Ex vivo Perfusion Optimization of Donor Liver Grafts for Transplantation and Cell Isolation

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Abstract

There is a constant demand for enormous numbers of high quality hepatocytes in the fields of cell transplantation, pharmacotoxicology, tissue engineering, and bioartificial assist devices. The scarcity of viable hepatocytes necessitates the use of suboptimal sources including damaged donor organs that are not transplantable. Many of these organs have potentially reversible pathologies however, that could be treated via ex vivo perfusion thereby increasing their cell yield. With the intent to translate organ recovery by perfusion into the clinic, we engineered a very simple room temperature-operated ex vivo organ perfusion system to test a rat liver model of uncontrolled non-heart beating donors. Seventeen times as many hepatocytes were recovered from livers exposed to an hour of warm ischemia (WI, 34°C) compared to untreated WI livers in only 3 hours of perfusion. Further, fresh liver hepatocyte yields were also increased by 32% post-perfusion, demonstrating that both damaged and healthy donor livers could benefit from this methodology. A linear correlation between cell yield and tissue ATP content was established. This enables an accurate prediction of cell recovery during preservation and can be used as a direct measure of organ viability and the trajectory of organ recovery during perfusion resuscitation. Further, a strong correlation between perfusion flow rate and cell yield was also established supporting the use of flow rates as low as possible without causing hypoperfusion or oxygen deprivation. Morphologically and functionally, perfusion-isolated hepatocytes generally performed comparably or better than fresh hepatocytes in cell suspension and plate culture. Cumulatively, these findings strongly support the ubiquitous use of organ perfusion systems in the clinic for optimal enhancement of donor grafts.

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Chapter 1: Introduction

There is a multi-billion dollar demand for viable organs in the fields of organ transplantation, cell-based therapeutics, basic research, toxicology and drug development[1-5]. As hepatocyte transplantation emerges as a treatment alternative to liver failure[6] and bioartificial liver assist devices (BAL) enter clinical trials[7], identifying suitable sources of hepatocytes for these methods is becoming an immediate bottleneck. The current donor shortage is severe; as few as 2% of waiting-list candidates may receive an organ in any one year[8]. Hepatocytes subsequently derive largely from untreated discarded organs that are severely compromised, resulting in low yields and low viabilities[9,10]. Donor livers obtained after Cardiac Death (DCD) are the single largest untapped human organ resource because of high incidents of primary non-function. They are also the most likely to be completely restored if treated to be used for transplantation[11] or as a source of hepatocytes[12,13]. In this work, we engineer a new approach to enhancing organ viability of both healthy and ischemically damaged organs through treatment with extracorporeal perfusion. Upon scaling these findings up to human organs, the contribution will be significant in developing the practical resources available for a highly desired supply of human hepatocytes[14]. This enabling methodology will impact all current fields of demand due to improved hepatocyte quality and function. It will unfetter the development and clinical applicability of hepatocyte transplantation and BAL methods, offering new treatment options to thousands of patients with liver failure.

Hepatic Failure and Treatment Options. Approximately 30,000 patients die each year from end-stage liver disease in the U.S. Approximately 80% of patients have decompensated chronic liver disease and become too ill to tolerate liver transplantation. The remaining 20% die of acute liver failure primarily due to ischemia/reperfusion injury, alcoholic liver disease, viral hepatitis, idiosyncratic drug reactions, and acetaminophen poisoning[15]. Orthotopic liver transplantation is the only clinically proven effective treatment for patients with end-stage liver disease. Unfortunately, it is both very costly and limited by the shortage of organ donors. The number of patients on the liver transplant waiting list in the U.S. stands at ~17,000, with new additions of about 10,000 per year, while the number of transplanted livers is only ~7,000 per year (UNOS OPTN data as of July 2007). Furthermore, ~2,500 patients per year die on the waiting list or become too sick to transplant before a donor is found. Living and split liver donor techniques may be promising alternatives, yet they have represented less than ~5% of

the total number of transplants performed in the U.S.[16], and present significant risks for donors.

Xenotransplantation could provide a limitless supply of donor organs, but previous attempts in humans resulted in hyperacute rejection and death[17]. Another approach under consideration is hepatocyte transplantation, where one donor liver could potentially treat several patients. This still incurs the need for immunosupression, however, fresh cells are rare and the efficiency of engraftment is generally low, particularly with cryopreserved cells. Further, an unpredictable lag time of as much as 48h before any clinical benefit occurs[18] may be too long in a rapidly deteriorating patient. Yet, this approach offers an attractive prospect for correcting non-acute conditions such as genetic defects of the liver[19].

Extracorporeal BALs containing functioning viable hepatocytes could provide temporary support for patients with fulminant hepatic failure and patients awaiting orthotopic liver transplantation. Furthermore, since the liver has the ability to regenerate, temporary support may obviate the need for transplantation in some patients, which would reallocate donor livers as well as eliminate the need for life-long immunosuppressive drugs that must be taken by all transplant recipients. Several hurdles must be overcome for the development of BALs. Some of the issues that the field continues to wrestle with are: (1) lack of reliable – and preferably human[20] - cell sources; and (2) deteriorating hepatocyte function in the bioreactor environment.

Quantifying Cell Requirements and Identifying Alternative Cell Sources. The cell mass required to support an animal model of hepatic failure has not been systematically determined. Prior studies have shown significant improvements in various parameters using as low as 2-3% of the normal liver mass of the animal [21,22]. Devices which have undergone clinical testing have used $6x10^9$ – $1x10^{11}$ porcine hepatocytes [23,24] or $4x10^{10}$ C3A cells [25]. In a pig model of hepatic failure, a BAL containing as few as $6x10^8$ pig hepatocytes (about 3-5% of the liver mass) significantly improved survival [26].

Clinical improvements have also been seen in hepatocyte transplantation studies using less than 10% of the host's liver mass. Intrasplenic transplantation of $2.5x10^7$ allogeneic rat hepatocytes (~5% of the liver mass in this model) prolonged the survival and improved blood chemistry of anhepatic rats [27]. In another study using reversibly transformed human hepatocytes, $5x10^7$ cells were injected intrasplenically into 90% hepatectomized rats [28]. In a recent study on humans with acute liver failure, intrasplenic

and intra-arterial injections of human hepatocytes (ranging from $10^9 - 4x10^{10}$ per patient, i.e. 1-10% of the total liver mass) transiently improved blood chemistry parameters and brain function after a lag time of about 48 h, but did not improve survival [18]. The lag time before any benefit is observed may reflect the time required for the engraftment of the cells in the host liver. Better survival of the injected cells may be possible if they were seeded in prevascularized polymeric scaffolds[29]. The relatively low number of hepatocytes needed for a therapeutic benefit suggests the possibility that the exogenously supplied hepatocytes may aid the regeneration of the native liver[22]. Assuming that the minimum cell mass necessary to support a patient undergoing acute liver failure is about 5-10% of the total liver weight sets the requirement to about 10^{10} cells/BAL device for humans.

Recent clinical studies using extracorporeal liver perfusion strongly suggest that human and baboon livers are superior to pig livers for treating acute liver with this modality[30]. Human hepatocytes are therefore preferred for the clinical liver assist device; however, they are scarce due to the competing demand of livers for orthotopic transplantation as well as for hepatocyte transplantation. In BAL devices containing human cells tested so far, only one used adult hepatocytes, while the remainder were C3A hepatoma cells[25,31]. One study suggests that C3A cells have lower levels of P450IA1 activity, ammonia removal, and amino acid metabolism than adult porcine hepatocytes [32]. Furthermore, when using immortalized cell lines, there are concerns with the possibility of transmission of tumorigenic products into the patient. Although porcine cells are available in large quantities, they also pose risks of hyperacute rejection[33], transmission of zoonoses[34], and potential mismatch between pig and human liver functions[35]. The first two could be addressed by dedicated breeding programs of transgenic animals[36], but there is little known about the third factor.

The lack of availability of adult human hepatocytes would be solved if they could be induced to replicate in vitro; however, this has not been conclusively reported yet. Human hepatocyte cell lines have been developed via spontaneous transformation [37] as well as via retroviral transfection of the simian virus 40 large T antigen [38]. Reversible transformation with the SV40 T antigen and Cre-Lox recombination has been used to grow human hepatocytes in vitro [28]. These cells, when transplanted into the spleen of 90% hepatectomized rats, improved biochemical and clinical parameters. However, quantitative characterization of these cells in vitro suggests relatively low levels of expression of liver-specific functions (Shinoda et al., unpublished data). While no one

has achieved the goal of generating a safe, fully functional yet clonal, immortalized, or genetically engineered human cell that can be substituted for primary hepatocytes, new promising avenues have arisen from recent studies with fetal hepatocytes [39-41] and stem cells [42-44]. These approaches, however, are possibly decades away from clinical application, and the availability of fetal and stem cells itself remains a controversial issue.

Marginal Organs form a Major untapped Donor Organ Pool. Currently, the primary source of donor livers is donors after brain death (DBD, formerly described as "heart-beating cadavers") who have irreversible loss of brain function but have functioning circulation and respiration. The most common cause of rejection from the donor pool is hepatic steatosis, due to significantly decreasing graft survival rates [45]. Based on prior surveys, steatosis prevalence was evaluated to be 10-25% of all donated livers[46,47], and approximately 65% of donor livers that are rejected for transplantation are steatotic[48] – hence the potential pool of steatotic livers that could be transplanted is ~1,000/year. The second, potentially much larger alternative source is donors after cardiac death (DCD, formerly described as "non-heart-beating cadavers")[49]. The use of DCD liver grafts, which suffer from varying durations of ischemia, is considered an experimental procedure[50], although the practice is much more common in kidney transplants[51]. The total number of potential DCDs is estimated to exceed 6,000 annually[11]. (A similar ballpark estimate of 10-20% more livers is also given by White and Prasad, 2006). About 1,000 of these would be "controlled" according to 1995 Maastricht criteria, which means they are obtained from hemodynamically stable individuals following a controlled clinical death[52]. In these cases, the ischemic time, hence damage to the organ, is limited, and these organs are prime candidates for transplantation or as cell sources.

Based on the estimates in the literature as outlined above, the potential pool of DCD livers is roughly twice the current demand (6,000 vs. 3,000). Therefore, the DCD organs rejected for transplantation could easily match the need as sources of human hepatocytes for other treatment modalities, such as BAL devices, even if the yield of viable cells obtained from these organs is only 5% of the total liver mass. Currently, reports on yields from discarded donor organs indicate low retrieval. Alexandrova et. al.[53] reported a mean specific yield of 2.6-3.6 million cells per gram of liver tissue, with a viability of 64%. Baccarini et. al[13] reported more than 7 million cells per gram liver tissue, with a mean 73% viability. Hughes et al.[9] obtained on average 2.2 10^6 cells/gram tissue

with 51% viability from livers with an average of 15 minutes of warm ischemia and 13 hrs of cold storage in University of Wisconsin (UW) solution. The results all demonstrate a negative correlation between ischemic time and cell viability. The use of organs rejected due to steatosis, cirrhosis and traumatic injury have been considered favorably as sources of hepatocytes for in vitro [54,55] applications, as well as in limited clinical trials[56] though with equally low yields. However, the estimated number of suitable livers is about 1,000 [55], significantly less as compared to ischemic livers.

Ex vivo liver perfusion optimizes healthy and discarded donor organs. The current gold standard for organ preservation is static cold storage (SCS) in University of Wisconsin solution[57-61] and is based on the minimization of metabolism and enzymic activity by temperature reduction. The logistical ease of use (placing the organ on ice after flushing), rapid organ mobilization and transport has favored this modality for the last 50 years. However, organ storage duration is limited by the gradual depletion of ATP supplies[62,63] which, combined with the inability to directly and objectively measure organ function, favor the use of ex vivo machine perfusion. This is particularly relevant today as the rising demands for viable organs necessitate the use of marginal or extended criteria donors[64,65], which do not fare as well under SCS conditions[11,66-70]. Hypothermic machine perfusion (HMP) simply circulates cold oxygenated UW solution through donor livers at low flow rates and has demonstrated that the sustenance of tissue ATP levels reduces cellular damage, enables longer storage times and improved graft performance upon transplantation[71-74]. More recently, temperature-dependent differences have confirmed that even better graft function occurs above 20°C[75-77], thereby necessitating the use of machine perfusion for optimal organ storage. Many groups favor creating an ex vivo environment that mimics the in vivo state[11,67] because of several perceived advantages of operating at normothermic conditions[78-83]. The first and most significant is the proven ability to recover ischemically damaged organs to transplantable state using normothermic perfusion[84-86]. Though this has only been achieved in a very select number of cases, the impact of this additional source of transplantable organs to the critically limited donor pool will significantly enhance treatment options to thousands of patients per annum[14]. Normothermic perfusion also enables the development of clinically relevant metrics of organ viability prior to transplantation, from which it would be possible to derive pathology-specific treatment protocols for optimal organ preservation. These quantitative measures of organ stability, likelihood of primary non-function or delayed

graft function, and recovery to transplantable-state would replace subjective decision making on questionable organs and dramatically improve the number of successful outcomes[80].

Despite these promising results, they derive from animal models only and the desired metrics are as yet ideals. Only in renal transplantation is machine perfusion (hypothermic) an accepted clinical practice in a few centers around the country. Despite increasing evidence that machine perfusion results in better grafts, early results that were arguably biased in favor of static cold storage did not demonstrate significant differences in preservation techniques[87-90]. Regarding human livers, only one report of hypothermic machine perfusion has been published[91].

Establishing organ perfusion as an accepted and standard methodology for all donor organs depends on the development of highly reliable, automated, simple systems that are comprehensively and accurately monitored using parameters that are tightly coupled to organ function and transplantability.

Thesis Contributions. The intent of this thesis was to engineer practical ex vivo perfusion systems that significantly enhance the current donor pool and eliminate the hepatocyte shortage by optimizing discarded organs. A major expectation of this work was to minimize the number of parameters required to establish the status of viability in perfused organs. As the energy content of the organ is known to be critical to its viability[62,92-95], and the perfusate medium serves primarily as a reservoir of nutrients, the approach taken in this work focuses on the hemodynamic parameters governing the rate of nutrient flow into and out of the liver, enabling comprehensive metabolic analyses to form the framework for perfusion optimization and control.

We begin with the development of a unique model that examines the metabolic fluxes across rat livers in vivo. This provides us with a basis upon which to evaluate the parameters of our perfusion systems. We measure 24 different extracellular metabolic fluxes, including amino acids, glucose, urea, albumin, ketone bodies, blood gases, and lactate and use them to estimate an additional 37 intracellular fluxes of intermediary metabolism with a mass balance approach, or Metabolic Flux Analysis (MFA). These data provide us with a comprehensive integrated snapshot of basic metabolism in a healthy liver. We assess the validity of the model to reflect global changes in metabolism by examining its ability to detect variations present in mild to moderate

hypermetabolism. We find that several features of this state of elevated resting energy expenditure are accurately portrayed in an appropriately dose-dependent fashion.

We use this model to evaluate the impact of a simple ex vivo perfusion system on normal liver function. We use these results to determine intrinsic shortcomings in the perfusate that need to be altered in order to allow sustainable and physiological organ function. The perfusion system is used as a technique to evaluate the same moderate model of liver hypermetabolism analyzed in vivo. A comparison between in vivo vs. ex vivo outcomes shows that the liver responds quickly and significantly to its new ex vivo surroundings and does not accurately portray its in vivo metabolic rate during the first hour of ex vivo perfusion. Rather, this first hour reflects an adjustment by the liver to its new environment. Combined, these data provide the basis for the rational development of a physiologically relevant perfusion system and an appreciation for the time to achieve organ stability at 37°C.

A normothermic extracorporeal liver perfusion (NELP) is established using a modified cell culture medium as the perfusate, and erythrocytes as oxygen carriers to allow physiological flow rates of oxygen delivery. A primary circuit contains the erythrocytes which are adjusted to the optimal hematocrit with a dialyzer that forms the connection to the second circuit and perfusate reservoir. The system is tested on fresh livers which are perfused for 6 hours, an average static cold storage time, prior to transplantation into syngeneic recipients. We show the animals survive beyond 1 month in good health.

We subsequently use the same perfusion system to evaluate whether ischemically damaged livers can be recovered. Livers are exposed to a severe model of warm ischemia (1hr, 34°C) and either experience 5 hours of static cold storage (SCS) or NELP followed by transplantation. All recipients of NELP livers survive, while none who received SCS do. We have not observed similar findings of recovery from such a severe WI model in the literature, except by Schön et. al. who performed the experiments in a porcine model[85].

MFA is performed on the Fresh and WI livers in perfusion to evaluate the overall performance of the organs in perfusion. The major differences between ischemic and fresh liver metabolism is noted, time to recovery is suggested, and methods of improving perfusion are discussed. Metabolites are generally assessed for linearity of uptake or production, rates of change, substrate depletion and excessive presence driving fluxes unnecessarily. The intrinsic differences between ischemic and fresh livers are observed and suggestions for treatment protocols specific to WI recovery are suggested.

A series of steps are undertaken to simplify the perfusion system based on the MFA findings. We first reduce temperature to reduce oxygen demand; we choose room temperature because this has shown both viable livers and allows the obviation of a heating or cooling system. No significant differences are observed between livers perfused at 37°C, 30°C and 20°C either during perfusion or after transplantation. Further, oxygen uptake rate by livers at room temperature is less than the oxygen that is supplied through solution alone if a 95%O_2/5%CO_2 mixture were to be used. Animals survive in good health beyond 1 month. Using hepatocyte isolation as an endpoint to allow greater quantification of differences observed, differences in duration of perfusion are examined. It is determined that full and optimum recovery of ischemically damaged hepatocytes occurs within 2-3 hours of perfusion, which also concurs with the aforementioned MFA findings.

An asanguineous perfusate is subsequently prepared, the heating apparatus removed and the circuit reduced to a primary loop only, comprising a reservoir, a pump, an oxygenator and a bubble trap prior to entering the liver in its chamber. Fresh livers are perfused with and without heparin pretreatment and no significant differences in cell yield and viability are observed.

Using a significantly simplified perfusion system, fresh livers are perfused for 3 hours (3hrRT), as are warm ischemic livers (WI+3hrRT) and subsequently the hepatocytes are isolated. The yield and viability is compared to fresh and warm ischemic livers without perfusion treatment, reflecting a normal isolation and the current discarded donor isolation. The results demonstrate that there is a complete recovery to fresh liver cell yield in WI+3hrRT livers suggesting 60 minutes of WI in a rat model does not induce a catastrophic degree of tissue damage and is generally reversible. Equally interesting is the observation that 3hrRT livers have a significantly higher cell yield than either fresh or WI+3hrRT livers and may relate to a period of ischemic preconditioning prior to inception of ex vivo perfusion. The cells procured from perfusion function equally or better than fresh livers in cell suspension and plate culture. We show that there is a significant linear correlation between cell yield post-Percoll purification and the ATP content of the organ tissue, normalized to total protein. This is the first time the relation between energy status and hepatocyte outcome has been so strongly correlated and enables the development of an objective marker of organ viability. A second correlation is observed between cell yield and final perfusate flow rate. The correlation is significantly linear in 3hrRT livers, less so in WI+3hrRT livers; both groups demonstrate cell yield is enhanced

at very low flow rates. Cumulatively, these data provide us with the next iterative steps for future improvement of the perfusion system, including ischemic preconditioning if appropriate, vasodilation, thrombolytics and an artificial oxygen carrier[96,97].

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Chapter 2: *In vivo* Metabolic Analysis of the Rat Liver

In this study, we develop an approach to evaluate the *in vivo* hemodynamic parameters of the rat hepatic blood supply and its major metabolic constituents for the purposes of establishing a platform upon which to base *ex vivo* perfusion parameters, and compare subsequent systems' performance. To investigate the validity of the model, we extensively compare our normal liver findings with current literature, and then develop a cohesive map of metabolic interactions using metabolic flux analysis (MFA). This tool allows us to portray a snapshot in time of organ metabolism and integrate our findings to appreciate large pathway activity such as gluconeogenesis, the pentose phosphate pathway, the urea and TCA cycles. To evaluate the sensitivity of the model to metabolic disturbances, we compare healthy liver metabolism to mild and moderate degrees of hypermetabolism. It is well known that thermal injury induces a hypermetabolic response that increases with the severity of injury. Sprague Dawley rats receive a sham, 20% or 40% total body surface area burn. The products of *in vivo* concentration measurements and flow rates, normalized to each liver's weight provide 24 fluxes with which to perform MFA, providing information regarding an additional 37 intracellular fluxes. Burn injury induces a mild metabolic response in 20% TBSA burn injuries. The primary finding is a reduction in hepatic gluconeogenesis, most likely in response to hyperinsulinemia, and a shift in nitrogen balance, favoring the formation of albumin and anaplerosis. A 40% TBSA burn injury, by contrast, causes significant metabolic alterations. Despite hyperinsulinemia, gluconeogenic fluxes are increased by significantly elevated lactate levels and increasing amino acid uptake, suggestive of insulin resistance. Nitrogen uptake is increased 1.5 times compared to *in vivo* values, with an increased and preferential use for anaplerosis; albumin production is reduced. These results accurately and cumulatively reflect the gross expected outcomes of hypermetabolism and provide new detailed insight as to the extent of hepatic reaction to, and impact on, metabolism. This is the first MFA analysis of whole-organ metabolism conducted on *in vivo* parameters and provides sufficiently useful data to enable detection of even subtle degrees of metabolic disturbances.

RESULTS

To determine the effect of burn injury on hepatic metabolism, rats were subjected to a dorsal burn corresponding to 20% of the total body surface area, or a combined dorsal and ventral burn corresponding to a total body surface area of 40%. Three days after burn, the animals were fasted overnight, and on the fourth day, portal vein and hepatic artery flow rates were measured and blood samples were taken. Sham controls consisted of rats that underwent the same procedures, but experienced an immersion in water at 20°C.

Metabolite Concentration Analysis

Table 1 displays the group averages of the measured extracellular metabolite concentrations in the portal vein (PV), hepatic artery (HA) and hepatic veins (SHVC) in addition to the flow rates and liver weights. Additional relevant blood chemistry values not used specifically for MFA are represented in Table 2. Nitrogen fluxes are accounted for in Table 3.

There were no elevations in liver enzymes (ALT, AST and ALP) or total bilirubin[1,2] amongst any of the vessels in all groups suggesting that no significant liver damage occurred within four days of burn injury. Plasma creatinine and urea nitrogen values remained within normal range for all rats suggesting unimpaired renal function due to burn injury.

Flow rates generally increased in the portal vein and decreased in the hepatic artery for burn groups compared to sham. Overall values ranged between 1.7-2.2ml/min/g liver in the portal vein and 0.07-0.05ml/min/g liver in the hepatic artery for a cumulative inflow of 1.77-2.25ml/min/g liver.

Trends common to all vessels likely signify the systemic impact due to burn. For example, a significant decrease in albumin and total hemoglobin levels occurred equally in both burn groups. Lactate levels were slightly decreased in the 20% TBSA burn group but elevated 4-fold in the 40% TBSA burn group. This particular finding, combined with reduced dissolved oxygen from low hemoglobin levels, low pH, and high carbon dioxide values suggest a mildly acidotic state induced by the liver in 40% TBSA burn animals.

Obvious dose-dependent changes in concentrations were generally not observed. The most apparent circumstance in which this occurred was a significant four-fold increase in SHVC insulin levels (Table 2). There was also a dose-dependent systemic decrease in VLDL and TG content reflected equally in all vessels (Table 2).

A general appreciation of amino acid metabolism was obtained by accounting for nitrogen. At the bottom of Table 1 is a summation of the total circulating amino acid nitrogen in each vessel. Arterial nitrogen, most representative of the systemic state of the body, demonstrates a slight decline in total nitrogen compared to sham (-3.5% and -1% in 20% and 40% TBSA burns respectively). The burn groups had 13 out of 20 amino acids with reduced trends compared to sham, except for arginine, ornithine and cysteine, which were increased systemically, and histidine, valine, tyrosine and phenylalanine, which were unchanged. In the PV, sham nitrogen content remained virtually unchanged from the HA, however the burn groups demonstrated a dose-related increase from intestinal contributions (3.3% and 8.9% in 20% and 40% TBSA burns respectively). SHVC nitrogen content of the burn groups was generally 5% greater than in sham. All groups had a SHVC nitrogen content significantly lower than either the HA or PV. Nitrogen flux across the liver (Table 3) shows that 20% TBSA burn livers took up as much nitrogen as sham livers but produced more albumin. 40% TBSA burn livers took up the surplus nitrogen but did not alter albumin production; rather they increased their urea nitrogen output suggesting the use of protein preferentially as an energy source.

In order to appreciate the effects of the liver on specific amino acids, influxes (Table 4) and fluxes across the liver were calculated (Table 5). Influxes took the combined contributions of the PV and HA into account, and estimated a total delivery rate of each metabolite to the liver. The efflux subtracted from the influx provided the flux across the liver. Though there were no significant differences in influx across any of the groups, dose-dependent trends of increasing influxes were observed for all amino acids except asparagine, aspartate, glutamate, glutamine, glycine, histidine, and serine, which were similar across groups. Increased uptake from this list of amino acids represents hepatic-specific requirements in the absence of mass-action driving fluxes. Glycine and serine had systemically low concentrations and demonstrated a dose-dependent significant increase in hepatic uptake. Glutamine and glutamate had systemically low concentrations; their uptake by the liver was insignificantly and equally increased. Asparagine and aspartate had systemically low concentrations. Here the flux across livers with the smaller burn was reduced compared to sham while the 40% TBSA burn fluxes were normal. Livers in the bigger burn group preferentially consumed more of these amino acids thereby increasing the flux and significantly lowering the output concentration. Histidine concentration did not vary with burn and its fluxes remained within normal range. Systemically increased concentrations, and the only cases of

significantly increased HA concentrations in burn, were those of arginine, cysteine and ornithine (40% TBSA burn only). These subsequently-increased influxes correlated strongly with *reduced* fluxes across the burn liver groups. Conversely, increases in PV concentrations outweighed concentration values in the HA that were within or below normal range. This resulted in substantially increased fluxes across the liver for proline, phenylalanine, lysine, alanine, threonine, and tyrosine. The BCAAs also had increasing influx trends due to increased PV concentrations. All BCAA fluxes demonstrated a slight upward trend in 20% TBSA burn compared with sham. In the 40% TBSA burn group however, each BCAA differed: valine had a significantly reduced flux, isoleucine was within normal range, and leucine was significantly increased. The resulting SHVC concentration values were below normal for the glucogenic amino acids, normal for the ketogenic ones and elevated for the BCAAs. To determine whether particular increases in influxes were necessary to determine whether the liver would take up or release an amino acid, ratios of burn influxes to sham influxes were calculated. These did not reveal any correlations between change in influx and liver action in any of the groups.

TABLE 1. Measured *In vivo* Hepatic Blood Flow and Metabolite Concentrations

	SUPRAHEPATIC VENA CAVA (SHVC, n=5)		
PARAMETER	SHAM	20% TBSA	40% TBSA
Blood flow (ml/min)	15±7.4	20±9.1	20±6.2
Total Hemoglobin (g/dL)	13±0.55	**11±1.4**	**11±1.2**
Oxyhemoglobin (%)	24±3.3	**34±7.3**	17±6.1*
Partial O_2 tension (mmHg)	27±0.63	41±16	21±6.2
Partial CO_2 tension (mmHg)	52±5.9	48±7.8	**67±5.5***
Dissolved O_2 (mlO_2/100ml blood)	4.2±0.5	5.2±1.2	**2.7±1.1***
Total CO_2 (mmol/L)	21±2.8	22±1.9	**25±0.45***
pH	7.28±0.01	7.29±0.04	**7.24±0.02***
Glucose (mg/dL)	210±57	165±33	144±12
Lactate (mmol/L)	1.0±0.17	**0.77±0.17**	**4.3±1.2***
β -Hydroxybutyric acid (µmol/L)	350±251	271±141	216±189
Acetoacetic acid (µmol/L)	116±36	132±12	**11±68***
Urea nitrogen (mg/dL)	14±5.0	15±1.1	17±2.0
Albumin (g/dL)	1.8±0.17	**1.2±0.11**	**1.2±0.09**
Ammonia (µmol/L)	41±3.4	29±12	**19±5.7**
Alanine (µmol/L)	261±26	**163±29**	**149±11**
Arginine (µmol/L)	62±1.5	**198±30**	**199±23**
Ornithine (µmol/L)	82±9.9	85±17	**124±11***
Asparagine (µmol/L)	28±0.83	**21±2.6**	**18±0.07**
Aspartate (µmol/L)	13±0.88	11±2.6	**8.3±0.62**
Cysteine (µmol/L)	12±0.46	**17±1.5**	**19±1.9**
Glutamate (µmol/L)	83±19	**51±6.8**	57±18
Glutamine (µmol/L)	305±42	**221±53**	**234±8.6**
Glycine (µmol/L)	194±32	**148±5.3**	**124±14***
Histidine (µmol/L)	56±6.6	50±10	60±7.1
Proline (µmol/L)	146±21	**114±4.7**	**117±14**
Serine (µmol/L)	170±12	**107±5.4**	**101±15**
Methionine (µmol/L)	38±1.1	**32±1.9**	**34±1.2**
Threonine (µmol/L)	191±24	167±37	**158±7.5**
Valine (µmol/L)	156±54	191±21	205±18
Tyrosine (µmol/L)	60±11	63±9.6	67±5.5
Isoleucine (µmol/L)	82±7.5	97±12	**103±11**
Phenylalanine (µmol/L)	54±2.8	52±7.7	51±2.9
Leucine (µmol/L)	241±35	266±33	240±41
Lysine (µmol/L)	210±42	201±33	207±24
Sum Amino Acid Nitrogen (µmol/L)	3683	3845	3873

Note:
Liver weights (g) in each group were SHAM: 9.4±0.76; 20% TBSA: **10.6±0.73**; 40% TBSA: 9.83±0.35.
Bolded items are significantly different ($p<0.05$) from SHAM.
* indicates the value for 40% TBSA is significantly different ($p<0.05$) compared to 20% TBSA burn

TABLE 1 *cont.* Measured *In vivo* Hepatic Blood Flow and Metabolite Concentrations

	PORTAL VEIN (PV, n=5)		
	SHAM	20% TBSA	40% TBSA
Blood flow (ml/min)	15±7.4	19±9.1	20±6.3
Total Hemoglobin (g/dL)	15±1.2	13±1.0	**13±0.41**
Oxyhemoglobin (%)	73±8.8	81±5.1	75±6.3
Partial O_2 tension (mmHg)	68±12	57±12	66±9.1
Partial CO_2 tension (mmHg)	61±12	62±14	57±2.0
Dissolved O_2 (mlO_2/100ml blood)	15±2.5	14±1.9	13±0.9
Total CO_2 (mmol/L)	19±2.9	**23±0.71**	**23±1.1**
pH	7.27±0.02	7.28±0.05	7.25±0.02
Glucose (mg/dL)	123±62	165±30	125±12*
Lactate (mmol/L)	1.0±0.18	0.75±0.25	**4.9±0.84***
β -Hydroxybutyric acid (µmol/L)	110±96	124±58	87±47
Acetoacetic acid (µmol/L)	110±73	143±95	98±88
Urea nitrogen (mg/dL)	13±5.0	16±2.2	16±2.7
Albumin (g/dL)	1.9±0.21	**1.4±0.13**	**1.3±0.11**
Ammonia (µmol/L)	109±33	65±30	74±5.0
Alanine (µmol/L)	397±13	401±74	412±69
Arginine (µmol/L)	141±93	219±36	222±5.1
Ornithine (µmol/L)	120±3.5	**106±3.3**	128±9.7*
Asparagine (µmol/L)	49±5.4	**33±2.5**	**31±0.65**
Aspartate (µmol/L)	21±7.6	13±0.54	13±3.0
Cysteine (µmol/L)	15±1.5	17±1.5	17±0.82
Glutamate (µmol/L)	68±7.7	**43±6.7**	53±16
Glutamine (µmol/L)	293±46	257±29	255±5.03
Glycine (µmol/L)	273±27	**216±18**	**213±25**
Histidine (µmol/L)	137±26	126±14	148±10*
Proline (µmol/L)	163±12	161±6.7	177±20
Serine (µmol/L)	199±13	**154±24**	**153±8.3**
Methionine (µmol/L)	44±7.0	43±4.6	43±1.8
Threonine (µmol/L)	204±41	208±39	211±27
Valine (µmol/L)	165±31	199±20	**205±2.9**
Tyrosine (µmol/L)	68±7.4	74±4.6	**80±5.3**
Isoleucine (µmol/L)	86±16	102±6.3	101±1.4
Phenylalanine (µmol/L)	59±3.9	**66±2.9**	**66±4.1**
Leucine (µmol/L)	261±33	**310±30**	**340±13**
Lysine (µmol/L)	220±38	242±23	**270±14**
Sum Amino Acid Nitrogen (µmol/L)	4880	5039	5313

Note:
Liver weights (g) in each group were SHAM: 9.4±0.76; 20% TBSA: **10.6±0.73**; 40% TBSA: 9.83±0.35.
Bolded items are significantly different ($p<0.05$) from SHAM.
* indicates the value for 40% TBSA is significantly different ($p<0.05$) compared to 20% TBSA burn

TABLE 1 *cont.* Measured *In vivo* Hepatic Blood Flow and Metabolite Concentrations

	HEPATIC ARTERY (HA, n=5)		
	SHAM	20% TBSA	40% TBSA
Blood flow (ml/min)	0.66±0.11	**0.46±0.05**	0.46±0.27
Total Hemoglobin (g/dL)	13±1.1	12±0.94	**9.9±1.2***
Oxyhemoglobin (%)	90±4.9	92±0.92	85±3.6*
Partial O_2 tension (mmHg)	114±27	115±15	96±13
Partial CO_2 tension (mmHg)	45±3.7	52±11	48±1.1
Dissolved O_2 (mlO_2/100ml blood)	15±1.7	16±1.1	**12±1.6***
Total CO_2 (mmol/L)	21±1.1	20±1.9	**25±1.8***
pH	7.28±0.03	7.29±0.04	7.25±0.02
Glucose (mg/dL)	164±57	141±28	144±18
Lactate (mmol/L)	0.94±0.55	0.55±0.16	**3.5±0.6***
β-Hydroxybutyric acid (µmol/L)	245±205	62±23	141±66
Acetoacetic acid (µmol/L)	122±21	101±67	**61±36**
Urea nitrogen (mg/dL)	14±4.3	13±1.7	17±2.6*
Albumin (g/dL)	1.9±0.10	**1.1±0.14**	**1.3±0.17**
Ammonia (µmol/L)	50±5.4	**38±1.4**	**23±2.4***
Alanine (µmol/L)	357±35	**302±14**	**284±33**
Arginine (µmol/L)	107±7.9	**195±37**	**185±11**
Ornithine (µmol/L)	105±20	117±1.8	**138±21**
Asparagine (µmol/L)	43±3.4	**35±2.3**	**33±3.4**
Aspartate (µmol/L)	11±1.2	8.6±3.7	9.4±2.0
Cysteine (µmol/L)	16±1.2	**21±0.7**	**25±1.1***
Glutamate (µmol/L)	78±5.2	**46±12**	**45±9.0**
Glutamine (µmol/L)	419±36	**359±29**	405±15*
Glycine (µmol/L)	218±5.4	**187±17**	**163±10***
Histidine (µmol/L)	65±0.17	59±5.4	67±3.5*
Proline (µmol/L)	169±5.6	**147±14**	**150±7.5**
Serine (µmol/L)	219±24	**159±9.6**	**178±19**
Methionine (µmol/L)	54±9.6	**42±4.1**	**40±5.5**
Threonine (µmol/L)	252±40	221±23	**203±15**
Valine (µmol/L)	202±69	193±22	193±27
Tyrosine (µmol/L)	80±10	89±1.6	82±5.4*
Isoleucine (µmol/L)	122±28	106±11	100±19
Phenylalanine (µmol/L)	65±4.4	63±2.0	67±1.1*
Leucine (µmol/L)	321±88	276±33	290±62
Lysine (µmol/L)	237±42	193±18	224±1*
Sum Amino Acid Nitrogen (µmol/L)	4809	4640	4759

Note:
Liver weights (g) in each group were SHAM: 9.4±0.76; 20% TBSA: **10.6±0.73**; 40% TBSA: 9.83±0.35.
Bolded items are significantly different ($p<0.05$) from SHAM.
* indicates the value for 40% TBSA is significantly different ($p<0.05$) compared to 20% TBSA burn

TABLE 2. Non-MFA *In vivo* Hepatic Blood Chemistry Values

	SUPRAHEPATIC VENA CAVA (SHVC) (SHVC)		
PARAMETER	SHAM (n=12)	20% TBSA (n=12)	40% TBSA (n=13)
Insulin (ng/ml)	1.1±0.01	**3.8±0.02**	**4.5±0.02***
AST (u/L) (87-114)	96±25.7	93±26.8	122±50.9
ALT (u/L) (28-40)	48±13.0	39±9.50	40±7.30
ALP (u/L) (136-188)	209±66.0	166±63.0	165±57.4
Total Bilirubin (mg/dL) (0.1-1.0)	0.32±0.06	0.31±0.03	0.32±0.06
Creatinine (mg/dL) (0.5-0.6)	0.25±0.09	0.23±0.049	0.22±0.04
Cholesterol (mg/dL) (55-89)	62±21.3	71±19.7	68±11.2
HDL (mg/dL)	33±7.41	32±8.4	32±7.27
LDL (mg/dL)	28±12.3	35±14.9	32±3.4
VLDL (mg/dL)	9±3.3	**5±1.10**	**4±0.45**
Triacylglycerol (mg/dL) (62-92)	37±17.0	**22±4.76**	**22±2.38**
	PORTAL VEIN (PV)		
PARAMETER	SHAM (n=12)	20% TBSA (n=12)	40% TBSA (n=13)
AST (u/L) (87-114)	95±30.0	110±2.36	47±45.4
ALT (u/L) (28-40)	53±15.1	47±7.20	43±7.92
ALP (u/L) (136-188)	231±81.7	188±86.9	198±58.9
Total Bilirubin (mg/dL) (0.1-1.0)	0.32±0.06	0.34±0.05	0.32±0.06
Creatinine (mg/dL) (0.5-0.6)	0.21±0.03	0.22±0.04	0.21±0.03
Cholesterol (mg/dL) (55-89)	68±20.0	**86.4±18.6**	82±8.50
HDL (mg/dL)	36±3.15	42±10.5	35±3.56
LDL (mg/dL)	26±11.1	37±12.2	**41±6.94**
VLDL (mg/dL)	11±4.47	**6±1.33**	**5±1.03**
Triacylglycerol (mg/dL) (62-92)	48±23.8	**27±8.15**	**24±4.80**
	HEPATIC ARTERY (HA)		
PARAMETER	SHAM (n=12)	20% TBSA (n=12)	40% TBSA (n=13)
AST (u/L) (87-114)	109±33.5	103±37.6	116±44.2
ALT (u/L) (28-40)	55±15.7	**40±10.5**	44±9.87
ALP (u/L) (136-188)	259±75.6	**156±93.5**	**186±42.1**
Total Bilirubin (mg/dL) (0.1-1.0)	0.32±0.06	0.32±0.04	0.35±0.05
Creatinine (mg/dL) (0.5-0.6)	0.21±0.03	0.23±0.05	0.25±0.08
Cholesterol (mg/dL) (55-89)	72±20.4	67±22.2	74±13.7
HDL (mg/dL)	32±9.82	32±11.6	34±4.40
LDL (mg/dL)	30±13.4	32±15.0	39±8.10
VLDL (mg/dL)	12±3.24	**7±2.4**	**5±0.89**
Triacylglycerol (mg/dL) (62-92)	51±21.2	**31±10.9**	**24±4.53**

Note
Bolded items are significantly different ($p<0.05$) from SHAM.
* value for 40% TBSA is significantly different ($p<0.05$) compared to 20% TBSA burn.

TABLE 3. Nitrogen Flux Across the Liver

	Sham	20% TBSA	40%TBSA
Net Urea Nitrogen Output (umol/min)	3.6	6.4	9
Net Albumin Nitrogen Output (umol/min)	318	344	298
Net Amino Acid Nitrogen Uptake (umol/min)	21	21	31

TABLE 4. Measured Influx Values

METABOLITE	PV+HA
Total Oxygen (ml O2/min/g liver)	0.26±0.14
Albumin (g/min/g liver)	0.03±0.01
Lactate (mmol/min/g liver)	0.07±0.03
Glucose (mg/min/g liver)	1.81±0.68
Alanine (umol/min/g liver)	0.64±0.28
Ammonia (umol/min/g liver)	0.15±0.04
Arginine (umol/min/g liver)	0.25±0.25
Asparagine (umol/min/g liver)	0.08±0.04
Aspartate (umol/min/g liver)	0.03±0.01
Cysteine (umol/min/g liver)	0.02±0.01
Glutamate (umol/min/g liver)	0.11±0.04
Glutamine (umol/min/g liver)	0.48±0.2
Glycine (umol/min/g liver)	0.43±0.17
Histidine (umol/min/g liver)	0.22±0.13
Isoleucine (umol/min/g liver)	0.15±0.08
Leucine (umol/min/g liver)	0.44±0.22
Lysine (umol/min/g liver)	0.36±0.18
Methionine (umol/min/g liver)	0.07±0.03
Ornithine (umol/min/g liver)	0.19±0.09
Phenylalanine (umol/min/g liver)	0.09±0.04
Proline (umol/min/g liver)	0.26±0.1
Serine (umol/min/g liver)	0.32±0.15
Threonine (umol/min/g liver)	0.33±0.14
Tyrosine (umol/min/g liver)	0.11±0.06
Valine(umol/min/g liver)	0.28±0.16

Note
In vivo influx is the combined portal vein (PV) and hepatic artery (HA) contribution to that flux. "Perfusion" is calculated according to the initial portal perfusate influx.

TABLE 5. Measured *In vivo* Metabolic Fluxes Across the Liver.

METABOLITE	SHAM (umol/hr/g)	20% TBSA (umol/hr/g)	40% TBSA (umol/hr/g)
Oxygen uptake	249±132	247±15	404±148*
Carbon Dioxide output	216±30	191±228	**291±20**
Glucose output	93±37	**28±17**	124±135
Lactate uptake	5.6±2.6	**-7.3±7.0**	**87±67***
Acetoacetate output	-1.3±0.6	1.1±2.7	-5.5±10
β-Hydroxybutyrate output	21±16	11±17	8.7±9.1
Urea output	28±23	25±38	**76±34**
Ammonia uptake	6.2±1.7	5.3±0.6	6.6±1.6
Alanine uptake	13±6.0	24±10	**31±8.0**
Arginine uptake	3.6±5.1	2.3±1.8	2.8±3.1
Ornithine uptake	3.4±0.9	2.6±2.5	**0.1±2.0**
Asparagine uptake	2.0±1.1	**0.9±0.2**	1.6±0.4*
Aspartate uptake	0.5±0.4	0.2±0.3	0.5±0.3
Cysteine output	0.2±0.0	**0.0±0.1**	**-0.3±0.1***
Glutamate output	0.8±1.3	1.1±1.1	1.0±3.4
Glutamine uptake	2.0±2.5	4.0±4.4	3.2±0.5
Glycine uptake	5.4±1.2	7.1±2.5	**9.2±2.6**
Histidine uptake	8.0±5.3	8.0±3.8	11±3.3
Proline uptake	0.7±2.9	**4.8±1.4**	**6.9±3.4**
Serine uptake	2.7±1.7	4.8±1.6	**6.4±2.7**
Methionine uptake	0.8±0.1	0.9±0.4	1.1±0.4
Threonine uptake	1.2±4.0	2.4±4.9	6.0±3.2
Valine uptake	0.8±0.4	1.2±2.8	**-0.1±0.4**
Tyrosine uptake	1.0±1.1	1.0±0.4	1.5±0.3
Isoleucine uptake	-0.2±0.7	0.5±1.3	-0.3±0.1
Phenylalanine uptake	0.5±0.4	**2.2±0.6**	**2.0±1.0**
Lysine uptake	1.5±0.4	5.1±5.7	**8.3±5.3**
Leucine uptake	-0.2±3.5	3.1±3.1	**13±7.0***

Note
Values in **bold** are significantly different from sham group ($p<0.05$)
* Values significantly different from 20% TBSA group ($p<0.05$)

Metabolic Flux Analysis

A total of 28 fluxes across the liver were calculated from the measured metabolite concentrations, flow rates and liver weights from each rat and averaged (Table 5). MFA then estimated the unmeasured internal metabolic fluxes Tables 6 and 7) using the measured external fluxes. The significant results are mapped in Figs 1-6. Figure 1 represents cumulatively how fluxes in each major pathway were altered (amino acid metabolism, gluconeogenesis, TCA cycle, lipid metabolism, oxidative phosphorylation, and urea cycle). The results show a significant reduction in gluconeogenesis for 20% burn in contrast to a significant increase in 40% burn. Dose dependent responses are seen in increased amino acid metabolism, and the reduced uptake of fatty acids and glycerol. In all other circumstances, the 40% TBSA burn group has increased fluxes compared to 20%, which approximates sham. Only the PPP suggests an increase in 20% burn compared to 40% burn and sham.

The metabolic flux distributions are depicted as comparisons of the 20% TBSA and 40% TBSA burn groups to the sham controls in Figures 2 and 3, respectively. The 20% TBSA burn group demonstrated few significant overall changes compared to sham (Figure 2). The most prominent finding was a major reduction in gluconeogenesis, despite being fasted, which favors an increase in this pathway. The concentration of glucose exiting the liver was low, but within range of sham values. The impact of reduced gluconeogenesis on oxidative metabolism was to retain TCA fluxes at sham values, but fluxes feeding into the TCA cycle were smaller or diverted. The hydrolysis of asparagine to aspartate and ammonia, for example, was significantly reduced, while acetoacetate production increased ($p<0.1$), a pathway that is favored when the concentration of acetyl CoA exceeds the oxidative capacity of the TCA cycle. Pyruvate tended to be redirected elsewhere, such as to the production of lactate, the plasma concentration of which was reduced systemically. Fluxes contributing to the PPP tended to increase, which coincided with previous results on burn metabolism using isolated perfused rat livers [3]. Though insignificant, amino acid metabolism was generally increased throughout (Table 6) except for asparagine, aspartate, ornithine, arginine and cysteine which were either decreased or unchanged.

By contrast, the 40% TBSA burn group showed a significant increase in gluconeogenesis (Figure 3); again the glucose concentration levels were lower, but within range of sham upon exiting the liver. Lactate conversion to pyruvate was significantly increased as was the uptake of several gluconeogenic amino acids. In

addition, increased uptake of ketogenic amino acids lysine and leucine occurred, resulting in a direct increase of glutamate fueling of the TCA cycle, and a subsequent increase in acetoacetate production. The urea cycle fluxes, as well as the fluxes connecting it to the TCA cycle were all significantly upregulated. The pentose phosphate pathway coincided with sham results.

Figure 4 illustrates the differences in hepatic metabolism of the two burn models. Here it can be appreciated that the energy requirement of the 40% TBSA burn is significantly increased compared to a 20% TBSA burn, with enhanced contributions to the TCA cycle deriving from lactate, acetyl-CoA and amino acids. The consequences of producing more ATP were twice as much oxygen consumption and carbon dioxide output, and three times as much urea output compared to a 20% TBSA burn. Pathways common to both burn groups were significant dose-dependent increases in uptake of proline, phenylalanine and alanine (Table 6; $p<0.01$ in 20% TBSA burn).

TABLE 6. Balanced Metabolites for MFA

Glucose-6-P	Citrulline
Fructose-6-P	Aspartate
Fructose-1, 6-P2	Alanine
Glyceraldehyde-3-P	Glutamate
PEP	Serine
Pyruvate	Cysteine
Oxaloacetate	Glycine
NADH	Propionyl-CoA
Acetyl-CoA	Acetoacetate
Citrate	Acetoacetyl-CoA
alpha-Ketoglutarate	O2
Succinyl-CoA	Tyrosine
Fumarate	Ribulose-5-P
FADH2	Ribose-5-P
Malate	Xylulose-5-P
Arginine	Erythrose-4-P
Ornithine	CO2
NH4+	

TABLE 7. Effect of burn injury on metabolic fluxes based on in situ (*in vivo*) and perfused liver data.

