PRECLINICAL AND CLINICAL EVALUATION OF TREPROSTINIL IN MINIMIZING HEPATIC ISCHEMIA AND REPERFUSION INJURY

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Liver is a vital organ in the human body with important exocrine, endocrine and metabolic functions. Many clinical conditions such as viral infections as well as certain toxic substances can lead to liver damage, resulting in end-stage liver diseases. Orthotopic liver transplantation (OLT) is the only treatment option for various end-stage liver diseases. During the process of harvesting and preservation, the liver grafts are subjected to injury due to cold ischemia, and during transplantation, the liver graft is further injured by warm reperfusion of the blood. These events are referred to as Ischemia/Reperfusion (I/R) injury. I/R injury is a major leading cause of primary graft nonfunction (PNF) that can occur in 4 - 23% of OLTs, resulting in urgent re-transplantation. The I/R injury is an antigen independent component of liver harvesting process, which is associated with vasoconstriction, upregulation of cytokines, platelet aggregation, increase in reactive oxygen species and neutrophils infiltration. One promising approach to minimize I/R injury is to use pharmacological agents to prevent the impact of cold ischemia and warm reperfusion. Our hypothesis is that treprostinil, a prostacyclin I_2 (PGI_2) analog, due to its vasodilatory property, anti-platelet activity and inhibition of the release proinflammatory cytokines will attenuate the I/R injury of the liver. Treprostinil diminished the hepatic injury, minimized the associated effect of I/R injury on hepatic drug transporters gene expressions and maintained activity of Abcb1 (Mdr1; P-gp) and Cyp3a in an animal model where the livers were preserved in treprostinil supplemented UW solution and then perfused in an *ex-vivo* isolated

perfused liver system. Incorporating treprostinil into the preservation solution may provide improved graft function. Clinically, liver transplant recipients tolerated continuous infusion of treprostinil for up to 5 days with an improved hepatic extraction of ICG, minimized need for ventilation support and hospitalizations without occurrence of any PNF. Given that treprostinil can be administered to liver transplanted patients safely, future studies should evaluate its efficacy in minimizing I/R injury of the livers. Improved preservation of the liver and decreasing I/R injury will be not only improving overall function of the livers transplanted, but will also increase the number of livers that can be transplanted.

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ABBREVIATIONS

ALT	Alanine aminotransferase
ANOVA	ANalysis Of VAriance
AST	Aspartate aminotransferase
AUC	Area Under the concentration-time Curve
Bcrp	Breast cancer resistance protein
Bsep	Bile salt export pump
Cftr	Cystic fibrosis transmembrane conductance regulator
CI	Cardiac index
CO	Cardiac output
Ct	Cycle threshold
CYP	Cytochrome P450
Cyt c	Cytochrome c
DBP	Diastolic blood pressure
DCD	Donation after cardiac death
DDLT	Deceased donor liver transplant
DLT	Dose-limiting toxicity
ESLD	End-stage liver disease
ET	Endothelin
FHF	Fulminant hepatic failure
FXR	Farnesoid X receptor
Gapdh	Glyceraldehyde-3-Phosphate Dehydrogenase
H&E	Hematoxylin and Eosin
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus

HNF4α	Hepatic nuclear factor
HR	Heart rate
I/R injury	Ischemia and Reperfusion injury
ICG-PDR	Indocyanine green plasma disappearance rate
ICU	Intensive care unit
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
INR	International normalized ratio
IPRL	Isolated Perfused Rat Liver
IV	Intravenous
KHB	Krebs–Henseleit buffer
Mdr1	Multi-drug resistance 1
MELD	Model for End-Stage Liver Disease
MIQE	Minimum information for publication of quantitative real-time PCR experiments
mPAP	Mean pulmonary arterial pressure
mPAP	mean pulmonary arterial presser
mRNA	Messenger ribonucleic acid
Mrp2	Multidrug resistance-associated protein 2
NO	Nitric oxide
Ntcp	Na+-taurocholate co-transporting polypeptide
Oat2	Organic anion transporter 2
Oatp1a1	Organic anion-transporting polypeptide 1a1
Oatp1a4	Organic anion-transporting polypeptide 1a4
Oatp1b2	Organic anion-transporting polypeptide 1b2
Oct1	Organic cation transporter 1
OLT	Orthotopic liver transplantation
PAF	Platelet-Activating Factor
PAH	Pulmonary arterial hypertension
PBS	Phosphate-Buffered Saline

PGE_1	Prostaglandin E1
PGI_2	Prostacyclin
PMNs	Poly Morphonuclear Neutrophils
PNF	Primary graft Non-Function
PT	Prothrombin time
RIN	RNA Integrity Number
RT-qPCR	Real-time quantitative polymerase chain reaction
RXRα	Retinoid X receptor
SBP	Systolic blood pressure
SC	Subcutaneous
SD rat	Sprague Dawley rat
SEC	Sinusoidal Endothelial Cells
SEM	Standard Error of the Mean
$t_{1/2}$	Half-live
TNFα	Tumor Necrosis Factor-alpha
TXA_2	Thromboxane
UGT	Uridine 5'-diphospho-glucuronosyltransferase
UPMC	University of Pittsburgh Medical Center
UW	University of Wisconsin
XDH	Xanthine dehydrogenase
XOD	Xanthine oxidase

1.0 INTRODUCTION

Liver is a vital and the largest internal organ in the human body. It has a wide variety of functions, such as synthetic, storage and detoxification. It regulates glycogen synthesis and storage as an essential source of energy. Additionally, it synthesizes proteins and clotting factors that support the colloidal osmotic pressure of blood and helps in stopping bleeding, respectively. The detoxification process is a combination of the uptake, the metabolic and the excretory functions of the liver, which are discussed in the following section.

1.1 HEPATIC DRUG DISPOSITION

Phase I and II pathways involve biotransformation of compounds to more polar products to facilitate their elimination from the body. Phase III (also known as drug transporters), play an important role in transferring molecules from one side of the cell wall to the other and are broadly classified into uptake and efflux drug transporters. Usually, large and charged compounds are normally transported by drug transporters. Figure 1-1 illustrates the overall interplay between phase I, II and III pathways in the hepatocytes.

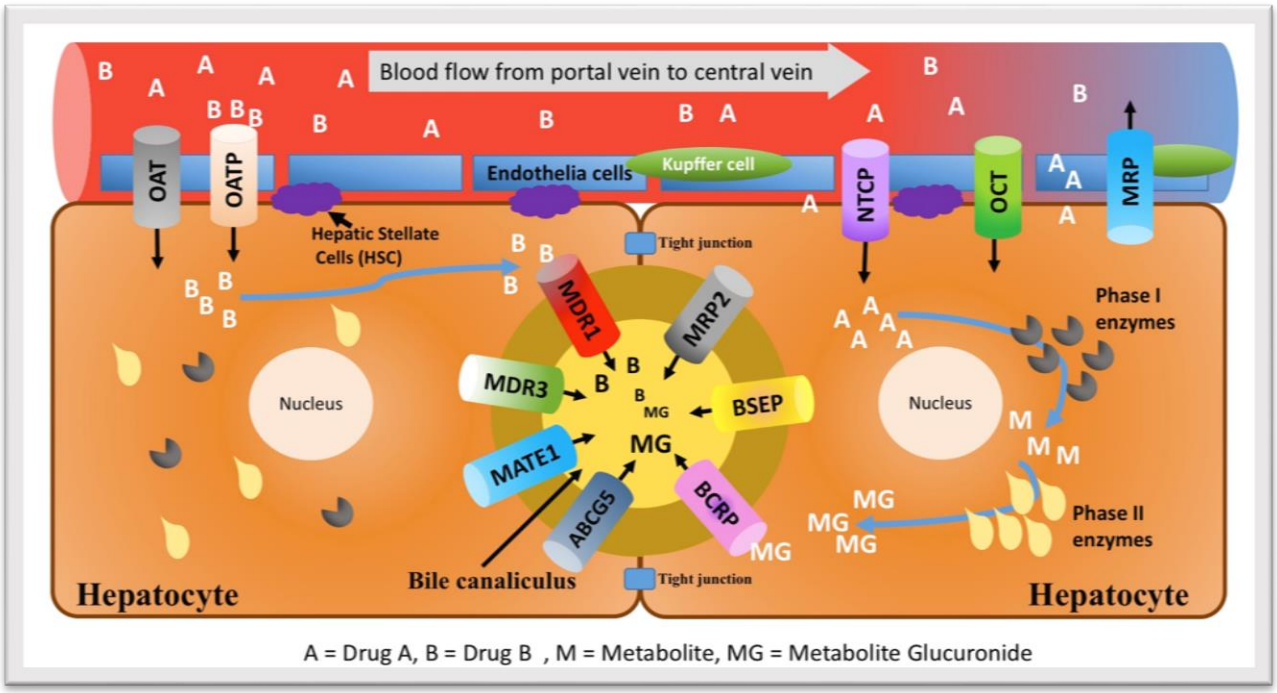


Figure 1-1. Hepatocyte showing the main cellular components and metabolizing enzymes.

