Normothermic machine perfusion for donor liver preservation

Herman Tolboom

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Normotherme machine-perfusie voor preservatie van donor levers
(met een samenvatting in het Nederlands)

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Voor mijn ouders

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Introduction

Introduction

The donor organ shortage

In 1967 a team lead by Dr. Thomas Starzl performed the first successful human liver transplantation in the United States [1]. The following year, Sir Roy Calne and coworkers were the first to perform a successful human liver transplantation in Europe [2]. Today, almost 50 years since its introduction, liver transplantation remains the only effective treatment for end-stage liver disease. Currently, up to 12.000 transplants are performed annually in Europe and the United States with an average 5-year survival of more than 70 % [3]. Although the number of patients on the waiting list continues to increase, the donor organ pool has remained stable over the years. With demand for donor organs consistently exceeding supply, an increasing number of patients will die before a donor organ is available.

The shortage of suitable donor livers could be alleviated by extending donor criteria to include livers from non-heart beating donors (NHBD) and donors with co-morbidities such as severe liver steatosis [3, 4]. However, current organ storage methods fall short when used for the preservation of these marginal donor organs [5]. Due to an increased risk of primary non-function and late complications, in particular biliary stricture, NHBD livers are only sparsely used for transplantation [6-7]. The use of severely steatotic donor livers is also associated with an increased risk of early graft failure; consequently, these organs are typically discarded [8,5].

Donor organ procurement

Currently, the majority of livers that are used for transplantation are obtained from brain dead heart beating donors (HBD). Typically, these are donors with disastrous cerebral injury with no chance of recovery, but with intact circulation. Donor organ retrieval occurs while the heart is still beating, hereby avoiding ischemia and ensuring optimal organ viability [9].

The use of NHBD donor livers could have significant impact on the donor organ shortage [6]. According to the Maastricht criteria, NHBD are divided into four categories: Category 1 includes donors that die on arrival at the hospital on whom no resuscitation is performed. Category 2 includes donors who were unsuccessfully resuscitated. Due to the unpredictable nature of death and hence ischemic time in these groups, Maastricht 1 and 2 donors are known as “uncontrolled” NHBD. Maastricht category 3 and 4 are formed by “controlled” NHBD, where cardiac arrest is anticipated and ischemia is therefore minimized.

Category 3 represents donors from whom life-supports is withdrawn, and organ retrieval occurs at a defined time after the heart has ceased to beat, whereas donors in category 4 are those diagnosed with brain-death when cardiac arrest occurs [10].

Organs from controlled NHBD are currently used with success for kidney transplantation [11], but their use is associated with higher rates of delayed graft function when traditional preservationmethods are used [12,13]. Liver transplantation is currently performed on a limited scale using controlled NHBD organs, yet although experience is limited, results suggest lower graft survival when compared to HBD organs [14, 15]. When cold preserved uncontrolled NHBD organs are used for transplantation, results are invariably poor [16].

Static cold storage

The current gold standard for organ preservation is static cold storage in preservation solutions such as University of Wisconsin (UW) solution. The key principle of static cold storage is cooling the organ down to 0-4 ^{o}C to slow down metabolism, reduce the need for oxygen and metabolic substrates, minimize ATP depletion [17]. To achieve this, the organ is flushed with ice-cold preservation solution during procurement and subsequently kept on ice, immersed in preservation solution, throughout the duration of preservation. Because every 10^{o}C drop in temperature is known to slow down metabolism 1.5–2 fold, healthy donor organs can be kept viable for extended periods of time while cut-off from circulation [18].

However, even under these conditions a significant degree of metabolic activity still exists. In the absence of oxygen and nutrients, metabolism becomes anaerobic and by-products of metabolism accumulate [17]. Cellular energy charges gradually become depleted and ATP-dependent processes, such as maintenance of transcellular electrolyte gradients, cannot take place. Eventually cell swelling, membrane leakage, mitochondrial degradation and the direct harmful effects of hypothermia lead to preservation damage [18, 19].

Commonly used cold-storage solutions contain a variety of components that counteract the effects of cold ischemia. These include oncotic agents and impermeants to prevent cell swelling, as well anti-oxidants and metabolic substrates. Nevertheless, preservation damage is directly related to the duration of preservation and the time organs can be preserved with hypothermic storage is therefore limited [18]. Even so, cold storage has given excellent results for the storage of healthy organs obtained from HBD, but results are less encouraging when used for the preservation of NHBD organs.

Mechanisms of damage to NHBD livers

The poor transplant outcome of NHBD livers is due to a combination of the effects of warm-ischemic damage sustained prior to explantation and the successive cold ischemia during cold storage. In fact, prolonged cold ischemia has been identified as the most important factor contributing to poor outcome in NHBD liver transplantation, with every additional hour of cold ischemic time increasing the risk of graft failure by 17% [20].

At body temperature even a short period of ischemia causes a rapid decline in intracellular energy stores and build-up of potentially toxic metabolites, in particular xantine oxidase. When the organ is subjected to an ensuing period of cold storage, the harmful effects of cold ischemia are superimposed on this. Upon reperfusion during reimplantation, the cumulative cellular damage sustained during ischemia, preservation and subsequent rewarming is aggravated by the formation of reactive oxygen species (ROS) due to the renewed abundance of oxygen. A cascade of deadly cellular responses subsequently leads to inflammation, cell death and ultimately, organ failure [21, 22].

Various strategies have been investigated to improve the preservation of especially marginal donor organs. In this regard, the single most promising approach to overcome the inherent limitations of cold storage is machine perfusion [18, 23, 24].

Machine perfusion

In the early days of organ transplantation, machine perfusion was the preservation method of choice due to an absence of suitable cold preservation solutions. Machine perfusion with diluted oxygenated blood was used by Starzl during the first successful human liver transplantation [25]. Improvements to cold preservation solutions [26], culminating in the introduction of University of Wisconsin (UW) solution by Belzer and Southard in the 1980's [27] now meant that machine perfusion was no longer necessary; due to it's simplicity, static cold storage (SCS) of donor organs became the clinical gold standard.

Continuous perfusion with an oxygenated solution can prevent ATP loss and therefore avoid the deleterious sequence of events that occurs after ATP depletion. [28-29, 30]. Addionally, a variety of compounds, including free-radical scavengers, vasoactive compounds, inflammatory mediators, calcium-channel blockers, hormones, bile salts and metabolic substrates can be continuously supplied to the organ during machine perfusion. Nowadays, there are two main approaches that are in use: Hypothermic machine perfusion (HMP) that occurs between 0-4^{o}C and normothermic machine perfusion (NMP) that is performed at temperatures that are in the physiological range [18]. Both strategies have their

advantages and limitations. HMP is undoubtedly the more straightforward approach due to its simplicity, whereas NMP promises greater resuscitative potential by maintaining the organ under (near-) physiological circumstances.

Hypothermic Machine Perfusion

Currently, the most commonly used machine perfusion method is HMP. Rather than immersing the organ preservation solution after an initial flush and then keeping it in an ice filled container, the graft is continuously perfused with ice-cold, oxygenated preservation solution. Hereby, HMP relies on the same principle as cold storage; cooling of the organ to slow down metabolism, but ads the benefit of continuous oxygen supply to the graft and wash-out of waste of metabolites.

As early as 1967 Belzer and colleagues successfully preserved canine kidneys for 72 hours using HMP with oxygenated plasma at 8–12°C [31]. Brettschneider demonstrated the first successful clinical application of HMP for liver preservation in 1968 [32]. A typical HMP setup consists of an insulated organ chamber in which the respective donor organ is continuously perfused with oxygenated cold preservation solution [33,34]. Various devices are currently available commercially, including a disposable perfusion device with an air driven pump [35].

Hypothermic machine perfusion (HMP) has yielded promising results in both extending the safe preservation period as well as improving the preservation quality of marginal liver grafts in small and large animal models [36-38]. Moreover, a recent multicenter clinical trial demonstrated superiority of HMP compared to SCS for preservation of NHBD kidneys [11] and promising results have also been obtained for NHBD liver preservation in a small clinical series [39]. However, extended HMP is known to cause endothelial damage [40-42] and it does not avoid damage specifically caused by hypothermia. Therefore the potential of HMP to improve marginal liver preservation may ultimately be limited.

Normothermic machine perfusion

Recently NMP has gained interest as method for improved preservation of marginal donor organs. Instead of relying on hypothermic conditions to reduce the organ's metabolic requirements, NMP maintains the organ under near-physiological conditions. By continuous perfusion with warm oxygenated perfusate, both cold ischemia and cold-induced damage can be avoided, leading to better graft quality and potentially prolonging safe preservation time without increased preservation damage [18, 43].

The idea of keeping organs alive outside the body by replicating in-vivo circumstances is far from new. In the early 1800's, Le Gallois suggested, "if one could substitute for the heart a kind of injection of arterial blood, either naturally or artificially made, one could succeed easily in maintaining alive indefinitely any part of the body whatsoever"[44]. Although this was not possible at the time due to technical limitations and a lacking understanding of asepsis, the theory suggested by Le Gallois is the first example of the underlying principle of NMP. The first modern application of NMP was demonstrated in series of experiments published in the 1930's by Carrell and Lindberg. Here, various organs were kept viable for several days by perfusing them with heated, oxygenated serum in a device specially designed for the purpose [44].

In this regard, NMP is a logical approach for marginal donor organ preservation; Rather than subjecting a pre-damaged organ to a further insult in the form of an additional period of hypothemia, circulation and oxygen supply to the organ are re-established. Hereby not only enabling replenishment of ATP stores, but also restoring normal hepatic function. Because normothermic conditions enable protein synthesis, reparative processes are possible. Also, by monitoring various metabolic parameters, assessment of graft viability and prediction of post-transplant function can take place[18, 24].

NMP has been successfully used experimentally in the early nineties using both swine and monkey livers, though the focus lay on establishing a liver-support therapy as bridging device for patients awaiting transplantation [45,46]. The first successful use of NMP for NHBD liver preservation was demonstrated by the group of Neuhaus at Charité, Berlin [47]. Here, orthotopic transplantation of porcine NHBD livers after 60 min. of warm ischemia followed by 4 hours of NMP was performed with excellent outcome. Further key developments, including successful 72 hour preservation of porcine livers, were described in a series papers by the group of Sir Friend at the University of Oxford [48-51]. This culminated in successful transplantation of porcine NHBD-livers after 60 min. of warm ischemia and 20 hours of NMP [19].

One of the major drawbacks of NMP is its complexity and associated costs. For cold storage of a donor organ, nothing more is needed that an insulated container filled with ice and the storage solution in which the organ is immersed. Due to the requirements of a fully metabolically active organ, NMP requires a far more elaborate setup. A typical circuit may include a perfusion chamber in which the organ is held during perfusion, a number of centrifugal pumps, a membrane oxygenator and a heat exchanger to control perfusate temperature [47, 48]. Typically, existing components from cardiopulmonary bypass or extracorporeal membrane oxygenation (ECMO) circuits are used. In the series of papers

published by the Berlin group, a hemodialysis circuit was added for detoxification, as well as a sophisticated perfusion chamber that simulated oscillating intra-abdominal pressure to improve sinusoidal perfusion [46, 47].

For NMP to become clinical reality, scaling down equipment to enable long-distance transportation to and from transplant centers could prove to be a major hurdle to take. A potential solution could lie in using a combination of NMP with a period of cold storage during transportation with. However, even a short period of cold storage prior to NMP has been shown to have a negative impact on the potential recovery of the organ [50, 51]. That subjecting an already ATP-depleted organ to the harsh conditions of cold ischemia negatively impacts potential recovery is hardly surprising, but whether a period of cold storage is feasible after recovery with NMP has taken place is unknown. This approach would enable recovery from ischemia during NMP, after which the organ could be transported in a traditional manner on ice. In a clinical setting, this could be achieved by ex-vivo machine perfusion in a dedicated device or in-situ normothermic recirculation using ECMO, followed by conventional cold storage during transportation [52, 53].

Subnormothermic Machine Perfusion

Whereas both HMP and NMP increase energy stores compared to SCS, NMP has additional regenerative potential by keeping the organ in a state that enables cytoprotective- and repair processes to take place. Whether the challenging near-physiologic conditions of NMP are optimal and necessary for recovery of ischemic livers is unknown. Machine perfusion at temperatures in the sub-normothermic range (20-30 ^{o}C) has been shown to improve preservation quality of marginal donor livers [54, 55] and improve cell quality during hepatocyte isolation [56]. An important potential benefit of sub-normothermic machine perfusion (SNMP), is that a lower preservation temperature leads to decreased metabolic requirement of the perfused organ [57]. Compared to NMP, the lower oxygen demand under subnormothermic conditions may therefore eliminate the need for an oxygen carrier [23], and enable the use of lower flow rates, which could reduce endothelial damage due to shear stress. Additionally, stringent temperature control and dialysis may be omitted. These changes could facilitate designing a less complex machine perfusion setup that combines the simplicity of HMP, with the resuscitative potential of NMP, while circumventing the latter's logistical limitations. SNMP could therefore prove to be a promising medium in between the two previously mentioned extremes.

Chapter 1

Rationale and experimental design

Rationale and experimental design

This thesis focuses on the exploration of NMP as method for preservation and resuscitation of ischemic rat livers. The experiments described were conducted from 2004-2008 during a research-fellowship at Massachusetts General Hospital's and Shriners Burns Institute's Center for Engineering in Medicine (CEM). Initially, work in the laboratory focused primarily on the study of liver metabolism and hepatic pre-conditioning strategies to improve transplant outcome. For this purpose, the time-honored isolated perfused rat liver (IPRL) model was used for in- and ex-vivo rat liver perfusion in order to study the dynamics of hepatic metabolism under various conditions. Upon learning about the work on extracorporeal liver perfusion described previously, we became interested in the idea of using the IPRL model to study liver preservation. Although outstanding work on NMP had been published previously, only large animal models such as monkey and pig were described and no small animal model for NMP existed. This limited NMP research to a select number of surgical groups with the expertise to conduct experiments on this scale. Therefore, we considered the development of a small animal model for NMP a valuable addition to the field. Hereby, we hoped to create a platform that was not associated with the high costs and complex logistics of large animal models and was therefore more amenable to laboratory work.

The first experimental section, **chapter two**, describes the development and characterization of a small animal model for NMP. The basis for our organ preservation system was formed by the IPRL, which has been used for decades as an analytical setup to study the shortterm dynamics of hepatic metabolism under various conditions [58, 59]. Traditionally, the IPLR uses a buffer solution without oxygen carrier as perfusate, therefore supra-physiological oxygen levels and flow rates are needed to provide adequate tissue oxygenation. Consequently, mainly due to the high oxidative and shear stress under these conditions, livers can be maintained in a steady state for only a limited time, and the perfused rat liver was therefore to be considered a dying organ [58]. In order to make the IPRL suitable for preservation, a number of modifications had to be made to enable long-term steady-state liver perfusion. The most important changes were: 1) providing an oxygen carrier that delivered sufficient oxygen at physiological flow rates 2) reducing priming volume 3) provide sufficient metabolic substrates to maintain liver metabolism under normothermic conditions 4) provide a means of detoxification and pH-stabilization.

For this purpose, we designed a system with a priming volume of approximately 55-60 ml, including an additional dialysis circuit. Our machine perfusion solution (MPS) was based on fortified tissue culture medium to which erythrocytes were added to a hematocrit of

approx. 20 %. With our model, we were able to maintain rat livers in a steady state for up to 12 hours, allowing subsequent orthotopic transplantation.

Chapter three describes the use of our newly developed NMP model for preservation of NHBD rat livers. After demonstrating stable extracorporeal preservation of fresh rat liver with NMP, the next step was to explore whether resuscitation of ischemic rat livers was possible with our system. To model NHBD, rat livers were subjected to 60 minutes of ex-vivo warm ischemia at 34 °C as previously described [37]. Subsequently, they were preserved by either static cold storage at 4 °C, or by NMP at 37 °C. Whether normothermic preservation of ischemic livers resulted in transplantable grafts, was evaluated by orthotopic transplantation into syngeneic recipients. Short-term graft function was evaluated during a two-hours ex-vivo sanguineous reperfusion.

Chapter four describes the use of a combination of NMP and SCS for rat NHBD liver perfusion. Previous results by others indicated that a period of cold storage prior to NMP revoked the latter's resuscitative potential. The observations during the experiments described in the previous chapter led us to believe that the initial 60-120 min. of perfusion were the most critical in the process of recovery from ischemia. Therefore, we hypothesized, that an initial period of NMP might be combined with a subsequent period of cold storage. To investigate this approach, we subjected rat livers to 45 min of ex-vivo warm ischemia and subsequently preserved them with a combination of 3 hours NMP at 37 °C, followed by 3 hours of cold storage in UW solution, or by 6 hours of cold storage UW solution alone. Graft viability was evaluated in an orthotopic transplantation model.

Chapter five describes SNMP for NHBD resuscitation. Machine perfusion at temperatures in the subnormothermic range may reduce the need for an oxygen carrier and allow a less complicated perfusion system to be used, while still maintaining the resuscitative potential of NMP. To evaluate whether resuscitation of ischemic liver was possible with SNMP, rat livers were subjected to one hour of ex-vivo ischemia and subsequently preserved with SNMP at either 20 or 30°C for 5 hours, followed by orthortopic transplantation. Short-term graft function was evaluated during ex-vivo reperfusion with diluted whole blood. As control ischemic and fresh liver were preserved with cold storage for 5 respectively 6 hours, as well as with NMP at 37°C.

Chapter six describes how data obtained during ex-vivo diluted blood reperfusion is correlated with transplant-survival of ischemic rat livers after NMP to identify a predictor of transplant outcome. As opposed to conventional organ preservation with SCS, an important potential benefit of both HMP and NMP is the possibility to measure the release

profiles of various markers of liver injury during perfusion. Hereby, organ viability can be assessed during preservation and could consequently be used to predict transplant outcome. A reliable viability indictor could not only improve transplant safety, it may also lead to less livers being discarded due to doubts about viability.

Chapter seven describes the use of metabolic flux analyis (MFA) to perform a dynamic metabolic analysis of fresh and ischemic livers during NMP. Identifying metabolic parameters critical to organ stability and recovery during NMP could help to optimize preservation conditions and perfusate composition. However, current studies typically focus on global indicators of energy recovery, such as glucose utilization, lactate accumulation, albumin synthesis and urea formation, which are by themselves non-specific. To evaluate the multiple pathways involved in recovery, MFA was used to correlate 28 measured and 34 calculated metabolite rates of uptake or release from hourly perfusion time points of ischemic and fresh organs during NMP.

Chapter eight describes the metabolic characterization variability of perfused livers as a function of ischemia. For this reason, dynamic analysis of metabolic activity during NMP was performed with multivariate statistical process monitoring (SPM) to analyze the behavior of fresh and ischemic livers during perfusion. To achieve this, we first evaluated perfusate metabolite levels during NMP of fresh livers that were transplanted successfully using multi-way principal component analysis (MPCA). MPCA was then applied to the perfusate metabolite levels during NMP of ischemic livers in the same manner. An index of ischemia then developed to assess whether an ischemic liver is beyond salvage and whether machine perfusion has rendered an injured organ sufficiently viable for transplantation.

Chapter nine describes how a partial-least squares (PLS) statistical modeling algorithm was developed to evaluate organ viability on-line based on blood gas analysis data. The results demonstrate that with the proposed methodology graft viability can be monitored and transplant success rate can be predicted online during preservation.

Chapter 2

A model for normothermic preservation of the rat liver

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Abstract

Current techniques for the preservation of donor livers typically rely on cold temperatures (~0 to 4°C) to slow down metabolic processes. Recently, normothermic extracorporeal liver perfusion (NELP) has regained interest as a potentially promising approach for long-term liver preservation. Unlike cold storage techniques, NELP attempts to maintain the liver in a near physiological environment, thus enabling normal metabolic as well as tissue repair processes to take place, which may help in the recovery of ischemically damaged and fatty donor livers, both of which represent significant untapped sources of donor livers. However, NELP is technically more complex than cold storage techniques, and its development is limited by the lack of standardized small animal models. Here we describe a rat NELP system that is both simple and cost-effective to run. We show that rat livers that underwent NELP for 6 hours could be routinely transplanted into syngeneic recipient rats with excellent one-month survival. During perfusion, the release of cytosolic enzymes, bile and urea production, as well as oxygen uptake rate, could be readily monitored, thus providing a comprehensive picture of hepatic function prior to transplantation. This system will help in the optimization of NELP in several ways, such as for the improvement of perfusion conditions and the development of quantitative metabolic criteria for hepatic viability

Introduction

Liver transplantation is currently the only established treatment for end-stage liver disease. The demand continuously exceeds the supply of donor livers, and a large number of patients still die while on the waiting list for a donor liver (www.unos.org). Various approaches are currently being investigated to alleviate the donor shortage, which can be categorized as (a) surgically based and (b) tissue engineering based. The former includes extending liver graft criteria to include marginal and rejected donor livers and split liver transplants from living donors. The latter includes extracorporeal liver support using whole xenogeneic or rejected human donor livers[46, 60], bioartificial livers, and hepatocyte transplantation techniques.

Normothermic extracorporeal liver perfusion (NELP) has been suggested as an alternative to cold storage of donor organs, in particular when applied to the preservation of marginal donor livers [18, 49]. Near-normothermic machine perfusion has been successfully used in experimental kidney preservation[61, 62], and recently normothermic perfusion has shown to be superior to simple cold storage in the preservation of DCD livers [43, 47, 49]. Unlike cold storage and cold machine perfusion techniques, which rely on the reduction of metabolic rates as a result of decrease in temperature, NELP attempts to maintain the organ in an environment that is as close to physiological as possible. NELP therefore has a number of potential benefits over cold storage, including the ability to restore oxidative metabolism in ischemically damaged organs, the removal of end-products of metabolism, and the possibility of pre-transplant diagnostic testing of the graft and the induction of repair processes. Possible other uses include immunomodulation and (pre)conditioning of the donor organ[43].

Although NELP is a promising technique for long-term preservation of donor livers, there are many obstacles that need to be overcome before it is a clinically feasible approach. The main issue is that NELP is more complicated than hypothermic preservation methods, and some other experimental preservation techniques, such as hypothermic perfusion and oxygen persufflation[63]. Due to its complexity, some investigators envision using NELP following a period of cold storage, but this approach has yielded mixed results[50, 51]. Hypothermic oxygenated machine perfusion has been successfully used in this manner, although a clear advantage over static cold storage could only be shown upon ex-vivo reperfusion[36, 64, 65]. Evaluation of the potential benefits of NELP has been impeded by the lack of standardized small animal models.

In this study, we describe a novel rat NELP system based on an extensively documented isolated perfused rat liver system that was originally developed for short-term perfusions [58, 59, 66]. We have modified the latter by (a) the use of a cell culture-based perfusate supplemented with plasma, (b) the use of autologous erythrocytes as oxygen carriers, and (c) the incorporation of continuous dialysis. We demonstrate using normal healthy rat livers, that this improved system can maintain livers metabolically stable for at least 6 hours, significantly extending the normothermic perfusion time from the typical 2-4 hours for perfused rat livers [58, 67]. Furthermore, we show that the perfused livers could be routinely transplanted into syngeneic recipients with excellent long-term survival. This system will be useful to optimize NELP and compare NELP with other liver preservation techniques, such as simple cold storage and cold machine perfusion.

Materials and Methods

Chemicals

Phenol red-free Williams Medium E was from Sigma Chemical, St. Louis, MO. Human insulin (Humulin) was from Eli Lily (Indianapolis, IN). Penicillin/streptomycin and L-glutamine stock solutions were from Gibco Invitrogen (Grand Island, NY). Hydrocortisone (solu cortef) was from Pharmacia &Upjohn, Kalamazoo, MI. Heparin was from APP (Schaumberg, IL).

Isolation of donor livers

Experiments were performed using male Lewis rats (Charles River Labs, Wilmington, MA) weighing 250-300 g. The animals were maintained in accordance with National Research Council guidelines and the experimental protocols were approved by the Subcommittee on Research Animal Care, Committee on Research, Massachusetts General Hospital.

The liver transplantation procedure used was the cuff technique first described by Kamada and Calne[68-70]. All the surgery was carried out by the same microsurgeon (H.T.) with a prior experience of more than 100 orthotopic liver transplants in the rat. Surgery was carried out using a Zeiss Opmi 1 microscope (Prescott, Monument, CO) with 6X magnification.

Briefly, donor operations began with a transverse abdominal incision. The bowel and the duodenum were retracted to the right using a moist gauze to expose the portal area of liver, the common bile duct (CBD) and the inferior vena cava (IVC). The right phrenic vein emptying into the supra-hepatic vena cava (SHVC) was ligated. The CBD was transected after distal clamping and insertion of 22 G polyethylene stent (Surflo, Terumo, Somerset, NJ). The IVC was divided from the right renal and adrenal veins. The portal vein (PV) was divided from the splenic and gastro-duodenal veins. One ml of saline containing 200 U

of heparin was injected through the penile vein. Several liver ligaments were dissected. The IVC was cross-clamped using a microvessel clip. The PV was clamped distally and a hemicircumferrential incision was made. An 18 G polyethylene cannula (Terumo) was inserted into the PV and the liver was flushed with 5 ml of cold (4^0C) university of Wisconsin (UW) solution (Viaspan, Barr Labs, Pamona, NY). The diaphragm was opened, the SHCV was transected and the liver was flushed with an additional 5 ml of UW solution. The liver was removed and placed in a bowl of ice-cold UW solution and cuffed as described previously[68]. Some livers were left in simple cold storage for 7 hours prior to transplantation. The others were used for normothermic extracorporeal liver perfusion.

Liver perfusion system

The perfusion system consists of a primary liver perfusion circuit and a secondary dialysis circuit (Figure 1A). The primary circuit consists of a jacketed liver perfusion chamber (Radnoti Glassware Technologies, Monrovia, CA) shown in Figure 1B, a Masterflex™ peristaltic pump (Cole Parmer, Vernon Hill, IL), a membrane oxygenator (Radnoti), heat exchanger (Radnoti), bubble trap (Radnoti), a second peristaltic pump (Cole Parmer), and a hollow fiber dialyzer with a 2200 cm^2 membrane area and a 30 kD nominal molecular weight cut-off (Spectrum Labs, Rancho Dominguez, CA). Temperature within the liver was maintained at 37.5^0C with a homeothermically controlled water bath (Lauda, Brinkmann, Westbury, NY) and was continuously monitored by two thermocouples (Omega, Stamford, CT), one located between the lobes of the liver, and the other in the PV cannula just before entering the vein. The oxygenator was gassed with a 95% O_2/5% CO_2 mixture. The secondary dialysis circuit consists of a 500 ml reservoir and peristaltic pump.

Normothermic extracorporeal liver perfusion

Dialysis medium consisted of phenol red-free Williams Medium E to which was added 2 U/l insulin, 40,000 U/l penicillin, 40,000 µg/l streptomycin, 0.292 g/l L-glutamine, 10 mg/l hydrocortisone and 1000 U/l heparin. Perfusate medium was prepared by supplementing the same medium used for dialysis with 25% (v/v) freshly isolated rat plasma, and then adding freshly isolated rat erythrocytes to a hematocrit of 16-18%. To obtain rat plasma and red blood cells, rats were anesthetised with isoflurane and 1 U heparin/g body weight was injected through the penile vein. Puncture of the abdominal aorta *using a 16 G intravenous catheter (Surflow, Terumo*, Somerset, NJ*)* was performed and the animal exsanguinated. Blood was kept on ice and then centrifuged in 50 ml tubes at 3200 rpm for 15 min at 4^0C. The plasma was removed and set aside. The buffy coat was discarded. Erythrocytes were twice washed with saline containing 5% dextrose (*Baxter, Deerfield, IL*) and centrifuged for 5 min at 3200 rpm. The total perfusate volume used for one liver perfusion was 55 to 60 ml. Dialysate volume was 500 ml.

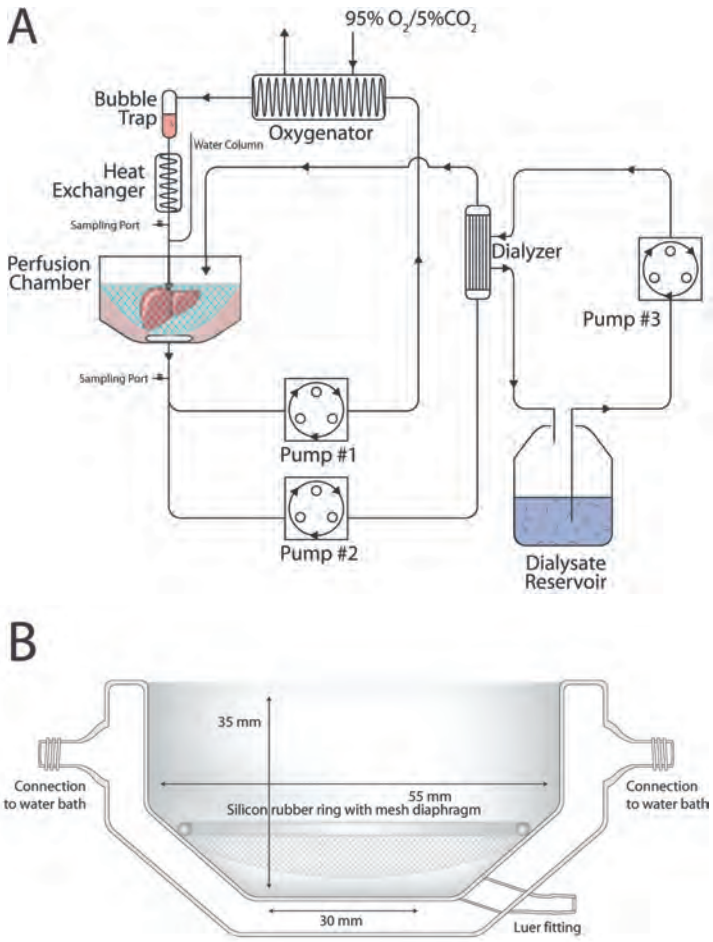

FIGURE 1 | Schematic of the normothermic liver perfusion system. (A) Perfusion circuit. (B) Detail of liver perfusion chamber.

The liver was immersed in perfusion medium placed in the jacketed perfusion chamber. An 18 G polyethylene catheter (Terumo) was placed inside the PV cuff and gently secured using a 6-0 silk suture. The CBD stent was connected to a PE 50 polyethylene catheter (Becton Dickinson) emptying into a pre-weighed microfuge tube for bile collection. The liver was perfused via the PV only and effluent flowed freely from the SHVC and IVC into the surrounding medium. Flow rate was maintained at 1.8 ml/min/g liver wet weight. The volume of perfusate in the primary circuit was visually monitored and kept constant by adjusting the flow rate of dialysate in the secondary circuit, typically ±20% of the flow rate in the primary circuit. After 3 hours of perfusion, the reservoir of dialysate was replaced by a similar reservoir containing 500 ml of fresh dialysate.

Analysis of perfusate levels of metabolites and liver enzymes

Perfusate samples (1 ml) were collected from the inlet of the liver in the primary circuit immediately prior to placing the liver in the perfusion system and hourly thereafter. Dialysate samples (1 ml) were collected from the reservoir in the secondary circuit at the same times. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose, and urea levels were measured using a Piccolo miniature blood chemistry analyzer (Abaxis, Union City, CA). Oxygen and CO_2 tensions were measured in perfusate samples (0.2 ml) taken from the in- and outflow (PV and IVC) of the liver every 10 min for the first hour of perfusion and subsequently every hour. Samples were analyzed immediately using a blood gas analyzer (Rapidlab, Chiron Diagnostics, Norwood, MA). The total (free and bound to hemoglobin) concentration of O_2 (g/dl) in the samples was determined according to the formula:

$$[O_2] = 0.0139 \times [Hb] \times FO2Hb + 0.00314 \times pO_2$$

where [Hb] is the hemoglobin concentration in g/dl, FO2Hb is the fraction oxygenated hemoglobin and pO_2 is the partial pressure of oxygen in mmHg. The hepatic oxygen uptake rate (HOUR) was determined by subtracting the total O_2 content in the outflow minus the inflow, then multiplying by the flow rate, and normalizing to the wet weight of the liver:

$$HOUR = ([O_2]_{in} - [O_2]_{out}) \times \text{flow rate} / \text{weight of liver}$$

Bile was collected continuously in pre-weighed microfuge tubes that were exchanged every hour. Wet weight of the liver was determined during the brief periods of cold storage before and at the end of the perfusion.

Light Microscopy

Liver tissue slices were fixed in 10% formalin, embedded in paraffin, sectioned to a 4 µm thickness, and stained with hematoxylin and eosin.

Transmission electron microscopy

Samples of liver tissue (approx.1mm^3) were fixed in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), washed in 0.1M cacodylate buffer and postfixed with a mixture of 1% osmium tetroxide + 1.5% potassium ferrocyanide for 2 hours, washed in water and stained in 1% aqueous uranyl acetate for 1 hour followed by dehydration in grades of alcohol (50%, 70%, 95%, 2 x 100%) and then infiltrated and embedded in TAAB Epon (Marivac Canada Inc., St. Laurent, Canada). Ultrathin sections (about 60-80 nm) were cut on a Reichert Ultracut-S microtome, picked up onto coppergrids, stained with 0.2% lead citrate and examined in a Tecnai *G^2 Spirit* BioTWIN transmission electron microscope. Images were taken with a 2k AMT CCD camera.

Transplantation surgery

At the end of the perfusion the liver was flushed with 20 ml of cold (0^0C) UW solution and placed on ice in a bowl containing UW solution. Typical cold storage time was around 60 min.

Briefly, the abdomen was opened with a midline incision and exposed with wound retractors. The bowel and the duodenum were retracted to the right using a moist gauze to expose the portal area of liver, the CBD and IVC. The right phrenic vein emptying into the SHVC was ligated. A 20 G polyethylene stent (Terumo) was inserted into the CBD at its bifurcation and secured with a 6-0 silk ligature, after which the CBD was transected. The hepatic artery and the suprarenal veins were divided. All ligaments of the liver were divided from the surrounding tissue. The PV was tied at its bifurcation and the IVC and PV were cross-clamped with microvessel clips. The SHVC was clamped with a Satinsky pediatric diaphragm clamp (Miltex, Lake Success, NY) and transected close to the liver. The PV and IVC were divided and the liver was removed. After removal of the liver, a gauze soaked in ice-cold UW solution was placed in the liver space and the donor liver was placed upon it. The SHVC was anastomosed using a 7-0 prolene running suture (Ethicon, Somerville, NY). The donor portal cuff was inserted into the lumen of the PV and secured with 6-0 silk. The microvessel clip on the PV as well as the Satinsky were removed. Reperfusion of the liver was established. The anhepatic phase of the procedure did not exceed 17 minutes. The cuff of the IVC was inserted into the lumen of the IVC and secured using 6-0 silk. The clip on the IVC was released. The CBD was connected. The wound was closed using 3-0 gut suture (Ethicon, Somerville, NY) and 8 ml/kg of warm (37^0C) lactated Ringer's solution with 5% dextrose and 2 ml/kg of $NaHCO_3$ 7% w/v were injected into the penile vein. The animals were put in a clean cage and allowed to recover from anesthesia under an infrared lamp for 30 min and were subsequently returned to the housing facility in single cage housing.

Post-operative blood sampling

To determine the post-operative levels of AST, ALT and total bilirubin, 100-200 µl of blood were collected on post-operative days 1, 3, 5 and 7 by tail vein puncture into a heparinized syringe under isoflurane anesthesia. The samples were immediately analyzed using a Piccolo miniature blood chemistry analyzer.

Statistics

Data are expressed as means ± standard error of the mean. Comparisons were made using the Student t test.

Results

Average operating parameter values for 6 perfusions are shown in Table 1, along with relevant values measured in vivo. The flow of perfusate through the portal vein of 1.80±0.12 ml/min/g was close to reported in vivo values, especially considering total (portal + arterial) blood flow. The main differences were the lower hematocrit, which was approximately 30% of the in vivo hematocrit, and the inflow pO2 of the perfusate, which was much higher than portal blood, and even arterial blood. The portal pressure was only slightly higher than values typically found in-vivo. CO_2 partial pressures were all within the reported physiological values.

TABLE 1 | Operating parameters during normothermic perfusion

	Normothermic Perfusion (n=6)	Published In Vivo Values [58,71]
Flow rate	1.80±0.12 ml/min/g	Portal: 1.2 to 1.5 ml/min/g Arterial: 0.2 to 0.3 ml/min/g
Hydrostatic pressure	12 to 15 cm H_2O	Portal: 8 to 10 cm H_2O
Hematocrit	17.63±0.58%	43%
Inlet pO_2	147.1±62.03 mmHg	Portal: 38 to 55 mmHg Arterial: 85 to 90 mmHg
Outlet pO_2	41.17±11.44 mmHg	37 to 42 mmHg
Inlet pCO_2	31.58±4.71 mmHg	Portal: 35 to 46 mmHg Arterial: 26 to 34 mmHg
Outlet pCO_2	36.64±5.20 mmHg	33 to 50 mmHg

Integrity of Liver During Perfusion

Levels of AST and ALT measured in the primary circuit increased 3 to 4 fold during the course of the perfusion (Figure 2). No ALT or AST could be found in the dialysate (data not shown). Liver wet weight slightly decreased from 9.74±0.81 g to 9.51±0.82 g during perfusion ($p<0.05$ by two-tailed paired t-test, n=11). Liver harvested 6 hours after normothermic perfusion showed a healthy appearance with no signs of degradation or vacuolization, except for a slight dilation of the sinusoids compared to livers in simple cold storage in UW solution for 6 hours (Figure 3a). TEM images demonstrate that livers stored under both conditions appear normal. Hepatocyte mitochondria appear elongated and not swollen, dominant rough endoplasmic reticulum. Sinusoids have open lumens and fenestrated endothelial walls. Cell nuclei appear round and healthy, and cells exhibit clear borders and obvious bile canaliculi (Figure 4).

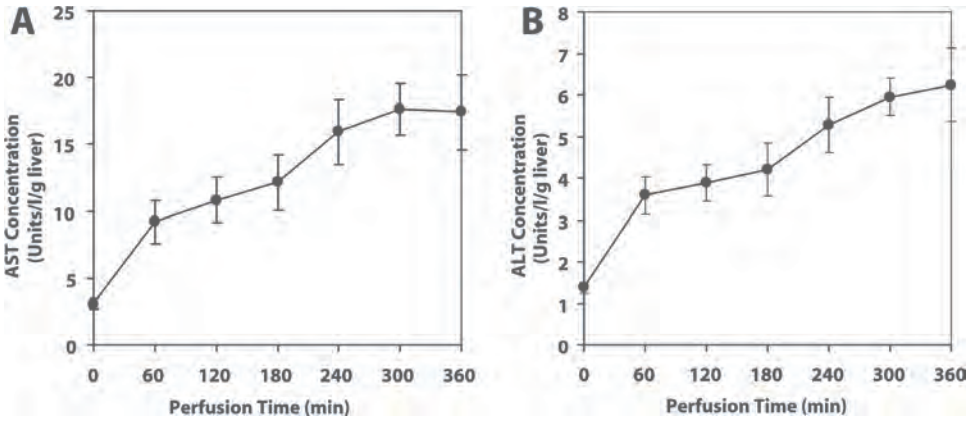

FIGURE 2 | Liver integrity during normothermic perfusion. (A) AST and (B) ALT levels in perfusate samples collected hourly from the primary circuit. Values are normalized to the wet weight of the liver. Values at 360 min were 17.39±6.34 U/l/g and 6.25±2.18 U/l/g for AST and ALT, respectively. N=6.

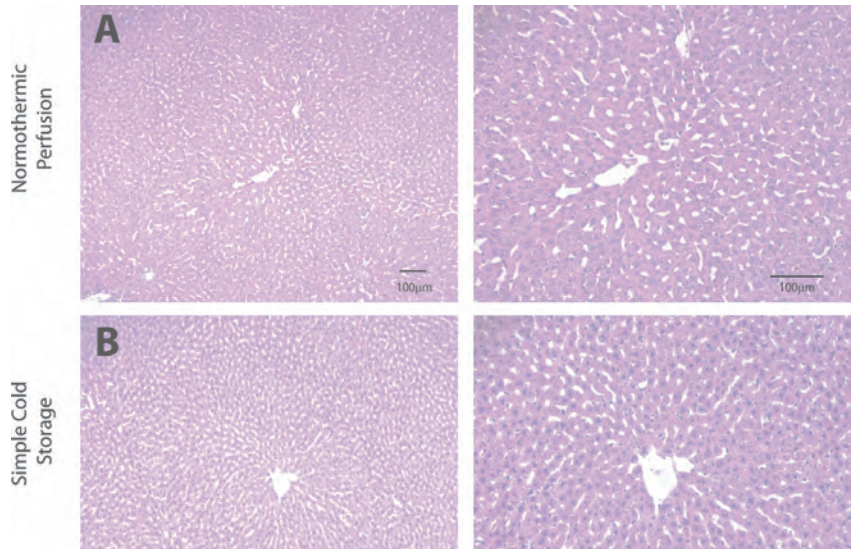

FIGURE 3 | Microscopic appearance of livers after (A) 6 hours of normothermic perfusion as compared to (B) those in simple cold storage in UW solution for 6 hours. Bar = 100 mm.

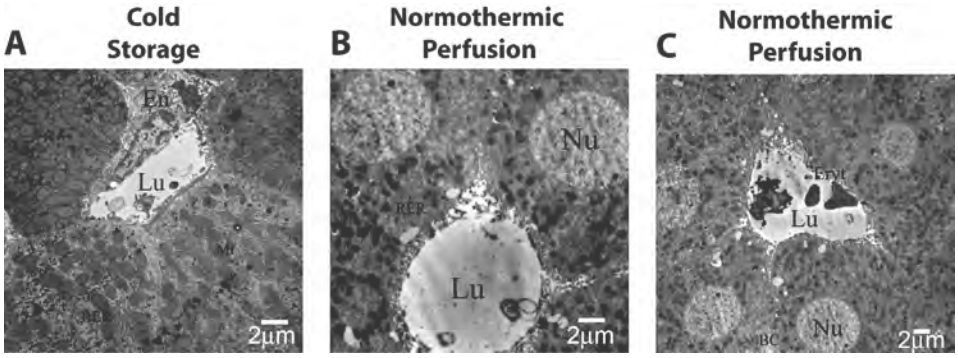

FIGURE 4 | Transmission electron micrograph of livers after (A) 6 hours of simple cold storage in UW solution as compared to (B, C) normothermic perfusion for 6 hours. Under both conditions the liver appears normal. Mitochondria (Mt) appear elongated and not swollen. Dominant rough endoplasmic reticulum (RER). Sinusoids appear have open lumens (Lu) and fenestrated endothelial wall. Cell nuclei (Nu) appear round and healthy, clear cell borders and obvious bile canaliculi (BC). The perfused liver shows a section of an erythrocyte (Eryt) and a thrombocyte in the lumen. Bar = 2 mm.

Metabolic Function Parameters During Perfusion

Bile production, oxygen uptake, glucose uptake, and urea secretion were monitored during the perfusions. Bile was secreted at a constant rate with an average total of 536±75 mg bile/g liver produced after 6 hours of perfusion (Figure 5A). The oxygen uptake rate declined rapidly during the first 60 min after initiation of the perfusion and reached a steady state value of 0.052±0.018 ml/min/g (2.32±0.08 µmol/min/g) liver for the remainder of the perfusion (Figure 5B). The glucose level in the perfusate decreased slightly from the initial value of 200 mg/dl to reach 182±12 mg/dl after 6 hours (Figure 5C). Urea levels exhibited a biphasic response (Figure 5D). In the early phase, urea levels were initially flat, and then increased almost linearly to reach a plateau at 0.6 mg/dl/g liver at 3 hours. At that time point, the dialysate was changed, causing the levels to suddenly decrease, followed by an increase at a rate similar to that observed in the 1 to 3 hour period, reaching a maximum level of 0.68±0.04 mg/dl/g at 6 hours.

Survival after Transplantation

Livers were transplanted into recipient rats after 6 hours of normothermic perfusion (n=11) or 6 hours of simple cold storage in UW solution at 0°C (n=5). In the perfused liver group one animal died during recipient surgery due to bleeding from the anastomosis of the SHVC and subsequent air embolism. All other animals (n=10) recovered rapidly from surgery and survived at least one month after transplantation. No signs of liver failure, such as jaundice, were observed. In the simple cold storage group, all animals also recovered from surgery and survived beyond one month.