#	REACTION	PATHWAY	*IN VIVO* FLUX DATA (umol/h/g liver) SHAM (n=5)	20% TBSA (n=5)	40% TBSA (n=5)
1	Glucose 6-phosphate ↔ Glucose	Gluconeogenesis	93±37	**28±17**	124±135
2	Fructose 6-phosphate ↔ Glucose 6-phosphate	Gluconeogenesis	99±86	58±30	129±101
3	Fructose 1,6-Bisphosphate ↔ Fructose6-phosphate	Gluconeogenesis	66±28	**29±17**	112±64*
4	2 Glyceraldehyde 3-P ↔ Fructose 1,6-Bisphosphate	Gluconeogenesis	57±26	27±18	108±55*
5	Phosphoenolpyruvate + NADH ↔Glyceraldehyde 3-P	Gluconeogenesis	95±33	**39±34**	**205±105***
6	Oxaloacetate ↔ CO2 +Phosphoenolpyruvate	Gluconeogenesis	90±32	37±35	**203±100***
7	Pyruvate + CO2 ↔ Oxaloacetate	Gluconeogenesis	31±18	13±30	**151±70***
8	Lactate ↔ Pyruvate + NADH	Lactate metabolism & TCA cycle	5.6±2.6	**-7.0±7.0**	**87±67***
9	Acetyl-CoA + Oxaloacetate →Citrate	Lactate metabolism & TCA cycle	68±47	68±11	120±53*
10	Citrate ↔ 2-oxo-Glutarate + NADH + CO2	Lactate metabolism & TCA cycle	75±46	70±12	123±52*
11	2-oxo-Glutarate → Succinyl-CoA + NADH + CO2	Lactate metabolism & TCA cycle	92±45	84±11	148±53*
12	Succinyl-CoA ↔ FADH2 +Fumarate	Lactate metabolism & TCA cycle	102±44	89±11	153±58*
13	Fumarate ↔ Malate	Lactate metabolism & TCA cycle	140±47	118±34	235±75*

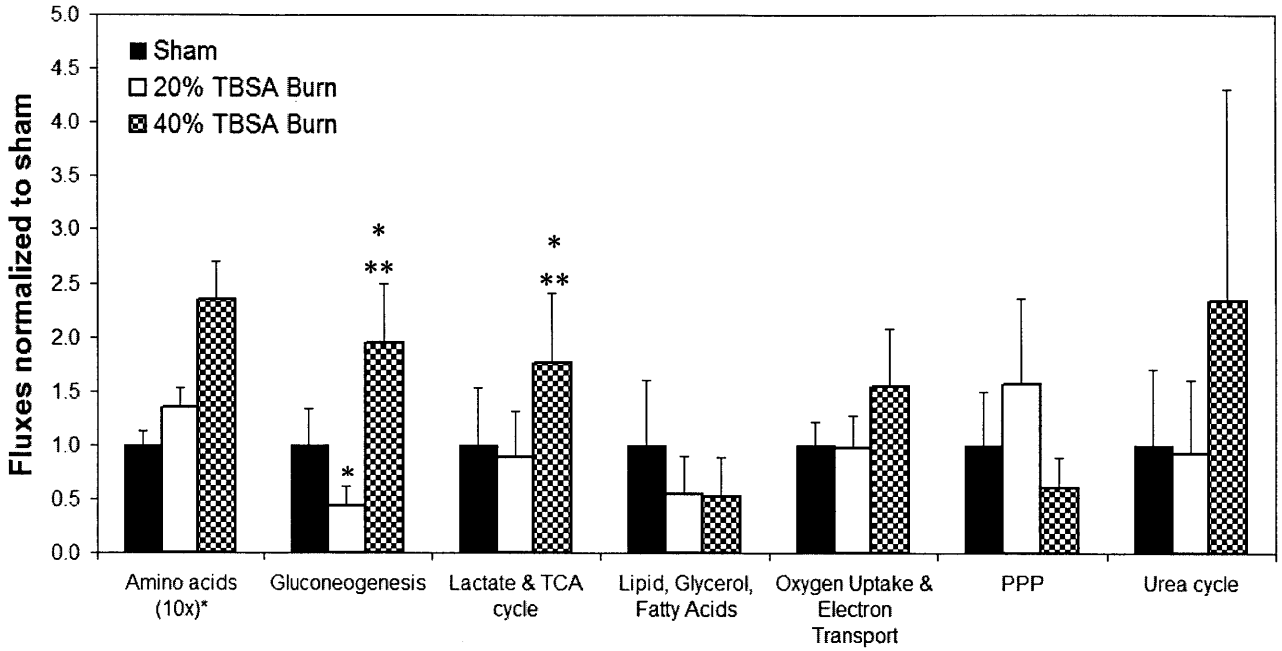
14	Malate ↔ Oxaloacetate + NADH	Lactate metabolism & TCA cycle	145±47	120±32	238±80*
15	Arginine → Ornithine + Urea	Urea cycle	28±23	25±38	**76±34**
16	Ornithine + CO2 + NH4 ↔Citrulline	Urea cycle	34±19	32±32	**75±29**
17	Citrulline + Aspartate →Arginine + Fumarate	Urea cycle	28±20	27±32	**73±29**
18	Arginine uptake	Aminoacid metabolism	3.6±5.1	2.3±1.8	2.8±3.1
19	Ammonia Output	Urea cycle	-6.2±1.7	-5.3±0.6	-6.6±1.6
20	Ornithine Output	Urea cycle	-3.4±0.9	-2.6±2.5	**-0.1±2**
21	Citrulline Output	Aminoacid metabolism	5.6±5.2	4.6±3.1	2.2±4.3
22	Alanine → Pyruvate + NH4 + NADH	Aminoacid metabolism	13±6.7	21±11	**33±9**
23	Alanine Output	Aminoacid metabolism	-13±6.2	-24±10	**-31±8**
24	Serine → Pyruvate + NH4	Aminoacid metabolism	6.8±11	1.6±18	25±16
25	Serine Uptake	Aminoacid metabolism	2.7±1.7	4.8±1.6	**6.4±2.7**
26	Cysteine → Pyruvate+ NH4 + NADH	Aminoacid metabolism	0.8±3.7	-2.8±5.9	3.3±5.4
27	Cysteine Output	Aminoacid metabolism	0.2±0.0	**0±0.1**	**-0.3±0.1***

28	Threonine → NADH + Glycine +Acetyl-CoA	Aminoacid metabolism	1.2±4	2.4±4.9	6±3.2
29	Glycine ↔ CO2 + NH4 +NADH	Aminoacid metabolism	4.2±8.2	1.3±13	18±11
30	Glycine Uptake	Aminoacid metabolism	5.4±1.2	7.1±2.5	**9.2±2.6**
31	Valine + 2-oxo-Glutarate → Glutamate + propionyl-CoA + 3 NADH +FADH2 + 2 CO2	Aminoacid metabolism	0.8±0.4	1.2±2.8	**-0.1±0.4**
32	Isoleucine + 2-oxo-Glutarate → Glutamate + propionyl-CoA + Acetyl-CoA + 2 NADH + FADH2 +CO2	Aminoacid metabolism	-0.2±0.7	0.5±1.3	-0.3±0.1
33	Leucine + 2-oxo-Glutarate → Glutamate + NADH + FADH2 Acetoacetate + Acetyl-CoA	Aminoacid metabolism	-0.2±3.5	3.1±3.1	**5.4±0.1**
34	Propionyl-CoA + CO2 ->Succinyl-CoA	Aminoacid metabolism	5.1±2.0	3.6±3.4	2.5±6.1
35	Lysine + 2 2-oxo-Glutarate → 2 Glutamate + 4 NADH + FADH2 + 2CO2 + Acetoacetatyl-CoA	Aminoacid metabolism	1.5±0.4	5.1±5.7	**4.6±2.2**
36	Phenylalanine + O2 → Tyrosine	Aminoacid metabolism	0.5±0.4	**2.2±0.6**	**2±1**
37	Tyrosine + 2 O2 →NH4 + CO2 + Fumarate + Acetoacetate + NADH	Aminoacid metabolism	3.7±3.7	0.1±5.4	6.4±6.8
38	Tyrosine Output	Aminoacid metabolism	-1.0±1.1	-1±0.4	-1.5±0.3
39	Glutamate ↔ 2-oxo-Glutarate + NADH +NH4	Aminoacid metabolism	15±7.5	27.4±13.7	**37±9**
40	Glutamate Output	Aminoacid metabolism	0.8±1.3	1.1±1.1	1±3.4
41	Glutamine → Glutamate + NH4	Aminoacid metabolism	2.0±2.5	4±4.4	3.2±0.5

42	Proline +0.5 O2→ Glutamate + 0.5 NADH	Aminoacid metabolism	0.7±2.9	**4.8±1.4**	**6.9±3.4**
43	Histidine → NH4 +Glutamate	Aminoacid metabolism	8.0±5.3	8±3.8	10.7±3.3
44	Methionine + Serine → Cysteine + NADH + Propionyl-CoA + CO2	Aminoacid metabolism	0.8±0.1	0.9±0.4	1.1±0.4
45	Aspartate↔Oxaloacetate + NH4 +NADH	Aminoacid metabolism	-24±17	-30±26	**-68±24**
46	Aspartate Uptake	Aminoacid metabolism	0.5±0.4	0.2±0.3	0.5±0.3
47	Asparagine → Aspartate + NH4	Aminoacid metabolism	2.0±1.1	**0.9±0.2**	1.6±0.4*
48	Palmitate→ 8 Acetyl-CoA +7 FADH2 + 7 NADH	Lipid, glycerol, fatty acid metabolism	11.1±7	8.5±4.2	11.8±8
49	2 Acetyl-CoA ↔ Acetoacetyl-CoA	Lipid, glycerol, fatty acid metabolism	11±16	2.9±19	-7.1±14
50	Acetoacetyl-CoA → Acetoacetate	Lipid, glycerol, fatty acid metabolism	15±16	8.6±18	-1.6±13
51	Acetoacetate Output	Lipid, glycerol, fatty acid metabolism	-1±1	1.1±2.7	**2.2±0.7**
52	Acetoacetate + NADH ↔b-Hydroxybutyrate	Lipid, glycerol, fatty acid metabolism	21±16	11±17	8.7±9.1
53	NADH + 0.5 O2 → NAD	Oxygen uptake and electron transport	302±181	327±31	527±205*
54	FADH2 + 0.5 O2 → FAD	Oxygen uptake and electron transport	182±87	158±25	245±97
55	O2 Uptake	Oxygen uptake and electron transport	249±132	247±15	404±148*
56	Glucose 6-phosphate→ 2 NADPH + CO2 +Ribulose 5-P	PPP	14±90	32±31	9.4±134

57	Ribulose 5-P ↔ Ribose 5-P	PPP	3±30	10±11	2.3±47
58	Ribulose 5-P ↔ Xylulose 5-P	PPP	18±61	24±19	10±81
59	Ribose 5-P + Xylulose 5-P ↔ Fructose6-P + Erythrose 4-P	PPP	10±30	12±10	5.6±40
60	Erythrose 4-P + Xylulose 5-P ↔ Glyceraldehyde 3-P + Fructose 6-P	PPP	15±31	14±9.1	8.2±36
61	CO2 Output	Oxygen uptake and electron transport	216±30	191±19	**291±20***

Note
Bolded items are significantly different ($p<0.05$) from SHAM.
* indicates the value for 40% TBSA is significantly different ($p<0.05$) compared to 20% TBSA burn.



* Significantly different from sham-burn group ($p<0.05$).
** Significantly different from 20% TBSA burn group ($p<0.05$).

Figure 1: Summary of the effects of burn injury on the major pathways in the hepatic metabolic network. Values shown are the averages of all fluxes in each pathway group, which were then normalized to the sham group. Pathways (from Table 6) are grouped as follows: Gluconeogenesis: #1-7, Lactate metabolism and TCA cycle: #8-14, Oxidative phosphorylation: #53-55, 61. Pentose phosphate pathway: #56-60. Amino acid metabolism: #18, 21-47, Urea cycle: #15-17, 19-20, Lipid metabolism: #48-52.

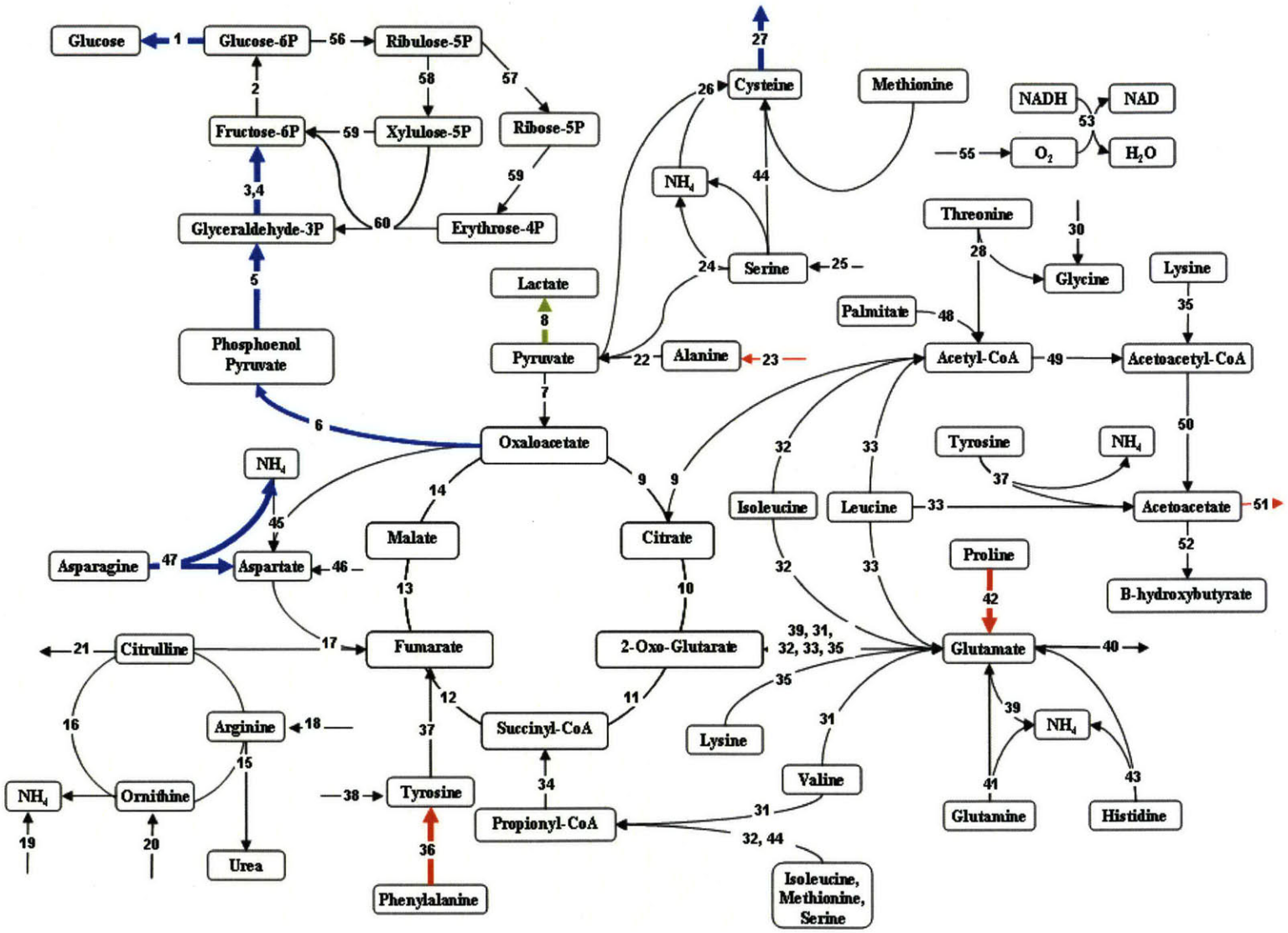


Figure 2: Flux directions reflect 20% TBSA results vs. sham controls. Red = Upregulated. Blue = Downregulated, Green = Reversed.

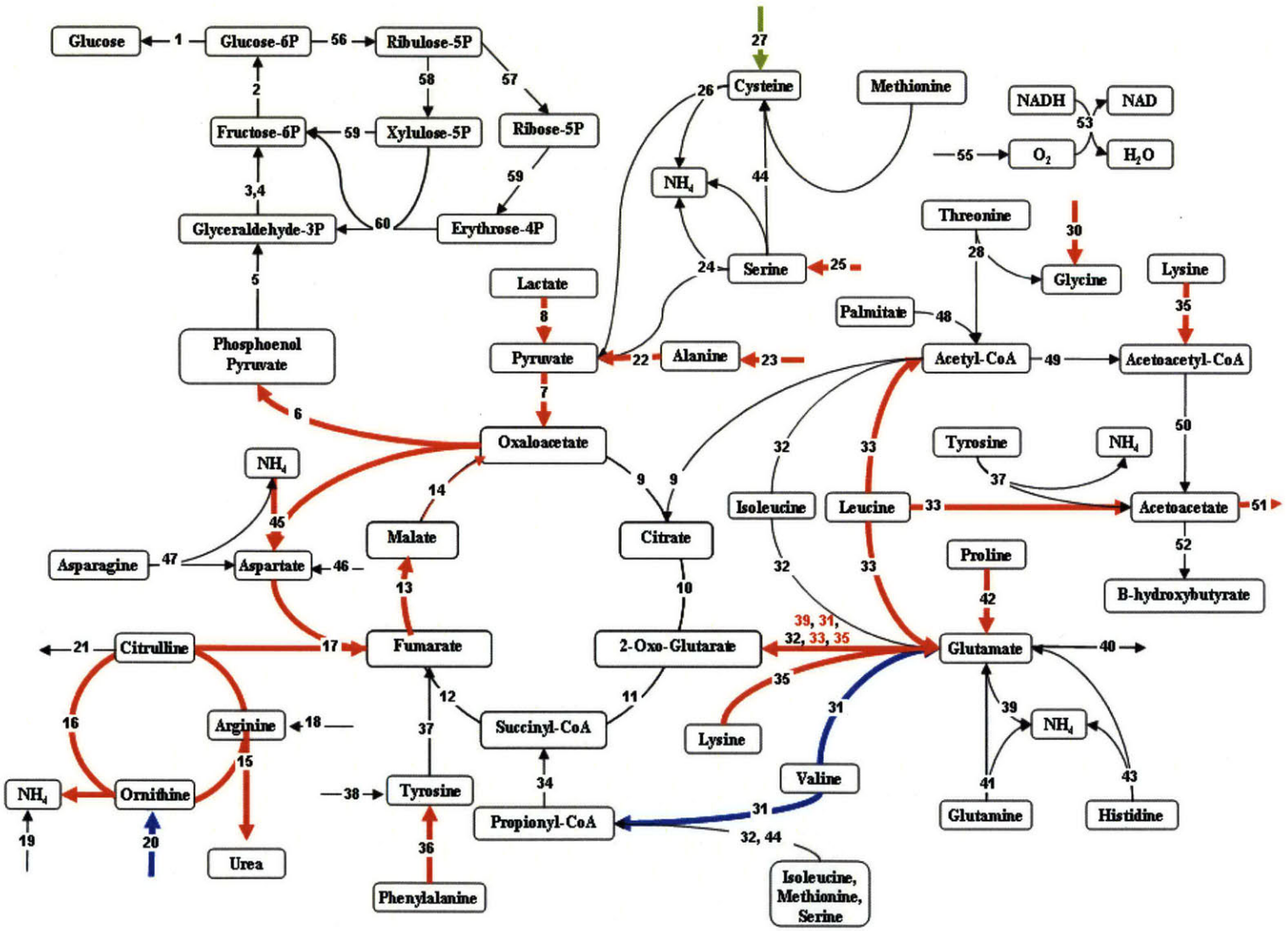


Figure 3: Flux directions reflect 40% TBSA results vs. sham controls. Red = Upregulated. Blue = Downregulated, Green = Reversed.

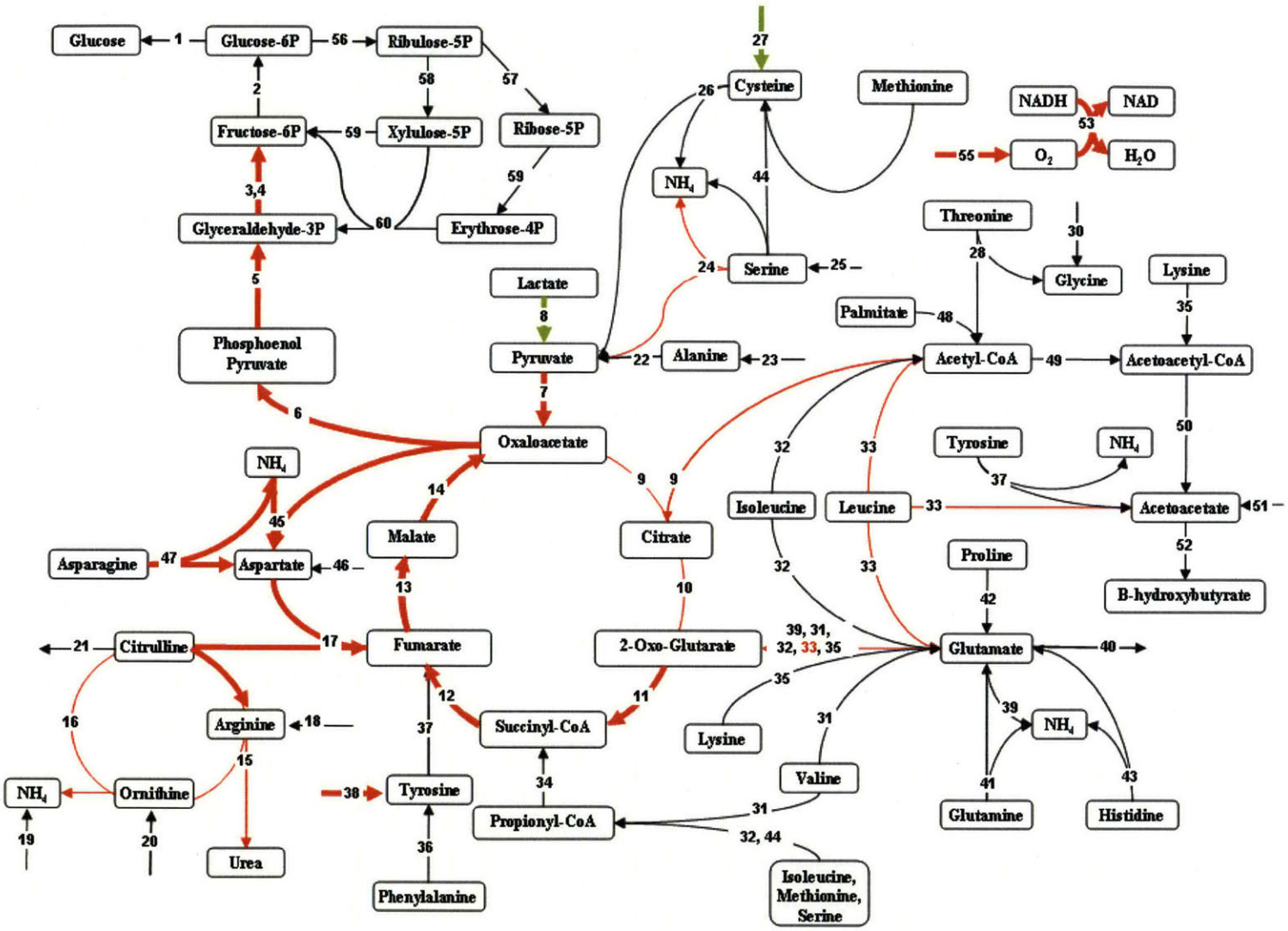


Figure 4: Flux directions reflect 40% vs. 20% TBSA burns. Red = Upregulated. Blue = Downregulated, Green = Reversed.

DISCUSSION

This investigation has examined the *in vivo* metabolic alterations in a rat model of a 20% and 40% TBSA burn. Although this was a terminal procedure not amenable to serial measurements, it provided the advantage of quantitatively characterizing the entire central carbon and nitrogen metabolism at once. Subsequently, this is the first MFA evaluation of *in vivo* data, and it has enabled us to connect the multiple alterations in metabolic pathways at the whole-organ scale to produce a comprehensive hepatic-specific map of the impact of thermal injury on metabolism. These results suggest that MFA is an appropriate methodology for organ-scale analyses. General pathway responses to metabolite-specific changes for a given burn severity may facilitate targeted treatment considerations.

An initial assessment of the validity of our measured metabolite concentrations and their trends in response to burn injury was made by comparison to published literature. Plasma metabolite values for sham rats were found to be within the normal range of venous and arterial blood measurements in several reports [1,4-9]. Flow rate values compared favorably with a total inflow of 1.74±0.2ml/min/g in normal livers observed by Daemen et al [10]. Both groups demonstrated a trend to increase portal flow and decrease arterial flow for ultimately insignificant increases in total hepatic blood flow [11,12]. Overt hepatic and renal damage was negated by normal liver function tests, creatinine and urea nitrogen values [13]. Fluxes across the liver were subsequently calculated.

The most pronounced observation in this analysis is the distinctly different responses in gluconeogenesis by the burn groups, which is most likely related to the dramatic increases in insulin levels observed. As the liver extracts insulin, the measurements obtained in the SHVC may only reflect as little as 40% of the amount delivered to it [14]. Glucose production in the 20% TBSA burn group was substantially reduced. Oxaloacetate, the conduit to forming phosphoenolpyruvate (Flux #6) was half the value of the sham control. Oxaloacetate flux from pyruvate occured at a tenth of the rate in sham and was 65 times smaller than in a 40% TBSA burn. Oxaloacetate formation from malate occured at the normal rate (Flux #14), but was largely shunted into the production of aspartate (Flux #45) thereby likely explaining the reduction in asparagine uptake in this group. While some liver sensitivity to insulin appeared to be retained in the 20% TBSA group, thereby minimizing its glucose output, the 40% TBSA

burn group demonstrated resistance to insulin by comparison. Despite even higher insulin levels than the 20% TBSA burn group, the bigger burn group responded with significantly increased gluconeogenesis, likely exacerbated upon fasting to increase the systemically low glucose concentrations. MFA has enabled the pinpointing of the multiple carbon sources mobilized for this increase (Table 6).

Increased insulin levels have been extensively associated with burn injury and insulin resistance [14-16]. A regression analysis of several parameters including insulin, glucagon, cortisol and triglycerides, demonstrated that in patients with low severity of injury (no fatalities, average TBSA ~37%), insulin was strongly correlated to glucose regulation without significant interplay of other factors [17]. Increased insulin release may in part be associated with the increase in certain circulating amino acids, notably arginine (Table 1), which is known to be a particularly strong stimulus for insulin production and release [18] and is present in 2-3 times the concentration of sham values. This alteration in amino acid concentration occurs in the larger context of increased catabolism of skeletal muscle postburn which is thought to contribute 70-80% of whole body insulin stimulated glucose uptake [19].

Impaired insulin signaling may also be related to dysregulation of fat metabolism and in particular the development of "ectopic" fat stores in the liver and muscle [20]. Reduced hepatic VLDL-TG release and fatty acid oxidation (Tables 2, 6) is known to occur in burn injury [21] and is observed in both burn groups. These measurements demonstrate a similarly strong dose-dependent response to burn severity as insulin. It has been proposed that this subsequent intracellular accumulation of fat, possibly contributing to liver enlargement seen here and in other reports of burn injury [22,23], may adversely impact the normal insulin signaling response to increased metabolic demand.

A complete nitrogen balance could not be performed in this analysis but general trends can be appreciated from the cumulative amino acid nitrogen concentration in plasma (Table 1). In the PV, both burn groups have an increase in total nitrogen due largely to intestinal contributions of arginine, lysine, phenylalanine, cysteine, the branched chain amino acids, and ornithine in 40% TBSA burns. Plasma nitrogen in the HA, representative of systemic circulating values, is slightly reduced compared to sham, which occurs despite increased protein catabolism [24]. In all groups, hepatic nitrogen uptake occurs such that the exiting nitrogen concentrations are significantly lower than in either PV or HA. Burn groups have on average 5% more nitrogen in the SHVC than sham. Table 3 shows that for a 20% TBSA burn, total nitrogen uptake is comparable to

sham. The use of this nitrogen however, favors an increase in albumin production. The 40% TBSA burn group responds to the increased available amino acids in the PV, however, the increased urea nitrogen output in this group suggests it is likely that the primary use of amino acids at this time in severe burn recovery is devoted to production of energy [25] and other acute phase proteins rather than albumin production, which remains low [26]. Albumin, a negative acute phase protein, is reduced systemically to the same degree in both burn groups [27,28]. The combined effects of increased catabolic rate, altered metabolism and a substantially increased transcapillary rate commonly seen in burn injury are known to contribute to plasma albumin reduction to varying degrees [29,30].

Particular amino acids and their trends were further analyzed and compared to other studies in both rats and humans. Cynober et. al. [31] analyzed venous blood samples on fasted humans at Day 4 post-burn. They found that alanine, glutamate, glutamine, glycine, proline, and serine were all significantly reduced below normal, while the BCAAs, tyrosine, and lysine were within normal range. Ornithine was within normal range, but then increased several days after burn. These concurred with our SHVC values. By contrast, they found an increase in phenylalanine, and no changes in methionine, asparagine and aspartate; we observed these trends only in the PV but not for asparagine which was reduced systemically. Cynober et. al. point out that phenylalanine is degraded primarily in the liver; from our flux analysis and concentration results we have no increase in tyrosine as a result nor an increase in phenylalanine suggesting unimpaired metabolism for this amino acid. Arterial plasma values for amino acids were obtained by Aulick, Snelling and Groves from the legs of patients with a range of burn severites [32-34]. There was general agreement amongst all studies and ours that aspartate, glutamate, glutamine, glycine, proline, serine, and threonine were decreased, while histidine was unchanged, and that in general, tyrosine, isoleucine, leucine and lysine were within normal range. Our results found a decline in alanine which agreed with Aulick's results but Snelling and Grove found it to be unchanged/increasing. Methionine and asparagine were decreased, while phenylalanine was normal in rats but, as in Cynober's results, these values were normal and increased in humans respectively. Valine in rats was normal while it varied in humans. Overall, human trends in amino acids illustrate a general increase in circulating phenylalanine, associated with increased proteolysis in the periphery [32,35], while in rats, sampling the aorta does not appear to have accumulated a significant increase at this point in the circulation; it is only apparent in the PV. Rats have a systemic depletion of asparagine

and methionine in burns which does not appear to be the case in humans. Other trends in concentration appear to be fairly comparable across species.

Amino acids that were preferentially taken up by the liver despite low circulating concentrations include glycine and serine, amino acids that can be produced interchangeably. From the flux results in Table 6, it would appear that glycine production is likely favored over serine in both burn groups. This is seen directly through an increase in glycine uptake (Flux #30) and production from threonine (Flux #28) resulting in an increase in glycine breakdown to produce NADH (Flux #29). Indirectly, serine uptake increases (Flux #25) but a disproportionate amount, if any, is converted into pyruvate (Flux #24) suggesting an alternative fate for serine not captured in this MFA model. Karner et. al. [9] suggested that glycine in rats is an important source of free nitrogen, replacing the role of glutamine in humans [24], and is rapidly consumed in burn injury in rats. Further, our results suggest that a correlation may exist between increased HA concentrations of amino acids and reduced hepatic fluxes. A converse correlation may also exist between increased PV content and a general increase in fluxes across the liver, demonstrating a mass-action driving effect. This has already been observed in the liver-specific degradation of phenylalanine. It is also particularly true for alanine, which has been well-documented as being a major source for gluconeogenesis in critically ill patients [11]. In both burn groups, alanine represents the largest amino acid flux at an uptake rate 2-3 times the value observed in sham. Less often reported but following a similar trend are proline and leucine, both of which appear to contribute substantially in a severe burn to the formation of glutamate (Flux #33 and #42) and the latter to acetyl-CoA (Flux #33) despite normally having minimal impact on liver metabolism as a BCAA [18]. Histidine appears to play no significant role in burn recovery. The effect of increasing the burn size to 40% TBSA further enhanced the overall amino acid metabolism (as seen in Figure 1), and more of the individual amino acid flux increases reached statistical significance (as seen in Figure 3). These results are consistent with a prior study reporting that 20% and 40% TBSA burns in rats cause dose-dependent increases in the activity of the hepatic amino acid transporter system A [36], which is one of the major cellular transport mechanisms of alanine and other neutral amino acids.

Increased production of lactate post thermal injury due to poor wound perfusion is well-understood [37] and forms an important gluconeogenic precursor in 40% TBSA burn livers. In addition to consuming at least double the amount of oxygen as any other

group, poor perfusion and impaired oxygenation may be limiting metabolism in the bigger burn animals. Total hemoglobin reduction, a typical consequence of heat-induced erythrocyte damage [11,38] appears to be critically reduced resulting in limited and potentially inadequate oxygen supply. Though oxygen delivery is not significantly different compared to either sham or 20% TBSA burn, most likely due to the compensatory increase in PV flow rate, the oxygen efflux appears to be exhausted and may be limiting the actual requirement. The results concur with the observed increase in TCA cycle fluxes in the 40% TBSA burn group (Figures 1 and 3), which are expected to generate the majority of the reducing equivalents (NADH and $FADH_2$) used as substrates for the electron transport chain. These findings are consistent with prior studies in rats and humans showing that burn injury increases whole-body resting energy expenditure [39,40]. Here we demonstrate the extent to which the liver is responsible for this energy increase.

In summary, we have performed the first *in vivo* metabolic flux analysis of the liver to characterize the hepatic metabolic response to experimental burn injury. We found that within 4 days post-burn significantly different metabolic changes in the liver result depending on burn severity. In a rat dorsal burn (20% TBSA) injury model, the response was mild and likely primarily impacted by elevated insulin levels curtailing gluconeogenesis. In a dorsal and ventral burn (40% TBSA) model, widespread changes were observed aimed at increasing gluconeogenesis, and resisting insulin. The responses included increased lactate, acetyl-CoA and amino acid utilization that increased the TCA cycle, electron transport chain activity, and urea output. These results agree with numerous reports in the literature regarding the changes brought about by burn injury and, with the use of MFA, have illustrated the importance of specific metabolites and pathways to burn recovery.

METHODS

Burn Injury Protocol

Male CD rats (Charles River Laboratories, Wilmington, MA) weighing 270-300g (n≥5 for each group) were housed in a temperature- (25°C) and light-controlled room (12-h light–dark cycle). The animals were cared for in accordance with the National Research Council guidelines. Experimental protocols were approved by the Subcommittee on Research Animal Care, Committee on Research, Massachusetts General Hospital. Water and rat chow were provided ad libitum. Animals were individually housed and allowed to adjust to their new surroundings for at least 2 days prior to inception of the experiment. On Day 0, rats were randomized into three groups and anesthetized with an intraperitoneal injection of ketamine (62.5 mg/kg of body weight) and xylazine (12.5 mg/kg of body weight). The dorsum of each rat was shaved with clippers, and those receiving a 40% total body surface area (TBSA) burn also had their abdomens shaved. The area to be burned was carefully marked. A full-thickness burn covering ~20% of the TBSA was administered through a 10-s immersion in boiling water [41]; the remaining 20% burn on the abdomen was administered through a 5-s immersion in boiling water [42]. Sham-treated animals were handled identically, except that room temperature water was used. Animals were then immediately resuscitated with an intraperitoneal saline injection (2.5 ml/kg per rat/%TBSA) and allowed to recover in individual cages. Animals were weighed every 24 hours and food consumption was monitored.

Measurement of Hepatic Flow Rates and Blood Sampling

It has been shown that whole-body parameters of hypermetabolism post-burn injury, including heart rate and temperature, are elevated and/or stable at Day 4 for rats of this age {Izamis, 2009 #54. Subsequently, on the third day following the burn injury, all rats were fasted overnight in preparation for blood sampling on the fourth day. This also coincided with the time point of *ex vivo* liver perfusions that were performed in earlier studies [43,44] enabling the comparison of *in vivo* and ILP results. Fasting served the dual purpose of providing a baseline measurement of the absolute rate of protein breakdown in the body by which to compare trauma-induced catabolism, and creating a gluconeogenic state for unidirectional fluxes in the MFA model.

On Day 4 each rat was anesthetized with ketamine (62.5mg/kg) and xylazine (12.5mg/kg) and the abdomen was shaved from sternum to groin. A transverse

abdominal incision was made and the xyphoid process clamped and retracted to expose the proximal abdominal contents. The intestines were moved aside and the caudal lobes of the liver gently elevated to reveal the portal triad. A perivascular ultrasonic flow-probe (Transonic Systems, Ithaca, NY) provided flow rates for the portal vein (PV) and hepatic artery (HA); acoustic resolution was improved with the use of lubricant gel. The sum of flow rates into the liver was assumed to equal the flow rate out via the suprahepatic vena cava (SHVC).

Following flow rate measurements, a removable clamp was placed on the inferior hepatic vena cava, immediately distal to the liver, and caudal to the renal and adrenal vasculature. After a few minutes, gentle retraction of the liver revealed a markedly reduced SHVC blood volume. A 23G heparinized syringe was used to withdraw ~1 ml of blood at the point of confluence of the hepatic veins into the SHVC. This technique did not cause extraneous blood loss. The clamp was carefully removed and then placed on the portal vein proximal to the liver. A 23G heparinized syringe was used to withdraw ~1 ml of blood from the vein under high pressure. The site was clamped again to prevent exsanguination post-sampling. To obtain a hepatic artery measurement, the abdominal aorta was rapidly isolated caudal to its bifurcation into the common iliacs and catheterized with a heparinized 18G catheter, again ~1 ml was collected. The liver was excised and a wet weight obtained.

Metabolite Analyses

All blood samples were immediately evaluated for blood gases () and subsequently analyzed for multiple metabolites using a rapid-assay device (Comprehensive Metabolic and Lipid Plus panels, Piccolo, Abaxis). The remaining blood was spun down at 3000 x g and the plasma frozen at -80°C. The samples were later analyzed for glucose, insulin, lactate, urea, albumin, acetoacetate and β-hydroxybutyrate, using biochemical assays, and amino acid composition (automated high-performance liquid chromatography system, Waters Co., Milford, MA) as described elsewhere [45].

Data Preprocessing

An outlier analysis was performed for each variable utilizing box-and-whisker diagrams in MATLAB (The Mathworks, Natick, MA); this resulted in deletion of less than 1% of data. The missing extracellular metabolite concentrations were replaced by the

median of the measurements from the other animals from each group, which is a standard procedure in data mining analysis [46]. Fluxes were calculated as:

$$v_i = F_i^{SHVC} c_i^{SHVC} - F_i^{PV} c_i^{PV} - F_i^{HA} c_i^{HA} \qquad (3)$$

where *F* is the flow rate and *c* is the concentration for metabolite *i* in the designated vessel (SHVC, PV, or HA). A second outlier analysis was performed for the fluxes, and 58 fluxes out of 405 total were deleted. The average value for each flux (for each group) was calculated for use in MFA, as discussed below.

Metabolic Flux Analysis (MFA)

MFA is based on fitting the measured set of fluxes to a stoichiometric model for the hepatic metabolic reaction network developed previously {Lee, 2000 #22}. This model captures the main biochemical reactions involved in central carbon and nitrogen metabolism under fasting conditions. Fasting is used to deplete glycogen stores so that glucose output reflects de novo gluconeogenesis without the confounding effects of glycogen breakdown. Furthermore, under this condition pyruvate dehydrogenase is inhibited, such that both glycogenolysis and pyruvate dehydrogenase fluxes can be set equal to zero. The mathematical model uses a total of 35 metabolites (Table 4) and 61 chemical reactions (Table 6), and was implemented and solved using MATLAB software as described previously [43].

The model estimates otherwise inaccessible intracellular reaction fluxes by performing a mass balance around each intracellular metabolite using measured extracellular fluxes. Assuming steady-state conditions (intracellular concentrations of metabolites are constant), these mass balances reduce to a system of linear equations. Briefly, the sum of fluxes to and from a metabolite or its "pool" is assumed to be zero:

$$S.v = 0 \qquad (1)$$

where the matrix S contains the stoichiometric coefficients of the biochemical reactions in the hepatic metabolic network. 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 1 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 reaction (S_u) fluxes, as follows:

$$S_u.v_u = - S_m.v_m \qquad (2)$$

The measured fluxes represent rates of uptake or release of extracellular metabolites and by solving Equation 2 they also give estimates of unknown intracellular fluxes.

MFA was performed on each individual rat's data, and the results from all the rats in each group pooled to determine average ± standard deviation. The validity of the statistical model was confirmed via the test of Wang and Stephanopoulos [47], at a threshold of $p<0.05$. This method statistically tests for the presence of gross errors that are inconsistent with other measurements, which lead to the violation of the pseudo steady-state assumption. Moreover, in the event of such errors, it can be used to identify the artifactual/erroneous measurements that lead to these inconsistencies. This approach was used to identify and eliminate several gross errors in the data.

Statistics

Comparisons were performed using ANOVA and 2-tailed Student's t-tests. $p<0.05$ was the criterion used for statistical significance.

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Chapter 3: Design of a Normothermic Extracorporeal Liver Perfusion System (NELP)

In this chapter we describe the design and implementation of an ex vivo perfusion system that is capable of successfully storing healthy transplantable livers for 6 hours.

The work described in section 3-2 was first published as part of:

Tolboom, H., Pouw, R., Uygun K., Tanimura, Y., Izamis, M.-L., *et. al.* A Model for Normothermic Preservation of the Rat Liver. Tissue Engineering. (2007) **13**, 2143-2151

The full publication is attached as Appendix 1.

3-1. MFA comparison of simple organ perfusion systems to in vivo parameters.

An in vivo model provides us with a basis upon which to establish a physiologically relevant perfusion system. We devise a simple way of creating such a system by comparing the metabolic profile of a preexisting design to those obtained in vivo and accordingly adjusting the parameters to enable stable, long-term function. We begin with a perfusion system used previously in our lab to evaluate liver metabolism. Its design is representative of many currently employed perfusion systems that have varied very little since Hems and Krebs[1] endorsed the use of Miller and Mortimore's designs[2,3] in 1966 to study various facets of liver physiology[4,5]. Blood or components of blood were originally used but this practice has fallen out of favor with some investigators[6,7] because of the additional logistical complexity it introduces as well as the likely extent to which it impacts metabolic measurements. The perfusion system we start with therefore comprises a peristaltic pump to flow perfusate through a jacketed oxygenator, heat exchanger, and bubble trap into the liver via the portal vein and out via the suprahepatic vena cava back to the perfusate reservoir. The perfusate comprises a cell culture medium that is oxygenated with $95\%O_2/5\%CO_2$. Using MFA, we compare its metabolic profile to in vivo results and arrive at the following major conclusions and requirements for a successful normothermic perfusion system:

- We evaluate the use of the perfusion system as a technique to capture in vivo organ metabolism by extrapolating observed metabolic rates in perfusion to appreciate what they were in vivo, minutes earlier. We take healthy livers and the same model of mild liver hypermetabolism (20% TBSA burn) and compare their metabolism using ex vivo perfusion and in vivo results. The data show that the liver responds quickly and significantly to its new ex vivo surroundings, suggesting that MFA cannot accurately portray its in vivo metabolic rate during the first hour of perfusion if the perfusate differs significantly from in vivo. This first hour reflects an adjustment by the liver to its new environment and should be taken into account when evaluating ex vivo organ stability.
- The metabolic rate of a normothermic perfusion requires a high oxygen delivery rate. Flow rates in the absence of oxygen carriers must be increased to ensure adequate oxygenation but at values 3x physiological, risk inducing mass-action effects that drive fluxes inappropriately or cause liver damage. We propose the addition of erythrocytes to the system to reduce flow rates to physiological values.

- To minimize the volume of erythrocytes used for both ethical and logistical reasons, we propose the formation of a primary, erythrocyte-containing, circuit and a secondary circuit connected to a perfusate reservoir.
- Communication between the circuits will be facilitated by a dialyzer with a cutoff weight to exclude albumin. This will serve as the "kidney" of the system enabling adequate mixing of nutrients and waste molecules from the primary to secondary circuit.
- The use of erythrocytes will enable the use of a $21\%O_2/5\%CO_2$ and balance nitrogen gas mixture if it is determined that high oxygen tension is contributing to free radical damage[8].
- We propose the use of a perfusate medium more closely resembling in vivo conditions and better suited to the culture of rat hepatocytes, such as phenol-red Williams Medium E[9].
- As bovine serum albumin is believed to be toxic to murine cells, we opt to exclude this from the perfusate and initially supplement only with flash frozen and filtered plasma from freshly drawn blood[6,10].
- To prevent significant leukocyte activation we propose the addition of the steroid hydrocortisone, a common finding in cell culture medium. We also propose the addition of penicillin and streptomycin to reduce bacterial contamination over long perfusion times[11].
- A significant difference between perfused livers and in vivo livers appears to be related to the inadequate presence of insulin in perfusate, or a high insulin/glucagon ratio, which strongly impacts the utilization of substrates. We therefore also propose the addition of insulin to perfusate.

Monitoring organ metabolism through ex vivo perfusion.

Much of the hypermetabolic response post-injury has been investigated through whole-body responses, and protein turnover in skeletal muscle or the wound bed[12-20]. These findings have captured the hyperdynamic circulatory changes, the increases in resting energy expenditure, and the hypercatabolism of muscle tissue impacting the patient's overall nitrogen balance, which form some of the distinguishing features of this disease state[21-24]. The need to identify and pharmacologically target specific pathways involved in the cause-effect relationships governing hypermetabolism has spurred multiple investigations into the role of the

liver in response to severe injury[25-34]. Our lab has investigated ways in which the liver effects changes in metabolism post-burn injury in a rat burn injury model using isolated organ perfusion systems (ILP)[35]. The development of the metabolic flux analysis (MFA) model[36-38] enabled the use of extracellular fluxes measured during ILP to estimate intracellular fluxes, and thereby create a cohesive map of pathways that contributed to time-dependent changes in hepatic metabolism[28,37-40]. The benefits of using ILP systems are the high degree of control over liver metabolism achieved by creating identical conditions of perfusate composition, temperature, flow rate, etc. between experiments[41]. Thus, hypermetabolic-induced differences would be best appreciated as "intrinsic" changes in metabolism compared to sham.

However, the extent to which the *ex vivo* setup and perfusate conditions impact liver metabolism, and subsequently the accuracy of ILP systems in depicting intrinsic metabolic changes, has yet to be completely investigated. The "invasiveness" of the process of perfusion is substantial. The animal must be heavily anesthetized, and access to the vasculature requires the animal is sacrificed. The liver is subsequently exposed to an artificial perfusate that inevitably differs significantly from blood incurring a response from the liver. The washout period between time of cannulation and sample measurement may contain the most informative data but is usually discarded because it is inherently noisy and difficult to capture. A rather substantial assumption is therefore made that the metabolite trajectories measured every ten minutes during the first hour of established perfusion are not significantly impacted by the liver's new environment. In order to evaluate the validity of this assumption, we compiled a detailed comparison of metabolic fluxes across livers in an ILP system versus livers *in vivo* using data from prior studies[42,43].

RESULTS

The results presented here are a meta-analysis of data obtained from healthy and hypermetabolic livers examined in Izamis et. al.[42] and Banta et. al.[43]. In each study, two groups of rats were subjected to either a sham burn or a dorsal burn corresponding to 20% of the total body surface area. Three days after burn, the animals were fasted overnight, and on the fourth day, samples were taken either via ex vivo perfusion, or directly from the vasculature representing the in vivo measurements.

Metabolic Flux Analysis: Isolated liver perfusion vs. in vivo

Intracellular fluxes were calculated using the MFA model of Lee et. al.[38] with data derived from the reported values measured in the isolated perfused rat liver study by Banta et. al. [39] and the *in vivo* samples by Izamis et. al.[42].

The results of the MFA comparison between ILP and in vivo are presented in Table 1 while Figure 1 summarizes the differences of major grouped pathways. From Figure 1 we can appreciate that perfusion significantly increased lipid metabolism, PPP activity, and gluconeogenesis. Amino acid metabolism and urea output were also increased in perfusion though this was not significant. Only lactate, TCA cycle, oxygen uptake and electron transport activity tended to be equal or less than livers in vivo; they were not significantly different. Figure 2 illustrates in detail the MFA results comparing sham ILP to sham in vivo. Here we see that perfusion causes a preferential uptake of certain amino acids: glutamine (Flux #41), which results in an increased extracellular glutamate pool (Flux #40), asparagine (Flux #47), citrulline (Flux #21), cysteine (Flux #26) and phenylalanine (Flux #36). While others, such as histidine (Flux #43), glycine (Flux #30), and alanine (Flux #23), are utilized less, and arginine output rather than uptake occurs (Flux #18). Perfusion creates a significantly elevated oxaloacetate pool originating from increased carboxylation of pyruvate (Flux #7), fueled by increased lactate and cysteine fluxes (Flux #8 and #26 respectively). This translates into an increased formation of phosphoenolpyruvate (PEP, Flux #6) and aspartate, which in turn cause an increase in the PPP and urea cycle respectively. Though fluxes through the gluconeogenic pathway are increased as a consequence, glucose output is in fact reduced compared to in vivo, with preference given to increasing the PPP instead. Oxaloacetate formation of aspartate is significantly contributed to by asparagine, resulting in a significantly increased formation of fumarate, an entry point into the TCA cycle, and arginine. Oxaloacetate entry into the TCA cycle via citrate production is reduced in perfusion. Citrate formation is similarly bypassed by an increased uptake of palmitate (Flux #48), which is preferentially used to produce ketone bodies (Fluxes #51, and #52) via acetyl-CoA. This decrease in citrate formation combined with a decreased succinyl-CoA formation via propionyl-CoA causes a significant reduction in the majority of the TCA cycle. However, with the increased fumarate production and the increased lactate flux, this average metabolic activity coincides with the seemingly unchanged values compared to in vivo seen in Figure 1. Urea output is increased as arginine is metabolized or released into the extracellular space. Ornithine and ammonia output occur rather than being recycled to citrulline.

Hemodynamics and Perfusate Composition

Influxes were compared between in vivo and ILP sham groups to evaluate the impact of perfusate composition and the rate of flow into the livers. Table 2 provides influx data in columns 1 and 2 where column 1 is the combined in vivo influx obtained from both the portal vein and hepatic artery, while column 2 is the initial perfusate influx values at t=0. Column 3 is the ratio between perfusate and in vivo influxes. Columns 4 and 5 are the corresponding fluxes across the livers and their ratio is expressed in column 6. A significantly higher inflow was used in ILP of average 5.5ml/min/g liver, compared to the average 1.8ml/min/g liver observed in *in vivo* conditions. This 3-fold difference enabled an oxygen delivery rate that was 40% of the in vivo value though oxygen uptake rates were comparable between groups. Ammonia and ornithine influxes were zero initially as they were not present in perfusate; they were observed to accumulate in perfusate, a common occurrence that typically continues until in vivo values are observed[4]. Lactate was the only other metabolite with an influx ratio of less than 1, but it was consumed at 16 times the rate of in vivo livers. High rates of lactate breakdown would suggest increased gluconeogenesis, but by contrast reduced glucose output was observed in perfusion. Glucose influx was 3-fold higher in perfusion than in vivo; there was no insulin in the perfusate.

All other influxes were elevated 3-71 times in vivo values. A scatterplot of the ratios of influxes and fluxes in Table 2 suggests a correlation suggesting that increasing influxes cause higher fluxes, and vice versa, indicative of a response to a mass action by the liver (Figure 3).

Metabolic flux analysis: Burn vs. Sham

Figure 4 generalizes both the metabolic response to burn as it compares with sham, and the significant differences between the in vivo and ILP burn groups. Overall, the trends observed post-burn tend to agree between groups, such as an increase in amino acid metabolism or a reduction in lipid metabolism however, the response by the ILP group seems generally damped by comparison. This is particularly evident in that only the in vivo model is capable of depicting any differences between sham and burn by a significantly reduced gluconeogenesis pathway. Gluconeogenesis, the PPP and lipid metabolism continue to differ significantly between ILP and in vivo post-burn suggesting livers respond very rapidly to the ex vivo environment and that it tends to overwhelm the underlying pathology being investigated. Figure 5 displays the MFA outcomes of sham vs. burn side by side for both in vivo and ILP and shows that at this level of detail, both techniques estimate substantially different liver responses to burn. It could be argued that both models demonstrate an increase in glutamate formation

though by different pathways; that acetoacetate production is favored over β-hydroxybutyrate; and that reduced lactate in ILP ultimately suggests reduced gluconeogenesis as seen in vivo. Figure 6 however demonstrates that ILP 20% TBSA burn livers have distinctly different metabolisms from in vivo livers. Intrinsic differences between sham and burn are unlikely to be distinguished in an organ largely impacted by a non-physiological perfusate stimulus.

Table 1: Effect of burn injury on metabolic fluxes based on in situ (in vivo) and perfused liver data.