Where, A and B are two different drugs that have different clearance pathways. Drug A passively diffuses into the hepatocyte and is metabolized by phase I pathway to M (main metabolic product) followed by conjugation process by phase II enzymes to MG that is excreted into the bile. Drug B is taken up by an active transport system (OATP; *SLCO*) into the hepatocytes and then effluxed into the bile canaliculus by MDR1 (*ABCB1*) without any chemical modification to the drug molecule. Some drugs undergo a combination of active uptake transport and metabolism. Some molecules or their metabolites can also be effluxed back into the circulation and may be cleared from the body by the kidneys.

1.1.1 Phase I: Drug metabolizing enzymes

The most common, phase I drug metabolizing enzymes are represented by cytochrome P450 (CYP) superfamily. CYPs are the major group of enzymes, that chemically modify drugs into their water soluble products to facilitate their excretion by kidney and/or liver.[1] In the late 1980s, Nebert developed and reported a nomenclature system for CYP enzymes. Human CYP genes comprise of more than 115 gene and pseudogene members and are one of the most extensively annotated mammalian genes that start from CYP1A1 and currently end with CYP51P3.[2, 3] In humans, CYPs are distributed throughout various tissues and organs; including peripheral blood cells, platelets, aorta, adrenal glands, adipose tissues, nasal tissue, vaginal tissues, seminal vesicles, brain, lung, kidneys, gut, and liver. Of all the various tissues, liver and small intestine contribute to the maximum extent to the overall metabolism and elimination of drugs. Among all the CYP enzymes in human liver, *CYP3A4* is the most abundant one, followed by *CYP2E1* and *CYP2C9*; representing around 22.1, 15.3 and 14.6 percent of the total CYPs (based on protein content), respectively (see Figure 1-2).[4] CYP enzymes may also be classified based on their major substrates, such as sterols, xenobiotics, fatty acids, eicosanoids, vitamins and others.[2] Some drugs secreted in the bile are reabsorbed back from the intestine; some metabolites secreted in the bile can be converted back to the drug by enzymes in the gut, and can be reabsorbed. This phenomenon is known as enterohepatic circulation, a process that prolongs the residence of a drug in the body. Liver plays a dominant role in the first-pass metabolism of several orally administered medications.[5]

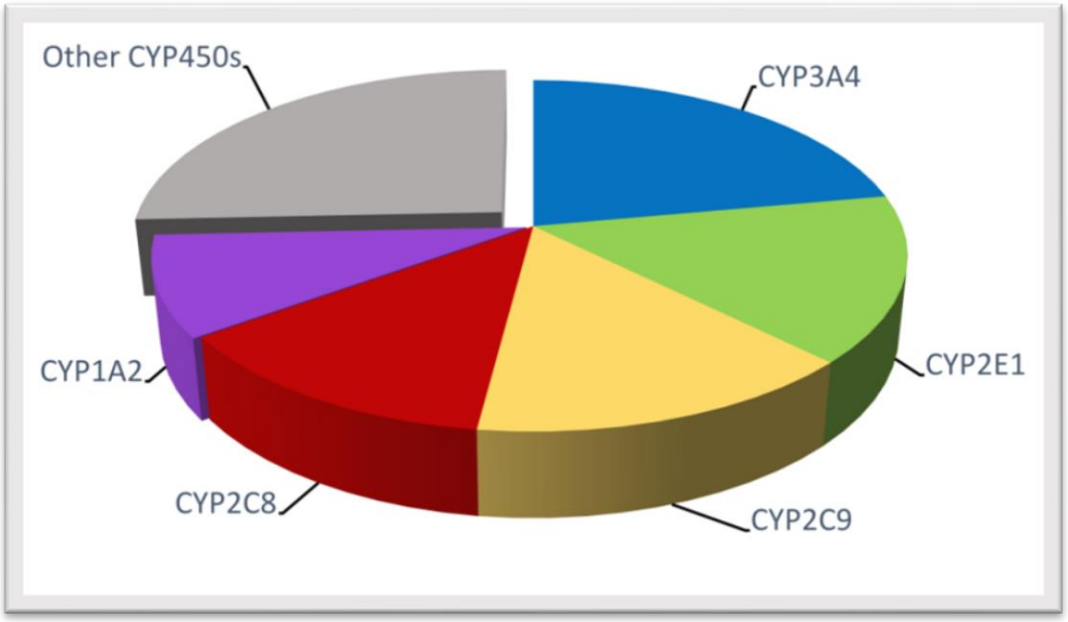


Figure 1-2: Pie chart showing the expression of various CYP enzymes in the liver in the human.

Figure plotted by data used from: Achour B, Barber J, Rostami-Hodjegan A. Expression of hepatic drug-metabolizing cytochrome P450 enzymes and their intercorrelations: a meta-analysis. Drug Metab Dispos 2014;42(8):1349-1356.[4]

CYPs expression is regulated in different compartments of the cell, nuclei or cytosol, by many factors. Nuclear receptor mediated regulation of gene expression occurs in the nucleus, which is the most critical regulatory pathway resulting in differential gene transcription. Aryl hydrocarbon receptor (AhR) is a transcription factor that is activated by several endogenous and exogenous ligands which activates the gene translation and synthesis of various CYPs.[6] Both pregnane X receptor (PXR; *NR1I2*) and constitutive androstane receptor (CAR; *NR1I3*) play similar roles in the regulation of expression of several important CYPs.[7-11] Induction of CYP enzymes will lead to increased clearance of certain drugs leading to decreased drug exposure and response. Rifampin and phenobarbital are examples of CYP inducers. On the other hand, inhibition of CYPs by endogenous or exogenous compounds leads to reduction in the ability of enzyme to clear drugs. CYP inhibitors can drastically increase the blood levels of various CYP substrates, leading to toxicity. Azole antifungals, HIV protease inhibitors, and certain HCV drugs are well-known inhibitors.[12, 13] A typical dose of tacrolimus in a transplant patient not on ritonavir is 3 mg, bid. In patients on lopinavir and ritonavir (Kaletra®), it is sufficient to give less than 1 mg once a week to achieve comparable trough blood concentrations of tacrolimus.[14] In the cytosol, cofactors such as NADPH-cytochrome P450 reductase, cytochrome b5 reductase and/or cytochrome c reductases are essential to carry out the biotransformation reactions. Iron is very important for CYPs synthesis, and is present in the center of the binding site between the enzyme and substrate.[15, 16] Thus, different status of these regulators will affect the functional activity of CYPs, resulting in inter- and intra-individual variability in the metabolic capacity within and among the population. Consequently, differences in the pharmacological responses to the same dose of a drug may result due to differences in metabolism and elimination of drugs.[17]

1.1.2 Phase II: Conjugation enzymes

During phase II drug metabolism, the drugs or metabolites from phase I pathway are enzymatically conjugated with a hydrophilic endogenous compound with the help of transferase enzymes. The most common phase II drug metabolizing enzymes are UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), N-acetyltransferases (NATs), glutathione S-transferases (GSTs), thiopurine S-methyltransferases (TPMTs), and catechol O-methyltransferases (COMTs).

