injury. Am J Surg 2001;181:160–166.

92. Clemens MG. Nitric oxide in liver injury. Hepatology 1999;30:1–5.

93. Vollmar B, Glasz J, Leiderer R, Post S, Menger MD. Hepatic microcirculatory perfusion failure is a determinant of liver dysfunction in warm ischemiareperfusion. Am J Pathol 1994;145:1421–1431.

94. Van Bossuyt H, Wisse E. Structural changes produced in kupffer cells in the rat liver by injection of lipopolysaccharide. Cell Tissue Res 1988;251: 205–214.

95. Bilzer M, Gerbes A. Preservation injury of the liver: mechanisms and novel therapeutic strategies. J Hepatol 2000;32:508–515.

96. Lentsch A, Kato A, Yoshidome H, McMasters K, Edwards M. Inflammatory mechanisms and therapeutic strategies for warm hepatic ischemia/reperfusion injury. Hepatology 2000;32:169–173.

97. Witthaut R, Farhood A, Smith CW, Jaeschke H. Complement and tumor necrosis factor-alpha contribute to Mac-1 (CD11b/CD18) up-regulation and systemic neutrophil activation during endotoxemia in vivo. J Leukoc Biol 1994;55:105–111.

98. Perry BC, Soltys D, Toledo AH, Toledo-Pereyra LH. Tumor necrosis factor-a in liver ischemia/reperfusion injury. J Invest Surg 2011;24:178–188.

99. Teoh NC, Farrell GC. Hepatic ischemia reperfusion injury: pathogenic mechanisms and basis for hepatoprotection. J Gastroenterol Hepatol 2003;18:891–902.

100. Lesurtel M, Graf R, Aleil B, Walther DJ, Tian Y, Jochum W, et al. Plateletderived serotonin mediates liver regeneration. Science 2006;312:104–107.

101. Nakamura K, Kageyama S, Kupliec Weglinski JW. Innate immunity in ischemia reperfusion injury and graft rejection. Curr Opin Organ Transplant. 2019, Dec 24 (6); 687-693.

102. Moers, C., Pirenne, J., Paul, A., Ploeg, R. J.Machine Preservation Trial Study Group. Machine perfusion or cold storage in deceased-donor kidney transplantation. N Engl J Med. 366, 770–771 (2012).

103. Guarrera JV, Estevez J, Boykin J, Boyce R, Rashid J, Sun s et al. ypothermic machine perfusion of liver grafts for transplantation: technical development in human discard and miniature swine models. Transp Proc 2005. 37. 323-325.

104. Guarrera JV, Henry SD, Samstein B, et al. Hypothermic machine preservation in human liver transplantation: The first clinical series. Am J Transplant 2010; 10: 372–381.

105. Guarrera JV, Henry SD, Samstein B, Reznik E, Musat C, Lukose TI, et al. Hypothermic machine preservation facilitates successful transplantation of “orphan” extended criteria donor livers. Am J Transplant 2015;15:161-169.

106. Henry SD, Nachber E, Tulipan J, Stone J, Bae C, Reznik L, et al. Hypothermic machine preservation reduces molecular markers of ischemia/reperfusion injury in human liver transplantation. Am J Transplant 2012;12:2477-2486.

107. Dutkowski P, Schonfeld S, Heinrich T, Junginger T. Rat liver preservation by hypothermic oscillating liver perfusion compared to simple cold storage. Cryobiology. 36, 61-67. 1998.

108. Dutkowski P, Schonfeld S, Heinrich T. Reduced oxidative stress during acellular reperfusion of the rat liver after hypothermic oscillating perfusion. Transplantation 1999; 68: 44–50.

109. Dutkowski P, Clavien P. Uploading cellular batteries: Caring for mitochondria is key. Liver Transpl 2018;24:462-4.

110. Schlegel A, Muller X, Dutkowski P. Hypothermic Liver Perfusion. Curr Opin Organ Transplant 2017;22:563-70.

111. Schlegel A, Kron P, Graf R, et al. Warm vs. cold perfusion techniques to rescue rodent liver grafts. J Hepatol 2014;61:1267-75.

112. Schlegel AA, Kalisvaart M, Muiesan P. Machine perfusion in liver transplantation: an essential treatment or just an expensive toy? Minerva Anestesiol 2018;84:236-45.

113. 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 of Hepatol. 60. 765-772. 2014.

114. Schlegel A, Muller X, Dutkowski P. Machine perfusion strategies in liver transplantation. HepatoBiliary Surg Nut. 2019;8(5): 490-501.

115. Muller X, Schlegel A, Kron P, Eshmuminov D, Wurdinger M, Meierhofer D et al. Novel real-time prediction of liver graft function during hypothermic oxygenated machine perfusion before liver transplantation. Annals of Surg. 2019(5);783-790.

116. 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;233:114-123.

117. 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;20:1296-1305.

118. 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;250:1-6.

119. 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;13:1441-1449.

120. Op den Dries S, Karimian N, Porte RJ. Normothermic machine perfusion of discarded liver grafts. Am J Transplant 2013;13:2504.

121. 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;20:987-999.

122. Jamieson RW, Zilvetti M, Roy D, Hughes D, Morovat A, Coussios CC, Friend PJ. Hepatic steatosis and normothermic perfusion-preliminary experiments in a porcine model. Transplantation 2011;92:289-295.

123. Goldaracena N, Speltzer VN, Echeverri J, Kaths JM, Cherepanov V, Persson R. Inducing Hepatitis C Virus Resistance After Pig Liver Transplantation-A Proof of Concept of LiverGraft Modification Using Warm Ex Vivo Perfusion. Am J Transplant. 2017 Apr;17(4):970-978.

124. Ravikumar R, Jassem W, Mergental H, et al. Liver transplantation after ex vivo normothermic machine preservation: a phase 1 (first-in-man) clinical trial. Am J Transplant. 2016;16:1779–1787.

125. Mergental, H. et al. Transplantation of declined liver allografts following normothermic ex-situ evaluation. Am. J. Transplant. 16, 3235–3245 (2016).

126. Selzner M, Goldaracena N, Echeverri J, et al. Normothermic ex vivo liver perfusion using steen solution as perfusate for human liver transplantation—first North American results. Liver Transpl. 2016;22:1501–1508.

127. 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 May;557(7703):50-56.

128. Hessheimer AJ, Coll E, Torres F, et al. Normothermic regional perfusion versus super rapid recovery in controlled donation after circulatory death liver transplantation. J Hepatol 2019;70:658-65.

129. Watson CJ, Hunt F, Messer S, et al. In situ normothermic perfusion of livers in controlled circulatory death donation may prevent ischemic cholangiopathy and improve graft survival. Am J Transplant 2019;19:1745-58.

130. 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 Feb;253:259–64.

131. O'Neill S, Roebuck A, Khoo E, Wigmore SJ, Harrison EM. A meta-analysis and metaregression of outcomes including biliary complications in donation after cardiac death liver transplantation. Transpl Int 2014 Nov;27:1159–74.

132. De Carlis R, Di Sandro S, Lauterio A, Ferla F, Dell`AcquaA, Zanierato M. Successful donation after cardiac death liver transplants with prolonged warm ischemia time using normothermic regional perfusion. Liver Transplantation 2017; 23: 166-173.

133. He X, Guo Z, Zhao Q, et al. The first case of ischemia-free organ transplantation in humans: A proof of concept. Am J Transplant 2018;18:737-44.