#	REACTION	PATHWAY	IN VIVO FLUX DATA (umol/h/g liver) SHAM (n=5)	IN VIVO FLUX DATA (umol/h/g liver) 20% TBSA (n=5)	PERFUSED LIVER DATA (umol/h/g liver)[1] SHAM (n=5)	PERFUSED LIVER DATA (umol/h/g liver)[1] 20% TBSA (n=5)
1	Glucose 6-phosphate ↔ Glucose	Gluconeogenesis	93±37	28±17[a]	52±6[b]	51±11
2	Fructose 6-phosphate ↔ Glucose 6-phosphate	Gluconeogenesis	99±86	58±30	174±74	201±48[c]
3	Fructose 1,6-Bisphosphate ↔ Fructose6-phosphate	Gluconeogenesis	66±28	29±17[a]	96±21	102±15[c]
4	2 Glyceraldehyde 3-P ↔ Fructose 1,6-Bisphosphate	Gluconeogenesis	57±26	27±18	97±20[b]	102±15[c]
5	Phosphoenolpyruvate + NADH ↔Glyceraldehyde 3-P	Gluconeogenesis	95±33	39±34[a]	157±20[b]	155±20[c]
6	Oxaloacetate ↔ CO2 +Phosphoenolpyruvate	Gluconeogenesis	90±32	37±35	157±20[b]	155±20[c]
7	Pyruvate + CO2 ↔ Oxaloacetate	Gluconeogenesis	31±18	13±30	124±20[b]	100±20[c]
8	Lactate ↔ Pyruvate + NADH	Lactate metabolism & TCA cycle	5.6±2.6	-7.0±7.0[a]	89±14[b]	67±14[c]
9	Acetyl-CoA + Oxaloacetate →Citrate	Lactate metabolism & TCA cycle	68±47	68±11	34±14	20±15[c]
10	Citrate ↔ 2-oxo-Glutarate + NADH + CO2	Lactate metabolism & TCA cycle	75±46	70±12	33±13	20±14[c]
11	2-oxo-Glutarate → Succinyl-CoA + NADH + CO2	Lactate metabolism & TCA cycle	92±45	84±11	50±13	49±14[c]
12	Succinyl-CoA ↔ FADH2 +Fumarate	Lactate metabolism & TCA cycle	102±44	89±11	49±13[b]	50±14[c]
13	Fumarate ↔ Malate	Lactate metabolism & TCA cycle	140±47	118±34	125±20	149±21

14	Malate ↔ Oxaloacetate + NADH	Lactate metabolism & TCA cycle	145±47	120±32	125±20	149±21
15	Arginine → Ornithine + Urea	Urea cycle	28±23	25±38	59±17	76±16 [c]
16	Ornithine + CO2 + NH4 ↔Citrulline	Urea cycle	34±19	32±32	42±15	61±15
17	Citrulline + Aspartate →Arginine + Fumarate	Urea cycle	28±20	27±32	69±15 [b]	92±16 [c]
18	Arginine uptake	Aminoacid metabolism	3.6±5.1	2.3±1.8	-16±7 [b]	-20±10 [c]
19	Ammonia Output	Urea cycle	-6.2±1.7	-5.3±0.6	7±1 [b]	9±3 [c]
20	Ornithine Output	Urea cycle	-3.4±0.9	-2.6±2.5	11±5 [b]	11±8 [c]
21	Citrulline Output	Aminoacid metabolism	5.6±5.2	4.6±3.1	-27±9 [b]	-31±13 [c]
22	Alanine → Pyruvate + NH4 + NADH	Aminoacid metabolism	13±6.7	21±11	7±4	6±3 [c]
23	Alanine Output	Aminoacid metabolism	-13±6.2	-24±10	-2±2 [b]	-2±2 [c]
24	Serine → Pyruvate + NH4	Aminoacid metabolism	6.8±11	1.6±18	21±10	22±10
25	Serine Uptake	Aminoacid metabolism	2.7±1.7	4.8±1.6	6±4	8±3
26	Cysteine → Pyruvate+ NH4 + NADH	Aminoacid metabolism	0.8±3.7	-2.8±5.9	7±3 [b]	6±3 [c]
27	Cysteine Output	Aminoacid metabolism	0.2±0.0	0±0.1 [a]	-1±1 [b]	-1±0 [c]
28	Threonine → NADH + Glycine +Acetyl-CoA	Aminoacid metabolism	1.2±4	2.4±4.9	-2±2	-1±1
29	Glycine ↔ CO2 + NH4 +NADH	Aminoacid metabolism	4.2±8.2	1.3±13	11±6	11±7
30	Glycine Uptake	Aminoacid metabolism	5.4±1.2	7.1±2.5	2±1 [b]	4±1
31	Valine + 2-oxo-Glutarate → Glutamate + propionyl-CoA + 3 NADH +FADH2 + 2 CO2	Aminoacid metabolism	0.8±0.4	1.2±2.8	0±1 [b]	0±1

32	Isoleucine + 2-oxo-Glutarate → Glutamate + propionyl-CoA + Acetyl-CoA + 2 NADH + FADH2 +CO2	Aminoacid metabolism	-0.2±0.7	0.5±1.3	0±1	0±1
33	Leucine + 2-oxo-Glutarate → Glutamate + NADH + FADH2 Acetoacetate + Acetyl-CoA	Aminoacid metabolism	-0.2±3.5	3.1±3.1	0±1	0±1
34	Propionyl-CoA + CO2 ->Succinyl-CoA	Aminoacid metabolism	5.1±2.0	3.6±3.4	0±2 [b]	1±2
35	Lysine + 2 2-oxo-Glutarate → 2 Glutamate + 4 NADH + FADH2 + 2CO2 + Acetoacetatyl-CoA	Aminoacid metabolism	1.5±0.4	5.1±5.7	2±2	2±1
36	Phenylalanine + O2 → Tyrosine	Aminoacid metabolism	0.5±0.4	2.2±0.6[a]	3±1 [b]	3±0 [c]
37	Tyrosine + 2 O2 →NH4 + CO2 + Fumarate + Acetoacetate + NADH	Aminoacid metabolism	3.7±3.7	0.1±5.4	8±3	8±3
38	Tyrosine Output	Aminoacid metabolism	-1.0±1.1	-1±0.4	0±2	0±1
39	Glutamate ↔ 2-oxo-Glutarate + NADH +NH4	Aminoacid metabolism	15±7.5	27±14	20±8	33±10
40	Glutamate Output	Aminoacid metabolism	0.8±1.3	1.1±1.1	7±1 [b]	16±5 [c,d]
41	Glutamine → Glutamate + NH4	Aminoacid metabolism	2.0±2.5	4±4.4	16±8 [b]	37±9 [c,d]
42	Proline +0.5 O2→ Glutamate + 0.5 NADH	Aminoacid metabolism	0.7±2.9	4.8±1.4[a]	2±2	3±1
43	Histidine → NH4 +Glutamate	Aminoacid metabolism	8.0±5.3	8±3.8	1±1 [b]	1±1 [c]
44	Methionine + Serine → Cysteine + NADH + Propionyl-CoA + CO2	Aminoacid metabolism	0.8±0.1	0.9±0.4	1±0	1±0
45	Aspartate↔Oxaloacetate + NH4 +NADH	Aminoacid metabolism	-24±17	-30±26	-56±14 [b]	-74±15 [c]
46	Aspartate Uptake	Aminoacid metabolism	0.5±0.4	0.2±0.3	0±1	-3±1 [c,d]
47	Asparagine → Aspartate + NH4	Aminoacid metabolism	2.0±1.1	0.9±0.2[a]	9±4 [b]	17±6 [c]
48	Palmitate→ 8 Acetyl-CoA +7 FADH2 + 7 NADH	Lipid, glycerol, fatty acid metabolism	11.1±7	8.5±4.2	22±3 [b]	18±3 [c]
49	2 Acetyl-CoA ↔ Acetoacetyl-CoA	Lipid, glycerol, fatty acid metabolism	11±16	2.9±19	69±10 [b]	62±15 [c]

50	Acetoacetyl-CoA → Acetoacetate	Lipid, glycerol, fatty acid metabolism	15±16	8.6±18	71±10 [b]	64±15 [c]
51	Acetoacetate Output	Lipid, glycerol, fatty acid metabolism	-1±1	1.1±2.7	18±5 [b]	27±14 [c]
52	Acetoacetate + NADH ↔b-Hydroxybutyrate	Lipid, glycerol, fatty acid metabolism	21±16	11±17	60±8 [b]	45±5 [c,d]
53	NADH + 0.5 O2 → NAD	Oxygen uptake and electron transport	302±181	327±31	236±48	212±42 [c]
54	FADH2 + 0.5 O2 → FAD	Oxygen uptake and electron transport	182±87	158±25	204±25	180±23
55	O2 Uptake	Oxygen uptake and electron transport	249±132	247±15	240±34	216±29
56	Glucose 6-phosphate→ 2 NADPH + CO2 +Ribulose 5-P	PPP	14±90	32±31	121±75	150±50 [c]
57	Ribulose 5-P ↔ Ribose 5-P	PPP	3±30	10±11	40±25	50±16 [c]
58	Ribulose 5-P ↔ Xylulose 5-P	PPP	18±61	24±19	79±51	100±34 [c]
59	Ribose 5-P + Xylulose 5-P ↔ Fructose6-P + Erythrose 4-P	PPP	10±30	12±10	40±26	50±17 [c]
60	Erythrose 4-P + Xylulose 5-P ↔ Glyceraldehyde 3-P + Fructose 6-P	PPP	15±31	14±9.1	39±27	50±17 [c]
61	CO2 Output	Oxygen uptake and electron transport	216±30	191±19	217±80	236±47

Note
[1] Data from ref. [39].
[a,b] Values different from in vivo sham, $p<0.05$
[c] Values different from in vivo 20% TBSA, $p<0.05$
[d] Values different from sham perfusion, $p<0.05$

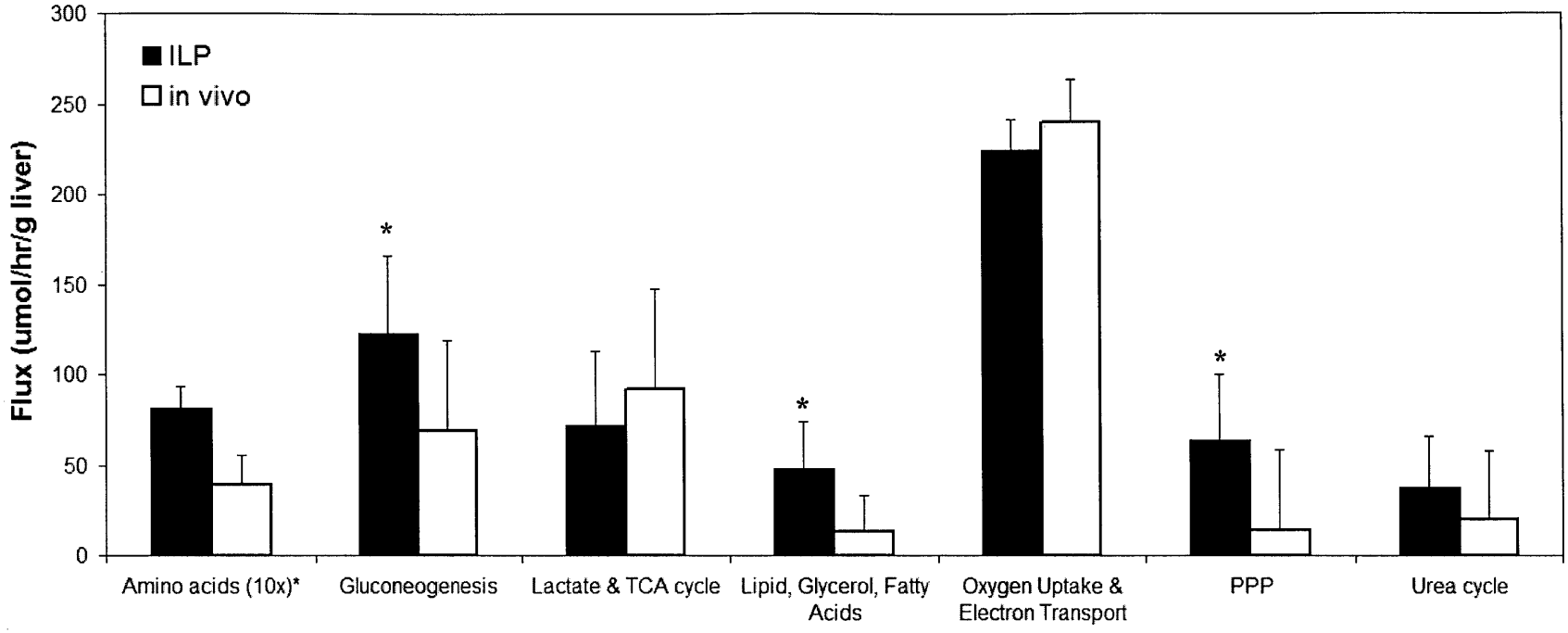

Figure 1: In vivo sham vs. ILP sham. A summary the major pathways in the hepatic metabolic network. Values shown are the averages of all fluxes in each pathway group. Flux numbers (from Table 1) averaged in each group are as follows: Gluconeogenesis: # 1-7. Lactate & TCA cycle: # 8-14. Oxidative phosphorylation: # 53-55, #61. Pentose phosphate pathway: #2, 3, 4, 5, 6. Amino acid metabolism: #18, # 21-47. Urea cycle: # 15-17, #19-20. Lipid metabolism: #48-52. PPP: # 56-60. *Indicates significant differences between *in vivo* and ILP response to burn ($p<0.05$).

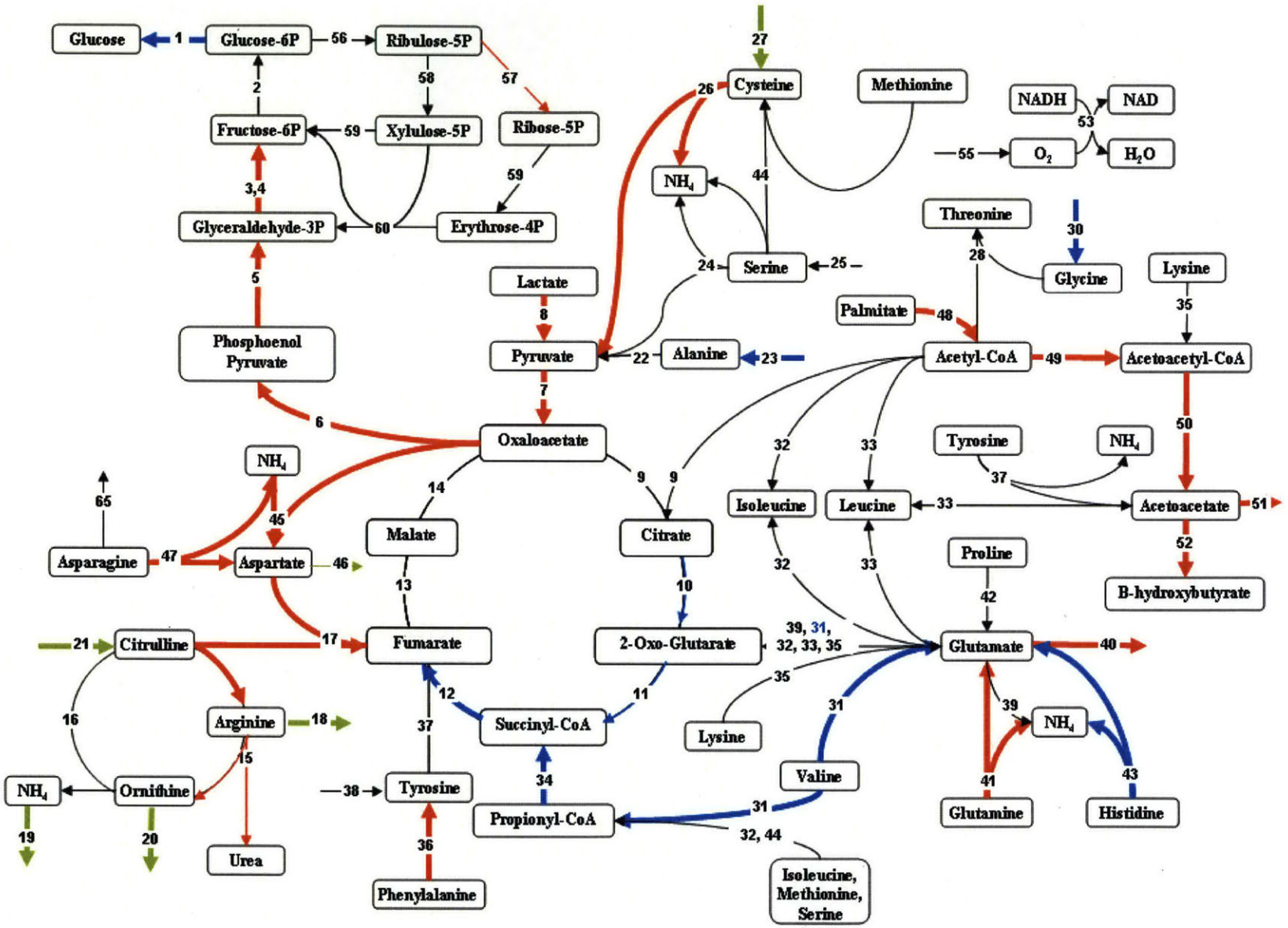

Figure 2: Flux directions reflect ILP results vs. in vivo. Red = Upregulated. Blue = Downregulated, Green = Reversed.

Table 2: Perfusate Influxes vs. Fluxes Across the Sham Liver

	INFLUX			FLUX		
PARAMETER	PV+HA	Perfusate	Perfusate / PV+HA	in vivo	ILP	Perfusate / PV+HA
Total Oxygen (ml O2/min/g liver)	0.26±0.14	0.1	0.4	249±132	240±30	1
Albumin (g/min/g liver)	0.03±0.01	0.17	6			
Lactate (mmol/min/g liver)	0.07±0.03	0.03	0.4	5.6±2.6	89±14*	16
Glucose (mg/min/g liver)	1.81±0.68	5.5	3	93±37	52±6.0*	0.6
Alanine (umol/min/g liver)	0.64±0.28	2.65	4	13±6.0	1.6±2.2*	0.1
Ammonia	0.15±0.04	0	0	6.2±1.7	-6.8±1.1*	-1
Arginine	0.25±0.25	4.15	17	3.6±5.1	16±7.0*	4
Asparagine	0.08±0.04	4.79	60	2.0±1.1	8.6±4.4*	4
Aspartate	0.03±0.01	0.35	12	0.5±0.4	-0.2±0.5	-0.4
Cysteine	0.02±0.01	1.42	71	0.2±0.0	1.4±1.2*	7
Glutamate	0.11±0.04	1.81	17	0.8±1.3	7.1±1.3*	9
Glutamine	0.48±0.2	11.08	23	2.0±2.5	16±8.0*	8
Glycine	0.43±0.17	2.1	45	5.4±1.2	2.4±1.4*	0.4
Histidine	0.22±0.13	1.63	7	8.0±5.3	0.8±1.0*	0.1
Isoleucine	0.15±0.08	2.2	15	-0.2±0.7	0.4±1.1	-2
Leucine	0.44±0.22	2.2	5	-0.2±3.5	-0.4±0.8	2
Lysine	0.36±0.18	2.88	8	1.5±0.4	1.8±1.7	1
Methionine	0.07±0.03	0.56	8	0.8±0.1	0.8±0.4	1
Ornithine	0.19±0.09	0	0	3.4±0.9	-11±5.0*	-3
Phenylalanine	0.09±0.04	1.07	12	0.5±0.4	3.2±1.0*	6
Proline	0.26±0.1	2.29	9	0.7±2.9	2.1±1.5	3
Serine	0.32±0.15	3.48	11	2.7±1.7	5.6±3.6	2
Threonine	0.33±0.14	2.23	7	1.2±4.0	-2.3±1.7	-2
Tyrosine	0.11±0.06	1.73	16	1.0±1.1	-0.5±1.7	-0.5
Valine	0.28±0.16	2.18	8	0.9±0.4	-0.4±0.9*	-0.4

Note
* Values significantly different from in vivo group ($p<0.05$)

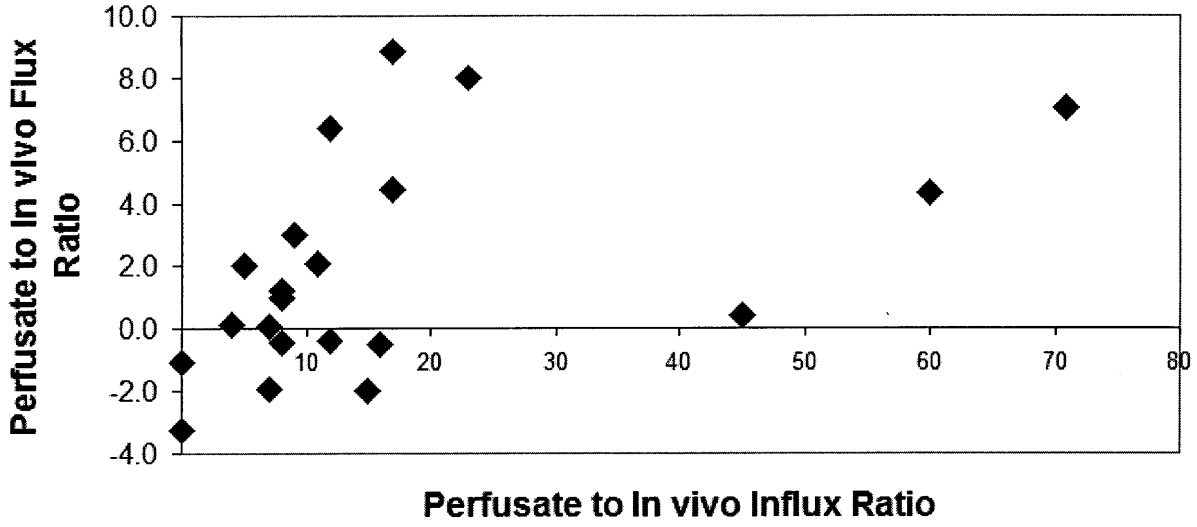

Figure 3: Scatterplot of the ratios of perfusate to in vivo influxes, and ILP to in vivo fluxes to determine if the liver is responsive to mass action effects from its inflow values.

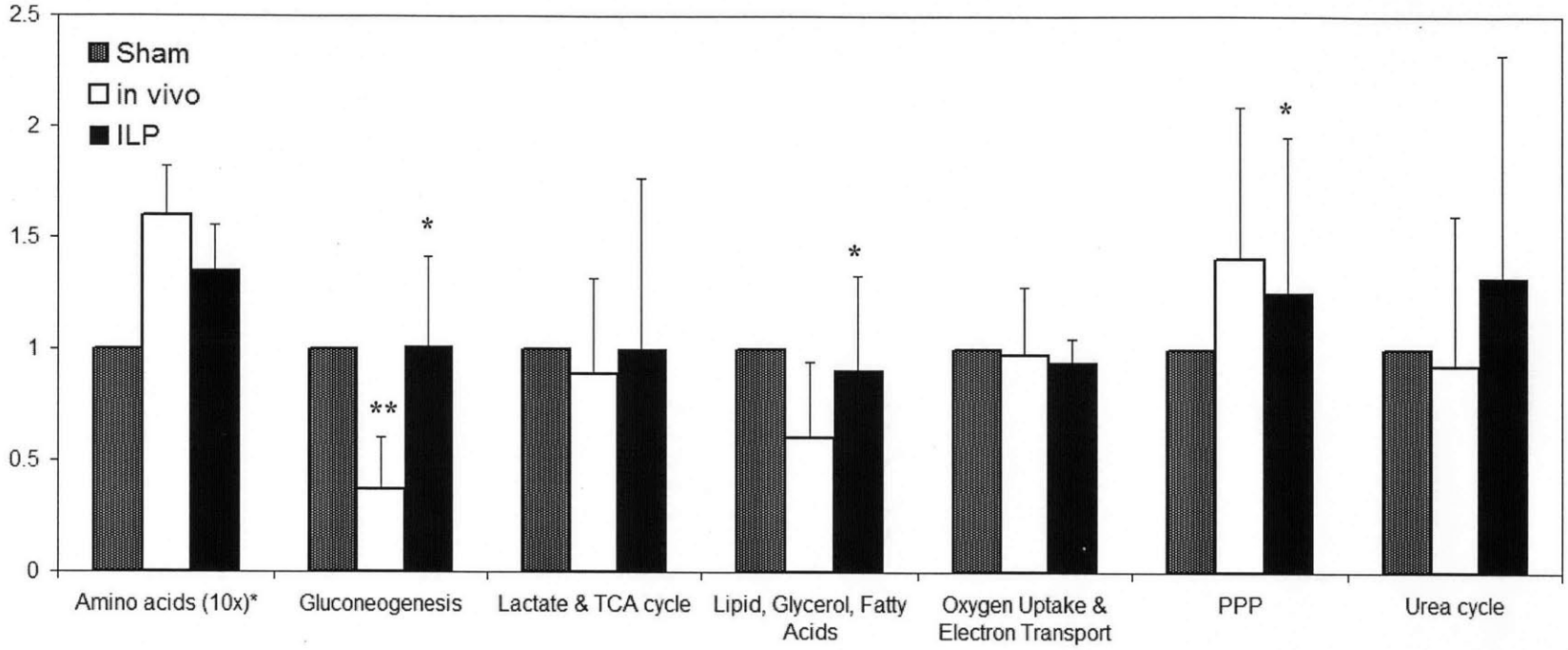

Figure 4: In vivo burn vs. ILP burn. A summary of the major pathways in hepatic metabolism after a burn injury, normalized to their sham values. *Indicates significant differences between *in vivo* and ILP response to burn ($p<0.05$). **Indicates a significant difference between sham and burn.

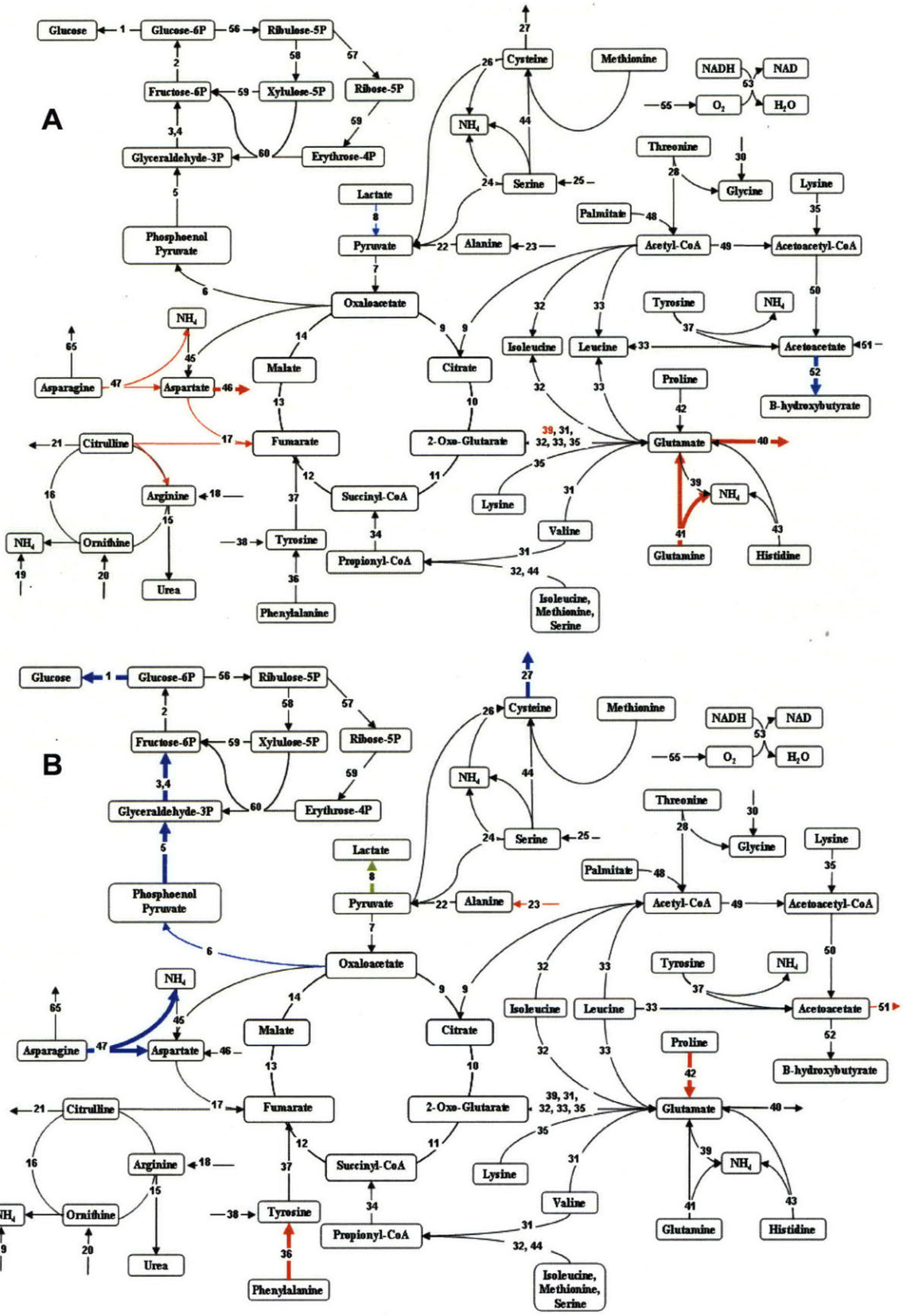

Figure 5: Flux directions reflect 20% vs. sham. **A:** ILP **B**: In vivo. Red = Upregulated. Blue = Downregulated, Green = Reversed

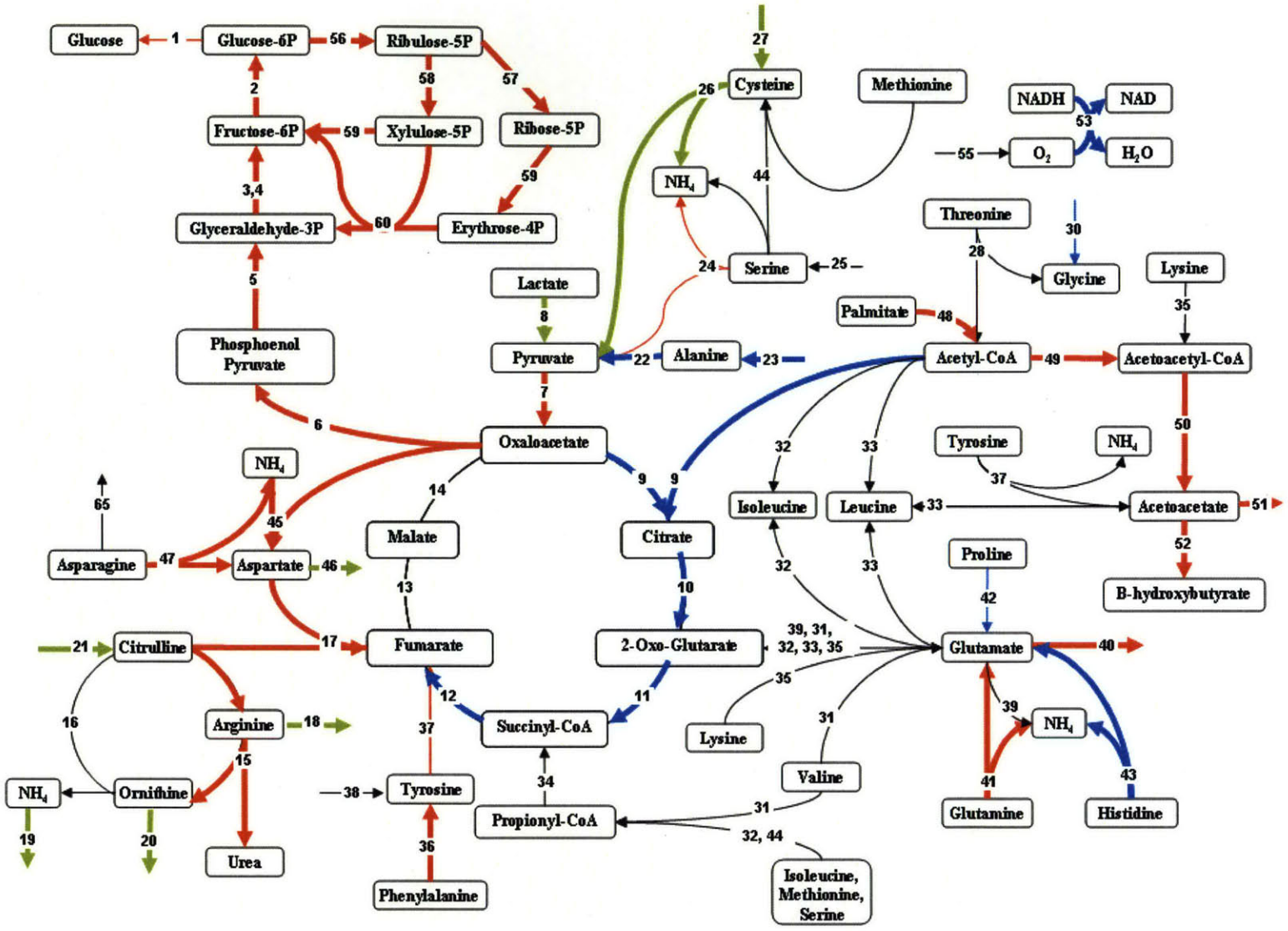

Figure 6: Flux direction reflects ILP 20% TBSA burn vs. in vivo. Red = Upregulated. Blue = Downregulated, Green = Reversed

DISCUSSION

The purpose of this study was to use a simple perfusion methodology as a basis for engineering an enhanced system capable of supporting a viable organ stably for extended periods of time. We took healthy livers and livers experiencing mild hypermetabolism (20% TBSA burn) and compared their metabolic profiles using ex vivo perfusion and in vivo results. The disparities between the groups yielded several important perfusion design considerations.

Evaluating the impact of a new ex vivo environment.

When evaluating the use of the perfusion system as a technique to capture in vivo organ metabolism we observed that within minutes of perfusion inception the metabolic composition of the perfusate had a profound impact on whole organ metabolism. A perfusate that is significantly different from blood has multiple clinical consequences: It implies that the original metabolic state of an organ, especially a diseased one, cannot be accurately gauged or subsequently treated; metrics for long-term organ stability and viability must be perfusion specific, and may not be limited to known metabolic parameters, frustrating the rapidity with which this will be translated to clinical use; approximately an hour is required for the organ to assume a new steady state in an ex vivo environment. A positive finding regarding non-physiologically relevant perfusates is their apparent ability to dampen, or potentially obviate the stimuli for, metabolic abnormalities. This may be useful in returning a liver to its metabolic baseline, potentially even enabling self-repair mechanisms. If, in addition, it were possible to altogether reduce metabolic activity pharmacologically, the synergistic effect may be to enable very long stable storage times. Designing a truly physiological perfusate that imparts no systemic effects on organ metabolism is an unlikely endeavor, but ensuring a quiescent environment that optimizes organ energy status should be strived for.

Meeting the oxygen requirements of the ex vivo perfused liver.

The metabolic rate of normothermic perfusion would logically require a high oxygen delivery rate. Flow rates in the absence of oxygen carriers must be increased to ensure adequate oxygenation[44] but at values 3x physiological, they risk inducing mass-action effects that drive fluxes inappropriately, consume resources unnecessarily, accumulate toxic byproducts more rapidly, and ultimately impart detrimental mechanical stress on the liver. Further, it has been suggested that the use of hyperbaric oxygen is ultimately damaging to the ultrastructural components of hepatocytes, the mitochondria in particular[8]. We propose the addition of erythrocytes to the system to reduce flow rates and oxygen tension to physiological

values. The use of erythrocytes however, introduces various levels of complexity to perfusion design. The first is the ethical procurement of enough blood to prime the perfusion circuit; the recommendation of a 10-20% volume of erythrocytes[45,46] for a rat model requires the exsanguination of 3 additional large rats for an 80ml perfusate volume, or the use of donated blood for human livers. Which leads to the second issue of the effects of aging on the function of the erythrocytes where the freshest possible are preferred, but are also the most metabolically active[1,5,47,48]. The issue of procurement therefore must also be considered and possibly incorporated into the routine of the procedure. Normal erythrocyte loss of viability and mechanical induction of lysis are feared to cause problems by varying hematocrit, causing microemboli and possible cytotoxic effects. Further, a study by Mischinger et. al. [6] strongly eschewed the use of erythrocytes demonstrating that even in their presence, the rate of oxygen uptake by livers in perfusion gradually declined over time (60 minutes) prior to plateauing at levels similar to asanguineous perfusates. It is of course entirely plausible that in the absence of lipids in the perfusate, and the removal of any external demands on the organ, the metabolic activity will be reduced enough to consider the removal of erythrocytes, however, none of these studies advocated low perfusion rates which will certainly be deleterious over long perfusion times[45] at 37°C. However, if the use of perfusion systems does not require function at physiological temperatures to evaluate organ viability, then it is entirely feasible to consider the removal of erythrocytes[49] (at the increased risk of hyperbaric damage).

To minimize the volume of erythrocytes used for both ethical and logistical reasons, we propose the formation of a primary, erythrocyte-containing, circuit and a secondary circuit connected to a perfusate reservoir. Communication between the circuits will be facilitated by a dialyzer with a cutoff weight designed to exclude albumin (<60kDa). This will serve as the "renal module" of the system enabling adequate mixing of nutrients and waste molecules from the primary to secondary circuit while retaining erythrocytes and large proteins in the primary circuit at hematocrits controlled by the flow rates of each circuit.

Evaluating the need for impermeants or colloids.

The addition substrates to reduce or prevent cell swelling are a popular topic in organ preservation as edema is a natural correlate of reduced ATP. Numerous impermeants and colloids have been considered with various degrees of success[50]. Bovine serum albumin (BSA) was used in this study. A natural colloid with substantial importance it has nevertheless contended with controversy in its use - some believe it to be toxic to murine cells, while others have shown no difference at short perfusion times[49,51]. We opt to exclude this from the perfusate

and initially supplement only with flash frozen and filtered plasma from freshly drawn blood[6,10]. Should the addition of this fragment be either redundant or deemed as conferring additional levels of unregulated complexity to perfusion, it will be removed altogether.

Improving the basic perfusate composition through metabolic analysis.

Findings due to perfusion-based metabolism analysis may be supplemented with additional prior results by Yamaguchi, Chen and Lee et. al. [28,35,38]. Evaluation of their outcomes reveals mixed consistency in results. While no attempts at seeking conclusions of hepatic hypermetabolism through ex vivo perfusion are sought in this work, the point of consistency and accuracy in perfusate content and metabolic analysis is highly valued in being able to derive meaningful results. The volatility in these measurements speaks to the difficulty of assessing organ function based on rapidly-changing organ metabolism. Nevertheless, through the development of systematic stable perfusion, identifying the combination of parameters that most strongly reflect viability will be possible.

The use of MFA improves the ability to compare perfusion outcomes based on intrinsic organ function and metabolic state rather than highly system-specific findings. Nevertheless, the impact of mass action driving effects further supports the need to evaluate organ function with certain standardized perfusion conditions. For example, subtle differences between in-lab models result in very different outcomes and interpretations. In Yamaguchi and Lee et. al.'s work, perfusate flow rates were between 2.7 and 3.4 ml/min/g liver compared to Banta et. al. at 5.5ml/min/g liver. Flow rates in perfusion were increased in Banta et. al. in order to ensure adequate oxygen delivery to the livers; oxygen uptake was found to be increased compared to Lee et. al. Further, Lee et. al. did not recirculate the perfusate implying a constant concentration at the influx compared to other groups which closed the perfusion circuit, inducing cumulative changes in metabolite concentrations over time. The latter groups observed tendencies to arrive at steady state values in 60 minutes, while mostly linear trends were observed in Lee et. al. Particular pathways impacted by large influxes can be appreciated in Table 2 and Figure 3. The rate of influx does not appear to correlate with liver function in all cases however, due to other likely mechanisms of metabolism control.

Hormonal impact of metabolism is an important consideration in enabling stable and non-futile metabolism. It was observed that in fasted sham in vivo livers, insulin levels were low, but 4-fold higher in fasted 20% burn rats [52], while MEM-based perfusate was not supplemented with insulin. Insulin was determined to be a strong factor in the reduction of gluconeogenesis and lipid metabolism, and subsequent increase consumption of amino acids in vivo.

In conclusion, these results suggest that ILP is amenable to depicting in vivo phenomena provided perfusate composition is used that mimics the ratios of metabolites present in vivo, and includes appropriate amounts of hormonal constituents such as insulin and glucagon. We subsequently propose the use of a perfusate medium more closely resembling in vivo conditions and better suited to the culture of rat hepatocytes, such as phenol-red Williams Medium E[9]. To prevent significant leukocyte activation we propose the addition of the steroid hydrocortisone, a common finding in cell culture medium. We also propose the addition of penicillin and streptomycin to reduce bacterial contamination over long perfusion times[11].

METHODS

Isolated Liver Perfusion

Surgical preparation and *in situ* liver perfusion have been described elsewhere [35,39]. Briefly, access to the hepatic vasculature was obtained after anesthesia induction and laparotomy. The portal vein and then the SHVC were cannulated with a 2.0-mm O.D. basket-tipped cannula (Harvard Apparatus). The hepatic artery and inferior vena cava were ligated. The perfusion apparatus (Harvard Apparatus) comprised a peristaltic pump, a membrane oxygenator (95% O_2, 5% CO_2), and heat exchanger (38°C). A flow rate of ~5.5 ml/min/g of liver was chosen to ensure adequate oxygen delivery. The liver was flushed with ~100 ml of perfusate, prior to closing the circuit. Perfusate samples (1 ml) were collected every 10 min for an hour and the final weight of the liver and perfusate volume were recorded. Perfusate comprised a standard solution of Minimum Essential Medium supplemented with amino acids, lactate, pyruvate, and 3% bovine serum albumin (BSA) as previously described [35]. Measured fluxes were calculated from the linear segment of the slope obtained by plotting the metabolite concentration in perfusate as a function of time.

Metabolite Analyses

All blood/perfusate samples were immediately evaluated for blood gases (Bayer Diagnostics, Pittsburgh, PA). The remaining sample was spun down at 3000 x g and the plasma frozen at -80°C. The samples were later analyzed for glucose, lactate, urea, albumin, acetoacetate and β-hydroxybutyrate, using biochemical assays. Amino acid composition was

determined using an automated high-performance liquid chromatography system (Waters Co., Milford, MA).

Statistical evaluation of the differences between perfusion and in vivo outcomes

The measured fluxes, calculated influxes and MFA results of the experiments conducted by Izamis et. al. and Banta et. al. were compared using a 2-tailed Student's t-test, and $p<0.05$ was the criterion used for statistical significance.

3-2. Transplantable organ preservation with NELP.

A normothermic extracorporeal liver perfusion (NELP) is established that is comparable to static cold storage (SCS) in the preservation of viable organs for successful transplantation. This system enables the investigation of the treatment of reversible pathologies such as ischemia[53] and steatosis[54] which could not be achieved with SCS, thereby significantly increasing the sources of hepatocytes and transplantable organs. In this study, we describe a novel rat NELP system (Figure 1) based on an extensively documented isolated perfused rat liver system that was originally developed for short-term perfusions[38,43,55,56]. 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. This improved system can maintain healthy rat livers metabolically stable for at least 6 hours, significantly extending the normothermic perfusion time from the typical 2-4 hours for perfused rat livers[56]. Furthermore, we show that the perfused livers could be routinely transplanted into syngeneic recipients with excellent long-term survival.

RESULTS

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, $n=11$). Hematoxylin and eosin evaluation of livers after 6 hours of 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 wall. Cell nuclei appear round and healthy, clear cell borders and obvious bile canaliculi (Figure 3).

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 4A). 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 4B). 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 4C). Urea levels exhibited a biphasic response (Figure 4D). 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 5). Both AST and ALT levels were increased 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 AST 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.

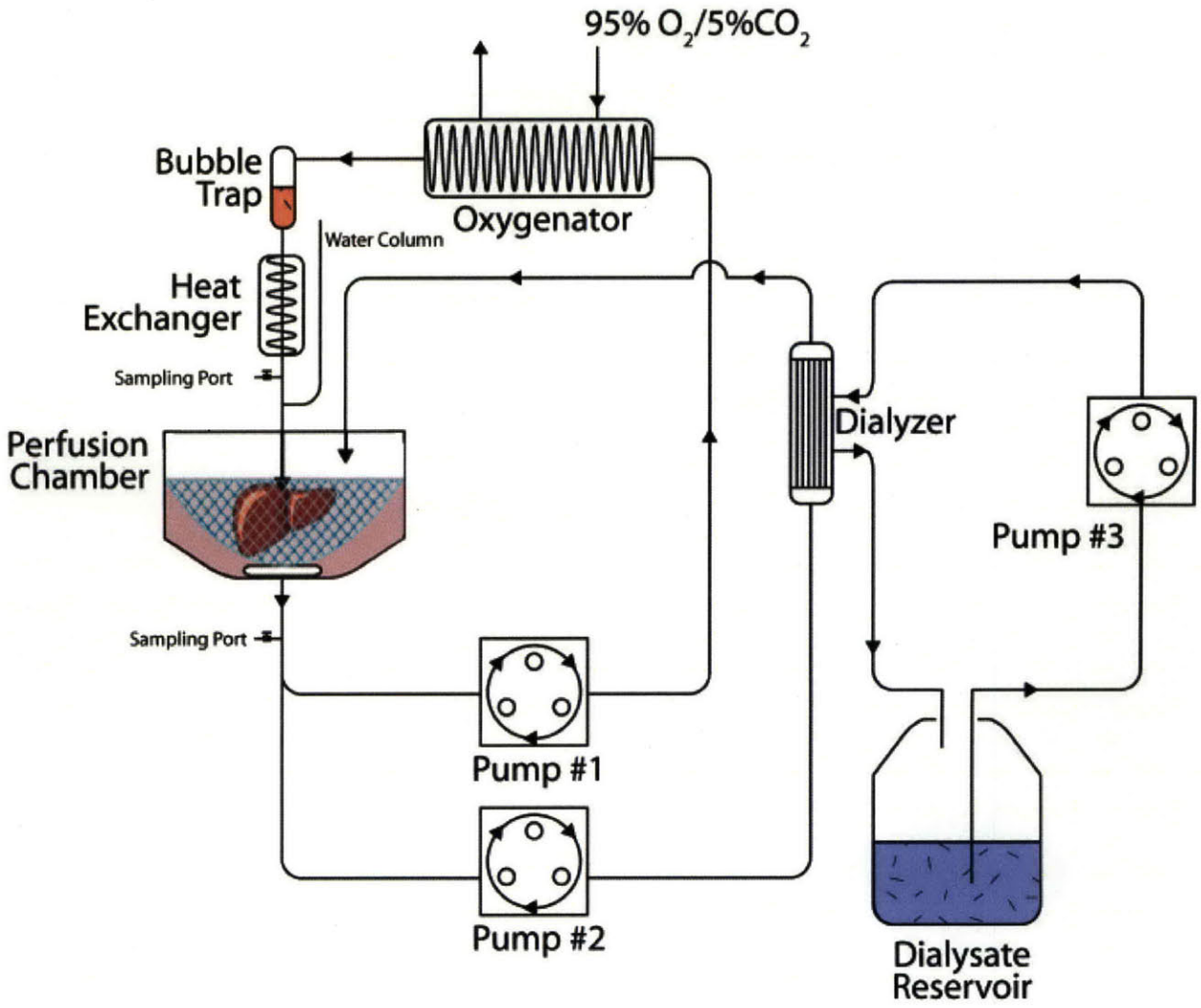

Figure 1: Normothermic Extracorporeal Liver Perfusion system with: (I) Primary Circuit (Pump #1, Oxygenator, Bubble Trap, Heat Exchanger, Manometer, Sampling Port leading directly to portal vein catheterized access of the liver in Perfusion Chamber), (II) Secondary Circuit (Pump #2, Dialyzer, Pump #3, Dialysate Reservoir).

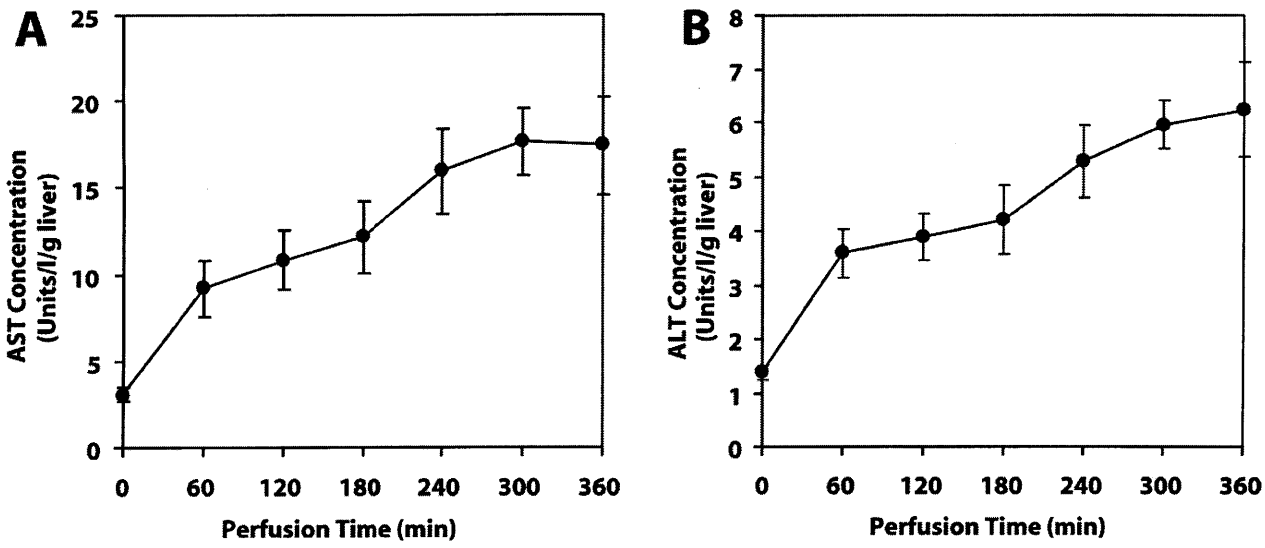

Figure 2: Liver integrity during normothermic perfusion. (A) Aspartate aminotransferase (AST) and (B) alanine aminotransferase (ALT) levels in perfusate samples collected hourly from the primary circuit. Values are normalized to the wet weight of the liver. Values at 360 min were 17.39±6.34 U/L per g for AST and 6.25±2.18 U/L per g for and ALT, n=6.

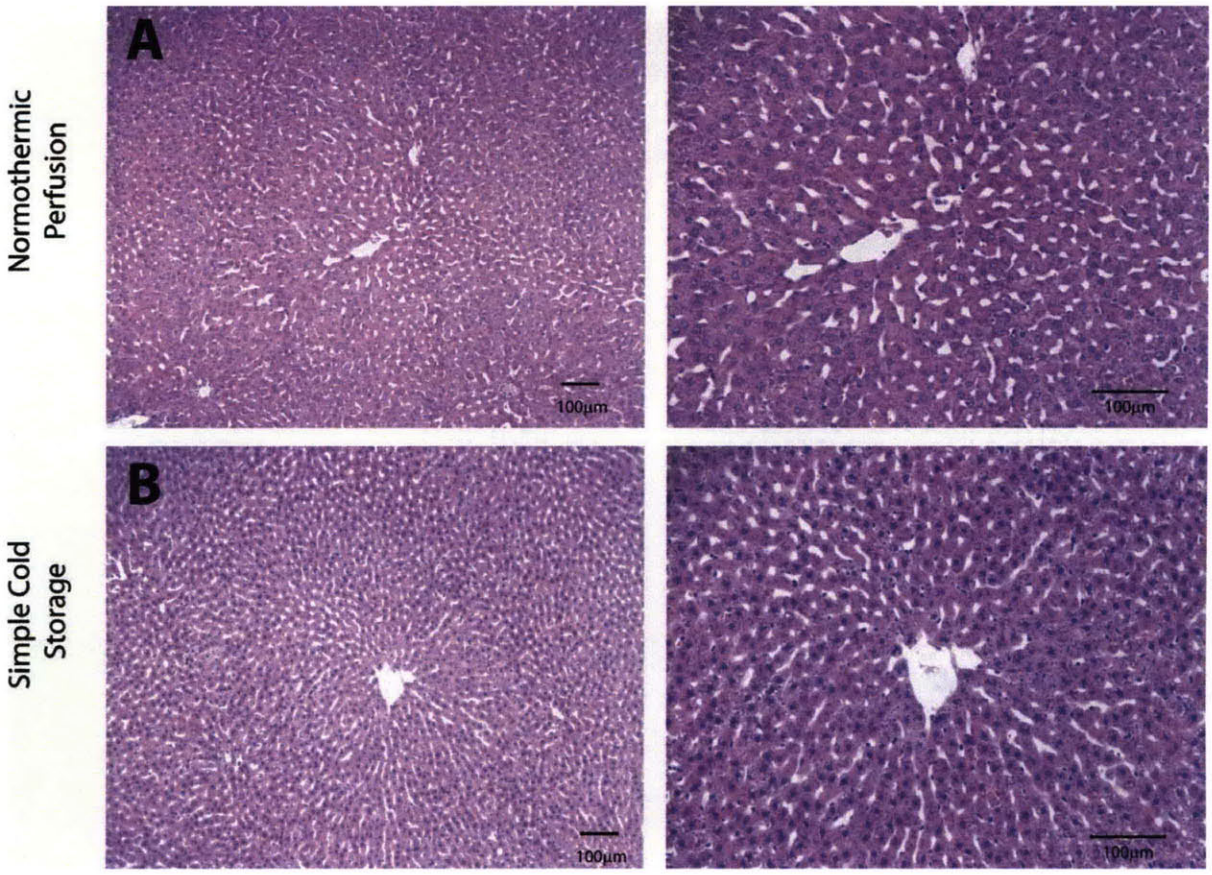

Figure 3: Microscopic appearance of livers after (A) 6 h of normothermic perfusion and (B) 6 h in simple cold storage in University of Wisconsin solution. Bar=100µm.