Glucuronidation is the major phase II drug metabolism pathway, with about 40-70% of human endogenous and exogenous compounds being conjugated to glucuronidated end products.[18] Conjugated products are more hydrophilic and are readily excreted from the body. In the cytoplasm, glucose 1-phosphate reacts with uridine triphosphate to form uridine diphosphate glucuronic acid (UDPGA), a co-substrate, and this is transferred into the endoplasmic reticulum (ER) by transmembrane proteins. In the ER, UGT attaches UDPGA to the appropriate substrate by nucleophilic attack, forming glucuronidated compounds. Currently, four families of UGTs have been identified in human: *UGT1*, *UGT2*, *UGT3*, *UGT8*. *UGT2*s have been sub divided into *UGT2A* and *UGT2B*.[19] UGTs metabolize a wide range of compounds and their substrates also overlap with each other. *UGT1A1* is the highly expressed phase II enzyme in human, which preferentially metabolizes bilirubin; *UGT1A1* also metabolizes certain phenols and estradiols.[20] Whereas, *UGT2B7* metabolizes opiates[21], *UGT1A3*, *UGT1A9*, and *UGT2A1* metabolize carboxylic acids. Various organs express UGTs; however, UGTs are normally highly expressed in the liver and gut.

The functional activity of the UGTs is controlled by the amount of enzymes available and the amount of co-substrate available to conjugate the drug or the metabolite. Some drugs such as

phenobarbital and rifampin are known to increase the expression of *UGTs* and decrease drug exposure. On the other hand, competition for UGTs may lead to inhibition for metabolism and increased exposure of corresponding substrates.

1.1.3 Phase III: Drug transporters

Drug transporters are transmembrane proteins that facilitate the transport of large and/or ionized molecules in and out of the cells. Phase III pathway is classified into two main super families; ATP-binding cassette (ABC) and solute carrier (SLC) transporters. ABC transporters are dependent on the energy (ATP) consumption to actively uptake or efflux the drug from one side of the cell membrane to another; whereas, SLCs facilitate the passage of certain solutes (e.g., sugars, amino acids, etc.) across the membrane and actively transport other solutes against their electrochemical gradients by coupling the process with other solute or ion. They are present in many locations; such as liver, kidney, intestine and brain.

Conceptually, uptake transporters help in transferring the molecules into the cells and efflux transporters pump them outside the cell. In the liver, the main uptake transporters are Na^+-taurocholate co-transporting polypeptide (NTCP; *SLC10A1*), organic cation transporter 1 (OCT1; *SLC22A1*), organic anion transporter 2 (OAT2; *SLC22A7*) and organic anion-transporting polypeptides (OATP1B1, OATP1B3, OATP2B1; *SLCO1B1*, *SLCO1B3*, *SLCO2B1*, respectively). The hepatic efflux transporters are multi drug resistance protein 1 or P-glycoprotein (MDR1; P-gp; ABCB1), bile salt export pump (BSEP; ABCB11), and multidrug resistance-associated protein 2 (MRP2; ABCC2). Other efflux transporters pump drugs toward the blood stream (circulation), which increase the total body residence of drugs, i.e. multidrug

resistance-associated protein 3, 4 and 6 (MRP3, 4 & 6; *ABCC3, 4 & 6*).[22-24] (please refer to Figure 1-1).

1.2 FACTORS AFFECTING DRUG DISPOSITION

Metabolism of drugs could be affected by several factors such as age, gender, pregnancy, medications, and genetic polymorphism (Figure 1-3). Typically, the neonatal expression of *CYP2C*, *CYP2E1*, and *CYP1A2* are nearly ten times lower than adults. However, in the elderly, even when most gene expressions are not altered, hepatic blood flow and oxygenation are decreased and fat deposition is increased, that could lead to reduced overall metabolic capacity for certain drugs in the elderly.[25-27] Furthermore, the activities of CYP2D6 and CYP1A2 have been reported to be higher in men than in women and vice versa for CYP3A4.[28] Another factor is pregnancy, which is associated with a number of physiological changes. Such as changes in body water, fat content, and hormones that can potentially alter absorption, distribution, metabolism and elimination of drugs.[29, 30] The activities of CYP2D6, CYP3A4, CYP2B6, and CYP2C9 increase during pregnancy resulting in shorter drug elimination half-life in pregnant women when compared to non-pregnant women. Concomitant administration of medications can affect the metabolism of each other. Food and Drug Administration (FDA) requires all drugs under development to be tested for any possible interaction, as substrate, inhibitors and/or inducers.[31] Inhibitors mainly work on the enzyme levels where they block or compete at the site of metabolism for a substrate. Types of inhibitors are; competitive (binds to the active site of free enzyme), uncompetitive (binds to the drug-enzyme complex to inhibit), noncompetitive (binds to different site other than site of the metabolism) or mixed. Inducers act by increasing the gene

transcription that will result in higher enzyme content. Last but not least, polymorphism in drug metabolizing enzymes and transporters is known to influence the clearance of several drugs. For example, voriconazole metabolism is mainly affected by *CYP2C19* polymorphisms, with voriconazole levels can be 4-5 times higher in poor metabolizers in comparison to extensive metabolizers receiving the same dose.[32]

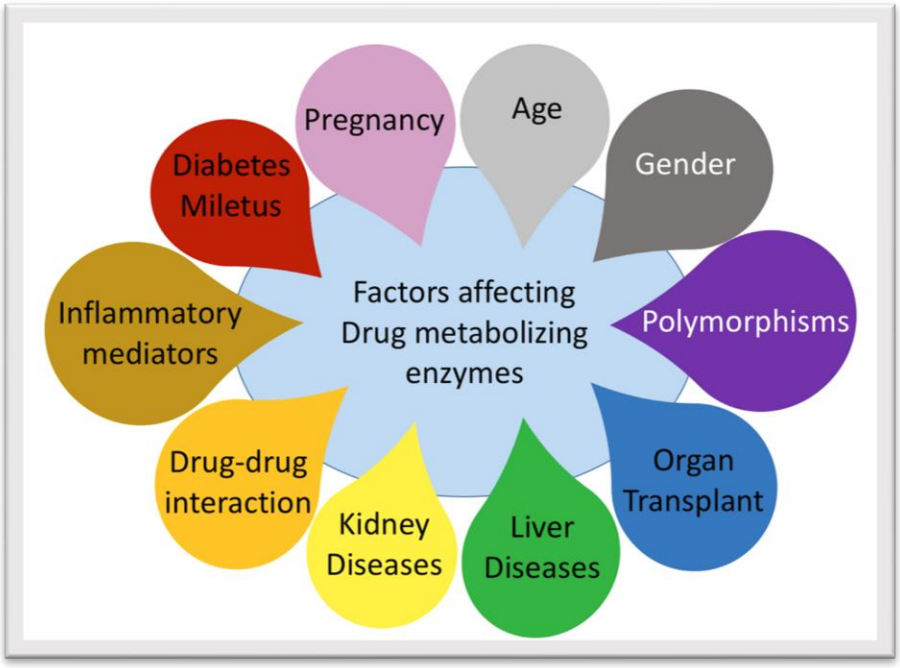


Figure 1-3: Factors affecting the drug metabolizing enzymes expression and functions.