Post-operative Liver Enzymes and Bilirubin

Transplant recipients were monitored for circulating liver enzyme and bilirubin levels to assess liver graft function (Figure 6). The levels of both AST and ALT were increased relative to baseline on post-operative day 1 in both groups, with values of 699±85 u/l and 431±80 u/l respectively in the NELP group, and 615±177 u/l and 372±138 u/l respectively for the SCS group. In the NELP group both ASL and ALT levels subsequently declined to reach 131±15 u/l and 72.7±6.6 u/l respectively on day 5, and increased again to reach 812±257 u/l and 373±133 u/l for AST and ALT, respectively, on day 7. In contrast, in the SCS group, the levels of AST and ALT peaked on day 5, reaching 182±182 u/l and 915±210 u/l, respectively, and then decreased subsequently on day 7. Comparing the NELP and SCS groups shows no statistically significant difference between AST and ALT levels, except on day 5 ($p<0.05$ by two-tailed n=3 unpaired t-test n=3). Total bilirubin levels, an indicator of liver function, were similar in both groups (N=3) on day 1, and increased to reach the maximum levels of 0.37±0.09 mg/dl and 1.73±0.83 mg/dl in the NELP and SCS groups, respectively, on day 7.

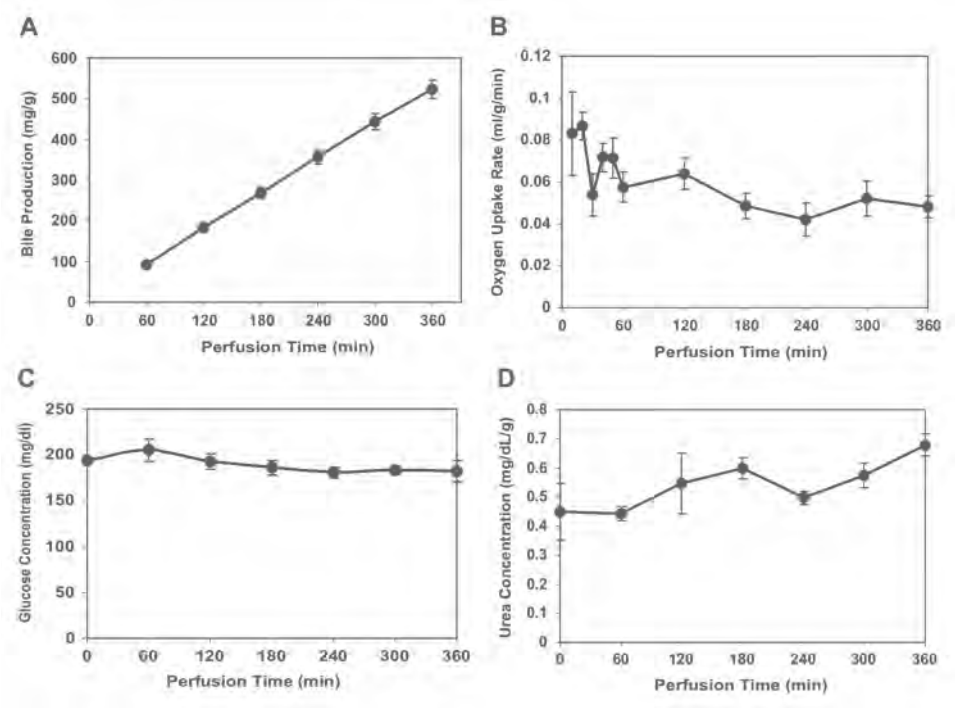

FIGURE 5 | Hepatic metabolic activity during perfusion. (A) Total bile produced normalized to wet liver weight. (B) HOUR normalized to wet liver weight. (C) Glucose levels during the perfusion. (D) Urea levels normalized to wet liver weight. N=6.

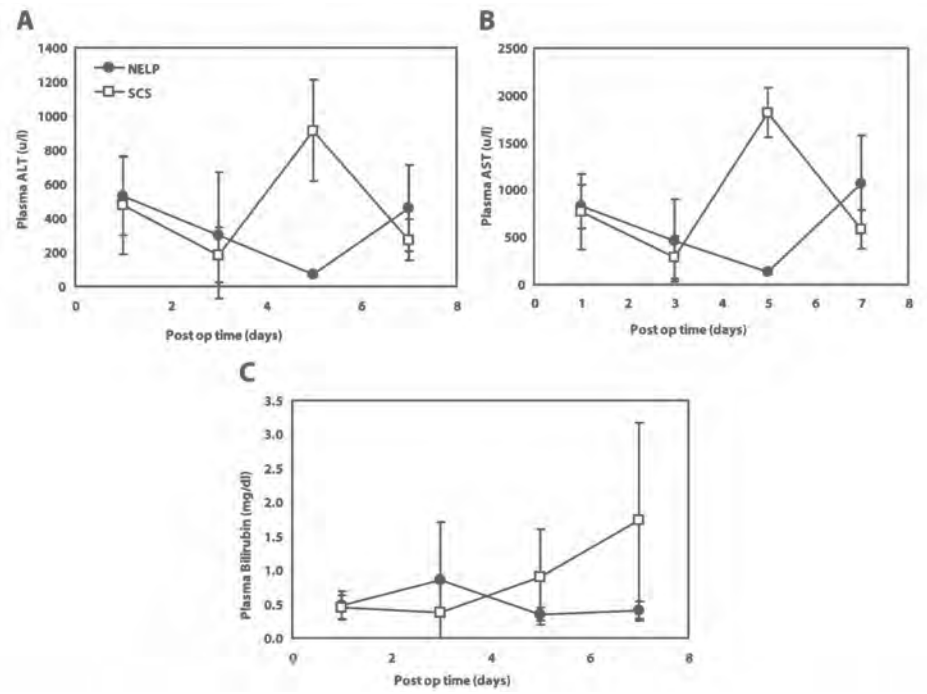

FIGURE 6 | Markers of hepatic integrity and function in the transplant recipient. (A, B) Post-operative plasma AST and ALT levels. (C) Total plasma bilirubin levels. N=3.

Discussion

We have modified the traditional isolated perfused liver system so that it can be applied to normothermic preservation of the rat liver. The most important changes that were made are: (a) the use of a cell culture-based perfusate supplemented with plasma and hormones, (b) the use of autologous erythrocytes as oxygen carriers, and (c) the incorporation of continuous dialysis. The lifespan of rat liver in traditional isolated perfused liver systems was typically less than 4 hours, and it was suggested to be considered a "dying organ"; the system was primarily to be used as short term analytical model[58]. Using this modified isolated liver perfusion system, rat livers could be normothermically preserved for 6 hours and subsequently transplanted with 100% success. Livers preserved in this manner were similar to livers preserved with conventional simple cold storage in UW solution with respect to gross morphology, histological appearance, recovery from surgery and long-term survival after orthotopic transplantation. Furthermore, liver metabolic function, as judged by bile production, hepatic oxygen uptake, and urea synthesis, were stable throughout the perfusion after an initial 60 min period of equilibration, making it relatively easy to assess the functional activity of donor livers prior to transplantation.

We chose the rat as model species because of relatively low cost (compared to large animals) and extensive body of published work with isolated rat perfusion systems. The blood supply of the rat liver is ~10% arterial and ~90% venous[71]. Although some studies indicate that certain areas of the liver are accessed by arterial blood only[72], the bulk of the literature suggests that it is possible to perfuse via the portal vein only[58], as well as to carry out the transplantation without reconstruction of the hepatic artery[70]. The transplantation procedure can be performed by one person with a total operating time around 50 min. Finally, inbred rat strains are widely available, which makes it possible to decouple immunological compatibility issues from handling, storage, and perfusion-related factors that affect the outcome of the transplanted livers. We have chosen to characterize our system using healthy liver grafts in order to establish optimal perfusion parameters, determine baseline metabolic values and establish transplant survival data.

Isolated liver perfusion has been extensively studied for over 90 years, although most studies have been carried out in the rat using asanguineous buffers as perfusate [59]. More recently, solutions containing a more complete panel of physiological substrates were used to assess various aspects of hepatic function[66, 67]. Cell culture-based perfusates have been used in reperfusion studies of the rat liver [73], hypothermic liver preservation [74] and near-normothermic kidney preservation[62]. Here we have chosen a basal perfusate medium consisting of Williams Medium E, which is routinely used for culturing hepatocytes. This medium contains both essential and non-essential amino acids, vitamins, glucose and the antioxidant glutathione. Insulin was added in order to mimic a "fed state" where the liver utilizes the available glucose in the medium. Hydrocortisone was added for its anti-inflammatory characteristics. The basal medium was supplemented with plasma, and washed rat erythrocytes were used as oxygen carriers. This approach ensured that no white blood cells or platelets are present in the perfusion solution, as these cells can become activated and cause undesirable reactions, such as clotting, the release inflammatory mediators and endothelial activation, in an extracorporeal perfusion setting. Plasma was added primarily to provide albumin and a high enough protein content to maintain oncotic pressure.

A key component of this perfusion system is the use of rat red blood cells as oxygen carriers. Unlike the traditional isolated perfused liver circuit, which typically utilizes a large circulating volume ranging from 150-300 ml[59], the primary perfusion circuit used in our system has a volume of 50 to 55 ml, making it possible to use blood from two 400 g rats providing 15 ml of blood each to achieve the hematocrit close to 20 deemed necessary for optimal liver function[58], for one perfusion experiment. Artificial oxygen carriers have successfully been used by others [61, 62], and in principle have the advantage of being inert and

could potentially exhibit more reproducible properties. However, current products, which mainly fall in the classes of polymerized hemoglobins and perfluorocarbons, are costly and have found limited use. Polymerized hemoglobins lose oxygen carrying capacity as they become oxidized into methemoglobin during perfusion because they lack the erythrocyte enzymatic machinery to reduce methemoglobin to hemoglobin. Perfluorocarbons, which are highly hydrophobic, must be incorporated as emulsions. They have been used with success as oxygen carrier for hypothermic machine preservation[70]. Our poor preliminary experience using perfluorocarbon based oxygen carriers in our setup, possibly based on instability of the used emulsion, lead us to not pursue their further use.

In our initial experiments without dialysis, we observed gradual acidosis, hypernatraemia, and depletion of metabolic substrates in the perfusate (unpublished observations). Our efforts to transplant livers perfused in this manner were unsuccessful, consistent with the poor survival reported previously with rat livers normothermically perfused without dialysis[75]. Long-term normothermic perfusion of the porcine liver has been demonstrated,[49] but transplantation of these livers has not been reported. The only study that demonstrates successful stable post-transplant survival employed a dialysis circuit[47]. Clearly, given the small volume of the primary perfusion circuit, a means to remove waste products and replenish nutrients was necessary; therefore, we incorporated a secondary dialysis circuit with a 10 times greater volume, which we found effective to stabilize the pH, electrolyte, and substrate levels in the primary perfusion circuit. The molecular cut-off weight (MCOW) of 30 kD is large enough to enable free exchange of electrolytes, nutrients and metabolites while retaining albumin in the perfusate, thus providing the necessary oncotic pressure.

In conclusion, we have developed a simple and cost-effective NELP system that successfully preserves rat livers for up to 6 hours. Normothermically preserved livers were histologically similar to livers preserved by simple cold storage in UW solution for the same duration, and could be orthotopically transplanted successfully. Although we do not claim to have improved on simple cold storage as a preservation method for healthy liver grafts, the system described herein is a cost-effective alternative to the porcine liver perfusion and transplantation models that have been employed in the field so far. It could be easily set up in most laboratories, which should speed up the development of NELP as a preservation method. More specifically, we envision that this system could be used to further optimize perfusion conditions to extend normothermic preservation times for normal livers, as well as to provide new opportunities for the preservation and recovery of marginal liver grafts.

Chapter 3

Recovery of warm ischemic rat liver grafts by normothermic extracorporeal perfusion

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Abstract

Background

Liver transplantation is currently the only established treatment for end-stage liver disease, but it is limited by a severe shortage of viable donor livers. Donors after cardiac death (DCD) are an untapped source that could significantly increase the pool of available livers. Preservation of these DCD livers by conventional static cold storage (SCS) is associated with an unacceptable risk of primary non-function and delayed graft failure. Normothermic extracorporeal liver perfusion (NELP) has been suggested as an improvement over SCS.

Methods

Livers recovered from male Lewis rats were subjected to 1hr of warm ischemia and preserved with 5hrs of SCS or NELP, and transplanted into syngeneic recipients. As additional controls, non-ischemic livers preserved with 6hrs of SCS or NELP and unpreserved ischemic livers were transplanted.

Results

Following NELP, ischemically damaged livers could be orthotopically transplanted into syngeneic recipients with 92% survival (N=13) after 4 weeks, which was comparable to control animals which received healthy livers preserved by SCS (N=9) or NELP (N=11) for 6hrs. On the other hand, animals from ischemia/SCS control group all died within 12hrs post-operatively (N=6). Similarly, animals that received ischemic livers without preservation all died within 24hrs after transplantation (N=6).

Conclusions

These results suggest that NELP has the potential to reclaim warm ischemic livers that would not be transplantable otherwise. The rat model in this study is a useful platform to further optimize NELP as a method of recovery and preservation of DCD livers.

Introduction

Transplantation is currently the only established treatment for end-stage liver disease, but it is limited by the shortage of available organs. Extending liver graft criteria to include marginal livers, such as those obtained from donors after cardiac death (DCD), could alleviate this problem[6]. It is estimated that about 6,000 ischemic livers [6, 76] could be reconditioned for transplantation, effectively doubling the availability of grafts. However, conventional static cold storage (SCS) of these marginal organs leads to unsatisfactory transplant outcome [6]; they exhibit a higher risk of primary non-function as well as delayed graft failure, especially due to biliary complications such as stricture[77]. It is thought that warm ischemic damage experienced by DCD livers leads to increased sensitivity to subsequent cold ischemia and rewarming injury associated with SCS.

Both hypothermic and normothermic machine perfusion have been suggested as methods to improve the preservation of DCD livers. The advantages of hypothermic perfusion over SCS have been previously demonstrated[37, 78-80]. Recently, functional recovery of ischemically damaged rat livers was shown using a combination of SCS followed by short-term hypothermic machine perfusion[36, 37, 64, 78-80]. However, extended hypothermic machine perfusion can cause endothelial damage [41] which may limit organ viability.

Normothermic extracorporeal liver perfusion (NELP) has been suggested as a method to avoid the problems associated with SCS and hypothermic perfusion[43, 47, 81]. Near-normothermic machine perfusion has been successfully used in experimental kidney preservation [62, 82], and recently normothermic perfusion was shown to be superior to SCS in the preservation of DCD livers[43, 47, 49]. A survival benefit after transplantation of DCD livers preserved normothermically has been demonstrated in one study using a porcine model[47].

The complexity and high cost of large animal models limits the number of thorough studies that can be conducted, making systematic characterization and optimization of NELP difficult. To provide an alternative model that is more amenable to research and development, we developed a small-scale NELP system where rat livers can be successfully transplanted after 6 hours of normothermic perfusion[83]. Herein, we investigated the potential of NELP to recover warm ischemic livers. We show that rat livers that underwent 60 minutes of ex-vivo warm ischemia (34°C) and then preserved by 5 hours of NELP could be successfully transplanted into syngeneic recipients. By contrast, recipients of similar livers stored by SCS for 5 hours, as well as those transplanted directly without having undergone preservation, did not survive.

Experimental Procedures

Isolation of donor liver

Experiments were performed using male Lewis rats weighing 250-300g (Charles River Labs, Wilmington, MA). The animals were maintained in accordance with National Research Council guidelines and the experimental protocols were approved by the Subcommittee on Research Animal Care, Massachusetts General Hospital. All animals were anesthetized with isoflurane using a Tech 4 vaporizer (Surgivet, Waukesha, WI) under sterile conditions. The donor liver surgery and is described in detail elsewhere[68, 83].

Warm ischemia induction

After isolation from the donor, the liver was weighed and then placed in a temperature controlled chamber filled with saline and maintained at 34±0.1°C for one hour. During this period the PV and IVC were cuffed as previously described[83].

Normothermic liver perfusion

The perfusate and dialysate comprised phenol red-free Williams Medium E (Sigma Chemical, St. Louis, MO) supplemented with: 2 u/l insulin (Humulin, Eli Lily, Indianapolis, IN), 100,000 u/l penicillin, 100 mg/l streptomycin sulfate (Gibco, Invitrogen, Grand Island, NY), 0.292 g/l l-glutamine (Gibco), 10 mg/l hydrocortisone (Solu-Cortef, Pharmacia & Upjohn, Kalamazoo, MI), and 1000 u/l heparin (APP, Schaumberg, IL). Fresh frozen rat plasma (25% v/v), and erythrocytes (18-20% v/v) were collected earlier [83] and added to the perfusate only. The total perfusate volume was 55-60 ml.

The perfusion system consisted of a primary liver perfusion circuit and a critical secondary dialysis circuit [83]. Briefly, the primary circuit included perfusate that recirculated via a peristaltic pump through a jacketed perfusion chamber, a membrane oxygenator, a heat exchanger and bubble trap. The oxygenator was gassed with a mixture of 74% N2/21% O2/5% CO2 and 100% O2 to maintain a constant pH. A fraction of the perfusate was diverted to the secondary circuit through a hollow fiber dialyzer with a 2200 cm2 membrane area and a 30 kD nominal molecular weight cut-off (Spectrum Labs, Rancho Dominguez, CA) at a rate of 3 ml/ min/g wet liver weight. The secondary circuit dialyzed the perfusate by counter-current exposure to 450ml of dialysate. The volumes of perfusate and dialysate were kept constant by varying the flow of dialysate through the dialyzer in the secondary circuit. Temperature within the system was maintained at 37.5°C.

After the warm ischemic period, the liver was flushed with 10ml of warm saline and immersed in perfusate in the perfusion chamber. The liver was perfused at a constant

flow rate via the portal vein and effluent flowed freely from the suprahepatic and inferior vena cava into the surrounding medium. While the recipient hepatectomy was prepared, the liver was disconnected from the circuit, rinsed in a bowl of saline at room temperature and weighed again prior to transplantation. The operating parameters of the perfusion system were: Flow rate: 1.84±0.05 ml/min/g; Portal hydrostatic pressure: 12-16 cm H2O (8-12 mmHg); Hematocrit: 17.8%±0.8; Inlet pO2: 128.4±8.1 mmHg; Outlet pO2: 47.9±1.7 mmHg; Inlet pCO2: 30.1±1.1 mmHg; Outlet pCO2: 34.6±1.6 mmHg.

Analysis of perfusate levels of metabolites and liver enzymes

Perfusate samples (1ml) were collected prior to placing the liver in the perfusion system and hourly thereafter. For each sample, 100µl aliquots were immediately analyzed using a Piccolo comprehensive metabolic panel (Abaxis, Union City, CA) for alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, electrolytes and glucose. The remainder was stored at -80°C for later analysis. Dialysate samples (1ml) were collected at the same times and stored at -80°C.

For analysis of the hepatic oxygen uptake rate (HOUR), 200 µl samples were taken from the PV and IVC of the liver every 10 minutes for the first hour of the perfusion and every hour subsequently. Samples were analyzed immediately using a blood gas analyzer (Rapidlab, Chiron Diagnostics, Norwood, MA). The total concentration of O2 (ml/dl) in the samples was determined according to the formula:

$$[O2] = 1.39 \times [Hb] \times FO2Hb + 0.00314 \times pO2$$

where [Hb] is the hemoglobin concentration in g/dl, FO2Hb is the fraction oxygenated hemoglobin and pO2 is the partial pressure of oxygen in mmHg. HOUR was determined as:

$$HOUR = (([O2]\ in - [O2]\ out)/100) \times \text{flow rate/weight of liver.}$$

Bile was collected continuously in pre-weighed microfuge tubes that were exchanged every hour.

Recipient surgery

The cuff technique developed by Kamada and Calne [68-70,83] was implemented and is described in detail elsewhere [83]. All recipient surgery was carried out by the same microsurgeon (H.T.). The anhepatic phase of the procedure was typically 13- 15 minutes and did not exceed 17 minutes. Animals were hydrated with 8ml/kg of warm (37°C) lactated Ringer's solution with 5% dextrose and 2ml/kg of NaHCO3 7%w/v (Abbott, North Chicago, IL) by penile vein injection.

The animals were put in single clean cages, allowed to recover from anesthesia under an infrared lamp for half an hour, and subsequently returned to regular housing. The first 12 hours post-operatively animals were checked every 2 hours and subsequently every 8 hours for one week.

Post-operative blood sampling

To determine the post-operative levels of AST, ALT and total bilirubin, 100-200µl of blood were drawn from the tail vein under isoflurane anesthesia on post-operative days 1, 3, 5, 7, 14, 21, 28 and immediately analyzed using a Piccolo blood chemistry analyzer. For these studies n was ≥4 for each group.

Simple cold storage

Warm ischemic livers (n=6) and freshly isolated livers (n=6) were flushed with 20ml of ice- cold (0°C) UW solution and placed on melting ice in a bowl containing UW solution for the duration of the SCS period; these livers were not perfused.

Diluted Whole Blood Reperfusion

For detailed evaluation of the graft response in the very early phase (0-2hrs) after transplantation, we employed a diluted whole-blood reperfusion model. This method was preferable as manipulation of animals for sampling immediately after transplantation could further stress the animals, affect survival, and introduce artefactual findings. The reperfusion circuit was identical to the normothermic perfusion system, but contained no secondary dialysis circuit. The livers were reperfused for 120 minutes and inflow (portal vein) and outflow (infrahepatic vena cava) sampling was performed every 15 minutes. The operating conditions for the reperfusion system were: Flow rate: 1.74±0.15 ml/min/g; Hematocrit 13.8±8.2; Inlet pO2: 263.5±111.9 mmHg; Outlet pO2: 75.1±49.7 mmHg; Inlet pCO2: 40.4± 14.9 mmHg; Outlet pCO2: 43.4±15.9 mmHg.

Liver tissue slices were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Apoptosis was evaluated through TUNEL staining (Roche, Indianapolis, IN).

Statistical Analysis

Data presented are means ± SE. All statistical analysis for differences performed with ANOVA at significance level of $\alpha=0.1$.

Results

Integrity and Function of Liver during Perfusion

ALT and AST activities as indicators of hepatocellular damage are shown in Figure 1A & B; both AST and ALT accumulated during the first 180 min of perfusion and then decreased. These values were several fold higher than those previously reported for freshly isolated livers not subjected to any warm ischemia [83]. Neither ALT nor AST were detected in the dialysate (data not shown).

Bile secretion and oxygen consumption describe the metabolic state of the liver. Bile was produced at a constant rate throughout the perfusion (Figure 1C). This rate was 40% lower than that previously reported for freshly isolated livers. The HOUR of warm ischemic livers declined rapidly during the first 60 minutes of the perfusion and then remained stable (Figure 1D). This behavior was very similar to that observed for freshly isolated livers. The HOUR's of the perfused warm ischemic and freshly isolated livers were very similar in the plateau region beyond 60 min.

The urea level in the perfusate showed a steady increase from 4.20 mg/dl at t=0 to 8.60 mg/dl at t=300 minutes, indicating a constant rate of urea production. This rate was consistently higher than that observed in perfused healthy livers (Figure 1E).

Figure 2 shows the histological appearance of warm ischemic livers after 5 hours of NELP. Ischemic livers treated with NELP show minimal to no damage compared to freshly isolated livers preserved either by SCS or NELP. In contrast, livers subjected to 1 hour of warm ischemia and subsequently preserved by SCS show swelling of hepatocytes (indicated by the arrows in the figure), widespread vacuolization and destruction of liver architecture (as indicated by the asterisks).

Survival after Transplantation

Warm ischemic livers were transplanted into recipient rats after 5 hours of NELP (n=13) or 5 hours of SCS in UW solution at 0°C (n=6). In addition, freshly isolated livers not subjected to any warm ischemia were transplanted after 6 hours of SCS (n=6) or NELP (n=11) and ischemic livers were transplanted directly without having undergone preservation (n=9). Transplantation of NELP treated ischemic livers was uneventful in all but one case, where bleeding at the anastomosis occurred. All animals recovered from anesthesia rapidly. The animal that bled during surgery died on day 4 postoperatively. The other recipient animals survived beyond one month and did not exhibit external signs of liver failure, such as jaundice.

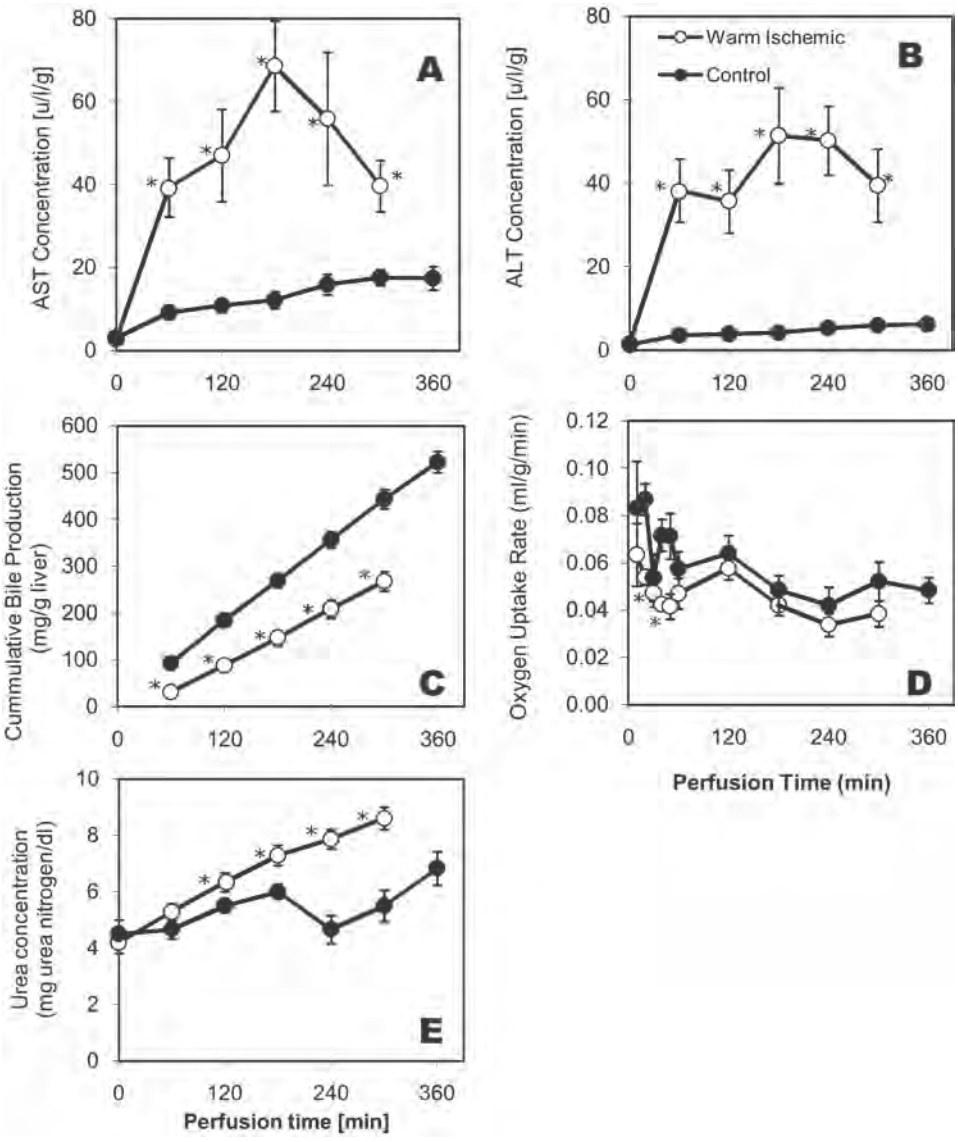

FIGURE 1 | Function and Integrity of warm ischemic livers during normothermic perfusion. (A) Asparate aminotransferase (AST) and (B) Alanine Aminotransferase (ALT) levels in perfusate samples collected hourly from the primary circuit; (C) Total bile accumulation normalized to wet liver weight; (D) Oxygen uptake rate normalized to wet liver weight. Data shown are averages of 6 ischemic livers ± Standard Error. Values for the warm ischemic livers are significantly lower than the controls for bile and oxygen uptake, and significantly higher for urea ($p<0.01$ by Analysis of Variance). Data for the control group (normothermic perfusion of non-ischemic livers) are from Tolboom et al. (19). * indicates statistical difference compared to healthy perfused livers at $p<0.1$.

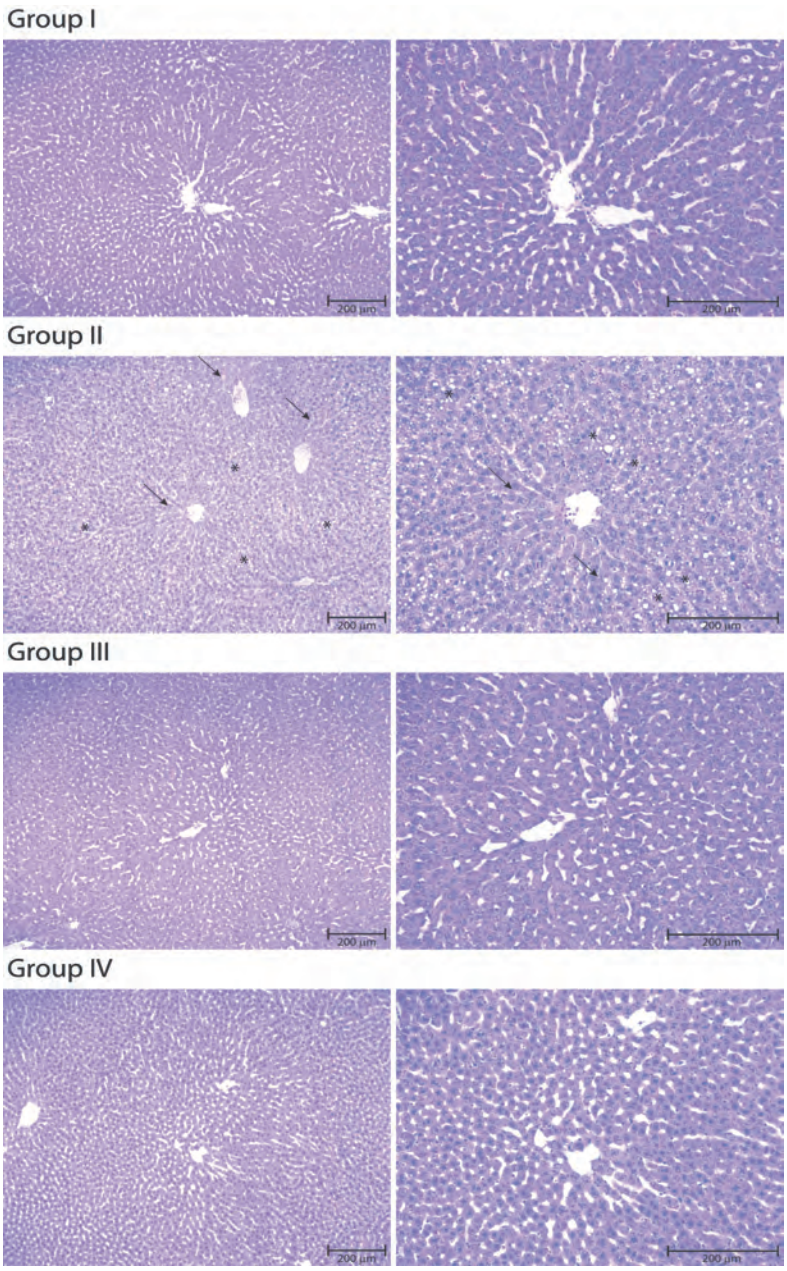

FIGURE 2 | Microscopic appearance of livers after preservation. A) Group I: Warm ischemic livers after 5 hours of Normothermic Extracorporeal Liver Perfusion (NELP). B) Group II: Warm ischemic livers after 5 hours of Static Cold Storage (SCS) in University of Wisconsin (UW) solution (Arrows indicate cell swelling and asterisks vacuolization and tissue destruction). C) Group III: Freshly isolated livers after 6 hours of NELP. D) Group IV: Freshly isolated livers after 6 hours of SCS in UW solution. Bar = 200 μm.

No surgical complications occurred during transplantation of ischemic livers preserved by SCS and recipients recovered rapidly from anesthesia, but within 6 hours all developed symptoms and died within 12 hours. Autopsy revealed patchy livers and serous fluid in the abdomen.

All recipients of directly transplanted ischemic livers died in a similar way within 24 hours post-operatively.

All controls that received freshly isolated livers preserved for 6 hours by SCS recovered rapidly from surgery and survived beyond one month (Figure 3).

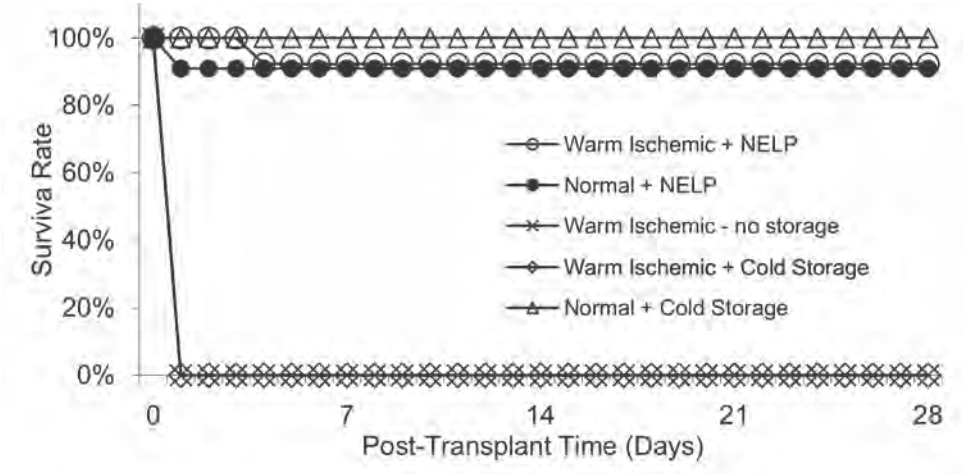

FIGURE 3 | Survival curves of recipient rats after transplantation of perfused warm ischemic livers, warm ischemic cold-stored livers, compared to healthy perfused livers, and healthy cold-stored livers. Data for the Normal+ Normothermic Extracorporeal Liver Perfusion (NELP) group are from Tolboom et al. (19).

Post-operative liver enzymes and bilirubin

The levels of both AST and ALT (Figures 4A and B) were elevated on day 1 post-operatively similar to the levels found in recipients of healthy cold stored livers. Overall, values of recipients for healthy cold-stored livers and DCD livers showed similar and normal levels implying successful transplantation. The AST levels were significantly lower for the perfused warm ischemic livers as compared to the healthy cold-stored livers on post-operative day 5. Both values were comparable to those observed in a hypothermic machine perfusion study [37].

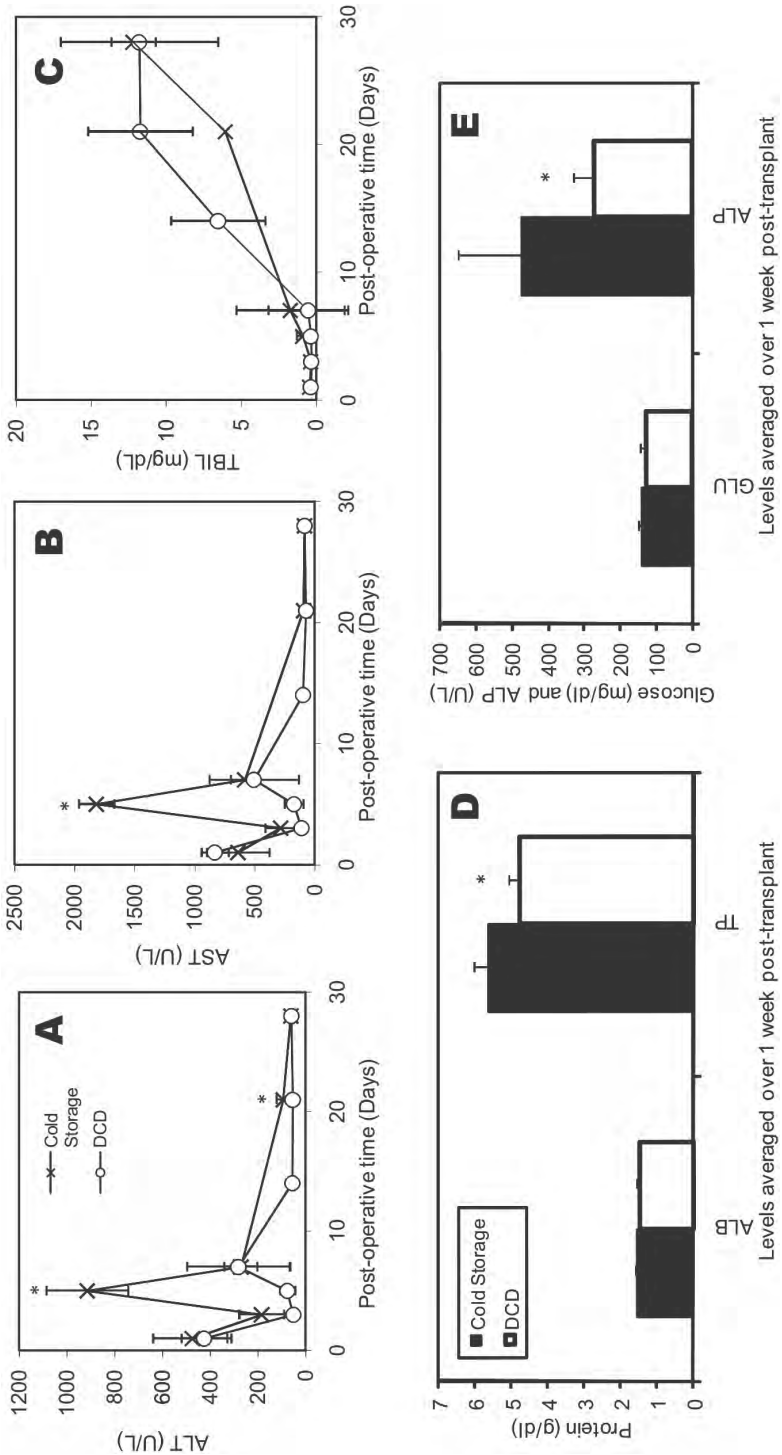

FIGURE 4 | Values of A) Asparate aminotransferase (AST), (B) Alanine Aminotransferase (ALT) (C) Total Bilirubin (TBIL) measured on days 1, 3, 5, 7, 14, 21 and 28 after transplantation of perfused warm ischemic livers compared to healthy cold-stored livers. (D) and (E): comparison of the serum levels of Albumin (ALB), Total Protein (TP), Glucose (GLU) and Alkaline Phosphatase (ALP) within 1 week after transplant. * indicates statistical difference compared to cold stored livers at $p<0.1$

The total bilirubin level, an indicator of liver function, was similar in both groups post- operatively and showed an increasing trend (Figure 4C). The increasing bilirubin value is within expected ranges and is likely an artifact of non-rearterialization and ensuing histopathologically observable bile duct proliferation [84]. It is worth noting that the elevated bilirubin levels are reported [84] to normalize after 6 weeks and survival is minimally affected. There was no statistical difference of bilirubin levels between the groups on any day.

The recipient serum analysis is displayed in Figures 4D and E. Albumin levels were similar for both SCS and WI-NELP groups (1.52 and 1.46 mg/dL respectively), though lower than systemic levels we previously obtained *in vivo* (1.91±.23 g/dl) (25). The total protein was higher in the SCS group (5.63 vs. 4.76 g/dl, both similar to the *in vivo* levels of 4.96±0.64 g/dl), suggestive of immunoglobulin elevation. Glucose levels were similar and within the normal *in vivo* range (241.64±.119.81 mg/dl), as were the electrolyte levels (results not shown). ALP, indicative of general tissue damage, was beyond the normal rat values (208.54±.66.019 U/L) for cold stored organs (472.75 U/L). NELP-treated ischemic livers were statistically lower than healthy DCD livers (273.36 U/L) and well within the normal range. These results are in agreement with slightly increased ALT and AST levels in recipients of cold stored organs, though the difference is statistically significant only for day 5. These results overall indicate that the function of NELP-perfused DCD livers is slightly better than cold stored healthy organs. This could be either due to avoiding the cold injury [41], or ischemic preconditioning effects of warm ischemia [85].

Short-term graft function

In order to evaluate the very-short-term graft function post-transplantation, we employed a diluted whole-blood reperfusion model. This was preferred to repetitive blood sampling shortly after transplantation., as the animals would not tolerate well additional manipulations. Figure 5 displays ALT and AST as markers of cellular damage, bile secretion as a viability indicator, as well as liver oxygen uptake rate as base-line indicator of metabolic activity. ALT levels for WI+NELP group was lower than WI-only and WI+SCS groups at all time points, and indifferent from normal livers after the first 45 minutes. Very similar trend were observed for AST, although the difference between WI+NELP and freshly isolated livers was statistically significant at all time points except t=45min. These results suggest that NELP improves early graft function and viability.

It was observed that bile secretion in the reperfusion system was well correlated with the survival results (Figure 5C). Post-transplant bile secretion has been previously shown to be strongly correlated to graft survival [86-88] and to cellular ATP levels [89, 90]. The average

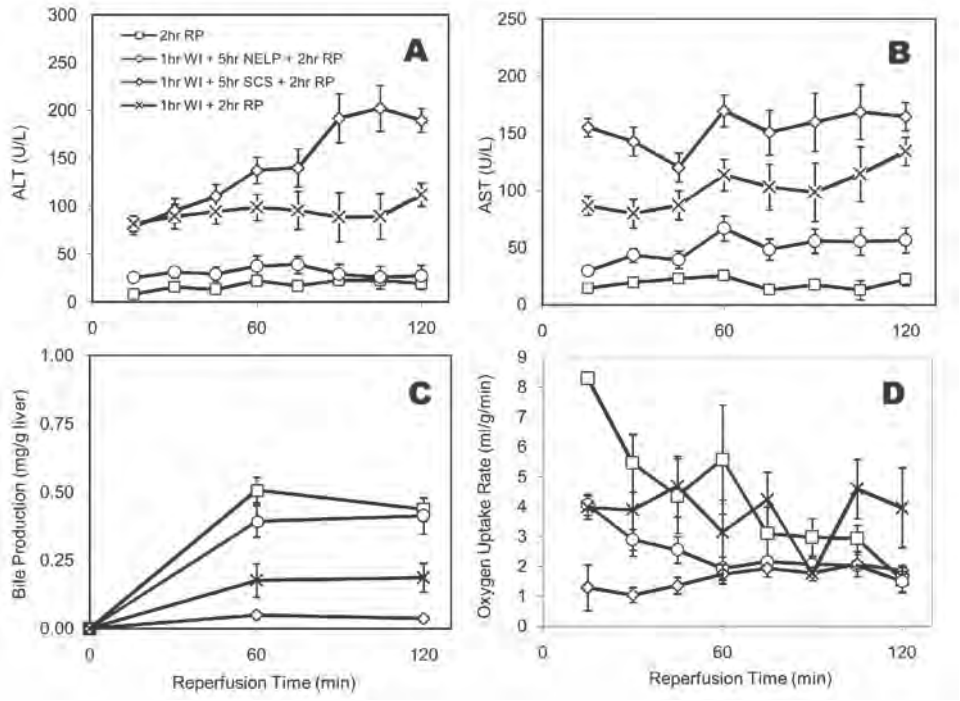

FIGURE 5 | Reperfusion results: (A) Alanine Aminotransferase (ALT), (B) Asparate aminotransferase (AST), (C) bile synthesis, and (D) oxygen uptake rate measured during reperfusion. See text for statistical analysis.

bile production during reperfusion of normal livers and NELP-treated WI livers was statistically not different (p=0.21); secretion for both groups was higher than the other two WI groups (p=0.01). Further, WI + SCS group showed lower bile secretion than WI-only group. Overall these results were as anticipated: it is known that substrate depletion causes reduction in bile synthesis, and the degree of reduction is proportional to ischemic injury [91].

As displayed in Figure 5D, reperfusion results in oxygen uptake rates that are different between groups. The average oxygen uptake was highest for the freshly isolated livers, statistically higher than that of WI+ NELP and WI +SCS groups, but not different from 1hr WI alone. There was no difference between WI+NELP and WI+SCS groups. Interestingly the WI-only livers displayed oxygen uptakes comparable to healthy livers. While oxygen uptake can be considered a bottom-line figure for respiration and metabolism, these results suggest that there is limited correlation between survival and early oxygen uptake rates.