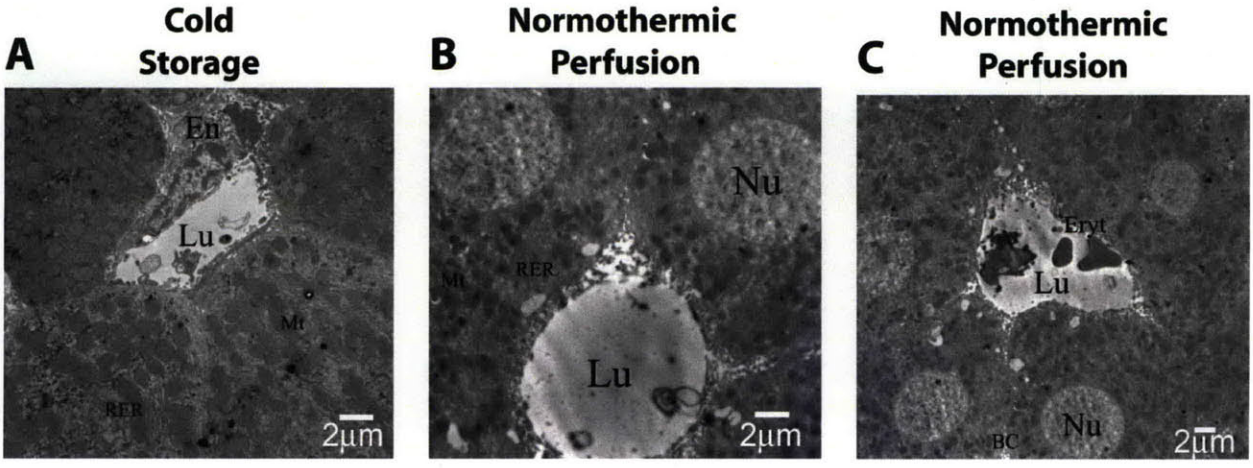

Figure 4: Transmission electron micrograph of livers after (A) 6 h of simple cold storage in University of Wisconsin solution and (B, C) 6 h of normothermic perfusion. Under both conditions, the liver appears normal. Mitochondria (Mt) appear elongated and not swollen, with dominant rough endoplasmic reticulum (RER). Sinusoids appear have open lumens (Lu) and fenestrated endothelial wall. Cell nuclei (Nu) appear round and healthy, with 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=2µm.

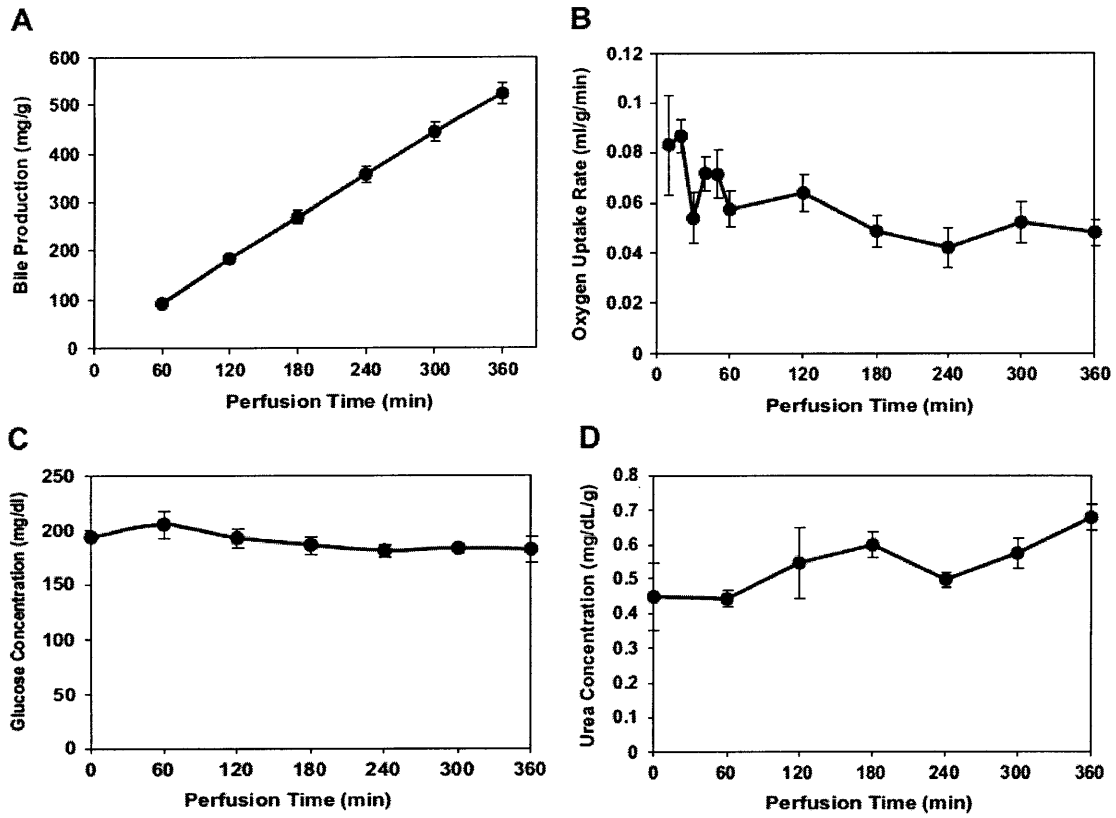

Figure 5: Hepatic metabolic activity during perfusion. (A) Total bile produced normalized to wet liver weight. (B) Hepatic oxygen uptake rate normalized to wet liver weight. (C) Glucose concentration during the perfusion. (D) Urea levels normalized to wet liver weight, n=6.

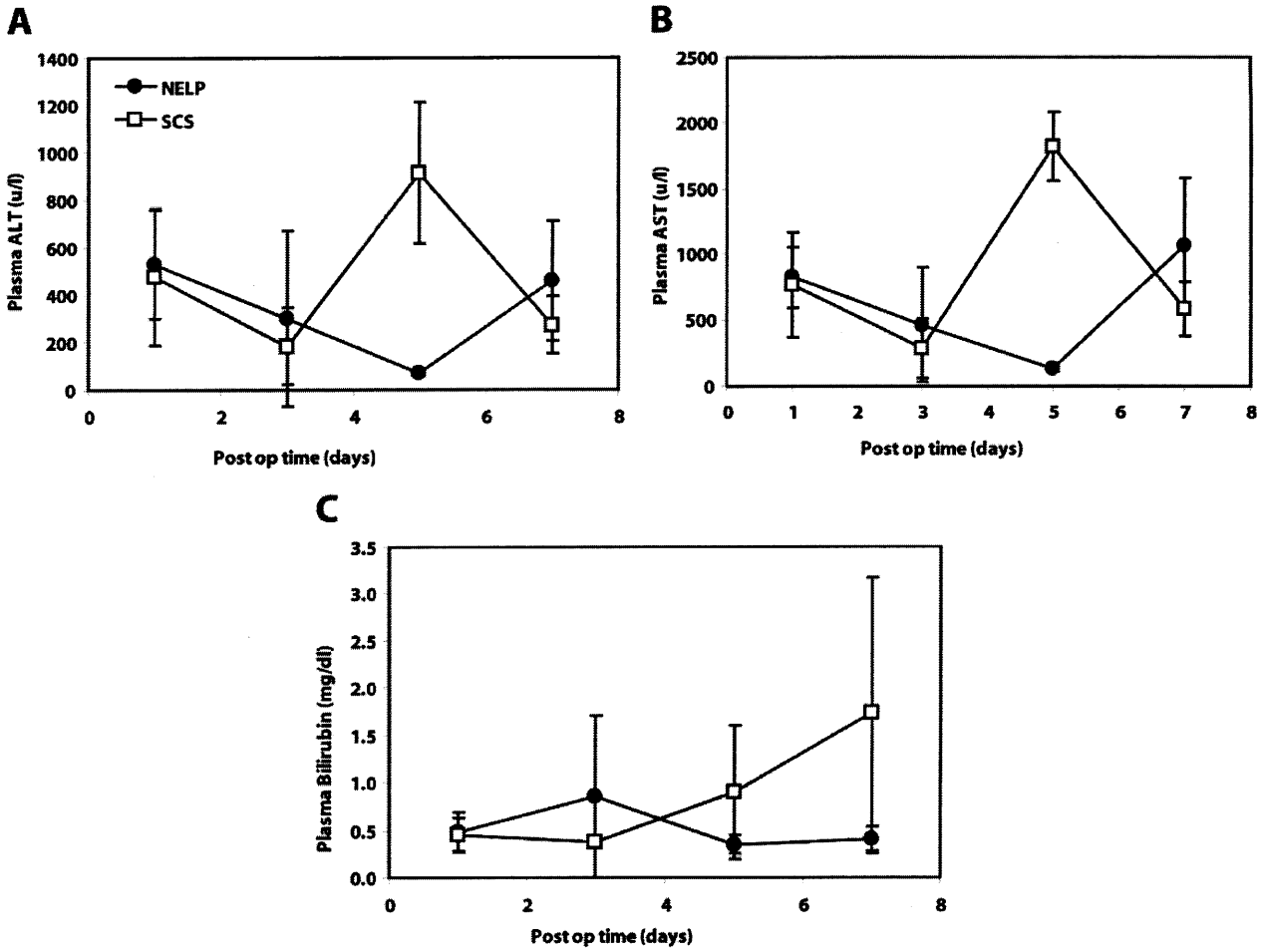

Figure 6: Markers of hepatic integrity and function in the transplant recipient. (A, B) Postoperative plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. (C) Total plasma bilirubin levels, n=3.

DISCUSSION

We 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. 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[57]. Although some studies indicate that certain areas of the liver are accessed by arterial blood only[58], the bulk of the literature suggests that it is possible to perfuse via the portal vein only[56,59], as well as to carry out the transplantation without reconstruction of the hepatic artery[60]. 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.

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 (16), the primary perfusion circuit used in our system has a volume of 50 to 55 ml, making it possible to use blood from two to three 400 g rats providing 15 ml of blood each to achieve the hematocrit close to 20 deemed necessary for optimal liver function[46] 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[60]. 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[63] with rat livers normothermically perfused without dialysis. Long-term normothermic perfusion of the porcine liver has been demonstrated[64], but transplantation of these livers has not been reported. The only study that demonstrates successful stable post-transplant survival employed a dialysis circuit[53]. 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.

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[65-67]. 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°C) 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 MasterflexTM 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°C 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 40C. 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.

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 O2 (g/dl) in the samples was determined according to the formula

$$[O2] = 0.0139 \times [Hb] \times FO2Hb + 0.00314 \times pO2$$

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. The hepatic oxygen uptake rate (HOUR) was determined by subtracting the total O2 content in the outflow minus the inflow, then multiplying by the flow rate, and normalizing to the wet weight of the liver:

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

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 (00C) 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, 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 (370C) lactated Ringer's solution with 5% dextrose and 2 ml/kg of NaHCO3 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 from 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.

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Chapter 4: Perfusion Recovery of Ischemic Livers for Transplantation

In this chapter we describe the application of NELP as a methodology to recover non-transplantable ischemically damaged organs to a viable state.

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The full publication is attached as Appendix 2. Permission to reuse this article has been granted by Wolters Kluwer Health.

We successfully recover organs damaged by warm ischemia to transplantable state using NELP. By contrast, static cold storage (SCS) has a 100% mortality rate. This illustrates the superior functional advantage of perfusion as a means of expanding the current donor pool. 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 [1]. It is estimated that about 6,000 ischemic livers [1,2] 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 [1]; they exhibit a higher risk of primary non-function as well as delayed graft failure, especially due to biliary complications such as stricture [3]. 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 [4-8]. Recently, functional recovery of ischemically damaged rat livers was shown using a combination of SCS followed by short term hypothermic machine perfusion[9,10]. However, extended hypothermic machine perfusion can cause endothelial damage [11], 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[12-14]. Near-normothermic machine perfusion has been successfully used in experimental kidney preservation[15-17], and recently normothermic perfusion was shown to be superior to SCS in the preservation of DCD livers[13,14,18]. A survival benefit after transplantation of DCD livers preserved normothermically has been demonstrated in one study using a porcine model[13].

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[19]. 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.

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 [19]. 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

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).

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 [20].

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 [21]. It is worth noting that the elevated bilirubin levels are reported [21] 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) [22]. 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 [11], or ischemic preconditioning effects of warm ischemia [23].

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 [24-26] and to cellular ATP levels [27,28]. The average 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 [29].

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 preservation of ischemic livers. However, the absence of apoptosis in the WI-only livers that result in primary non-function when transplanted, suggest that apoptosis is not a determinant factor for graft survival.

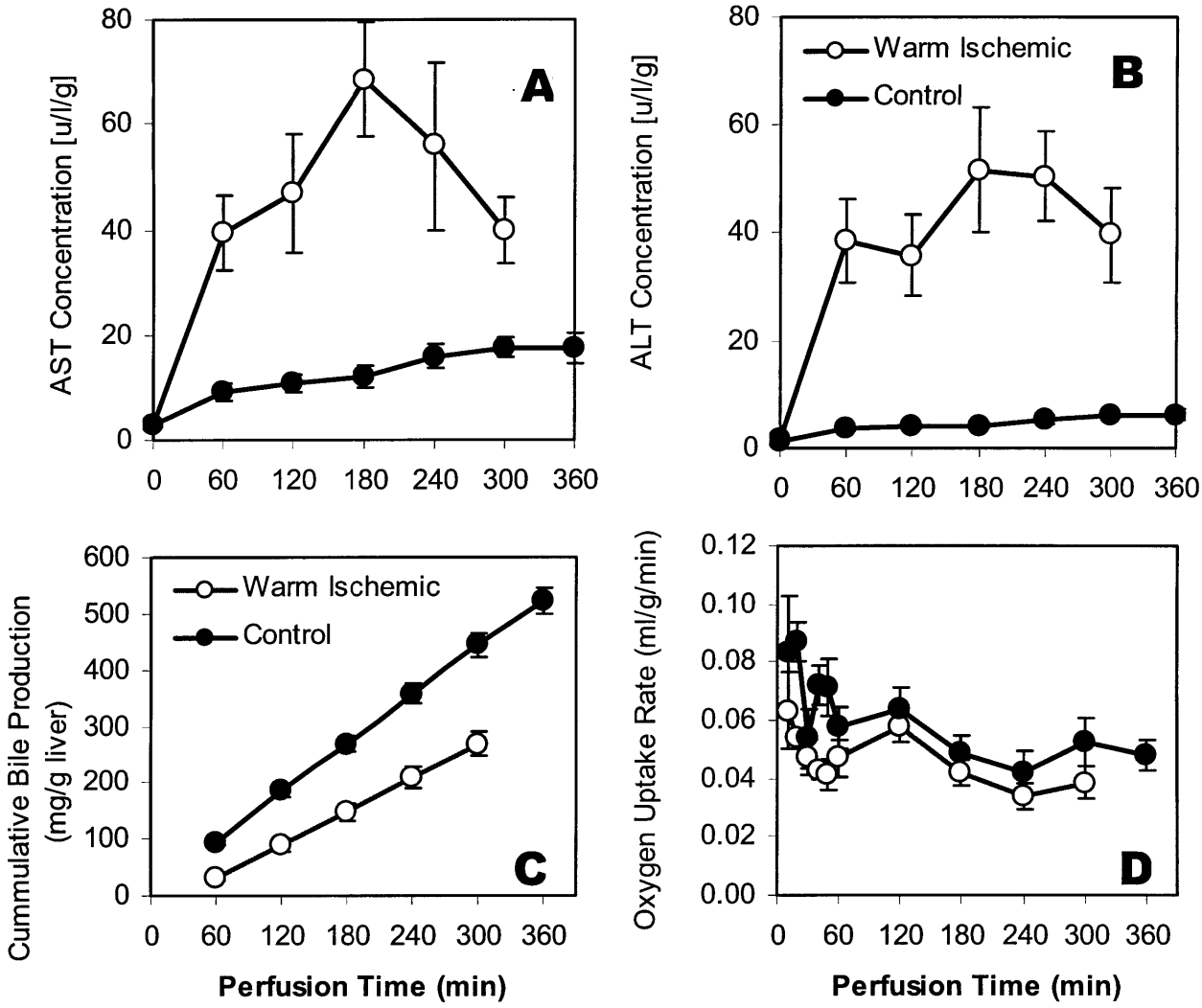

Figure 1: Function and Integrity of warm ischemic livers during normothermic perfusion. (A) Asparate aminotransferase (AST) and (B) Alanine Aminotransferase (ALT) levels in perfusate samples collected hourly from the primary circuit; (C) Total bile accumulation normalized to wet liver weight; (D) Oxygen 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 $p<0.1$.

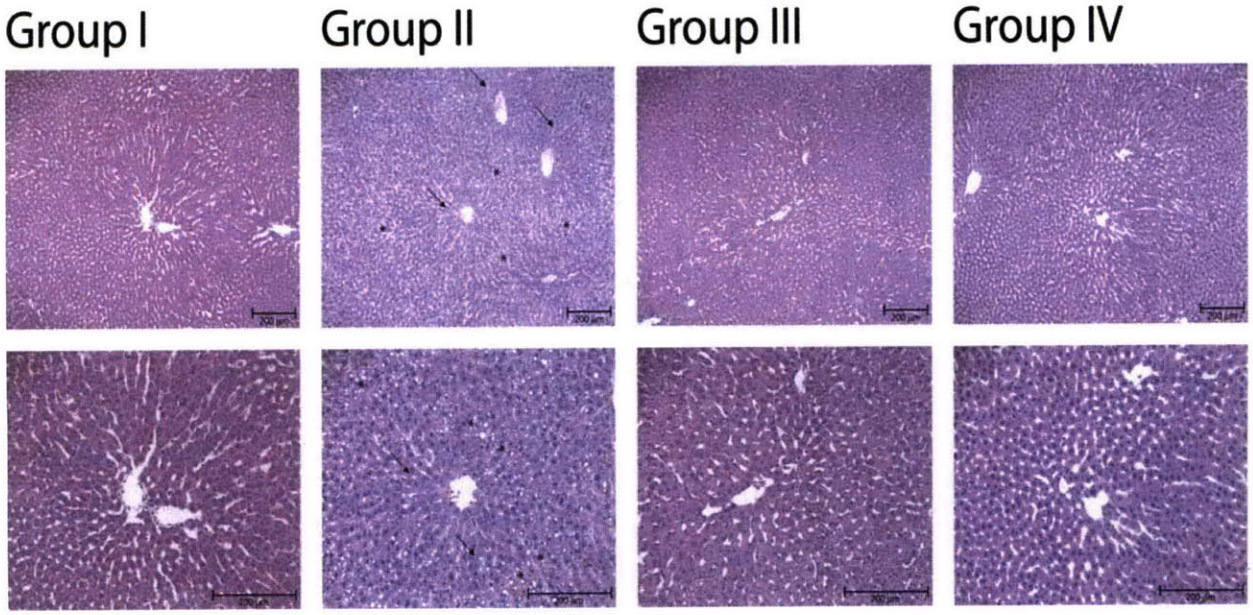

Figure 2: Microscopic appearance of livers after preservation. A) Group I: Warm ischemic livers after 5 hours of Normothermic Extracorporeal Liver Perfusion (NELP). B) Group II: Warm ischemic livers after 5 hours of Static Cold Storage (SCS) in University of Wisconsin (UW) solution (Arrows indicate cell 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.

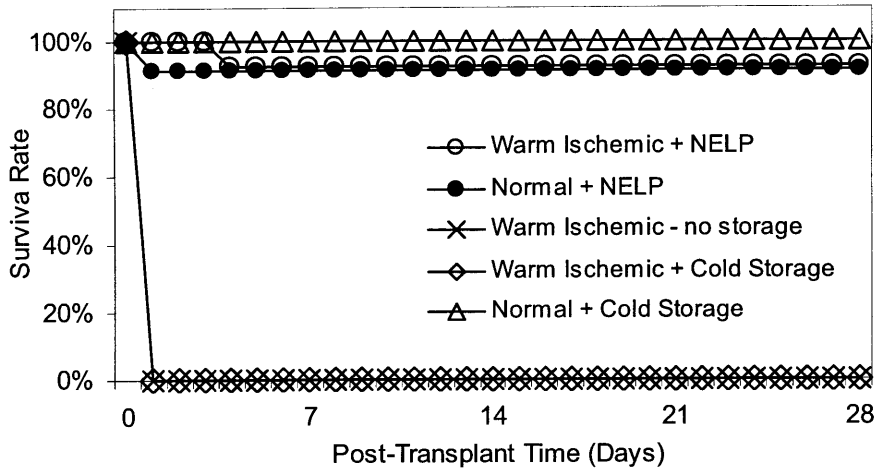

Figure 3: Survival curves of recipient rats after transplantation of perfused warm ischemic livers, warm ischemic cold-stored livers, compared to healthy perfused livers, and healthy cold-stored livers. Data for the Normal+ Normothermic Extracorporeal Liver Perfusion (NELP) group are from Tolboom et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

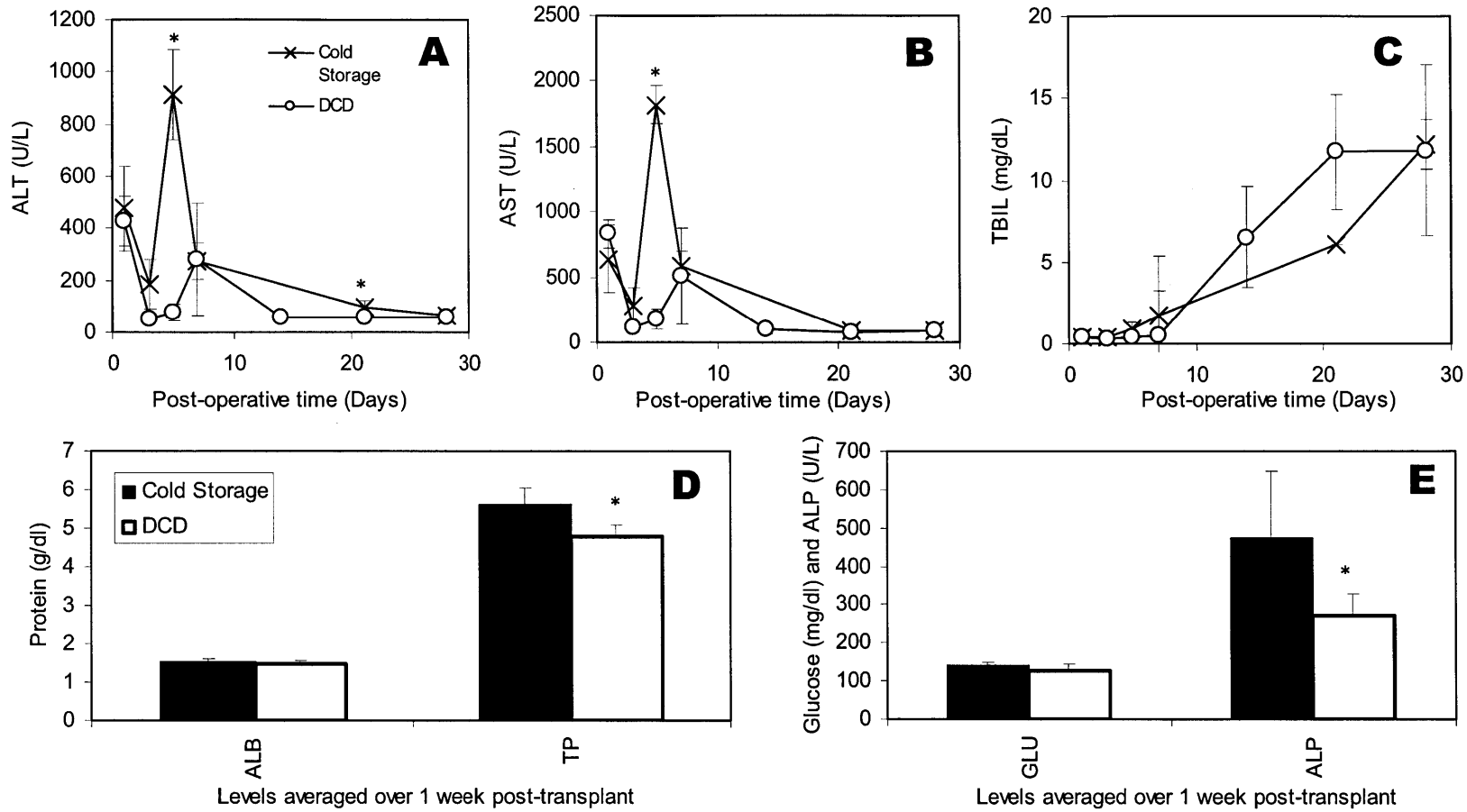

Figure 4: Values of A) Asparate aminotransferase (AST), (B) Alanine Aminotransferase (ALT) (C) Total Bilirubin (TBIL) measured on days 1, 3, 5, 7, 14, 21 and 28 after transplantation of perfused warm ischemic livers compared to healthy cold-stored livers. (D) and (E): comparison of the serum levels of 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 $p<0.1$.

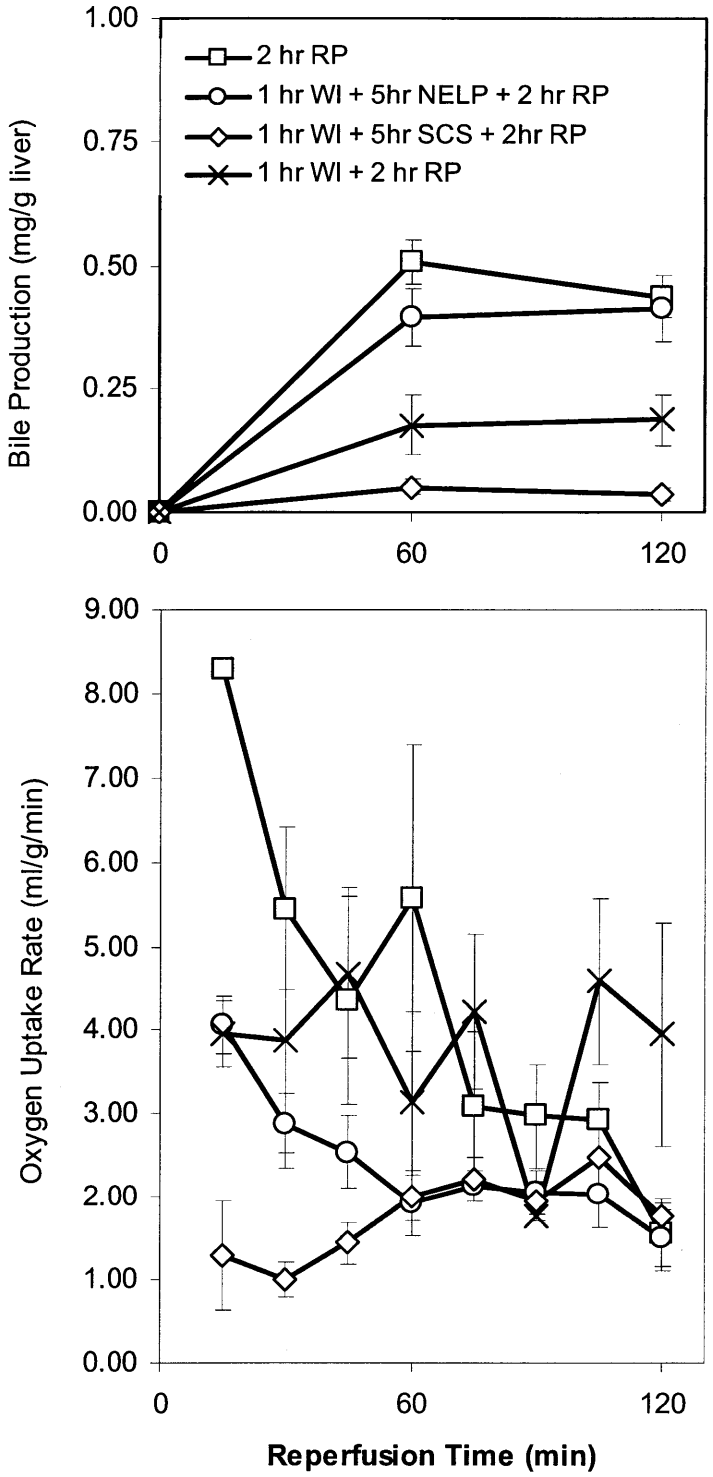

Figure 5: Reperfusion results: (A) Alanine Aminotransferase (ALT), (B) Asparate aminotransferase (AST), (C) bile synthesis, and (D) oxygen uptake rate measured during reperfusion. See text for statistical analysis.

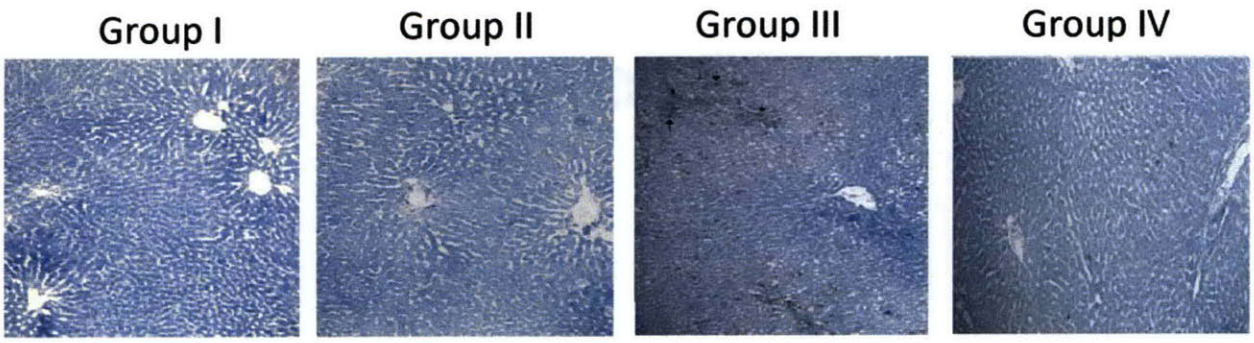

Figure 6: TUNEL of livers after preservation and reperfusion. A) Group I: Freshly isolated liver after reperfusion. B) Group II: Warm ischemic livers after reperfusion. C) Group III: Warm ischemic livers after 5 hours of Static Cold Storage (SCS) in University of Wisconsin (UW) solution after reperfusion.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

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 [30-32].

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 [33,34].

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 [35,36], we have chosen to perfuse via the portal vein only, as usually done in the traditional isolated perfused rat liver systems [35,37] , 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 [38,39]. 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 [21]. 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 [21]; 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 [5]. 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 [40] 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 metabolic resuscitation through restoration of an oxygen supply and removal of waste products. This is suggested by the correlation between bile secretion and survival whereby the restoration of metabolic activity, perhaps ATP levels and/or other metabolites, may play a role in the protective effects of NELP

A second hypothesis was that NELP reduced apoptosis, either through reduced Kupffer cell activity (due to presence of hydrocortisone in the perfusate) which is known to be correlated to improved graft survival[41] or ROS reduction (through glutathione which is present in Williams E) which was also found to correlate with graft viability[42]. 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.

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 [43]. 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.

METHODS

Isolation of donor livers

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[44,45].

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[19].

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[19] 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[19]. 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% N_2/ 21% O_2/ 5% CO_2 and 100% O_2 to maintain a constant pH. A fraction of the perfusate was diverted to the secondary circuit through 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) 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 H_2O (8-12 mmHg); Hematocrit: 17.8%±0.8; Inlet pO_2: 128.4±8.1 mmHg; Outlet pO_2: 47.9±1.7 mmHg; Inlet pCO_2: 30.1±1.1 mmHg; Outlet pCO_2: 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 O_2 (ml/dl) in the samples was determined according to the formula:

$$[O_2] = 1.39 \times [Hb] \times FO_2Hb + 0.00314 \times pO_2$$

where [Hb] is the hemoglobin concentration in g/dl, FO_2Hb is the fraction oxygenated hemoglobin and pO_2 is the partial pressure of oxygen in mmHg. HOUR was determined as:

$$HOUR = (([O_2]_{in} - [O_2]_{out})/100) \times \text{flow rate/weight of liver.}$$

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

Recipient surgery

The cuff technique developed by Kamada and Calne [38,39,45] was implemented and is described in detail elsewhere [19]. 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 $NaHCO_3$ 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 pO_2: 263.5±111.9 mmHg; Outlet pO_2: 75.1±49.7 mmHg; Inlet pCO_2: 40.4± 14.9 mmHg; Outlet pCO_2: 43.4±15.9 mmHg.

Histology

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 α=0.1.

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Chapter 5: NELP Optimization

5-1. MFA comparison of Fresh and Warm Ischemic Livers in NELP

MFA is performed on Fresh and WI livers in perfusion to evaluate the overall performance of the organs in perfusion. The major differences between ischemic and fresh liver metabolism is noted, time to recovery is suggested, and methods of improving perfusion are discussed. As transplantation is currently the only treatment option for end-stage liver disease and it enjoys a high success rate, there has been a significant increase in the demand for organs as older and more clinically complex recipient candidates are considered. An area of keen investigation is into the use of NELP as a means of resuscitating controlled and uncontrolled donors after cardiac death (DCD), as they represent a virtually unlimited source of organs[1]. Currently, unless very strict criteria regarding organ quality are enforced, clinical outcomes using these marginal organs have generally been poor[2]. However, porcine and murine models of DCD livers that have experienced an hour or more of warm ischemia have shown that when stored with NELP, the organs fare significantly better than organs exposed to static cold storage[3,4], and furthermore, NELP is necessary for their successful transplantation[5-7].

Multiple parameters of organ damage[8,9] and function[10] are currently measured during perfusion to gauge organ viability and potential transplantability. The restoration of organ energy charge and minimal lactate formation, for example, are suggestive of adequate mitochondrial function, and avoidance of microcirculatory failure[11,12]. Other factors frequently considered are trends in glucose metabolism, oxygen consumption, albumin or other complex protein syntheses, as well as rates of excess amino acid breakdown and urea formation[13,14]. As perfusate itself serves as a metabolic reservoir, it is reasonable and desirable to use whole-organ metabolism as a basis for developing a standardized integrated index of viability.

In this study, we perform a temporal analysis of 28 metabolites measured during perfusion in a rat model of NELP treatment of DCD livers[6], and use the stoichiometric metabolic flux analysis (MFA) model[15], to provide a comprehensive overview of ex vivo machine-perfused liver metabolism. This enables comparison of the metabolic state of ischemically damaged livers to fresh livers after several hours of perfusion, as well as to an MFA model describing in vivo liver metabolism[16].

The major findings of this work are:

- Recovery from ischemic damage occurs within 2 hours of perfusion signified by the restoration to Fresh livers levels of oxygen uptake rate and a significant shift in metabolic activity

- Fresh livers differ from WI livers metabolically; even upon the "restoration" of WI livers in their second phase of perfusion. WI livers appear to be overall less metabolically active than Fresh livers.
- The only distinct increase in WI liver activity is in the consumption and depletion of arginine highly suggestive of attempts at vasodilation; WI specific therapeutic protocols should focus on reversing microcirculatory damage and ischemia reperfusion injury.
- Both perfusion groups consume less oxygen, metabolize fewer lipids and have reduced TCA cycles compared to in vivo livers.
- Livers may have overall less of a requirement for aerobic respiration or, they may be mildly ischemic.
- The use of erythrocytes is paradoxically a limitation to increasing the oxygen delivery rate. The increased viscosity at higher hematocrits causes greater problems of increased portal pressure and self-limits the rate of flow.
- Based on these results, a significant optimization strategy targeted particularly at the isolation of hepatocytes from donor organs, where physiological performance of the entire organ is not required, would be to reduce the temperature of perfusion to 20°C. This would reduce the metabolic activity and hence the need for oxygen, potentially also obviating the need for erythrocytes. The result is a highly simplified perfusion system designed for ischemic cell recovery.

RESULTS

In Chapters 3 and 4 we demonstrated that ex vivo normothermic perfusion of freshly isolated rat livers results in transplantable organs, and further that the same perfusion system is necessary for the restoration of ischemic rat livers to transplantable condition[17,18]. In this work we examine the temporal profiles of 28 metabolites measured hourly during perfusion. These data provide information on the time frame of recovery of ischemically damaged organs, organ stability during perfusion, and the impact of perfusion on organ metabolism through comparison to in vivo values (see Appendix A for these reference values).

Moreover, since the trends in metabolite uptake or production in all groups were generally linear (see Statistical Identification of Linear Response Phases During Perfusion in the Methods section of the selected time segments) it was possible to raise the steady state assumption for the fluxes hence enabling the use of MFA. MFA provided a comprehensive overview of organ function during perfusion by enabling the evaluation of several intracellular

fluxes from the measured extracellular fluxes. We also compared the MFA results to an in vivo MFA study[19] conducted previously to identify critical differences that could be corrected to enhance the restorative capacity of the perfusion system. It should be noted that the in vivo MFA study[20] used for comparison here 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.

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 0.05-0.057ml 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 from t=0-3hrs after which consumption began to very slowly 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, is 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). Subtle changes in trends could be appreciated such as an increase in concentration within the first hour of perfusion in both groups to values significantly above the starting value of Williams Medium E, which is similar to the in vivo upper bound level. WI livers continued to increase the glucose concentration, while Fresh livers reduced it during the second hour. During t=3-5hrs, both groups had similar glucose concentrations that demonstrated a gradual steady increase from 2.18-2.24g/L for Fresh livers and to 2.29-2.37g/L for WI livers.

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 does not contain any lactate.

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, R^2=0.6). 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 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 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 significant output by WI livers at t=4hrs, well above the in vivo upper bound value. A linear increase in ornithine output by Fresh livers resulted in a perfusate concentration within in vivo range at t=5hrs. Despite WE being deficient in lactate, ornithine, ammonia, urea, albumin, and ornithine, the liver generally increased the concentrations of each of these significantly during perfusion. However, in the case of histidine (Figure 3I) and serine (not shown), 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 were all produced linearly during perfusion.

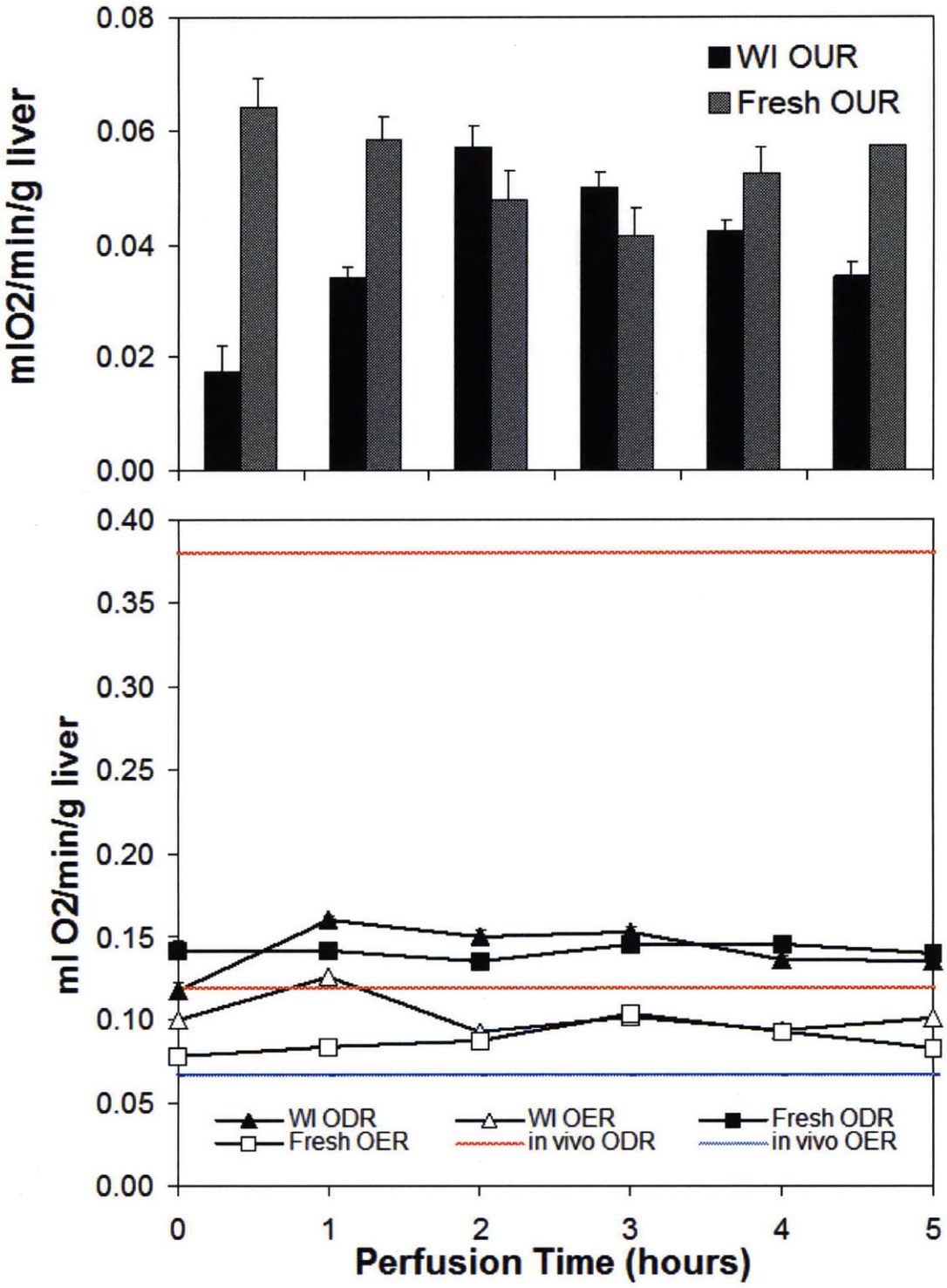

Figure 1. A) Oxygen uptake rate (OUR) for WI and Fresh livers. B) Oxygen delivery rate (ODR) and exit rate (OER) in perfused livers compared to those in vivo. Red and blue lines represent average ODR and OUR +/- 1std dev. *Indicates significantly different from Fresh ($p<0.05$)

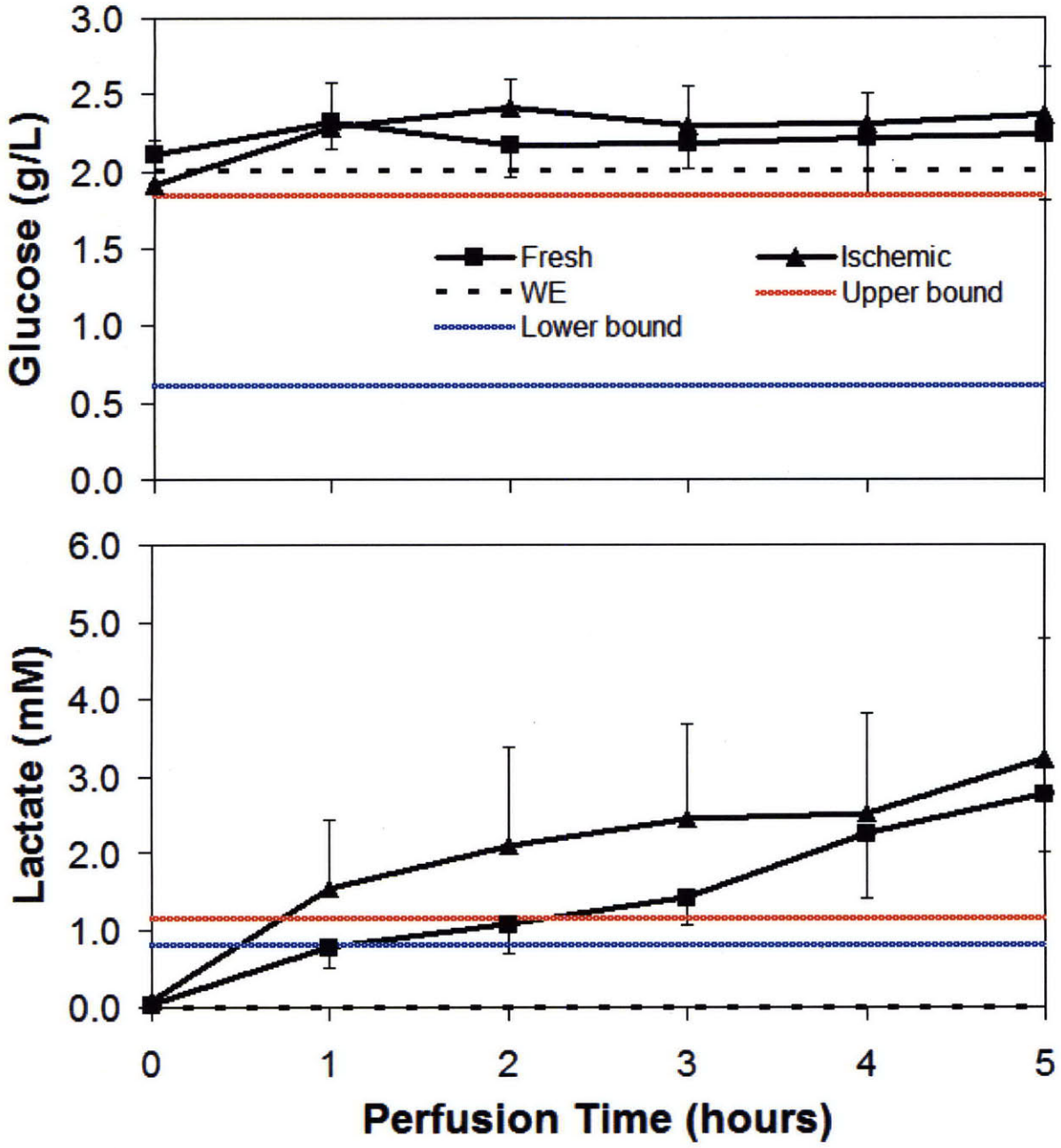

Figure 2: Average concentrations during NELP of WI and Fresh livers, compared to Williams Medium E (WE), and in vivo upper bound values (ave + 1 std dev) and lower bound values (ave – 1 std dev).

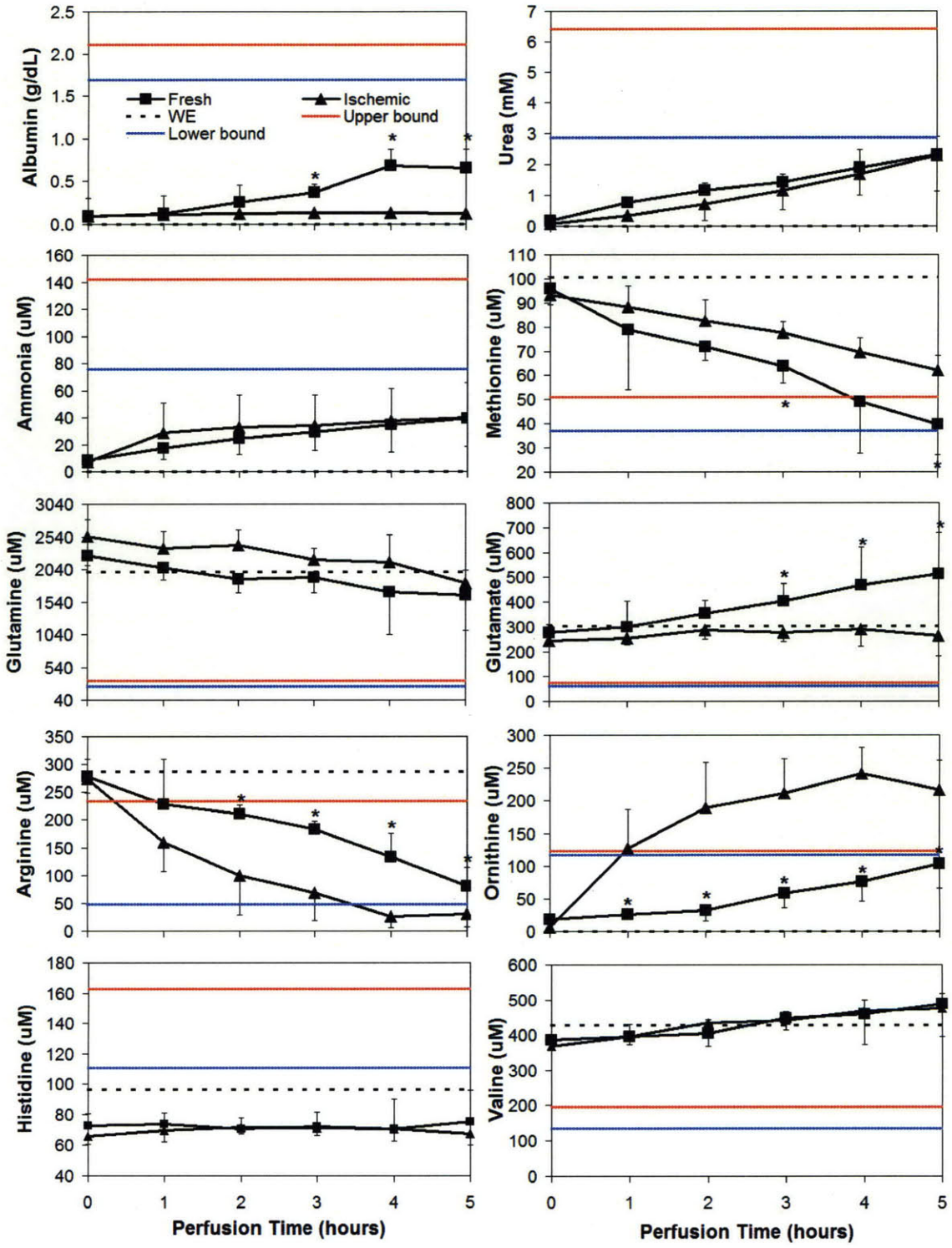

Figure 3: Average amino acid metabolism during NELP of WI and Fresh livers, compared to Williams Medium E (WE), and in vivo upper bound values (ave + 1 std dev) and lower bound values (ave – 1 std dev). * Indicates significantly different from Ischemic ($p<0.05$).

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 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. Table 1 delineates the results of MFA for all groups.

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 NELP 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% ($p<0.1$).

Between 2-5hrs of ischemic liver perfusion (Figure 5) more differences were apparent between fresh and ischemic livers than at 0-2hrs. Ischemic livers appeared to be predominantly 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 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 remain 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 to the perfusate, a comparison of perfused and in vivo livers was performed using MFA (Figures 6-8). 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.

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 ischemic 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, amino acid metabolism picked up to include lysine, glutamine, phenylalanine, tyrosine and aspartate catabolism. Glutamate 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 (Figure 8) 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 2). 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 2) 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 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 26μM, 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. The systematic production of branched chain amino acids appeared to be a perfusion artifact. 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).

Table 1. Metabolic Flux Analysis.