1.3 LIVER DISEASES AND DRUG DISPOSITION

Liver diseases can be broadly classified by duration (acute vs. chronic), etiology (viral, alcohol, and others) or severity (cirrhosis vs. end stage liver disease). Various liver diseases are known to affect the metabolism of drugs as well as endogenous compounds. There are several reasons for the observed changes in drug metabolism in patients with liver disease. Altered hepatic blood flow, altered expression of drug metabolizing enzymes, altered availability of co-substrates, and altered binding of drugs to plasma proteins can account for the observed changes in drug metabolism in patients with liver disease. Plasma concentrations of midazolam are more than two folds higher in patients with nonalcoholic steatohepatitis (NASH), when compared to normal health subjects. The observed increase in plasma concentration of midazolam is due to NASH mediated decrease in CYP3A hepatic metabolism. Additionally, more than 50% reduction in the plasma concentration of 4β-hydroxycholesterol, which is used as an endogenous biomarker for CYP3A4 activity, was also reported in patients with simple steatosis.[33] The clearance of several drugs that are metabolized in the liver is decreased in cirrhosis. Liver cirrhosis decreases the clearance of voriconazole, a drug that is completely metabolized in the liver. Patients with hepatic insufficiency must be closely monitored when dosed with voriconazole to prevent drug associated toxicities.[32, 34] The protease inhibitors (PI), such as telaprevir and boceprevir have resulted in improved outcomes for HCV genotype 1 patients. Telaprevir is both a substrate and an inhibitor of CYP3A4 and can also saturate or inhibit P-glycoprotein in the gut. However, clinically significant drug-drug interactions involving these drugs may limit the use of these drugs and may affect the safety of their use along with other drugs.[35]

1.4 LIVER TRANSPLANTION

Liver transplantation is the only therapeutic option for patients with end stage liver diseases (ESLD). A long and rich history accompanies the field of solid organ transplantation. Organ transplantation was feasible with the ability of sewing blood vessels together that was first described by Alexis Carrel.[36] This was followed by further improvements in the vascular anastomotic techniques. In 1954, the first successful kidney transplantation was performed.[37] This procedure involved transplantation of kidney from a live identical twins without any immunosuppressants. In 1967, Thomas E. Starzl performed the first successful liver transplantation.[36, 38, 39] Since that time, the surgical procedures and technology used in organ transplantation have been significantly improved.

Liver transplantation is the acceptable treatment procedure for those indications that lead to irreversible liver failure.[40] The main indication for liver transplantation in the United States of America is hepatitis C virus, Figure 1-4.[41] Livers for transplantation usually come from two main sources: deceased donors (DD) or living donors (LD). Currently, there are more than 15,000 patients who are listed in the active waiting list for liver transplantation.[42] In the most recent OPTN/SRTR Annual Data Report (2012) for liver organs indicated that of the 6256 liver transplants (out of 12,427 patients in the waiting active list) that were performed in the US, only 246 (~4%) livers were from living donors whereas the rest came from DD, accounting for more than 95%.[43] The allocation of liver grafts for transplantation is mainly based on the severity of the sickness of the patient. Patient with Child-Turcotte-Pugh (CTP) score > 7 or a model for end-stage liver disease (MELD) score of 10 or more is recommended by the American Association for the Study of Liver Disease guidelines to be listed in the liver transplant waiting list. The adaptation of using MELD score was beneficial to continuously update the status of the sickness

of the patients. MELD score calculated by measuring the serum creatinine (mg/dL), international normalized ratio (INR), bilirubin (mg/dL) and serum sodium (mEq/L) and using Equation 1-1 and Equation 1-2.[44]

Equation 1-1: $MELD_{(i)} = 0.957 * [Log_e (creatinine)] + 0.378 * [Log_e (bilirubin)] + 1.120 * [Log_e (INR)] + 0.643$

Equation 1-2: $MELD = MELD_{(i)} + [1.32 * (137-Sodium)] – [0.033 * MELD_{(i)} * (137-Sodium)]$

Where $MELD_{(i)}$ is the score that is calculated initially for any patient aged 12 years or older and the second MELD equation is used for those patients who get a score of 11 or higher.[45] In the United States, the median waiting times for ESLD patients with MELD score between 11–18, 19–24 and >25 are 21 months, 4 months and 20 days, respectively.[46]

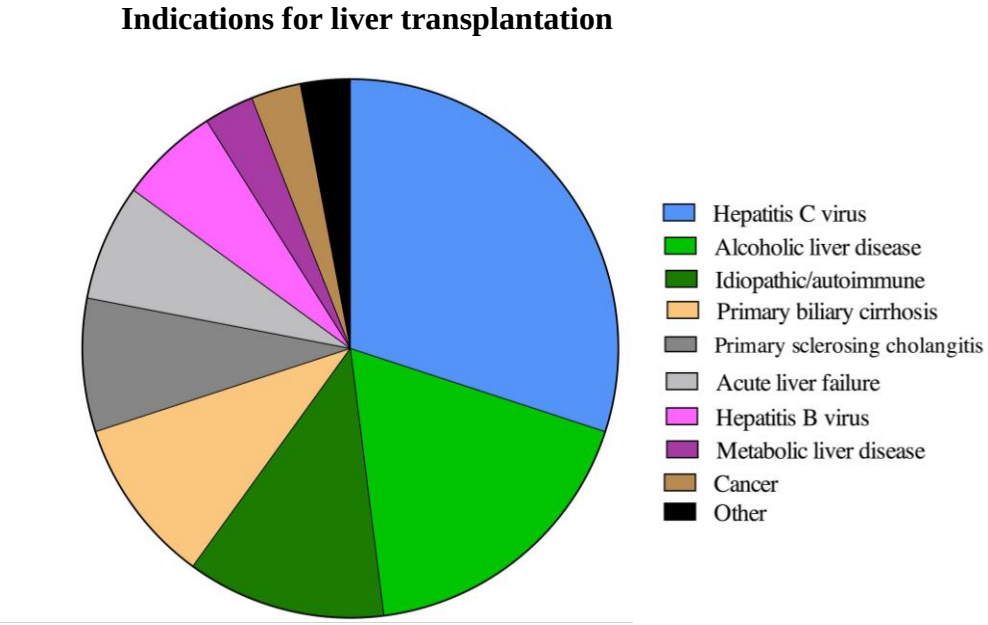


Figure 1-4: Prevalence of liver transplant indications

Percentage distribution of most common indications for adult liver transplantation in the US. (*Data from* Luu L. Liver Transplants. Drug & Diseases 2015).[41]

During the process of liver harvesting and transport, the blood nutrient supply and oxygen content that goes to the liver grafts are diminished which initiates the damages that is known as the ischemic injury. This injury is seen to be aggravated during the transplantation process that is accompanying the liver graft supplementation with oxygen and nutrient which is normally referred as the reperfusion injury. Ischemia and reperfusion (I/R) injury is resulted from the damages from the two events combined (more details in section 1.6).[47, 48] In the deceased donors, liver grafts are more susceptible to I/R injury because of the longer duration of cold preservation and lack of oxygen and nutrients. Additionally, this injury is worsened when the hepatic blood flow is re-established. I/R injury is also associated with release of proinflammatory cytokines that will lead to lower activity of drug metabolizing enzymes, such as CYP2C9 and CYP2B6.[49] Commonly used immunosuppressants in solid organ transplantation cyclosporine, tacrolimus, and mycophenolic acids are metabolized by CYP3A or UGT or transported by ABCB1 (MDR1; P-gp).[50] Muder *et al.* (2002) have shown that cefoperazone and sulbactam pharmacokinetics in liver transplants were significantly altered compared to normal subjects, indicating impaired biliary secretion capacity during early post-transplant period.[51] Also, the blood cyclosporine A concentration has been documented to be increased by liver dysfunction after liver transplantation, is attributed to impaired secretion of metabolites formed and cross reactivity of metabolites in immunoassay used.[52] Furthermore, cyclosporine A is a well-known inhibitor of P-gp, and can potentially alter the bioavailability of drugs which are P-gp substrate.[53] Several studies have shown that I/R injury decreases the *in-vivo* and *in-vitro* activity levels of several hepatic CYP enzymes and intestinal drug transporters.[54, 55] Another side effect of I/R damage is that the bile formation can be significantly impaired due to decrease in transporters activity.[55]