Figure 6 displays the TUNEL staining results at the end of reperfusion. Apoptosis was absent in healthy and NELP-treated ischemic livers, and limited in WI-only livers. By comparison, WI+SCS group demonstrated significant staining. These results confirm that NELP is an effective method for preservion of ischemic livers. However, the absence of apoptosis in the WI-only livers that result in primary non-function when transplanted, suggests that apoptosis is not a determinant factor for graft survival.

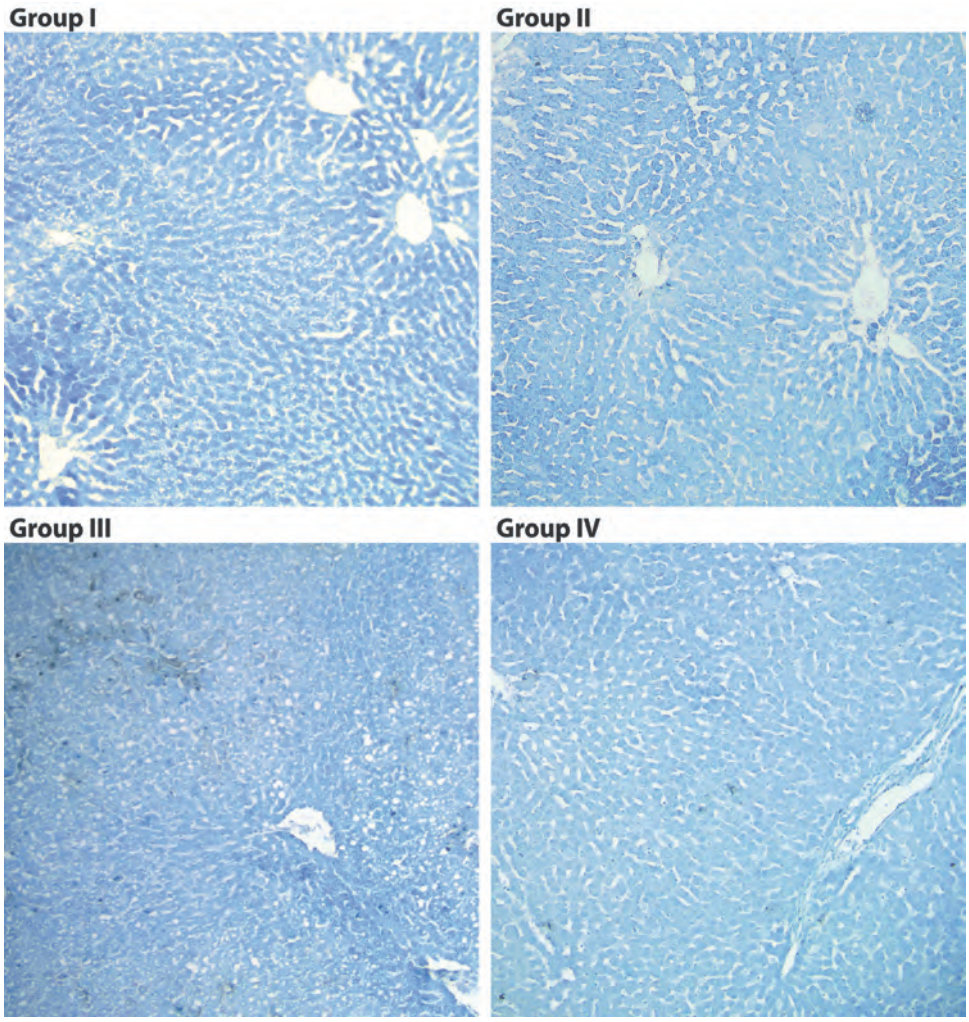

FIGURE 6 | TUNEL of livers after preservation and reperfusion. A) Group I: Freshly isolated liver after reperfusion. B) Group II: Warm ischemic livers after reperfusion. C) Group III: Warm ischemic livers after 5 hours of Static Cold Storage (SCS) in University of Wisconsin (UW) solution after reperfusion. D) Group IV: Warm ischemic livers after 5 hours of Normothermic Extracorporeal Liver Perfusion (NELP) followed by reperfusion. Magnification (10×).

Discussion

We have demonstrated that livers subjected to 60 min of ex-vivo warm ischemia can be resuscitated with NELP and transplanted with excellent graft function and long term survival of the recipient, comparable to that of recipients of perfused fresh livers and fresh livers preserved with cold storage. Animals that received ischemic livers that were either preserved with cold storage or experienced no storage time at all, died within 24 hours of transplantation.

Diluted whole-blood reperfusion experiments were performed to assess early graft function. It was observed that function of NELP-treated ischemic livers matched that of freshly isolated livers, whereas the function of untreated ischemic grafts was significantly worse. The transaminase levels and bile trends in the reperfusion system correlated very well with our survival results. In addition, the results indicate that the reperfusion system can be used to simulate liver transplantation for rapid optimization of NELP conditions.

Hypothermic machine perfusion provides the organ with constant supply oxygen and nutrients while waste is removed. However, the basic approach to preservation still relies upon slowing down metabolic rates, and herein does not differ from SCS. Under hypothermic conditions, a delicate equilibrium exists between maintaining perfusate flow sufficient to ensure adequate tissue oxygenation and damage of the sinusoidal endothelium due to barotrauma and shear stress that may limit it usefulness [40-42].

Normothermic machine perfusion is fundamentally different from hypothermic perfusion because its aim is to not only re-establish perfusion of the liver, but also closely mimic the in- vivo conditions and maintain the liver in a metabolically active state. The organ's metabolic activity can be continuously monitored throughout the preservation period, making it possible to assess its viability and function, providing potential markers that could be used to predict viability after transplantation. Furthermore, once oxidative metabolism has been sufficiently restored and intracellular energy supplies have been replenished, induction of repair and even regenerative processes might be possible. Other applications that have been suggested for normothermic machine perfusion include preconditioning, such as the induction of heat-shock proteins, and immunomodulation, such as induction of resistance against recurrent hepatitis C infection of the liver graft and possibly the reversal of hepatic steatosis [92, 93].

Although previous NELP efforts have predominantly used larger animal models, the rat was our model of choice in order to keep our approach simple and inexpensive. Since the blood supply of the rat liver is mostly venous [58, 71], we have chosen to perfuse via the portal

vein only, as usually done in the traditional isolated perfused rat liver systems [58, 59], which further helped to simplify the setup. For the same reason, the orthotopic liver transplant without reconstruction of the hepatic artery was performed using the cuff technique first described by Kamada [68, 69]. By using an inbred strain of rats, issues associated with immunoreactivity during perfusion and after transplantation were avoided. A limitation of this approach is the lack of rearterialization, which is known to introduce certain artifacts, including histopathologically observable biliary proliferation [94] The recipient rats in our studies displayed the same complications (results not shown), as well as the increased serum bilirubin which is known to return to normal levels after 6 weeks in this model [94]; however, survival was not affected by this phenomenon. This artifact makes it difficult to identify other biliary complications, such as biliary strictures, which is an important long term issue with DCD transplantation.

In order to model DCD we subjected livers to ex-vivo warm ischemia in a homeothermically controlled bath filled with warm saline as previously described [37]. The benefit of this method is precise control of the ischemic time and temperature due to the fact that the explantation of the organ occurred before the period of ischemia. We have chosen 60 minutes as clinically relevant time scale of prolonged ischemia. The temperature of 34 C was chosen to simulate a degree of reduction of the core temperature after cardiac arrest. Although we have also tested the effect of warm ischemia prior to liver explantation [36] we found that this approach introduces more variability due to poor control of the temperature history of the liver and variation of the duration of the donor surgery. Additionally we found that body-core temperature in the rat dropped much faster that what could be expected in a larger animal model or in humans, reducing the impact of the ischemia. We have chosen to heparinize animals before explantation for the same reason of consistency. During normothermic perfusion of the ischemic livers, the initial peak in the release of both AST and ALT suggests that hepatocellular damage occurred during the period of warm ischemia but stopped upon perfusion of the liver. Post-operative values of the AST, ALT were comparable, if not lower than those of recipients of fresh livers preserved with either NELP or SCS for a similar period. The lower bile secretion of the ischemic livers suggests that the damage sustained during warm ischemia may affect the biliary epithelium more than the hepatic parenchyma. Interestingly, the urea production of the ischemic livers was higher than that of freshly resected livers, which may reflect an increased nitrogen availability caused by proteolysis secondary to cellular damage. The fact that the oxygen uptake rate was similar to that of fresh livers indicates that the machinery responsible for oxidative metabolism was largely intact and mitochondrial function maintained in warm ischemic livers.

One of the possible hypotheses to explain the beneficial effect of NELP in reconditioning DCD organs is reduced apoptosis, which could be through reduced Kupffer cell activity (due to presence of hydrocortisone in the perfusate) which is known to be correlated to improved graft survival [95]or ROS reduction (through glutathione which is present in Williams E) which was also found to correlate with graft viability [96]. However, the TUNEL results of reperfused livers displayed that WI-only and WI+NELP groups had both very limited apoptosis, and yet the survival in recipients of WI-only livers were nil. This result indicates that suppression of Kupffer cells is an unlikely cause of survival in our system. However, it is possible that other inflammatory mechanisms not present in our model are involved. On the other hand, the correlation between bile secretion and survival suggests that restoration of metabolic activity, perhaps ATP levels and/or other metabolites, may play a role in the protective effects of NELP.

Conclusions

The goal of this study was to evaluate the possibility of resuscitating livers after warm ischemia with normothermic perfusion in a modified isolated perfused rat liver system. We have shown that livers subjected to one hour of ex vivo warm ischemia can be reclaimed using warm perfusion technology. Post transplant survival of rats that received these perfused livers was far superior to that of animals that received ischemic livers that did not undergo any preservation, and those preserved with traditional SCS. Our system provides an effective to investigate the various aspects of warm perfusion for preservation and resuscitation of the DCD liver grafts, as well as a model to study liver metabolism [97]. We envision that a similar, scaled up version of our system could be used in a clinical setting enabling the use of DCD livers for transplantation.

In addition, this study establishes that the dilute whole-blood reperfusion system can be used as a model simulating rat liver transplantation, and transaminase levels and bile synthesis are all adequate markers of viability

Chapter 4

Sequential cold storage and normothermic perfusion of the ischemic rat liver

Herman Tolboom, Maria-Louisa Izamis, John M. Milwid, Korkut Uygun, François Berthiaume, Martin L. Yarmush

Transplantation Proceedings 2008 Jun; 40(5):1306-9

Abstract

Extending transplant criteria to include livers obtained from donor after cardiac death (DCD) could increase the liver donor pool, but conventional simple cold storage of these ischemic organs can lead to poor graft function after transplantation. Experimental normothermic machine perfusion has previously proven to be useful for the recovery and preservation of DCD livers, but it is more complicated than conventional cold storage, and is therefore perhaps not practical during the entire preservation period. In clinical situations, the combined use of simple cold storage and normothermic perfusion preservation of DCD livers might be more realistic, but even a brief period of cold storage prior to normothermic preservation has been suggested to have a negative impact on graft viability. In this study we show that rat livers subjected to 45 minutes of ex-vivo warm ischemia followed by 2 hours of simple cold storage can be reclaimed by 4 hours of normothermic machine perfusion. These livers could be orthotopically transplanted into syngeneic recipients with 100% survival after 4 weeks (N=10), similar to the survival of animals that received fresh livers that were stored on ice in University of Wisconsin (UW) solution for 6 hours (N=6). On the other hand, rats that received ischemic livers preserved on ice in UW solution for 6 hours (N = 6) all died within 12 hours after transplantation. These results suggest that normothermic perfusion can be used to reclaim DCD livers subjected to an additional period of cold ischemia during hypothermic storage

Introduction

The current shortage of suitable donor livers could be alleviated significantly by extending liver graft criteria to include livers obtained from donors after cardiac death (DCD). However, conventional with simple cold storage (SCS) of these DCD livers is associated with a higher risk of primary non-function and delayed graft failure [6, 76]. Normothermic Extracorporeal Liver Perfusion (NELP) has been suggested as an alternative [47, 81]. However, if machine perfusion of donor organs is to be realized clinically, a brief period of static cold storage between donor organ recovery and normothermic preservation is desirable; This period would enable the recovery of the organ as usual, after which it could be transported to a specialized transplantation center, where subsequent recovery of the organ with NELP can occur. The addition of an intermittent period of SCS prior to NELP, however, has been suggested negatively impact the viability of these DCD grafts due to their increased sensitivity to cold ischemia [50].

To assess the feasibility this hybrid approach, we have evaluated post-operative graft function of orthotopically transplanted ischemic rat livers that were preserved with a combination of cold storage in University of Wisconsin (UW) solution and normothermic machine perfusion. Recipients of these livers all survived beyond 4 weeks. By comparison, animals that received warm ischemic livers preserved with simple cold storage alone all died within 24 hours after transplantation.

Experimental Procedures

For a details on the isolation of the donor liver, machine perfusion protocols, isolated reperfusion, transplantation sampling and processing of perfusate and tissue samples, please refer to chapters two and three and references [83, 98] respectively.

Results

ALT and AST levels were 3-4 fold higher during perfusion ($p<0.01$) for the experiment groups, as compared to perfused non-DCD livers. The values were very similar to the values obtained in perfusion of 1-hr warm ischemic livers without any intermittent cold storage. ALT levels reached a plateau after the first hour in all cases, and AST levels started to decline after the third hour. ALT or AST were not detected in the dialysate. Bile production rate was constant through perfusion, indicating consistent metabolic activity, but was lower than that of non-DCD livers ($p<0.01$). The oxygen uptake rate of the livers that were subjected to warm ischemia remained roughly constant during perfusion at an average value of 0.04ml/min g liver, though a slight decrease was observed ($p>0.1$).

The oxygen uptake rate of the fresh livers followed the same pattern, was slightly but not significantly higher ($p>0.1$).

Transplant surgery was without complications in all cases. The animals that received perfused ischemic livers (N=10) and the control group receiving fresh cold stored livers (N=6), survived beyond one month (100% survival in both groups), and plasma billirubin levels remained within physiological limits for up to 6 months (data not shown) The animals that received cold stored ischemic liver all died within 24 hours postoperatively (N=6). Examination of the abdomen revealed a patchy liver and serous fluid was present in the abdomen (0% survival).

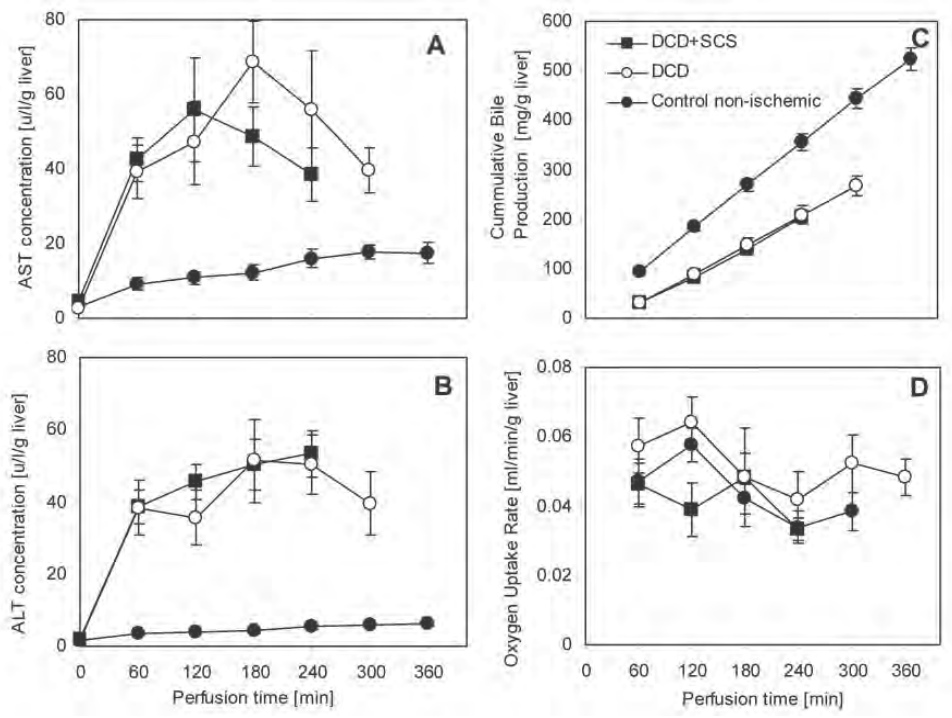

FIGURE 1 | Integrity and metabolic functioning of warm ischemic livers during NELP. The DCD group was subjected to 1 hr warm ischemia, DCD +SCS groups was subjected to 45 mins of warm ischemia and 2 hrs of SCS (A) AST and (B) ALT levels in perfusate samples collected hourly from the primary circuit. (C) Total bile accumulation; (D) Oxygen uptake rate. Data shown are averages of 6 warm ischemic livers ± SEM. ALT & AST values for the warm ischemic livers are significantly higher than the controls ($p<0.01$). OUR was not statistically different between the experiment group and other groups ($p>0.1$), where as bile production was significantly reduced ($p<0.01$) compared to fresh livers. Values are normalized to the wet weight of the liver.

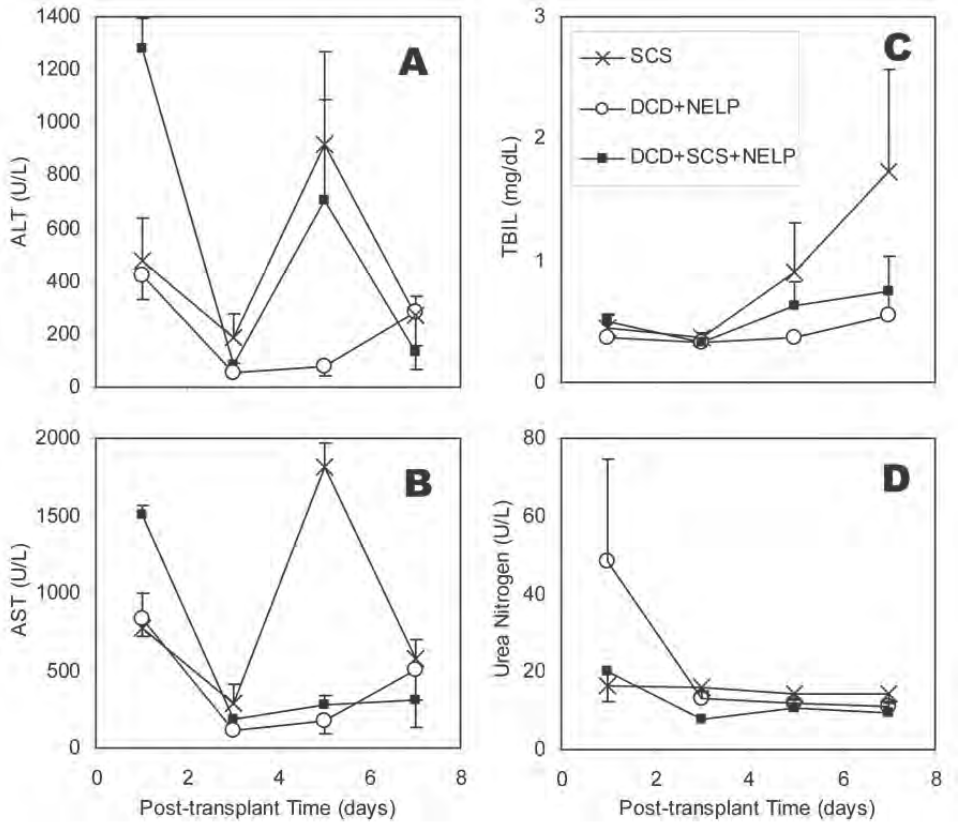

FIGURE 2 | Values of ALT (A), AST (B), Bilirubin (C), and Blood Urea Nitrogen (D) measured on days 1, 3, 5 and 7 after transplantation of perfused warm ischemic livers compared to healthy perfused livers (controls) and healthy cold-stored livers. Data shown are the averages of 3 recipients per group ± SEM. Overall values for recipients of DCD livers treated with NELP post cold storage were statistically indifferent from other DCD livers. However, on day 1 both ALT and AST levels are significantly higher in DCD+SCS group. BUN was lower when compared to recipients of NELP treated normal livers.

Conclusions

We have shown that livers subjected to forty five minutes of ex vivo warm ischemia followed by two hours of simple cold storage in UW solution can be resuscitated and preserved with normothermic extracorporeal perfusion. These livers showed functional recovery during normothermic perfusion, but overall hepatic metabolism was lower than that of fresh perfused livers. The survival after transplantation of rats that received these perfused livers was far superior to that of animals that received ischemically damaged

livers preserved with traditional cold storage and was similar to that of animals receiving fresh livers. The results of this study indicate that ischemic rat livers can be recovered and preserved with NELP even after a brief period of cold storage.

Chapter 5

Subnormothermic machine perfusion at both 20°C and 30°C recovers ischemic rat livers for successful transplantation

Herman Tolboom, Maria-Louisa Izamis, Nripen Sharma, Jack M. Milwid, Basak Uygun, François Berthiaume, Korkut Uygun, Martin L. Yarmush

Journal of Surgical Research 2012 Jun 1;175(1):149-56.

Abstract

Background

Utilizing livers from donors after cardiac death could significantly expand the donor pool. We have previously shown that normothermic (37ºC) extracorporeal liver perfusion significantly improves transplantation outcomes of ischemic rat livers. Here we investigate whether recovery of ischemic livers is possible using sub-normothermic machine perfusion at 20ºC and 30ºC.

Methods

Livers from male Lewis rats were divided into five groups after 1hr of warm ischemia (WI): 1) WI only, 2) 5hrs of static cold storage (SCS) or 5hrs of MP at 3) 20ºC, 4) 30ºC and 5) 37ºC. Long-term graft performance was evaluated for 28 days post-transplantation. Acute graft performance was evaluated during a 2hr normothermic sanguineous reperfusion ex vivo. Fresh livers with 5hrs of SCS were positive transplant controls while fresh livers were positive reperfusion controls.

Results

Following MP (Groups 3, 4 and 5), ischemically damaged livers could be orthotopically transplanted into syngeneic recipients with 100% survival (N≥4) after 4 weeks. On the other hand, animals from WI only, or WI + SCS groups all died within 24hrs of transplantation. Fresh livers preserved using SCS had the highest ALT, AST and the lowest bile production during reperfusion, while at 28 days post-transplantation, livers preserved at 20ºC and 30ºC had the highest total bilirubin values.

Conclusions

MP at both 20ºC and 30ºC eliminated temperature control in perfusion systems and recovered ischemically damaged rat livers. Post-operatively, low transaminases suggest a beneficial effect of sub-normothermic perfusion, while rising total bilirubin levels suggest inadequate prevention of ischemia- or hypothermia-induced biliary damage.

Introduction

It is estimated that approximately 15% of deaths due to chronic liver disease and cirrhosis in the US could be prevented by whole organ transplantation [99] however, the lack of donor organs limits the prevalence of this treatment option. A significant number of donors experience cardiac death (DCD). Organs from these donors are rarely considered for transplantation as static cold storage (SCS) [50] the current gold standard of organ preservation, is incapable of reversing the ischemic damage these organs have sustained. Encouraging results in animal models have shown that both normothermic (37ºC) extracorporeal machine perfusion (37MP)[18, 47, 81], and hypothermic (4ºC) machine perfusion (4MP), which aims to augment SCS, can recover ischemically damaged organs [79, 80]. However, 4MP has been shown to compromise graft integrity with cold-induced damage [41] and 37MP requires a labor-intensive and complex heated perfusion system, hindering its translation into the clinical setting. Further, it is unknown whether normothermic conditions are necessary or, in fact, optimal.

Reducing perfusion temperature into the subnormothermic range may enable a less complex system to be used by reducing the need for strict temperature control as well as lowering oxygen demand during perfusion. Therefore, we investigated whether MP-based recovery of warm ischemic (WI) livers was also possible at both 20ºC and 30ºC and evaluated survival in a rat model of orthotopic liver transplantation, as well short-term graft function in an ex-vivo perfusion setup. After orthotopic liver transplantation into syngeneic recipients, long-term graft performance was evaluated over the span of 28 days. Short-term graft function was determined using a 2hr ex-vivo reperfusion model of 50% saline and 50% whole blood post-MP recovery. Early graft evaluation during reperfusion revealed comparable performance by WI+MP livers to fresh livers, while long-term post-transplantation evaluation showed 100% survival in WI+MP livers. It is conceivable therefore, that MP may be reduced in complexity without compromising function, thereby bringing it a step closer to translation into standard clinical practice.

Experimental Procedures

Groups Experiments were performed using male Lewis rats weighing 250-300g (Charles River Labs). The animals were maintained in accordance with National Research Council guidelines and the experimental protocols were approved by the Subcommittee on Research Animal Care, Committee on Research, Massachusetts General Hospital. Animals were divided into five groups based on how they were treated after 1hr of WI: 1) WI only;

2) 5hrs of SCS; 5hrs of machine perfusion at 3) 20ºC, 4) 30ºC and 5) 37ºC. All groups were subsequently transplanted and monitored for 28 days, or subjected to a 2hr normothermic sanguineous reperfusion in order to assess early graft function[100]. Each group comprised n≥4 livers. Fresh livers with 6hrs of SCS were positive transplant controls while fresh livers were positive reperfusion controls.

For a details on the isolation of the donor liver, machine perfusion protocols, isolated reperfusion, transplantation and processing of perfusate and tissue samples, please refer to chapters two and three and references [83, 98] respectively.

Statistical Analysis

Data presented are means ± SEM. Statistical analysis was performed using 2-way (Analysis of Variance) ANOVA at $\alpha=0.05$, with Tukey-Kramer correction for multiple comparisons.

Results

Survival after Transplantation

Transplantation of all livers was successful and the animals recovered from anesthesia rapidly. 100% of the animals that received MP livers survived beyond one month in good health and without any external signs of jaundice (Table 1). Within 12 hours of transplantation, all recipients of SCS-preserved livers died. Autopsy revealed non-homogeneously perfused livers and serous fluid in the abdomen. All recipients of directly transplanted ischemic livers died later, within 24 hours of transplantation. All controls that received freshly isolated livers preserved for 6 hours by SCS recovered rapidly from surgery and survived beyond one month.

TABLE 1 | Survival of recipient rats after transplantation.

	Survival (Days)
Transplantation	
Fresh+SCS	>28
WI	<1
WI+SCS	<0.5
WI+20MP	>28
WI+30MP	>28
WI+37MP	>28

Integrity and Function of Liver during Perfusion

ALT and AST activities, measured hourly during perfusion and used to indicate presence of hepatocellular damage, are displayed in Figure 1A & B. 37MP and 30MP livers have no significant differences between trends of ALT and AST release ($p>0.05$), both of which illustrate a gradual increase and peak at t=3-4 hours, followed by a gradual decline. By contrast, 20MP livers demonstrated an increasing trend in AST release such that there was a significant difference between groups at t=4 hours ($p=0.015$), prior to a decrease in release such that AST levels were comparable amongst all groups at t=6hrs. ALT released by 20MP livers did not differ significantly from 30MP or 37MP on an hourly basis ($p=0.053$), but overall the release was statistically higher ($p=0.016$).

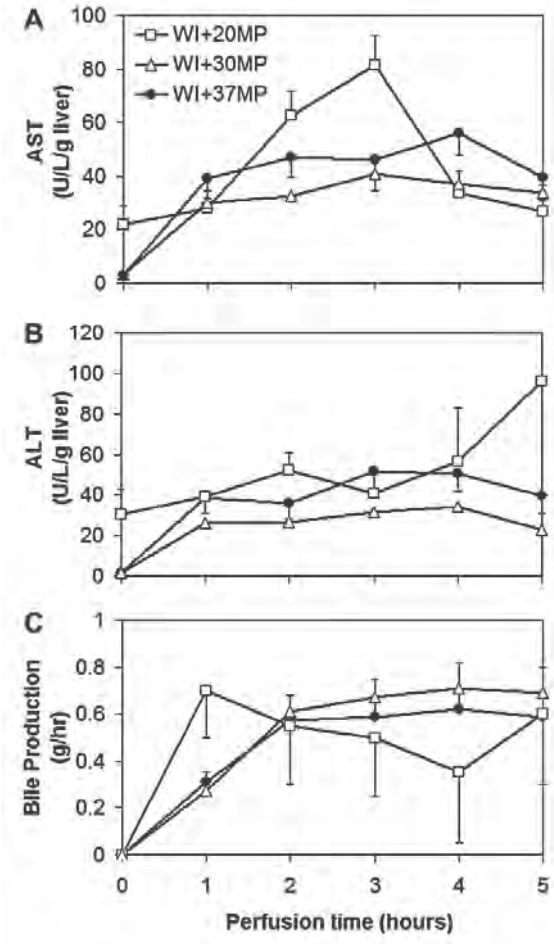

FIGURE 1 | Function and integrity of warm ischemic livers during normothermic perfusion. (A) Asparate aminotransferase (AST) and (B) Alanine Aminotransferase (ALT) levels in perfusate samples collected hourly from the primary circuit and (C) bile synthesis.

Rate of bile secretion, describing liver function, was observed to have a similar trend between 30MP and 37MP livers, increasing sharply for the first 2 hours of perfusion before stabilizing at 0.6±0.02g/hr and 0.7±0.02g/hr respectively (p=0.02) for the remainder of the experiment. Though not significantly different from the other groups, 20MP livers exhibited a very different trend by increasing bile production to 0.7±0.2g/hrs within the first hour of perfusion, and then subsequently declining linearly over time to 0.4±0.3g/hr at t=5 hours, before finally increasing to the same secretion rate of 0.6±0.3g/hr as 37MP livers at t=6 hours.

The metabolic state of the liver was assessed through its oxygen uptake rate, which was generally stable during perfusion (Figure 2A). Average oxygen uptake rate (OUR) of 20MP livers was 43% lower compared to 37MP livers (Table 2, p=0.02).

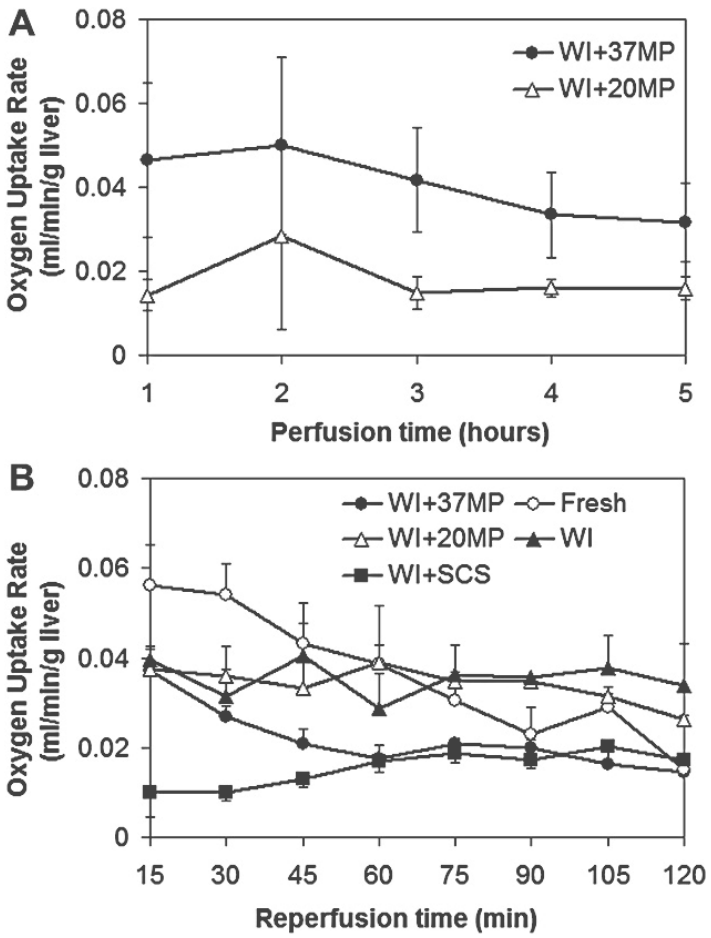

FIGURE 2 | Oxygen uptake rate measured during (A) perfusion and (B) reperfusion.

TABLE 2 | Average oxygen supply and uptake rate of livers after preservation followed by perfusion and reperfusion.

	ODR (ml/min/g liver)	OER (ml/min/g liver)	OUR (ml/min/g liver)
Perfusion			
WI+20MP	0.119±0.018	0.101±0.014	0.018±0.011
WI+20MP	ND	ND	ND
WI+20MP	0.141±0.018*	0.098±0.02	0.042±0.017
Reperfusion			
Fresh	0.112±0.012	0.076±0.024	0.036±0.02
WI	0.098±0.013**	0.062±0.008	0.036±0.015
WI+SCS	0.106±0.01	0.091±0.008***	0.015±0.006**
WI+20MP	0.017±0.009	0.082±0.012	0.034±0.005
WI+30MP	ND	ND	ND
WI+37MP	0.094±0.01**	0.073±0.013	0.022±0.004**

ODR: Oxygen Delivery Rate
OER: Oxygen Exit Rate
OUR: Oxygen Uptake Rate
*Significantly different from WI+20MP in perfusion ($p<0.05$)
,* Significantly different from other groups in reperfusion ($p<0.05$)

Short-term graft performance post-machine perfusion

In order to evaluate graft function immediately post-perfusion we employed a reperfusion system using diluted whole-blood. OUR was highest in fresh livers at the onset of reperfusion at 0.07±0.009 mlO_2/min/g liver, while WI+SCS livers had the lowest OUR at 0.013±0.005 mlO_2/min/g liver (Figure 2B). At the end of 2 hours of reperfusion, all livers occupied a narrow range of OUR values of 0.015-0.04 mlO_2/min/g liver. Fresh livers showed a linear decline in OUR throughout reperfusion while WI+SCS livers demonstrated a gradual increase in uptake. MP livers and WI livers both commenced at the same OUR of 0.039±0.015 mlO_2/min/g liver and over the course of the first hour achieved a steady value. Table 2 reveals that the lowest oxygen exit rate (OER) was 0.062±0.008 mlO_2/min/g liver, which illustrates usefully that though there were some oxygen delivery rates (ODR)s that were significantly lower than others, none of the livers were oxygen deprived. Curiously, the average OUR during reperfusion was similar between fresh, WI+20MP, and WI livers, while it was significantly lower in WI+37MP livers (approximately half the value observed in perfusion) and lowest in WI+SCS livers.

Figure 3 shows ALT and AST secretion for MP groups during reperfusion were similar to fresh livers and significantly lower than WI-only livers at all time points (p=0.004 and p=0.03 respectively), while WI+SCS groups were higher than WI-only livers (p=0.16 and p=0.056 respectively). The rate of ALT and AST release was decreased during the second hour of reperfusion relative to the first in all groups. ALT and AST values for MP livers during reperfusion were generally consistent with the values observed during machine perfusion.

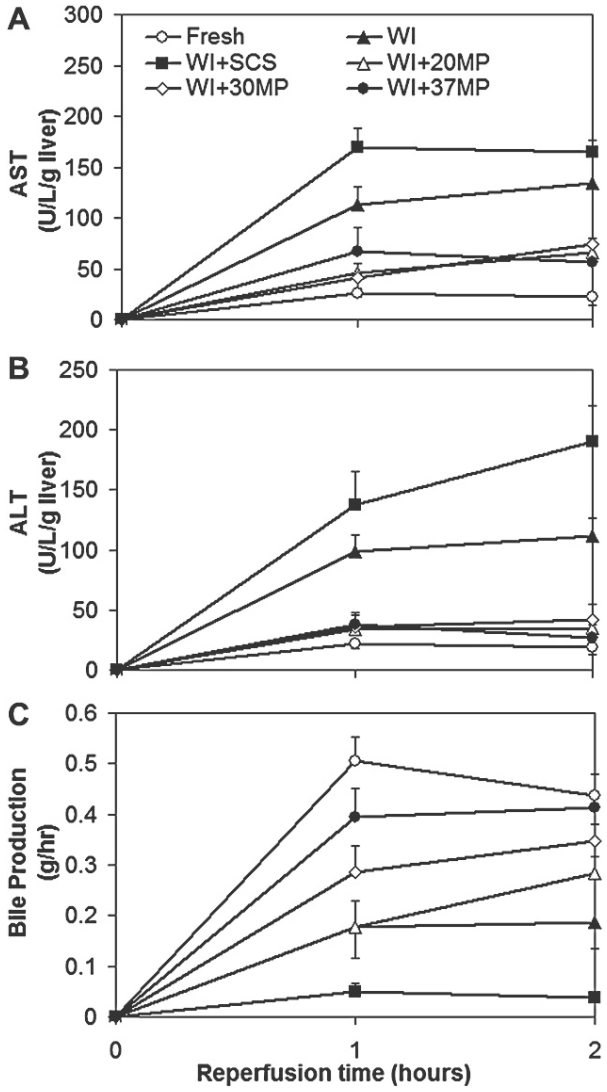

FIGURE 3 | Reperfusion results: (A) Alanine Aminotransferase (ALT), (B) Asparate aminotransferase (AST) and (C) bile synthesis.

Bile production during reperfusion was highest in fresh livers and decreased as a function of decreasing temperature, though the differences between fresh and MP groups were not significant. WI livers produced bile at a rate comparable to 20MP in the first hour but then reached a plateau in the second hour, making significantly less than fresh livers ($p=0.014$). WI+SCS livers produced significantly less bile throughout reperfusion compared to fresh or MP livers ($p=0.001$). MP livers produced less bile per hour during reperfusion than during machine perfusion.

H&E histology of livers biopsied immediately after reperfusion (Figure 4) show that MP livers retained a homogeneous architecture that appears comparable to fresh livers. Edema appeared more prevalent in 37MP livers, and seemed to decrease with temperature, showing minimal to none at 20°C. WI livers showed focal areas of necrosis, which was exacerbated in WI+SCS livers. Figure 5 illustrates that apoptosis was negligible in all reperfused livers.

Post-operative liver enzymes and bilirubin

Serological analysis was performed on the transplant recipients over the course of 28 days. Both ALT and AST levels (Figures 6A and B) were elevated on day 1 post-operatively for all groups compared to perfusion and reperfusion values. Fresh+SCS livers displayed a spike in both enzyme levels on day 5; a much less pronounced spike was also evident in 37MP-resuscitated ischemic livers on day 7. By contrast, 30MP and 20MP livers consistently displayed the lowest levels throughout the observation period.

Post-operative TBIL levels increased in all groups, though more so in 20MP and 30MP livers, such that recipients of 30MP livers had significantly elevated levels compared to recipients of Fresh+SCS livers at 28 days ($p<0.01$). No other differences were statistically significant.

Discussion

Machine perfusion of donor livers is gaining recognition as an organ preservation technique capable of both expanding the current donor pool and producing grafts superior to conventional static cold storage methods [9, 23, 24, 101, 102]. Recovery of damaged donor organs such as those which are ischemic [19, 37, 38, 47, 98] or steatotic [55, 103, 104] has been demonstrated in animal models, enabling clinical application of machine perfusion at 4°C [11, 39]. To our knowledge, this study is the first to demonstrate successful transplantation of ischemic livers recovered with subnormothermic machine perfusion.

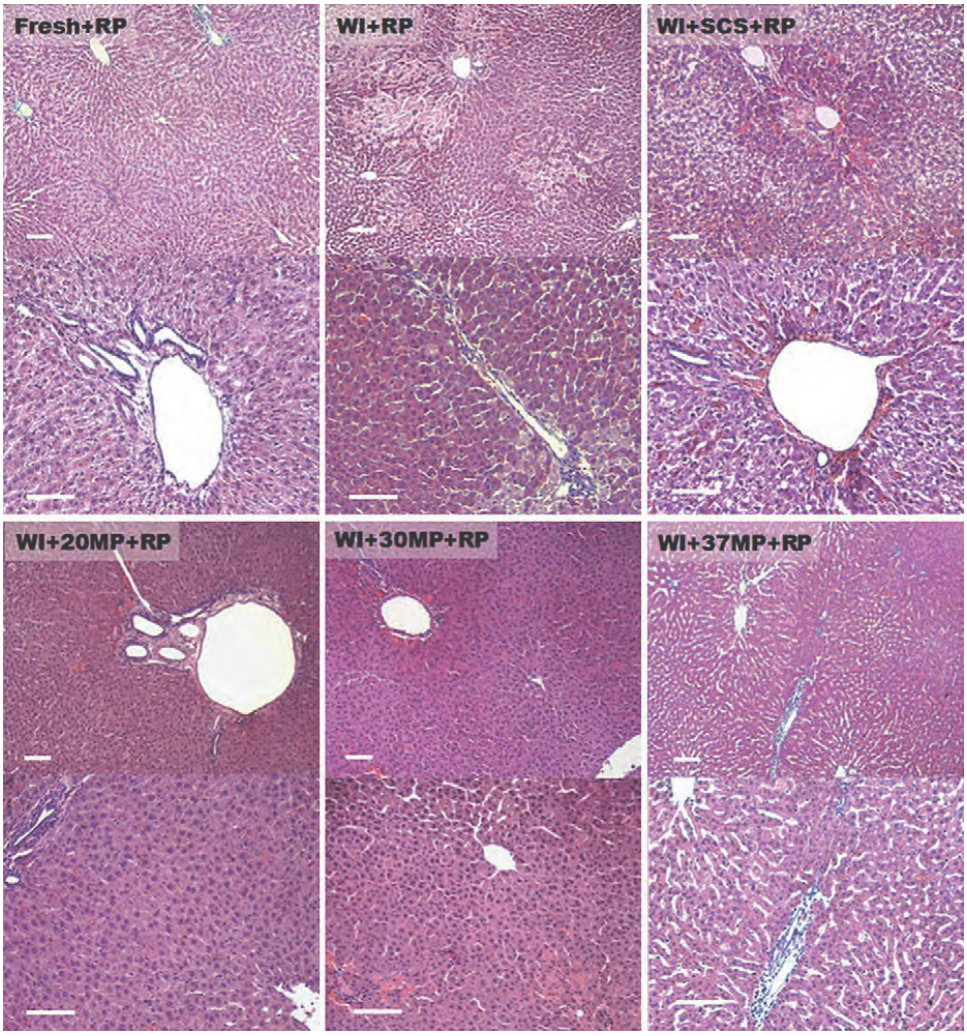

FIGURE 4 | H&E staining of livers after preservation and reperfusion at 10x (top row) and 20x (bottom row) magnification. A) Group I: Fresh livers . B) Group II: Warm ischemic livers. C) Group III: Warm ischemic livers after 5 hours of Static Cold Storage in UW solution). D) Group IV: Warm ischemic livers after 20C MP. E) Warm ischemic livers after 30C MP. F) Warm ischemic livers after 37C MP.

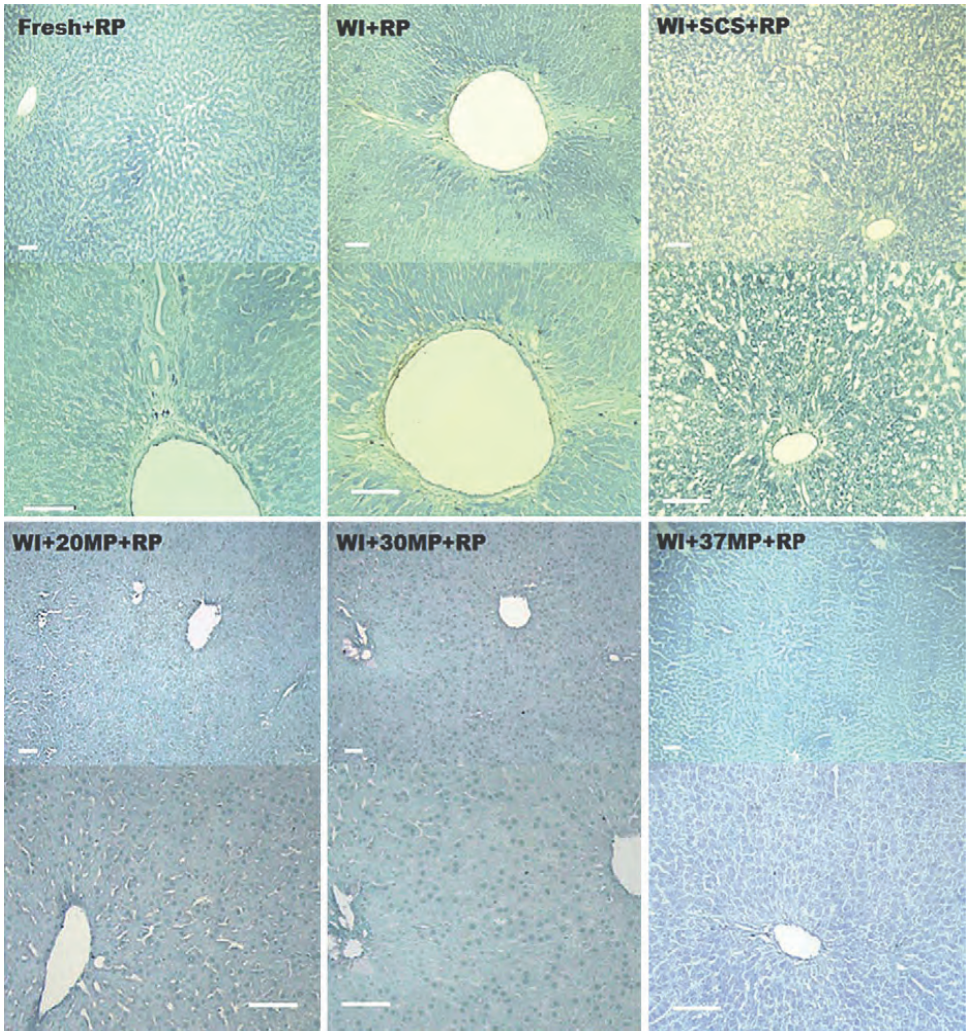

FIGURE 5 | TUNEL of livers after preservation and reperfusion at 10x (top row) and 20x (bottom row) magnification. A) Group I: Fresh livers. B) Group II: Warm ischemic livers. C) Group III: Warm ischemic livers after 5 hours of Static Cold Storage (SCS) in University of Wisconsin (UW) solution). D) Group IV: Warm ischemic livers after 20C MP. E) Warm ischemic livers after 30C MP. F) Warm ischemic livers after 37C MP.