			IN VIVO FLUX DATA (umol/h/g liver)			
#	REACTION	PATHWAY	FRESH T=1-5hr (n=11)	ISCHEMIC T=0-2hr (n=7)	ISCHEMIC T=2-5hr (n=7)	IN VIVO (n=6)
1	Glucose 6-phosphate ↔ Glucose	Gluconeogenesis	-31±15*	88±201	**44±17***	93±37
2	Fructose 6-phosphate ↔ Glucose 6-phosphate	Gluconeogenesis	24±85	-26±71*	22±25	79±82
3	Fructose 1,6-Bisphosphate ↔ Fructose6-phosphate	Gluconeogenesis	-5±21*	**-43±51***	6±8*†	31±22
4	2 Glyceraldehyde 3-P ↔ Fructose 1,6-Bisphosphate	Gluconeogenesis	-5±21*	**-43±51***	6±8*†	31±22
5	Phosphoenolpyruvate + NADH ↔Glyceraldehyde 3-P Oxaloacetate ↔ CO2 +Phosphoenolpyruvate (Gluconeogenic)	Gluconeogenesis	-24±26*	**-94±99***	**4±14***†	37±29
6	Phosphoenolpyruvate + ADP → Pyruvate (Glycolytic) Pyruvate + CO2 ↔ Oxaloacetate (Gluconeogenic)	Gluconeogenesis	-24±26*	**-94±99***	**4±14***†	37±29
7	Pyruvate + CoA + NAD+ → Acetyl-CoA + CO2 + NADH (Glycolytic)	Gluconeogenesis	-37±23*	**-110±101***	-24±12*†	24±19
8	Lactate ↔ Pyruvate + NADH	Lactate metabolism & TCA cycle	-57±21*	**-124±98***	-40±12*†	6±3
9	Acetyl-CoA + Oxaloacetate →Citrate	Lactate metabolism & TCA cycle	31±14*	20±26*	**12±9***	71±47
10	Citrate ↔ 2-oxo-Glutarate + NADH + CO2	Lactate metabolism & TCA cycle	31±14*	20±26*	**12±9***	71±47

11	2-oxo-Glutarate → Succinyl-CoA + NADH + CO2	Lactate metabolism & TCA cycle	35±14*	30±26*	34±9*	80±46
12	Succinyl-CoA ↔ FADH2 +Fumarate	Lactate metabolism & TCA cycle	32±14*	26±26*	32±9*	82±46
13	Fumarate ↔ Malate	Lactate metabolism & TCA cycle	69±19*	56±27*	56±17*	107±53
14	Malate ↔ Oxaloacetate + NADH	Lactate metabolism & TCA cycle	69±19*	56±27*	56±17*	107±53
15	Arginine → Ornithine + Urea	Urea cycle	35±12	35±4	**57±4*†**	28±23
16	Ornithine + CO2 + NH4 ↔Citrulline	Urea cycle	31±10	25±8	**56±4*†**	32±20
17	Citrulline + Aspartate →Arginine + Fumarate	Urea cycle	29±10	25±8	20±13	25±20
18	Arginine uptake	Aminoacid metabolism	4.3±1.6	**9.3±6.7**	2.6±0.6*†	3.6±5.1
19	Ammonia Output	Urea cycle	0.7±0.2*	0.8±0.9*	**0.5±0.1***	-6.2±1.7
20	Ornithine Output	Urea cycle	2.3±1.2*	**9.3±6***	**0.7±1.1*†**	-3.4±0.9
21	Citrulline Output	Aminoacid metabolism	2±1.9*	-0.1±9	2±1.2*	7±5.2
22	Alanine → Pyruvate + NH4 + NADH	Aminoacid metabolism	8.6±2.4	4.9±8.3	7.2±1.8	12±6.8
23	Alanine Output	Aminoacid metabolism	-7.1±1.4*	-3.9±7.6*	-7.2±1.8*	-12.7±6.2
24	Serine → Pyruvate + NH4	Aminoacid metabolism	8.6±6.3	6.7±13.5	6.6±1.5	6.4±11.5
25	Serine Uptake	Aminoacid metabolism	0.2±0.3*	**-1.2±1.1***	**0.5±0.2*†**	2.7±1.7

26	Cysteine → Pyruvate+ NH4 + NADH	Aminoacid metabolism	2.9±3.2	3±4.8	2.1±0.5	-0.1±3.8
27	Cysteine Output	Aminoacid metabolism	-0.1±2.7	-1.4±1.9	-1.2±0.5*	0.2±0
28	Threonine → NADH + Glycine +Acetyl-CoA	Aminoacid metabolism	0.4±0.7	0.6±8.2	**2.1±1.2**	1.2±4
29	Glycine ↔ CO2 + NH4 +NADH	Aminoacid metabolism	8.2±4.2	7.5±10.2	6.9±1.4	5.2±8
30	Glycine Uptake	Aminoacid metabolism	4.8±0.9	5±0.1	4.8±0.8	5.4±1.2
31	Valine + 2-oxo-Glutarate → Glutamate + propionyl-CoA + 3 NADH +FADH2 + 2 CO2	Aminoacid metabolism	-2.2±0.9*	-2.9±1*	**-0.9±1.4*†**	0.8±0.4
32	Isoleucine + 2-oxo-Glutarate → Glutamate + propionyl-CoA + Acetyl-CoA + 2 NADH + FADH2 +CO2	Aminoacid metabolism	-1.7±0.6*	-1.4±0.3*	-1.7±0.7*	-0.2±0.7
33	Leucine + 2-oxo-Glutarate → Glutamate + NADH + FADH2 Acetoacetate + Acetyl-CoA	Aminoacid metabolism	-2.6±2.1	-4±3.5	-2.4±1	-0.2±3.5
34	Propionyl-CoA + CO2 ->Succinyl-CoA	Aminoacid metabolism	-2.6±1.1*	-3.7±1*	-1.7±1.6*†	1.4±0.8
35	Lysine + 2 2-oxo-Glutarate → 2 Glutamate + 4 NADH + FADH2 + 2CO2 + Acetoacetatyl-CoA	Aminoacid metabolism	7.2±4.2*	2.9±6	3.7±1.6*	1.5±0.4
36	Phenylalanine + O2 → Tyrosine	Aminoacid metabolism	2.1±0.6*	2.6±0.4*	**0.9±0.2*†**	0.5±0.4
37	Tyrosine + 2 O2 →NH4 + CO2 + Fumarate + Acetoacetate + NADH	Aminoacid metabolism	7.5±2.2*	5±4.8	**3.6±0.2**	0.8±3.9
38	Tyrosine Output	Aminoacid metabolism	-3.9±0.4*	**-1.5±2**	**-2.7±0.1***	-1±1.1
39	Glutamate ↔ 2-oxo-Glutarate + NADH +NH4	Aminoacid metabolism	12.1±9.4	7.2±24.9	**24.2±6.8***	12.8±7.6
40	Glutamate Output	Aminoacid metabolism	5.4±3.5*	**2.3±0.1***	**0.2±0.9†**	0.8±1.3
41	Glutamine → Glutamate + NH4	Aminoacid metabolism	5.7±3.3*	9.3±25.9	**20.4±5.6***	2±2.5

42	Proline +0.5 O2→ Glutamate + 0.5 NADH	Aminoacid metabolism	2.6±0.4*	2±1.9	**1.6±0.9**	0.7±2.9
43	Histidine → NH4 +Glutamate	Aminoacid metabolism	-0.2±0.4*	-0.2±0.3*	0.1±0.2*	8±5.3
44	Methionine + Serine → Cysteine + NADH + Propionyl-CoA + CO2	Aminoacid metabolism	1.3±0.2*	**0.6±0.1***	**0.9±0.2**†	0.8±0.1
45	Aspartate↔Oxaloacetate + NH4 +NADH	Aminoacid metabolism	-24±8.7	-20.3±10.9	-15.9±12.9	-23±16.7
46	Aspartate Uptake	Aminoacid metabolism	2.5±0.7*	2.1±1.9	2.6±0.1*	0.5±0.4
47	Asparagine → Aspartate + NH4	Aminoacid metabolism	0.8±0.4*	**1.2±0**	**1.4±0.3**	2±1.1
48	Palmitate→ 8 Acetyl-CoA +7 FADH2 + 7 NADH	Lipid, glycerol, fatty acid metabolism	4.2±2.3*	4.1±4*	3.2±1.3*	13.2±6.7
49	2 Acetyl-CoA ↔ Acetoacetyl-CoA	Lipid, glycerol, fatty acid metabolism	-0.8±4.9*	3.9±14.1	**5.5±2.1**	17.6±16.4
50	Acetoacetyl-CoA → Acetoacetate	Lipid, glycerol, fatty acid metabolism	6.5±3.5*	6.7±13.2	9.2±1.3	19.2±16.4
51	Acetoacetate Output	Lipid, glycerol, fatty acid metabolism	11.4±1.8*	7.7±11.9	10.4±0.9*	-1.3±0.6
52	Acetoacetate + NADH ↔b-Hydroxybutyrate	Lipid, glycerol, fatty acid metabolism	0±0*	**0±0***	0±0*	21±16
53	NADH + 0.5 O2 → NAD	Oxygen uptake and electron transport	164±52*	109±87*	119±32*	317±178
54	FADH2 + 0.5 O2 → FAD	Oxygen uptake and electron transport	62±26*	49±44*	53±16*	176±87
55	O2 Uptake	Oxygen uptake and electron transport	132±38*	92±63*	**95±24***	249±132

56	Glucose 6-phosphate→ 2 NADPH + CO2 +Ribulose 5-P	PPP	43±100	25±61	25±29	73±97
57	Ribulose 5-P ↔ Ribose 5-P	PPP	14±33	8±20	8±10	24±32
58	Ribulose 5-P ↔ Xylulose 5-P	PPP	29±67	17±40	16±19	49±65
59	Ribose 5-P + Xylulose 5-P ↔ Fructose6-P + Erythrose 4-P	PPP	14±33	8±20	8±10	24±32
60	Erythrose 4-P + Xylulose 5-P ↔ Glyceraldehyde 3-P + Fructose 6-P	PPP	14±33	8±20	8±10	24±32
61	CO2 Output	Oxygen uptake and electron transport	120±97*	81±39*	**60±23**	216±30
62	Glycogen ↔ Glucose-6-P	Glucose metabolism	-13±26*	139±208*	-34±14*†	87±43

Note
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

Table 2. 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	0.11-0.2	0.00
Arginine	0-0.5	0.53
Asparagine	0.04-0.12	0.28
Aspartate	0.02-0.04	0.41
Cysteine	0.01-0.03	0.61
Glutamate	0.07-0.15	0.56
Glutamine	0.28-0.68	3.64
Glycine	0.26-0.6	1.23
Histidine	0.1-0.35	0.18
Isoleucine	0.07-0.23	0.70
Leucine	0.21-0.66	1.05
Lysine	0.18-0.54	1.10
Methionine	0.04-0.1	0.18
Ornithine	0.11-0.28	0.00
Phenylalanine	0.05-0.13	0.28
Proline	0.16-0.36	0.48
Serine	0.17-0.48	0.18
Threonine	0.19-0.47	0.62
Tyrosine	0.05-0.17	0.51
Valine	0.12-0.43	0.79

Note
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.

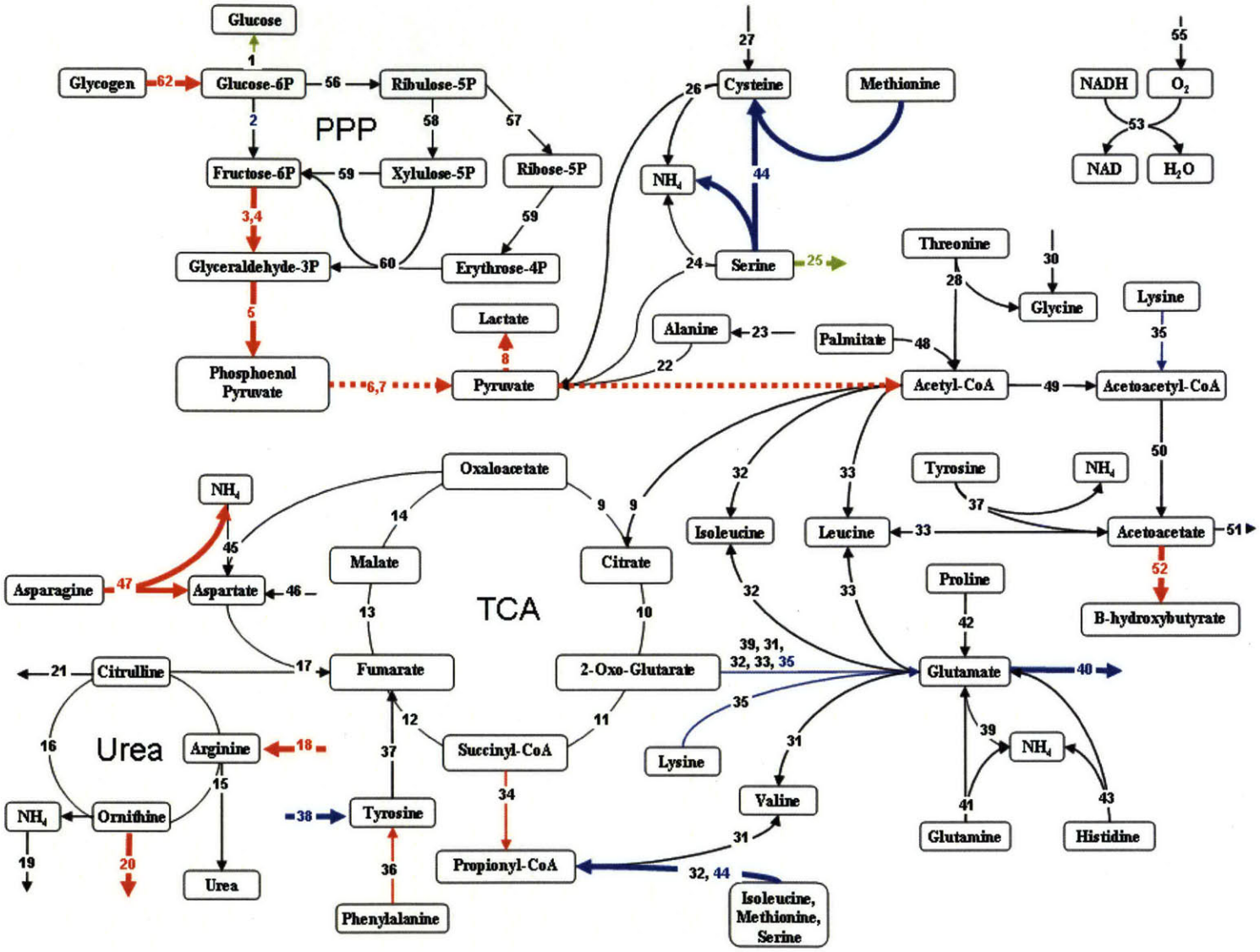

Figure 4. Flux directions reflect Phase I (t=0-2hrs) of WI vs. Fresh livers. Red arrows = significantly increased. Blue = significantly reduced. Green = reversed. Bold lines = $p<0.05$, Thin lines = $p<0.1$, Dotted lines = glycolysis.

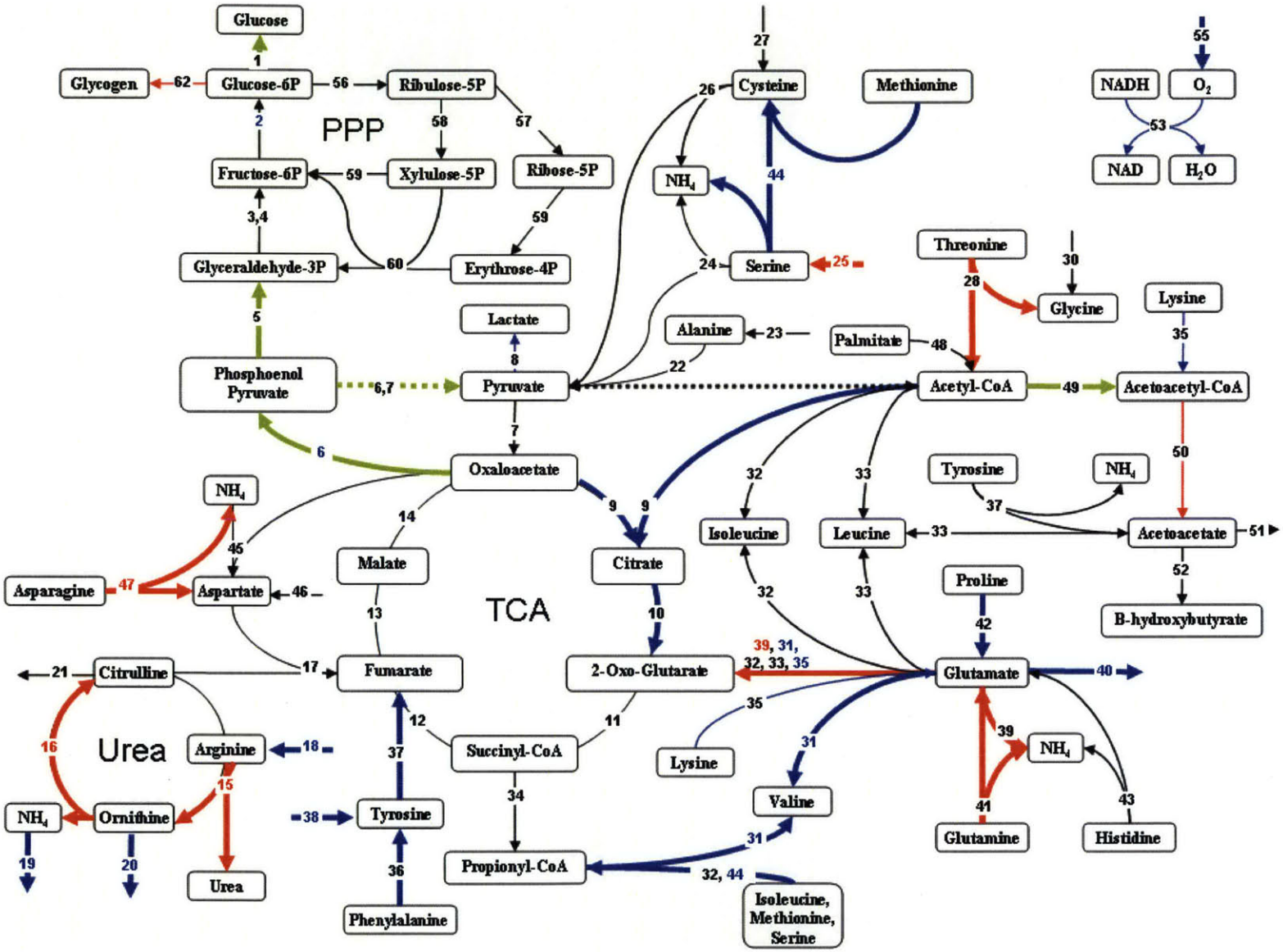

Figure 5. Flux directions reflect Phase II (t=3-5hrs) of WI vs. Fresh livers. Red arrows = significantly increased. Blue = significantly reduced. Green = reversed. Bold lines = $p<0.05$, Thin lines = $p<0.1$, Dotted lines = glycolysis

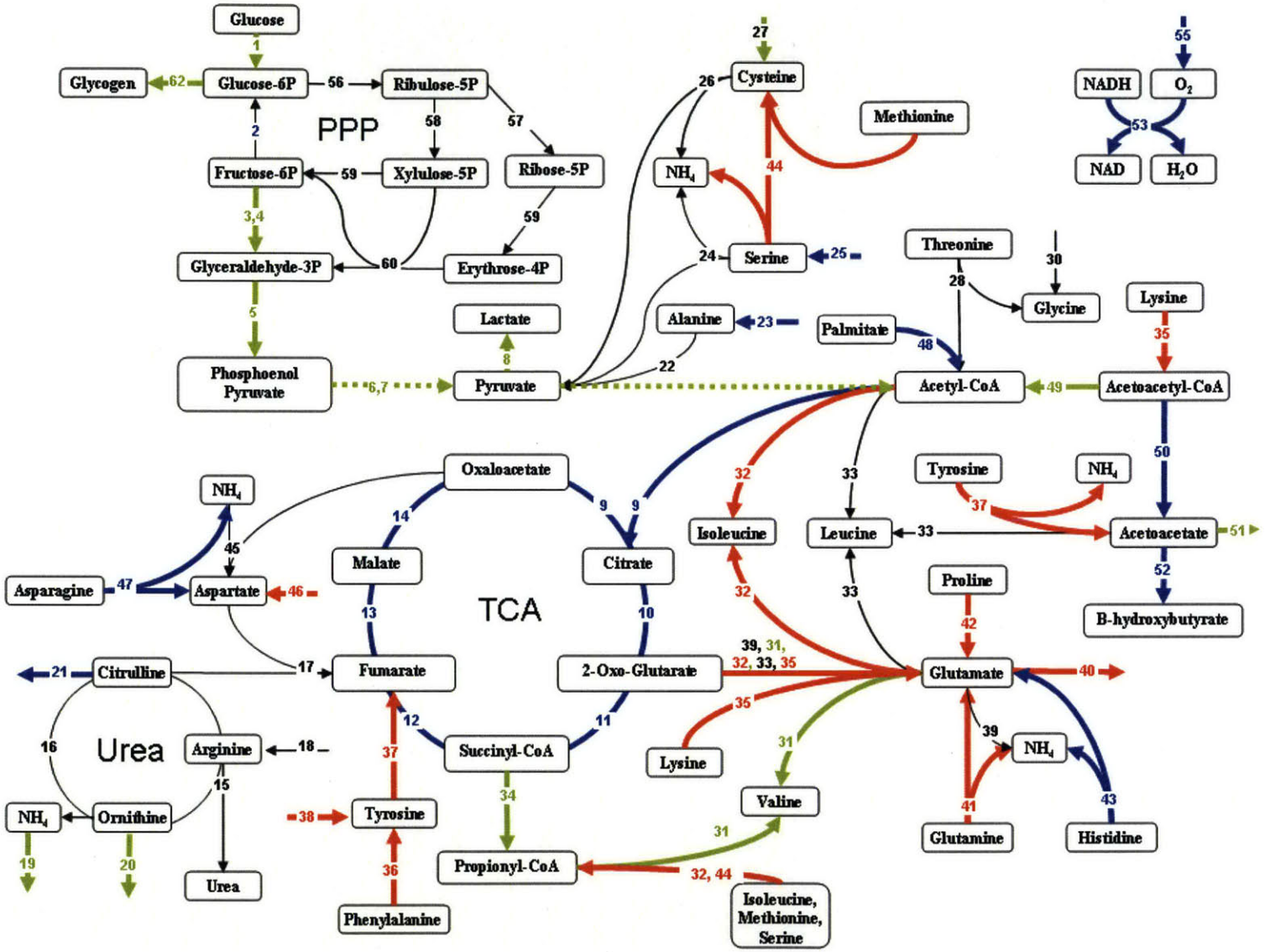

Figure 6. Flux directions reflect Fresh vs. in vivo. Red arrows = significantly increased. Blue = significantly reduced. Green = reversed. Bold lines = $p<0.05$, Thin lines = $p<0.1$, Dotted lines = glycolysis

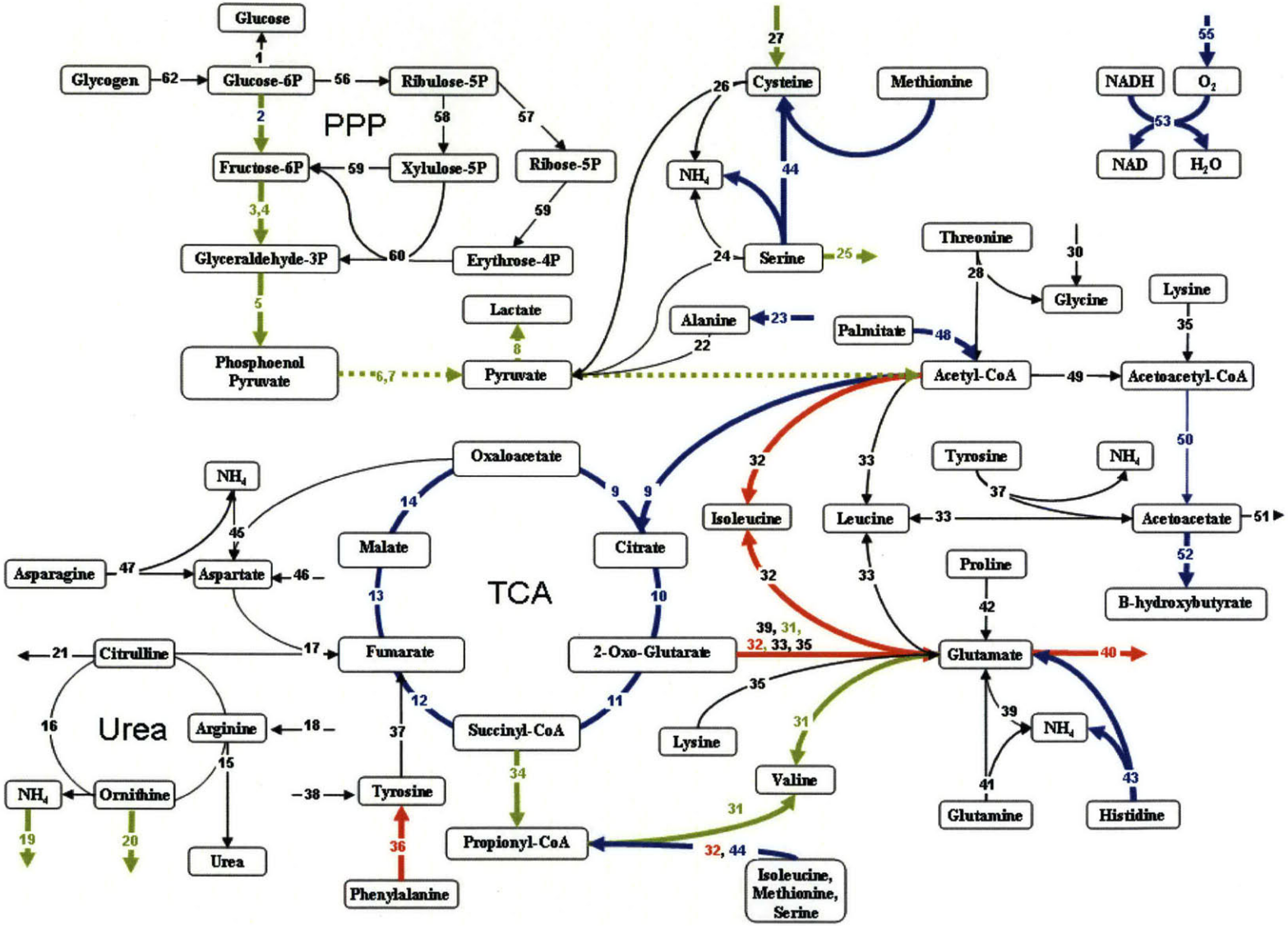

Figure 7. Flux directions reflect phase I (t=0-2hrs) of WI vs. in vivo. Red arrows = significantly increased. Blue = significantly reduced. Green = reversed. Bold lines = $p<0.05$, Thin lines = $p<0.1$, Dotted lines = glycolysis

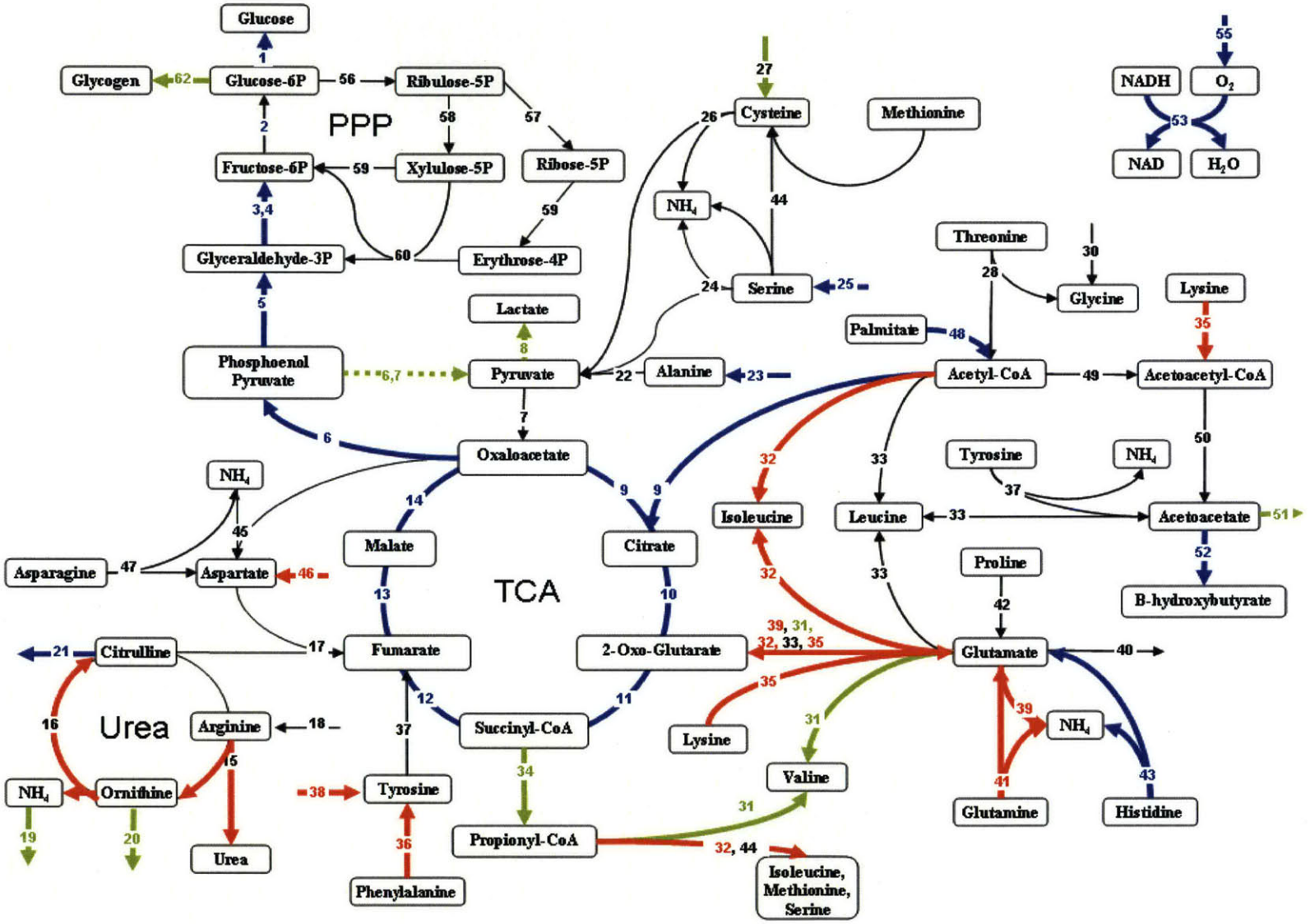

Figure 8. Flux directions reflect phase II (t=3-5hrs) of WI vs. in vivo. Red arrows = significantly increased. Blue = significantly reduced. Green = reversed. Bold lines = $p<0.05$, Thin lines = $p<0.1$, Dotted lines = glycolysis

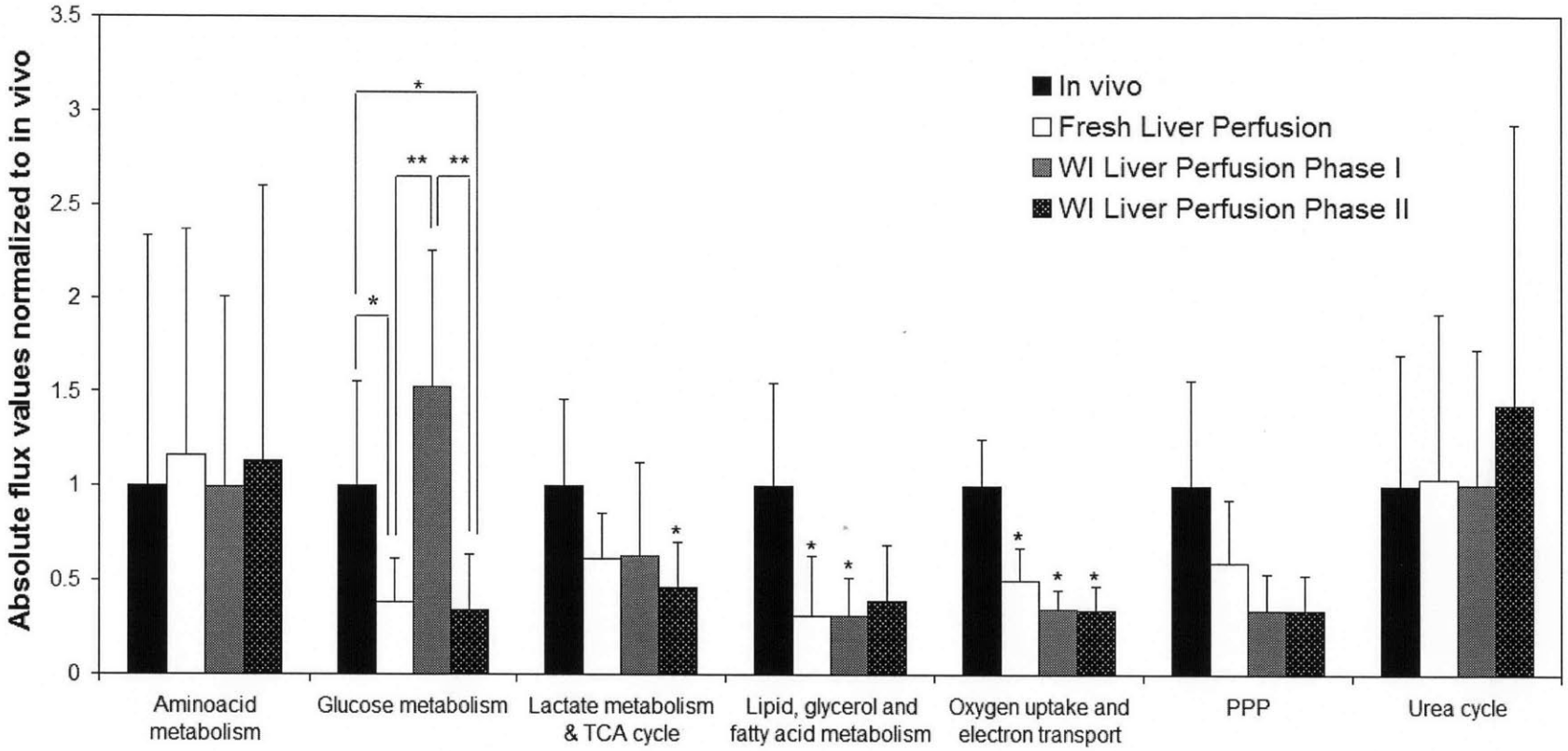

Figure 9: Summary of major pathways in perfused livers normalized to in vivo values.

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[21,22]. To facilitate the translation of this 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 biosynthetic capacity of the liver and evaluate the impact of perfusate nutrient supply and toxin dilution. We conducted our study on hourly perfusate samples from Fresh and WI livers that were successfully transplanted after 5-6hrs of NELP. 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 short-comings, and establish significant differences between Fresh and WI livers useful in the future design of treatment protocols.

WI livers demonstrated two distinct phases of metabolic stability, separated at t=2hrs, during a 5hr normothermic perfusion. The first phase appeared to include both a trajectory of ischemic organ metabolism by well-fed livers[23] and recovery. Livers depicted substantial dependence on glycogen breakdown for both glucose synthesis and glycolysis, marked lactate production and arginine uptake reflecting dependence on preexisting nutrient stores, anaerobic respiration and the likely recruitment of nitric oxide metabolism to sustain viability[24,25]. Woods and Krebs[23] further observed a reduction in the production of both glucose and lactate when glycogen stores were depleted within 135 minutes of perfusion. During this same time frame however, oxygen uptake rate by WI livers increased and matched the values of Fresh livers demonstrating increased access to, and metabolism of, viable cells. WI livers subsequently moved into their second stable phase of perfusion. It is possible that the restoration of OUR of WI livers to Fresh liver values within 2 hours marks a "recovery" time during perfusion. Phase II of WI livers more closely resembled a high insulin state with reduced gluconeogenesis and glycogen storage, and an increased uptake of select amino acids, particularly glutamine, to fuel the TCA and urea cycles.

The differences that existed between WI and Fresh livers in extracellular metabolite accumulation or consumption, such as methionine uptake, or albumin and glutamate production, were sustained throughout perfusion and did not show evidence of coinciding over time. Though not critical to recipient survival, these differences summarily reflect the fact that ischemic livers are functioning suboptimally and are not fully recovered at the time of transplantation. While it is evidently not necessary to fully restore all organ function for transplantability, enhancing organ

performance to a predefined level of viability will be necessary in the clinically relevant circumstances of unknown ischemic duration. These metabolic analyses support the need to facilitate the restoration of the endothelin/nitric oxide balance through the arginine pathway, though other markers of ischemic injury should also be included for future viability analyses[26]. Evidence of the possible impact of vasodilation may partly explain the increased oxygen uptake rate during t=0-2hrs[25,27,28] though other effects of perfusion can be further optimized to enhance recovery including: facilitation of debris washout by exposing otherwise live tissue using higher but non-detrimental flow rates and thrombolytics; the resuscitation of cellular activity through more rapid ATP restoration, and reduction of the inflammatory ischemia-reperfusion cascade to minimize cell loss[26,29,30].

Fresh livers served to illustrate how a healthy organ functioned in ex vivo perfusion and highlighted baseline factors to be enhanced in future perfusions. Fresh livers in perfusion demonstrated stable changes in metabolite concentrations throughout perfusion and no evidence of substrate limitation within 5 hours. Greatest stability was gained within the first hour, likely reflecting a period of adjustment to the ex vivo environment. MFA depicted the expected response to a fed/high insulin state with 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 as might be expected in a high energy, low-lipid, state. Amino acid metabolism differed substantially from in vivo trends, some of which reflects perfusion-specific artifacts. For example, Fisher and Kerly,[31] who performed analyses of amino acid metabolism in healthy, albeit fasted, perfused rat livers, observed a steady increase in perfusate content of branched chain amino acids valine, isoleucine and leucine, as seen in both the Fresh and WI livers. Further, these authors found both histidine and glutamate to be unchanged in perfusion. Both trends held true in WI livers, while Fresh livers demonstrated a net production of glutamate. This may be related to a normal transamination of excessive perfusate amino acids in conjunction with an otherwise reduced TCA cycle.

One major consideration in the improvement of the perfusion system would be the removal of erythrocytes from the perfusate. Red blood cells increase the overall complexity of the perfusion setup significantly, affect the quantitative metabolic analyses, and may also incur damage in the form of variable hematocrit on hepatic function, hemolysis and the tendency to embolize[32]. OUR was overall significantly reduced in perfused livers compared to in vivo livers but OERs were higher than in vivo values suggesting livers were not oxygen-limited. OUR in fresh livers demonstrated a steady decline from t=0hrs to t=3hrs after which uptake plateaued. It

is well-documented that livers perfused with erythrocyte-containing perfusion media consume less oxygen over time[32-35], which can be further modulated by perfusate temperature[36] The final steady state oxygen uptake of these livers is typically equivalent to OUR in livers perfused without erythrocytes, provided a delivery rate can be achieved at low-shear flow rates[32]. This decline in OUR has been correlated with a reduction in erythrocyte viability,[37] though it may also be associated with a change in the metabolic rate of the liver as it adjusts to its new, metabolically less-demanding, environment. Further, erythrocytes are also metabolically active. Though initial lactate content of erythrocytes was minimized by freshly harvesting blood hours before perfusion and washing 3 times with 0.9% sodium dextrose[33,38] they were nevertheless actively glycosylating and subsequently continuing to produce lactate during perfusion. The extent to which they impacted perfusate glucose and lactate levels was not accounted for in these experiments though Fisher and Kerly[31] performed controls whereby normothermic perfusions were carried out without livers giving an average uptake of glucose of approximately 60mg/hr in the presence of a hemoglobin concentration of 26mg/100ml blood. As our perfusion contained an average hemoglobin concentration of 5-7mg/100ml blood, the total expected uptake of glucose was approximately 12mg/hr, impacting the perfusate glucose concentration of 2000mg/L negligibly. Similar reasoning applied to the formation of lactate, however this had a greater impact on the total concentration at perfusion end. Again 60mg of lactate were produced in an hour in the study of Fisher and Kerly. This is equivalent to 0.13mmoles produced per hour or 0.65mmoles in 5 hours, for a total change in perfusate concentration of 1.3mM or roughly one third of the lactate observed at the end of our perfusions. This reasoning supports the removal of erythrocytes from the perfusate for more accurate metabolic analyses. As erythrocyte-free media have been successfully implemented with high oxygen uptake rates at a range of temperatures from 22°C-37°C,[32,39] organ perfusion may benefit substantially from this simplification.

In conclusion, this study describes an integrated metabolic analysis of the performance of transplantable Fresh and Ischemic livers in normothermic machine perfusion. Stable function is observed throughout perfusion and recovery of WI livers may occur with the restoration of oxygen uptake rate within the first 2 hours. These data provide the basis for rational NELP optimization, such as the removal of erythrocytes; and design of treatment protocols, such as the inclusion of substrates specific to the amelioration of ischemia reperfusion injury with emphasis on reversing microcirculatory collapse. Further, the data and analysis presented here may enable future meta-analyses such as data mining for an index of organ viability and Flux

Balance Analysis[40,41], necessary steps to build automated feedback control systems for real-time organ support.

MATERIALS AND METHODS

Animals.

Male Lewis rats weighing 200-300g were obtained from Charles River Laboratories (Wilmington, MA) and maintained in accordance with National Research Council guidelines. The Subcommittee on Research Animal Care, Committee on Research, Massachusetts General Hospital approved the experimental protocols. All animals were allowed to acclimatize for at least 2 days prior to any experimentation. Rats were divided into two groups: 1) Fresh livers (n=11) and 2) warm ischemic (WI) livers (n=7).

Ex Vivo Perfusion Model

Isolation of Donor Livers.

Full details of the procedure are describe elsewhere.[4] Briefly, a transverse abdominal incision was made and the intestines retracted to expose the portal vein (PV), the common bile duct (CBD), and the inferior vena cava (IVC). The CBD was cannulated (22 G polyethylene stent, Surflo, Terumo, Somerset, NJ) and the IVC freed from the right renal and adrenal veins. The portal vein (PV) was freed from the splenic and gastroduodenal veins. The right phrenic vein emptying into the supra-hepatic vena cava (SHVC) was ligated. Heparin (200 U) was injected through the penile vein. The IVC and PV were clamped. For Fresh livers, an 18 G polyethylene cannula (Terumo) was inserted into the PV, and the liver was flushed with 5mL of cold (4°C) University of Wisconsin (UW) solution (Viaspan, Barr Laboratories, Pamona, NY). The diaphragm was opened, the SHCV was transected, and the liver was flushed with an additional 5mL of UW solution. The liver was removed, weighed, and placed in a bowl of ice-cold UW solution to be cuffed. For WI livers, flushing of the organ via the PV was omitted. After isolation from the donor, these livers were then weighed and placed in a temperature-controlled chamber filled with saline and maintained at 34±0.1°C for 1 hr. During this period the portal vein (PV) and inferior vena cava (IVC) were cuffed.

Normothermic Liver Perfusion and Metabolite Sampling.

The perfusate and dialysate comprised phenol red-free Williams Medium E (Sigma Chemical, St. Louis, MO) supplemented with 2 u/L insulin (28.85units/mg Humulin, Eli Lily, Indianapolis, IN), 100,000 u/L penicillin, 100mg/L streptomycin sulfate (Gibco, Invitrogen,Grand Island,NY), 0.292 g/L L-glutamine (Gibco), 10mg/L hydrocortisone (Solu-Cortef, Pharmacia & Upjohn, Kalamazoo, MI), and 1000 u/L heparin (APP, Schaumberg, IL). Fresh rat plasma (25% v/v) and erythrocytes (18–20% v/v) were collected earlier[17] and added to the perfusate. The total perfusate volume was 55 to 60 mL, while that of the dialysate was 450-475ml. The primary circuit comprised perfusate that recirculated by means of a peristaltic pump through a jacketed perfusion chamber, a membrane oxygenator, a heat exchanger, and a bubble trap. The oxygenator was gassed with a mixture of 74%N_2/21%O_2/5%CO_2 and 100%O_2 to maintain a constant pH. A fraction of the perfusate was diverted to the secondary circuit through a hollow fiber dialyzer with a 2200 cm^2 membrane area and a 30 kDa nominal molecular cutoff weight (SpectrumLabs, Rancho Dominguez, CA) at a rate of 3mL/min/g wet liver weight. The secondary circuit dialyzed the perfusate by counter-current exposure to dialysate. The volumes of perfusate and dialysate were kept constant by controlling the flow of dialysate through the dialyzer in the secondary circuit. Temperature within the system was maintained at 37.5°C. Upon completion of cuffing of Fresh livers (~5min) and after the period of warm ischemia for WI livers, they were immersed in perfusate in the perfusion chamber. Livers were perfused at a constant flow rate through the portal vein[17] while maintaining portal pressure between 10 and 12cmH_2O. The effluent flowed freely from the SHVC and IVC into the surrounding medium. Perfusate and dialysate samples (1mL) were collected hourly from the liver effluent and reservoir respectively. Perfusate samples were first spun down at 3000g before storing the supernatant at -80°C. Oxygen delivery and exit rate were determined from samples taken immediately prior to entry to the portal vein and from the IVC. When the recipient hepatectomy was prepared, the liver was disconnected from the circuit, rinsed in a bowl of saline at room temperature, and weighed again before transplantation.

Metabolite Analysis

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:

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

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):

$$HOUR = OER - ODR$$

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

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:

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

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[42]. 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[43]. 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[44]. 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. Concentrations were calculated based on the primary circuit volume of approximately 60ml supplemented with fresh frozen plasma and erythrocytes. 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 distinct phases in liver metabolism during perfusion. The concentration profiles and R^2 values obtained from linear regression for each metabolite were checked to confirm that each metabolite changed in an approximately linear fashion, necessary to calculate fluxes accurately. Box-and-whisker plots of the resulting R^2 values for each time period and experimental group 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. Fresh livers were found to be generally stable throughout perfusion, with the greatest linearity between 1-5hrs.

Calculation of Fluxes.

Fluxes were calculated as the gradient of concentration of each metabolite (i.e. slope of the linear regression curve)[45] 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.

Date 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. This process is a necessity to eliminate artefactual values that increase variability.

Metabolic Flux Analysis.

MFA was performed based on a stoichiometric model for the metabolic reaction network developed and tested in more detail previously[46,47] and is referred to in Chapters 2 and 3.

In this work, the model consistency and validity of the steady state assumption was confirmed by the method of Wang and Stephanopoulos [48]. 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 the measured fluxes can be eliminated iteratively to identify any issues. This approach was used to identify two artefactual oxygen uptake measurements, which when eliminated resolved the issues observed.

Statistical Analysis.

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

5-2. Evaluation of Perfusate Temperature on Transplant Success

We demonstrate that sanguineous perfusion at room temperature results in transplantable organs and that all metrics of organ function are similar if not improved compared to NELP. Further we significantly simplify the requirements of the perfusion system by removing the need for any temperature regulation. Metabolic analysis of livers perfused at 37°C demonstrates that the presence of erythrocytes may require compromising on parameters known to cause damage. The presence of erythrocytes enable the use of physiological oxygen tensions, reducing the risk of oxidative damage at hyperbaric oxygen pressures, but subsequently require a higher hematocrit in order to deliver oxygen at the desirable rates. Increasing the hematocrit however, also increases the viscosity and the portal pressure, which necessitates a reduction in flow rates. The reduction in oxygen uptake rate by perfused livers could not easily be dissociated from the likelihood of ischemia because of significantly elevated lactate levels present in perfusate. The source of lactate may well have been the hepatocytes, but it could also simply have derived from the erythrocytes. In order to completely allay the seemingly perilous quest to provide the liver with ample oxygen while minimizing damage, the solution was to attempt sanguineous perfusion at room temperature thereby reducing both the metabolic demand of the liver and its oxygen requirements, while potentially even eliminating the need for erythrocytes.

A major limitation to the use of perfusion systems in the clinic is their complexity which makes them both generally immobile and resource intensive whether they operate at hypothermic conditions (~4°C), which utilizes the static cold storage modality of reducing the organ's metabolic needs [49-55], or normothermic conditions (37°C) which aims at maintaining the donor organ under near-physiological circumstances for oxidative metabolism to take place as usual [6,7,35,56-61]. Subnormothermic Machine Perfusion (SNMP, 20ºC) potentially stands to confer a substantial practical and functional advantage over these methods, rendering it steps closer to clinical applicability.

In 1980 [62] the first successful transplantation of healthy livers after perfusion at 10°C was reported. Subsequent analyses of liver function tests, metabolism, histology and transplantation of healthy livers perfused at 10°C-20°C demonstrated superior liver function compared to those stored on ice or perfused at temperatures above or below that range in the absence of oxygen carriers [63,64]. In addition, beneficial effects of hypothermia in ischemia/reperfusion injury were observed in the range of 4°C to 26°C [65]. Yet, no study had investigated the use of

subnormothermic perfusion-resuscitation of DCD organs using recipient survival as the endpoint.

In this work, we show excellent one-month survival of transplanted rat livers that underwent 60 minutes of ex-vivo warm ischemia (34°C) and preservation with 5 hours of SNMP. This success is similar to ischemic livers resuscitated with NMP [6,7]. By contrast, control recipients of ischemic livers stored by CS for 5 hours, as well as those who received an ischemic liver without preservation, did not survive. To assess early graft function, a separate set of experiments was conducted using an isolated reperfusion model. These tests also demonstrated superior performance of livers preserved with SNMP and NMP compared to those not machine-perfused. The lack of a need for perfusion temperature control, in addition to greatly decreased organ oxygen demand, promises to dramatically simplify machine perfusion of donor organs increasing the likelihood of incorporation into clinical practice.

RESULTS

Integrity and Function of Liver during Perfusion

ALT and AST activities as indicators of hepatocellular damage are displayed in Figure 1A & B and are several fold lower in freshly isolated livers compared to warm ischemic livers ($p=0.0031$ and 0.0247 respectively). There was no statistical difference in either ALT or AST for room-temperature vs. normothermically perfused ischemic livers ($p=0.1552$ and 0.6864, respectively). Change with time in ALT or AST during perfusion was statistically not significant ($p=0.1793$ and 0.1142, respectively), indicating stability of the organ during perfusion.

Bile secretion, describing liver function, was produced at a constant rate throughout all perfusions (Figure 1C). Ischemically damaged livers produced bile at a rate ~37% lower than Fresh + NMP livers ($p=0.0229$). Bile levels were not significantly different from WI + NMP livers ($p=0.6071$).

The metabolic state of the liver was assessed through its oxygen uptake rate. The HOUR of warm ischemic livers perfused at room-temperature was significantly lower compared to normothermically perfused fresh or ischemic liver rates (~35%, $p=0.0130$ and 0.0219 respectively). Statistically there was no change in HOUR throughout perfusion ($p=0.1142$).

Survival after Transplantation

Transplantation of all SNMP treated ischemic livers was uneventful. All animals recovered from anesthesia rapidly. Like other recipients of MP and fresh livers, SNMP recipients survived beyond one month and did not exhibit external signs of liver failure, such as jaundice.

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. 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 CS recovered rapidly from surgery and survived beyond one month (Figure 2).

Post-operative liver enzymes and bilirubin

Serological analysis was performed on recipients of the successful preservation modalities. Both ALT and AST (Figures 3A and B) were elevated on day 1 post-operatively for all groups similar to the levels found in recipients of healthy cold stored livers. Fresh, UW cold-stored livers displayed a spike in both enzyme levels on day 5; a much less pronounced spike was also evident in NMP-resuscitated ischemic livers on day 7, whereas the SNMP livers displayed consistent levels throughout the observation period. Enzyme levels for SNMP recipients were statistically lower compared to both recipients of cold-stored fresh livers (ALT: $p= 0.0393$, AST: $p=0.0042$) and normothermically perfused ischemic livers (ALT: $p= 0.0023$, AST: $p= 0.0670$).