Many factors that are inherent after liver transplantation can affect phase I, II and III pathways. In the case of living donor liver transplant recipients, the size of transplanted graft is much smaller than normal livers and livers in deceased donor liver transplant recipients. Consequently, the intrinsic metabolic capacity is significantly lower in recipients of living donor liver transplant. However, the hepatic blood flow will be higher per unit weight of the liver in these patients.[56] It has been shown that the dose of an immunosuppressive drug required to reach a therapeutic target level is significantly correlated with graft weight/standard liver volume, warm ischemia time, and cold ischemic time.[57] Also, the metabolic function can be altered when graft rejection occurs, either acutely or chronically. It is documented that the expression of *ABCB1* transporter and *CYP3A4* in the intestine is increased and decreased, respectively, when there is chronic rejection of the liver graft with an increase expression of proinflammatory cytokines (COX2, IL-2, IL-6, IL-8, IL-10, and TNFα).[58]

1.5 COMPLICATIONS OF LIVER TRANSPLANTATION

Post liver implantation, there is a high risk for several complications. One major risk and most unfavorable event is the acute and chronic rejection of the graft. It is critical to adjust the dose of immunosuppressive medications, to get the optimum efficacy of preventing the organ rejection by host immune system, while minimizing the side effects of those immunosuppressants. Another complication following live transplantation is the primary graft non-function (PNF), which is observed in 4 - 23% of the transplants.[59-64] The PNF is a condition that is responsible for 81% of re-transplantation, most of the times in the first week post transplantation.[48, 65] Azoulay et al. (2002) have illustrated that first re-transplantation accounts

for only 12%, which indicates the risk of graft failure in the beginning and higher chance of getting multiple re-transplantations.[66] Another study has shown that 22% of the re-transplantation were due to PNF.[67] Furthermore, there is a strong evidence that ischemia and reperfusion (I/R) injury is a major leading cause of PNF, which aggravate the demand for liver grafts. Thus, it is crucial to fill the gap and decrease the number of patients in the waiting list.

1.6 PATHOLOGY OF HEPATIC ISCHEMIA-REPERFUSION INJURY

Toledo-Pereyra et al. in 1975 was the first to identify the clinical importance of the ischemia injury in liver transplantations. Ischemia/reperfusion (I/R) injury happens during the process of liver harvesting, transport and transplantation.[47, 48] The ischemic injury results from a lack of blood flow to the organ, leading to a reduced oxygen and nutrient supply, whereas the warm reperfusion injury results from increased supply of oxygen to the organ. Mechanisms of I/R injury have been studied by many investigators utilizing the experimental models and clinical observations.[68] A complex of pathways are integrated together to mediate the I/R damage. The liver is initially subjected to lack of oxygen and nutrient supply during the cold ischemia phase that causes vasoconstriction, proinflammatory cytokines release, mitochondrial stress, depletions in the adenosine triphosphate (ATP), increase in the reactive oxygen species (ROS; also known as oxygen free radical "OFR"), imbalance in H+, Na+, Ca2+ homeostasis and sinusoidal endothelial cells (SEC) as well as Kupffer cells (KC) swelling.[48, 68-71] Figure 1-5 shows different routes that will lead to apoptosis, which is a programmatic cell death, or necrosis, which causes premature cell death. Furthermore, the reactive oxygen species are released/sourced mainly from stressed mitochondria and/or imbalance between xanthine dehydrogenase/xanthine oxidase

(XDH/XOD) enzymes.[70] Those different pathways will damage the hepatocytes and hepatic endothelial cells. As a consequence, the overall liver graft function will be impaired. More details regarding the mechanisms involved in the hepatic ischemia and reperfusion injury will be discussed below. The complexity of the condition is behind the reason of this unresolved problem in the clinical practice till today.

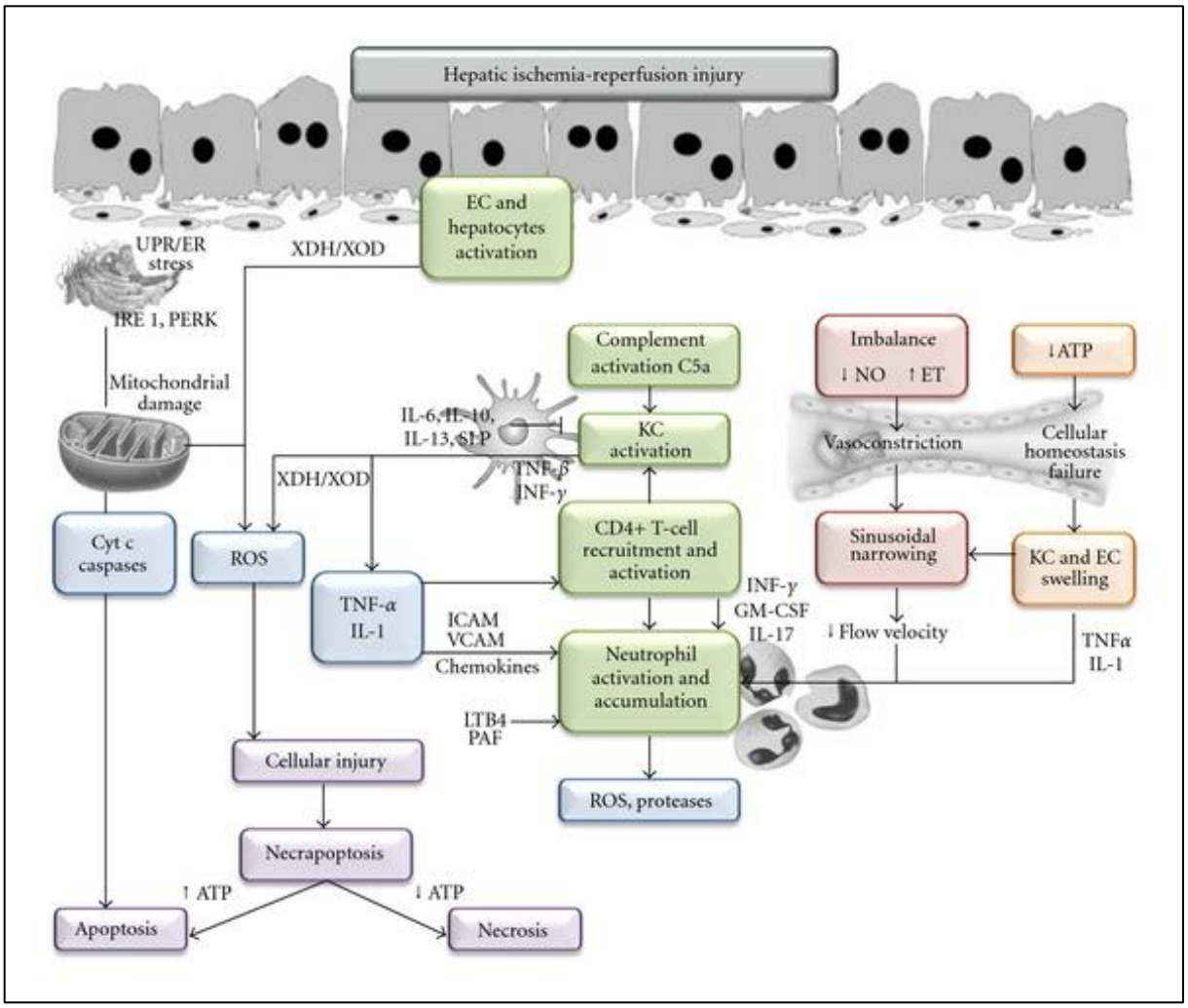


Figure 1-5. Shows integrated I/R injury mechanisms that occur in the liver grafts.