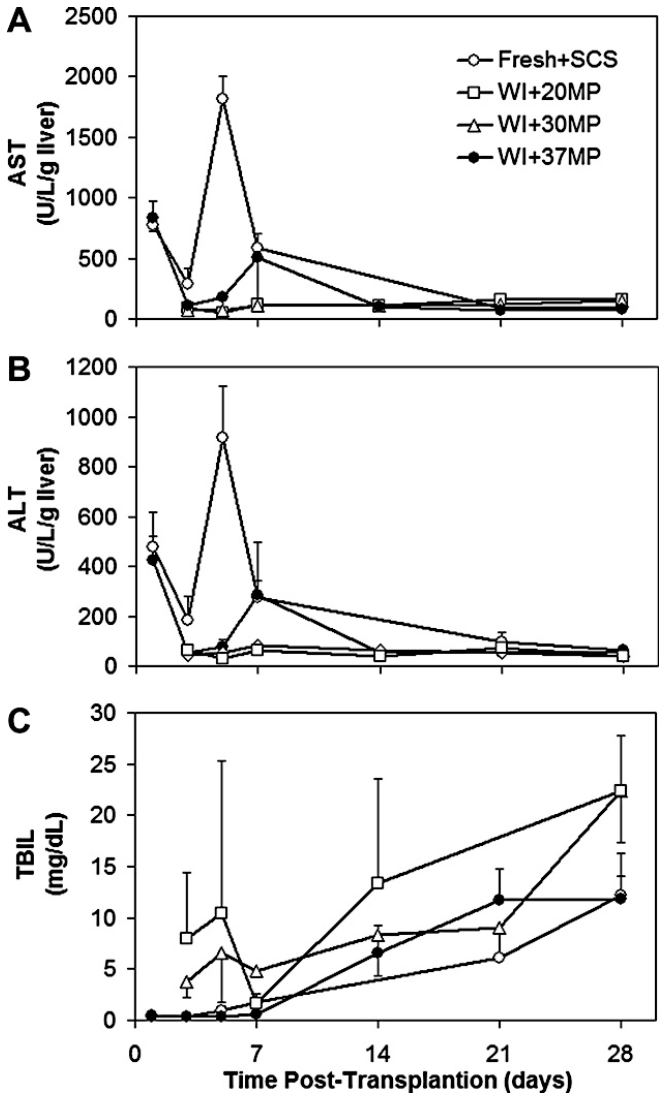

FIGURE 6 | Values of A) Aspartate aminotransferase (AST), (B) Alanine Aminotransferase (ALT) (C) Total Bilirubin (TBIL) measured after transplantation of healthy cold-stored livers compared to machine perfused warm ischemic livers at 20C, 30C or 37C.

Room-temperature perfusion has already proven advantageous through retention of hepatic and biliary structural integrity [57, 105], and improved protection of marginal livers [55]. Here we demonstrate that ischemic rat livers recovered by machine perfusion at 20°C and 30°C result in 100% transplantable grafts, measured 28 days postoperatively. Clinically, the recipients looked as well as those that had received livers recovered at 37°C or fresh livers stored using SCS. Interestingly, evidence that SCS further exacerbated warm ischemic damage [41, 54] was seen both in the rapidity with which recipients of these control livers died (within 12 hours of transplantation, compared to WI-only recipients that died within 24 hours of surgery), and the extent of ALT and AST release during reperfusion, which was highest in WI+SCS livers.

Perfusion of warm ischemic livers at 20°C and 30°C did not incur further cellular damage as very little ALT and AST release occurred, which stabilized during the first hour of perfusion. Further, organ function appeared intact with stable oxygen uptake rate and bile production within the first few hours of perfusion. WI+20MP livers consumed significantly less oxygen than WI+37MP, as would be expected with a temperature-induced decline in metabolic rate [105]. From OER values in Table 2, it can be seen that neither WI+20MP livers nor WI+37MP livers used all the available oxygen delivered to them, therefore they were not oxygen limited. Perfusate without erythrocytes, oxygenated with 95%O_2 and 5%CO_2, at the physiological flow rates employed in these experiments of 1.8±1.2 ml/min/g liver (in vivo: 1.7-2.3 ml/min/g liver [106] would deliver approximately 0.022 mlO_2/min/g liver (using a modified Fick's formula), which is higher than WI livers perfused at 20°C require. Future perfusions at 20°C may therefore be further reduced in complexity by removing the need for oxygen carriers.

Reperfusion data provided valuable insight as to the time frame and likely sources of warm ischemic organ failure post-transplantation. Reperfusion of WI livers demonstrated a metabolic rate of activity through OUR that was comparable to fresh or perfusion-recovered livers. This is not surprising from the perspective that these organs are still viable, as successful transplantation after machine perfusion demonstrates. However, reperfusion with (diluted) whole blood at 37°C caused a significant release of ALT and AST, and a greater reduction in the production of bile than in WI+37MP livers. Similarly, histological analysis of liver tissue after 2 hours of reperfusion showed evidence of necrosis in WI and WI+SCS groups, not seen in any of the other groups; none of the groups showed any signs of apoptosis within this time frame. It is likely therefore, that machine perfusion is capable of ameliorating ischemia reperfusion injury initiated immediately post-transplantation by factors present within the recipient's blood supply [107].

Serological analysis of recipients post-transplantation over the course of 28 days revealed that ALT and AST levels were lowest in 20°C and 30°C perfused livers. Though all grafts showed an increasing trend in total bilirubin, likely exacerbated by the non-arterialized orthotopic liver transplantation method used [94], WI+20MP and WI+30MP livers were highest (but statistically insignificant), possibly suggestive of temperature-associated damage to the biliary epithelium. Recent work by Brockmann et. al. [19] has demonstrated a more suitable porcine DCD-liver model of MP resuscitation that includes re-arterialization of the liver at transplantation, which will enable better evaluation of biliary epithelium recovery during MP.

Conclusion

We demonstrated that transplantable grafts can be recovered from warm ischemic rat livers by machine perfusion at both 20°C and 30°C. Subnormothermic perfusion may remove the need for stringent temperature control, and may also eliminate dependence on oxygen carriers in perfusate, providing critical simplifications for the successful translation of this technology into clinical practice.

Chapter 6

Diluted blood reperfusion as a model for transplantation of ischemic rat livers: ALT is a direct indicator of viability

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Abstract

Donors after cardiac death present a significant pool of untapped organs for transplantation, and use of machine perfusion strategies has been an active focus area in experimental transplantation. However, despite 2 decades of research, a gold standard has yet to emerge for machine perfusion systems and protocols. Whole blood reperfusion has been used as a surrogate for organ transplantation, especially as a model for the short-term response posttransplantation, and for optimization of perfusion systems. Although it is known that there is a strong correlation between liver function in whole-blood reperfusion and survival, the exact nature of these correlations, and to what extent they can be considered as an indicator of viability for transplantation/recipient survival, remain unclear. In this work, we demonstrate that diluted whole-blood reperfusion can be used as a direct model for transplantation of ischemic rat liver grafts. Specifically, we show that recipient survival can be predicted based simply on the value of alanine aminotransferase during perfusion, providing quantitative criteria of viability for use in this animal model. These results indicate that in the rat model graft survival is highly correlated with hepatocellular damage.

Introduction

Chronic liver disease and cirrhosis cause about 27,000 deaths annually in the US, about 4,000 of which could be saved with a transplantation[99]. The donor organ availability could be significantly increased if livers obtained from donors after cardiac death (DCD) could be rendered usable, which is not possible with simple cold storage (CS) [50]. Normothermic extracorporeal Machine Perfusion (NMP) [18, 98] as well as Hypothermic Machine Perfusion (HMP) have been suggested as alternatives [37, 79, 80]. However, despite two decades of research, a golden standard is yet to emerge for machine perfusion systems and protocols.

Whole blood reperfusion has been used as a surrogate for organ transplantation, especially as a model for the short-term response post transplantation [98], for optimization of perfusion systems. Whole blood reperfusion is ideal for rapid optimization of perfusion systems and protocols for perfusion-resuscitation of DCD organs, without the need for effort-intense, time-consuming and expensive transplantation and survival studies. While it is known that there is a strong correlation between liver function in whole-blood reperfusion and survival, the exact nature of these correlations, and to what extent they can be considered as an indicator of viability for transplantation/recipient survival, remain unclear.

In this work, we present a data-mining study that correlates survival to diluted-blood reperfusion parameters in machine perfused ischemic rat livers. Moreover, we present a simple criterion of viability for prediction of the graft survival, and optimization of perfusion protocols resuscitation of ischemic rat liver grafts.

Experimental Procedures

Three DCD liver transplantation modalities were considered: Direct transplantation, transplantation after 5hrs of Cold Storage (CS) in UW solution, and transplantation after 5hrs of Normothermic Machine Perfusion (NMP). Fresh livers, cold stored for 6hrs were used as control group.

For each group, transplantation studies were performed to evaluate recipient survival for up to 4 weeks, and diluted whole-blood reperfusion studies were performed to obtain in ex-vivo reperfusion data [98]. For purposes of practicality, the most commonly used parameters used in liver viability/perfusion studies were considered: Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Oxygen Uptake Rate (OUR) and bile secretion.

For a details on the isolation of the donor liver, machine perfusion protocols, isolated reperfusion, transplantation and processing of perfusate and tissue samples, please refer to chapters two and three and references [83, 98] respectively.

Nummerical Procedures

A data mining study was performed to correlate liver parameters (ALT, AST, bile, OUR) to recipient survival at 28 days post transplantation. A combined classifier and variable selection process was employed to create a mathematical model that predicts recipient survival based on a few of these parameters.

Training, cross-validation, and selection of classifiers to create a quantitative criterion of perfused graft viability were performed in WEKA data mining software [108]. The following classifiers were tested: BF Tree, J48 Tree, Conjunctive Rule, Bayesian Network, Neural Network, Kstar, Radial Basis Function (RBF) Network, Sequential Minimal Optimization algorithm for training a support vector classifier (SMO). These classifiers span the majority of the classes of available statistical prediction models, from simple decision trees to sophisticated artificial neural networks. Each of the parameters obtained during reperfusion was considered as an attribute, for identifying and training the appropriate statistical models for predicting transplant success based on the reperfusion measurements.

For each classifier, attribute selection was performed to create a quantitative criterion of viability. The attributes were selected using best first ranking algorithm in WEKA to identify the optimum set of attribute (with 10-replicates each and default parameters in WEKA otherwise). The estimated real-world accuracy of each classifier was then evaluated via ten ten-fold cross validations, where all randomizable variables in the model, as well as the partitioning of data into 10 folds, were randomized. N-fold cross validation is a multi-sampling procedure where the data is separated into N sets, and N-1 of the sets is used for model training whereas the remaining set is used for validation; the training and validation is repeated N-times to produce a statistical estimate of classifier accuracy. This method uses all the data for validation, and hence is a reliable estimator of real-world accuracy [108-110].

For each cross-validation, two accuracy criteria are reported: classification accuracy, and average relative error. Classification accuracy is simply the percent of correctly classified instances (i.e. survival/failure for each reperfusion experiment) based on the final prediction of the tested method. Average relative error further considers the quality of

the predictions by the classifier. For instance, if the classifier predicts that survival of a certain instance is correct at a 51% probability, the classification accuracy will be 100%, but relative error will be 49%. Thus, average relative error enables the comparison of the accuracies of classifiers even if the classification accuracies are similar or the same.

Results

As displayed in Figure 1, surgery of the resuscitated ischemic livers was without complications in all but one case, where bleeding at the anastomosis occurred and the animal died on Day 4 (N=13). Since the recipient death was due to surgical issues rather than graft function this animal was not accounted for in the survival statistics. All the remaining animals recovered from anesthesia rapidly and survived beyond one month, and did not exhibit external signs of liver failure, such as jaundice. Controls that received freshly isolated livers preserved for 6 hours by CS similarly recovered rapidly from surgery and showed excellent survival (N=6). No surgical complications occurred during transplantation of ischemic livers preserved by CS and recipients recovered rapidly from anesthesia, but within 6 hours all developed symptoms and died within 12 hours (N=6). Autopsy revealed patchy livers and serous fluid in the abdomen. The recipients of directly transplanted ischemic did not survive beyond 24 hours post-operatively (N=6).

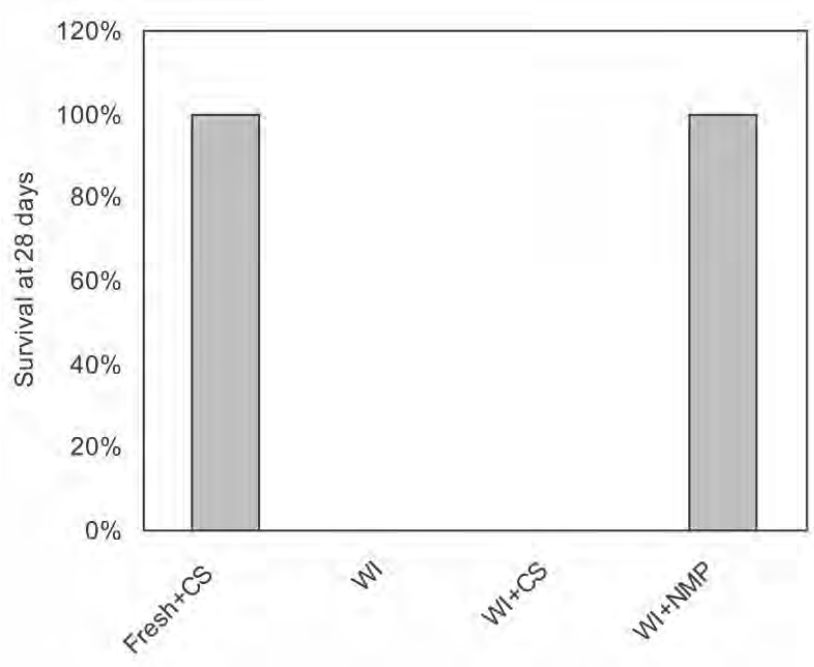

FIGURE 1 | Recipient survival post-transplantation.

The variable elimination process was performed for each model considered: The cross-validation results for the models evaluated are displayed in Table 1. Most models were successful in correctly predicting recipient survival with a classification Accuracy of 100%. Best-First Tree classifier, however, not only had a zero average relative error, but also is one of the simplest classifiers tested, and as such was chosen as the best classifier based on the Occam's Razor principle. The decision criterion developed via BF Tree classifier is:

ALT (@30 mins) < 54.69107: survival
ALT (@30 mins) ≥ 54.69107: failure

Since this 100% accurate prediction can be achieved by multiple classifiers, these results clearly demonstrate that the transplant survival can be predicted, based simply on the ALT levels during whole blood reperfusion.

TABLE 1 | Comparison of Tested Models.

Method	Classification Accuracy (%)	Relative Absolute Error (%)	Selected Variable
BF Tree	100 ± 0	0 ± 0	ALT 30
J48 Tree	91.67 ± 0	15.48 ± 0	ALT 15
Conjunctive Rule	93.33 ± 7.66	8.72 ± 8.72	ALT 30
Bayesian Network	100 ± 0	17.58 ± 5.06	ALT 30
Neural Network	100 ± 0	10.77 ± 0.46	ALT 30
Kstar	100 ± 0	10.16 ± 0.8	ALT 30
RBF Network	98.333 ± 3.51	5.35 ± 5.64	ALT 30
SMO	100 ± 0	2.74 ± 3.52	AST45

The next question that arises is whether ALT (measured at 30 minute mark) is the only successful indicator. To answer this question, ALT 30 was eliminated was from the list of potential variables, and the variable selection process was repeated, using the most successful classifier identified before (BF Tree). This process was continued until the cross-validation accuracy decreased below a heuristically chosen 85%.

As displayed in Table 2, ALT 30, ALT 120, and AST 15 all yield similarly perfect cross-validated prediction accuracies. This was followed by ALT 15, AST 45, and AST 75, which all resulted in one incorrect classification in cross validation. The next iteration identified AST 105, with an accuracy of 87.5%, followed by Bile 60 at 83.33% and the iterations were stopped.

TABLE 2 | Variables Measured During Ex Vivo Perfusion and Selected as Indicators of Viability.

Measure	Classification Accuracy (%)	Average Relative Accuracy (%)
ALT 30	%100 ± 0	%100 ± 0
ALT 120	%100 ± 0	%100 ± 0
AST 15	%100 ± 0	%100 ± 0
ALT 15	%91.67 ± 0	%84.15 ± 0
AST 45	%91.67 ± 0	%84.15 ± 0
AST 75	%91.67 ± 0	%84.15 ± 0
AST 105	%87.5 ± 4.39	%61.95 ± 6.73
BILE 60	%83.33 ± 0	%47.16 ± 0
OUR 30 + OUR 105	%79.17 ± 5.89	%60.12 ± 6.74

Since OUR was not selected at all during this process, a last variable selection was performed with only using the OUR measurements. This process resulted in selection of two OUR measurements (minutes 30 and 105) with a cross validated classification accuracy of 57%.

To test whether the selection of attributes is purely an artifact of measurement errors, we analyzed the standard deviations (normalized to the means) for all parameters. If the algorithm selects simply the variables with lowest measurement error/variability, the attribute selection would be in order of increasing standard deviations. As displayed in Table 3, OUR (90 mins) and AST (75 minutes) are the attributes with the smallest standard deviations and yet they are not selected by the algorithm, where as ALT 120 has one of the highest measurement deviation and yet is one of the highest ranked parameters. Therefore, it can be deduced that attributes selection is not simply due to low measurement errors, but rather a result of the underlying physiological phenomena.

TABLE 3 | Normalized Standard Deviations.

Attribute / Measurement Time	Fresh	WI	WI+SCS	WI+NMP	AVERAGE
ALT 15	0.576	0.173	0.570	0.123	0.360
ALT 30	0.259	0.244	0.333	0.339	0.294
ALT 45	0.113	0.234	0.587	0.453	0.347
ALT 60	0.307	0.242	0.354	0.504	0.352
ALT 75	0.364	0.359	0.312	0.410	0.361
ALT 90	0.143	0.501	0.234	0.635	0.378
ALT 105	0.648	0.464	0.264	0.803	0.545
ALT 120	0.506	0.190	0.273	0.703	0.418
AST 15	0.463	0.162	0.752	0.225	0.401
AST 30	0.470	0.222	0.507	0.497	0.424
AST 45	0.341	0.035	0.158	0.564	0.275
AST 60	0.213	0.266	0.185	0.624	0.322
AST 75	0.187	0.300	0.068	0.418	0.243
AST 90	0.330	0.415	0.108	0.459	0.328
AST 105	0.521	0.493	0.094	0.481	0.397
AST 120	0.652	0.406	0.125	0.272	0.364
BILE 60	0.160	0.605	0.529	0.256	0.387
BILE 120	0.169	0.483	0.568	0.279	0.375
OUR 15	0.217	0.140	0.720	0.120	0.299
OUR 30	0.249	0.563	0.341	0.172	0.331
OUR 45	0.407	0.308	0.291	0.247	0.313
OUR 60	0.462	0.493	0.261	0.284	0.375
OUR 75	0.408	0.310	0.214	0.119	0.263
OUR 90	0.316	0.000	0.212	0.179	0.177
OUR 105	0.214	0.307	0.284	0.262	0.267
OUR 120	0.390	0.478	0.134	0.326	0.332

Discussion

It is interesting to note that a single measurement seems to be sufficient as the indicator of viability, as previous attempts at similar decision criteria have lead to much more sophisticated formulations [109, 110]. ALT is an enzyme that is found in high concentrations in hepatocytes and it's clinically considered the single best indicator of liver failure, hence it's selection is not surprising; however the fact that ALT alone is sufficient indicates graft survival is strongly correlated to hepatocellular damage. By contrast, microcirculatory failure would have been indicated by selection of oxygen uptake due to poor perfusion of the liver, and selection of bilirubin could indicate either damage of the biliary epithelium or metabolic dysfunction. Our results indicate that neither of these latter scenarios are the case.

It was observed that AST can also be employed to predict viability; however the fact that ALT and AST are essentially interchangeable indicates that AST release is also primarily due to hepatocellular damage, rather than general cell damage which would result on the use of only AST as an indicator.

Another interesting finding is that specific time points appear to provide better indicators of graft viability. The standard deviation analysis shows this is not a simple matter of measurement error; however, available data does not provide further insight into the dynamics of organ damage/function during whole blood reperfusion, and further experiments is necessary to identify whether these time points correspond to key events in ischemia/reperfusion injury.

It is important to note that since purely statistical methods are employed here, the use of the criteria only applies to warm-ischemic rat liver transplantation (without rearterialization), with diluted whole-blood reperfusion as the testing platform. Especially clinical and large animal models have significant differences compared to this model, and the decision criteria developed here should not be applied before further, thorough validation.

Conclusion

In this work, we demonstrate that diluted whole-blood reperfusion can be used as a direct model for transplantation of ischemic rat liver grafts. Moreover, it was shown that recipient survival can be predicted based simply on the value of ALT during perfusion, and quantitative criteria of viability was developed for use in this animal model. These results indicate that in the rat model graft survival is highly correlated to hepatocellular damage.

Chapter 7

Resuscitation of ischemic donor livers with normothermic machine perfusion: a dynamic metabolic analysis of treatment in rats

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Manuscript status: submitted

Abstract

Normothermic machine perfusion (NMP) has previously been demonstrated to restore damaged warm ischemic livers to transplantable condition in animal models. However, the mechanisms of recovery are unclear, preventing rational optimization of perfusion systems and slowing clinical translation of machine perfusion. In this study, organ recovery time and major perfusate shortcomings were evaluated using a comprehensive metabolic analysis of organ function in perfusion prior to successful transplantation.

Two groups, Fresh livers and livers subjected to 1hr of warm ischemia (WI) received NMP for a total preservation time of 6hrs, followed by successful transplantation. 24 metabolic fluxes were directly measured and 38 stoichiometrically-related fluxes were estimated via a mass balance model of the major pathways of energy metabolism. This analysis revealed stable metabolism in Fresh livers throughout perfusion while identifying two distinct metabolic states in WI livers, separated at t=2hrs, coinciding with recovery of oxygen uptake rates to Fresh liver values. This finding strongly suggests successful organ resuscitation within 2 hrs of perfusion. Overall perfused livers regulated metabolism of perfusate substrates according to their metabolic needs, despite supraphysiological levels of some metabolites. Compared to in vivo livers, perfused livers had a generally reduced rate of metabolism seen through lowered oxygen uptake, lipid oxidation and TCA cycle activity. This reduced metabolic activity is suggestive of a response to mild hypoxia, despite sanguineous perfusion.

This study establishes the first integrative metabolic basis for the dynamics of recovery during perfusion treatment of marginal livers. Our initial findings support enhanced oxygen delivery for both timely recovery and long-term sustenance. These results are expected to lead the optimization of the treatment protocols and perfusion media from a metabolic perspective, facilitating translation to clinical use

Introduction

Transplantation is currently the only treatment option for end-stage liver disease but it is limited by the lack of high quality donor organs. An area of keen investigation is into the use of machine perfusion (MP) as a means of resuscitating presently non-transplantable donor organs after cardiac death (DCD), thereby significantly increasing the donor organ pool[111]. Porcine and murine models of DCD livers are significantly improved by MP compared to organs preserved by the gold standard of static cold storage[43, 83], and furthermore, normothermic MP is necessary for their successful transplantation[47, 98, 112].

While MP is particularly well-suited to dynamic evaluation of organ function, standardized quantitative analyses have yet to be established. Optimization of perfusion parameters such as temperature, flow rate, perfusion media and the ideal oxygen carrier[113] continue to dominate most research efforts. Two fundamental questions remain unanswered: i) what should the liver be perfused with and ii) how long should it be perfused for?

Optimizing the perfusate addresses the optimization of the metabolic state of the liver during MP. It is generally accepted that recovery of energy levels during perfusion is expected and will enhance transplant success by alleviating some aspects of ischemia-reperfusion injury. However, existing studies typically focus on a few global indicators of energy recovery, which by themselves are nonspecific, and so do not enable definitive conclusions to be reached regarding the limitations of the perfusion system and protocol employed. Lactate accumulation, for example, occurs during anaerobic metabolism from hypo-perfusion[114], normal erythrocyte metabolism[115] and in the absence of an initial lactate concentration in the perfusion medium[116]. Other frequently used parameters, such as trends in glucose metabolism, degree of oxygen consumption, ability to produce albumin or other complex proteins, rates of excess amino acid breakdown and urea formation[48, 86, 91] similarly reflect multiple likely pathways of activity that will provide more specific information if evaluated in a cohesive manner.

Endeavoring towards a standardized approach to organ recovery and rational perfusion optimization, we employed a comprehensive "Metabolic Flux Analysis" model of whole-organ metabolism[66] to dynamically assess the function of the liver during perfusion. The mathematical model stoichiometrically correlates 28 measured and 34 calculated metabolite rates of uptake or release (called fluxes) from hourly perfusion time points of organs that were subsequently successfully transplanted (>1 month survival). Using the temporal profiles of the measured fluxes, a significant metabolic turnaround, signaling recovery of warm ischemic livers, was observed within 2 hours of perfusion; oxygen

uptake rate (OUR) most dramatically illustrated this event. Metabolic flux analysis of perfused ischemic livers to perfused fresh livers and in vivo livers enabled highlighting the impact of MP on organ function and identification of system-specific shortcomings. This methodology and data enables future perfusion optimizations for long-term organ storage and recovery.

Materials and Methods

For a details on the isolation of the donor liver, machine perfusion protocols, isolated reperfusion, transplantation sampling and processing of perfusate and tissue samples, please refer to chapters two and three and references [83, 98] respectively.

Biochemical Assays

Blood gases were determined immediately using a blood gas analyzer (Rapidlab, Chiron Diagnostics, Norwood, MA). Oxygen concentration delivered and removed from the liver was calculated using the following equation:

$$[O_2] = (1.39 \times [Hb] \times FO_2Hb) + 0.00314 \times pO_2 \quad (1)$$

which expresses the concentration of oxygen (ml/dL of blood) as the sum of oxygen bound to hemoglobin and free in plasma. [Hb] (g/dL) is the concentration of hemoglobin, FO_2Hb is the fraction of oxyhemoglobin present, 1.39 (mlO_2/g Hb) is the binding capacity of oxygen to hemoglobin and 0.00314 (mlO_2/dL/mmHg) is the solubility coefficient of oxygen in plasma, which is dependent on the partial oxygen tension in the blood, pO_2 (mmHg). The rate at which oxygen is delivered to the liver (ODR) and exits the liver (OER) is subsequently dependent on the flow rate, $\dot{V}$ (ml/min), and the difference between the two normalized to the weight of the liver W(g) provides the hepatic oxygen uptake rate (HOUR):

$$HOUR = ODR - OER$$

$$HOUR = \frac{[O_2]_{inlet} \times \dot{V} - [O_2]_{outlet} \times \dot{V}}{W_{Liver}} \quad (2)$$

Similarly, total carbon dioxide release rate (CRR) at each time point was calculated based on the total carbon dioxide measures in the samples via Piccolo Blood Chemistry Analyzer (Abaxis) as:

$$CRR = \frac{([tCO_2]_{outlet} - [tCO_2]_{inlet}) \times \dot{V}}{W_{Liver}} \quad (3)$$

Urea was assayed by reaction with diacetyl monoxime using a commercial assay kit (BUN, Sigma-Aldrich, St. Louis, MO). Ketone bodies were measured enzymatically, by following the appearance of NADH in the conversion to acetoacetate and the disappearance of NADH in the conversion to β-hydroxybutyrate in the presence of β-hydroxybutyrate dehydrogenase[117]. Nineteen of the common amino acids (except tryptophan) and ammonia were fluorescently labeled using the AccQ-Tag system (Waters Co., Milford, MA), separated by high-performance liquid chromatography (HPLC; Model 2690, Waters Co.) and quantified by a fluorescence detector (Model 474, Waters Co.), as previously described[66]. Lactate was measured using the enzymatic conversion to pyruvate and hydrogen peroxide with lactate oxidase from a commercially available kit (Trinity Biotech, Berkeley Heights, NJ). Albumin concentration was determined by an enzyme-linked immunosorbent assay using a polyclonal antibody to rat albumin[118]. A standard curve was derived using chromatographically purified rat albumin (Cappel Laboratories, Aurora, OH) dissolved in medium. Note that the dialyzer molecular cutoff weight was determined so that albumin could not pass through, and subsequently did not appear in the secondary dialysate circuit. Glucose measurements were quantified with an enzymatic assay kit through conversion to 6-phospho-gluconate (Glucose assay kit, Sigma).

Statistical Identification of Linear Response Phases During Perfusion

Linear regressions were performed on the temporal concentration profiles of each of the metabolites at different time-periods of the perfusion (e.g. 0-5 hrs, 0-2 hrs etc). This was done in order to identify whether there were multiple distinct metabolic phases during perfusion. This linear-response phase analysis served the dual purpose of establishing times to attain stability or recovery in perfusion, as well as being a necessary prerequisite to calculating fluxes accurately for metabolic flux analysis (see below for details). Box-and-whisker plots of the resulting R^2 values for each time period and experimental group were evaluated to identify the segments of time with highest R^2 and minimum variation (Appendix B). Ischemic livers were found to exhibit two distinct stable phases between 0-2hrs and 2-5hrs of perfusion. Fresh livers were found to be generally stable; greatest linearity was seen between 1-5hrs, suggesting some degree of equilibration with the perfusate during the first hour.

Note that all metabolites displayed a generally linear trendline within the identified periods. However, for a few metabolites the concentration profiles were flat, which led to low R^2 values despite good fit by visual inspection (for example, see Figure 2 Glucose, Figure 3 Albumin, Glutamate, and Histidine). These outlier R^2 values therefore were not considered as a violation of the linearity assumption above. To avoid the analysis being

influenced by such artifacts, the median and quartile analysis, which is more robust against such outliers, was employed as opposed to the mean and standard deviation, which is more susceptible.

Calculation of Fluxes

Fluxes were calculated as the gradient of concentration of each metabolite (i.e. slope of the linear regression curve)[119] over the selected segments of perfusion, normalized to the weight of the liver and averaged for each group. Oxygen and carbon dioxide fluxes were determined every hour for each liver and were then averaged for each group over the selected segments of time.

Data Preprocessing and Outlier Analysis

An initial outlier analysis was performed for each measurement by plotting box-and-whisker diagrams in MATLAB and eliminating obvious errors (e.g. negative values). This was followed by a more stringent analysis where for each group any measurement value above/below mean ± 2x inter-quartile range for that group were considered outliers.

Metabolic Flux Analysis (MFA)

MFA was performed based on a stoichiometric model for the metabolic reaction network developed and tested in more detail previously[119]. The model allows for the estimation of otherwise inaccessible intracellular reaction fluxes by performing a mass balance around each intracellular metabolite using measured extracellular fluxes. The model was originally developed for perfused hypermetabolic rat livers[119], and currently uses a total of 28 metabolites and 62 chemical reactions, including TCA and urea cycles, amino acid metabolism, gluconeogenesis/glycolysis. The model does not incorporate complete fatty acid and lipid metabolism, and while the pentose phosphate pathway (PPP) is included, DNA synthesis/liver regeneration are assumed to be negligible. The details of these assumptions can be found elsewhere[119]. The primary accommodation of the model for the gluconeogenic state is the preferential formation of oxaloacetate and phosphoenolpyruvate from pyruvate rather than acetyl-CoA, which ultimately favors gluconeogenesis. By contrast, in a glycolytic state, glycolysis is predominant and favors the formation of acetyl-CoA via pyruvate. Fluxes 6 and 7 vary therefore depending on the state of carbohydrate metabolism and are represented as dotted lines, rather than solid lines, in the glycolytic case. It is noted here that all perfused livers derived from fed rats while in vivo results were obtained using fasted rats. WE insulin levels were approximately 10-fold higher than what was observed in fed rats[106, 120] and without glucagon supplementation.

Briefly, in MFA the change in the concentration of intracellular metabolites is assumed to be zero (pseudo steady-state assumption) hence the sum of fluxes of each metabolite's uptake, synthesis and utilization equals zero:

$$S.v = 0 \quad (4)$$

The matrix S contains the stoichiometric coefficients of the incorporated reactions. Each element S_{ij} of S is the coefficient of metabolite i in reaction j, and each v_j of vector *v* is the net flux or conversion rate of reaction j. Equation 4 is separated into measured (v_m) and unknown fluxes (v_u), as well as the matrices containing stoichiometric coefficients of known (S_m) and unknown reactions (S_u), as follows:

$$S_u.v_u = - S_m.v_m \quad (5)$$

The measured fluxes represent rates of uptake or release of extracellular metabolites and by solving Equation 5 they also give estimates of intracellular fluxes, therefore enabling an intracellular analysis based on extracellular changes.

It should be noted that if the number of stoichometric balances (i.e. independent rows, or equivalently, the rank of matrix S_u) in Eqn. 5 are equal to the number of unknown fluxes then there is a single solution. Ideally, the unknown fluxes are fewer; in this case the solution of equation 5 becomes a regression problem. Moreover, in this case the consistency of measured fluxes within each other and the model can be validated.

In this work, the model consistency and validity of the steady state assumption was confirmed by the method of Wang and Stephanopoulos[121]. Briefly, this approach tests if the errors from the regression for a chi-square distribution, which indicates a normal, expectable measurement error distribution. If the regression errors do not follow a chi-square distribution at $p<0.05$, then it is possible to identify the problematic measurements by an iterative elimination process [121] and eliminate artifactual/inconsistent measurements.. This approach was used to identify two artefactual oxygen uptake measurements, which when eliminated resolved the issues observed.

Statistical Analysis

All statistical comparisons between individual fluxes were performed using 2-tailed Student's t-test ($p<0.05$).

Groups Studied

It should be noted that the in vivo MFA study [106] used for comparison was performed with a different rat strain (Sprague Dawley); although strain-to-strain variations are expected to be minimal in the basic metabolic pathways considered in the model, these comparisons are best interpreted for their overall significance rather than any specific differences in detail.

Results

We previously demonstrated that ex vivo normothermic perfusion of freshly isolated rat livers results in transplantable organs, and further that the same perfusion system is necessary and sufficient for the restoration of 1 hr warm ischemic rat livers to transplantable condition[83, 98]. In this work we evaluated metabolites measured hourly during perfusion of successfully transplanted organs, to gauge the perfusion recovery time and organ stability. The systemic impact of perfusion on organ metabolism was evaluated through metabolic analysis and comparison to in vivo results (see Appendix A for the reference values)[106].

Oxygen Consumption

Samples of perfusate were taken hourly at the portal vein, to reflect oxygen delivery rate (ODR), and at the infra-hepatic vena cava, to determine oxygen exit rate (OER). Oxygen uptake rate (OUR) was determined as the difference between ODR and OER (Figure 1A). ODR and OER were also compared to in vivo conditions (Figure 1B). WI livers consumed significantly less oxygen than Fresh livers during the first hour of perfusion, but by the second hour, consumption had increased to 0.050-0.057ml O_2/min/g liver and was comparable between groups. WI livers showed a slow but steady decline in OUR from t=2-5hrs, the difference between WI and Fresh livers again becoming statistically different at t=5hrs. Fresh livers also demonstrated a decline in OUR initially until t=3hrs after which consumption began to gradually increase again. Figure 1B illustrates that the ODR in perfusion averages at 0.14 ml O_2/min/g liver in both groups, and falls within one standard deviation of the average in vivo ODR. The OER however, was significantly higher than in vivo, which has negligible variation in value, demonstrating that despite reduced oxygen supply, the liver does not consume all that is available to it in perfusion, regardless of ischemic injury.

Glucose

Concentration profiles of glucose in both WI and Fresh livers were generally stable at a value slightly above the original perfusate glucose content of 2g/L (Figure 2A).

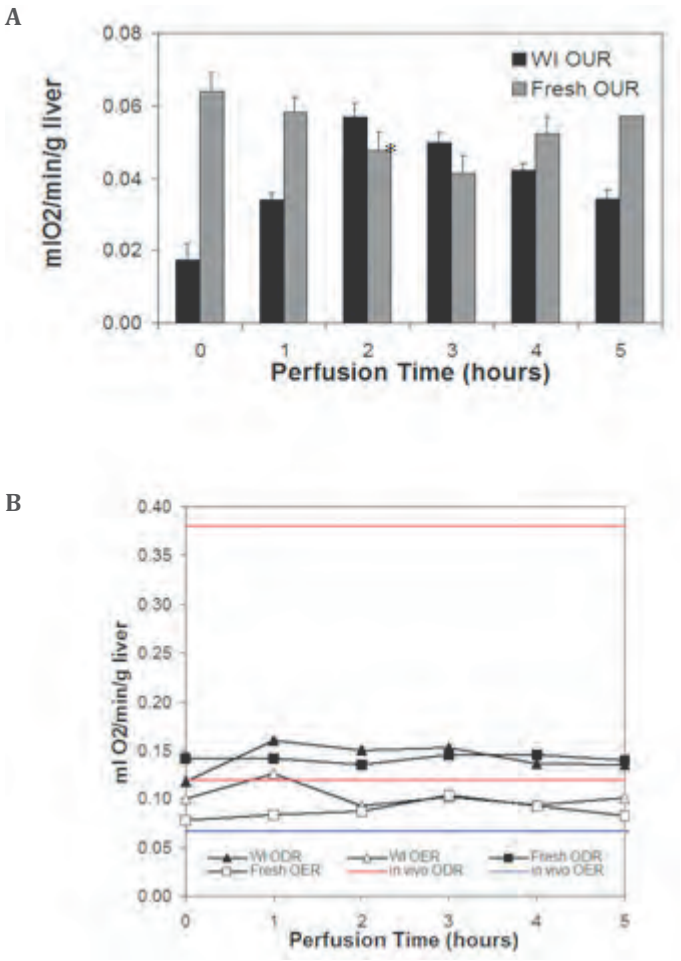


FIGURE 1 | A) Oxygen uptake rate (OUR) for WI and Fresh livers. B) Oxygen delivery rate (ODR) and exit rate (OER) in perfused livers compared to those in vivo. Red and blue lines represent the range in ODR and OER (average +/- 1std dev); the range for OER is negligible. *Indicates significantly different from Fresh ($p<0.05$)

Lactate WI

Livers produced more lactate in the first hour than Fresh livers, exceeding the in vivo upper bound value within the first hour (Figure 2B). The rate of production declined after the first hour resulting in concentrations comparable to Fresh livers by the end of perfusion. Fresh livers produced lactate linearly throughout, also exceeding the in vivo upper limits though this occurred later, a little after 2 hours of perfusion. Note that WE perfusate does not contain any lactate.

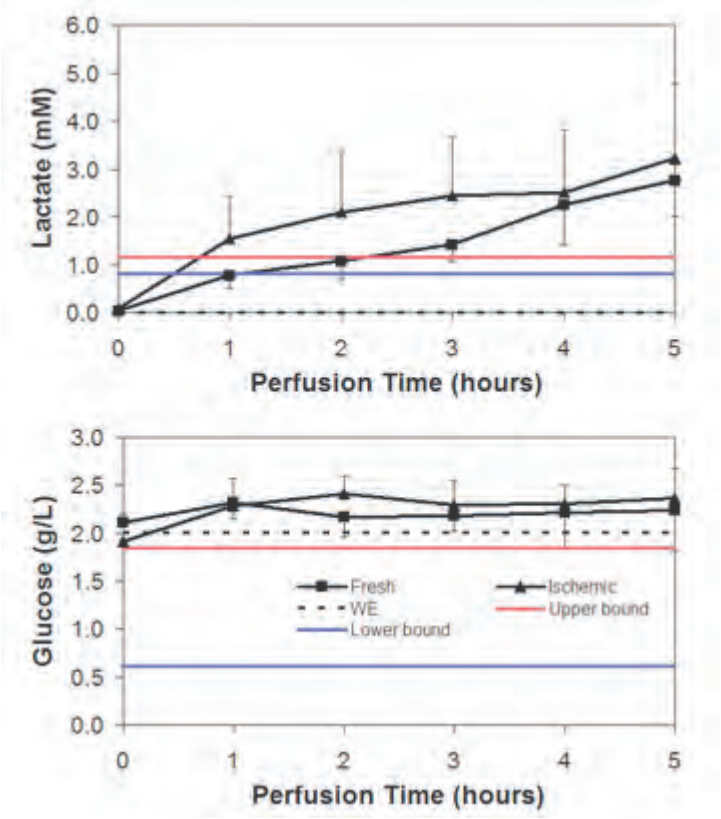


FIGURE 2 | Average concentrations during NELP of WI and Fresh livers. Dotted line is Williams Medium E (WE), red line is in vivo upper bound (ave + 1 std dev) and blue is in vivo lower bound (ave – 1 std dev).