The post-operative total bilirubin level was similar for SNMP-treated ischemic livers, cold-stored fresh livers ($p= 0.0547$) and NMP-treated ischemic livers ($p= 0.2466$) (Figure 3C). The bilirubin value increased with time for all groups ($p<<0.01$ in all cases).

Short-term graft performance post transplantation

In order to evaluate graft function immediately post-perfusion we employed a reperfusion system using diluted whole-blood.

Figure 4 shows ALT and AST secretion for WI+SNMP group was lower than WI-only and WI+CS groups at all time points (all $p<0.05$), and comparable to Fresh+NMP and WI+NMP groups (all $p>0.05$). These results indicate SNMP is significantly better than CS in the short term, and is equivalent to NMP in terms of reducing hepatocellular damage. The time-course

changes in ALT and AST release rates during reperfusion were not statistically significant (p= 0.3927 and p= 0.5477 respectively).

The average bile production during reperfusion of WI+SNMP livers was statistically lower than fresh livers (p=0.0006) but not different than any of the other groups (all p>0.05) (Figure 4C); however, the WI+SNMP group displayed a trend of increasing bile production, whereas in all other groups bile production remained constant. SNMP livers lag behind NMP livers in bile production rates.

As displayed in Figure 4D, the oxygen uptake rate of SNMP-treated WI livers was similar to that of NMP-treated WI livers (p=0.3101), but lower compared to fresh livers (p=0.0140). The changes in oxygen uptake rate over time during reperfusion were statistically not significant (p= 0.1458).

Figure 5 displays the H&E staining results at the end of reperfusion. NMP and SNMP-preserved WI livers appear healthy and very similar to fresh livers in H&E images, whereas WI and WI +SCS livers show necrosis, with loss of macro and micro architectural integrity observed in the latter. Apoptosis was negligible in all reperfused livers.

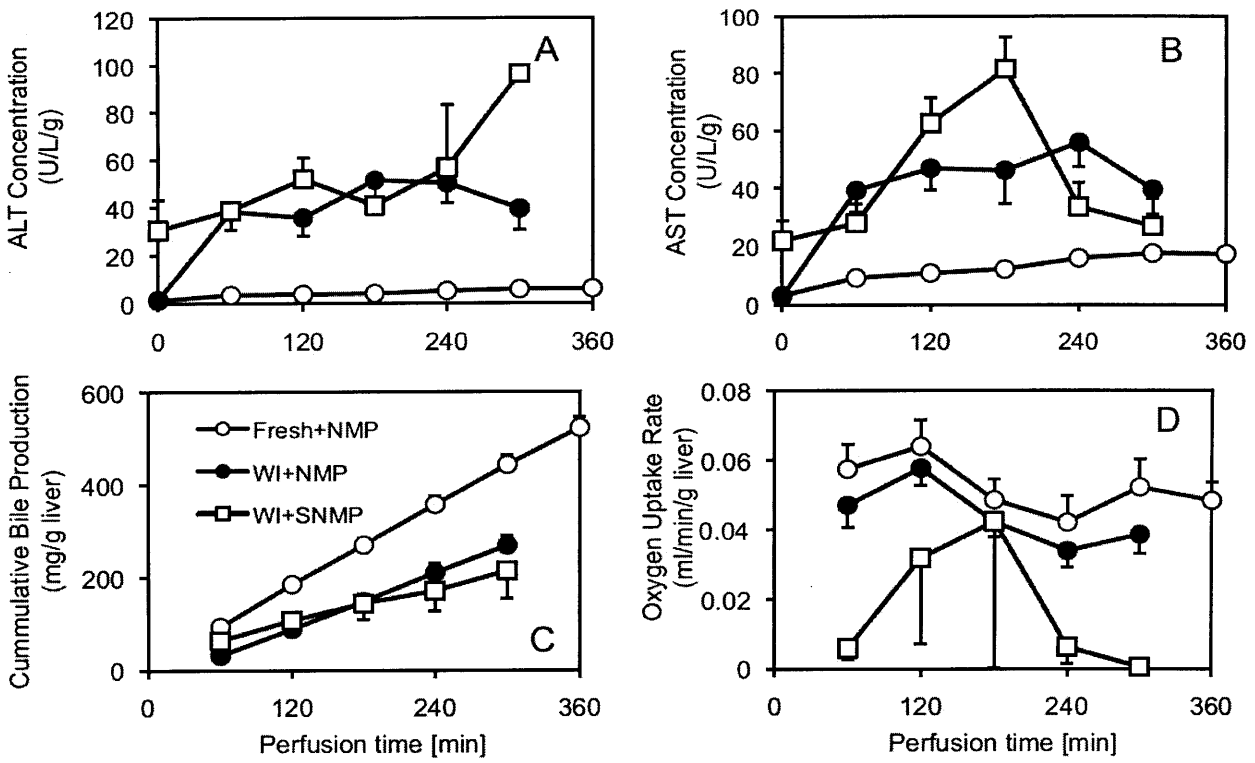

Figure 1: Function and integrity of warm ischemic livers during SNMP. (A) ALT and (B) AST levels in perfusate samples collected hourly from the primary circuit; (C) Total bile accumulation normalized to wet liver weight; (D) Oxygen uptake rate normalized to wet liver weight. Data shown are means ± SEM. Values for the SNMP livers are significantly higher than the fresh + NMP controls for ALT and AST, and significantly lower for bile and oxygen uptake ($p<0.05$). Only difference between NMP and SNMP was in the oxygen uptake rates.

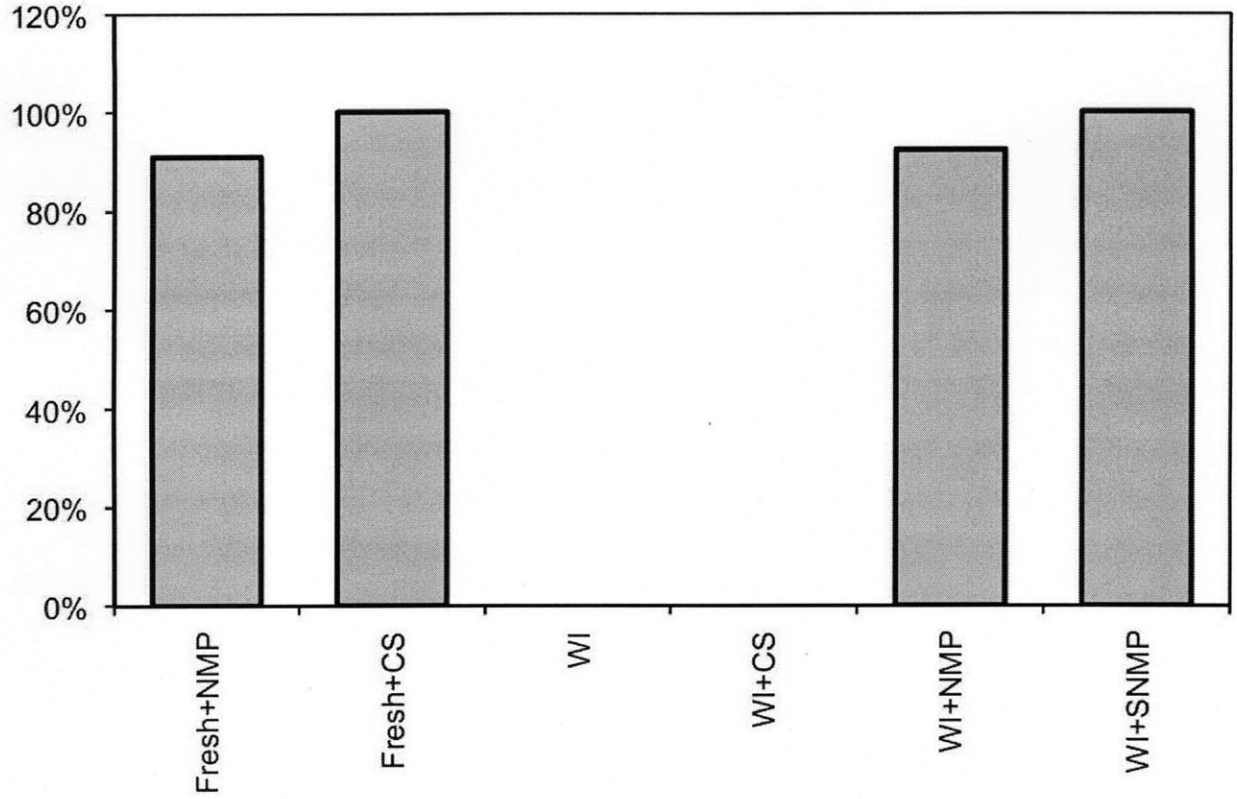

Figure 2: Survival curves of recipient rats after transplantation of perfused warm ischemic livers, warm ischemic cold-stored livers, compared to healthy perfused livers, and healthy cold-stored livers.

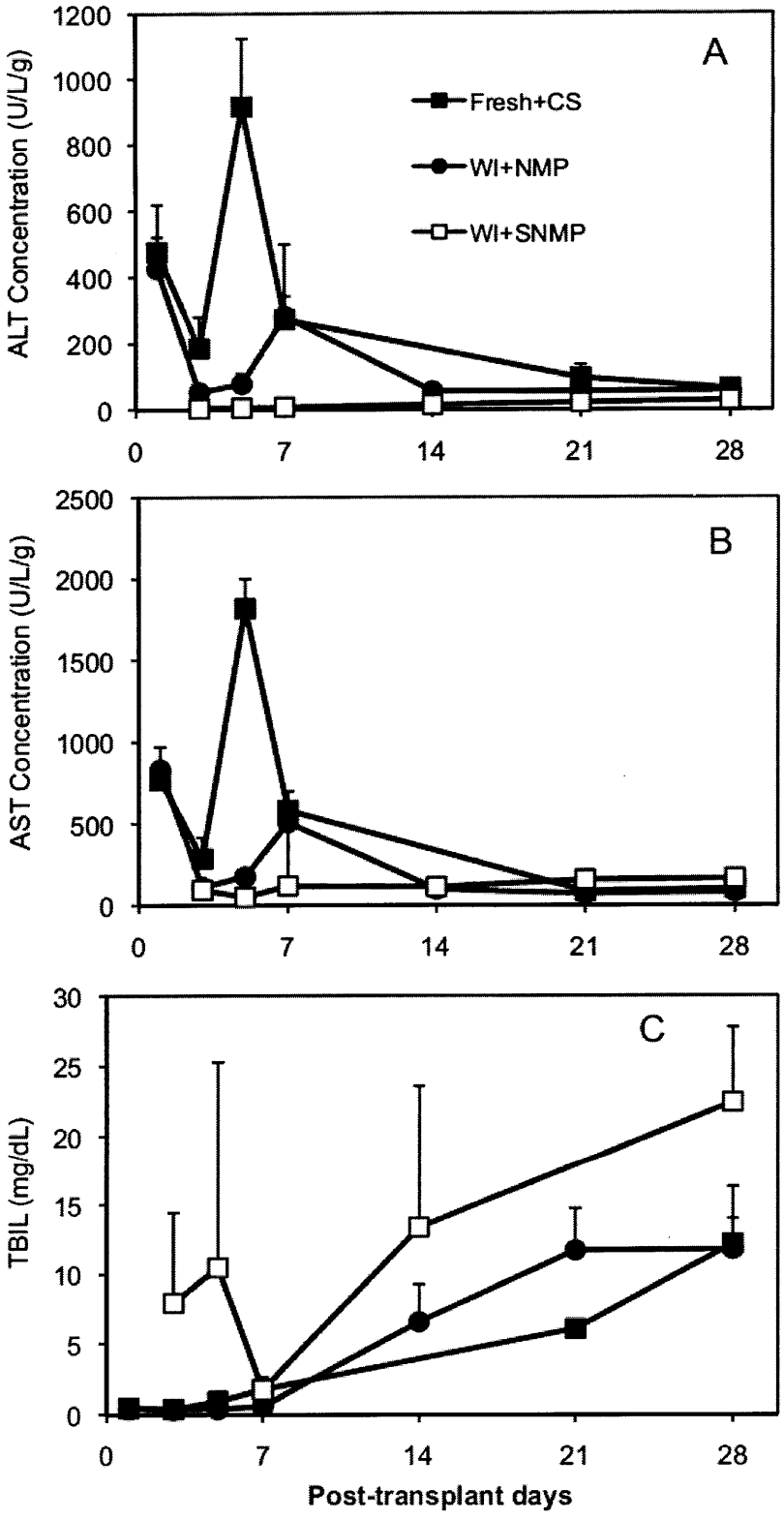

Figure 3: Post-transplantation values of ALT (A), AST (B) and Bilirubin (C), of perfused warm ischemic livers compared to healthy cold-stored livers.

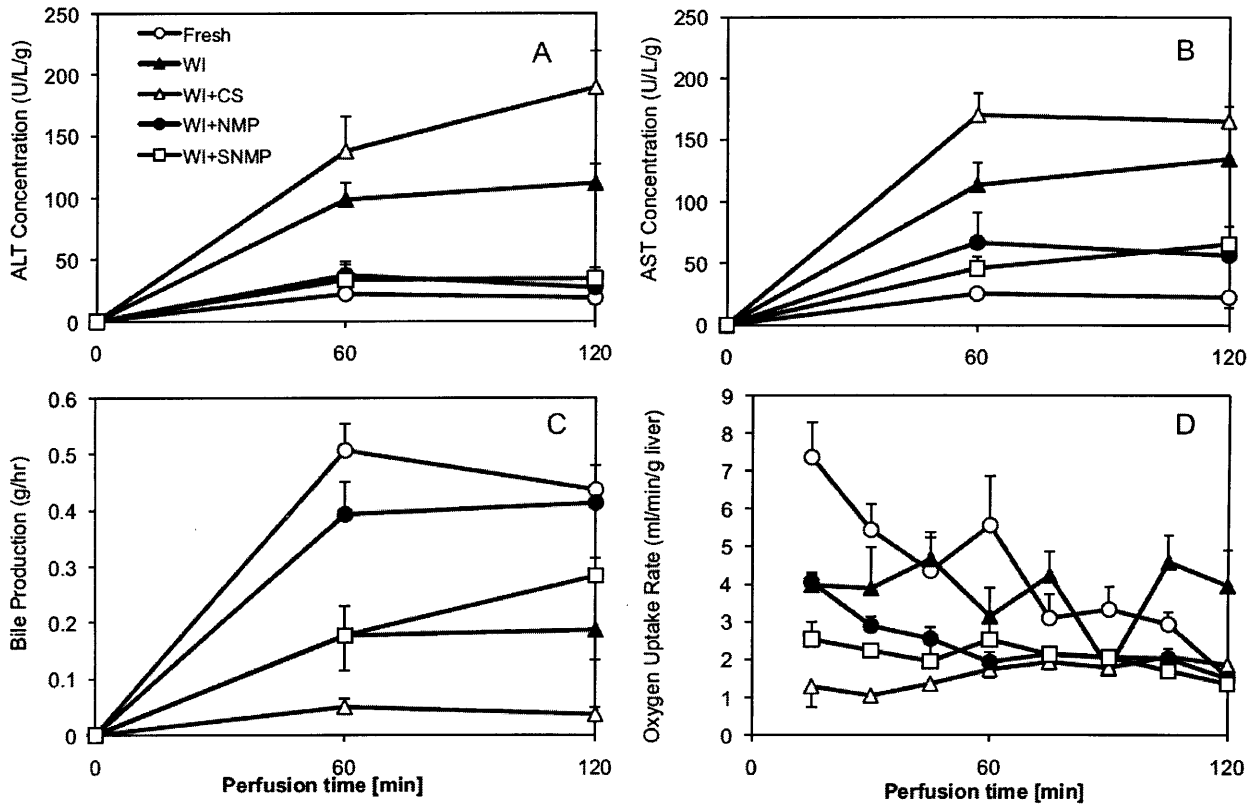

Figure 4: Reperfusion results: (A) ALT, (B) AST, (C) bile synthesis, and (D) oxygen uptake rate measured during reperfusion. Data shown are means ± SEM. ALT and AST values for the SNMP livers are statistically not different from fresh livers and normothermically perfused ischemic livers ($p>0.05$).

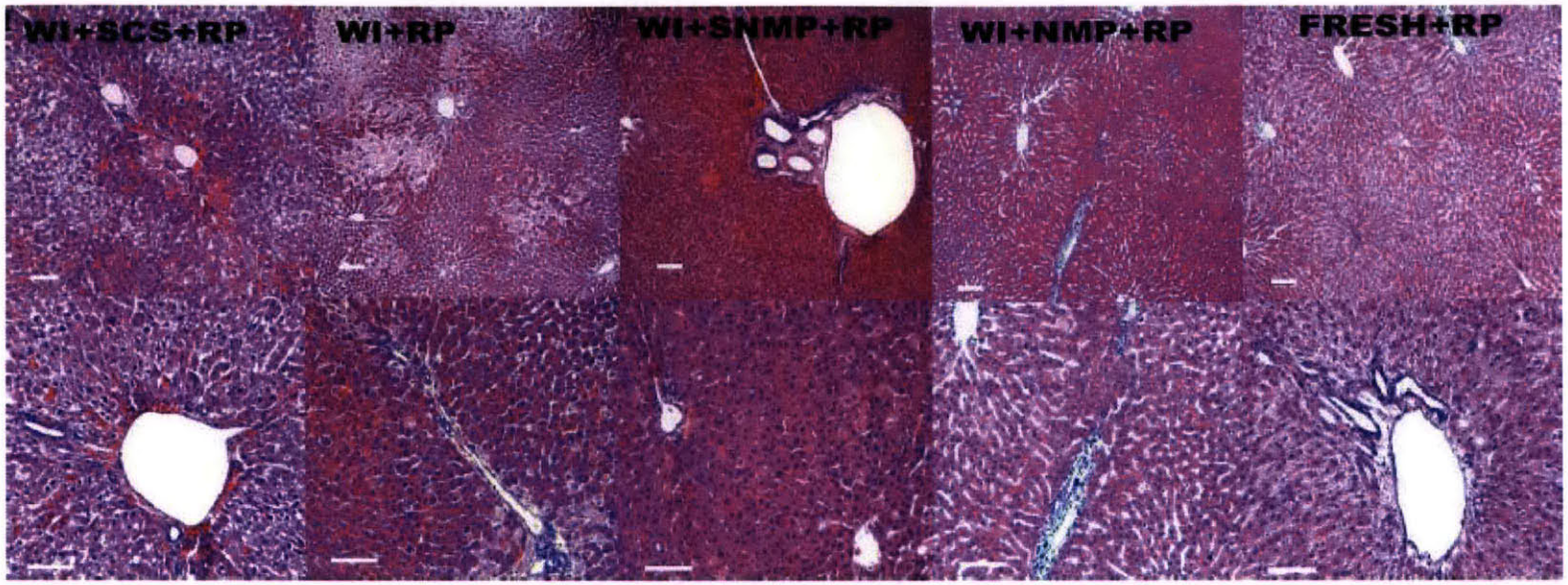

Figure 5. Microscopic appearance of livers after preservation and reperfusion. Top: 10X, Bottom: 20X. Bar = 200 μm.

DISCUSSION

We found that, in concurrence with our previous NMP studies[6], ischemic grafts preserved with SNMP outperformed the ones preserved with CS. This was demonstrated by the 100% one-month survival of the recipients of SNMP-preserved grafts, compared to the 0% survival of those in the CS group. Postoperatively, the profiles of transaminases and billirubin of the SNMP groups were similar or improved compared to those of previously observed NMP preserved ischemic livers, and healthy CS preserved controls. Similar trends were observed during the reperfusion experiments: Both the metabolic function and histological appearance of SNMP groups was similar to that of NMP livers and CS preserved healthy controls.

In general the livers preserved/resuscitated with subnormothermic perfusion displayed lower ALT and AST release during both perfusion and reperfusion. The recipient serum-enzyme levels were also lower. However, it was noted that the bile production of SNMP livers during reperfusion was slightly (but not statistically) lower compared to NMP-resuscitated livers, and postoperative serum total bilirubin levels were slightly (but not statistically) elevated in recipients of SNMP-treated grafts suggestive of exacerbated biliary damage associated with SNMP. However the rat model is not ideal for analysis of this issue, since the most commonly used non-arterialized OLT method is often associated with postoperative damage to the biliary system [66]. Though the recent work of Brockmann et. al. [67] has demonstrated a more suitable porcine DCD-liver model of NMP resuscitation, the long-term effects of machine perfusion on the biliary epithelium remain to be elucidated.

The use of machine perfusion in liver resuscitation after ischemic damage has gained significant traction over the years. The use of hypothermic machine perfusion demonstrates that despite the likelihood of cold-induced damage [68], perfusion results in superior organs compared to static cold storage [69,70]. Further, the establishment of NMP systems implies transplantable outcomes with the prevention of cold storage damage [7,59] during which a number of real-time organ treatments can occur [71,72]. The benefit of SNMP has now been demonstrated here and elsewhere [64,73,74] as a winning simplification strategy, removing the need for temperature regulation. Here we demonstrate that SNMP was equal to or better than NMP in most of the tests performed.

Fujita et. al. [63] reported the change in oxygen uptake rate of livers perfused at different temperatures, using Krebs Henseleit medium. Their model suggests that the oxygen uptake rate at 20°C is 32.4% of the oxygen consumed at 37°C NMP. The actual measured oxygen uptake rate of the grafts during SNMP was about 35% of NMP, only slightly higher than Fujita et. al.'s prediction. This is important however as it is yet another suggestion that livers at physiological

temperatures were oxygen deprived; had they been consuming enough oxygen Fujita et. al.'s results could have coincided. These findings suggest that exsanguinous perfusion or use of artificial oxygen carriers will prove sufficient in delivering the requisite oxygen to a liver perfused at room temperature, thus eliminating the need for erythrocytes. The removal of erythrocytes would further obviate the necessity of continuous dialysis of the perfusate [4]. Coupled with the absence of the need for temperature regulation, this could enable the use of a much less complicated perfusion system such as the portable and disposable perfusion system proposed by Doorschodt et. al. [75] for hypothermic kidney perfusion.

In summary, this work demonstrates for the first time in literature, that a rat model of subnormothermic extracorporeal machine perfusion can successfully resuscitate 1hr ischemic livers for transplantation, comparable to that of normothermic perfusion. Clinically, SNMP can provide a practical model of machine perfusion while retaining the resuscitative benefits of NMP with the ease of operation of HMP.

METHODS

Groups

Six groups that received transplants were compared: i. 1hr WI followed by transplantation (n=9); 1hr WI followed by ii. 5hr SNMP (n=5), iii. NMP (n=8), iv. CS in UW solution at 0°C (n=6); fresh livers transplanted after v. 6hr CS (n=6) or vi. NMP (n=11). Data from groups iii, iv, v, and vi were derived from a prior publication using identical perfusion and surgical methods [6]. For all groups that received 1hr WI, additional experiments were done employing a reperfusion method using diluted whole-blood [76] to provide acute post-transplantation results; the results were compared to the reperfusion of a fresh liver.

Isolation of donor livers

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) and heparinized under sterile conditions. The donor liver isolation procedure is a modification of Delrivière's technique [77] and is described in detail elsewhere [4]. Liver excision occurred by ligation of the HA, clamping of the IVC and PV, followed by transection of the SHVC, PV, HA and IVC. The liver was not flushed.

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. Ex vivo ischemia ensured a constant temperature [78] and enabled a severe model of warm ischemia [79]. During this period the PV and IVC were cuffed as previously described [4].

Subnormothermic and 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 [4] 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 secondary dialysis circuit as described previously [4]. This design enabled a small blood supply to provide a high hematocrit in the primary circuit and retained the benefit of a large reservoir for nutrients and waste dilution via the secondary circuit. 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% N_2/ 21% O_2/ 5% CO_2 and 100% O_2 to maintain a constant pH. Inflow was maintained at approximately 2 ml/min/g liver, and 10-14 cmH_2O portal pressure. A fraction of the perfusate was diverted to the secondary circuit via continuous peristaltic flow through 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) at an average 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. Room temperature was recorded as 20°C throughout SNMP perfusions, or maintained at 37°C via the water-jacketed apparatus for NMP.

Fresh livers were immersed in ice-cold UW, cuffed in less than 10 minutes and then either stored on ice or flushed with saline prior to being placed in the perfusion system. After the warm ischemic period, livers were flushed with 10ml of warm saline and connected to the

perfusion system. Livers were 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. At the end of perfusion, flow was terminated; the liver was disconnected from the circuit, rinsed in a bowl of saline at room temperature and weighed again prior to transplantation.

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 O_2 (ml/dl) in the samples was determined according to the formula:

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

where [Hb] is the hemoglobin concentration in g/dl, FO_2Hb is the fraction oxygenated hemoglobin and pO_2 is the partial pressure of oxygen in mmHg. HOUR was determined as:

$$HOUR = (([O_2]_{in} - [O_2]_{out})/100) \times \text{flow rate/weight of liver.} \quad [2]$$

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

Recipient surgery

A modification of the cuff technique designed by Kamada and Calne [77,80,81] was implemented and is described in detail elsewhere [4]. 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 $NaHCO_3$ 7%w/v (Abbott, North Chicago, IL) by penile vein injection.

The animals were put singly in clean cages, allowed to recover from anesthesia under an infrared lamp for half an hour, and subsequently returned to regular housing. During the first

12 hours post-operatively animals were checked every 2 hours and subsequently every 8 hours for one week, and daily afterwards.

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 ≥3 for each group.

Simple cold storage

Warm ischemic livers (n=6) and freshly isolated livers (n=6) were flushed with 20ml of ice-cold (0-4°C) UW solution and placed on melting ice in a bowl containing UW solution for the duration of the CS 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 an extracorporeal 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 artifactual findings. The primary circuit was identical to the room temperature perfusion system with the exception that the temperature was set at 37°C and the secondary circuit comprising the dialyzer was disconnected from the perfusion apparatus. The livers were reperfused for 120 minutes and inflow (portal vein) and outflow (infrahepatic vena cava) sampling was performed every 15 minutes. Details of this system can be found elsewhere[4].

Histology

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 ± SEM. Statistical analysis for comparison of groups in survival was done by the log-rank test. The rest of the analysis was performed using 2-way ANOVA in MATLAB (v7.7.0) at $\alpha=0.05$, with Tukey-Kramer correction for multiple comparisons.

For all measured variables (ALT, AST, bile, O_2) group comparisons were performed on overall time responses, rather than individual time points such that the differences between groups could be identified.

5-3. Evaluation of NELP Duration on Hepatoycte Yield

We use hepatocyte isolation as an endpoint to quantify the extent of perfusion success. In a simple series of experiments we show that maximum cell yield is obtained at short perfusion durations of 2 hours when using NELP. Based on our MFA analyses of ischemic liver recovery that demonstrated a turnaround in liver metabolism at t=2 hours, we hypothesized that this corresponded with the time to organ recovery. In order to evaluate this claim, we conducted a series of perfusion experiments in which we varied the duration of perfusion from 5 hours to 3, 2, and 1 hours and measured the number of hepatocytes procured per gram of liver weight.

RESULTS

Upon completion of 5, 3, 2 or 1 hours of liver perfusion using NELP, at each time point, perfusion was stopped and the livers were subjected to a normal two-step collagenase-based isolation technique. Figure 1 illustrates that though there are no significant differences between the groups, the trends are higher in cell yield earlier in perfusion.

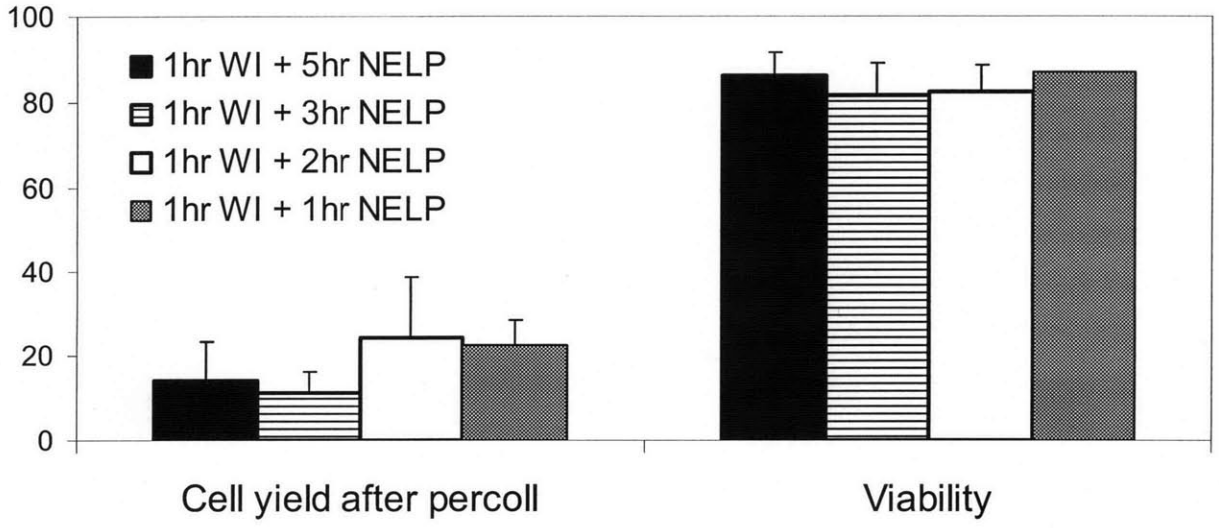

Figure 1. Cell yield and viability after NELP, n=5.

DISCUSSION

The enhancement of warm ischemic livers within the first 2 hours, and the correlation this has with increased oxygen uptake rate ties in well with the concept of debris washout, enhanced cell access and energy recovery. It is unclear however, why livers do not subsequently go on to exhibit Fresh-like liver metabolic rates in MFA, or indeed, why fewer cells are recovered as soon as an hour later (t=3 hrs). It is possible that the two phases of ischemia reperfusion injury (IRI) are at play here, where the first is microcirculatory collapse and the second is neutrophil mediated damage. The first is known to occur within minutes of reperfusion, while the second begins a few hours later[27,30,82-84]. The consequences are cell death due to "necroapoptosis" – a term used to define the seemingly combined signals of induced and programmed cell death in this unusual form of tissue damage. Therefore, capturing cells before leukocyte mediated oxidative damage would ensure maximal cell yield. Usefully, these findings imply that cell recovery can occur within 1-2 hours if perfusing at 37°C. Less optimistic however, is the suggestion that livers transplanted after resuscitation with NELP may still be in the throes of combating IRI. While these livers have been shown to result in long-term viability in excess of 1 month after transplantation, they have also been transplanted into healthy syngeneic recipients who have intact immune systems capable of handling this stress. Further careful analysis of recipients of perfusion-recovered ischemic livers may provide insight as to both the severity of the injury as well as the immunomodulatory factors responsible for ameliorating the damage.

METHODS

Hepatocyte Isolation

A two-step collagenase perfusion technique described by Seglen[85], and modified by Dunn et al.[86] was used to isolate hepatocytes. Briefly, using aseptic technique, after gaining portal vein access with an 18G catheter, warm oxygenated KRB+EDTA was flowed through the livers at approximately 17ml/min. For Fresh livers, the IVC was immediately dissected and the liver subsequently removed from the animal into a petri dish until perfusion was completed. Perfused and WI livers were already in petri dishes with cuffed PVs. A collagenase (type IV, Sigma, C5138-1G) solution with KRB and $CaCl_2$ was introduced to perfusion as the KRB solution was depleted and allowed to flow until successful digestion was observed. The livers were then moved to a sterile hood on ice where approximately 10mL of sterile, cold KRB were added. The liver capsule was gently broken to release the cells which were then passed through

a 250um filter followed by a 60um filter. The suspension was divided into 50 mL conical tubes and centrifuged at low speed (15g-21g, 4°C, no brake, 5 minutes). The supernatant was aspirated and the pellet resuspended with 10ml KRB. An initial cell count and viability was performed. A volume of 24mL of cold Percoll solution (9 parts Percoll : 1 part 1.5M NaCl, pH 5-5.5) was used for every 25mL of cell suspension. Cells were added at a concentration of 5 million cells/mL and inverted several times before being centrifuged (49-58 g, 4°C, no brake, 10 minutes). The buffy coat and supernatant were discarded and resuspended to 10mL in DMEM + 10% FBS + 100,000 u/L penicillin + 100mg/L streptomycin sulfate, after which a final count was performed using Trypan Blue exclusion.

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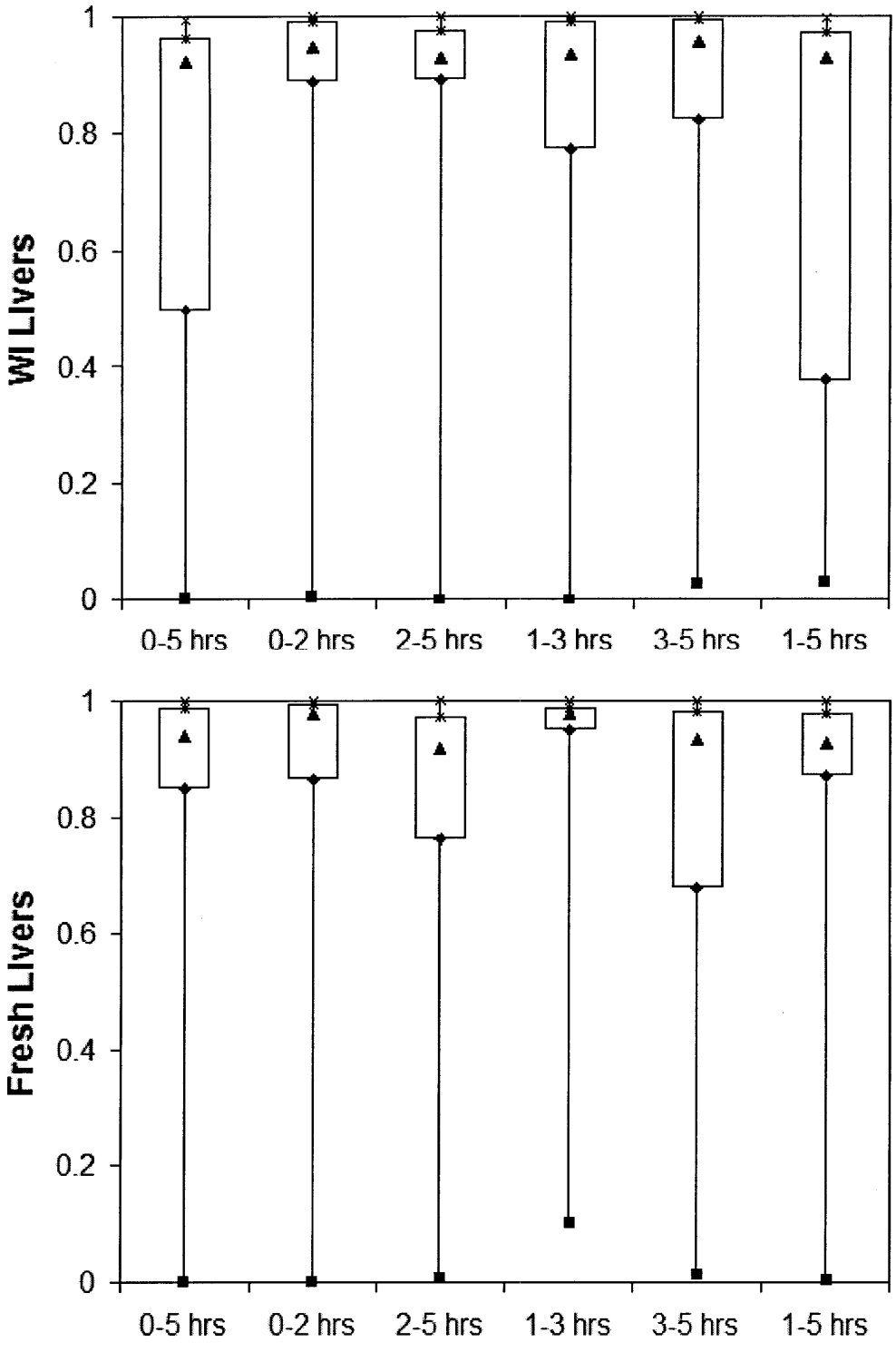
Appendix A. In vivo and Perfusate (WE) Reference Concentrations

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

[1] Izamis ML, et. al. In vivo metabolic flux analysis of the liver: Effect of burn injury in rats. 2010.
[2] 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 6: Perfusion-Enhanced Cell Yields from Fresh and Ischemic Grafts

We design a room temperature-operated ex vivo organ perfusion system with an off-the-shelf perfusate medium for the easy and rapid recovery of hepatocytes. The major findings of this study are:

- We demonstrate using this method that donor pretreatment with heparin is unnecessary for high cell yields and viabilities.
- We obtain seventeen times as many hepatocytes from livers exposed to an hour of WI (34°C) compared to untreated WI livers.
- We increase fresh liver hepatocyte yields by 32%.
- We demonstrate a linear correlation between post-purified (Percoll treated) cell yield and tissue ATP content, normalized to total protein. This enables direct measures of organ viability and organ recovery during perfusion resuscitation.
- A strong correlation between perfusion flow rate and cell yield is established supporting the use of flow rates as low as possible without causing hypoperfusion or oxygen deprivation.
- Morphologically and functionally, perfusion-isolated hepatocytes performed comparably or better than fresh hepatocytes in cell suspension and plate culture.

There is a tremendous need for high-quality hepatocytes in the fields of cell-based therapeutics, toxicology, drug metabolism, tissue engineering and basic research. Optimizing cell yield and viability from both transplantable as well as discarded donors would have a dramatic impact on meeting this demand. Ischemically damaged organs present a promising, virtually limitless supply of hepatocytes, based on evidence that ex vivo perfusion of these organs recovers functionality to a significant degree[1,2]. Additional findings show the resuscitative benefits of ex vivo sanguineous perfusions can also be achieved at room temperature[3] where a reduced metabolic rate numerically obviates the need for erythrocytes thereby enabling a significantly simplified perfusion design. A dynamic study of organ metabolism during normothermic perfusion further speculated that the recovery of ischemic livers occurs within 2-3 hours of perfusion inception[4].

Based on these data we developed an ex vivo organ perfusion system comprising only a pump that takes an off-the-shelf perfusate from a reservoir at room temperature through an oxygenator and bubble trap to the liver, before being recycled. The simplicity of the design lends itself readily to translation into clinical practice where it

stands to recover an order of magnitude more hepatocytes from ischemic livers than currently obtained[5,6] at viabilities greater than 90% in less than 3 hours of un-manned perfusion. Further, it has the ability to enhance hepatocyte performance in healthy livers procured for donation, promoting the ubiquitous use of perfusion techniques in donor organ storage.

RESULTS

Our goal was to investigate a clinically translatable perfusion technology for the enhanced recovery of transplantable-grade cells from all donor organs, particularly donors after uncontrolled cardiac death. Using a highly simplified ex vivo perfusion system we recovered rat hepatocytes from non-pharmacologically pre-treated rat donors who experienced 10 minutes or 60 minutes of warm ischemia prior to perfusion. The results were compared to freshly isolated hepatocytes and hepatocytes procured from livers exposed to 60 minutes of warm ischemia without treatment.

Livers that experienced 10 minutes of ischemia (cooled from 37°C to approximately 20°C for cuffing purposes prior to ex vivo perfusion) and livers that experienced 60 minutes of WI (34°C) were subsequently perfused at 20°C (room temperature, RT) for 3 hours prior to hepatocyte isolation. Figure 2a illustrates that there was no significant difference in the number of hepatocytes obtained from WI livers treated with 3hr RT perfusion (34±11 million cells/g liver tissue, n=9) and Fresh livers (34±9 million cells/g liver tissue, n=15). By contrast, untreated WI livers yielded significantly fewer hepatocytes (1.7±0.6 million cells/g liver tissue, n=8). Further, fresh livers that experienced 10 minutes of ischemia prior to 3hr RT perfusion resulted in significantly more hepatocytes than untreated Fresh livers (45±10 million cells/g liver tissue, n=12). All hepatocyte isolations were treated with Percoll to obtain a viability ≥90% (Figure 2c).

Hepatocyte function was tested under two conditions: 6hr cell suspension culture and 2 week double layer collagen gel sandwich plate culture. Cell viability and mitochondrial activity were measured in suspension using trypan blue exclusion and MTT reduction to formazan (Figure 3A and C respectively). While freshly isolated hepatocytes started with relatively high viability during the first hour of suspension, their viability was the lowest at t=6hrs. Similarly, while mitochondrial activity of fresh livers was generally higher than that of perfusion-treated groups, they also showed the

greatest decline at t=6hrs, whereas there were no differences between initial and final values in the perfused liver groups. Cell damage in suspension was measured with ALT and AST. Figures 3B and D demonstrate that both perfusion groups start with significantly higher ALT and AST values compared to Fresh cells; ALT remains relatively constant in WI+3hrRT cells, gradually degrades in 3hrRT cells and inclines in Fresh cells. AST continues to increase during the first 2 hours before reaching a plateau in WI+3hrRT cells, is stable in 3hrRT cells and inclines steadily for Fresh livers. Cytochrome P450 activity was measured by dealkylation of benzyloxy resorufin (CYP4502B2), pentoxy resorufin (CYP4502B1), ethoxy resorufin (CYP4501A1) and methoxy resorufin (CYP4501A2) after 3,3'-methylene-bis(4-hydroxycoumarin) activation (Figure 3E). CYP450 activity was comparable amongst all groups except for 3hrRT cells which exceeded Fresh EROD and MROD activity significantly. Metabolic activity was evaluated at t=6hrs and it was observed that all groups produced equal amounts of glucose (2g/L at baseline), and urea (0mM at baseline), while WI+3hrRT cells produced significantly less albumin than either Fresh or 3hrRT cells.

In plate culture, perfused liver cells showed excellent confluency at day 14 when observed using phase contrast imaging (Figure 4C); better than Fresh hepatocytes (Figure 4A) and structurally better-preserved compared to WI cells (Figure 4B). Viability and mitochondrial activity were comparable amongst all groups and displayed a mild but insignificant decline over the two-week culture period. CYP450 activity did not differ significantly across groups. Certain trends could be observed, such as Fresh cells generally peaking in activity within 7 days, while perfused livers appeared to either remain constant throughout culture or gradually pick up in activity, peaking at Day 14. Daily metabolism of albumin shows that Fresh and 3hrRT cells behaved comparably but WI+3hrRT cells did not produce as much. 3hrRT cells produced significantly more urea than either WI+3hrRT or Fresh cells, which were comparable. All groups metabolized glucose equally.

Performance of livers in perfusion was evaluated via several metrics. Figure 5 illustrates the metabolic characteristics of ex vivo livers perfused at room temperature. The rates of glucose consumption, and lactate[7] and urea production were comparable in both groups, and neither demonstrated a net production of albumin (Figures 5A, E, G and C respectively). The liver's ability to control pH[8] was demonstrated from the very beginning of perfusion by 3hrRT livers, while WI+3hrRT livers recovered this function within the first hour (Figure 5B). Both livers extracted almost all available oxygen in

perfusate (Figure 5D). Overall, 3hrRT livers tended to consumed slightly more oxygen (0.016±0.003mlO2/min/gliver) than WI+3hrRT livers (0.012±0.003mlO2/min/gliver), though the difference was insignificant at each measured time point (Figure 5F). Bile production was delayed in WI+3hrRT, frequently initiating at t=2hrs compared to the instantaneous production by 3hrRT livers. Subsequently the cumulative amount of bile from WI+3hrRT livers was less at t=3hrs than that produced by 3hrRT livers (Figure 5H). Liver weights in WI+3hrRT livers averaged 7.3±0.6g before ischemia, 7.9±0.91g after 1hr of warm ischemia, and 7.6±0.7g after perfusion. Liver weights in 3hrRT weighed 7.2±0.5g before perfusion and 7.9±0.6g after perfusion. None of these increases in weight were significant.

To evaluate the impact of ATP normalized to total protein on cell yield, its content was measured in liver tissue biopsied from organs immediately after harvest, 1hr of WI, 1hr WI+3hrRT and 3hrRT. Figure 1B illustrates that there is a significantly high correlation between ATP content and purified cell yield per gram of liver tissue (Pearson's correlation 0.96). As the ATP measurements were made on biopsied tissue sections, a comparison to initial cell yields was also made (Figure 6) but this shows a weaker correlation. Percoll subsequently removed 48% of the total yield in Fresh livers, 47% in 3hrRT livers, 51% in WI+3hrRT livers and 96% in WI livers.

Further differences between WI+3hrRT and 3hrRT livers were highlighted by perfusion hemodynamics. A scatter-plot of final flow rate vs. cell yield and pressure was created for both perfusion groups (Figures 7B and C). Both plots show that generally higher yields are obtained at lower flow rates; this relation is distinctly linear in 3hrRT livers (Pearson's correlation -0.94). Pressure across the livers was held generally constant and demonstrated no correlation to cell yield in these experiments. The ratio of pressure drop across the liver and flow rate, initially measured every 5 minutes for the first half hour, and then every half hour thereafter, provided a value of hepatic resistance (Figure 7A). 3hrRT (n=13) livers were generally reduced in resistance compared to WI+3hrRT (n=14) livers though there was no consistent statistical difference between the groups. Flow rates for 3hr RT livers started at 8.5±1.6ml/min, with initial pressures at 4.5±2.4cmH_2O. WI+3hrRT livers experienced a similar initial flow rate of 8.6±2.4ml/min with initial pressures at 6.25±4.8cmH_2O. By the end of perfusion, 3hrRT livers saw flow rates of 13±0.8ml/min and pressures of 1.9±0.8 cmH_2O while WI+3hrRT livers experienced flow rates of 12.6±1.2ml/min and pressures of 3.3±1.8 cmH_2O. This

demonstrates that despite slightly lower flow rates in WI+3hrRT, pressure across these livers remained higher.

Histological evaluation using TEM allowed visualization of the presence of edema in WI livers (Figure 8), with generally reduced cytoplasm-to-nuclear space, a suggestion of rounded or swollen mitochondria and the presence of vacuoles. These were also present in WI+3hrRT livers though to a lesser extent and gross edema was significantly reduced by comparison. However, sinusoids appeared generally dilated and congested while hepatocyte sinusoidal cell boundaries were indistinct. 3hrRT cells did not show significant vacuolation or edema but also suggested evidence of sinusoidal congestion and indistinct sinusoidal hepatocyte borders. At 0.5microns (Figure 9), nuclear and mitochondrial membranes appeared intact, and their contents comparable to Fresh livers. Mitochondria appeared more swollen in the WI and perfused livers compared to Fresh livers. Rough endoplasmic reticulum and glycogen rosettes appeared comparable in Fresh and WI livers, less-distinct and discontinuous in perfused livers.