Complex network of pathways that occur in ischemia and reperfusion injury in the liver. Endothelial cell (EC), endothelin (ET), unfolded protein response/endoplasmic reticulum (UPR/ER), inositol-requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK), secretory leukocyte protease inhibitor (SLP), intracellular cell adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL), interferon (INF), tumor necrosis factor (TNF), platelet-activating factor (PAF), leucotriene B4 (LTB4), Kupffer cell (KC), xanthine/xanthine oxidase (X/XOD), cytochrome c (Cyt c). (*From* Mendes-Braz M, Elias-Miro M, Jimenez-Castro MB, et al. The current state of knowledge of hepatic ischemia-reperfusion injury based on its study in experimental models. Journal of biomedicine & biotechnology 2012;2012:298657; open access).[68]

1.6.1 Organ microcirculatory disruption

Nitic oxide (NO) and endothelin imbalance has been documented during the development of I/R injury. It has been demonstrated that thromboxane (TxA_2) increases and prostacyclin (PGI_2) decreases, resulting in vasoconstriction by stellate cell contraction in the sinusoidal lumen that subsequently results in microcirculatory dysfunction. Pretreatment of the liver with an endothelin receptor antagonist, such as bosentan or tezosentan, has shown protective effect against I/R injury induced endothelial damage.[64] However, controversial opinions have emerged in regard to the involvement of NO as a beneficial versus harmful compound in mediating the I/R injury.[48] These views were due to diversity in the experimental models, animal species and tissue and cellular types used to evaluate the contribution of NO in I/R injury. Elias-Miró et al. (2011) have suggested that NO, which is related to the NO production from the inducible synthase (iNOS), is involved in worsening the overall hepatic status. Another study has shown that NO that comes from endothelial NO synthase (eNOS) has beneficial effects.[69] Furthermore, excess NO level has been associated with an increase in the apoptosis by inducing cytochrome c (Cyt c) release and caspase activation.[72] The endogenous NO source could play cytoprotective role; but, NO does not minimize the I/R injury when supplemented exogenously.[48, 73] In clinical and preclinical studies NO decreases the blood flow and ultimately less O_2 is supplied to the organ.

1.6.2 Reactive oxygen species

Reactive oxygen species (ROS) plays an important role in hepatic ischemia and reperfusion injury. There are several sources of ROS, either intra or extra- hepatocellular. It has been suggested that xanthine dehydrogenase and oxidase (XDH/XOD) are the main pathway for ROS

production. This was proven by altering the I/R injury via utilizing specific XDH/XOD enzymes inhibitors, such as allopurinol.[65] On the contrary, Jaeschke and Mitchell (1989) have shown utilizing an isolated perfused liver system that ROS was mainly derived from mitochondrial production after the ischemic stress.[74] Also, other studies have shown that ROS generation in the liver was attenuated by inactivation of kupffer cells, using gadolinium chloride ($GdCl_3$), but not allopurinol. Endothelia cells and hepatocytes are involved to a lesser extent in ROS production due to the slow-base release of ROS in comparison to kupffer cells. Duration of the cold ischemia is a main determinant of which is the primary pathway responsible for injury. Longer ischemia times will clearly induce the mitochondria to release ROS, and it is considered as the main source of ROS.[75] The ultimate consequences of ROS release are activation of several transduction pathways, such as nuclear factor kB (NF-kB), caspase activation leading to hepatocytes and endothelial cells necrosis and apoptosis.[47] Also, ROS lead to loss of microvascular integrity and decreased blood flow to organs.

1.6.3 Cytokines

Substantial amounts of proinflammatory cytokines are released into the circulation by activated Kupffer cells. Those proinflammatory cytokines are tumor necrosis factor – alpha (TNF-α), interleukin-1 (IL-1), IL-6, and platelet-activating factor (PAF). Hepatocytes and endothelial cells injury, neutrophil activation and cellular apoptosis through caspase activation are linked to higher levels of TNF-α.[76] Parenchymal and hepatic microvascular destruction are associated with neutrophils activation and accumulation within the liver due to overexpression of intercellular adhesion molecule 1 (ICAM-1) and P-selectin that result from TNF-α and IL-1 signaling.[76, 77] Gene therapy to suppress both TNF-α and IL-1 or IL-1 alone have successfully attenuated the I/R

injury in animal models. Vascular swelling and lower membrane flexibility could be another explanation for the neutrophil trap. Rats that were treated with antiselectin therapy to decrease the intestinal reperfusion injury were found to have less neutrophil infiltration in the liver organ compared to untreated group.[78] Release of the proinflammatory cytokine IL-6 is relatively delayed after reperfusion, when compared to release of TNF-α and IL-1. It is documented that IL-6 inhibition promotes hepatocyte proliferation. Higher serum levels of TNF-α and IL-6 were seen in the cases of liver donation after cardiac death (DCD), where the heart has stopped before the liver procurement from the donor. Higher level of TNF-α and IL-6 was associated higher incidence of PNF.[63] Significant increase in bile flow and hepatic tissue levels of ATP were observed when PAF antagonist was used. Furthermore, rats treated with PAF antagonist was protected from I/R injury that is characterized by less neutrophils infiltration, reduced hepatic aminotransferases and increased animal survival rate.[79] Complement activation is also considered a critical event in the development of hepatic I/R injury. It is normally activated by substantial release of the cellular proteins in the early phase. Macrophage-1 (Mac-1) receptor on neutrophils is upregulated by complement factor C5a leading to recruitment into the sinusoids.[64] Anti-inflammatory cytokines, such as prostaglandins, IL-10 and IL-13, are released by Kupffer cells as a protective measure from ischemia. Pig, dog and rat animal studies have demonstrated that the use of prostaglandin E1 (PGE-1) and prostaglandin I2 (PGI-2) have significantly reduced ICAM-1, plasma liver enzymes, TNF-α, P- and E-selectin.[76] Hepatic edema, injury and neutrophil recruitment was reduced by IL-10 gene transfer. These effects were combined by decrease in TNF-α transcription and NF-kB inactivation. A summary of most important factors that play role in hepatic I/R injury and its direct observed effects is listed in Table 1-1.

Table 1-1: Summary of major factors and their documented effects

Mediator	Documented effect	
	Increased ↑	**Decreased ↓**
iNO	apoptosis, Cyt c, caspase activation	---
eNO	hepatic cell protection	inflammatory cytokines, PAF
Endothelin	vasoconstriction, hepatocellular and endothelial injury	blood flow
XDH/XOD	ROS, NF-kB activation, caspase activation, necrosis and apoptosis	---
Mitochondria stress	ROS, NF-kB activation, caspase activation, necrosis and apoptosis	---
TNF-α	neutrophil activation, ROS, apoptosis, ICAM-1, IL-8	IL-10
IL-1	TNF-α , ICAM-1, IL-8	IL-10
IL-6	TNF-α , IL-1	---
PAF	TNF-α, ALT, AST, ROS, neutrophil recruitment	---
IL-12	TNF-α, ALT, AST, ROS, neutrophil recruitment, edema	---
IL-18	NF-kB activation, apoptosis, neutrophil recruitment, edema, ALT, AST	IL-10
PG-E1	---	TNF-α, ICAM-1, VCAM-1, P- and E-selectin, ALT
PG-I2	---	ALT, AST
IL-10	---	NF-kB activation, TNF-α, neutrophil recruitment, edema
IL-13	---	TNF-α, neutrophil recruitment, edema
C5a	ROS, Mac-1, cellular injury	---

1.7 EXPERIMENTAL MODELS TO EVALUATE HEPATIC ISCHEMIA AND REPERFUSION INJURY

Several experimental models of liver transplantation have been used to evaluate I/R injury in the rat. Rat to rat orthotopic liver transplantation (OLT) or warm ischemia model or cold static liver preservation followed by warm reperfusion in an isolated perfused system have been performed previously. Rat OLT model is the most clinically relevant, reliable and technically applicable model, but requires more time and experience to perform the transplant surgery. In rat OLT, normally it is difficult to standardize the procedure between rats, specially anastomosis, prevent excessive bleeding and thrombosis.[68] Normothermic ischemia of the liver graft, either completely or partially, is also widely used to simulate clinically observed warm ischemia. However, it lacks the cold ischemia that is clinically unavoidable in OLT. A normothermic continues perfusion preservation system, till the liver is transplanted showed more favorable results in preventing I/R injury, but the use of blood products or oxygen carrier is essential in such a system. Hypothermic perfusion does have an advantage of preventing ATP depletion and therefore minimize some of the I/R damages in contrast to normothermic perfusion.