Nitrogen Metabolism

Albumin concentration (Figure 3A) increased steadily in Fresh liver perfusions, reaching a maximum of 0.68g/dL at t=4hrs, approximately 40% of the in vivo lower bound value. By contrast, WI livers produced little to no albumin during this time span (0.0055 g/dL/hr). Urea concentration increased similarly and linearly in both groups (Figure 3B) at a rate of 4.1 mM/hr, R^2=0.99 for Fresh livers and 4.4mM/hr, R^2=0.98 for WI livers. Urea concentration also did not reach the in vivo lower bound concentration of approximately 2.8mM at the end of perfusion. Ammonia concentration increased similarly in perfusate from zero to 40uM in both groups (Figure 3C). The perfusate values of ammonia were significantly below the in vivo lower bound of approximately 76uM. The amino acids methionine (Figure 3D), tyrosine, proline, lysine and phenylalanine (not shown) were all consumed at significantly lower rates in WI livers than in Fresh livers. Glutamine uptake (Figure 3E) occurred at a stable rate that was similar for both WI and Fresh livers. Rates

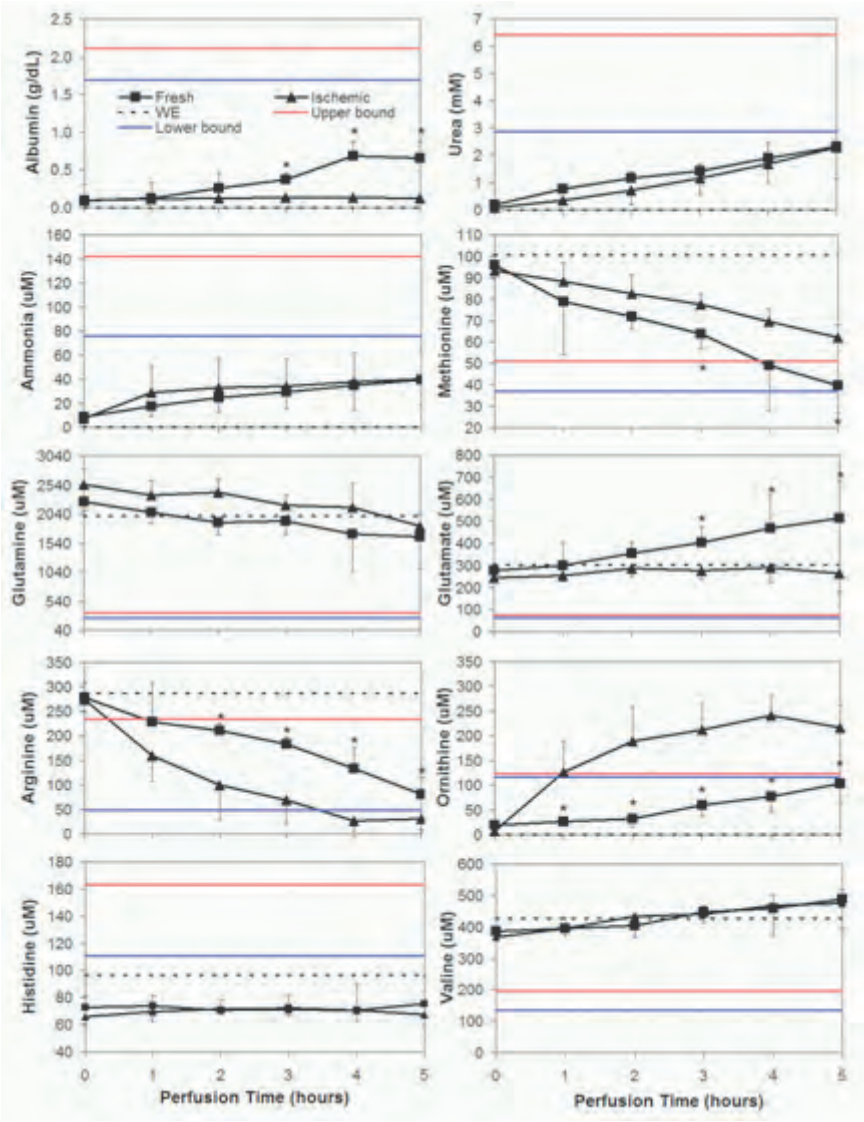


FIGURE 3 | Average amino acid metabolism during NELP of WI and Fresh livers. Dotted line is Williams Medium E (WE), red line is in vivo upper bound (ave + 1 std dev) and blue is in vivo lower bound (ave – 1 std dev). * Indicates significantly different from Ischemic ($p<0.05$).

of uptake between WI and Fresh livers were also similar for aspartate, alanine, glycine, asparagine, cysteine and threonine (not shown). WI and Fresh livers differed significantly in glutamate metabolism (Figure 3F). Glutamate concentration increased linearly in Fresh livers but was relatively unchanged in WI livers and remained within the value present in WE. Arginine (Figure 3G) by contrast was consumed at a significantly higher rate by

WI livers, to the extent that it became substrate depleted at t=4hrs. A reciprocal increase in ornithine was observed (Figure 3H); a plateau was reached in the output by WI livers at t=4hrs, well above the in vivo upper bound value. By contrast, a linear increase in ornithine output by Fresh livers resulted in a perfusate concentration within in vivo range at t=5hrs. WE was deficient in lactate, ornithine, ammonia, urea, albumin, and ornithine at the onset of perfusion, and the liver generally increased the concentrations of each of these metabolites during perfusion. However, in the case of histidine (Figure 3I) and serine (not shown), both present in WE at values significantly below the in vivo lower bound, neither were utilized or contributed to during perfusion. The branched chain amino acids valine (Figure 3J), isoleucine and leucine (not shown) were all produced linearly during perfusion by both groups.

MFA of Fresh vs. Ischemic Perfused Livers

In order to identify whether there were distinct phases in liver metabolism during perfusion, linear regressions were performed on the temporal concentration profiles of each of the metabolites. Box-and-whisker plots of the resulting R^2 values were evaluated for least variation across different segments of time (Appendix B). Ischemic livers were found to exhibit stable but distinctly different metabolic rates between 0-2hrs and 2-5hrs of perfusion; MFA was conducted separately for both phases. Fresh livers were generally stable throughout perfusion; MFA was performed on the segment with greatest linearity, determined as being t=1-5hrs.

In Figure 4, the first two hours of ischemic liver perfusion are compared to fresh liver metabolism using MFA. The map suggests ischemic livers were significantly more glycogenolytic than fresh livers at NMP onset, breaking down glycogen for glycolysis and glucose release. Glycolysis appeared to result in a 116% increase in the production of lactate (Flux #8). Oxygen uptake rate (Fluxes 53-55) and the TCA cycle were comparable between groups. Ischemic livers demonstrated a preferential uptake of the amino acid arginine (118% increased in Flux #18) and a reciprocal, 304% increase, in the release of ornithine into the extracellular space (Flux #20). Ischemic livers also showed a 46% increase in the formation of asparagine from aspartate (Flux #47). Phenylalanine uptake was increased by 24% ($p<0.1$, Flux #36) while tyrosine uptake was reduced by 63% ($p<0.05$, Flux #38) resulting in an overall reduction of fumarate production (Flux #37). Methionine and serine metabolism were significantly reduced (Flux #44); extracellular serine release was observed at this time also (Flux #25). Glutamate production (Flux #40) was 58% of that found in fresh livers, a significant reduction due likely to a decline in contribution from lysine and 2-oxo-glutarate (Flux #35), which were reduced by 61% ($p<0.1$).

TABLE 1 | Measured Influx Values

Parameter	PV+HA	Perfusate
Albumin (g/min/g liver)	0.02-0.05	0.00
Lactate (mmol/min/g liver)	0.03-0.1	0.00
Glucose (mg/min/g liver)	1.13-2.5	3.68
Alanine (umol/min/g liver)	0.36-0.92	1.86
Ammonia (umol/min/g liver)	0.11-0.2	0.00
Arginine (umol/min/g liver)	0-0.5	0.53
Asparagine (umol/min/g liver)	0.04-0.12	0.28
Aspartate (umol/min/g liver)	0.02-0.04	0.41
Cysteine (umol/min/g liver)	0.01-0.03	0.61
Glutamate (umol/min/g liver)	0.07-0.15	0.56
Glutamine (umol/min/g liver)	0.28-0.68	3.64
Glycine (umol/min/g liver)	0.26-0.6	1.23
Histidine (umol/min/g liver)	0.1-0.35	0.18
Isoleucine (umol/min/g liver)	0.07-0.23	0.70
Leucine (umol/min/g liver)	0.21-0.66	1.05
Lysine (umol/min/g liver)	0.18-0.54	1.10
Methionine (umol/min/g liver)	0.04-0.1	0.18
Ornithine (umol/min/g liver)	0.11-0.28	0.00
Phenylalanine (umol/min/g liver)	0.05-0.13	0.28
Proline (umol/min/g liver)	0.16-0.36	0.48
Serine (umol/min/g liver)	0.17-0.48	0.18
Threonine (umol/min/g liver)	0.19-0.47	0.62
Tyrosine (umol/min/g liver)	0.05-0.17	0.51
Valine (umol/min/g liver)	0.12-0.43	0.79

In vivo influx is the combined portal vein (PV) and hepatic artery (HA) contribution to that flux (ave – 1 std dev, ave + 1 std dev).

Perfusate influx is calculated according to the initial perfusate concentrations and a flow rate of 1.8ml/min/g liver.

Between 2-5hrs of WI perfusion (Figure 5) more differences were apparent between Fresh and WI than at 0-2hrs. WI livers appeared to be mildly gluconeogenic and demonstrated a 30% reduction in lactate output compared to fresh livers, despite a further decline in oxygen uptake rate (Figure 1A). Contributions to the TCA cycle via phenylalanine conversion to tyrosine were reduced, such that fumarate production via this pathway (Flux #37) was only 50% of Fresh liver flux values. Reduced acetyl-CoA and oxaloacetate (Flux #9) resulted in a 61% reduction of citrate formation, while threonine conversion to acetyl-CoA was increased 470%. Glutamate output was further reduced to within 4% of

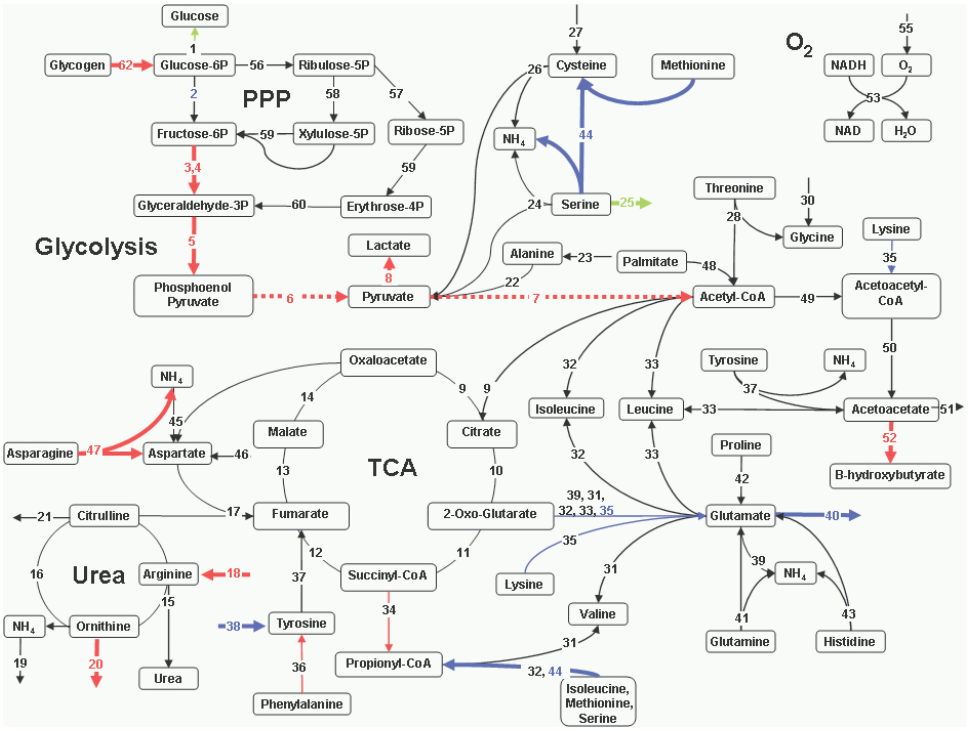


FIGURE 4 | MFA of Fresh perfused livers compared to in vivo livers; in vivo MFA forms the black baseline. Red arrows are significantly increased fluxes. Blue arrows are significantly reduced fluxes and green are reversed. Bold lines = $p<0.05$, Thin lines = $p<0.1$, Dotted lines = glycolysis.

Fresh liver fluxes; contributions to its formation from both lysine (50% of fresh livers, Flux #35) and proline (40% of fresh livers, Flux #42) impacted its production substantially. There was however, a 260% increase in glutamate formation via glutamine (Flux #41) which resulted in a 100% increase in glutamate formation of 2-oxo-glutarate (Flux #39). This increased flux converged on the TCA cycle at a point of reduced incoming fluxes from citrate, such that the downstream pathway of the cycle was restored to a value similar to that of Fresh livers. Asparagine to aspartate production was further increased to a rate 60% greater than Fresh livers, while tyrosine, methionine and serine metabolism remained reduced, though extracellular serine was then actively consumed at a higher rate (Flux #25). Arginine uptake and ornithine output were significantly reduced compared to Fresh livers, but the increased metabolism of asparagine, threonine and glutamine resulted in an overall increased urea cycle.

MFA of perfused vs. in vivo livers

In order to appreciate the impact of perfusion itself on liver metabolism and evaluate potential limitations of the perfusate, a comparison of perfused and in vivo livers was

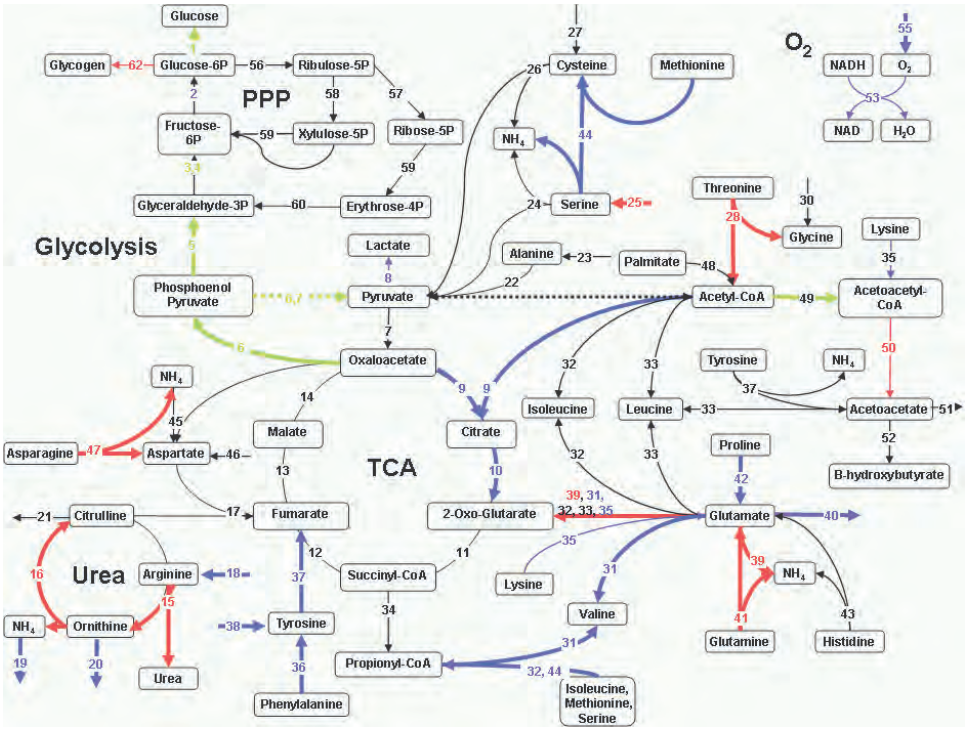


FIGURE 5 | MFA of phase I (t=0-2hrs) for WI livers compared to in vivo livers; in vivo MFA forms the black baseline. Red arrows are significantly increased fluxes. Blue arrows are significantly reduced fluxes and green are reversed. Bold lines = $p<0.05$, Thin lines = $p<0.1$, Dotted lines = glycolysis.

performed using MFA (Figures 6-8) and results from a previous study[106]. Figure 9 summarizes the MFA findings demonstrating that perfused liver activity of the major pathways of metabolism including oxygen uptake, electron transport, lipid oxidation, the TCA cycle and the PPP was reduced compared to in vivo values. Lactate production was increased in perfusion while amino acids and urea cycle were similar to in vivo livers. The latter findings correlate well with the MFA maps that demonstrate either very little amino acid variation from in vivo as in Figure 7, or a synchronous increased uptake in some and decreased uptake in others (Figures 6 and 8). All perfused livers released branched chain amino acids and had negligible histidine uptake compared to in vivo livers. Further, in the absence of any lipids in the perfusate, and with the unusual formation of branched chain amino acids, the model predicted formation of propionyl-CoA from succinyl-CoA (Flux #34). Perfused livers were generally glycolytic compared to fasted gluconeogenic in vivo livers, though extracellular glucose content varied little in concentration during perfusion.

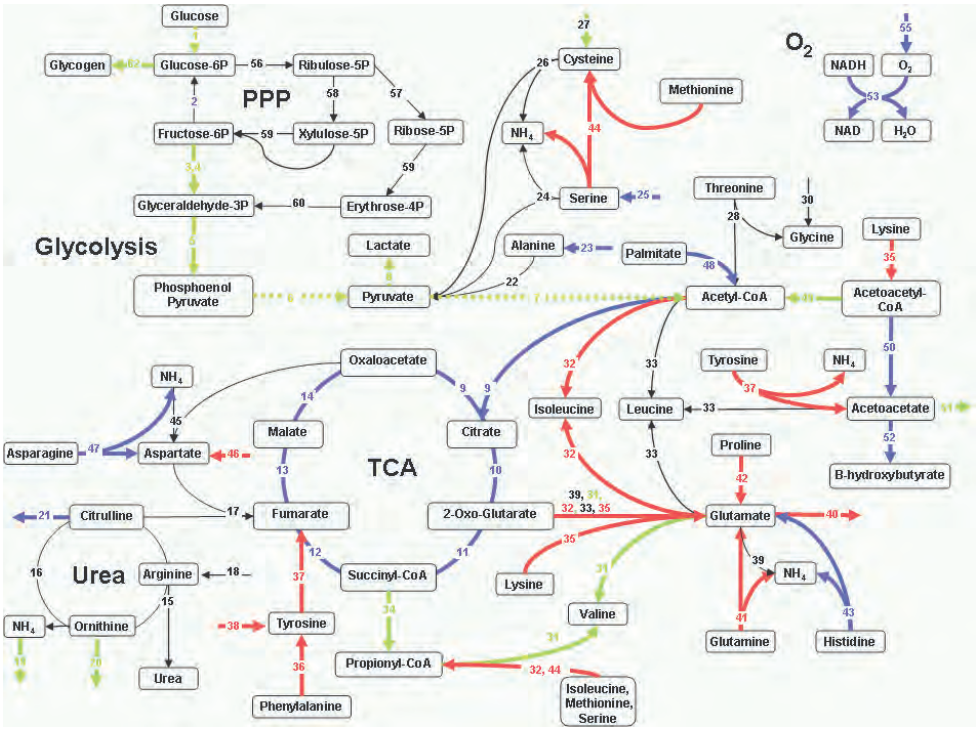


FIGURE 6 | MFA of phase II (t=3-5hrs) for WI livers compared to in vivo livers; in vivo MFA forms the black baseline. Red arrows are significantly increased fluxes. Blue arrows are significantly reduced fluxes and green are reversed. Bold lines = $p<0.05$, Thin lines = $p<0.1$, Dotted lines = glycolysis.

Fresh livers compared to in vivo livers (Figure 6) represent the response of a healthy liver in perfusion. Amino acid metabolism was substantially altered. Extracellular glutamate production and 2-oxo-glutarate levels were significantly increased by the catabolism of proline, aspartate, lysine and glutamine (Fluxes #31, #32, #33, and #35). Phenylalanine and tyrosine metabolism were significantly increased contributing to fumarate production, while increased serine and methionine consumption contributed to the pyruvate pool. Conversely, asparagine, histidine, serine and alanine uptake were significantly reduced. The urea cycle remained within in vivo range.

During the first two hours of WI liver perfusion, glutamate output was significantly increased but in contrast to Fresh livers, there were no major amino acid changes except an increase in phenylalanine and a decrease in methionine, serine and alanine (Figure 7). Between the 2nd and 5th hours of perfusion (Figure 8) amino acid metabolism picked up to include lysine, glutamine, phenylalanine, tyrosine and aspartate catabolism. Glutamate

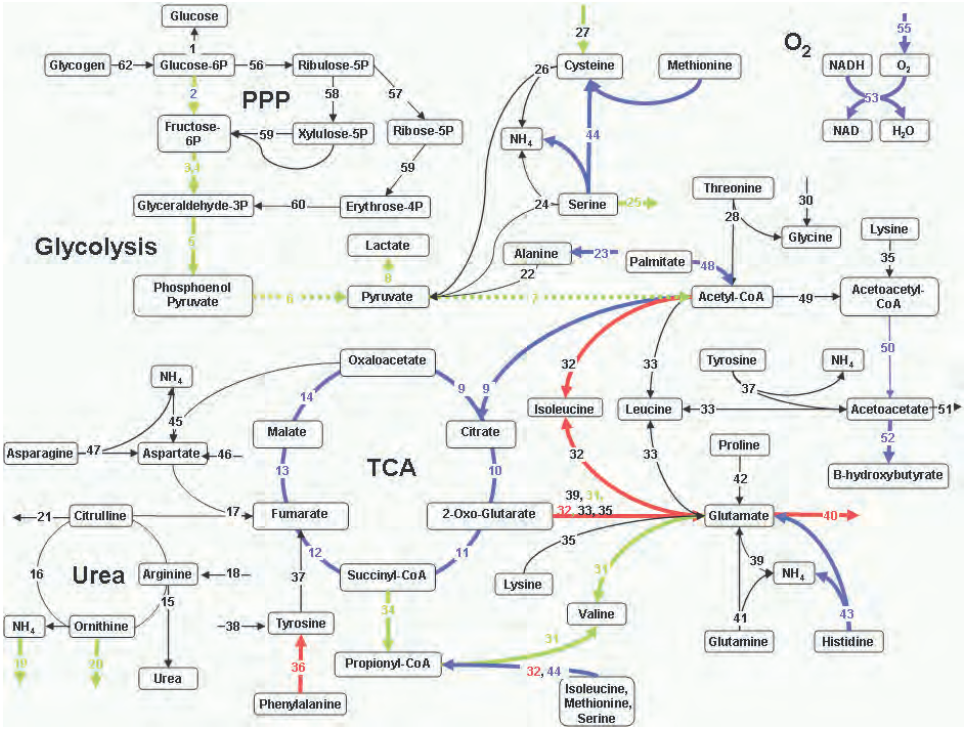


FIGURE 7 | MFA of phase I (t=0-2hrs) for WI livers compared to Fresh livers; Fresh liver MFA forms the black baseline. Red arrows are significantly increased fluxes. Blue arrows are significantly reduced fluxes and green are reversed. Bold lines = $p<0.05$, Thin lines = $p<0.1$, Dotted lines = glycolysis.

was oxidized rather than released into the extracellular pool, increasing the 2-oxo-glutarate flux such that downstream TCA activity was comparable to the first two hours of perfusion and the urea cycle was increased. WI livers also reverted to gluconeogenesis and possible glycogen formation, though at significantly reduced rates compared to fasted in vivo livers. Lactate formation was reduced 67% from the first two hours of perfusion.

The impact of perfusate content can be appreciated from the concentration of metabolites in Williams Medium E, and the portal influxes (Appendix A and Table 1). The content, and subsequent influx, of Williams Medium E exceeded in vivo values for all amino acids except serine and histidine, and their uptake in perfusion was negligible. The converse, exceedingly high influxes (Table 1) as seen in the cases of cysteine, aspartate and glutamine, which were 20x, 10x and 5x higher than the upper bound in vivo influx respectively, resulted in 5-10x higher uptake rates than in vivo. Influxes 1-4x higher than the upper bound in vivo value had variable, less substrate-driven responses by the livers in each of

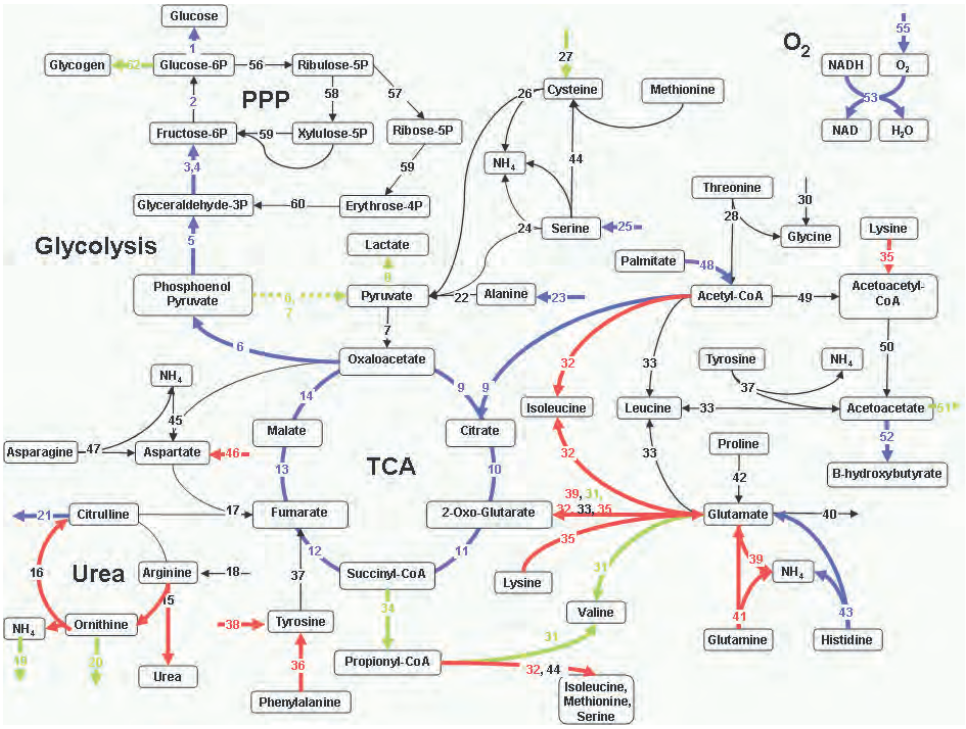


FIGURE 8 | MFA of phase II (t=3-5hrs) for WI livers compared to Fresh livers; Fresh liver MFA forms the black baseline. Red arrows are significantly increased fluxes. Blue arrows are significantly reduced fluxes and green are reversed. Bold lines = $p<0.05$, Thin lines = $p<0.1$, Dotted lines = glycolysis.

the groups. Arginine was taken up, and ornithine reciprocally released, at a high rate in WI livers and finally demonstrated a plateau when perfusate concentration reached 26mM, suggestive of substrate depletion at t=4hrs. Only tyrosine in Fresh livers demonstrated a similar final concentration, though its concentration profile was linear for the entire duration of the perfusion. All other amino acids exhibited linear concentration profiles and had yet to be depleted at t=5hrs. By contrast, methionine metabolism depended on the state of the liver, being upregulated in Fresh liver perfusions but down-regulated in WI perfusions. The metabolism of certain amino acids remained within in vivo ranges across all groups, for example, glycine (Fluxes #28-30) and even aspartate (Fluxes #17, #45, #47) despite the increased aspartate uptake (Flux #46).

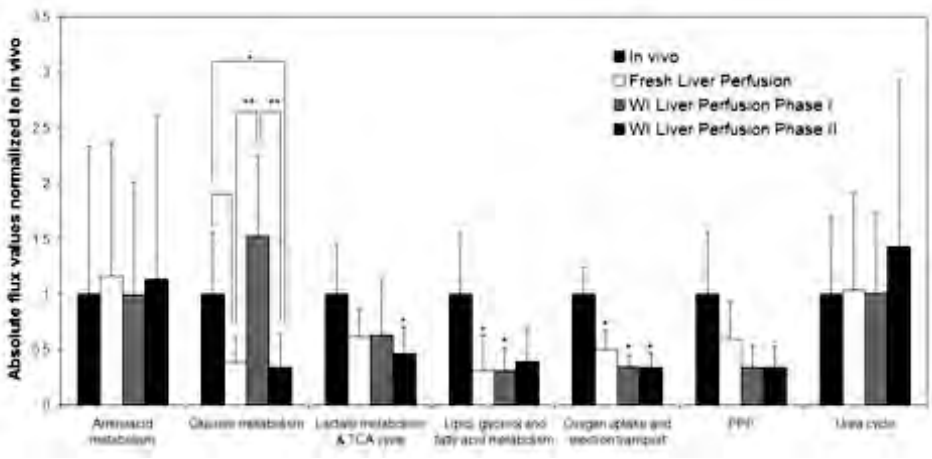


FIGURE 9 | Summary of major pathways in perfused livers normalized to in vivo values.
Bolded items are significantly different ($p<0.05$) from FRESH.
* Items significantly different ($p<0.05$) from IN VIVO.
† Items significantly different from ISCHEMIC T=0-2hrs

Discussion

Perfusion systems are capable of significantly impacting the availability of transplantable organs by optimally supporting donor organs during storage, and recovering reversibly damaged tissues through perfusate-based treatment protocols[122, 123]. To facilitate the translation of perfusion technology to clinical use, comprehensive and dynamic analyses of organ function during perfusion are needed that identify parameters critical to organ stability and recovery. As a first step in this direction, we performed a comprehensive metabolic analysis to capture the time of ischemic liver recovery and evaluate the impact of perfusate on organ stability. We conducted our study on hourly perfusate samples from Fresh and WI livers that were successfully transplanted after 5-6hrs of NMP. Through the measurement of 28 metabolites and the calculation of an additional 34 fluxes using MFA, we were able to evaluate the stability of our perfusion system, identify perfusate shortcomings, and establish significant differences between Fresh and WI livers useful in the future design of treatment protocols.

Fresh livers highlighted baseline factors to be considered in future perfusions. They demonstrated functional stability through linear changes in metabolite concentrations during perfusion; greatest stability/linearity was seen after the first hour of perfusion, likely reflecting a period of adjustment to the ex vivo environment. There was no evidence of substrate limitation in the perfusate at t=6 hours. MFA depicted the expected response

to a fed/high insulin state by showing glucose uptake, glycogen storage and glycolysis. Alanine and lactate uptake, the usual precursors for gluconeogenesis were down-regulated and produced, respectively. The TCA cycle was reduced, as were ketone body production and fatty acid oxidation, altogether requiring a minimum of oxygen, concomitant with a high energy state.

Restoration of ischemic livers during perfusion appears to be correlated to a distinct change in liver metabolism at t=2hrs as seen using linear regression analysis on the temporal concentration profiles of each of the metabolites. The restoration of oxygen uptake rates to Fresh liver values at t=2hrs, followed by a relative plateau in oxygen consumption was the most dramatic change amongst the metabolites measured and coincides with Pastor's description [36]that this marks the full recovery of the organ from ischemic damage. It is interesting to note that In MP applications typically, longer perfusion durations are chosen[47, 98] whereas these results suggest a much shorter time to transplantation may be sufficient. It is further possible that optimizing the delivery of oxygen during perfusion may result in even shorter ischemic organ recovery times[36]., as discussed below.

The value of optimal oxygen delivery, in that it is delivered to all cells at an acceptable rate, is appreciated upon closer inspection of the data. From in vivo data[106] it can be deduced that a healthy rat liver consumes approximately 0.25ml O_2/min/g liver in the fasted state. From equation 1 above, using a pO2 of approximately 500mHg in perfusion, and assuming a 90% oxygenation of the hemoglobin present, the maximum value of hemoglobin the liver needs to ensure sufficient oxygenation at physiological flow rates and portal pressures is approximately 8g/dL or 23%. Riedel et. al.[124] verified experimentally that the optimal hematocrit in perfusion was approximately 20%, above which oxygen uptake rates did not increase with flow rates while portal pressures became destructively high. Similarly, we found that in order to sustain a physiological perfusion pressure in our system, adjustment of the hematocrit through dialysis was optimal at values between 15-20%. Nevertheless, despite meeting the oxygen requirements of the liver, perfused livers did not take up all the oxygen available to them, suggesting an overall damped rate of metabolic activity. Several publications concur with our findings of lower-than-physiological oxygen uptake rates[43, 48, 125] to the extent that Mischinger et. al.[115] favor removing erythrocytes altogether, resulting in a significantly simpler perfusion setup. Before this can be done however, it is necessary to determine why the livers are not consuming all the available oxygen.

It is possible that livers in perfusion are functionally incapable of maximizing oxygen extraction. There may be several mechanisms potentially involved in this response: The first, alluded to in the discussion on Fresh livers above, may simply be that livers

in perfusion do not need to be as metabolically active in their non-demanding ex vivo environment. Alternatively, the delivery of perfusate oxygen at the lowest range of typical in vivo values for prolonged periods of time (hours) (Figure 1) may be resulting in a systemic hypoxia-induced mitochondrial inhibition[126]. Third the metabolic consequences of ischemia reperfusion injury may be causing localized areas of hypoperfusion in the microvasculature[127, 128].

Several factors have been implicated in the metabolic response to hypoxia, which serve to minimize organ dependence on oxygen by reducing anabolic pathways and increasing catabolic pathways, using anaerobic respiration as the end-point:

Hypoxia-inducible factor (HIF)[126], as the name suggests, is rapidly incurred during periods of low or insufficient oxygen. HIF strongly induces glycolysis, also referred to as the Pasteur effect[129], as seen dominantly in Figure 4, Fluxes #2-6, but also in Figures 6, and 7. HIF is also responsible for the preferential formation of lactate from pyruvate (Flux #8) by upregulation of lactate dehydrogenase. This serves to enable the production of ATP, while decreasing the contribution of acetyl-CoA to the TCA cycle (Flux #9), effectively inhibiting it[130] (Fluxes 9-14), thereby reducing cellular dependence on oxygen. Also, HIF induces pyruvate dehydrogenase (PDH) kinase-1 which phosphorylates and inactivates PDH, the mitochondrial enzyme that converts pyruvate into acetyl-CoA. Our findings do not suggest that the rate of formation of acetyl-CoA from pyruvate is impeded by warm ischemia, however in all perfused livers, the TCA cycle is reduced compared to in vivo. Therefore, the results of MFA generally agree with these expected effects of HIF, suggesting the persistence of hypoxia in both ischemic and fresh livers.

AMP-activated protein kinase (AMPK) is also stimulated during hypoxia and ischemia, and functions to suppress the overall metabolic rate and hence energy requirements of cells for self-preservation[131]. AMPK is associated with increasing glycolysis and minimizing gluconeogenesis[132] thereby largely coinciding with the effects of HIF and the MFA findings. It is also associated with increasing lipid oxidation and decreasing fat storage, notably opposing many insulin pathways[133, 134], which is not observed in the MFA. The preferential reduction in palmitate oxidation (Flux #48) seen in Figures 4-8 illustrates the likely domination of a high insulin state over AMPK, controlling lipid metabolism. It is unclear at this point whether promoting the reduction of lipid oxidation through higher insulin values is an effective means of reducing the damaging consequences of lipid peroxidation at the expense of reduced ATP production; subsequently further optimization of perfusate insulin and fatty acid levels is necessary.

Nitric oxide (NO) has been identified as another important determinant of metabolism during hypoxia, facilitating the distribution of available oxygen both at the mitochondrial and vascular scale[135, 136]. In the mitochondrion, there is an increased NO accumulation at low O_2 levels, when cytochrome c oxidase is primarily reduced. NO actively competes with the enzyme and suppresses the respiratory rate, which coincides with the effects of HIF[106, 135-139]. Increased intracellular NO has also been shown to reduce protein synthesis, which requires energy expenditure, during hypoxia[131], in particular inhibiting the formation of albumin, the effects of which may last for several hours after NO exposure[140]. From our findings, albumin synthesis in WI livers remained flat for the duration of perfusion (Figure 3), while Fresh livers began to recover by t=2 hours, logically suggesting they had less NO exposure. At the vascular level, the accumulation of NO intracellularly results in reduced NO secretion, and subsequent vasoconstriction that serve to extend the ischemic time despite initiation of reperfusion[141]. Alteration of NO production is also supported by the comparatively increased extracellular arginine uptake seen in WI livers, with a reciprocal increase in ornithine production. Arginine is a precursor for NO, but the balance between arginases and nitric oxide synthases are dramatically altered during hypoxia [142-144] possibly favoring an ineffective shuttling of arginine to ornithine as opposed to the desirable production of NO.

Considering issues with liver oxygenation during perfusion, one possible consideration is to reduce the liver's dependence on oxygen even further by reducing the perfusate temperature, which has also been associated with less post-ischemic vasoconstriction[145]. We[146] and others[105] have shown that room temperature perfusion significantly reduces whole organ metabolism but still results in viable, transplantable organs. This could also ensure that use of erythrocytes as oxygen carriers is not necessary. The combination of erythrocyte removal and room temperature perfusion would simplify the machine perfusion approach substantially, addressing a major factor in the reluctance to utilize machine perfusion systems clinically. Further measures to ensure patent microvasculature[114] and desirable flow rates would be the addition of thrombolytics, edema-reducing colloids or impermeants, and vasodilators, including arginine[147-149].

The likely combined effects of the factors HIF, AMPK and NO, in addition to the reduced metabolic demand on the ex vivo liver, effectively minimize the perfusate requirements to keeping the liver alive and assessing its function. Amino acids may offer the greatest room for perfusate optimization. Their metabolism in perfused livers served to reflect the livers' abilities to restore perfusate content to physiological levels; utilize particular amino acids for specific applications, such as the vasodilatation function of arginine; and illustrate previously observed perfusion-specific artifacts. For example, Fisher and

Kerly,[150] who performed analyses of amino acid metabolism in healthy fasted perfused rat livers, observed a steady increase in perfusate content of branched chain amino acids (BCAAs) valine, isoleucine and leucine, as seen in both the Fresh and WI livers in this work. Further, these authors found both histidine and glutamate to be unchanged in perfusion; both trends held true in WI livers. Fresh livers demonstrated a net production of glutamate, which may be related to a normal transamination of excessive perfusate amino acids in conjunction with an otherwise reduced TCA cycle. Cumulatively, these findings strongly support a high degree of substrate specificity by the liver in perfusion and a regulated response to supraphysiological perfusate content. A reduction in the concentration of some amino acids, including the BCAAs, histidine and glutamate, may serve to reduce the metabolic activity of the liver by way of their removal as urea, and simplify the requirements of the organ during its recovery phase.

Conclusions

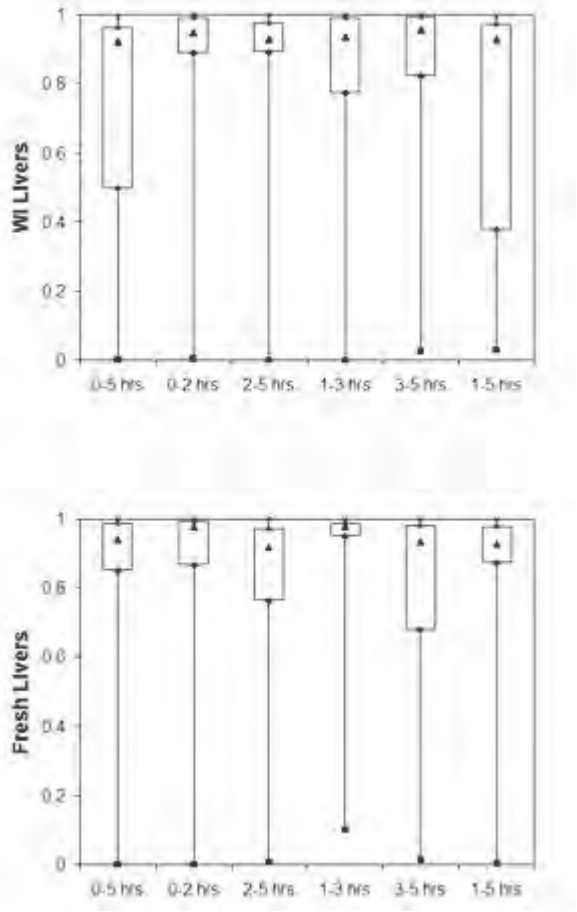
In summary, this comprehensive metabolic analysis of the performance of Fresh and Ischemic livers in normothermic MP demonstrates sustained organ stability and the restoration of WI livers to a likely transplantable state within 2 hours of perfusion. It was observed that lipid oxidation was suppressed, likely due to the high insulin levels, while amino acids were extensively metabolized. It was also observed that perfused livers did not consume all the available oxygen and were hypoxic independent of ischemic injury; the metabolic analysis suggests enhancing microcirculation via vasodilators, anti-thrombotics and other solutions might be a profitable approach.

The data presented in this study provides the basis for rational NMP optimization, indicating a primary focus should be on optimizing the delivery of oxygen to hepatocytes in both groups as early in perfusion as possible. Further, the data and analyses presented here may enable future meta-analyses such as data mining for an index of organ viability and Flux Balance Analysis[151, 152], or indicators of organ viability[100 110] necessary steps to build automated feedback control systems for real-time organ support.

APPENDIX A | In vivo and Perfusate (WE) Reference Concentrations

Metabolite	Portal Vein[1]	Williams Medium E[2]
Acetoacetic acid (μM)	110±73	0
Alanine (μM)	397±13	1010
Albumin (g/dL)	1.9±0.21	0
Ammonia (μM)	109±33	0
Arginine (μM)	141±93	287
Asparagine (μM)	49±5.4	151
Aspartate (μM)	21±7.6	225
b-Hydroxybutyric acid (μM)	110±96	0
Cysteine (μM)	15±1.5	330
Glucose (g/dL)	123±62	0
Glutamate (μM)	68±7.7	302
Glutamine (μM)	293±46	2000
Glycine (μM)	273±27	666
Histidine (μM)	137±26	97
Isoleucine (μM)	86±16	381
Lactate (mM)	1.0±0.18	0
Leucine (μM)	261±33	572
Lysine (μM)	220±38	598
Methionine (μM)	44±7.0	101
Ornithine (μM)	120±3.5	0
Phenylalanine (μM)	59±3.9	151
Proline (μM)	163±12	261
Serine (μM)	199±13	95
Threonine (μM)	204±41	336
Tyrosine (μM)	68±7.4	278
Urea nitrogen (mM)	4.6±1.8	0
Valine (μM)	165±31	427

[1062] Sigma-Aldrich cat. #W1878



APPENDIX B | Box-and-whisker plots of linear regressions performed on the temporal concentration profiles of 28 metabolites measured for WI and Fresh livers.
Legend: ■ Minimum. ♦ First quartile. ▲ Median. * Third quartile. ´ Maximum.

Chapter 8

A metabolic index of ischemic injury for perfusion-recovery of cadaveric rat livers

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Abstract

With over 110,000 patients waiting for an organ transplantation, the current crisis in organ transplantation is based on a lack of donors after brain-death (DBD). A very large alternative pool of donor organs that remain untapped are the donors after cardiac death (DCD), recovered after cardiac activity has ceased and therefore sustained some ischemic injury. Machine perfusion has been proposed as a novel modality of organ preservation and treatment to render such cadaveric organs, and in particular livers, transplantable. Two key issues that remain unaddressed are how to assess whether a DCD liver is damaged beyond repair, and whether machine perfusion has rendered an injured organ sufficiently viable for transplantation. In this work, we present a metabolic analysis of the transient response of cadaveric rat livers during normothermic machine perfusion (NMP), and develop an index of ischemia that enables evaluation of the organ ischemic injury level. Further, we perform a discriminant analysis to construct a classification algorithm with >0.98 specificity to identify whether a given perfused liver is ischemic or fresh, in effect a precursor for an index of transplantability and a basis for the use of statistical process control measures for automated feedback control of treatment of ischemic injury in DCD livers. The analyses yield an index based on squared prediction error (SPE) as log(SPE) > 1.35 indicating ischemia. The differences between metabolic functions of fresh and ischemic livers during perfusion are outlined and the metabolites that varied significantly for ischemic livers are identified as ornithine, arginine, albumin and tyrosine.

Introduction

About 110,000 patients are currently on the organ transplant waiting list in the US, with the number increasing by 5% every year (United Network for Organ Sharing, www.unos.org , as of July 2011). The major untapped pool of donor organs that could be used to alleviate this crisis in organ transplantation are the organs obtained from Donors after Cardiac Death (DCD)[76]. For the liver, which this work focuses on, an estimated 4,000 waitlisted patients perish every year due to a lack of transplantable organs [146], while the estimated pool of DCD livers with ischemic time <60mins is on the order of 6,000 grafts per year [77].

In the absence of cardiac output, ischemic damage increases in severity as a function of time. Beyond a certain cutoff (about 15 mins for the heart and 30 mins for the liver) graft survival in the recipient falls dramatically [4]. Preclinical studies with extracorporeal machine perfusion systems in porcine and murine models of DCD livers ,including from our group [98, 146], indicate that up to 60 minutes of warm ischemic damage can be successfully reversed, whereas static cold storage in preservation medium, the current clinical gold standard, simply exacerbates the damage and recipient animals do not survive. Research in machine perfusion systems is subsequently a very active field in donor organ recovery and preservation.

In humans however, cardiac death frequently occurs in uncontrolled environments (uDCD). Without objective metrics of ischemic duration and organ viability, uDCD organs cannot be safely transplanted. Another benefit of machine perfusion is that data can easily be procured and analyzed, providing those necessary metrics that describe organ status. Normothermic (37°C) Machine Perfusion (NMP) in particular allows the organ to be metabolically active producing measurable changes in perfusate metabolite composition that are comparable to its in vivo counterpart.

Since hepatic metabolism is a highly integrated network which features many metabolites that are auto- and cross-correlated in time, univariate techniques (such as ANOVA) which ignore the correlation structure between the metabolites and assume that these variables are independent of each other are inadequate to handle the problem complexity [153]. A suitable framework for developing an index of ischemia, and more broadly quantitative methods of organ quality control, is multivariate statistical process monitoring (SPM) methodologies[153]. Multivariate SPM techniques can look at the whole picture to identify commonalities between different perfusions, correlations among variables as a function of ischemia, and trends in time.