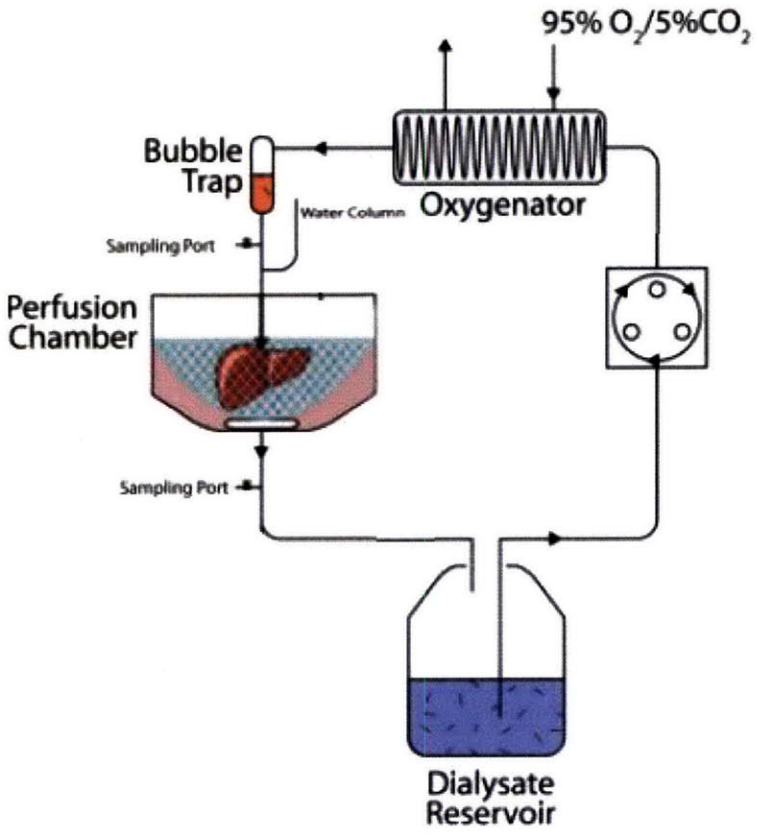


Figure 1: Simplified liver perfusion system

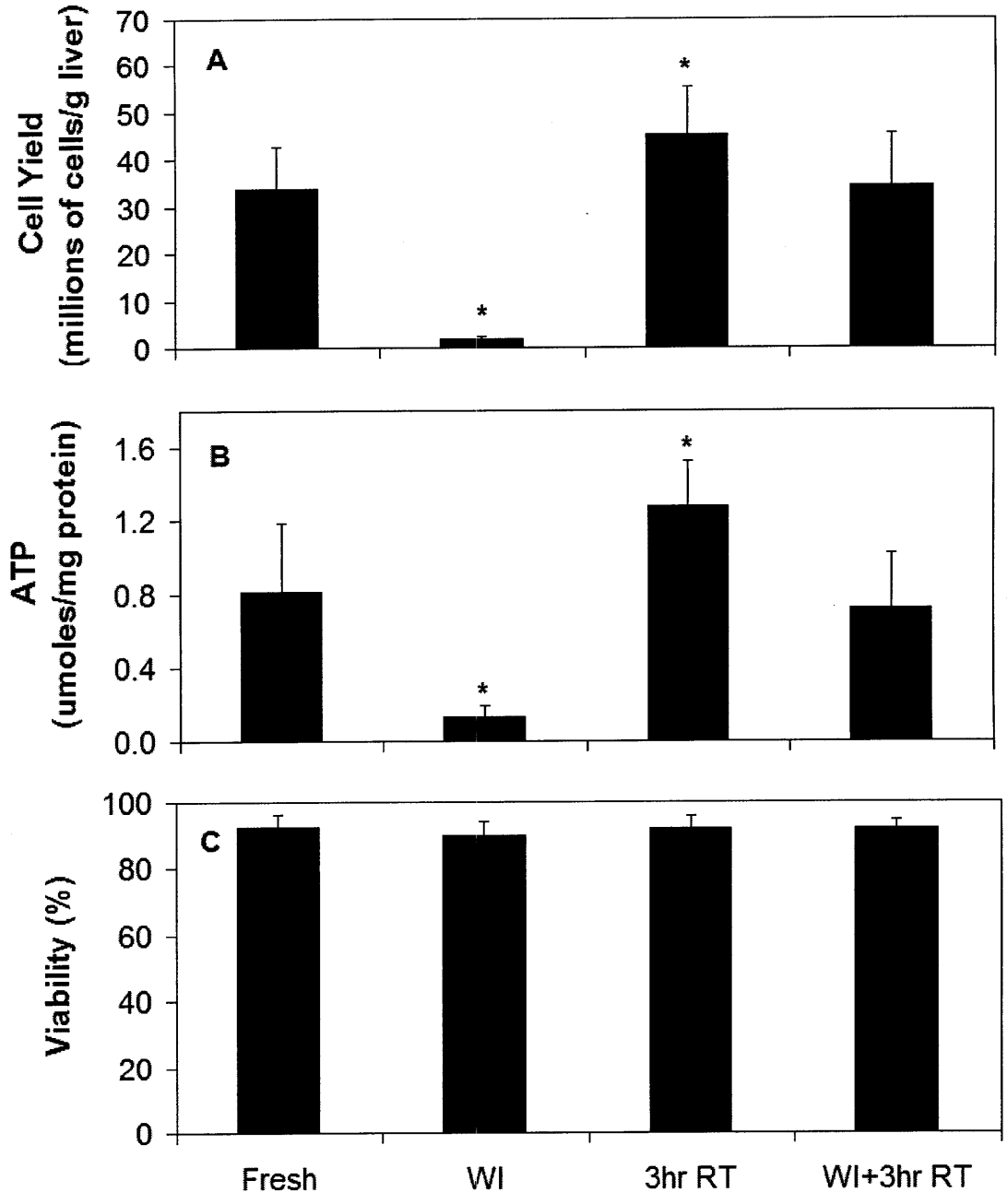


Figure 2: A) Cell yield post-Percoll purification normalized to wet liver weight. B) ATP content of biopsied tissue normalized to the total protein content. C) Viability of cells post-Percoll purification. Data shown are means ± std dev. Values for WI and 3hrRT livers are significantly different from other groups ($p<0.05$); there is no statistical difference between Fresh and WI+3hrRT groups

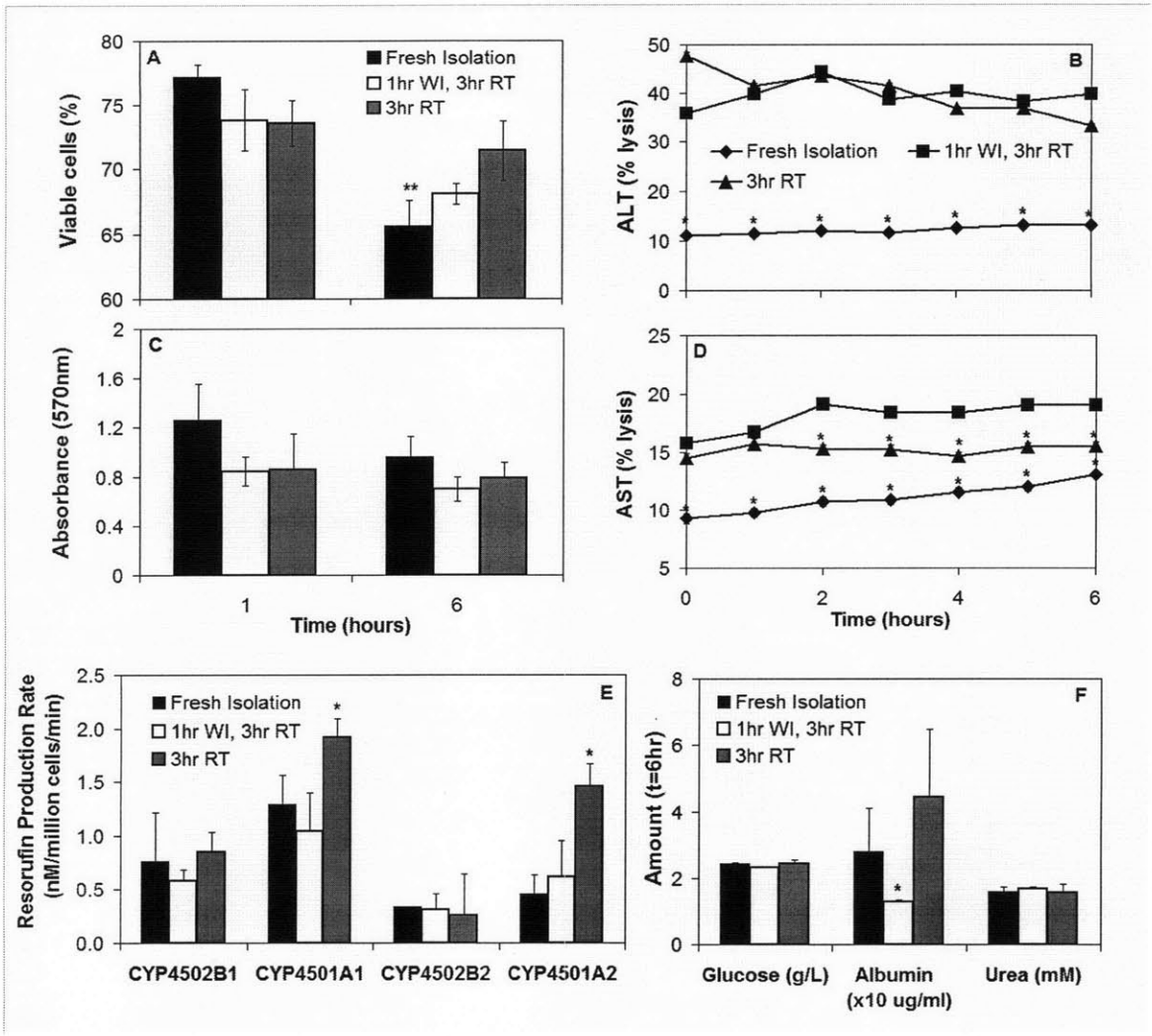
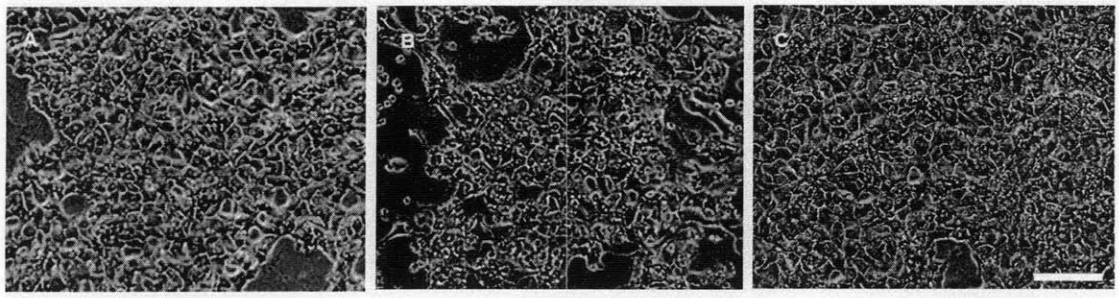


Figure 3: Cell function in suspension for WI+3hrRT and 3hrRT groups compared to Fresh hepatocytes. A) Percent viability of cells using Trypan Blue exclusion. Fresh hepatocytes are significantly reduced in viability at t=6hrs ($p<0.05$). B) ALT; perfused groups have significantly higher starting values ($p<0.05$). C) Mitochondrial activity measured by absorbance of formazan produced from MTT at 570nm. D) AST; significant differences exist between all groups ($p<0.05$). E) CYP450 activity measured using resorufin production by dealkylation of benzyloxy resorufin (CYP4502B2), pentoxy resorufin (CYP4502B1), ethoxy resorufin (CYP4501A1) and methoxy resorufin (CYP4501A2) after 3,3'-methylene-bis(4-hydroxycoumarin) activation. Activity is significantly higher in 3hrRT cells for CYP4501A1 and CYP4501A2 ($p<0.05$). F) Metabolic assays of glucose, albumin and urea. Albumin is significantly reduced in WI+3hrRT cells at t=6hrs ($p<0.05$). All data shown are means ± std dev.


A
B
C

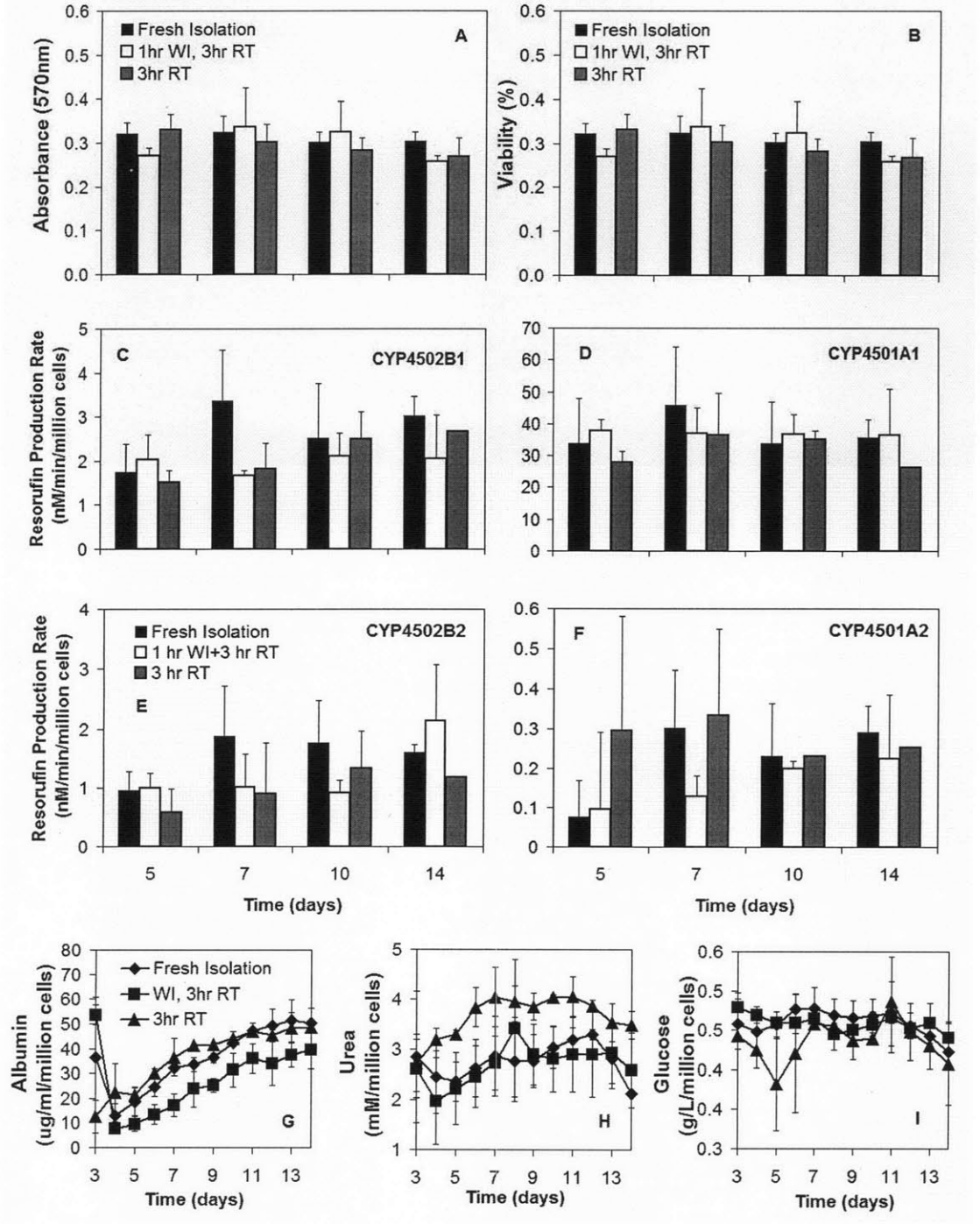

Absorbance (570nm)
Fresh Isolation
1hr WI, 3hr RT
3hr RT
A
Viability (%)
B
Resorufin Production Rate (nM/min/million cells)
C
CYP4502B1
D
CYP4501A1
1 hr WI+3 hr RT
3 hr RT
E
CYP4502B2
F
CYP4501A2
Time (days)
Albumin (ug/ml/million cells)
WI, 3hr RT
G
Urea (mM/million cells)
H
Glucose (g/L/million cells)
I

Figure 4: Cell function in double layer collagen gel sandwich plate culture. A-C) Phase contrast images for Fresh, WI and WI+3hrRT livers respectively. D) Mitochondrial activity measured by absorbance of formazan produced from MTT at 570nm. E) Viability of cells using Hoechst 33452/Ethidium homodimer-1 double stain. F-I) CYP450 isoenzyme activity for days 5, 7, 10 and 14 of culture. J-L) Metabolic assays for Albumin, urea, glucose. Urea production is significantly increased in 3hrRT livers ($p<0.05$).

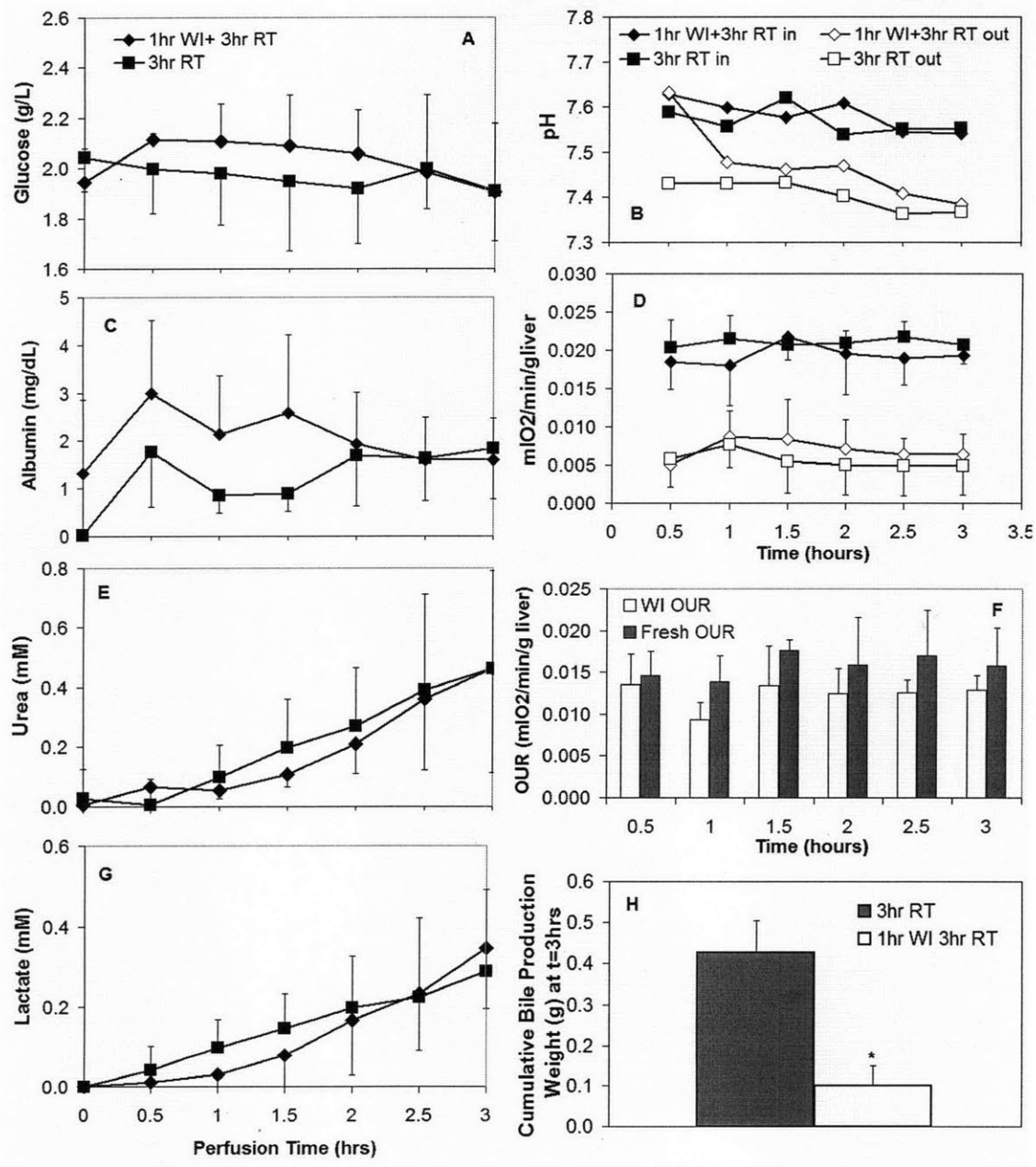


Figure 5: Liver performance in perfusion. A) Glucose consumption over time. B) pH of perfusate entering and exiting the liver. pH out at t=0.5hrs is significantly different between 1hr WI+3hrRT livers ($p<0.05$). C) Albumin production over time. D) Oxygen delivery and exit from the liver measured as a function of the partial oxygen tension and flow rate of perfusate. E) Urea production over time. F) Oxygen uptake rate over time. G) Lactate production over time. H) Bile weight accumulated by t=6hrs.

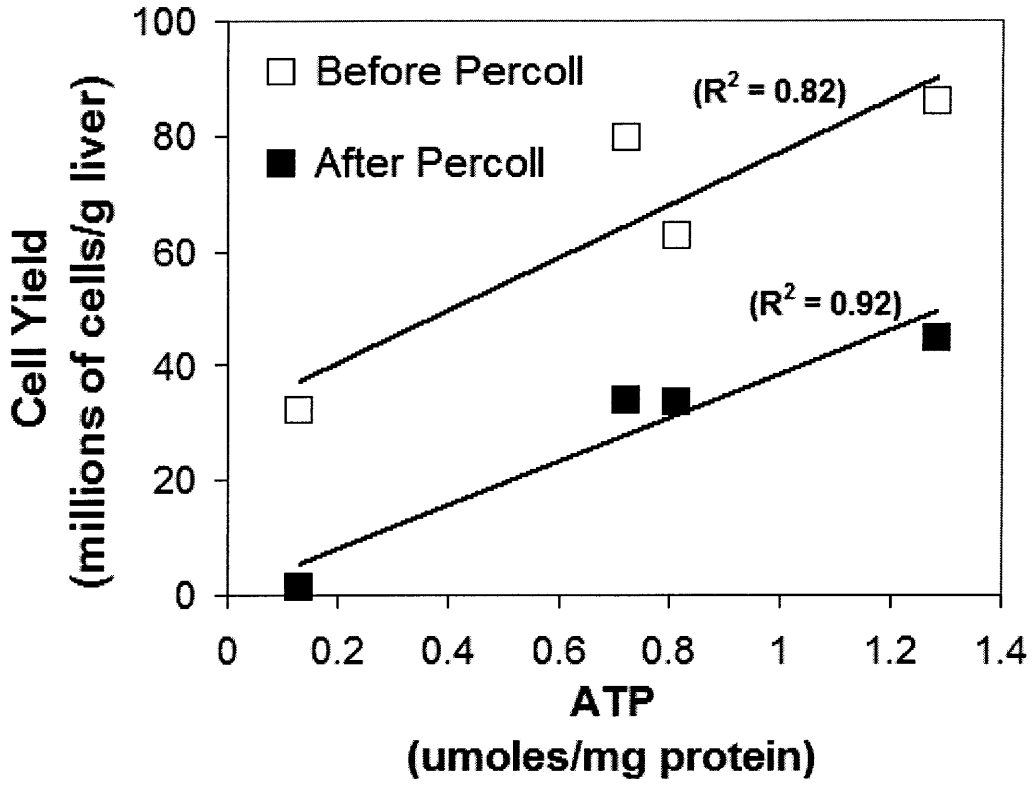


Figure 6: Scatter-plot of average cell yields obtained in each group both before and after Percoll purification vs. average ATP content normalized to total protein.

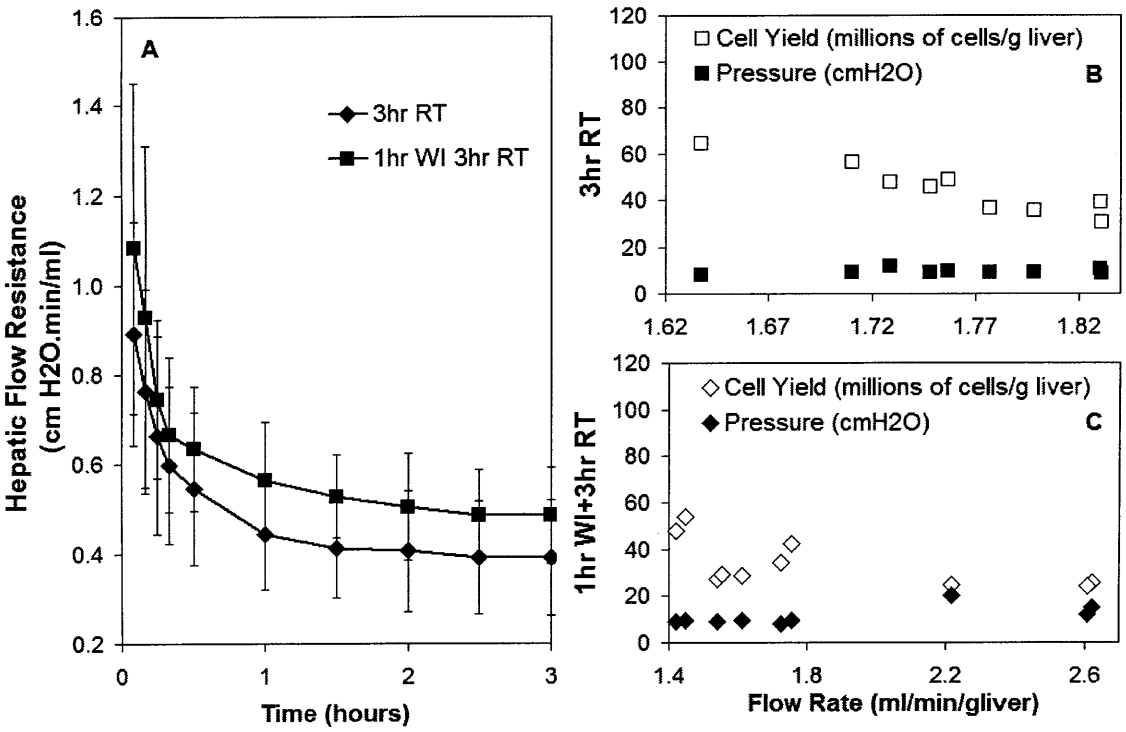


Figure 7: Hemodynamic relations in perfusion. A) Hepatic resistance of both WI+3hrRT and 3hrRT livers. B) Scatter-plot of individual experiment cell yields and pressures (n=9) vs. corresponding flow rates for 3hrRT livers. Linear correlation between cell yield and flow rate is significant (Pearsons -0.97). C) Scatter-plot of individual experiment cell yields and pressures vs. corresponding flow rates for WI+3hrRT livers (n=10).

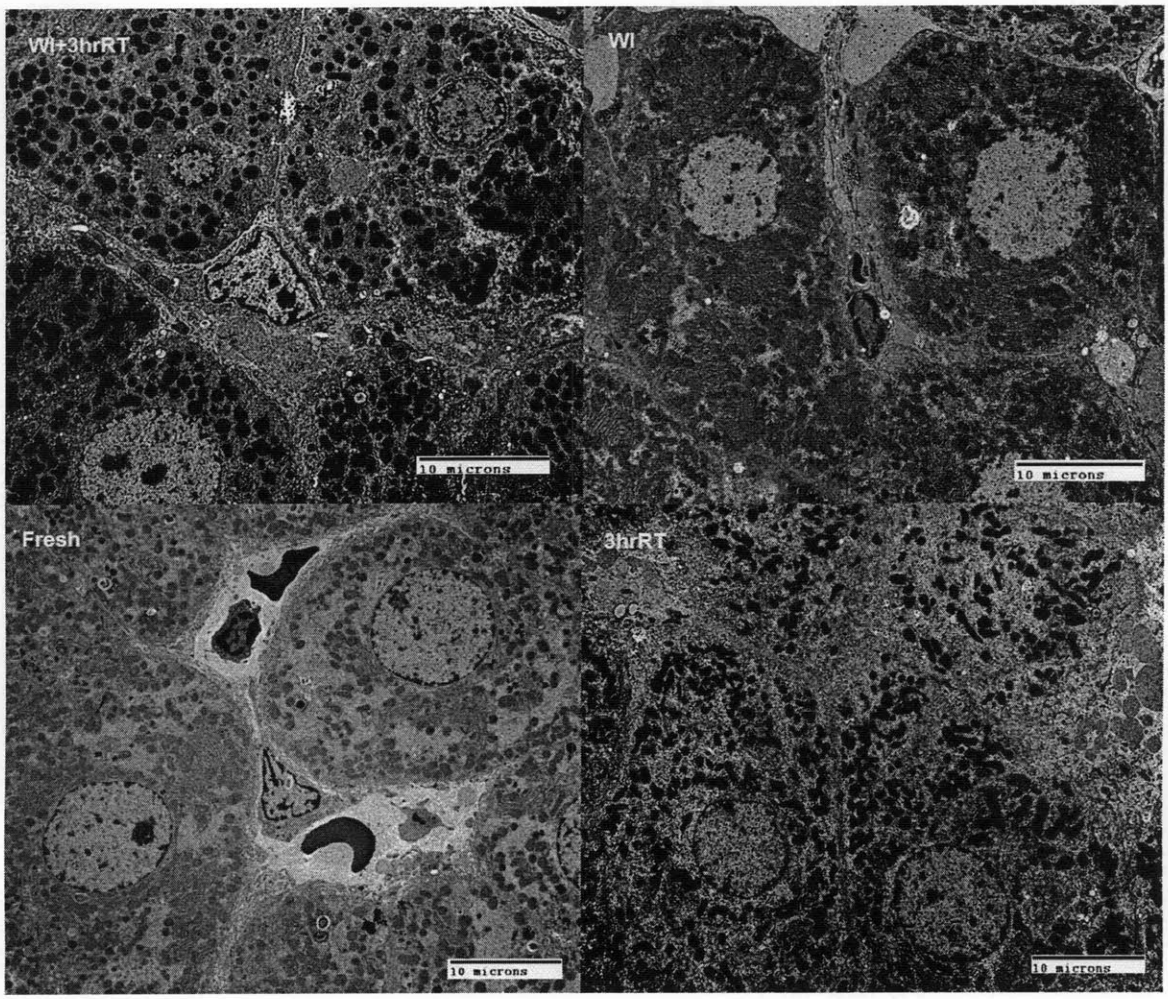


Figure 8: Transmission electron microscopy (10μm) revealing gross edema in WI livers, vacuolation, diminished cytoplasm-to-nucleus ratio and congested sinusoids. Perfused livers have sinusoidal congestion, and variegated cell borders.

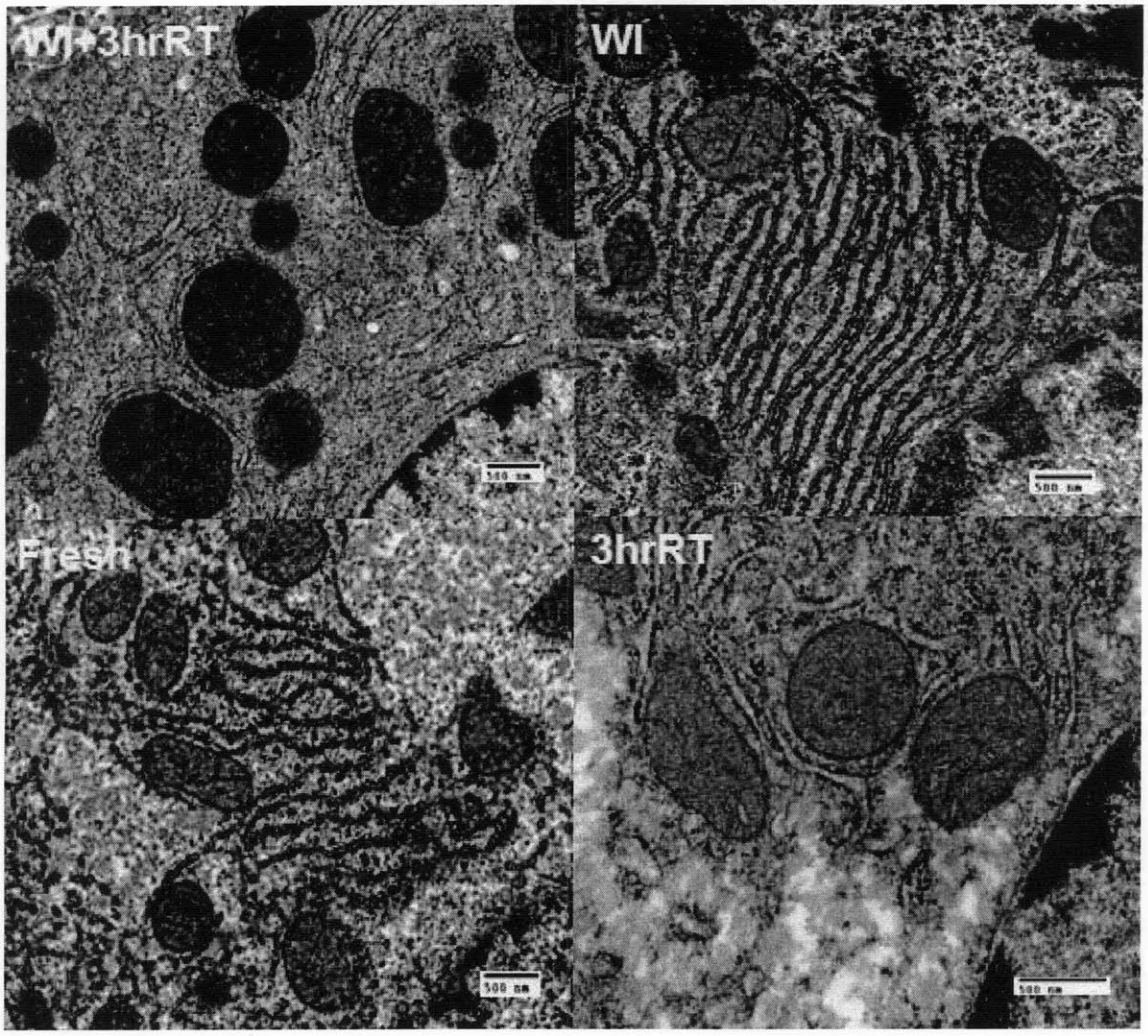


Figure 9: Transmission electron microscopy (500nm) shows WI and perfused livers have swollen mitochondria and potentially disrupted endoplasmic reticulum.

DISCUSSION

Our goal was to develop a highly simplified ex vivo organ perfusion system to recover and temporarily store all donor livers for the purposes of hepatocyte isolations or transplantations. By choosing to operate at room temperature[3] our system comprised only a pump, a reservoir of perfusate, an oxygenator and a bubble trap. We subsequently achieved cell yields from a model of uncontrolled warm ischemia in rat livers that were comparable to fresh hepatocyte isolations. Both WI+3hrRT and Fresh livers yielded approximately 34 million cells/g liver with viabilities in excess of 90% post-Percoll purification, compared to non-perfused WI livers, which yielded only 2 million cells/g liver[5,6], or 6% of the total possible outcome. Further, the perfusion of healthy fresh livers (3hrRT) for the same duration resulted in 32% more hepatocytes than Fresh livers not treated by perfusion. Cell procurement

The quality of the hepatocytes was determined through a select series of standard techniques[9] to demonstrate competency in the likely uses of cell-based therapies, pharmaceutical tests and research. The ability to attain a viability above 90%[10] was preferred for optimal function in culture settings, though higher than the 60% cutoff required for cell transplantation[11]. The number of cells lost during the Percoll step reflected, to some extent, the degree of pre-existing damage and possibly the extent to which it had been reversed in perfusion; healthy liver cell yields were reduced to 52-53%, ischemia-resuscitated yields were reduced to 49%, while the ischemic group retained only 4% of its initial yield.

Cell suspension was used to reveal the impact of cell source on function[6,9], but also as a rapid means of assessing batch performance[10]. Viability in suspension was assessed by hourly measurements of the production of formazan from MTT, and trypan blue exclusion. Perfused cells retained their viability better than Fresh cells over the course of 6 hours despite having higher initial values for ALT and AST. These signs of cell damage may have originated in perfusion or the process may have left the cells more susceptible to damage in the subsequent cell isolation phase. Nevertheless, perfused hepatocytes ceased further ALT and AST production once in suspension while Fresh hepatocytes gradually increased their levels. Perfused cells generally appeared to function as well as, if not better, than Fresh cells. Albumin was the exception; its production was highest in 3hrRT cells, but lowest in WI+3hrRT cells. Urea was produced equally in all groups; a clinical marker for ammonia removal[12], while biotransformation

capacity was evaluated through CYP450 enzyme activity with significantly enhanced performance by 3hrRT cells[13].

Plate culture, useful for long-term evaluation of cell function, demonstrated similar trends as in cell suspension. Here, WI+3hrRT hepatocytes initially produced very low amounts of albumin; however production was recovered by Day 2 and was comparable to the other groups for the remaining 12 days. Plate culture also served to verify that cells were kept free of microbiological contamination during perfusion. Overall, these results demonstrate a simple basis upon which to procure, and further enhance, a significant source of high-quality, high-functioning hepatocytes from currently available resources.

The striking correlation between post-Percoll cell yield and ATP content normalized to total protein suggests that direct or indirect measurements of ATP during perfusion will enable the observation of the trajectory to optimal cell yield. This will also allow dynamic testing of organs from uncontrolled cardiac death providing an objective metric of hepatocyte viability. The correlation of ATP with cell yield was stronger after cell purification suggesting that cells with low ATP content are unlikely to have fully intact energy-dependent processes. These include simple membrane functions controlling cell density, thereby being eliminated with Percoll. However, as can be seen by the significant recovery of WI cells, perfusion enables restoration of ATP and ATP-dependent functions and should be further optimized to this end.

The cell yield in WI+3hrRT livers was almost exactly the same values in Fresh livers and may be purely coincidental, or it may relate to a similar phenomenon that 3hrRT livers are seemingly able to overcome. Our observations in this study support the likely combined effects, though to varying degrees, of microcirculatory collapse, mild ischemia reperfusion injury (IRI) and ischemic preconditioning on the final cell yield.

For Fresh livers, access to the portal vein represents two major insults that may cause an immediate vasoconstrictive response to shock: 1) A sudden drop in blood pressure and 2) Asanguineous reperfusion. The consequences of shock under these circumstances may persist for the duration of washout, without the time to recover before the advent of collagenase[14]. It is possible that localized areas of hypoperfused cells result in suboptimal cellular ATP levels. WI induction for 60 minutes in the rat model significantly depleted ATP levels, caused generalized edema, ultrastructural changes, and capillary narrowing, as expected[15-18]. The extent of damage from subsequent IRI is known to result in essentially non-transplantable grafts from untreated WI livers[1].

Evidence of extensive vasoconstriction in our livers upon initiation of ex vivo perfusion was observed by the elevated portal pressures at low flow rates. This coincides with the acute "no-reflow" phase of IRI which occurs within minutes of flow initiation due to endothelial dysfunction creating an endothelin/NO imbalance, exacerbated by resident leukocytes[19-22]. For the first 20 minutes, perfusate was not recirculated allowing accumulated toxins and debris to be washed out. This washout phase contributed significantly to the decline in portal pressure though hepatic resistance was never reduced to 3hrRT values suggesting continued microcirculatory compromise throughout perfusion. The presence of hydrocortisone in the perfusate may also have conferred positive anti-inflammatory effects on resident leukocytes. Leukocyte activity in "late" phase IRI was further curtailed by cutting perfusion short at t=3hrs. Cumulatively, it may be concluded that rat livers exposed to 60 minutes of WI are generally recovered by perfusion due to the benefits of a large reservoir of nutrients devoid of inciting toxins. However, only partial amelioration of microcirculatory collapse occurred, potentially resulting in a liver not unlike a Fresh liver at the time of KRB+EDTA flushing. The use of endothelin receptor agonists, thrombolytics and muscle relaxants may enable further optimization of cell yields[23-25]. The "pH paradox" is another IRI phenomenon that can be optimized in perfusion by lowering pH to abrogate cell death, a trend already in progress by anaerobic glycolysis and hydrolysis of ATP[26,27]. 3hrRT livers potentially benefited from ischemic preconditioning (IP), having been exposed to the suspected optimal 10 minute ischemic duration[21,28,29]. Their increased ATP levels, enhanced cell yield, lower hepatic resistance, and somewhat improved metabolic performance is strongly associated with the protective mechanisms of IP[30-33].

Removing erythrocytes from perfusate was favored for a number of reasons[34] including the significant reduction in complexity of system setup and analysis. It was justified by the reduced metabolic rates of livers perfused at room temperature. In our previous reports, livers perfused at 37°C consumed on average 0.05mlO_2/min/gliver, necessitating a minimum hemoglobin concentration of 7g/dL as a partial oxygen tension of 600mmHg could only supply 0.03mlO_2/min/gliver at physiological flow rates of approximately 1.8ml/min/g liver. However, measures of oxygen uptake rate were correlated to temperature by Fujita et. al.[35] who showed that at room temperature, oxygen uptake rate of fresh livers in our system would approximate 0.017mlO_2/min/gliver, below the amount of oxygen deliverable by solution at physiological flow rates. In practice, oxygen delivery rate averaged 0.021±0.002

mlO_2/min/gliver while uptake averaged 0.016±0.003 mlO_2/min/gliver. These uptake rates are slightly lower than we predicted. They are also lower than results obtained in a study by Höper and Kessler[36] who also demonstrated feasibility of asanguineous liver perfusion at room temperature. Further, the livers perfused at room temperature in a sanguineous system also actually slightly exceeded our observations[3]. Combined with the finding that our oxygen exit rates were very low, it is likely that our livers were oxygen deprived during perfusion. This is particularly important in light of the correlation between cell yield and low flow rates (maximum cell yield occurred at 1.6ml/min/g liver), and hence even lower oxygen delivery rates. Interestingly, Höper and Kessler[36] reported ischemic times of 7 minutes or less before initiation of perfusion, and they recommended perfusate flow rates of 2.2ml/min/g liver which are twice our suggested flow rates, but they reported ATP levels that were below fresh liver controls. It is unclear whether ischemic preconditioning was not achieved in these livers or whether the higher flow rates resulted in fewer viable cells with high ATP content. It is reasonable to suggest though that a strategy for optimal perfusion of fresh livers should likely include an ischemic preconditioning period of 10-15 minutes, and an artifical oxygen carrier in perfusate to sustain perfusion simplicity while meeting the oxygen requirements of the liver with very low flow rates.

In conclusion, these results demonstrate that a simple ex vivo perfusion system operating at room temperature provides a hands-free technology which, when applied to all donor organs for recovery and assessment, can significantly optimize cell yield and function.

MATERIALS AND METHODS

Experimental Groups

Experiments were conducted on female Lewis rats (160g-180g) which were kept in accordance with National Research Council guidelines. The Subcommittee on Research Animal Care, Committee on Research, Massachusetts General Hospital approved the experimental protocols. Animals were randomly divided into four groups: 1) Livers that were exposed to an hour of WI at 34°C (**WI**), 2) Livers that were perfused for 3 hours at room temperature after 1hr WI (**WI+3hrRT**), 3) Fresh livers (**Fresh**), and 4) Fresh livers perfused for 3 hours at room temperature (**3hrRT**).

Hepatectomy

Livers were excised according to the technique of Delrivière et. al.[37]. Briefly, a transverse abdominal incision was made and the intestines retracted to expose the portal vein (PV), the common bile duct (CBD), and the inferior vena cava (IVC). The CBD was cannulated (12cm, 22 G polyethylene stent, Surflo, Terumo, Somerset, NJ) and the IVC freed from the right renal and adrenal veins. The portal vein (PV) was freed from the splenic and gastroduodenal veins. The right phrenic vein emptying into the supra-hepatic vena cava (SHVC) was ligated. The hepatic artery was then ligated and the IVC clamped. Finally, the PV was clamped and the clock started for ischemic duration. The diaphragm was opened, the SHVC was transected, and the liver was removed, and weighed. Fresh livers were then immediately prepared for biopsy. 3hrRT livers were placed in a bowl of room temperature saline to be cuffed at the PV and IVC prior to perfusion with an average ischemic time of 10 minutes. WI livers were placed in a temperature-controlled chamber filled with saline and maintained at 34±0.1°C for 1 hr during which time they were cuffed.

Perfusion Circuit

The perfusate comprised 750ml phenol red-free Williams Medium E (Sigma Chemical, St. Louis, MO) supplemented with 2 u/L insulin (28.85units/mg Humulin, Eli Lily, Indianapolis, IN), 100,000 u/L penicillin, 100mg/L streptomycin sulfate (Gibco, Invitrogen,Grand Island,NY), 0.292 g/L L-glutamine (Gibco), 10mg/L hydrocortisone (Solu-Cortef, Pharmacia & Upjohn, Kalamazoo, MI), and 1000 u/L heparin (APP, Schaumberg, IL). The circuit comprised a peristaltic pump which brought perfusate from a reservoir to a membrane oxygenator, through a bubble trap and to an 18G catheter for portal flow into the liver. The liver was positioned on a fine flexible mesh surface, permeable to perfusate, in a perfusion chamber. Effluent flowed freely from the PV and SHVC into the chamber where it was then returned to the perfusate reservoir (Figure 1). The oxygenator was gassed with a mixture of 95%O_2/5%CO_2. Upon completion of cuffing of Fresh livers and after the period of warm ischemia for WI livers, organs were immersed in a drained perfusion chamber into which perfusate dripped at 4ml/min; perfusion chamber outflow was redirected to a catch basin where effluent from the liver was collected for the first 20 minutes before the circuit was closed. Using a simple manometer, portal pressure was recorded every 5 minutes during this flushing, as were flow rates which were gradually increased over time in accordance with the drop in

pressures observed. It was desirable to achieve *in vivo* flow rates of ~1.8ml/min/g liver[38] but preference was given to sustaining an absolute pressure below 4-6cmH_2O. At t=30mins and every half hour after, additional sampling commenced with 1.2ml perfusate aliquots collected and stored at -80°C for later analysis. Inflow and outflow blood gas analysis was also performed at this time for pO2 and pH measurements, corrected to 20°C (Rapidlab, Chiron Diagnostics, Norwood, MA). Bile was collected in a tube outside the perfusion chamber and both it and the livers were weighed at the end of perfusion.

Hepatocyte Isolation

A two-step collagenase perfusion technique described by Seglen (1976), and modifiedby Dunn et al. (1991) was used to isolate hepatocytes. Briefly, using aseptic technique, after gaining portal vein access with an 18G catheter, warm oxygenated KRB+EDTA was flowed through the livers at approximately 17ml/min. For Fresh livers, the IVC was immediately dissected and the liver subsequently removed from the animal into a petri dish till perfusion was completed. Perfused and WI livers were already in petri dishes with cuffed PVs. A collagenase (type IV, Sigma, C5138-1G) solution with KRB and $CaCl_2$ was introduced to perfusion as the KRB solution was depleted and allowed to flow until successful digestion was observed. The livers were then moved to a sterile hood on ice where approximately 10mL of sterile, cold KRB were added. The liver capsule was gently broken to release the cells which were then passed through a 250um filter followed by a 60um filter. The suspension was divided into 50 mL conical tubes and centrifuged at low speed (300- 350 RPM, 4°C, no brake, 5 minutes). The supernatant was aspirated and the pellet resuspended with 10ml KRB. An initial cell count and viability was performed. A volume of 24mL of cold Percoll solution (9 parts Percoll : 1 part 1.5M NaCl, pH 5-5.5) was used for every 25mL of cell suspension. Cells were added at a concentration of 5 million cells/mL and inverted before being centrifuged (50 g, 4°C, no brake, 5-10 minutes). The buffy coat and supernatant were discarded and resuspended to 10mL in DMEM + FBS + 100,000 u/L penicillin + 100mg/L streptomycin sulfate, after which a final count was performed.

Cell Suspension

Cells were diluted to 1 million/ml in Williams Medium E and aliquoted into 1.6mL microcentrifuge tubes, 4 separate vials were used for each assay except for Trypan Blue

exclusion where time permitted only 2. The tubes were subsequently rotated at slow speeds in a 37°C incubator, except light-blocked vials for CYP450 activity which were allowed to settle between readings so as to reduce pipetting errors.

ALT and AST.

4 vials each were prepared for ALT and AST. At every hourly time point starting at t=0hrs, 15ul from each vial was placed into a 96 well plate on ice. At t=6 hours, Triton-X 100 was diluted to 1% concentration with a volume of the remaining cells. The cells were lysed by rapid pipetting, and then diluted 1:4 with PBS; 15ul from each vial was finally added to the 96 well plate as the positive control. 150ul of reagent at room temperature was then rapidly pipetted into the wells and a kinetic endpoint assay provided enzyme activity per minute (TR71121 and 7200-006, Thermo Electron, Pittsburgh, PA). The results were subsequently normalized to the completely lysed cells.

CYP450 activity.

4 vials for each of the CYP450 enzymes to be tested were prepared; these included benzyloxy resorufin (CYP4502B2), pentoxy resorufin (CYP4502B1), ethoxy resorufin (CYP4501A1) and methoxy resorufin (CYP4501A2). 20μL of 6mM stock solution 3,3'-methylene-bis(4-hydroxycoumarin) was added to each vial (Sigma M1390) and allowed to incubate for 20 minutes. 10μL of 1mM solutions of each of the isoenzymes was then added to the vials. The vials were inverted several times and a 50μL sample was taken at t=0 minutes and stored on ice, away from light. Samples were taken again at 10, 20, 30 and 40mins. A standard was prepared by serial dilution of 1000nM resorufin. Samples and standards were read with a fluorescence plate reader (Ex530, Em590) and recorded as rates of resorufin production per million cells.

Glucose, Albumin and Urea

4 vials were incubated for the entire 6hr duration, spun down and the supernatant was stored at -80°C. Standard assay reagents were used for glucose (Stanbio 1075-825) and urea (Stanbio proc. No. 0580), and an elisa was used to detect albumin.

Viability

2 vials were counted hourly using Trypan Blue exclusion to test for viability.

Mitochondrial activity

The MTT assay was performed hourly by pipetting approximately 50,000 cells (50μL) from 4 vials into 4 wells on a 96-well plate and diluting with 50μL of Williams Medium E for 4x100μL of cell suspension. 1 vial of MTT was thawed (Biotium, Inc., 30006) and 10μL added to each well. Samples were mixed gently while incubating at 37°C, protected from light. The plates were then spun at 800rpm, the supernatant removed, and 200μL of DMSO added to dissolve the Formazan. Absorbance was read at OD_{570}-OD_{630}.

Plate Culture

Cells were plated in standard 6-well plates using a double layer collagen gel sandwich plate culture[39] with C+H as the culture medium; phenol red-free medium was used in the CYP450 assays. Enough plates were prepared for 3 wells to be devoted to each assay on days 5, 7, 10 and 14.

Viability

Hoechst 33452 and Ethidium homodimer-1 double stain was used to detect all nuclei, and dead nuclei, obtaining a measure of viability. Briefly, 10μL of a 1mM solution of Ethd-1 and 5μL of Hoechst 33452 were added to 5ml of PBS, while protecting from light exposure. Medium from the cells was washed with PBS and 1ml of the dye added to each well. The cells were incubated at 37C for 10-15 minutes before processing using a Zeiss axiovert 200 microscope. Thirty five snapshots were taken at distinct locations within each well for each dye, overlaid on corresponding phase contrast images. Cell nuclei were subsequently counted using CellProfiler (Broad Institute, Cambridge, MA).

Mitochondrial activity

100μL of MTT were added to each well and incubated for 1hr at 37°C. The medium was subsequently aspirated from the wells and 1mL of 6mg/mL collagenase was added and incubated at 37°C for 15 minutes and pipetted rigorously to dissolve all collagen. 1mL of DMSO was then added and the contents rigorously pipetted up and down to dissolve the Formazan present. 250μL samples were subsequently placed in

triplicate on a 96 well plate (3 wells x 3 samples) and absorbance was read at OD_{570}-OD_{630}.

CYP450 activity

48 hours before the assay, 1mL of a 2μM solution of 3-methylcholanthrene inducer was added to each well. This medium was left on the cells for the subsequent 48 hours. 5μM and 80μM solutions of substrate solution and dicumarol respectively were prepared to a volume of 5ml (1ml/well) by diluting in EBSS. After 48 hours, the medium was aspirated and 1ml of warm EBSS was added to each plate. After 15 minutes, the EBSS was aspirated and 1ml of the substrate+dicumarol solution was added to each well. 50μL of medium was subsequently removed at t=5,15 25, and 35 minutes and placed in a 96-well plate protected from light. Fluorescence intensity was subsequently measured (Ex530, Em590).

Glucose, Albumin, Urea.

Media from plates prepared for assay analysis on Day 14 were collected daily and stored for metabolic analysis using the same techniques as above.

Tissue Biopsies

Tissue sections were rapidly frozen in liquid nitrogen upon resection of Fresh livers, after perfusion was complete for WI+3hrRT and 3hrRT livers, and after 1hr of warm ischemia for WI livers. Remaining lobes were then perfused with 15mL of Karnovsky's solution for histological preparation.

ATP

ATP content was measured in tissue segments homogenized with a mortar and pestle under liquid nitrogen and resuspended in 500μL of nucleotide releasing buffer (Biovision, #K354-100). Each sample was spun down at 16rpms for 2 minutes. 100μL of sample was subsequently placed in a cuvet and the assay continued as prescribed in the kit. Data were plotted against a standard and normalized to the total protein present in the sample supernatant using a standard Bradford assay.

Transmission Electron Microscopy

Constructs were fixed overnight in modified Karnovsky fixative (2.5% Glutaraldehyde, 2.5% formaldehyde, 0.1M cacodylate buffer, pH 7.2), washed with 0.1M buffer, post fixed in 1% osmium tetroxide in 0.1M cacodylate buffer, and dehydrated in a graded series of ethanol. The samples were infiltrated and embedded in a mixture of Spurrs resin and Quetol according to Ellis[40]. 60-80nm cross sections and en face sections were cut on an Ultracut E microtome (Reichert, Depew, NY) using a diamond knife. Thin sections were stained with 5% uranyl acetate and Reynolds lead citrate. The sections were viewed with a JEOL JEM 1010 transmission electron microscope (JEOL, Tokyo, Japan) and images were digitally captured on an AMT XR-41B CCD camera system (Advanced Microscopy Techniques Inc., Danvers, MA)[41].

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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 (~0–4°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 and 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 the lack of standardized small animal models limits its development. Here we describe a rat NELP system that is simple and cost-effective to run. We show that rat livers that underwent NELP for 6 h could be routinely transplanted into syngeneic recipient rats with excellent 1-month survival. During perfusion, the release of cytosolic enzymes, bile and urea production, and oxygen uptake rate could be readily monitored, thus providing a comprehensive picture of hepatic function before 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. Demand continuously exceeds the supply of donor livers, and a large number of patients die while on the waiting list for a donor liver (www.unos.org). Various approaches are being investigated to alleviate the donor shortage, which can be categorized as surgically based or 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,[1,2] 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.[3,4] Near-normothermic machine perfusion has been successfully used in experimental kidney preservation,[5–7] and normothermic perfusion has recently been shown to be superior to simple cold storage (SCS) in the preservation of livers donated after cardiac death.[4,8,9] 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 pretransplantation diagnostic testing of the graft and the induction of repair processes. Possible other uses include immunomodulation and (pre)conditioning of the donor organ.[9]

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

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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.[10] Because its complexity, some investigators envision using NELP after a period of cold storage, but this approach has yielded mixed results.[11,12] 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.[13,14] The lack of standardized small animal models has impeded evaluation of the potential benefits of NELP.

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 perfusion.[15–18] We modified the latter by using a cell culture-based perfusate supplemented with plasma, autologous erythrocytes as oxygen carriers, and the incorporation of continuous dialysis. We demonstrate, using normal healthy rat livers, that this improved system can maintain livers in a metabolically stable condition for at least 6 h, significantly extending the normothermic perfusion time from the typical 2 to 4 hours for perfused rat livers.[15] Furthermore, we show that the perfused livers can be routinely transplanted into syngeneic recipients with excellent long-term survival. This system will be useful in optimizing NELP and comparing NELP with other liver preservation techniques, such as SCS 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 Laboratories, Wilmington, MA) weighing 250 to 300 g. The animals were maintained in accordance with National Research Council guidelines, and the Subcommittee on Research Animal Care, Committee on Research, Massachusetts General Hospital approved the experimental protocols.