Isolated perfused rat liver (IPRL) is a well-established experimental model that has been widely used to study rat liver physiology and pathophysiology. IPRL was first introduced by Claude Bernard in 1855 and since then the model has continuously been improved.[80] The results that were generated from IPRL experiment have been highly valuable, especially with the reperfusion experiments. The structural and functional organization in the isolated livers are preserved and hepatocytes polarity is maintained in this system. IPRL has been used to evaluate the cellular damage and assesses the organ function. Among all other models, this model has been used the most for ischemia and reperfusion injury assessment after preserving the liver graft

for a targeted duration. Several groups have used IPRL to investigate the extent of cellular injury, characterize the metabolism of compounds and oxygen consumption in the perfusate. One major advantage of using IPRL is the ability to control the conditions that result in reliable and reproducible results. It also minimizes the use of the laboratory animals and associated resources. Around one half of the numbers of animals is needed for the IPRL experiments, that usually result in more power for the study due to lower variability. Krebs–Henseleit buffer (KHB) is widely used as the perfusion buffer, since it provides energy source, osmotic pressure, oxygen carrying and buffering capacity.[81-83]

However, IPRL system comes with certain disadvantages that should be taken into consideration. The perfusate solution composition cannot allow similar partial oxygen pressure observed when using blood, but this can be overcome by increasing the perfusate flow, to 3 mL/min/g liver weight. Also, the lack of leukocytes in the perfusion solution will relatively limit some the I/R injury mechanisms that result from neutrophils activation. The use plasma protein such as albumin in the IPRL system increases the possibility of endotoxins contamination that could have toxic effect on the hepatocytes.[82, 84]

The use of animals to investigate and evaluate the I/R injury and different interventions, respectively, offers significant advantages. A lot of animals can be reproduced fast and easily managed to test various type of interventions, including gene therapy approaches. Also, extrapolation of observations from the animal study results to humans is reasonable. One of the most commonly used species is rodents (mice or rats) because of their ease of availability, reasonable costs and availability of previous data on I/R injury in this model. Furthermore, rats have a similar portal and hepatic venous systems that are comparable to the human liver. However, it is very important to remember that rodents are different anatomically and they may

metabolize chemicals/drugs faster compared to humans.[85] Unlike the small animals, larger species, such as dogs and pigs, have more similarity to humans but they are harder to manage, need more expertise to run the studies and are more costly to use.[86]

1.8 PHARMACOLOGICAL INTERVENTIONS AGAINST HEPATIC ISCHEMIA AND REPERFUSION

Numerous animal and human studies have focused on implementing techniques to minimize the I/R injury and its harmful consequences. Some investigators have tested surgical manipulations and others use various molecules to target the known pathophysiological pathways. One method is to improve the composition of the University of Wisconsin (UW) used for organ preservation. Another method is pharmacological treatment of the donors or recipients to prevent the I/R injury of the transplanted organ. Several studies have been conducted to test many chemical entities in hepatic, intestinal or cardiac I/R models. Gene therapy approach was also utilized by several investigators in order to benefit from the advances in that field. Our main focus in this section and through-out the dissertation is pharmacological treatment to minimize I/R injury of the liver grafts.

1.8.1 Pharmacological strategies to minimize I/R injury

Ischemia and reperfusion injury is a field that has been thoroughly investigated and many studies have been carried-out to develop a pharmacological intervention to inhibit the harmful effects of the I/R injury; however, no successful treatment has emerged to prevent I/R damage. Static cold

storage method is the most commonly used preservation techniques in solid organ transplantation. Several categories of pharmacological interventions have been evaluated in this model. Trimetazidine, which is anti-ischemia metabolic agent, is one of the oldest anti-ischemic drugs that has been tested more than thirty-five years ago for cardiac ischemia. Trimetazidine, which inhibit β-oxidation of free fatty acid, has been recommended recently to be used clinically to minimize ischemic heart disease.[87] Also, its cytoprotective effect on the liver through targeting mitochondria, energy metabolism, oxidative stress and improvements of microcirculation has been tested after partial hepatic occlusion.[88] However, trimetazidine is not ready to be used as a standard of care in liver transplantation. Adenosine and NO agonists, endothelin antagonists, prostaglandins and prostacyclin's have been used to target microcirculation and microvascular protection. FK3311 has significantly increased the survival rate and minimized the hepatic injury markers when donor rats were treated.[89] Inactivation of Kupffer cells with gadolinium chloride ($GdCl_3$) was another method to minimize I/R injury that was achieved by treating the donor rats. Taurine, a ubiquitous sulfur-containing β-amino acid, attenuated the I/R injury in a rat liver transplantation model due to its suppression of Kupffer cell.[90] Inhibiting the complement signaling was also approached with limited studies. Zhang et al. (2011) have shown that depletion of complement by cobra venom factor can decrease the severity of liver dysfunction in rats.[91] The addition of serine protease inhibitors and streptokinase (anti-thrombolytics) has been shown to minimize warm ischemic injury of the liver after cardiac death experimental models.[92] In isolated perfused livers, the addition of epidermal growth factor (EGF) and insulin growth factor (IGF-I) has been shown to reduce hepatic injury. Many groups have used anti-inflammatory drugs or free radical scavengers to attenuate the I/R injury. Steroids have reduced the release of proinflammatory cytokines and are associated with lower morbidity clinically.[63]

Diannexin, a neutrophil inactivator, has increased the survival rate of rats after orthotropic liver transplantation.[93] Further pharmacological classes that were investigated includes anti-apoptosis agent, heat shock protein inducer and others.

Limited number of pharmacological agents have been tested in the clinical settings. Steroids are one of the classes that was tested in deceased donor liver transplantation. Kotsch et al. (2008) have shown that methylprednisolone significantly downregulated proinflammatory cytokine (TNF-α and IL-6) release that is associated with I/R injury. However, there was no differences in incidence of PNF between the control and treatment groups.[94] A more recent study has failed to show the beneficial effect of donor treatment with methylprednisolone on minimizing I/R injury. The primary endpoint was used in this study to detect the difference in serum alanine and aspartate aminotransferase and the secondary end point was the survival rate and biopsy-confirmed acute rejection inciedences.[95] Another clinical study has shown that IDN-6556, a novel irreversible broad-spectrum caspase inhibitor, minimized the peak values of transaminases and serum concentrations of apoptosis marker; however, there was no significant difference between the groups in survival rate and PNF after OLT.[96] Hilmi et al. (2010) have used N-acetylcysteine as a free radical scavenger in patients undergoing OLT to prevent I/R injury associated side effects on the liver as well as kidneys. However, they have concluded that N-acetylcysteine did not show any improvement between the intervention vs placebo groups.[97] In a prospective, randomized and placebo-controlled study, Lang et al. (2007) have shown that patients supplemented with an inhaled NO during liver transplantation have reduced hospital stays and serum transaminases.[98] Meta-analyses that was performed on eighteen randomized clinical trials concluded that some medications showed protective role in I/R injury, but their recommendation restricted any further larger clinical studies.[99] Most of these clinical studies

have shown little or partial effectiveness but was not enough to convince the medical filed to be adopted as routine procedure for liver transplantation. A comprehensive summary of previous animal and clinical studies are listed in Table 1-2. The next section will focus on the use of prostaglandin and prostacyclin and their analogs in the field of liver transplantation.