The aim of this work is the development of an index of ischemia to evaluate DCD liver injury based on the organ's dynamic metabolic activity during machine perfusion. To create such an index, we first construct a multi-way principal component analysis (MPCA) liver perfusion model that captures the correlation structure between the metabolites during perfusion of fresh livers that were later successfully transplanted with >1 month survival, and defines the multivariate confidence limits for the metabolite concentrations in the perfusion medium. Quantitative evaluation of the process of recovery from ischemic injury by NMP is then done on an online basis, enabling the perfusionist to apply the necessary interventions that will optimize recovery. This is done by projecting the metabolite concentrations for the damaged livers onto the fresh liver MPCA model, revealing the differences in metabolic functioning of DCD livers compared to fresh livers. We then quantify the similarity of ischemic livers to fresh livers by employing the Squared Prediction Error (SPE) statistic, hence constructing an index of ischemia.

Further, we introduce an online multi-way partial least squares discriminant analysis (MPLSDA) model to predict the end-of-perfusion quality (i.e. ischemic or not) of a perfused liver during perfusion. This analysis complements the ischemia index by creating a means of classifying any given liver definitively as ischemic or fresh, and it is proposed as the basis for a future decision criterion of DCD liver transplantability.

Materials and methods

For a details on the isolation of the donor liver, machine perfusion protocols, isolated reperfusion, transplantation and processing of perfusate and tissue samples, please refer to chapters two and three and references [83, 98] respectively.

Statistical Preprocessing

Data consisted of 25 metabolites measured hourly for each perfusion (Table 1). 11 Fresh livers and 7 WI livers were perfused in total. During the initial data preprocessing obvious outliers (e.g. negative concentrations) were eliminated and replaced by the mean trajectory estimates 15. The data sizes that are used for the statistical analysis is 11 x 25 x 6 for fresh livers and 7 x 25 x 6 for damaged livers. All variables (i.e. the metabolite concentrations) were mean-centered and unit-variance scaled for further statistical analyses.

Multi-way Principal Component Analysis (MPCA)

While the differences in metabolic activity between fresh and ischemic livers can be compared on a per-metabolite (i.e. univariate) basis, since the liver is very capable of

TABLE 1 | List of metabolites used in data analysis

1	ACAC	10	Glutamine	19	Phenylalanine
2	Alanine	11	Glycine	20	Proline
3	Albumin	12	Histidine	21	Serine
4	Ammonia	13	Isoleucine	22	Threonine
5	Arginine	14	Lactate	23	Tyrosine
6	Asparagine	15	Leucine	24	Urea
7	Aspartate	16	Lysine	25	Valine
8	Glucose	17	Methionine		
9	Glutamate	18	Ornithine		

converting these metabolites into each other such an analysis would have problems with identifying that a large number of small differences could also mean a significant alteration in an entire metabolic pathway; for instance a significant alteration in urea cycle can easily come through a combination of changes in citrulline, ornithine, urea, and arginine concentrations in perfusion media with individual p-values that are not significant, but the overall fluxes over the entire pathway significantly altered; hence a univariate analysis such as ANOVA or t-test would lead to a Type II error, i.e. a missed alarm or false negative. Alternatively a difference in a single metabolite could be falsely interpreted to mean significant alteration in an entire pathway, which is a Type I error (false alarm or false positive). In other words, evaluation of a highly interconnected network such as the hepatic metabolism based on a univariate analysis that does not account for the cross-correlations between variables is expected to lead to a significant amount of Type I and Type II errors.

Multivariate methodologies such as principal component analysis (PCA) have been proposed for the analysis of such datasets with many correlated variables. PCA captures the correlation structure between the variables and forms a model plane with fewer dimensions which explain the largest variations in the data, in effect distilling the many correlated variables (such as metabolites in a pathway) into a few variables that are uncorrelated with each other.

Briefly, PCA captures the correlation structure between the variables in **X** [(I x J), j = 1,..., J variables; i = 1,...,I independent samples] and forms a model plane with fewer dimensions (R principal component (PC) directions) using only the R largest variance directions. R is

chosen such that adding additional components to the model does not provide additional significant information. Instead of working with highly correlated collinear variables (**X**), PCA yields fewer and uncorrelated projections (scores (**T**)).

$$\mathbf{X} = \mathbf{T}\mathbf{P}^{\mathrm{T}} + \mathbf{E} = \hat{\mathbf{X}} + \mathbf{E} \qquad (1)$$

PCA is performed by singular value decomposition in the covariance of **X** and the loadings **P** (J x R) are derived. The R eigenvectors are the R highest variance directions. Scores, **T** (I x R), are the new uncorrelated variable projections onto the newly derived PCA plane. **E** is the residual matrix. Score biplots can then be used to reveal how similar *I* independent samples are to each other, reveal possible clustering among the samples and also used in determination of outlier samples. Loading plots show the correlation among *J* variables and identifies the variables that have high influence on each model score vector **t**.

For Statistical Process Monitoring (SPM) of dynamic datasets, PCA has been extended to multiway-PCA (MPCA). Multiway-PCA is used for the analysis of three dimensional data arrays $\underline{\mathbf{X}}$ (I x J x K) where I independent processes are referred to as batches (i = 1,..., I). A multi-way PCA model is equivalent to an ordinary PCA model performed on a 2D matrix constructed by unfolding the three-way data array [hz]. An (I x J x K) data array can be unfolded by preserving the batch direction I and augmenting the J variable measurements taken at each time point *k* (k = 1,..., K) side by side resulting in an I x JK matrix, or by preserving the variable direction *J* and augmenting the variable trajectories for different batches resulting in a IK x J matrix (Figure 1). The former unfolding direction is the best to use in the SPM of batch processes since it considers the batch-to-batch variations and determines the ranges of variable trajectories during the batch. The latter unfolding technique provides information on the extent of the progress of a batch and can be used to determine the completion time of a batch[154].

For the statistical modeling of fresh liver perfusions with MPCA, an MPCA model was built using the remaining fresh liver samples and the confidence intervals around the model plane were determined. Later, the liver samples that were exposed to 1hr warm ischemia (WI) were projected onto the fresh liver model plane, and new scores and residuals were calculated using:

$$\hat{\mathbf{t}} = \mathbf{x}_{new}\mathbf{P}; \quad \mathbf{e}_{new} = \mathbf{x}_{new} - \hat{\mathbf{t}}\mathbf{P} \qquad (2)$$

P loadings (size (JK x R) for batch processes) are used to calculate the new scores **t** (1 x R) using the new batch trajectory $\mathbf{x}_{new}$ (1 x JK).

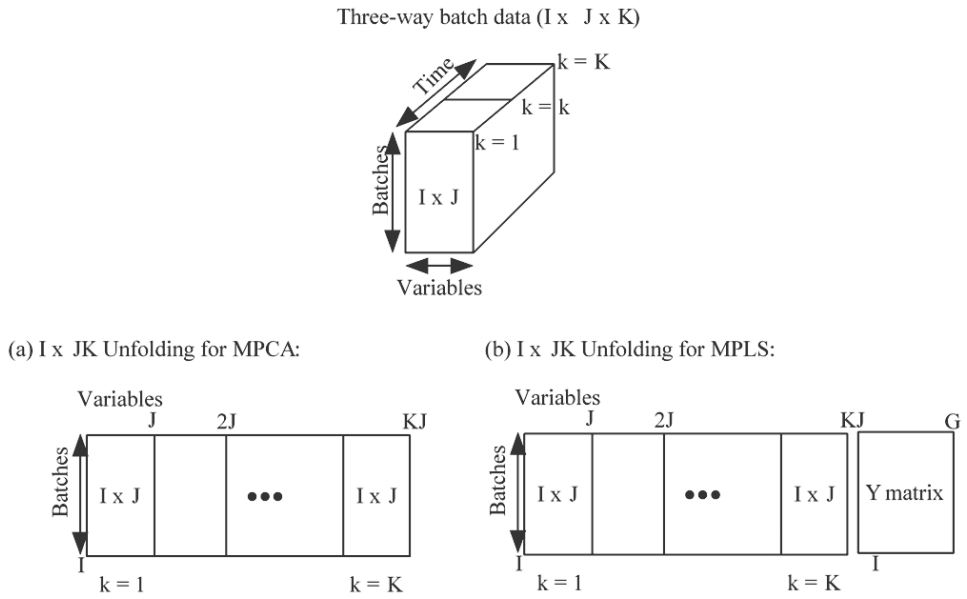

FIGURE 1 | Unfolding of the (I x J x K) dynamic batch process data for MPCA and MPLS analyses.

Squared Prediction Error (SPE)

There are several measures to quantify similarity between two batch operations with multiple measurements at each time point, and evaluate if they are within normal operational bounds (also termed confidence limits). Multivariate statistics such as the Squared Prediction Error (also called Q-statistic) are used to determine the state of the batch progression and detect possible deviations in the new batches ($\mathbf{x}_{new}$, (1 x JK)) from the reference batches[153-157].

SPE, in particular, captures the large deviations from the normal operating (NO) process that are not explained by the model and is calculated for the new batch using the residuals $\mathbf{e}_{new}$[158],

$$SPE = \mathbf{e}_i \mathbf{e}_i^T = \sum_{c=1}^{KJ} E_{i,c}^2 \qquad (3)$$

SPE statistic considers the residual space and determines the variation of a sample that is not explained by the model, hence, a large SPE statistic indicates that the observation under consideration contains a different correlation structure and is not explained by the model. For instance, if ischemic livers contain a correlation structure that is not captured by the fresh liver model, it will be indicated by a large SPE value. As such, SPE effectively distills a single quantitative variable from a large number of highly correlated metabolite profiles, and comparison of a perfused liver to a set of ideal-condition livers can be accomplished simply by comparing the SPE values to each other.

An offline analysis of the metabolite trajectories (i.e. analysis at the end of a batch when data for all time points is ready) such as the SPE statistic determines the events that have taken place during the process. An online analysis (i.e. during perfusion) is more desirable since it provides information on the events taking place as the perfusion progresses and an estimate of the liver viability based on the current conditions. Accordingly, online SPM enables regulation of the process (e.g. adjusting the operational parameters such as oxygenation, flow rate etc.) to improve the final organ viability. For online analysis each independent score is calculated at time k using,

$$\mathbf{t}_k = \mathbf{x}_{new,jk}\mathbf{P}_{jk}\left(\mathbf{P}^T\mathbf{P}\right)^{-1} \quad (4)$$

where $\mathrm{P_{jk}}, (\mathrm{kJ} \times \mathrm{R})$ are the model loadings until time interval k. Residuals are then calculated using $\mathrm{e_{new}} = \mathrm{x_{new}} - \mathrm{t_k P_k^T}$.

For such an online analysis, SPE statistic can be calculated at each time point k (k = 1K) for the new batch as:

$$SPE_k = \sum_{c=(k-1)J+1}^{kJ} \mathbf{e}_{new,c}^2 \sim \chi^2_{2m^2/\upsilon,\alpha} \quad (5)$$

$\chi^2_{2\mathrm{m}^2/\upsilon,\alpha}$ is the critical value of the chi-squared variable with $2m^2/\upsilon$ degrees of freedom at significance level α m and υ are the sample mean (m) and variance (υ) of the SPE sample at each time k[157].

Contribution plots can be used as a diagnostic tool to identify the variables that are contributing to the deviation. The variable contributions to SPE statistic for batch *i* are calculated using [159],

$$\sum_{k}^{K}\sum_{j}^{J}\mathbf{e}_{jk}^2 \quad (6)$$

In order to quantify the total variation of each variable in the residual space, contributions to the normalized error vector are calculated as [160],

$$\text{Normalized error } \mathbf{R}_{jk} = \frac{\mathbf{e}_{jk}}{\hat{\mathbf{s}}_{jk}} \quad (7)$$

where $\mathbf{e}_{jk}$ is the j[th] element of the residual vector $\mathbf{e}_{new}$ at time k and $\hat{\mathbf{s}}_{jk}$ is the standard deviation of the j[th] variable for the training set of fresh livers.

Multiway Partial Least Square Discriminant Analysis (MPLSDA)

While SPE_k statistic can evaluate whether a liver is within normal operation range (i.e. how ischemic it is), for livers that are mildly ischemic, it is possible to build on the basis of MPCA to perform a complementary discriminant analysis to classify a given liver either as fresh or ischemic definitively.

To be able to perform online classification of livers as ischemic or fresh, an algorithm that can discriminate different metabolic states from the dynamic metabolite data collected during perfusions is required. Partial least squares or projections to latent structures (PLS) is a regression extension of PCA and is used to connect the information in two blocks of variables, namely the predictor block **X** and response block **Y**. PLS gives a way of predicting the **y**-values from the **x**-values, a generalization of multiple regression, by correlating **X** to **Y** and finding the orthogonal directions that maximize the correlation between **X** and **Y**. An algorithm for Nonlinear Iterative Partial Least Squares (NIPALS) is provided in [161].

$$\begin{aligned} \mathbf{X} &= \mathbf{T}\mathbf{P}^{\mathrm{T}} + \mathbf{E} \\ \mathbf{Y} &= \mathbf{U}\mathbf{Q}^{\mathrm{T}} + \mathbf{F} \\ \mathbf{U} &= \mathbf{T}\mathbf{B} + \mathbf{H} \quad (\mathbf{B}\ \text{diagonal}) \end{aligned} \tag{8}$$

Interrelation gives the predictive formulation for **y**.

$$\mathbf{Y} = \mathbf{T}\mathbf{B}\mathbf{Q}^{\mathrm{T}} + \mathbf{F}^{*} \tag{9}$$

Similar to MPCA, PLS is extended to multi-way PLS (MPLS) for batch processes. MPLS is equivalent to performing PLS where **X** predictor set is the unfolded batch data and **Y** is a 2D matrix of end-of-batch quality variables. The predictor set (**X**) consists of the same 3-D dynamic data collected at each sampling time and analyzed for the 25 metabolite concentrations collected, and unfolded into a I x JK matrix as shown in Figure 1(b). For new data coming from a new perfusion, $\mathbf{x}_{new}$, MPLS computes the response variables $\mathbf{y}_{new}$ using the model regression coefficients.

For prediction of the state of a liver as belonging to one of the a priori known classes, partial least squares methodology is named partial least squares discriminant analysis (PLSDA). PLSDA is used to classify observations or independent experiments in the predictor set **X** as belonging to one of several a-priori known classes in **Y** based on their relationship. The response matrix **Y** represents the class membership of each row in **X** by a set of dummy variables. The dummy **Y** matrix consists of *G* columns for *G* classes with 1s and 0s such that

the g^{th} column is 1 and the other columns are 0 for observations of class g (Figure 1(b)). For the analysis of the dynamic metabolite data a multiway PLSDA (MPLSDA) model is fit between **X** matrix and the dummy **Y** matrix (I x G, G = 2 for WI or fresh) and discriminant plane that separates perfusions i according to their membership in a certain class is found. When the unfolded new data $\mathbf{x}_{new}$ is projected onto the MPLSDA model, vector is calculated. The predicted class is the numerical maximum of the normalized vector.

For the analysis of fresh and ischemic livers, the (I x JK) unfolded predictor matrix (**X**) consists of the metabolite trajectories for the selected ten batches; the response matrix (**Y**) consists of two columns representing the class membership of each of the ten batches. For a fresh liver perfusion batch (class 1), the corresponding row of **Y** is [1, 0], whereas, for a WI liver batch the row is set to [0, 1].

Multi-sampling cross validation

The predictive power of MPLSDA was evaluated by case-resampling cross validation technique [162, 163]. In this approach, the data set is sampled randomly multiple times to create training and validation data sets. For each sampling, five samples from fresh livers group and five samples from the WI livers are selected randomly and the remaining samples, 5 fresh and 2 WI livers, are used for testing the model. The maximum selection threshold for each batch is set to 2; the maximum selection threshold is the maximum number of times same perfusion can be used in the same model building. The selection threshold is added to avoid the same samples being selected most of the time.

Results

MPCA and Outlier Analysis

MPCA was used to create a model of the variability and the correlation structure of the fresh livers metabolic function during perfusion. The data that is used for the statistical analysis with MPCA is 11 x 25 x 6 for fresh livers and 7 x 25 x 6 for damaged livers. Several (12 chosen out of 25) metabolite profiles during the perfusions of fresh livers are shown in Figure 2. As it can be observed, the mean variable trajectories change linearly with time indicating that the system was stable during perfusion. An MPCA model with R = 3 components was built after the data is unfolded into a (I x JK) matrix to model the batch-to-batch variation (Figure 1(a)). First three principal components account for more than 65% of the variability in the data.

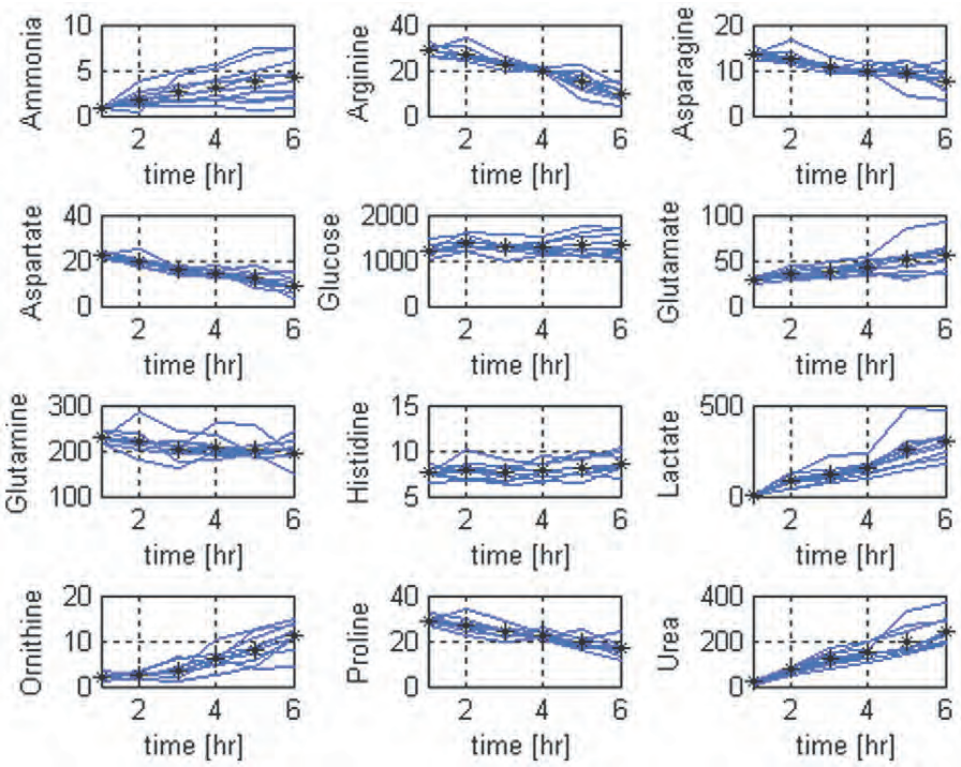

FIGURE 2 | Selected metabolite concentration profiles of fresh livers during 6 hours of normothermic extracorporeal liver perfusion. Blue solid lines show the profiles for the different perfusions of fresh livers and the black stars show the mean values.

When an MPCA model was built using all the liver samples (n = 18, 11 F and 7 WI) on a 18 x 25 x 6 data set, a score biplot ($\mathbf{t}_1$) vs ($\mathbf{t}_2$) reveals the two clusters; one for fresh livers (F) and one for WI livers (Figure 3). Formation of two different clusters is an indication of different metabolic functioning between the two groups. Within the groups each liver has similar metabolic functioning, however, liver samples #7 (F) and #15 (WI) fall away from the two clusters outside the confidence levels and therefore were accepted as outlier samples and are not used further in modeling.

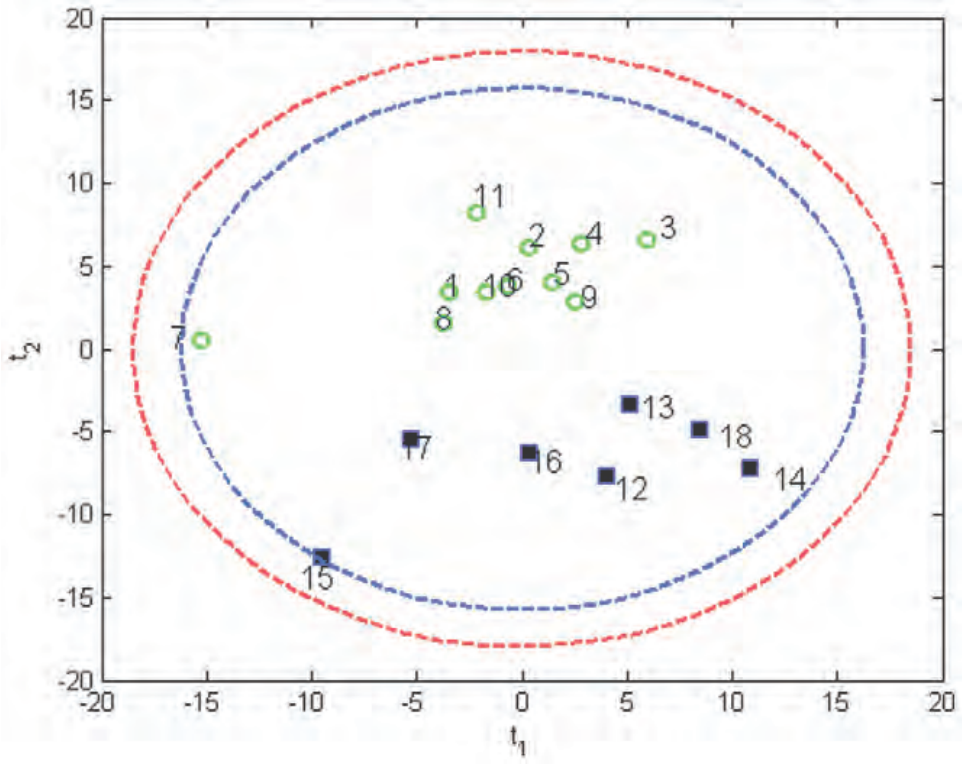

FIGURE 3 | Score bi-plot of all liver samples measured, with 95% and 99% confidence limits. Fresh liver sample 7 and warm ischemic liver sample 15 fall away from the two clusters representing fresh livers and warm ischemic livers. (1-11 fresh samples denoted by green circles, 12-18 warm ischemic samples denoted by squares).

Index of Ischemia

The MPCA model allows the calculation of SPE statistics for each perfusion (using the residuals in Eq. 3) for fresh and WI perfused livers. Figure 4 depicts the projection of (offline) SPE values for all livers, along with the confidence limits for fresh livers, demonstrating that SPE is clearly able to distinguish between fresh and warm ischemic perfused livers.

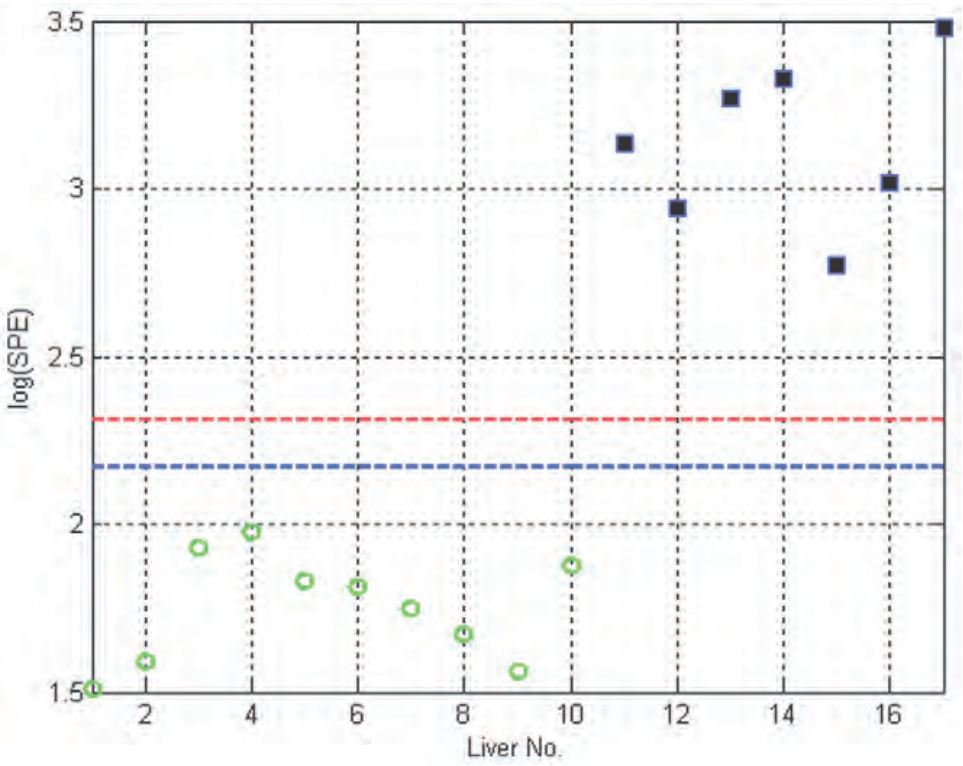

FIGURE 4 | Projection of warm ischemic (WI) liver samples onto the MPCA fresh liver model. Fresh livers are denoted by green circles and are inside the confidence limits (blue:95%, red: 99%), whereas, the WI livers, denoted by squares are outside the confidence limits.

Perhaps more interesting is the online SPE_k statistics which are calculated at each time point using Eq. 5. Figure 5 depicts the time-evolution of the SPE_k during perfusion for each liver; again note that the SPE_k statistics are online, meaning that they are evaluated at time point *k* using only the data available for that liver until that point. As shown in Figure 5, fresh livers display high variations in SPE_k values within the first 2 hours, and stabilize afterwards. In somewhat of a contrast the changes in SPE_k for the WI livers as a function of time are less dramatic. However, interestingly the liver-to-liver variation between SPE_k values decreases noticeably in time for ischemic grafts (std (SPE_k) = [0.2391, 0.3951, 0.2268, 0.2484, 0.1044, 0.1463] for t = 1,..., 6hrs respectively), and it appears that by the end of perfusion all ischemic livers are in a very similar metabolic state although their initial states are quite different. Still, despite this "standardization", the SPE_k values for the WI livers always remain outside the 99% confidence limits of fresh livers from the beginning of the perfusion till the end, clearly demonstrating that the perfusion-resuscitation of these organs' metabolism does not render them equivalent to that of fresh livers.

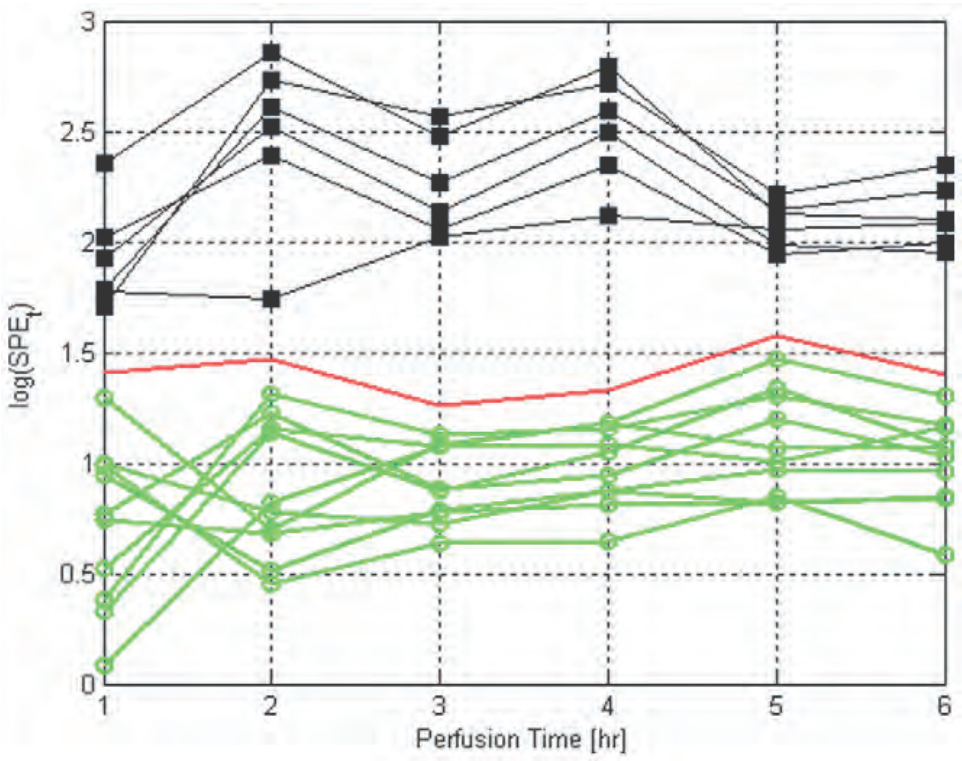

FIGURE 5 | Online SPE statistic for fresh and warm ischemic livers, denoted by green circles and black squares, respectively. The solid line shows the 99% confidence limits.

As Figure 5 demonstrates, the SPE_k statistic quantifies and captures the similarities and differences between ischemic and fresh perfused livers as a group, as well as liver-to-liver variances. It is a single number that can be calculated during perfusion from the metabolite concentrations (see appendix for the full equation used for calculations in Figure 5), and compared to existing data to evaluate how fresh or ischemic an organ is. Moreover it is a continuous number; hence, it has the resolution and ability to differentiate between different degrees of ischemic injury. In fact, as displayed in Figure 5, it is capable of catching small metabolic differences, likely small variations due to rat-to-rat differences in metabolism, last time of eating prior to sacrifice, and small differences in the exact ischemia duration. As such, we propose its use as an index of ischemic injury for rat livers. Moreover, as can be observed in Figure 5 for fresh livers, the average confidence limits $\log(SPE_k)$ is ~1.35. Accordingly, we suggest that $\log(SPE_k)$ as a heuristic limit for ischemia, with values >1.35 indicating ischemia.

TABLE 2 | Variable contributions to SPE_k statistic

WI Liver No.	k = 1 hr	k = 2 hr	k = 3 hr	k = 4 hr	k = 5 hr	k = 6 hr
1	Lactate	Ornithine	Arginine, Lactate, Ornithine	Arginine, Glutamate	Albumin, Ornithine	Glutamate, Ornithine, Tyrosine
2	Glutamine	Ornithine	Arginine, Lactate	Arginine, Glutamate	Albumin, Ornithine	Glutamate
3	Lactate	Ornithine	Arginine, Ornithine	Arginine, Glutamate, Ornithine	Albumin, Ornithine	Glutamate, Ornithine, Tyrosine
4	Alanine	Ornithine	Arginine, Lactate, Ornithine	Arginine, Glutamate	Albumin, Ornithine	Ornithine
5	Lactate	Ornithine	Tyrosine	Albumin, Glutamate	Albumin, Ornithine	Glutamate, Tyrosine
6	Aspartate	Ornithine	Arginine, Ornithine	Arginine	Albumin, Ornithine	Glutamate, Ornithine
7	Glutamine	Ornithine	Arginine, Ornithine	Arginine, Glutamate, Ornithine	Ornithine	Glutamate, Tyrosine

Table 2 summarizes the key metabolites that contribute to the SPE_k. Note that only the variables with higher than the 3σ limits of normal contributions are tabulated. "Normal contributions" are calculated using the fresh liver perfusion profiles. Ornithine (#18), arginine (#5), albumin (#3), and tyrosine (#23) concentrations are significantly different between all fresh and WI livers, although these differences vary from time point to time points. Lactate, glutamine and glutamate are other major contributors to the SPE_k values. Figure 6 shows the contributions of selected metabolites to the normalized error vector for warm ischemic livers (see Eqns 6 and 7); note that only metabolites with high contributions are shown for clarity. Although albumin is one of the variables with large contribution values, it is not shown in the plot for scalability. Contributions from each perfused graft are stacked with the colors indicating different livers. The advantage of normalized error vector is that it can differentiate between positive and negative variable contributions; a positive value for a particular metabolite indicates that its concentration is higher than the fresh livers. Overall, albumin concentrations in the WI livers are always less than the

concentrations for fresh livers. An interesting note is that ornithine levels start negative, and switch to positive at 2hrs and on. Arginine concentration starts decreasing after the third hour and drops to a minimum at time = 4 hr. Tyrosine levels increase towards the end of the perfusion.

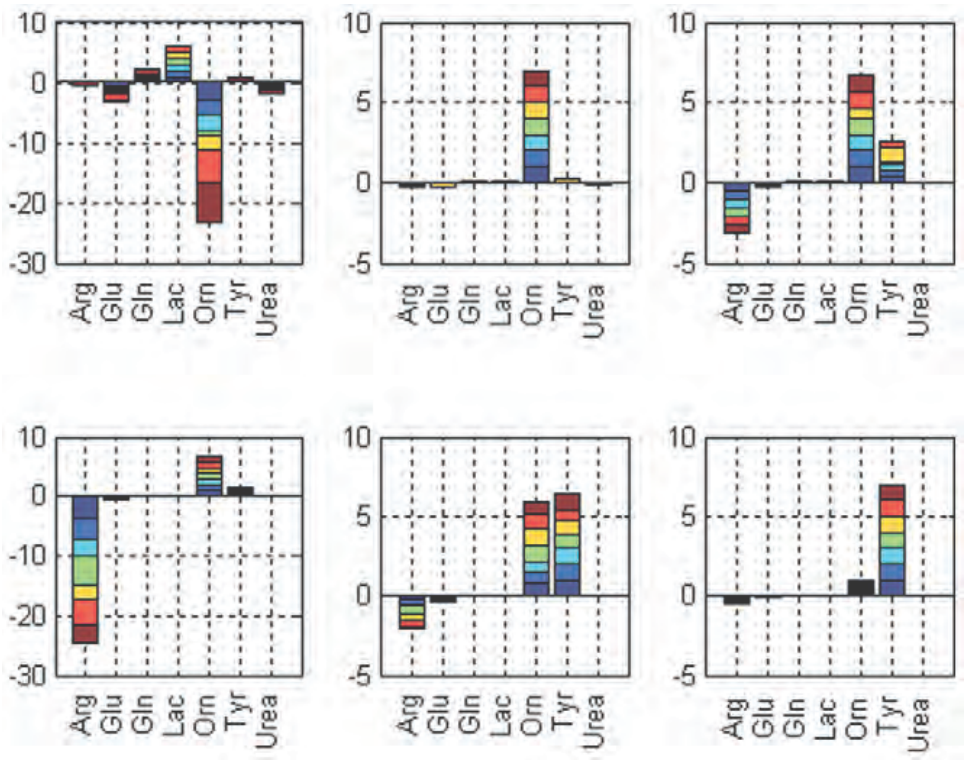

FIGURE 6 | Contributions of selected metabolites to the normalized error vector at each time point for WI livers. Contribution values for each WI liver are stacked in each bar. Six figures correspond to k = 1,...,6 hr time points.

Classification

While SPE_k provides a continuous index to evaluate a given liver, the $\log(SPE_k)>1.35$ criterion suggested above is a heuristic. The relatively wide margin of SPE_k values (Figure 5) can make classification problematic in the circumstance that a liver is equally different from ischemic and fresh livers. Accordingly, to complement the index we built and employed an MPLS-DA model to solve this corollary classification problem, and evaluated its accuracy by multi-sampling cross validation.

An MPLSDA model with five fresh liver samples and five warm ischemic liver samples using three principal components was developed and found to explain more than 60 % of

the variation in **X** and 99 % variation in **Y**. The results of offline classification using 100 runs are illustrated in Figure 7. The perfusions used in the modeling are plotted along with the projections. The perfusions that were not used in model building (five fresh livers and two WI livers in each run) were used to evaluate the accuracy of the model. As it can be observed, the model correctly classifies all of the liver samples in all 100 runs as fresh or warm ischemic (accuracy, specificity and sensitivity = 1). The test was repeated with 50 and 500 runs as well and the results did not change (results not shown).

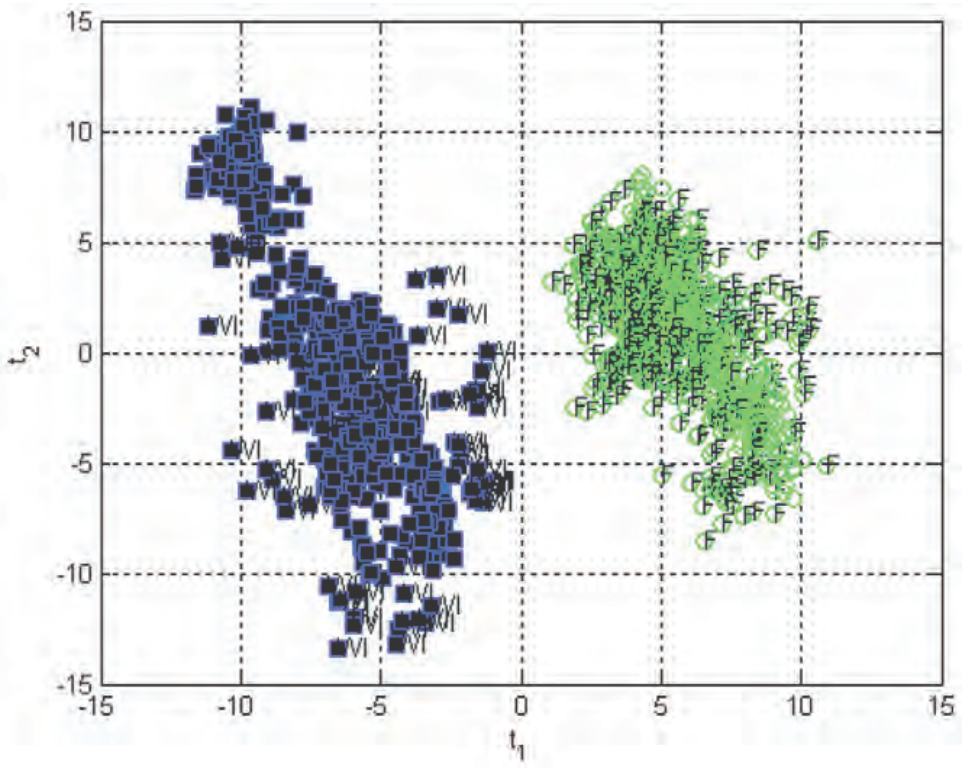

FIGURE 7 | Offline classification of fresh (F) and warm ischemic (WI) liver samples using case resampling cross-validation. During cross-validations some of the liver samples were left out to be used in model testing. These samples are projected onto the PLSDA model and they are denoted as WI (squared) and F (circles). All of the test samples for all 100 models were clustered correctly.

Figure 8 shows the most important variables in determination of class membership for each liver sample. Variable importance in the projection (VIP) was calculated by summing the squared PLS weights over all dimensions. The variables with VIP > 1 are effective on the response **Y**. The most important variables that separate WI livers from fresh livers are albumin (#3), arginine (#5), glutamate (#9), ornithine (#18), and tyrosine (#23), similar to the analysis before with SPE_k except acetoacetate is also chosen for MPLSDA.

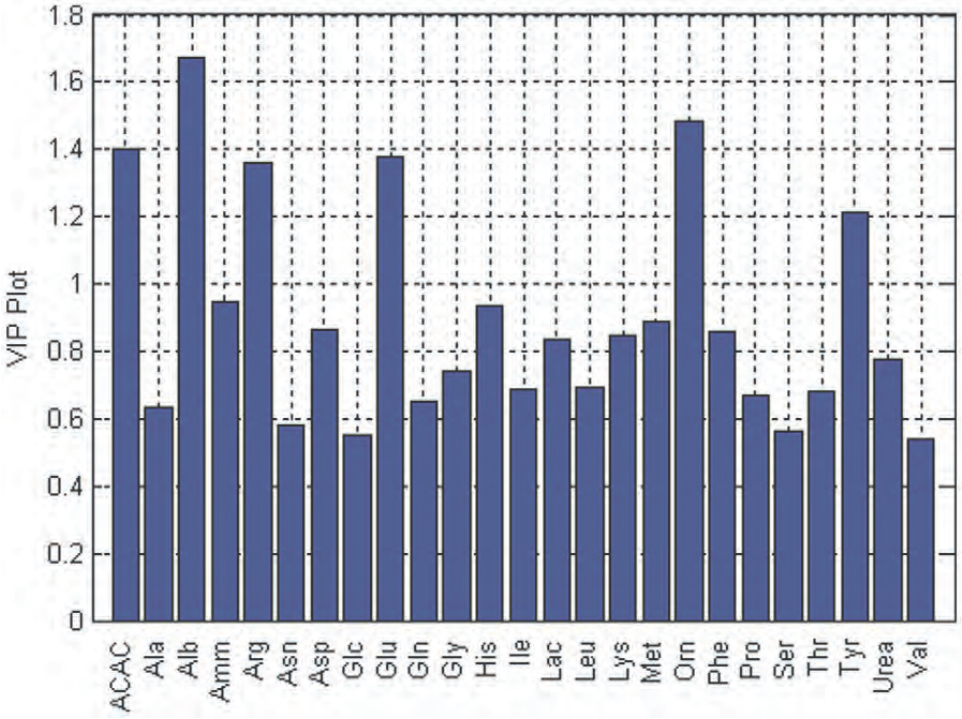

FIGURE 8 | Important variables found in MPLSDA. The most important variables that mark the differentiation of warm ischemic livers from fresh perfused livers have VIP values greater than 1.

With the ability of MPLSDA confirmed in offline mode, an on-line implementation was then tested. Similar to the online ischemia index, the basic idea is to be able to classify a perfused graft as healthy or ischemic during perfusion so that a decision can be made about the organ quality. Such a determination will ultimately be necessary to identify the need for further treatments, and to build a decision criterion for fitness of the graft for transplantation.

The prediction power of MPLS was also tested with case-resampling cross-validation, results of classifications are given in Table 3 for 100 resampling runs; all the misclassifications occur during the first two hours and after the third hour usually there are no misclassifications (Figure 9). Detailed specificity and sensitivity values for each time point are displayed in Table 4. As also displayed in Table 4, to ensure that the specificity and sensitivity evaluations are not dependent on the cross validation parameters, analysis was repeated with three different numbers of resampling runs; indeed the variation due to number of replicates is minimal. Moreover, specificity and sensitivity are always

above 0.98 which is excellent for online classification. It is notable that misclassifications during online classification are more common in the first 3hrs, although the difference is essentially negligible.

TABLE 3 | Sensitivity and specificity analysis for online quality prediction analysis via MPLS for 100 cross-validation runs.

		Actual	
		WI	Fresh
Classified as	WI	599	10
	Fresh	1	2990

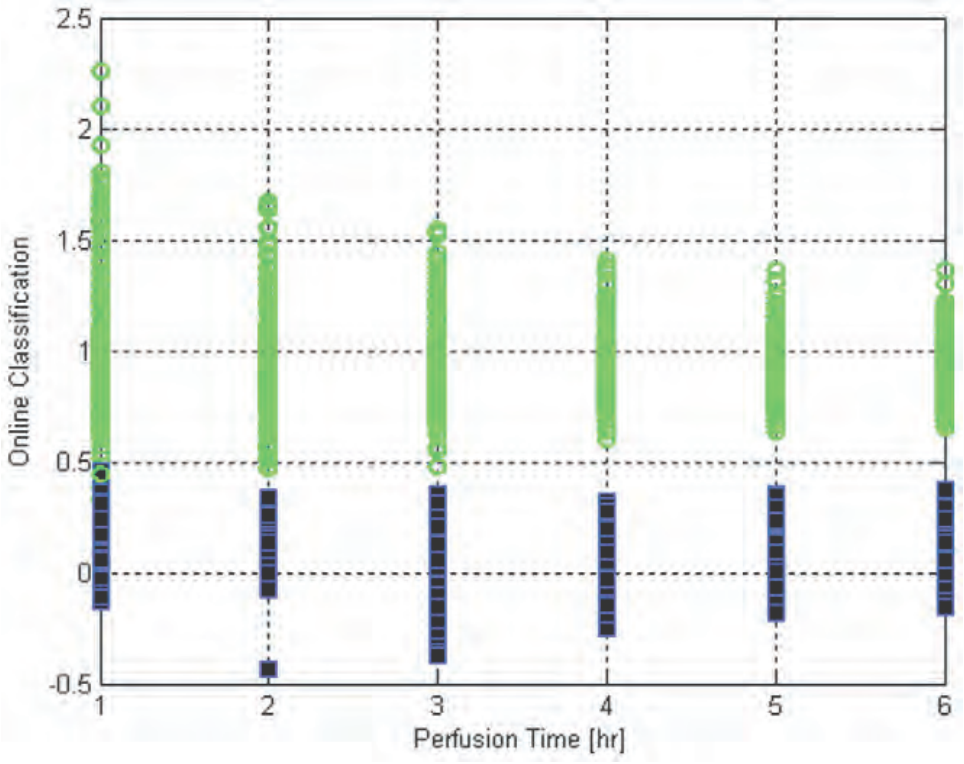

FIGURE 9 | Online classification for fresh and warm ischemic metabolite profiles using case resampling cross validation. Fresh liver samples and warm ischemic livers are represented by circles (>0.5) and squares (<0.5), respectively. Results shown for 50 runs with 10 random livers selected equally from fresh and WI samples.