The liver transplantation procedure used was the cuff technique first described by Kamada and Kalne.[19–21] The same microsurgeon (HT), with prior experience of more than 100 orthotopic liver transplants in the rat, performed all surgeries. Surgeries were performed using a Zeiss Opmi 1 microscope (Prescott, Monument, CO) with 6× magnification.

Briefly, donor operations began with a transverse abdominal incision. The bowel and the duodenum were retracted to the right using 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 a 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 gastroduodenal 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 hemicircumferential 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°C) University of Wisconsin (UW) solution (Viaspan, Barr Laboratories, 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.[19] Some livers were left in SCS for 7 h before 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 (Fig. 1A). The primary circuit consists of a jacketed liver perfusion chamber (Radnoti Glassware Technologies, Monrovia, CA) (Fig. 1B), a Masterflex peristaltic pump (Cole Parmer, Vernon Hill, IL), a membrane oxygenator (Radnoti), a heat exchanger (Radnoti), a bubble trap (Radnoti), a second peristaltic pump (Cole Parmer), and a hollow fiber dialyzer with a 2200-cm^2 membrane area and a 30-kDa nominal molecular weight cut-off (Spectrum Laboratories, Rancho Dominguez, CA). Temperature within the liver was maintained at 37.5°C with a homeothermically controlled water bath (Lauda, Brinkmann, Westbury, NY) and was continuously monitored using 2 thermocouples (Omega, Stamford, CT): 1 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% oxygen (O_2)/5%carbon dioxide (CO_2) mixture. The secondary dialysis circuit consisted 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% to 18%. To obtain rat plasma and red blood cells, rats were anesthetized with isoflurane, and 1 U heparin/g of body

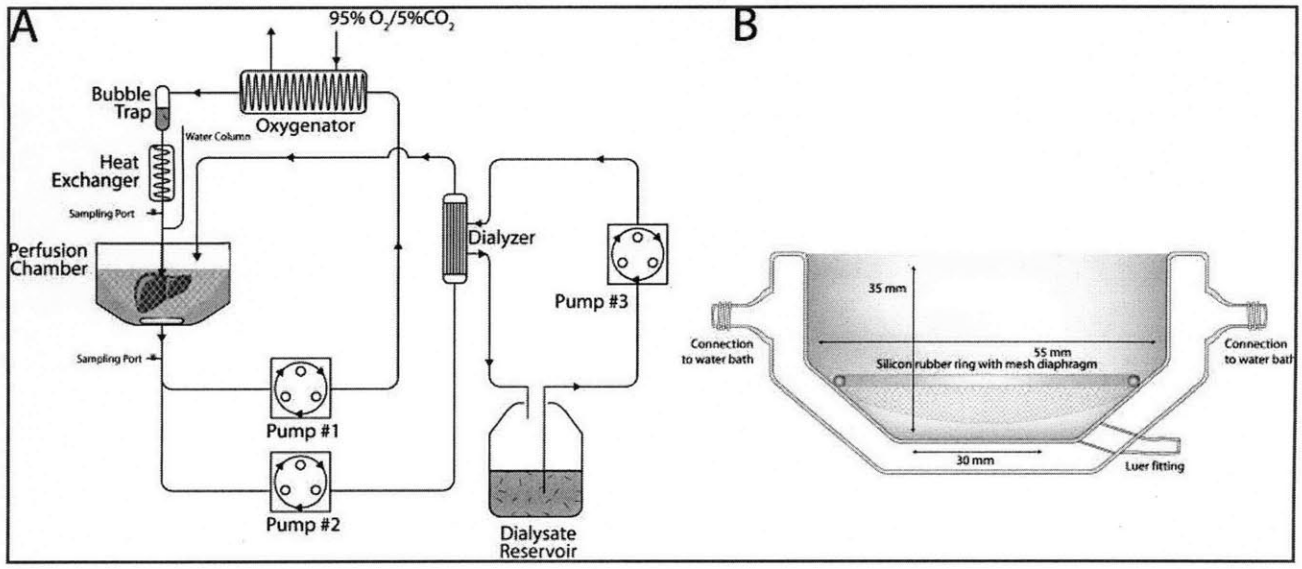

FIG. 1. Schematic of the normothermic liver perfusion system. (**A**) Perfusion circuit. (**B**) Detail of liver perfusion chamber. Color images available online at www.liebertpub.com/ten.

weight was injected through the penile vein. Puncture of the abdominal aorta using a 16 G intravenous catheter (Surflow, Terumo) was performed, and the animal exsanguinated. Blood was kept on ice and then centrifuged in 50-mL tubes at 3200 revolutions per min (rpm) for 15 min at 4°C. 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 1 liver perfusion was 55 to 60 mL. Dialysate volume was 500 mL.

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, Franklin Lakes, NJ) emptying into a preweighed 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 per g of 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 h 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 before 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

$$[O_2] = 0.0139 \times [Hb] \times FO_2Hb + 0.00314 \times pO_2$$

where [Hb] is the hemoglobin concentration in g/dL, FO_2Hb is the fraction of 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:

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

Bile was collected continuously in preweighed microfuge tubes that were exchanged every hour. Wet weight of the liver was determined during the brief periods of cold storage before and after 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 (~1 mm^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 0.1 M cacodylate buffer and postfixed with a mixture of 1% osmium tetroxide and 1.5% potassium ferrocyanide for 2 h, washed in water and stained in 1% aqueous uranyl acetate for 1 h followed by dehydration in grades of alcohol (50%, 70%, 95%, 2×100%), and then infiltrated and embedded in TAAB Epon (Marivac Canada Inc., St. Laurent, Canada). Ultrathin sections (~60–80 nm) were cut on a Reichert Ultracut-S microtome, picked up onto copper grids, stained with 0.2% lead citrate, and examined using a TEM (Tecnai) G^2 Spirit BioTWIN transmission electron microscope (FEI, Hillsboro, OR). Images were taken with a 2 k AMT CCD camera (AMT, Danvers, MA).

Transplantation surgery

At the end of the perfusion, the liver was flushed with 20 mL of cold (0°C) UW solution and placed on ice in a bowl containing UW solution. Typical cold storage time was approximately 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 moist gauze to expose the portal area of liver, the CBD, and the 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, 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 and the Satinsky clamp were removed. Reperfusion of the liver was established. The anhepatic phase of the procedure did not exceed 17 min. 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°C) lactated Ringer's solution with 5% dextrose and 2 mL/kg of sodium bicarbonate 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, after which they were returned to the housing facility in single-cage housing.

Postoperative blood sampling

To determine the postoperative levels of AST, ALT, and total bilirubin, 100 to 200 μL of blood was collected on postoperative days 1, 3, 5, and 7 using 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 errors 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 per 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.

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 (Fig. 2). No ALT or AST could be found in the dialysate (data not shown). Liver wet weight decreased slightly, from 9.74 ± 0.81 g to 9.51 ± 0.82 g, during perfusion ($p < .05$ according to two-tailed paired *t*-test, $n = 11$). Liver harvested 6 h after normothermic perfusion showed a healthy appearance with no signs of degradation or vacuolization except for a slightly greater dilation of the sinusoids than with livers in SCS in UW solution for 6 h (Fig. 3A). Transmission electron microscope images demonstrated that livers stored under both conditions appeared normal. Hepatocyte mitochondria appeared elongated and not swollen, with a dominant rough endoplasmic reticulum. Sinusoids had open lumens and fenestrated endothelial walls. Cell nuclei appeared round and healthy, and cells exhibited clear borders and obvious bile canaliculi (Fig. 4).

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 h of perfusion (Fig. 5A). The oxygen uptake rate declined rapidly during the first

TABLE 1. OPERATING PARAMETERS DURING NORMOTHERMIC PERFUSION

	Normothermic Perfusion (n = 6)	*Published* In Vivo *Values*	*Reference for* In Vivo *Value*
Flow rate	1.80 ± 0.12 ml/min/g	Portal: 1.2 to 1.5 mL/min per g Arterial: 0.2 to 0.3 mL/min per g	22, 29
Hydrostatic pressure	12 to 15 cm H_2O	Portal: 8 to 10 cm H_2O	15, 30
Hematocrit	17.63 ± 0.58%	43%	www.criver.com/techdocs/84jan_tb/t84tab01.html
Inlet pO_2	147.1 ± 62.03 mmHg	Portal: 38 to 55 mmHg Arterial: 85 to 90 mmHg	31, 32
Outlet pO_2	41.17 ± 11.44 mmHg	37 to 42 mmHg	32
Inlet pCO_2	31.58 ± 4.71 mmHg	Portal: 35 to 46 mmHg Arterial: 26 to 34 mmHg	30, 32
Outlet pCO_2	36.64 ± 5.20 mmHg	33 to 50 mmHg	32

H_2O, water; pO_2, partial pressure of oxygen; pCO_2, partial pressure of carbon dioxide.

60 min after initiation of the perfusion and reached a steady state value of 0.052 ± 0.018 mL/min per g (2.32 ± 0.08 μmol/min per g) of liver for the remainder of the perfusion (Fig. 5B). The glucose level in the perfusate decreased slightly from the initial value of 200 mg/dL to 182 ± 12 mg/dL after 6 h (Fig. 5C). Urea levels exhibited a biphasic response (Fig. 5D). In the early phase, urea levels were initially flat and then increased almost linearly to reach a plateau at 0.6 mg/dL per g of liver at 3 h. At that point, the dialysate was changed, causing the levels to decrease suddenly, followed by an increase at a rate similar to that observed in the 1- to 3-h period, reaching a maximum level of 0.68 ± 0.04 mg/dL per g at 6 h.

Survival after transplantation

Livers were transplanted into recipient rats after 6 h of normothermic perfusion ($n = 11$) or 6 h of SCS in UW solution at 0°C ($n = 5$). In the perfused liver group, 1 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 1 month after transplantation. No signs of liver failure, such as jaundice, were observed. In the SCS group, all animals also recovered from surgery and survived beyond 1 month.

Postoperative liver enzymes and bilirubin

Transplant recipients were monitored for circulating liver enzyme and bilirubin levels to assess liver graft function (Fig. 6). The levels of AST and ALT increased from baseline on postoperative day 1 in both groups, with values of 699 ± 85 μ/L and 431 ± 80 μ/L, respectively, in the NELP group, and 615 ± 177 μ/L and 372 ± 138 μ/L, respectively, for the SCS group. In the NELP group, ASL and ALT levels subsequently declined, to 131 ± 15 μ/L and 72.7 ± 6.6 μ/L, respectively, on day 5, and increased again, to 812 ± 257 μ/L and 373 ± 133 μ/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 μ/L and 915 ± 210 μ/L, respectively, and then decreased on day 7. There was no statistically significant difference between AST and ALT levels in the NELP and SCS groups, except on day 5

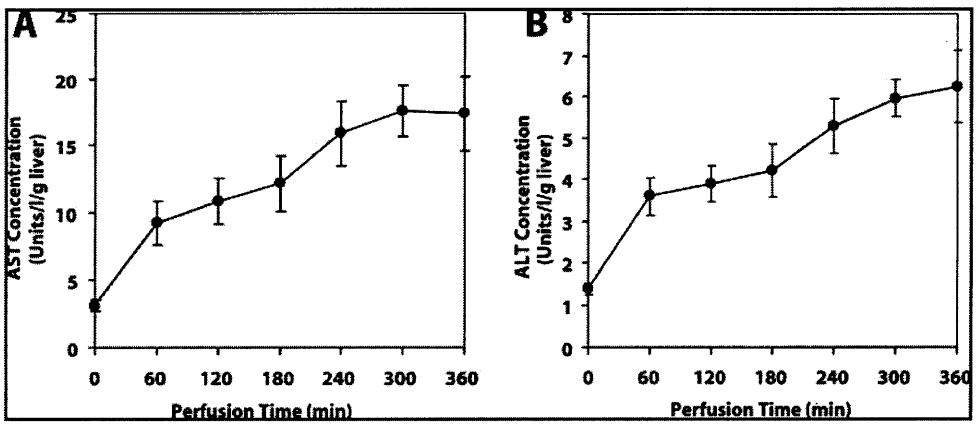

FIG. 2. Liver integrity during normothermic perfusion. (**A**) Aspartate aminotransferase (AST) and (**B**) alanine aminotransferase (ALT) levels in perfusate samples collected hourly from the primary circuit. Values are normalized to the wet weight of the liver. Values at 360 min were 17.39 ± 6.34 U/L per g for AST and 6.25 ± 2.18 U/L per g for and ALT, $n = 6$.

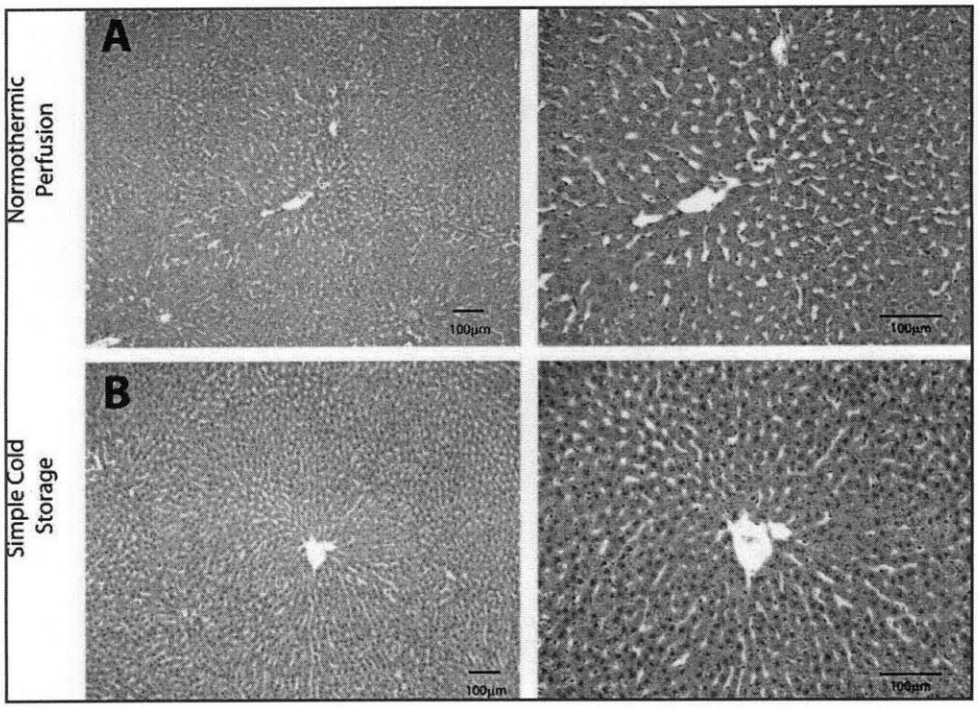

FIG. 3. Microscopic appearance of livers after (**A**) 6 h of normothermic perfusion and (**B**) 6 h in simple cold storage in University of Wisconsin solution. Bar = 100 µm. Color images available online at www.liebertpub.com/ten.

($p < .05$ according to 2-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 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.

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 were the use of a cell culture–based perfusate supplemented with plasma and hormones, the use of autologous erythrocytes as oxygen carriers, and the incorporation of continuous dialysis. The lifespan of rat liver in traditional isolated perfused liver systems was typically less than 4 h, and it was suggested that it be considered a "dying organ"; the system was primarily to be used as short-term analytical model.[15] Using this modified isolated liver perfusion system, rat livers could be normothermically preserved for 6 h and subsequently transplanted with 100% success. Livers

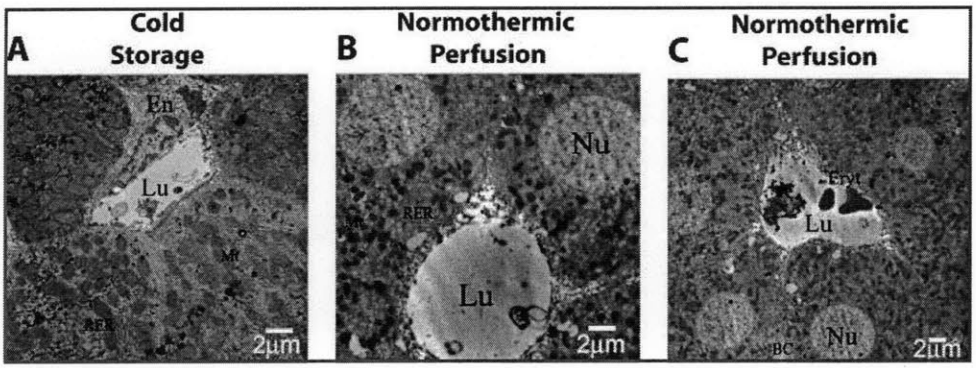

FIG. 4. Transmission electron micrograph of livers after (**A**) 6 h of simple cold storage in University of Wisconsin solution and (**B, C**) 6 h of normothermic perfusion. Under both conditions, the liver appears normal. Mitochondria (Mt) appear elongated and not swollen, with dominant rough endoplasmic reticulum (RER). Sinusoids appear have open lumens (Lu) and fenestrated endothelial wall. Cell nuclei (Nu) appear round and healthy, with 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 µm.

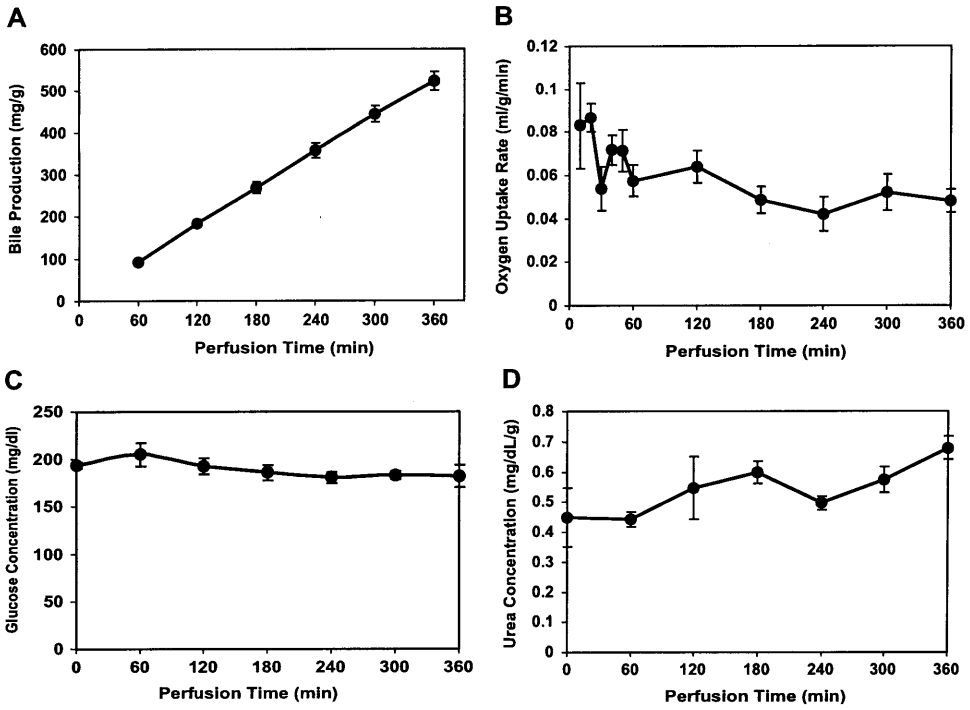

FIG. 5. Hepatic metabolic activity during perfusion. (**A**) Total bile produced normalized to wet liver weight. (**B**) Hepatic oxygen uptake rate normalized to wet liver weight. (**C**) Glucose levels during the perfusion. (**D**) Urea levels normalized to wet liver weight. $n = 6$.

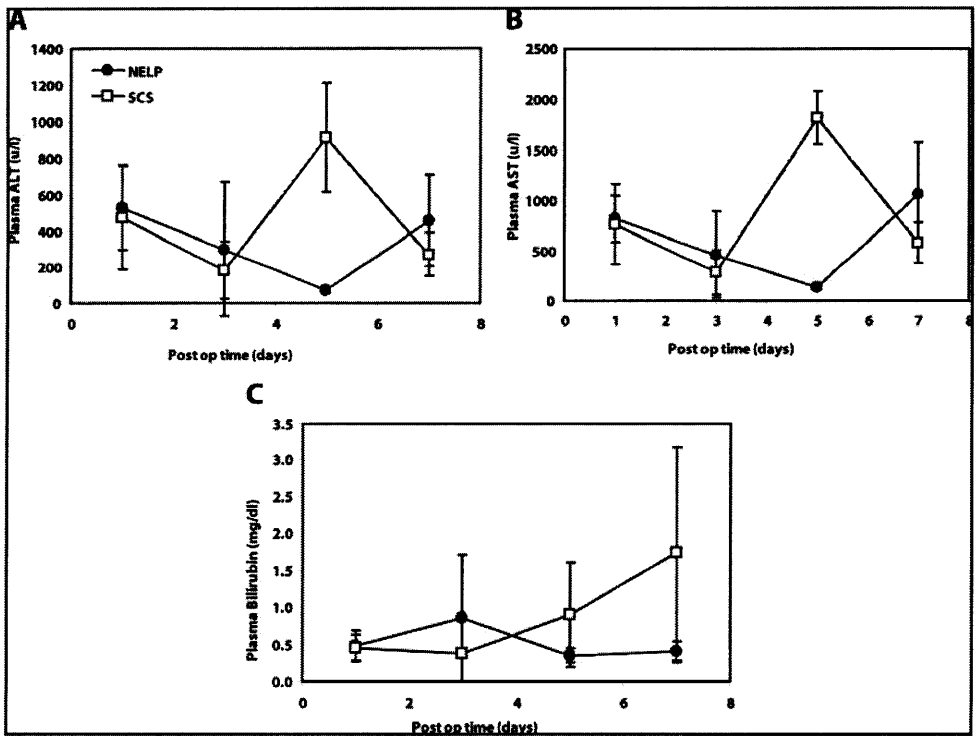

FIG. 6. Markers of hepatic integrity and function in the transplant recipient. (**A, B**) Postoperative plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. (**C**) Total plasma bilirubin levels. $n = 3$.

preserved in this manner were similar to livers preserved with conventional SCS 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 according to bile production, hepatic oxygen uptake, and urea synthesis, were stable throughout the perfusion after an initial 60-min period of equilibration, making it easy to assess the functional activity of donor livers before transplantation.

We chose the rat as the model species because of low cost (compared with large animals) and extensive body of published work with isolated rat perfusion systems. The blood supply of the rat liver is approximately 10% arterial and 90% venous.[22] Although some studies indicate that certain areas of the liver are accessed by arterial blood only,[23] the bulk of the literature suggests that it is possible to perfuse via the portal vein only,[15] as well as to perform the transplantation without reconstruction of the hepatic artery.[21] One person can perform the transplantation procedure, with a total operating time of approximately 50 min. Finally, inbred rat strains are widely available, which makes it possible to decouple immunological compatibility factors from handling, storage, and perfusion-related factors that affect the outcome of the transplanted livers. We chose to characterize our system using healthy liver grafts to establish optimal perfusion parameters, determine baseline metabolic values, and establish transplant survival data.

Isolated liver perfusion has been extensively studied for more than 90 years, although most studies have been conducted in the rat using asanguineous buffers as perfusate.[16] More recently, solutions containing a more-complete panel of physiological substrates were used to assess various aspects of hepatic function.[17,18] Cell culture–based perfusates have been used in reperfusion studies of the rat liver,[24] hypothermic liver preservation,[25] and near-normothermic kidney preservation.[7] Here, we have chosen a basal perfusate medium consisting of Williams Medium E, which is routinely used for culturing hepatocytes. This medium contains essential and nonessential amino acids, vitamins, glucose, and the antioxidant glutathione. Insulin was added to mimic a "fed state," in which the liver uses 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 were present in the perfusion solution, because 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 uses a large circulating volume ranging from 150 to 300 mL,[16] 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[15] for one perfusion experiment. Others have successfully used artificial oxygen carriers,[5,26] which 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 been found to have 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 carriers for hypothermic machine preservation.[27] Our poor preliminary experience using perfluorocarbon-based oxygen carriers in our setup, possibly based on instability of the used emulsion, led us not to pursue their further use.

In our initial experiments without dialysis, we observed gradual acidosis, hypernatremia, and depletion of metabolic substrates in the perfusate (unpublished data). Our efforts to transplant livers perfused in this manner were unsuccessful, consistent with the poor survival reported previously[28] with rat livers normothermically perfused without dialysis. Long-term normothermic perfusion of the porcine liver has been demonstrated,[4] but transplantation of these livers has not been reported. The only study that has demonstrated successful, stable post-transplant survival employed a dialysis circuit.[8] 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 volume that was 10 times as great, which we found effective to stabilize the pH, electrolyte, and substrate levels in the primary perfusion circuit. The molecular cut-off weight of 30 kDa 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 h. Normothermically preserved livers were histologically similar to livers preserved using SCS in UW solution for the same duration and could be orthotopically transplanted successfully. Although we do not claim to have improved on SCS 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.

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Recovery of Warm Ischemic Rat Liver Grafts by Normothermic Extracorporeal Perfusion

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Liver transplantation is currently the only established treatment of 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 nonfunction and delayed graft failure. Normothermic extracorporeal liver perfusion (NELP) has been suggested as an improvement over SCS. Livers recovered from male Lewis rats were subjected to 1 hr of warm ischemia and preserved with 5 hr of SCS or NELP, and transplanted into syngeneic recipients. As additional controls, non-ischemic livers preserved with 6 hr of SCS or NELP and unpreserved ischemic livers were transplanted. After NELP, ischemically damaged livers could be orthotopically transplanted into syngeneic recipients with 92% survival (n=13) after 4 weeks, which was comparable with control animals that received healthy livers preserved by SCS (n=9) or NELP (n=11) for 6 hr. On the other hand, animals from ischemia/SCS control group all died within 12 hr postoperatively (n=6). Similarly, animals that received ischemic livers without preservation all died within 24 hr after transplantation (n=6). 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.

Keywords: Transplantation, Reperfusion injury, Machine perfusion, Preservation, Preconditioning.

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Transplantation is currently the only established treatment of 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 (*1*). It is estimated that approximately 6000 ischemic livers (*1, 2*) 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 (*1*); they exhibit a higher risk of primary nonfunction and delayed graft failure, especially because of biliary complications such as stricture (*3*). It is believed 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 (*4–8*). Recently, functional recovery of ischemically damaged rat livers was shown using a combination of SCS followed by short term hypothermic machine perfusion (*9, 10*). However, extended hypothermic machine perfusion can cause endothelial damage (*11*), 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 (*12–14*). Near-normothermic machine perfusion has been successfully used in experimental kidney preservation (*15–17*), and recently normothermic perfusion was shown to be superior to SCS in the preservation of DCD livers (*13, 14, 18*). A survival benefit after transplantation of DCD livers preserved normothermically has been demonstrated in one study using a porcine model (*13*).

The complexity and high cost of large animal models limit 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 hr of normothermic perfusion (*19*). Herein, we investigated the potential of NELP to recover warm ischemic livers. We show that rat livers that underwent 60 min of ex-vivo warm ischemia (34°C) and then preserved by 5 hr of NELP could be successfully transplanted into syngeneic recipients. By contrast, recipients of similar livers stored by SCS for 5 hr, and those transplanted directly without having undergone preservation, did not survive.

EXPERIMENTAL PROCEDURES

Isolation of Donor Livers

Experiments were performed using male Lewis rats weighing 250–300 g (Charles River Labs, Wilmington, MA). The animals were maintained in accordance with National Research Council guidelines and the experimental protocols

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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 is described in detail elsewhere (*19*, *20*).

Warm Ischemia Induction

After isolation from the donor, the liver was weighed and placed in a temperature-controlled chamber filled with saline and maintained at (34±0.1)°C for 1 hr. During this period the portal vein (PV) and inferior vena cava (IVC) were cuffed as previously described (*19*).

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 (*19*) and added to the perfusate only. The total perfusate volume was 55 to 60 mL.

The perfusion system consisted of a primary liver perfusion circuit and a critical secondary dialysis circuit (*19*). Briefly, the primary circuit included perfusate that recirculated by means of a peristaltic pump through a jacketed perfusion chamber, a membrane oxygenator, a heat exchanger, and a bubble trap. The oxygenator was gassed with a mixture of 74% N_2/21% O_2/5% CO_2 and 100% O_2 to maintain a constant pH. A fraction of the perfusate was diverted to the secondary circuit through a hollow fiber dialyzer with a 2200 cm^2 membrane area and a 30 kDa nominal molecular weight cutoff (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 450 mL 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 10 mL of warm saline and immersed in perfusate in the perfusion chamber. The liver was perfused at a constant flow rate through the portal vein and effluent flowed freely from the suprahepatic and inferior vena cava into the surrounding medium. When the recipient hepatectomy was prepared, the liver was disconnected from the circuit, rinsed in a bowl of saline at room temperature, and weighed again before transplantation. The operating parameters of the perfusion system were as follows: flow rate, 1.84±0.05 mL/min/g; portal hydrostatic pressure, 12 to 16 cm H_2O (8–12 mm Hg); hematocrit, 17.8%±0.8; inlet pO_2, 128.4±8.1 mm Hg; outlet pO_2, 47.9±1.7 mm Hg; inlet pCO_2, 30.1±1.1 mm Hg; outlet pCO_2: 34.6±1.6 mm Hg.

Analysis of Perfusate Levels of Metabolites and Liver Enzymes

Perfusate samples (1 mL) were collected before placing the liver in the perfusion system and hourly thereafter. For each sample, 100 μL 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 (1 mL) 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 min 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 O_2 (mL/dL) in the samples was determined according to the formula:

$$[O_2] = 1.39 \times [Hb] \times FO_2Hb + 0.00314 \times pO_2$$

where [Hb] is the hemoglobin concentration in g/dL, FO_2Hb is the fraction oxygenated hemoglobin, and pO_2 is the partial pressure of oxygen in mm Hg. HOUR was determined as follows:

$$HOUR = (([O_2]_{in} - [O_2]_{out})/100) \times \text{flowrate/weight of liver.}$$

Bile was collected continuously in preweighed microfuge tubes that were exchanged every hour.

Recipient Surgery

The cuff technique developed by Kamada and Calne (*20–22*) was implemented and is described in detail elsewhere (*19*). All recipient surgery was carried out by the same microsurgeon (H.T.). The anhepatic phase of the procedure was typically 13 to 15 min and did not exceed 17 min. Animals were hydrated with 8 mL/kg of warm (37°C) lactated Ringer's solution with 5% dextrose and 2 mL/kg of $NaHCO_3$ 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 hr postoperatively animals were checked every 2 hr and subsequently every 8 hr for 1 week.

Postoperative Blood Sampling

To determine the postoperative levels of AST, ALT, and total bilirubin, 100–200 μL of blood were drawn from the tail vein under isoflurane anesthesia on postoperative days 1, 3, 5, 7, 14, 21, 28, and immediately analyzed using a Piccolo blood chemistry analyzer. For these studies, n was more than or equal to 4 for each group.

Simple Cold Storage

Warm ischemic livers (n=6) and freshly isolated livers (n=6) were flushed with 20 mL 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 early phase (0–2 hr) after transplantation, we used 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 artifactual findings. The reperfusion circuit was identical to the normothermic perfusion system, but contained no secondary dialysis circuit. The livers were reperfused for 120 min and inflow (portal vein) and outflow (infrahepatic vena cava) sampling was performed every 15 min. The operating conditions for the reperfusion system were as follows: flow rate, 1.74±0.15 mL/min/g; hematocrit, 13.8±8.2; inlet pO_2, 263.5±111.9 mm Hg; outlet pO_2, 75.1±49.7 mm Hg; inlet pCO_2, 40.4±14.9 mm Hg; outlet pCO_2, 43.4±15.9 mm Hg.

Histology

Liver tissue slices were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Apoptosis was evaluated through terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) staining (Roche, Indianapolis, IN).

Statistical Analysis

Data presented are mean±SE. All statistical analysis for differences performed with ANOVA at significance level of α equal to 0.1.

RESULTS

Integrity and Function of Liver During Perfusion

ALT and AST activities as indicators of hepatocellular damage are shown in Figure 1(A and B); both AST and ALT accumulated during the first 180 min of perfusion and then decreased. These values were several folds higher than those previously reported for freshly isolated livers not subjected to any warm ischemia (*19*). 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 (Fig. 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 min of the perfusion and then remained stable (Fig. 1D). This behavior was similar to that observed for freshly isolated livers. The HOURs of the perfused warm ischemic and freshly isolated livers were 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 $t=300$ min, indicating a constant rate of urea production. This rate was con-

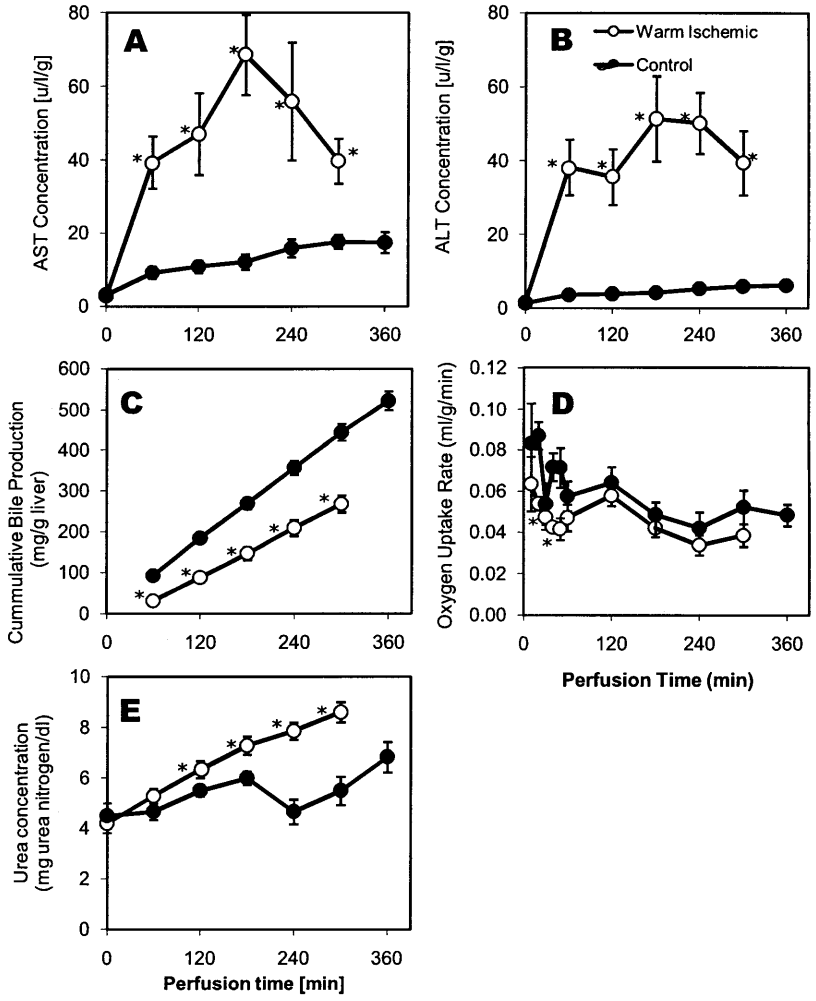

FIGURE 1. Function and integrity of warm ischemic livers during normothermic perfusion. (A) Asparate aminotransferase (AST) and (B) alanine aminotransferase (ALT) levels in perfusate samples collected hourly from the primary circuit. (C) Total bile accumulation normalized to wet liver weight. (D) Oxygen uptake rate normalized to wet liver weight. (E) Urea concentration in the perfusate. Data shown are averages of six ischemic livers±standard error (SE). 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 nonischemic livers] are from Tolboom H, Pouw R, Uygun K, et al. A model for normothermic preservation of the rat liver. *Tissue Eng* 2007; 13(8): 2143). *Indicates statistical difference compared with healthy perfused livers at $P<0.1$.

sistently higher than that observed in perfused healthy livers (Fig. 1E).

Figure 2 shows the histologic appearance of warm ischemic livers after 5 hr of NELP. Ischemic livers treated with NELP show minimal to no damage compared with freshly isolated livers preserved by SCS or NELP. In contrast, livers subjected to 1 hr 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).

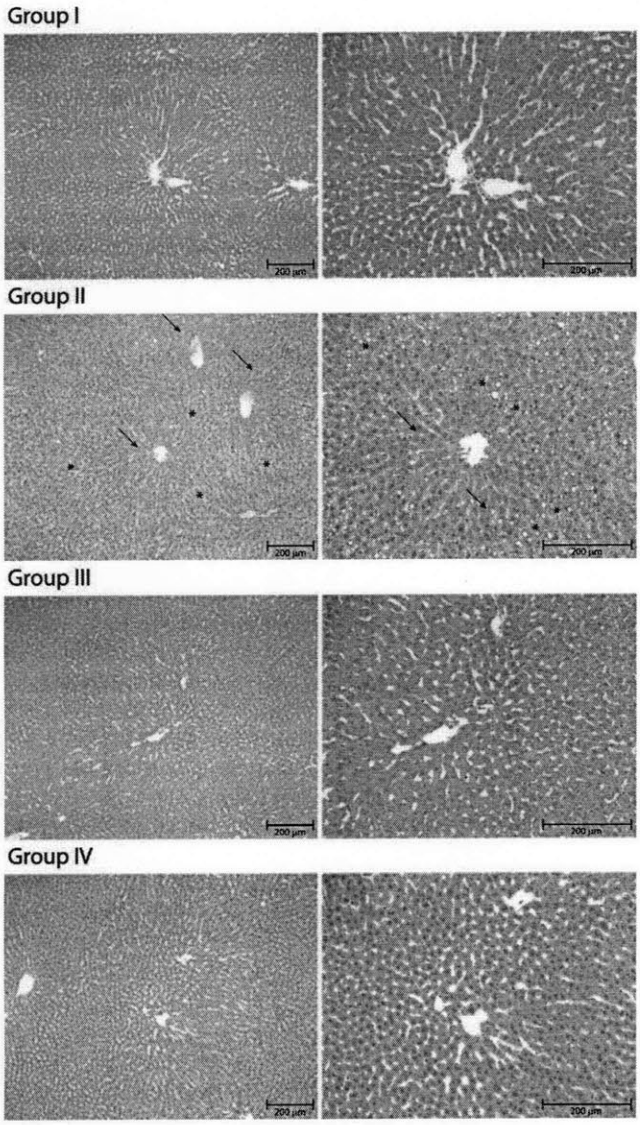

FIGURE 2. Microscopic appearance of livers after preservation. (A) Group I: Warm ischemic livers after 5 hr of normothermic extracorporeal liver perfusion. (B) Group II: Warm ischemic livers after 5 hr of static cold storage in University of Wisconsin solution (*arrows* indicate cell swelling and asterisks vacuolization and tissue destruction). (C) Group III: Freshly isolated livers after 6 hr of normothermic extracorporeal liver perfusion. (D) Group IV: Freshly isolated livers after 6 hr of static cold storage in University of Wisconsin solution. Bar=200 μm.

Survival After Transplantation

Warm ischemic livers were transplanted into recipient rats after 5 hr of NELP (n=13) or 5 hr 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 hr 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 1 month and did not exhibit external signs of liver failure, such as jaundice.

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

All recipients of directly transplanted ischemic livers died in a similar way within 24 hr postoperatively.

All controls that received freshly isolated livers preserved for 6 hr by SCS recovered rapidly from surgery and survived beyond 1 month (Fig. 3).

Postoperative Liver Enzymes and Bilirubin

The levels of both AST and ALT (Fig. 4A and B) were elevated on day 1 postoperatively 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 with the healthy cold-stored livers on postoperative day 5. Both values were comparable with those observed in a hypothermic machine perfusion study (5).

The total bilirubin level, an indicator of liver function, was similar in both groups postoperatively and showed an increasing trend (Fig. 4C). The increasing bilirubin value is

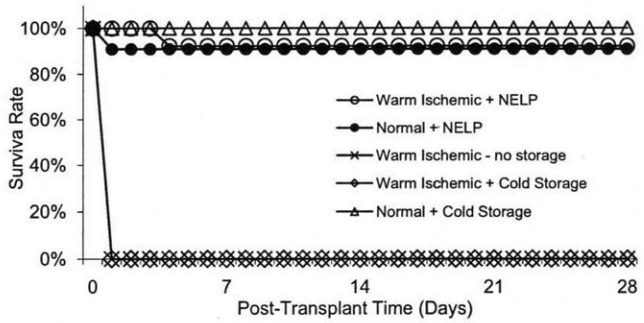

FIGURE 3. Survival curves of recipient rats after transplantation of perfused warm ischemic livers, warm ischemic cold-stored livers, compared with healthy perfused livers, and healthy cold-stored livers (Data for the normal+normothermic extracorporeal liver perfusion group are from Tolboom H, Pouw R, Uygun K, et al. A model for normothermic preservation of the rat liver. *Tissue Eng* 2007; 13(8): 2143).

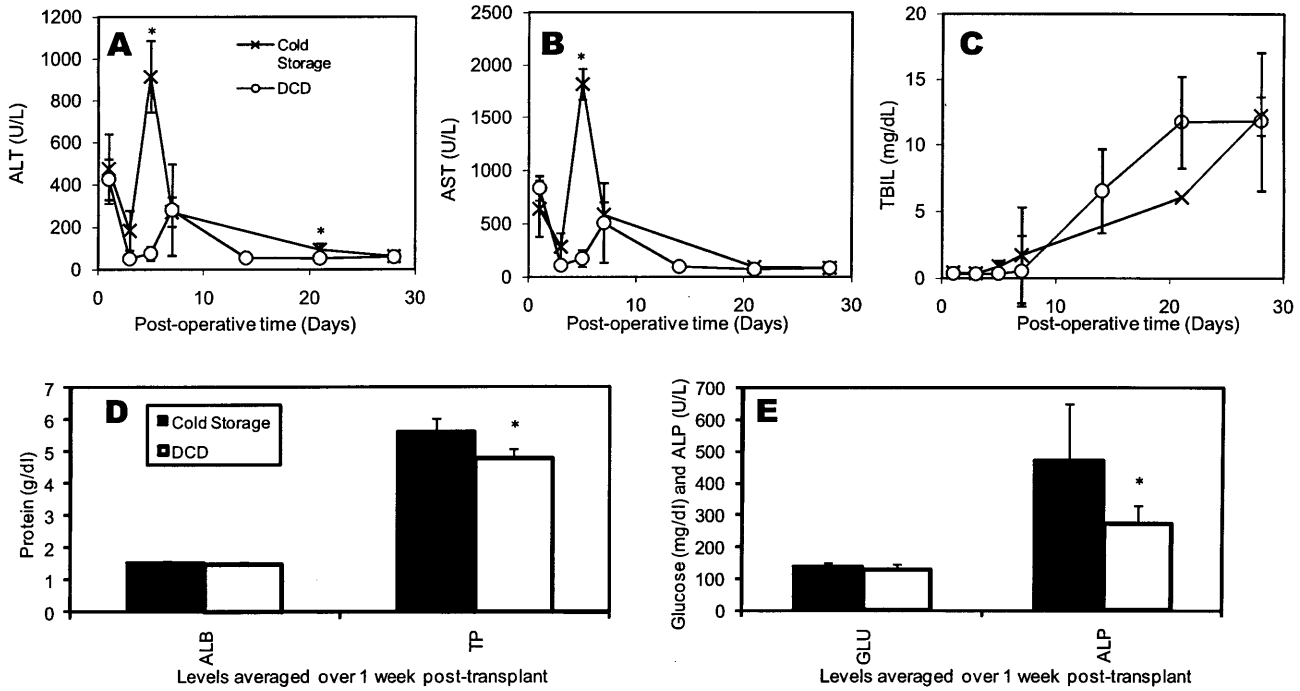

FIGURE 4. Values of (A) asparate aminotransferase, (B) alanine aminotransferase, and (C) total bilirubin measured on days 1, 3, 5, 7, 14, 21, and 28 after transplantation of perfused warm ischemic livers compared with healthy cold-stored livers. (D, E) Comparison of the serum levels of albumin, total protein, glucose, and alkaline phosphatase within 1 week after transplant. *Indicates statistical difference compared with cold-stored livers at $P<0.1$.

within expected ranges and is likely an artifact of nonrearterialization and ensuing histopathologically observable bile duct proliferation (*23*). It is worth noting that the elevated bilirubin levels are reported (*23*) 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 Figure 4(D 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±0.23 g/dL; Izamis et al., submitted). 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). Alkaline phosphatase, 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 due to avoiding the cold injury (*11*) or ischemic preconditioning effects of warm ischemia (*24*).

Short-Term Graft Function

To evaluate the short-term graft function posttransplantation, we used 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, and liver oxygen uptake rate as baseline indicator of metabolic activity. ALT levels for WI and NELP group was lower than WI-only and WI and SCS groups at all time points, and indifferent from normal livers after the first 45 min. Similar trends were observed for AST, although the difference between WI+NELP and freshly isolated livers was statistically significant at all time points except $t=45$min. 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 (Fig. 5C). Posttransplant bile secretion has been previously shown to be strongly correlated with graft survival (*25–27*) and with cellular ATP levels (*28, 29*). The average 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 ($P\ll0.01$). Furthermore, 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 (*30*).

As displayed in Figure 5(D), 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 1 hr WI alone. There was no difference between WI+NELP and WI+SCS groups. Inter-

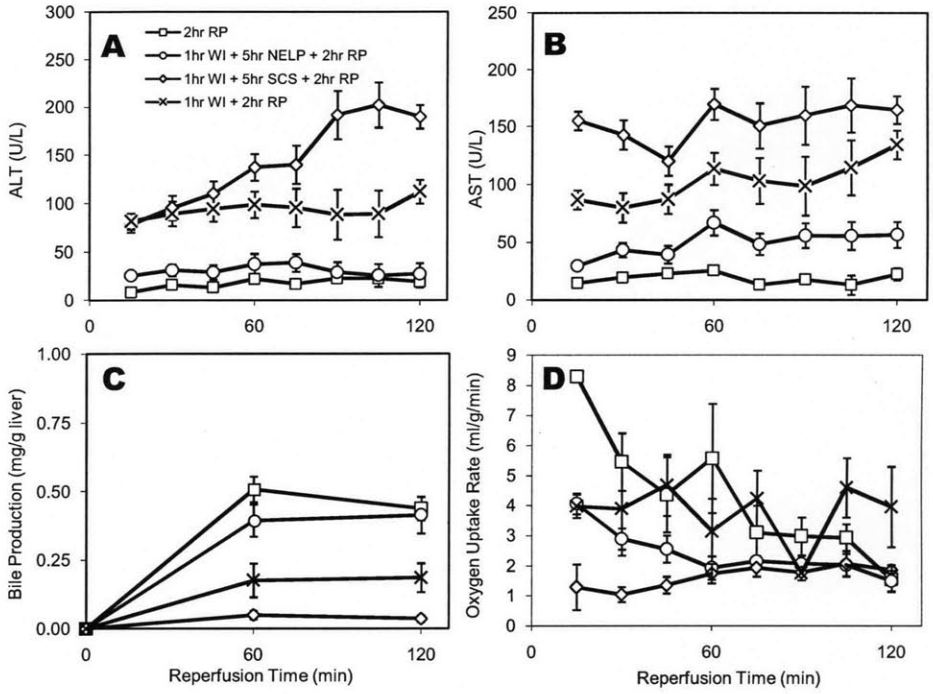

FIGURE 5. Reperfusion results: (A) alanine aminotransferase, (B) asparate aminotransferase, (C) bile synthesis, and (D) oxygen uptake rate measured during reperfusion. See text for statistical analysis.

estingly the WI-only livers displayed oxygen uptakes comparable with healthy livers. Although 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 preservation of ischemic livers. However, the absence of apoptosis in the WI-only livers that result in primary nonfunction when transplanted, suggest that apoptosis is not a determinant factor for graft survival.

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 with that of recipients of perfused fresh livers and fresh livers preserved with cold storage. Animals that received ischemic livers that were preserved with cold storage or experienced no storage time at all, died within 24 hr 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 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 of oxygen and nutrients while waste is

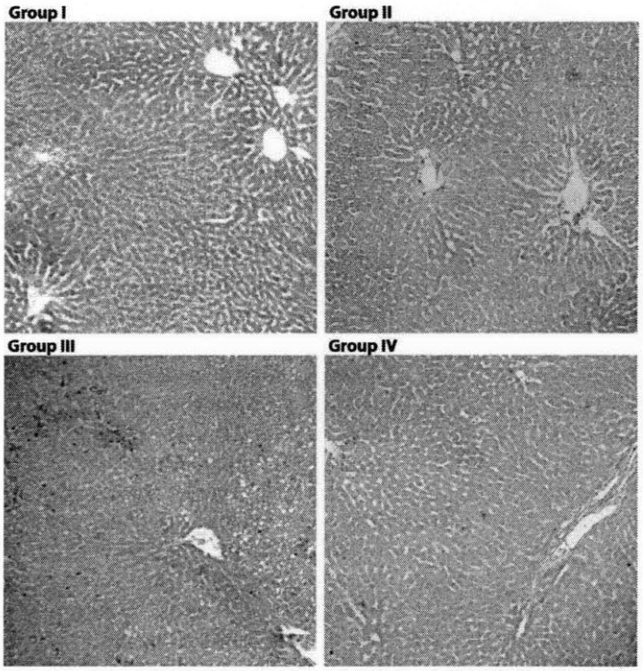

FIGURE 6. Terminal deoxynucleotide tranferase-mediated dUTP nick-end labeling of livers after preservation and reperfusion. (A) Group I: Freshly isolated liver after reperfusion. (B) Group II: Warm ischemic livers after reperfusion. (C) Group III: Warm ischemic livers after 5 hr of static cold storage in University of Wisconsin solution after reperfusion. (D) Group IV: Warm ischemic livers after 5 hr of Normothermic Extracorporeal Liver Perfusion followed by reperfusion. Magnification ×10.

removed. However, the basic approach to preservation still relies on 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 (*31–33*).

Normothermic machine perfusion is fundamentally different from hypothermic perfusion because its aim is to not only reestablish 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 (*34, 35*).

Although previous NELP efforts have predominantly used larger animal models, the rat was our model of choice to keep our approach simple and inexpensive. Because the blood supply of the rat liver is mostly venous (*36, 37*), we have chosen to perfuse through the portal vein only, as usually performed in the traditional isolated perfused rat liver systems (*36, 38*), 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 and coworkers (*20, 21*). 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 (*23*). The recipient rats in our studies displayed the same complications (results not shown), and the increased serum bilirubin, which is known to return to normal levels after 6 weeks in this model (*23*); 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.

To model DCD, we subjected livers to ex-vivo warm ischemia in a homeothermically controlled bath filled with warm saline as previously described (*5*). The benefit of this method is precise control of the ischemic time and temperature because the explantation of the organ occurred before the period of ischemia. We have chosen 60 min 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 before liver explantation (*39*) 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 than 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 on perfusion of the liver. Postoperative values of the AST and ALT were comparable, if not lower than those of recipients of fresh livers preserved with 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 with improved graft survival (*9*) or ROS reduction (through glutathione which is present in Williams E) which was also found to correlate with graft viability (*40*). However, the TUNEL results of reperfused livers demonstrate that there is no appreciable difference in apoptosis between WI-only and WI+NELP groups. Although both groups showed limited apoptosis, the survival of unperfused ischemic livers was zero. 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 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 1 hr of ex vivo warm ischemia can be reclaimed using warm perfusion technology. Posttransplant 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 method to investigate the various aspects of warm perfusion for preservation and resuscitation of the DCD liver grafts, and a model to study liver metabolism (*41*). 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.

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