TABLE 4 | Sensitivity and specificity for online predictions via MPLS. Results are provided for 50, 100 and 1000 runs.

Runs	Criterion	k = 1 hr	k = 2 hr	k = 3 hr	k = 4 hr	k = 5 hr	k = 6 hr
50	Sensitivity	1	1	0.980	1	1	1
	Specificity	0.988	0.984	0.992	1	1	1
100	Sensitivity	1	1	0.990	1	1	1
	Specificity	0.986	0.9940	1	1	1	1
1000	Sensitivity	0.995	1	0.997	1	0.999	1
	Specificity	0.984	0.990	0.997	1	1	1

Discussion and conclusion

In this work we introduce statistical process monitoring methodologies for evaluation of ischemic injury of cadaveric livers via their metabolic functions during extracorporeal perfusion. We focused specifically on hepatic metabolism because of the large integrated body of data with which to systemically gauge organ functional status, compared to the more traditionally employed singular tests of cellular injury, such as liver enzyme release (ALT & AST)[100], oxygen uptake[98] or ATP[164]. Our goal was to test the hypothesis that liver metabolic performance can be employed to evaluate the degree of ischemic injury cadaveric organs have sustained, with the ultimate goal of developing a metabolic feedback control scheme for optimizing organ viability and transplantability on-line.

The multivariate analysis performed demonstrated that fresh and ischemic rat livers display clearly distinguishable metabolic activities. Further, it was possible to distill these differences into a single metric, the multivariate SPE_k statistic, which we; we therefore conclude that the SPE_k statistic can be employed as an accurate and continuous index of ischemic injury for perfused rat livers. Moreover, it was demonstrated that this statistic can be computed online during perfusion, and hence can be used in the future to build a more sophisticated feedback-control scheme for regulation of perfusate supplements. Based on our data, a cutoff value for SPE_k was determined to be used as a heuristic limit of ischemia. This analysis can be further expanded to explore the correlations between metabolic function and traditional tests of cellular injury, and translated to human livers for a clinically applicable index of ischemia.

We also performed a complementary discriminant analysis to classify new livers with unknown conditions as either fresh or ischemic. Whereas the SPE_k creates a continuous index that is ideal for utilization in on-line regulation/optimization of organ function and viability, discriminant analysis approach enables a final determination to be made if the

organ is ready for transplantation or not. Specificity and sensitivity of the MPLSDA model we created were both >0.98, the accuracy of which was tested through multi-sampling cross validation. As in the index of ischemia/SPE_k, both offline and online analysis was performed and demonstrated to be successful in discriminating new perfusion data. It was noted that online MPLSDA had a few misses in the first 2 hours, and that these errors disappeared completely at t=3 hr and on. Two possible explanations here are i) fully sufficient data for discriminant analysis becomes available only after three time points of evaluation, or ii) the variability among fresh livers diminishes after the second hour when their functions stabilize, hence allowing easier separation of fresh livers from ischemic livers. If the latter case is valid, this *might* indicate that all the perfused livers have recovered from hypoxia sufficiently to perform measured metabolic functions, and therefore perfusion has achieved its goal at t=3 hrs and further perfusion is not necessary. In fact, the ability to identify when the liver is ready for transplantation would be very valuable for clinical utility of perfusion systems as such a measure is currently not available. However, it is important to note though that while the models proposed and developed in this work can identify the degree of ischemia, they cannot be used for evaluation of transplantability since all the livers perfused were successfully transplanted. However, this model can be the basis for determining transplantability if data from extended ischemic organs where recipients fail is included in the data. Testing by further transplantation at shorter perfusion durations is necessary for development of such an index.

The multivariate SPE_k statistic identified arginine, ornithine, albumin, tyrosine, lactate, glutamate, with the addition on acetoacetate in the MPLSDA model, as key metabolites that distinguished fresh from ischemic livers. The majority of these metabolites are directly associated with ischemia-induced pathway alterations. Arginine, for instance, is a precursor to both the vasodilator nitric oxide (NO) and ornithine. The balance between arginases (producing ornithine) and nitric oxide synthases (producing NO and citrulline) are dramatically altered during hypoxia [142-144]. This may account for the significantly higher rate of arginine depletion observed in WI livers compared to Fresh livers (figure 6), with a concomitant increase in ornithine production (Figures 1 and 6). Tyrosine, normally catalyzed in the liver to acetoacetate and fumarate, acts a precursor to nitrotyrosine, a derivative of altered NO metabolism, the increased presence of which may be associated with suboptimal levels of arginine [165]. Further investigations to elucidate the role of arginine in hepatic microvasculature preservation, and verification of the dominant by-products of tyrosine metabolism during perfusion of Fresh and WI livers, will enable optimal priming of the perfusate to minimize reperfusion injury. Albumin is a negative acute phase protein such that its production decreases when hepatocytes are stressed. Interestingly, a known trigger that reduces albumin synthesis is increased intracellular

NO as a measure of reducing energy expenditure during hypoxia [131]; the effects may last for several hours [140]. From our findings, albumin synthesis in WI livers remained flat for the duration of perfusion, while Fresh livers began to recover by t=2 hours. Lactate is a by-product of anaerobic metabolism; while cells will secrete lactate into a perfusate devoid of any lactate, lactate production exceeding this rate by WI livers in the first hour of perfusion suggests a response to their inadequate oxygen supply (Figures 1 and 6). Glutamate production as a marker of amino acid transamination increases throughout perfusion of Fresh livers, but is constant in WI livers. This would be consistent with the overall reduction in protein synthesis by WI livers, requiring less amino acid degradation, or an upregulation of the urea cycle by these livers, driven by their increased arginine consumption.

The analyses performed in this work all confirm that a metabolic index of ischemic injury is a feasible idea for evaluation of perfused ischemic livers, and such a measure would be of significant use in utilization of DCD livers for transplantation. This study demonstrates the power of SPM methodologies in achieving this goal; however, further work is needed to reach an index of transplantability. The data gathered here can also be used for more sophisticated metabolic analyses which reveal more details of the cellular function.

Chapter 9

Identification of optimum perfusion duration of machine-perfused rat livers for maximization of transplant success

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Submitted

Abstract

With the crisis in donor organ availability, recent studies on liver preservation focus on new, dynamic modalities such as machine perfusion. Machine perfusion protocols maintain the functionality of livers ex-vivo for extended periods of time and improve the quality of damaged livers via enabling recovery of energy levels and other aspects of ischemia. Compared to static cold storage, machine perfusion provides oxygen and nutrition enabling recovery of the organ. Despite years of research however, little is known about key factors such as how to evaluate the degree of recovery during perfusion and what is the ideal perfusion duration to maximize the graft recovery and transplantation success. Development of such approaches are complicated by the fact that every donor liver is different and therefore it is very likely that the optimum perfusion time, as well as other operating parameters and specific treatments, will be different for every organ. In this study, we present a novel approach that enables assessment of liver viability during perfusion, the determination of the optimum duration of perfusion and the prediction of clinical outcome post-transplantation. 11 rat livers that were perfused up to 12 hrs were transplanted with mixed survival results observed at 4 weeks. A Partial-Least Squares (PLS) based statistical modeling algorithm was developed to evaluate organ viability on-line based on Blood Gas Analysis data. The results demonstrate that with the proposed methodology, the graft viability can be monitored and transplant success rate can be predicted, online during perfusion, with cross-validated sensitivity and specificity over 91%. Moreover, it is shown that the PLS-based statistical model can be used to accurately predict the viability of the graft ahead of time, and therefore enables the identification of the optimum time to transplant the graft for maximum transplant success.

Introduction

It is estimated that every year 27,000 people die because of liver-failure; currently the twelfth leading cause of death in the US [99]. The only known treatment is liver transplantation. According to the Organ Procurement and Transplantation Network, over 10,000 patients are added to the waiting list and less than 7,000 receive transplants each year. It is further estimated that about 5,000 ischemic livers obtained from Donors after Cardiac Death (DCD) could be reconditioned for transplantation, dramatically increasing the availability of grafts [76]; currently these organs are discarded due to lower survival rates for these marginal grafts. We [98, 112, 146] and others [36, 38, 41, 64, 98, 112, 146] have demonstrated this is possible in extracorporeal machine perfusion systems.

The decision to use a non-ideal donor liver for transplantation is a difficult choice that to date lacks an accurate measure[166]. A large number of DCD organs, for instance kidneys, are not transplanted due to fear of graft failure based on qualitative tests that are open to interpretation [167]. Employing a quantitative, objective criterion instead could drastically enhance transplant success: For kidneys, machine perfusion of DCD organs has been practiced clinically for over a decade. It has been reported from one hospital that employing viability assessment during perfusion (based on a formula employing total glutathione S-transferase in the perfusate, the intrarenal vascular resistance and flow characteristics over time) has increased the DCD graft survival from 45.5% to 88.4%[168], almost reaching the normal kidney transplant results (90.5%).

A similar viability assessment method for livers, unfortunately, remains missing[166], a major drawback of static cold storage. The viability decision is made based on texture of organ and quality of perfusion at retrieval[169], and in some cases histology[145]. The development of a measure of viability for DCD grafts would clearly make decision making for transplantation much easier, and potentially save more patients. A similar issue exists for determination of transplantable steatotic livers as well[170].

An important benefit of machine perfusion, in particular normothermic perfusion, is that it allows the evaluation of the function of the graft prior to transplant, in direct comparison to in vivo values. Demonstrating the connection between viability and liver function during extracorporeal perfusion, a study using normothermic perfusion of porcine livers was able to correlate recipient survival to bilirubin content in bile [86]. A second swine study also identified bile production as a potential predictor of graft viability prior to transplantation[88]. However, these studies are simple univariate statistical analysis of correlations between transplant success and bile secretion that establish

normal limits. Moreover, bile component analysis requires HPLC, Mass Spectrometry or a similar sophisticated analysis which is expensive, time consuming and therefore currently impractical within the usual transplant settings.

The ideal method in perfused graft viability assessment would be based on online measurements that can be performed accurately, quickly and at a relatively low cost, such as Blood Gas Analyses (BGA) or cell injury measured via transaminase release. Rather than relying on patient statistics, an ideal approach would evaluate the dynamic patterns of organ function during normothermic perfusion, compare it to successful and unsuccessful transplants in a manner that allows a statistical analysis of transplant success, and therefore provide an estimate for the likelihood of graft success to the transplant surgeon as a decision support system. Moreover, such a dynamic, real-time assessment method would enable feedback for decision making related to the perfusion operational parameters, such as oxygenation, pH, metabolites or other pharmaceutical/nutritional supplements, which have so far been selected either heuristically or based on convenience[59].

One such key operational parameter that would appear to be most basic is the optimum perfusion duration. It is well known that the existing organ preservation systems are limited in duration; static cold storage is limited to ~12 hrs, after which the success rate falls to 59.5%, vs. 83.5% of marginally ischemic grafts (<30mins) and 92% of nonmarginal organs[171]. Similar limits are observed for perfusion, with the record successful perfusion-preservation confirmed with transplantation at 20 hrs [19]. While many studies demonstrate that perfusion, typically in the order of hours, is necessary for resuscitation of a DCD organ, it is also well known that even in perfusion the organ state is far from physiological and the organ function deteriorates if it is kept on perfusion for an extended amount of time. Accordingly, the organ viability and transplant success probability very likely goes through an optimum, before which the recovery from ischemia is incomplete, and beyond which the organ begins to deteriorate due to damage by the perfusion system. This optimum is also certain to vary from organ to organ, as everything from donor age and genetics to lifestyle and recently eaten food and used medications could affect the recovery from ischemic injury. Yet, based on a literature search and to our knowledge, there is exactly one report testing effect of perfusion duration on organ viability[172], evaluated via indirect indicators such as enzyme release (ALT, LDH)[100] and tissue ATP levels[164] rather than transplantation. While unique in providing evidence that as little as 1 hour may be sufficient for perfusion-resuscitation of ischemic grafts, this single study remains far short of providing an organ-specific predictor of transplant success.

With the on-line data obtained during machine perfusion, a suitable methodology for such dynamic organ viability assessment is statistical process monitoring (SPM) based on partial least squares (PLS) methodology and its extension for dynamic processes: multi-way PLS (MPLS). The key advantages of MPLS methodology that renders it ideal as the basis for perfused liver viability are i) its dynamic nature, enabling prediction of transplant success on-line during perfusion as opposed to static methods such as ANOVA that require awaiting the end of perfusion and completion of data acquisition; ii) again unlike univariate methods such as ANOVA, the ability to handle multiple, highly correlated variables (such as oxygen, lactate and pH in ischemia) and quantify which are critical in determining viability, iii) its statistical nature, which enables readily accommodating organ-to-organ variations and other uncertainties in the system mathematically, and lastly iv) that MPLS constructs a statistical model of liver dynamic response to perfusion, which therefore enables performing in-silico predictions of the liver trajectory during perfusion. These PLS-based predictions of the trajectory of the organ in the following hours can then be used to detect any emerging trends that indicate deterioration of organ viability, and therefore enable identifying the optimum perfusion duration, and potentially other operational variables, for each organ based on its function on-line during perfusion.

This work presents a novel approach and a two-step MPLS/PLS algorithm for the prediction of perfused liver survival, based on measurements taken during perfusions of rat livers for 6 and 12 hours, with recipients of first group surviving and latter group mostly failing. Via an online MPLS analysis, blood gas data taken hourly is used to estimate the post-transplantation survival rates dynamically during perfusion, which are compared with the actual post-transplantation clinical outcomes for each animal. More specifically, with this work we test the following hypotheses: i) blood gas analyses obtained during perfusion is correlated to graft survival post-transplant, ii) there are significant variations between the responses of the livers in perfusion, organ-to-organ and temporally, and iii) these variations may lead to the same organ being viable for transplantation at one time point and unviable at another during perfusion.

Materials and methods

For a details on the isolation of the donor liver, machine perfusion protocols, isolated reperfusion, transplantation sampling and processing of perfusate and tissue samples, please refer to chapters two and three and references [83, 98] respectively.

Numerical Methods

Briefly, the proposed algorithm is a two-fold PLS/MPLS approach with the PLS being used for future trajectory predictions and MPLS for online multi-way assessment of dynamic perfusion quality as the perfusion is progressing, as detailed below.

Statistical Preprocessing

Blood samples taken hourly during perfusions were analyzed for the eleven variables given in Table 2. During the initial data preprocessing the missing data, which constitute the 1.35% of the total, were replaced by the mean of all trajectories. The data were further normalized using mean centering and unit variance scaling{Eriksson L, 2006 #434}. An outlier analysis was performed via MPLS (see *Outlier Organ Analysis* below for details) to determine the existence of unusual perfusions within both successful and unsuccessful perfusions. Liver #1 of the successful perfusions group was found to exhibit unusual variations and declared an outlier perfusion, therefore, was eliminated from data and not used in further analysis.

TABLE 2 | Variables measured in Blood Gas Analysis

Variable #	Variable Name	Abbreviation
1	Acidity	pH
2	Carbondioxide partial pressure	pCO_2
3	Oxygen partial pressure	pO_2
4	Percent hemoglobin	ctHb
5	Hematocrit	Hct
6	Fraction of oxygenated hemoglobin	FO_2Hb
7	Fraction of carboxyhemoglobin	FCOHb
8	Fraction of methemoglobin	FMetHb
9	Deoxyhemoglobin	FHHb
10	Oxygen Exit Rate	OER
11	Total hemoglobin bound oxygen	Bound O_2

Partial Least Squares (PLS) and Multi-way PLS (MPLS)

Partial least squares or projections to latent structures (PLS) is a regression method that is used to connect the information in two blocks of variables, namely the predictor block **X** and response block **Y** (Eqn 4). PLS gives a way of predicting the **y**-values from the **x**-values, a generalization of multiple regression, by correlating **X** to **Y** and finding the orthogonal directions that maximize the correlation between **X** and **Y**. An algorithm for Nonlinear Iterative Partial Least Squares (NIPALS) is provided in [161].

$$\begin{aligned} \mathbf{X} &= \mathbf{TP}^{\perp} + \mathbf{E} \\ \mathbf{Y} &= \mathbf{UQ}^{T} + \mathbf{F} \\ \mathbf{U} &= \mathbf{TB} + \mathbf{H} \qquad (\mathbf{B} \text{ diagonal}) \end{aligned} \tag{1}$$

Interrelation gives the predictive formulation for **y** as in Eq 5.

$$\mathbf{Y} = \mathbf{TBQ}^{i} + \mathbf{F}^{*} \quad (2)$$

T and **U** are called the score matrices; **P** and **Q** the loading matrices and **E** and **F** are the residuals for **X** and **Y**, respectively. The matrices are denoted by bold capital letters and the vectors are denoted by bold letters in the text.

In this manuscript, **y** is a vector that consists of binary values which correspond to the viability of the perfused organ. For the set of perfusions that are used in model building, the **y** vector contains 1s for the grafts that were successfully transplanted with > 1 month survival, whereas, the perfusions with poor outcomes are represented in **y** as 0s.

PLS has been extended to multi-way PLS (MPLS) for batch processes. Batch processes consist of 3D data where each dimension accounts for batches, variables and time, respectively. MPLS is equivalent to performing a PLS where **X** predictor set is the unfolded batch data and **Y** is a 2D matrix of end-of-batch quality variables. A 3D representation of the batch data and the 2D (I by JK) unfolding for MPLS applications are presented in Figure 1[155-157, 173].

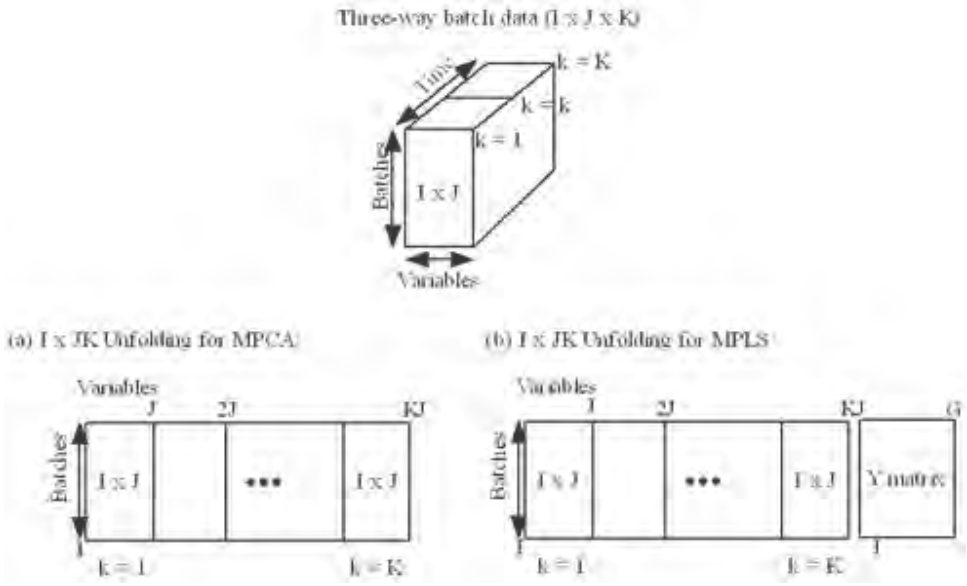

FIGURE 1 | Representation of the 3D batch process data and (I x JK) unfolding for MPLS.

Each perfusion can be considered as a batch-wise operation since each perfusion has a finite duration and follows a certain processing recipe. Furthermore, the trajectories of variables present dynamic time dependencies. Since the transplantation success is dependent on the end-of-perfusion graft quality, which is affected by the changes that take place during the perfusion, it is possible to build a statistical model that captures this dependency and correlates the dynamic trends during the perfusion to the outcome of transplantation. An MPLS-based model that captures the variation in blood gas analysis measurements during the perfusions and correlates it with survival statistics of the recipient animals can therefore be used to predict the transplant success based on the perfusion measurements obtained.

Variable Importance on Projections (VIP)

VIP plot represents the contribution of each of the predictor variables in X in fitting the PLS model. VIP is a weighted sum of squares of the PLS weights taking into account the amount of explained Y-variance in each principial component dimension. Wold et al. [161] consider a value less than 0.8 to be small for the VIP. VIP score for the jth variable can be calculated by .[153, 161]

$$VIP_j = \sqrt{\frac{p\sum_{r=1}^{R}\left[SS(b_r t_r)\left(w_{jr}/\|w_r\|\right)^2\right]}{\sum_{r=1}^{R} SS(b_r t_r)}} \qquad (3)$$

where $SS(b_r t_r) = b_r^2 t_r^T t_r$, t_r is the r^{th} column of scores T, and b_r is the r^{th} element of column vector **b** that explains the relation between **y** and $\mathbf{t}_r$.

Online MPLS Analysis

Rather than wait for the end of fixed perfusion duration, it is desirable to perform quality assessment online, which requires an algorithm that can discriminate these two states on the basis of the dynamic perfusion data available thus far in the perfusion. For the analysis of the dynamic metabolite data the MPLS model is fit between **X** matrix (I x JK; I batches, J variables and K time points) and the **y** vector (I x 1) and the plane that correlates dynamic perfusion profiles of grafts to their post-transplantation survival outcome is found. The model, i.e. **P, W, B** and **Q** matrices, is used to determine the viability state of a new observation, $\mathbf{x}_{new}$ (1 x JK) as 1 (good) or 0 (poor). When the unfolded new data $\mathbf{x}_{new}$ is projected onto the MPLS model, $\hat{\mathbf{y}}_{new}$ is calculated. The predicted state $\hat{\mathbf{y}}_{new}$ is calculated as

$$\hat{\mathbf{t}}_{new} = \mathbf{x}_{new}\mathbf{W}(\mathbf{P}^T\mathbf{W})^{-1};\ \hat{\mathbf{y}}_{new} = \hat{\mathbf{t}}_{new}\mathbf{B}\mathbf{Q}^T \qquad (4)$$

where **W** loadings are called weights and express the correlation between **U** and **X**. For the online calculations at time point k where only $\mathbf{x}_{new}$ (1 x kJ) part of the whole trajectory is available, **W** (kJ x R) part of the whole **W** matrix is used (*see Appendix for details on calculation procedure*).

One-step-ahead future trajectory estimation. Online MPLS analysis predicts the survival outcome of a perfusion based on the available perfusion data. A logical next step would then be to determine if additional perfusion time would have a positive effect on the survival outcome. This necessitates the reasonably accurate prediction of measured variable trajectories based on available data, which is then followed by online MPLS analysis to determine the projected survival rate as detailed above.

In this work, the PLS methodology is also utilized as an estimation algorithm where future variable trajectories are estimated at a given time point based on the dynamic correlation structure available until that time. The one-step-ahead prediction scheme is shown in Figure 2. Time lagged windows are combined together and form the **X** predictor matrix. For a model order of 2, which is employed here, three time windows are used that belong to the current time t, t-1 and t-2. The **Y** response matrix consists of the measurements obtained in the next time period, t+1. Therefore, the PLS model captures the system dynamics during the time period from t-2 to t and relates it to the system response in the next time period, t+1.

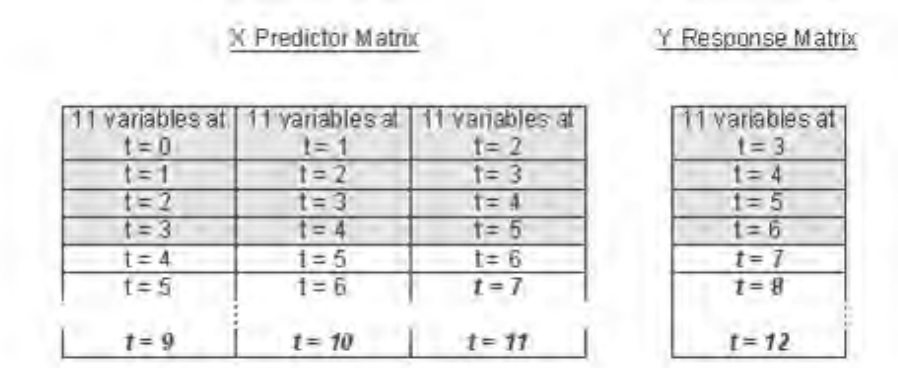

FIGURE 2 | One-step-ahead estimation scheme with PLS. Three time windows; t-2, t-1 and t, in the predictor matrix are used to predict the data at t+1. The PLS model is built using the predictor matrix **X** and the response matrix **Y** (X 4 x 33, Y 4 x 11, gray shaded part) that consist of all the available data until time = 6hr. The remaining future trajectory is estimated starting with the estimation of data for time = 7 hr based on the data for t = 4 to t = 6; the estimates for t = 7 are used in the estimation of the next time point time = 8 hr, and the procedure is repeated until time = 12 hr.

The PLS model is built using the predictor matrix **X** and the response matrix **Y** (X 4 x 33, Y 4 x 11, gray shaded part) that consist of all the available data until time = 6 hr (Figure 2). The remaining future trajectory is estimated starting with the estimation of data for time = 7 hr based on the data for t = 4 to t = 6; the estimates for t = 7 are used in the estimation of the next time point time = 8 hr, and the procedure is repeated until the estimates for time = 12 hr are obtained at time = 6hr. In order to estimate the BGA measurements for the 7th hour, $\mathbf{x}_{new}$ is projected as $\mathbf{x}_{new}$ = [**x** (t = 4hrs), **x** (t=5hrs), **x** (t= 6hrs)] where the size of the new **x** vector is (1 x 33) and the **y** vector that consists of the estimates for the 7th hour is a vector of size (1 x 11) where eleven is the total number of variables (J)

As a control for the proposed projection strategy, the same $\mathbf{x}_{new}$ vector can be predicted via ordinary least squares (OLS) method to determine $\hat{\mathbf{y}}$ as,

$$\hat{\mathbf{a}} = \left(\mathbf{X}^T\mathbf{X}\right)^{-1}\mathbf{X}^T\mathbf{Y};\ \hat{\mathbf{y}} = \hat{\mathbf{a}}\,\mathbf{x}_{new} \tag{5}$$

where the **X** matrix is the same matrix used in PLS model building, after the correlation coefficients $\hat{\mathbf{a}}$ are calculated, the new **x** is projected and consequently $\hat{\mathbf{y}}$ is found.

The MPLS model is used for the quality assessment of the perfusions based on the estimated data with either method. Using these estimated projections, MPLS analysis can be continued until a later time point to determine estimated transplant success. The optimum time required for a perfusion can therefore be easily deduced from these viability estimates and is the time point where estimated quality does not improve further with time or starts deteriorating.

Multi-sampling Cross Validation

The predictive power of MPLS models was evaluated using case-resampling cross validation technique [162, 163]. The data was sampled randomly multiple times to create different training and testing data sets. At each sampling, four samples from successfully transplanted group and four samples from the non-survival group were selected randomly and used to build an MPLS model. The remaining samples were used for testing the model. Maximum selection threshold, the maximum number of times the same perfusion sample can be used in one model was set to 2. The maximum selection threshold prevents any bias from being introduced as a result of same samples being selected most of the time.

Multisampling cross-validations were performed 500 times, which we observed were sufficiently large replicates such that the results did not change significantly with further increase in runs (results not shown). To ensure selection of samples was randomized, each 500-run cross validation was repeated three times.

Results

Outlier Organ Analysis

As part of the multivariate analysis with MPLS, we performed an outlier analysis on the organ level to see if any perfusions were significantly different than any others. As in all the MPLS models in this manuscript, four successful perfusions and four poor perfusions were selected randomly among the whole set and the remaining perfusions were used in model testing. A score (**t**) biplot ($\mathbf{t}_1$ vs $\mathbf{t}_2$) shows that the MPLS model built with 500 such samplings successfully captures two different classes among the eight perfusions and also determines that the first successful perfusion exhibits some variation that is not common to the rest of the successful perfusions, therefore, it clusters away from the main successful perfusions cluster and forms a separate group with a different orientation (Figure 3a). Therefore, the first successful perfusion was considered an outlier and eliminated from consideration in the remaining text. An MPLS model was built without the outlier perfusion and the new score biplot is shown in Figure 3b.

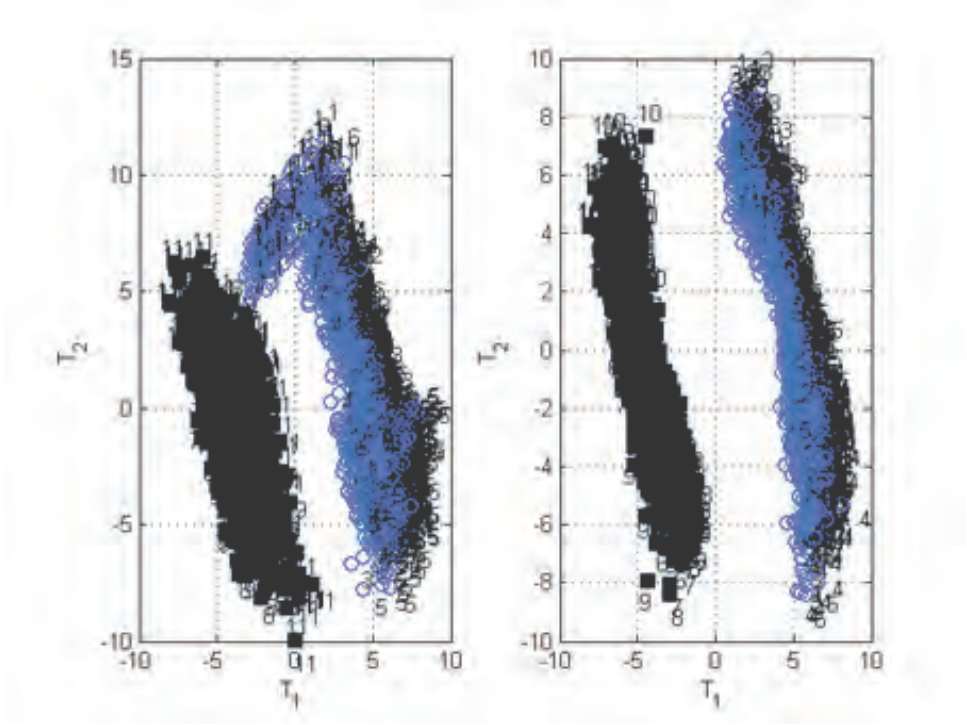

FIGURE 3 | Score biplot (t_1 vs t_2). (a) Outlier analysis performed with MPLS, the figure illustrates the two classes corresponding to successful (blue circles) and unsuccessful (black squares) perfusions. Liver #1 exhibits variations that are different than other successful perfusions and forms a separate group. (b) After liver #1 is removed from the original set, there are no remaining outliers.

Online Quality and Survival Assessment

The goal here was to capture the variation during perfusion of each liver and correlate this with the end-of-perfusion liver viability and survival rate of the recipient animal. For the analysis of successful and poor perfusions, the (I x JK) unfolded predictor matrix (**X**) consisted of the blood gas trajectories for selected eight batches. The rows of the response vector (**y**) were set to 1 for each successful perfusion and to 0 for each poor perfusion. An MPLS model with four successful perfusions and four poor perfusions using three principal components was found to explain more than 75% of the total variation in **X** and 99 % variation in **y**.

Online MPLS model yields predictions of **y** using available data until that time point. Figure 4 shows the predicted survival rates after transplantation for each perfused liver at the end of 6 hours, which are the average outcomes for 500 MPLS runs. With a cutoff of success taken as online survival estimate>0.6 (the worst estimate value for a successful transplant, as seen in Liver #1 in Figure 4), the online MPLS model accurately predicts the survival rates for the successful and unsuccessful perfusions. Notably, during perfusion two successful livers (#1 and #3) actually degrade significantly, but are then able to recover back. Paralleling this, non-surviving liver #3 recovers to nearly viable levels at t=4, but fails to continue on this recovery trajectory.

The sensitivity and specificity of online classification testing for 500 runs are displayed in Table 3. The specificity for the test is 1 for all three repetitions, hence the test is found to always correctly classify an unsuccessful perfusion. The sensitivity is higher than 93%, meaning ~7% of the time a viable liver will be misidentified as unviable for transplantation.

Survival Prediction Based on Estimated Perfusion Trajectories

Since previous results demonstrate successful estimation of liver viability, the next question is if this method can be used to determine if perfusion-recovery is complete, or if longer perfusion would be beneficial, or if the liver is unrecoverable and perfusion should be aborted. To perform this analysis, we performed one step-ahead estimation at each time point after t=6 hrs, and continued performing online survival estimates.

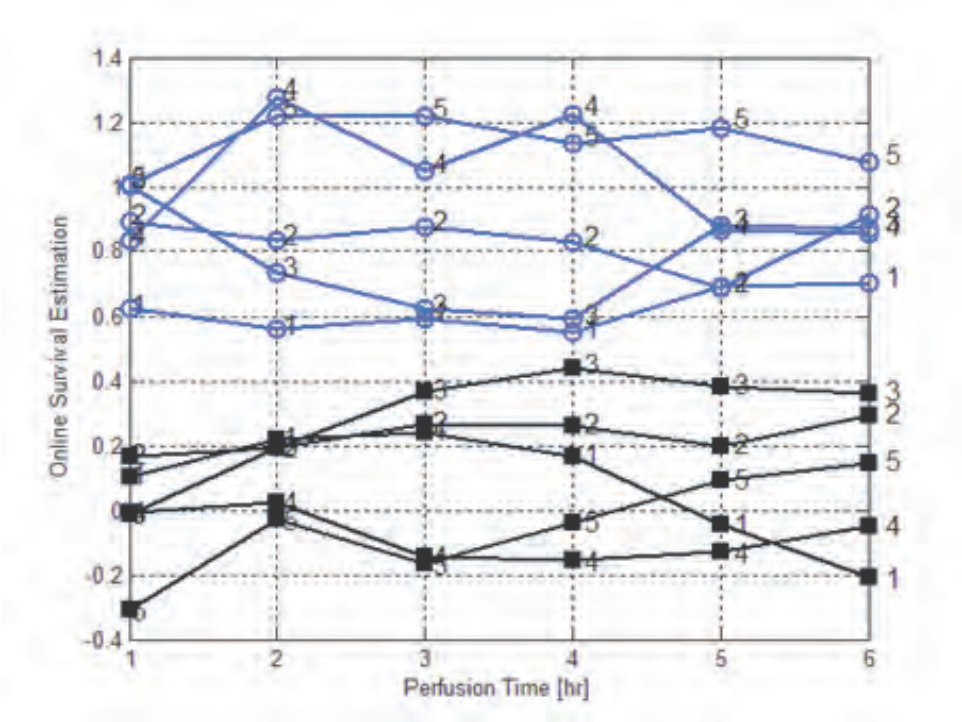

FIGURE 4 | Average survival rate predictions using online MPLS based on 6hr perfusion data.

TABLE 3 | Sensitivity and Specificity for MPLS Classifications Using 6hr and 12hr Perfusion Trajectories. Analysis Based on 500 Runs Repeated 3 Times (given in columns).

6hr Perfusions	Sensitivity	0.9410	0.9487	0.9360
	Specificity	1	1	1
12hr Perfusions with Estimates	Sensitivity	0.9278	0.9720	0.9172
	Specificity	0.9667	0.9800	0.9700

To test the accuracy of one-step-ahead estimation scheme with PLS, we first compared this approach with Ordinary Least Squares estimate. As an example, the estimated trajectory of unsuccessful perfusion #1 is shown in Figure 5. The actual trajectory is shown in blue lines and the actual measurements are denoted by 'x'. The red down triangles show the estimated values using PLS and the black circles denote the OLS estimates. For variables: pH, pO_2, FO_2Hb, the estimation error for OLS is significantly larger than the estimation error for PLS. The sum of squared errors of the variables for the whole trajectory of #1 is smaller for PLS estimates than OLS estimates (PLSError:OLSError = 1:3.87) and therefore demonstrating that the PLS method is better; therefore, we used the PLS approach used to estimate the future trajectories (7-12hrs) of all perfusions.

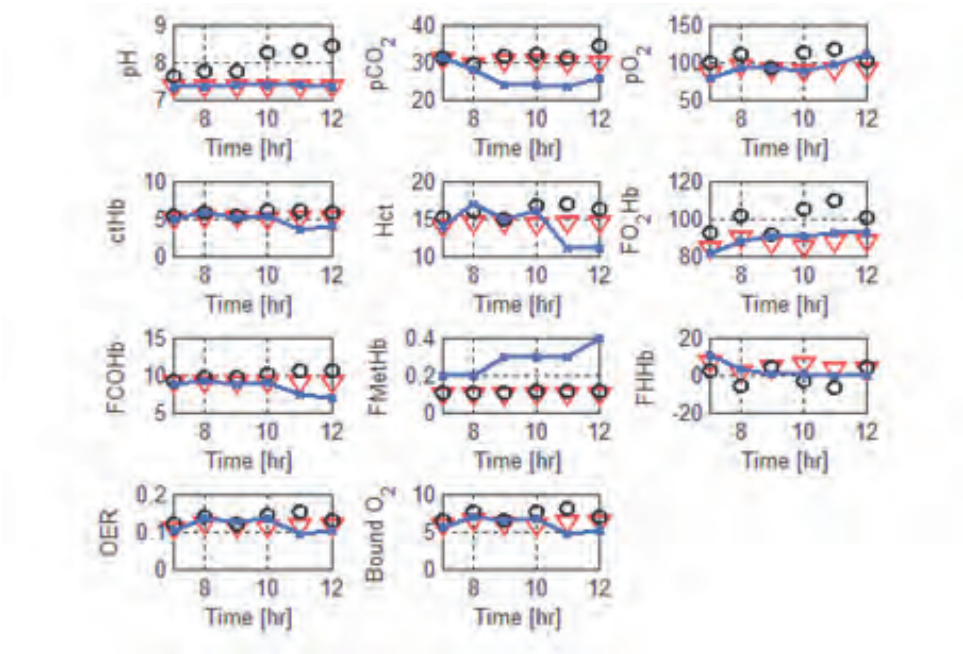

FIGURE 5 | One-step-ahead estimates for the perfusion of liver #1 (survival < 3 days). Each figure corresponds to a different variable and shows the variable estimates until the end of the 12th hour. Blue lines and 'x' denote the actual measurements. The PLS estimates are shown with red down triangles, whereas, the OLS estimates are shown with black circles.

The estimated survival rates based on these organ trajectory predictions, which are the average of 500 cross validation runs, are depicted in Figure 6. All the livers that have been successfully transplanted were correctly predicted as being in better conditions than the unsuccessful perfusions with higher survival rate estimations than the unsuccessful

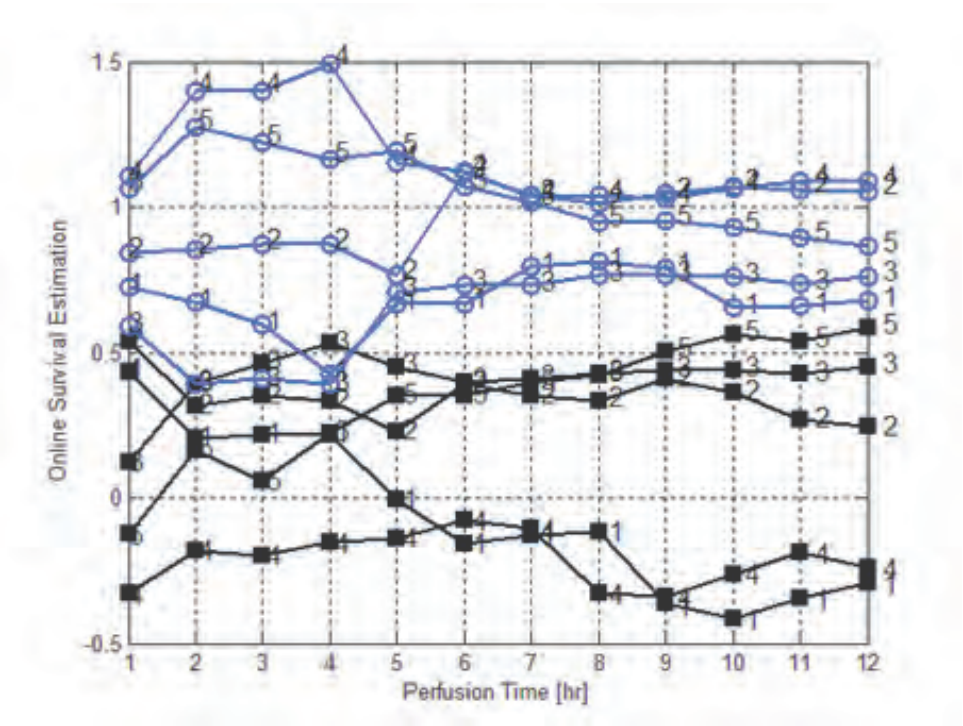

FIGURE 6 | Average survival rate assessment using online MPLS based on estimated 12hr perfusion data.

perfusions via online MPLS. As in Figure 4, the viability change as a function of time can be clearly appreciated with unsuccessful livers #3 and #5 improving to the verge of successful transplantability at the end of 12 hours, and it is possible that these two livers could become transplantable with a few more hours of perfusion. On the other end of the spectrum, unsuccessful liver #1 starts of quite viable (0.4) and rapidly declines during perfusion. Similar temporal patterns are observed with successful graft perfusions as well. Another note is that for successful perfusions, the online viability assessment indicates that the graft viability rarely improves significantly after the 6th hour of perfusion.

As displayed in Table 3, accuracy in prediction of viability with the 12 hr MPLS model is slightly lower than the 6hr MPLS model, which is as expected since the estimates are involved in the 12 hr trajectories. However, the sensitivity and specificity values remain higher than 91% and 96%, respectively, which are still excellent for a diagnostic test.

Classification and Variable Importance on Projection (VIP)

In addition to classifying the livers, a corollary information that the MPLS model reveals is the relative importance of each measured variable on determining the outcome, based on the VIP results, as displayed in the bar chart in Figure 7. The parameters that are different between these two groups and affect the classification have VIP values > 1 are pO_2 and FCOHb, followed by FHHb, FO_2Hb and FMetHb. Of these, pO_2 and FO_2Hb in combination reflect the amount of oxygen that is available for delivery to the tissues. FCOHb, FHHb, and FMetHb in the context of machine perfusion, are indicators of hemoglobin that has been rendered unusable for delivery oxygen to the tissues.

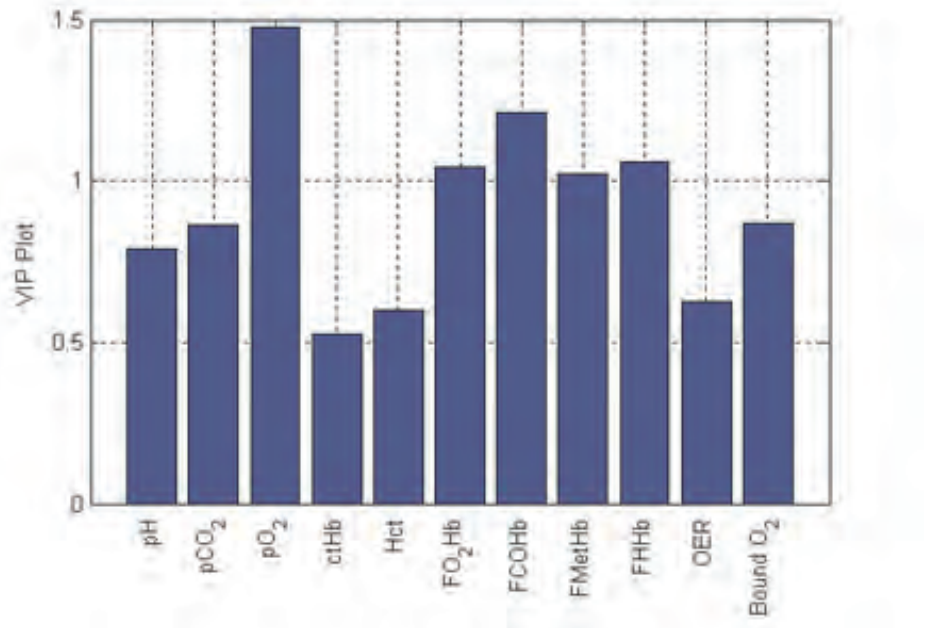

FIGURE 7 | The variable importance on projection (VIP) plot. The variables that yielded the class separation in MPLS are pO_2, FCOHb, FHHb, FO_2Hb, and FMetHb.

Discussion and conclusions

In this work, we present a novel approach to analyze the viability of livers during extracorporeal machine perfusion from the blood gas analysis, which enables the accurate prediction of post-transplantation survival success and the optimum perfusion duration. The algorithm involves a tandem PLS/MPLS methodology to predict the future organ trajectories and consequently estimate the evolution of organ viability as perfusion progresses. Prediction results were compared with actual transplantation results, with the cross-validation analysis indicating an excellent greater than 91% sensitivity and specificity in all cases.

The analysis revealed that there is dramatic variability organ-to-organ and temporally in the same organ during perfusion. It was observed that two of the livers that were successfully transplanted were actually in poor viability for the first four hours, and only recovered during the last two hours of perfusion, and terminating perfusion earlier for transplantation would have likely resulted in graft failure. Two unsuccessful livers were noted to have improved to near-transplantable viability at 12 hours, and analysis indicates they could be rendered transplantable if perfused longer. On the other hand several livers did actually deteriorate during perfusion and could benefit from an earlier cutoff of perfusion, around 4 hours. At least one liver was unusable from the start of perfusion and should actually have been abandoned without further perfusion. The exact source of these variations are difficult to identify, and could range from the initial state of the liver (such as the metabolic state of the donor), the variations in surgical time (such as the duration of ischemia during cuffing and preparation for transplant, catheter placement and consequent microperfusion quality) or variability in the perfusion operation (such as minor variations in the pH, oxygen, temperature regulation). What is certain is that with human livers, the variation in donors will be much higher than these inbred, lab controlled rats, and the operator and equipment factors will also be less easy to standardize. As such, this analysis confirms that the liver-to-liver variation in organ perfusion is a significant factor that has to be taken into consideration if machine perfusion is to become a clinical reality. Moreover, it demonstrates that these variations can be captured by simple and cost effective analytical measurements, such as blood gas analysis which is a very cheap and routine clinical procedure, and that these variations are highly correlated to transplant success.

It was also noteworthy that about viability of about 50% of the livers could be determined by the first data point at 1 hour of perfusion. This indicates that by the end of 1 hour of perfusion these livers have already largely stabilized one way or the other. Therefore, for future studies, especially those focusing in cadaveric livers, more attention should be directed towards the early perfusion function, and more frequent sampling is advisable.

Analysis of the variables with the largest weights in determination of viability indicates that the supply of oxygen within the system was the key factor. This indicates that the perfused livers may actually be still hypoxic, as was also indicated by a recent metabolomic analysis (results not published). This is actually an ideal example for demonstration of how a feedback control system based on an organ viability indication scheme as developed here could be used effectively to enhance graft viability, in this case by simply increasing the oxygen supply as needed to increase viability. A secondary collection of important factors involved unusable hemoglobin in the system; this indicates that the detrimental effect of

perfusion observed on some livers was due to a reduction in total functional hemoglobin, and hence reduced delivery of oxygen. Use of artificial blood carriers that are less prone to denaturing when exposed to air, or replenishment of the perfusate every few hours are possible solutions to resolve such issues.

This work provides statistical evidence that liver function is correlated to transplant success. From a quantitative systems biology perspective, such correlations could be improved upon by coupling with sophisticated metabolic analysis approaches, such as metabolic flux analysis[174], flux balance analysis[106, 174, 175], as well as pathway analysis and other data-mining type approaches [144, 176], such that the correlations to viability at the macroscopic level (such as oxygen) can be traced down to specific pathways (beta-oxidation, TCA activity etc.) to develop specific interventions to improve organ viability.

In this manuscript, we restricted the study to a proof-of-concept trial where the hypothesis that BGA readings can be used to predict transplant success was tested and found to be correct in the rat model. Some of the results of the analysis however, such as the determination of the optimum perfusion duration, or feedback regulation of identified variables require additional confirmation by transplantations, which would require an online implementation of the algorithm developed here; this work is currently in progress. Another critical direction for future work is testing this work with cadaveric livers, which is the ultimate goal of this work.

Discussion and future Perspectives

Discussion and future Perspectives

The work described in this thesis is not intended to replace the excellent work of others, but rather to supplement it. By successfully establishing a miniaturized NMP setup, we hope to have made NMP available to a broader scientific public and less limited to the operation theatre. Although the work is based on preservation of the liver, it is likely applicable to other organs.

The second chapter of this thesis describes in detail how our perfusion model was established. Previously described large animal models have mostly used diluted whole blood as perfusate [47, 48, 177]. However, we hypothesized that the presence of leucocytes and thrombocytes may be undesirable in an extracorporeal setting, because ischemia is known to lead to endothelial activation [17]. Reperfusion with whole blood may therefore lead to recruitment of neutrophils, activation of clotting cascades and inflammation [21]. Additionally, our initial results with diluted whole blood were poor. Therefore, isolated washed autologous erythrocytes were used as oxygen carrier, these were combined with a tissue culture medium optimized for hepatocyte culture and autologous plasma, to create our machine perfusion solution (MPS). As opposed to buffer solutions commonly used for the IPRL, the use of a tissue culture medium provides the liver with a broad panel of metabolites, including essential and no-essential amino acids, as well as anti-oxidants and vitamins. This way, optimally supporting liver metabolism [78, 79] and potentially stimulating protein synthesis and repair processes.

In order to reduce the number of animals required to provide erythrocytes, reduction of the priming volume of the system was crucial. However, due to the limited buffering capacity of a small circulating volume, our initial perfusion attempts suffered from gradual perfusate acidosis and depletion of metabolic substrates. Therefore, we added a secondary dialysis circuit to our setup. By using the same hepatocyte medium that formed the basis of our MPS, as dialysate, the liver was provided with an ample pool of substrates, while at the same time preventing accumulation of metabolites and preventing acidosis. The necessity of dialysis during perfusion was disputed at the time and the recent landmark study by Brockmann did not include dialysis [19, 178]. However, at the time our experiments were planned, the only previous study demonstrating survival transplantation after normothermic perfusion regarded dialysis as crucial to its success [47].

With our newly developed model, we were able to demonstrate stable liver function during 6 hours of NMP at physiological flow rates, indicated by stable oxygen uptake, linear bile secretion rate and stable synthetic function. After perfusion, the histological appearance

was indistinguishable from that of fresh-excised control livers. Electron microscopy revealed normal morphology of mitochondria and nuclei. Orthotopic transplantation into syngeneic recipients resulted in 100% one-month transplant survival, hereby eliminating the idea that the perfused rat liver is to be considered a dying organ. Extending perfusion to 12 hours also resulted in transplantable grafts with excellent one-month survival in a limited number of cases, but due to technical and logistic problems, the results were difficult to reproduce and therefore not published.

Resuscitation of ischemic rat livers

The results in the third chapter describe NMP for resuscitation of rat livers subjected to 60 min of warm ischemia. During NMP ischemic livers showed an initial sharp increase in the release of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the first 120 minutes of perfusion, after which levels normalized throughout the remaining duration of the perfusion, indicating that little or no further damage occurred during perfusion. Interestingly, this time profile is concurrent with the ALT/AST peak observed during clinical hypothermic machine perfusion [39] and after liver transplantation [23].

Cumulative bile secretion of ischemic livers was about 30 % lower than that of fresh livers. Whereas fresh livers secreted bile almost immediately at a constant rate, bile secretion of ischemic livers was virtually absent during the first hour of NMP, after which the rate of secretion constant. Since bile secretion is an energy dependent process [179], this suggests that the replenishment of depleted adenosinetriphospate (ATP) stores occurs within the first hour of perfusion, after which a steady state seems to occur. When comparing the synthetic function of the ischemic and fresh livers, an increase urea production could indicate proteolysis due to cellular damage.

Reperfusion experiments with diluted whole blood showed similar results, although the metabolic function of the NMP recovered livers was lower than that of healthy controls, the release profiles of ALT and AST and bile secretion ischemic livers were very similar. Non-treated ischemic livers showed significantly higher transaminase profiles and lower bile secretion. When comparing the bile secretion of ischemic and fresh excised livers during the first two hours of NMP, bile secretion was about half in the ischemic group. During ex-vivo reperfusion, bile secretion of ischemic livers is about a third of that of fresh excised livers. These results indicate that a controlled reperfusion in absence blood components such as leucocytes leads to better recovery of function, confirming the validity of using isolated erythrocytes rather that whole blood in our MPS. However, the presence of hydrocortisone in the MPS solution vs. the absence during reperfusion may have contributed to these findings.

After transplantation of NMP resuscitated ischemic livers, survival of recipients was close to 100% at 28 days, which was similar to recipients of healthy cold-stored control livers. The animals that received cold stored ischemic livers all died with 12 hours. The recipients of ischemic livers that were directly transplanted all died within 24 hours.

An interesting observation was that recipients of ischemic NMP preserved livers had lower serum levels of ALT and AST the first week after transplantation. However, the serum levels of the transaminases normalized in both groups and were not significantly different at 28 days after transplantation. Initial bilirubin levels showed a similar, albeit not as dramatic trend. All groups subsequently demonstrated a gradual increase in serum bilirubin levels, which at day 28 after transplantation levels were not significantly different from each other.

These results suggest an early protective effect of ischemia when paired with NMP. It is known that non-lethal cellular stress, such in-vivo ischemia, can induce protective mechanisms such as upregulation of heat shock protein (Hsp) expression [180-183]. Although this possibility remains to be investigated, Hsp overexpression could be part of the underlying mechanism in the findings we observed. Another plausible explanation for this phenomenon is quiescence of Kupffer cells and the subsequent suppression of inflammatory response by the hydrocortisone in the perfusate [95]. On the other hand, dexamethasone was present in much higher concentration in the UW solution, and this effect was not observed in the cold-storage group.

The results of both our reperfusion and survival experiments show that 1) NMP is an effective preservation method for ischemic rat livers; 2) an additional period of cold ischemia after warm ischemia is detrimental to organ viability 3) since ischemia alone resulted in 100% recipient death, NMP does not only prevent further damage by avoiding further (cold) ischemia it actually reverses the negative impact of warm ischemia.

Most likely, these results can be explained by a combination of controlled reperfusion in the absence of whole blood, avoidance of cold-storage induced damage, as well as restoration mitochondrial function and replenishment of cellular energy stores [184, 185]. Furthermore, NMP may enable induction of cytoprotective mechanisms that alleviate ischemia-reperfusion damage associated with transplantation, but the underlying mechanism remain to be elucidated. Although in-depth analysis was not performed due to technical difficulties, we observed higher ATP levels in livers preserved with NMP (unpublished results).

Combined cold storage and NMP

Chapter four describes how rat livers subjected to 45 min of warm ischemia could be resuscitated with an initial 3 hours of NMP, followed by an additional 3 hours of cold storage. Although livers were subjected to a shorter period of ischemia, the release profiles of ALT and AST and metabolic behavior during perfusion were similar to the results in the previous chapter.

After transplantation, the post-operative enzyme levels of the recipients of livers preserved with combined NMP and cold storage were elevated on day 1, but remained below those of SCS liver recipients beyond that point until the last time point on day 7 after transplantation. Compared to the animals that received ischemic livers preserved with NMP alone, enzyme levels were higher during the first week of follow-up. Although the ischemic period in both groups was different (45 vs. 60 min) and the total preservation time was longer (360 vs. 300 min.), these results indicate that although a combination of both modalities is possible, it is probably not ideal.

Transplant survival was 100% at 30 days for the combined perfusion and cold storage group, this was comparable to the animals that received fresh livers stored in UW solution for 6 hours. The animals that received ischemic livers preserved with SCS all died within 24 hours after transplantation.

The one-month postoperative survival of 100% clearly shows that the restorative effect of NMP is not lost when combined with an equal period of cold storage. Moreover, it shows that replenishment of intracellular energy stores, as indicated by bile secretion, likely occurs within the first few hours of ex-vivo reperfusion. Preliminary transplant results (unpublished data) of ischemic livers preserved by a period of initial cold storage followed by NMP were invariably poor and consequently, this approach was not further pursued.

Subnormothermic machine perfusion

During preservation, the livers preserved with SNMP at 30^{o}C displayed no significant differences in levels of ALT and AST release when compared to livers ischemic livers preserved with NMP. By contrast, livers preserved with SNMP at 20^{o}C showed an increasing trend in AST release, this was only significantly different between groups at one time-point. ALT released did not differ significantly over the course of time between groups, but overall the release was higher in the 20^{o}C SNMP group. Bile secretion was not significantly different amongst the three groups, however the oxygen uptake rate of livers perfused at 20°C was 43% lower compared to NMP preserved livers.

During ex-vivo reperfusion, bile secretion decreased with lower machine perfusion temperature. The release of transaminases was not significantly affected and approached the low levels observed in fresh excised livers. Interestingly, oxygen uptake during reperfusion of livers preserved with NMP was lower than that of those preserved with SNMP at 20°C. Subsequent histological analysis showed evidence of necrosis in WI and WI+SCS groups, not seen in any of the other groups; none of the groups showed any signs of apoptosis within this time frame. A follow up of postoperative transaminase profiles over the course of 28 days postoperatively showed lowest ALT and AST levels in the livers perfused at 20°C and 30°C. Curiously, although all groups showed an increasing trend in total bilirubin, the increase was the highest, although statistically non-significant, in the livers preserved at 20°C and 30°C.

These findings demonstrate that resuscitation of ischemic rat liver can occur at temperatures ranging from ambient room to body temperature. Recovery of the hepatic parenchyma is optimal at the lower end of the spectrum, but damage to the biliary epithelium is best ameliorated under physiological conditions. It is noteworthy, that during SNMP at 20°C, the livers oxygen requirement is lower than the capacity to deliver oxygen if one was to omit erythrocytes. Therefore it is likely, that subnormothermic perfusions at 20°C may eliminate the need for an oxygen carrier. However, since erythrocytes are also known to act as scavengers of free radicals in presence of supra-physiological oxygen levels [186], the addition of a radial scavenger such as superoxide dismutase will likely be necessary when an perfusate not containing erythrocytes is used.

Comprehensive metabolic analysis during perfusion

Metabolic flux analysis (MFA) demonstrated sustained organ stability during perfusion. Behavior of the WI livers was similar to that of fresh livers from 120 min of perfusion onward. These findings confirm our hypothesis that the primary resuscitative effect of NMP occurs in the first two hours of perfusion. Functional stability was demonstrated through linear changes in metabolite concentrations after the first hour of perfusion. MFA revealed an expected response to the high perfusate insulin concentrations by showing glucose uptake, glycogen storage and glycolysis. The typical precursors for gluconeogenesis, alanine and lactate uptake were down regulated. The citric acid cycle (TCA), ketone body production and fatty acid oxidation, were all reduced, concurrent with a high-energy state. Resuscitation of ischemic livers demonstrated a distinct change in liver metabolism at t=2hrs. During the initial two hours of perfusion, ischemic livers were significantly more glycogenolytic than fresh livers at NMP onset; the oxygen uptake rate and the TCA cycle were comparable between groups. Between 2-5hrs of WI perfusion WI livers appeared to be gluconeogenic and demonstrated a 30% reduction in lactate output

compared to fresh livers. In both groups we observed a suppression of lipid oxidation, likely due to the high perfusate insulin levels, and extensive metabolization of amino acids. There was no evidence of substrate limitation throughout perfusion.

Curiously, although both fresh and ischemic livers did not utilize all the oxygen provided, MFA revealed a level of hypoxia independent of ischemic injury. Although oxygen uptake rates and bile secretion indicated stable liver function during perfusion, metabolic analysis showed that oxygen delivery was sub-optimal in both groups. This may be due to microcirculatory disturbances caused by thrombosis or dysregulation of the vasomotor control, but may also be a result of the portal vein only route of perfusion. Additionally, elevated levels of free hemoglobin, due to progressive mechanical damage to erythrocytes, are know to cause vasoconstriction by scavenging nitric oxide and thus disturbing the endothelin-nitric oxide balance[187]. Therefore, one of the first targets for improving our NMP model is optimization of hepatic microcirculation to improve oxygen delivery. This may be achieved by perfusate enrichment with vasodilators such nitric oxide (NO)-donors [188, 189], and compounds such as prostacyclin [177] or by dual vessel perfusion using both the portal vein and hepatic artery.

Viability assessment during NMP

An advantage of MP over conventional organ preservation with SCS is the possibility to measure the release profiles of various markers of liver injury during perfusion. Apart from the most commonly used markers for hepatic injury, ALT and AST, various biomarkers have been shown to correlate with hepatic viability during machine perfusion. These include beta-galactosidase [190], glutamate dehydrogenase (GLDH) [191], glutathione-S-transferase-a (a-GST) [192] and hyaluronic acid [193]. Our results indicate that in our model of ischemic liver resuscitation using NMP, recipient survival could be predicted using a single parameter, the release of ALT. ALT is an enzyme that is found in high concentrations in hepatocytes and is considered an accurate indicator of liver failure clinically. It is important to note that purely statistical methods were employed, therefore, the uses of ALT as predictor of viability applies only to this specific combination of models used.

Metabolic analysis for MPS optimization

Multivariate analysis demonstrated that fresh and ischemic rat livers display clearly distinguishable metabolic behavior. Furthermore, using the multivariate square prediction error (SPE_k) statistic, it was possible to construct a single value index of ischemia. Since this analysis can be performed on an online basis, the degree of resuscitation can be evaluated during perfusion. Hereby allowing possible interventions, such as alterations of perfusate composition, perfusion temperature, oxygen levels etc., during preservation in order to

enhance recovery. Extending the analysis to data of perfused ischemic organs determined to fail after transplantation, the same approach could be used to predict transplantability. Assessment of this index of ischemia revealed that from t=2 hr onward, the metabolic profile of the ischemic livers had assumed that of non-ischemic controls. This supports our previous finding that the greatest effects of resuscitation is seen during the first 2 hours of perfusion which explains the success of the combined approach of short-term MPS, followed by cold storage. Such an index of ischemic injury, based solely on organ function, could be combined with previously mentioned indicators if hepatic viability, and thus could simplify the surgical team's decision process whether to use or to reject, an organ for transplantation. This could not only lead to better transplant outcome, it could also reduce the number of organs that are discarded unnecessarily.

Prediction of graft viability and optimum perfusion duration

Here we provide statistical evidence that liver function is correlated to transplant success and that blood gas readings can be used to predict transplant success and identify the optimum perfusion duration. Cross-validation with actual transplantation results indicated excellent sensitivity and specificity in all cases. These correlations could be improved upon by coupling metabolic analysis approaches, such that the correlations to viability at the macroscopic level (such as oxygen uptake) can be traced down to specific pathways which could be used to develop interventions to improve organ viability. Interestingly, about viability of 50% of the livers could be determined by the first data point at 1 hour of perfusion. Therefore, in the context of resuscitating ischemic liver grafts, particular attention should be directed towards the early perfusion function where pharmacological or metabolic supplementation may be crucial to whether the organ can be salvaged or not.

Limitations of our approach

One of the evident limitations of our approach is the use of portal vein only route of perfusion the use of a non-arterialized transplant model. These decisions were largely dictated by technical limitations at the time. Although non-arterialization of transplanted rat liver is common and accepted [69], it is known to result in biliary proliferation of the transplanted livers since blood supply to peribiliary vascular plexus mainly takes place through the hepatic artery [94]. This is likely to have influenced our post-operative findings, in particular the gradually increasing serum bilirubin levels we observed in all our recipients. Since biliary complications are the most common long-term complication associated with the used of NHBD, our model would increase in clinical relevance when future experiments are performed with reconstruction of the hepatic artery. Furthermore, portal vein-only perfusion is likely partially responsible for the hypoxia revealed by the dynamic metabolic analyis during perfusion. Therefore, to improve perfusion results and

for added validity of the model, dual vessel perfusion should be included in future NMP and SNMP studies.

A further limitation of our experimental design is the use of ex-vivo warm ischemia to model NHBD and the use of heparin before explantation of the livers. Ex-vivo warm ischemia was preferred over in-situ warm ischemia as to have precise control over the temperature and duration of ischemia and has been previously been used by others [37]. The clinical validity of administration of heparin before induction of ischemia can be debated. In case of controlled NHBD, administration of heparin before cardiac arrest is imaginable, whether it is ethically acceptable can be disputed. Nevertheless heparinization has been previously used in NHBD animal models [37] and administration of heparin has been used clinically before onset of cardiac arrest in controlled NHBD [7].

The preservation times described in this thesis are relatively short, but were dictated mainly by logistical and mechanical constraints. In particular, hemolysis by mechanical damage to erythrocytes was a limiting factor in our experiments and we observed a gradual decrease in hematocrit and increase in perfusate methemoglobin levels as perfusion time elapsed. This was likely due to the use of standard peristaltic pumps in our perfusion circuit and the blood-air contact that occurred in our perfusion chamber. In large animal studies, these issues seem to be less of a limiting factor due to the availability of a wide array of specialized components, such as centrifugal pumps, oxygenators, bioactive tubing etc. designed for cardio-pulmonary bypass. Downscaling components to maintain a low priming volume was one of the biggest challenges we faced during the establishment of our model. Nevertheless, we were able to successfully transplant livers that had been perfused for up to twelve hours under normothermic conditions (unpublished data).

Future directions

The possibility of donor pool expansion, longer safe preservation times, and prediction of graft viability make NMP and SNMP the most promising of currently developed preservation modalities. The recent study by Brockmann [19] has demonstrated successful transplantation of both fresh and NHBD-pig livers after up to 20 hours of machine perfusion. In this regard, theoretically, there is no upper limit to the duration of normothermic preservation without compromising organ viability. These promising results, along with the successful clinical application of extracorporeal membrane oxygenation for resuscitation of uncontrolled NHBD livers [53], clearly justify clinical evaluation of NMP.

For NMP to become clinical reality, not only will superior graft quality have to be demonstrated, but a clear benefit will also have to be shown in regard to reduction of waiting list associated mortality. For this, a machine perfusion device will have to be developed that is simple to operate, portable and cost-effective. Technology will need to be incorporated that enables real-time monitoring metabolic of function, as well as non-invasive methods to assess hepatic viability, such laser-doppler flowmetry to assess hepatic microcirculation and near-infrared spectroscopy (NIRS) to assess hepatic energy charge [188]. To enable transportation to and from transplant centers, down-scaling of components will have to occur, since most circuits that are in use today originate from not quite portable cardiopulmonary bypass equipment. These issues present technological and logistical challenges that need to be overcome before NMP can be considered a viable alternative to SCS. That this is not science fiction, but is likely to occur in the near future, is illustrated by the fact that similar technology is currently being evaluated clinically in both the United States and Europe for normothermic cardiac preservation [194].

If the promising results with SNMP can be reproduced in large animal models, less elaborate technology may be necessary, enabling the use of existing liver perfusion devices [34] and perhaps even of disposable devices such as proposed by v. Gulik [35]. Alternatively, in-situ normothermic reperfusion with ECMO could be performed at the site of explantation, after which the organ could be stored on ice during transportation. Although this approach was initially limited in its success at salvaging NHBD livers [52], recent results indicate 1-year transplant survival after transplantation of uncontrolled NHBD livers that is comparable to that of heart beating brain dead donors [53].

Further development of machine perfusion solutions will also aid acceptance of NMP by clinicians. Although the results NMP with diluted blood are impressive, it is not an ideal perfusate for an ischemic organ due to the likely interaction of the organ's activated endothelium with blood components. This could be partially overcome by the use of leucocyte depletion filters, as is routine practice in cardiopulmonary bypass and experimental cardiac machine perfusion. However, since the organ donor is likely also to be the source of blood to base the MPS on, there are further potential problems that need to be overcome. When NMP is used for perfusion organs from brain dead donors, the onset brain death is known to cause a so called cytokine storm, releasing into the blood soluble compounds that negatively impact organ quality [195]. Also, if NMP becomes a mainstream approach and is adopted for preservation of other organs, it is likely that the blood supply of a single donor, potentially already diminished by blood loss during surgery or trauma, could become a limiting factor. Therefore, alternative oxygen carriers, such as polymerized

hemoglobins, need to be explored [187, 196]. Alternatively, oxygen carrier may no longer be of critical importance if SNMP can be applied clinically.

Due to the high metabolic demand of an organ perfused under normothermic conditions, sufficient metabolic substrates will need to be available to the organ. This could be achieved by formulating an enriched, off-the-shelf MPS such as Polysol [78], which has been used with success experimentally for hypothermic machine perfusion of various organs. Alternatively, an MPS could be based on a buffer solution, to which nutritional supplements can be added on demand as dictated by diminishing levels or predicted benefit [177]. Furthermore, supplements may include therapeutic targets to attenuate tissue injury and organ dysfunction, such as antioxidative, antiadhesive, and anti-inflammatory compounds [188].

The potential uses of NMP are not limited to improved NHBD preservation. NMP opens the door to a wide array of possible applications such as ex-vivo induction of protective mechanisms, including synthesis of heat-shock proteins [92] and improving graft quality of steatotic livers by ex-vivo "defatting" during machine perfusion [103, 197] as well as for genetic modification of donor organs to reduce post-transplant immune response [198]. Organs that prove to be unsuitable for orthotopic transplantation could be used for hepatic support procedures [199] or hepatocyte transplantation [200]. In this context, Uygun and colleagues recently described organ re-engineering, a potential further use of NMP [201]. Here, a process of liver de-cellularization followed subsequent re-population with autologous hepatocytes was demonstrated to yield transplantable liver grafts that support hepatic function and is proposed as potential method to change otherwise discarded livers into transplantable grafts.

NMP will also benefit from greater understanding of the mechanisms underlying it's resuscitative properties. For the development of new technology, the porcine model is clearly most suited due to anatomical similarities to humans. However, optimization of NMP, including finding the ideal perfusion conditions and perfusion solution, can only occur when deeper insight into gained into the mechanisms underlying NMP. Combined with the described metabolic tools and including planned improvements such as dual vessel perfusion, greater biocompatibility, automated control and feedback systems, as well as the possibility to perform intra-vital microscopy during perfusion and reperfusion, we believe our model is an ideal platform for this.

Nederlandse samenvatting

Samenvatting Nederlands

De in dit proefschrift beschreven experimenten zijn uitgevoerd van 2004-2008 toen ik als research fellow in het Center for Engineering in Medicine van het Massachusetts General Hospital en Shriners Burns Institute werkte. Aanvankelijk was het werk in het lab voornamelijk gericht op het bestuderen van de stofwisseling van de lever. Voor dit doeleinde werden levers van ratten onder verschillende omstandigheden ex-vivo geperfundeerd. Gaandeweg raakte ik geïnteresserd in de mogelijkheid het model dat we hiervoor gebruikten, the isolated perfused rat liver (IPRL), als basis voor een verbeterde leverpreservatiemethode te gebruiken. Deze methode, normothermic machine perfusion (NMP), zou de nadelen van gangbare methoden om de lever voor transplantatie te bewaren kunnen omzeilen en daardoor leiden tot betere levensvatbaarheid van donor levers. Uiteindelijk zou NMP kunnen worden toegepast om levers die normaal niet als donororgaan geschikt zijn, bijvoorbeeld na schade door een periode van zuurstofgebrek, voor transplantatie bruikbaar te maken.

De eerste succesvolle menselijke levertransplantatie werd in 1967 aan de universiteit van Colorado door een team onder leiding van Thomas Starzl uitgevoerd. Sindsdien is een levertransplantatie nog altijd de enige curatieve behandeling voor patïenten in het eindstadium van leverfalen. Jaarlijks worden in de Verenigde Staten en Europa tot 12.000 levertransplantaties gedaan met een 5-jaars overleving van meer dan 70%. Helaas overlijdt elk jaar een toenemend aantal patïenten voordat een donorlever beschikbaar is, door een groeiend tekort aan beschikbare donorlevers.

Tegenwoordig zijn de meeste donororganen afkomstig van hersendode donoren. Meestal zijn dit patïenten in coma na zwaar hersenletsel die kunstmatig in leven worden gehouden en bij wie hersendood is vastgesteld. Als toestemming is verleend om de organen voor transplantatie te gebruiken, wordt begonnen de lever en andere bruikbare organen te verwijderen terwijl het hart van de donor nog klopt en de organen van zuurstof voorziet. Deze donoren worden daarom heart-beating donors (HBD) genoemd. Helaas is het aantal HBD ontoereikend om in de behoefte aan donororganen te voorzien.

Het tekort aan beschikbare donorlevers zou kunnen worden teruggedrongen door levers te gebruiken van donoren die overleden zijn na hartstilstand, zogenaamde non-heart beating donors (NHBD). Het gebruik van NHBD-levers is echter geassocieerd met een verhoogd risiko voor de ontvanger van het orgaan. Bij HBD wordt de lever onmiddellijk na het afklemmen van de bloedtoevoer met een speciale koude bewaarvloeistof gespoeld. Hierdoor wordt de lever afgekoeld tot net boven het vriespunt. Het doel hiervan

is de zuurstofbehoefte van de lever te minimaliseren tot de bloedtoevoer tijdens de transplantatie hersteld wordt. Deze periode van zuurstofgebrek wordt "koude ischemie" genoemd.

De ontwikkeling van gespecialiseerde bewaarvloeistoffen, in het bijzonder University of Wisconsin (UW) solution door Folkert Belzer in de jaren tachtig, heeft ertoe geleid dat gezonde donorlevers ondergedompeld in bewaarvloeistof tot 12 uur bewaard kunnen worden voor transplantatie. Hoewel doeltreffend, goedkoop en eenvoudig uitvoerbaar, heeft deze methode, simple of static cold storage (SCS), helaas ook een aantal nadelen. De bewaartemperatuur van net boven het vriespunt zorgt ervoor dat de zuurstofbehoefte een fractie is van die op lichaamstemperatuur, maar zelfs bij 0-4 ^{o}C is deze echter niet nul. Door zuurstofgebrek schakelt de lever van aëroob naar anaërobe stofwisseling om en raken de intracellulaire energievoorraden geleidelijk uitgeput. Omdat circulatie afwezig is, hopen potentieel giftige afvalstoffen zich gaandeweg op. Verder leidt de combinatie van koude temperaturen en zuurstofgebrek tot het optreden van zogenaamde oxidatieve stress. Uiteindelijk leidt dit tot verlies van ingrediënten over de celmembraan, celzwelling en uiteindelijk celdood. Hoewel tegenwoordig gebruikte bewaarvloeistoffen verschillende stoffen bevatten die de schadelijke effecten van koude ischemie tegengaan, is de tijd dat koude ischemie door de lever verdragen wordt beperkt. Een verder nadeel van SCS is dat terwijl het orgaan in bewaarvloeistof ondergedompeld is, er geen inschatting gemaakt kan worden van de levensvatbaarheid van het orgaan.

Bij NHBD treedt hartstilstand op voordat de organen verwijderd worden. Hierdoor is een periode van zuurstofgebrek bij lichaamstemperatuur, zogenaamde warme ischemie, overmijdelijk. Deze periode van warme ischemie leidt ertoe dat organen van NHBD gevoeliger zijn voor de schadelijke gevolgen van koude ischemie, waardoor de kans op complicaties na transplantatie onaanvaardbaar hoog is. Daarom worden levers van NBHD in de regel niet voor transplantatie gebruikt.

Het tekort aan donororganen heeft een zoektocht op gang gebracht naar alternatieve bewaarmethoden, die beter geschikt zijn voor NHBD levers en veilige transplantatie van deze ischemische levers mogelijk maakt. NMP is een van deze methoden. In plaats van de lever op ijs te bewaren om de stofwisseling te remmen en zuurstofbehoefte te minimalisieren, wordt het orgaan bewaard onder omstandigheden die de normale fysiologische omgeving benaderen. Om dit mogelijk te maken wordt de lever gedurende de bewaarperiode door een pomp continu van een warme, zuurstof- en voedingstoffenrijke bewaarvloeistof voorzien. Op deze manier wordt zuurstofgebrek tijdens de bewaarperiode vermeden, kunnen afvalstoffen weggespoeld worden en wordt oxidatieve stress geminimaliseerd.

In de context van NHBD levers, stelt NMP het orgaan in staat zich te herstellen van de gevolgen van warme ischemie en maakt op deze manier veilige transplantatie van deze organen mogelijk. Een bijkomend voordeel van NMP is, is dat het een inschatting mogelijk maakt van de levensvatbaarheid van het orgaan en daarmee het risiko op een mislukte transplantatie gereduceerd wordt.

Hoewel de eerste NMP-experimenten in de jaren dertig al door Carrel en Lindberg beschreven waren en op het gebied van lever NMP indrukwekkende resultaten waren gepubliceerd, bestond toen ik met mijn promotieonderzoek begon, nog geen klein diermodel voor NMP . Hierdoor was toegang tot onderzoek op het gebied van normotherme leverpersfusie door de hoge kosten en complexe logistiek beperkt tot slechts enkele gespecialiseerde chirugische onderzoeksgroepen. Daarom was ons aanvankelijke doel een klein diermodel voor NMP te ontwikkelen dat geschikt was om in een laboratorium gebruikt te worden en gecombineerd zou kunnen worden met zowel ex-vivo reperfusie experimenten als transplantatiestudies en daarmee en een bijdrage zou kunnen leveren aan de ontwikkeling van dit onderzoeksgebied.

Hoofdstuk twee beschrijft de ontwikkeling van een klein proefdier model voor NMP. Als basis voor ons model werd het IPRL model gebruikt. Omdat het IPRL-model van origine voor kortdurende experimenten gebruikt werd, waren een aantal wijzigingen noodzakelijk om het model geschikt te maken voor leverpreservatie. De belangrijkste hiervan waren: 1) de toevoeging van een zuurstofdrager om de lever tijdens de perfusie van genoeg zuurstof te voorzien 2) een verkleining van het circulerende volume om het gebruik van autologe rode bloedcellen als zuurstofdrager mogelijk te maken 3) een bron van voedingstoffen te bieden 4) afvoer van door de lever geproduceerde afvalstoffen mogelijk te maken. Om dit te bereiken, onwierpen we een perfusiesysteem met een volume van circa 60 ml, inclusief een dialysecircuit dat diende om afvalstoffen te verwijderen en tegelijkertijd voedingstoffen te leveren. Als perfusievloeistof gebruikten we een weefselkweekmedium in combinatie met erytrocyten als zuurstofdrager, verrijkt met autoloog plasma. Deze wijzigingen stelden ons in staat gezonde rattenlevers tot 12 uur stabiel bij 37°C te perfunderen en vervolgens probleemloos te transplanteren.

Hoofdstuk drie beschrijft de preservatie van ischemische rattenlevers. Hiervoor werden levers geëxplanteerd en vervolgens aan een periode van warme ischemie blootgesteld door ze een uur in in een physiologische zoutoplossing van 34 °C te bewaren. Vervolgens werden ze gedurende 5 uur ofwel met SCS bewaard bij 4°C, of met NMP bij 37 °C en aansluitend orthotoop getransplanteerd. Van de dieren die ischemische levers

getransplanteerd kregen die met NMP bewaard waren, overleefde bijna alle meer dan 4 weken na de transplantatie. De dieren die ischemische levers onvingen die met koude preservatie bewaard waren overleefden na transplantatie minder dan 24 uur.

Een nadeel van NMP is dat het technisch lastig uitvoerbaar zou kunnen zijn tijdens transport naar een transplantatiecentrum. Dit zou kunnen worden opgelost door een NMP voor of na een periode van SCS plaats te laten vinden. De resultaten die in hoofstuk drie worden beschreven, suggereren dat de schadelijke gevolgen van een periode van ischemie in de eerste 60-120 minuten van NMP ongedaan worden gemaakt. Wij veronderstelden daarom dat een korte periode van NMP, genoeg om de lever van een periode van ischemie te laten herstellen, gevolgd door een periode van SCS tijdens tranport van het orgaan, een oplossing zou kunnen bieden. Hoofdstuk vier beschrijft hoe ischemische rattenlevers na 3 uur NMP gevolgd door drie uur koude preservatie probleemloos getransplanteerd konden worden. Dieren die ischemische levers ontvingen met meer dan 6 uur bewaartijd met SCS overleefden alle minder dan 24 uur.

Hoewel we met NMP bij 37 °C uitstekende resultaten hadden, vroegen we ons af, of 37 °C de optimale temperatuur voor MP was. Een lagere temperatuur tijdens preservatie zou kunnen leiden tot een verminderde zuurstofbehoefte, minder verbruik van voedingstoffen en een verminderde productie van afvalstoffen. Hierdoor zou een eenvoudiger perfusiesysteem zonder dialysecircuit gebruikt kunnen worden en mogelijk zelfs een zuurstofdrager overbodig worden. In Hoofdstuk vijf beschrijven we hoe rattenlevers na 60 minuten warme ischemie ook na 6 uur machine-perfusie op kamertemperatuur (20C) en bij 30 °C probleemloos getransplanteerd konden worden.

Hoofdstuk 6 beschrijft hoe eveneens met behulp van statistische modellen, het gedrag van gezonde en ischemische levers tijdens machinepreservatie kan worden vergeleken met de resulaten na transplantatie van dezelfde groepen. Dit heeft ons in staat gesteld om voor ons model parameters aan te wijzen die duidend zijn voor de gezondheid van de lever tijdens perfusie. Een of meerdere tijdens de machine meetbare parameters zou kunnen leiden tot veiligere transplantaties door een betere risiko inschatting mogelijk te maken. In ons specifieke model bleek het niveau van het leverenzym alanine-amino transferase, het best te correleren met het overleven na levertransplantatie.

Hoofdstuk 7 beschrijft hoe met behulp van een wiskundig model aan de hand van 28 tijdens de ex-vivo perfusies gemeten en 34 berekende metabole parameters, de stofwisseling van de geperfundeerde lever in kaart kan worden gebracht. Door de metabole profielen

van ischemische en gezonde levers te vergelijken, kan inzicht worden verkregen in de verschillen tussen beide en kunnen gerichte veranderingen aan de samenstelling van het perfusaat en de condities gedurende de perfusie worden gemaakt.

Hoofstuk acht beschijft hoe met behulp van wiskundige modellen de algemene gezondheid van donorlevers kan worden berekend door de tijdens machineperfusie in kaart gebrachte stofwisseling van de geperfundeerde levers te correleren met succesvolle transplantatie. Door vervolgens hetzelfde proces te volgen bij ischemische levers, kan de levensvatbaarbeid van deze organen worden ingeschat op basis van hun metabole profiel. Op zijn beurt zou dit kunnen worden gebruikt om de kans dat de transplantatie van het orgaan wel of niet slaagt te voorspellen.

Hoofstuk negen beschrijft tenslotte hoe door analyse van de o.a. zuurstofopname door de lever tijdens perfusies, gecorreleerd aan het wel of niet succesvol kunnen transplanteren van de levers, een ischatting gemaakt kan worden over de levensvatbaarheid van het orgaan tijdens de perfusie.

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Addenda

List of abbreviations

ALB	Albumin
ALP	Alkaline Phosphotase
ALT	Alanine Aminotransferase
ANOVA	Analysis of Variance
AST	Aspartate Aminotransferase
ATP	Adenosinetriphospate
CBD	Common Bile Duct
DCD	Donors after Cardiac Death
ECMO	Extracorporeal Machine Perfusion
GLU	Glucose
HA	Hepatic Artery
HBD	Heart Beating Donor
HMP	Hypothermic Machine Perfusion
HOUR	Hepatic Oxygen Uptake Rate
IPRL	Isolated Perfused Rat Liver
IVC	Inferior Vena Cava
MFA	Metabolic Flux Analysis
MP	Machine Perfusion
MPCA	Multi-way Principal Component Analysis
MPS	Machine Perfusion Solution
NECMO	Normothermic Extracorporeal Membrane Oxygenation
NELP	Normothermic Extracorporal Liver Perfusion
NHBD	Non-Heart Beating Donor
NMP	Normothermic Machine Perfusion
ODR	Oxygen Delivery Rate
OER	Oxygen Exit Rate
OUR	Oxygen Uptake Rate
PLS	Partial-Least Squares
PV	Portal Vein
ROS	Reactive Oxygen Species
SCS	Static Cold Storage
SEM	Standard Error of the Mean
SNMP	Sub-Normothermic Machine Perfusion

SHVC	Suprahepatic Vena Cava
SPE	Square Prediction Error
SPM	Statistical Process Monitoring
TBIL	Total Bilirubin
TCA	Citric Acid Cycle
TP	Total Protein
UW	University of Wisconsin
WI	Warm Ischemia

List of Publications

A model for normothermic preservation of the rat liver.
Tolboom H, Pouw R, Uygun K, Tanimura Y, Izamis ML, Berthiaume F, Yarmush ML.
Tissue Engineering 2007 Aug; 13(8):2143-51

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Subnormothermic machine perfusion at both 20°C and 30°C recovers ischemic rat livers for successful transplantation.
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Diluted blood reperfusion as a model for transplantation of ischemic rat livers : ALT is a direct indicator of viability.
Uygun K, Tolboom H, Izamis ML, Uygun B, Sharma N,Yagi H, Soto-Gutierrez A, Hertl M, Berthiaume F, Yarmush ML.
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Resuscitation of Ischemic Donor Livers with Normothermic Machine Perfusion: A Dynamic Metabolic Analysis of Treatment in Rats
Izamis ML, Tolboom H, Uygun B, Berthiaume F, Uygun K , Yarmush ML.
In preparation

A metabolic index of ischemic injury for perfusion-recovery of cadaveric rat livers.
Perk S, Izamis ML, Tolboom H, Uygun B, Berthiaume F, Yarmush ML, Uygun K.
PLoS One. 2011;6(12)

Identification of Optimum Perfusion Duration of Machine-Perfused Rat Livers for Maximization of Transplant Success.
Perk S*, Izamis ML*, Tolboom H, Uygun B, Yarmush ML, Uygun K.
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Curriculum Vitae

Herman Tolboom werd geboren op 14 februari 1977 te Nijmegen. Een belangrijk deel van zijn jeugd bracht hij door in Lesotho, Zuidelijk Afrika. Na het behalen van het VWO-diploma aan het Kandinsky College te Nijmegen en een jaar studie farmacie wegens uitloting, begon hij in 1996 met de studie geneeskunde aan de Universiteit van Utrecht. Tijdens zijn studie was hij student-assistent bij de afdelingen Functionele Anatomie en Farmacologie en Toxicologie. Zijn wetenschappelijke stage doorliep hij bij de afdeling Vasculaire Geneeskunde (begeleider Prof. Dr. M.C. Verhaar). Na het behalen van het artsexamen in 2003, maakte begeleiding door Prof. Dr. I.H.M. Borel Rinkes (afdeling Algemene Heelkunde, UMCU) en een Fulbright fellowship het mogelijk om in november van dat jaar research fellow te worden aan het Center for Engineering in Medicine (Hoofd: Prof. Dr. M.L. Yarmush), Shriners Burns Hospital and Massachusetts General Hospital, Harvard Medical School (Boston, USA). Het onderzoek werd in het voorjaar van 2008 afgerond en vormt thans de basis voor dit proefschrift. Na deze periode van onderzoek volgde, als onderdeel van de opleiding in de chirurgie, een assistentschap bij de afdeling Viscerale en Transplantatiechirurgie (Hoofd: Prof. Dr. P.A. Clavien) van het Universiteitsziekenhuis Zürich (USZ). Thans werkt hij als arts-assistent en wetenschappelijk onderzoeker bij de afdeling Cardio-thoracale- en Vaatchirurgie (Hoofd: Prof. Dr. V. Falk) van het USZ.

Herman Tolboom was born in 1977, on the 14th of February, in the city of Nijmegen, The Netherlands. A significant part of his youth, he spent in Lesotho, Southern Africa. After completing his secondary education at the Kandinsky College (Nijmegen), followed by one year of Pharmacy, he entered the School of Medicine at Utrecht University in 1996. He completed his Master's in 2001 and received his MD in 2003. During his studies he was teaching assistant in the departments of Functional Anatomy, and Pharmacology and Toxicology, and did his first research in the field of Vascular Medicine (supervisor, Prof. Dr. M.C. Verhaar). Supervised by Prof. Dr. I.H.M. Borel Rinkes (department of General Surgery, Utrecht University Medical Centre) and supported by a Fulbright fellowship, he started a research fellowship at the Center for Engineering in Medicine (Head, Prof. Dr. M.L. Yarmush), Shriners Burns Hospital and Massachusetts General Hospital, Harvard Medical School (Boston, USA) in November 2003. The research was concluded in the spring of 2008 and forms the basis of this thesis. Upon his return to Europe, he started surgical training at the department of Visceral and Transplantation Surgery (Head, Prof. Dr. P.A. Clavien) of the University Hospital Zürich (USZ). At present, he is a cardiac surgery trainee and researcher at the department of Cardiovascular Surgery (Head, Prof. Dr. V. Falk) at USZ.