1.8.2 Prostaglandins and prostacyclin experience in I/R injury

Various preclinical and human studies have shown the pathology and etiology of I/R injury in orthotopic liver transplantation. One major event that happens in this condition is the decrease in the ratio of prostacyclin (PGI_2) and thromboxane (TxA_2), which promotes neutrophil adhesion to endothelial cells and activates platelet aggregation. Prostaglandins (PG) and PGI_2 have been as potential therapeutic agents in OLT.[100] PGs have antiplatelet activity, but results do not show any increase in bleeding incidence when they are used.[101-103] Prostaglandin E1 (PGE_1) when administered to patients with liver PNF has improved the survival rate from 33% to 90% compared to untreated patients.[104] The PGE_1 infusion was started 4 to 34 hours of transplantation and maintained for up to a week. Greig et al. have shown that PGE_1 significantly reduced the peak transaminases. Additionally, the hepatic tissue necrosis was decreased and blood coagulation factors were enhanced. However, they have recommended further evaluation of PGE_1 in larger randomized studies. Another major issue in the liver transplantation population is the incidence of positive cytotoxic crossmatch of around 15%, which is associated with poor outcomes. Patients with positive crossmatch test (present) have 4 folds' higher incidence of retransplantation, two times higher mortality and graft loss when compared to crossmatch negative patients.[105] Those adverse events were overcome by treating the patients with a combination of PGE_1 and high doses of prednisone.[106] Takaya and colleagues, have also shown

the beneficial effects of using PGE_1 concomitant with FK506 in crossmatch negative patients.[107] In one study PGE_1 use has resulted in decreasing PNF incidence from 5.9%, in historic controls, to 1.1%.[108] With all of the evidence, Henley et al. (1995) carried-out a large randomized-placebo controlled, double blinded clinical study to investigate starting PGE_1 infusion intraoperatively during the anhepatic phase of the OLT procedure. The study included 160 subjects (78 PGE_1; 82 placebo) and showed significantly reduced hospital and intensive care unit stay by 20% and 40%, respectively.[109] However, the use of PGE_1 did not decrease the PNF, graft and patient survival rates. Another study that was a randomized, double blind, placebo-controlled, multicenter trial has been reported by Klein et al. (1996). They have also used PGE_1 during the OLT procedure but the start of the infusion was delayed till the restoration of the portal and arterial flow.[110] In line with the previous studies, this study also documented that PGE_1 is effective in improving the early renal function and decreasing the length of ICU stay. However, all other findings were not statistically significant, including the incidence of PNF that was around 6.8% in both treated and control groups. Neumann and colleagues have published 3 placebo-controlled studies to evaluate the effectiveness of infusing PGE_1 and PGI_2 following reperfusion and continued for 6 to 7 days after OLT. They have shown that all patients were alive after one month and the transaminase levels tended to be lower ($p < 0.1$), but was not significant.[111-113] Those studies have demonstrated that PGE_1 and PGI_2 improved hepatic-splanchnic oxygenation that was assessed by measuring the hepatic venous oxygen saturation ($SvhO_2$) levels after 1 and 2 days. This finding suggest that both PGE_1 and PGI_2 improves early microvascular blood flow. In order to tackle the problem (I/R injury) before its occurrence, Klein and colleague have treated the liver with 500 µg bolus of epoprostenol (PGI_2) or placebo at the time of organ harvesting.[114] Donor pretreatment with epoprostenol significantly reduced both

peak AST and ALT levels in comparison to controls. Most recently, Barthel et al. (2012) have shown that continuous infusion of iloprost, a PGI_2 analog, for one week has decreased the PNF incidences by 75% and improved allograft synthetic function.[115] They started a larger prospective, double-blinded, randomized, placebo-controlled multicenter study to assess seven days iloprost infusion after liver transplantation in minimizing PNF incidence and overall liver transplantation outcome. The study is ongoing and the data have not been reported yet.[116] In a Cochrane review, there was no evidence of increased risk of complications when prostaglandins were infused during the liver transplantation.[101] Most of the prostaglandin dosing regimens were gradually increased every 10 - 30 minutes till they reach the targeted dosing, in general and in liver transplant patients.[104, 106-111, 113] The use of prostaglandin and prostacyclin class of agents could improve the clinical outcome by decreasing morbidity and mortality that are associated with liver transplantation. In previous studies, acute kidney injury was significantly reduced when prostaglandins were used. The renal protection by prostaglandin and prostacyclin might be beneficial in decreasing the calcineurin inhibitors induced nephrotoxicity. Major drawbacks of using prostaglandins as standard of care in liver transplant patients is its chemical instability, short half-life and high cost.

Table 1-2 : Selected list of small and large animals and human clinical studies that implemented pharmacological treatment method to minimize the I/R injury in orthotopic liver transplantation.[63, 68, 88, 89, 92, 93, 95-98, 106-110, 112-114, 117-123]

Drug (class)	Intervention Timing	Species	Effect
Trimetazidine (anti-oxidant)	Before LTx	Rats	↓ liver injury, ↑ survival rate
Gadolinium chloride (KC suppresser)	Before liver harvesting	Rats	↓ PNF, ↓ ICAM-1
Connecting segment-1 (fibronectin blocker)	During organ preservation	Rats	↓ neutrophils, ↓ TNF-α, ↓ interferon, ↓ iNO
Prostaglandin E1	Before and after LTx	Rats	↓ neutrophils, ↓ liver injury, ↑ histological finding
FK3311 (complement inhibitor)	During organ preservation	Rats	↑ survival rate, ↓ liver injury
Diannexin (neutrophil inactivator)	During and after organ harvesting	Rats	↑ survival rate, ↓ TNF-α, ↓ICAM-1 ↓ liver injury, ↑ histology
L-arginine (NO substrate)	During organ harvesting	Pigs	↑ portal blood flow, ↓ necrosis, ↓ ischemic cholangitis
E5880 (PAF antagonist)	During harvesting and after LTx	Pigs	↑survival rate, ↓ neutrophils, ↓liver injury, ↑ histological finding
IDN-6556 (Caspase inhibitor)	Before organ harvesting	Human	↔ survival rate, ↔ PNF ↓ transaminase
N-acetylcysteine (Anti-oxidant)	During and after organ harvesting	Human, Pigs	↔ survival rate, ↔ transaminase
Methylprednisolone (anti-inflammatory agent)	Before organ harvesting	Human	↔ survival rate, ↔ transaminase
Thymoglobulin (anti-inflammatory antibody)	During and after LTx	Human	↓ transaminase, ↔ survival rate
Inhaled NO (endogenous NO inhibitor)	During OLT	Human	↓ transaminase, ↓ length of stay, ↔ inflammatory markers
Tacrolimus (immunosuppressant)	Organ flushed before LTx	Human	↓ peak AST
Alprostadil (PGE_1)	Before, during or after LTx	Humans	↓ transaminases
Epoprostenol (PGI_2)	During LTx	Humans	↓ transaminases
Iloprost (PGI_2)		Human	↓ PNF, ↑ synthetic functions

1.8.3 Treprostinil

Treprostinil (Remodulin®) is a chemically stable analog of PGI_2, a naturally occurring prostanoid with potent pulmonary and systemic vasodilatory, and anti-platelet aggregatory actions *in-vitro* and *in-vivo*, as well as cytoprotective properties. Treprostinil was developed by United Therapeutics Corporation for the treatment of pulmonary arterial hypertension (PAH), an orphan disease with a global prevalence of approximately 50,000 patients. In 2002, the subcutaneous (SC) treprostinil was approved by the U.S. Food and Drug Administration. Several other countries have also approved the use of SC treprostinil; including Canada and most of the European Economic Areas. Currently, more than 1700 patients with PAH and health volunteers have been dosed with treprostinil under controlled studies, with exposures ranging from single acute to chronic administration of treprostinil that revealed its safety profile. In 2004, the intravenous (IV) treprostinil, which is bioequivalent to the subcutaneous treprostinil formulation has been approved in the U.S. for patients with PAH.[124] United Therapeutics Corporation has continued to develop other route of administrations of treprostinil. An inhalation formulation of treprostinil (Tyvaso®) and an extended release tablet for oral administration of treprostinil (Orenitram®) have been approved by U.S. FDA in 2009 and 2013, respectively. Treprostinil has preferable pharmacokinetic profile in comparison to other prostaglandins and prostacyclin analogs. The elimination half-life is estimated to be around 4 hours, which is 8 times longer than other approved PGI_2.[125] It is primarily metabolized by CYP2C8 and CYP2C9 that results in several metabolites. Seventy nine percent of [^{14}C]-treprostinil dose is recovered in the urine, but only 4% is unchanged, and ~13% is recovered in the feces.[126] The apparent plasma clearance for doses ranging between 2.5-15 ng/kg/min was around 10 mL/kg/min. The pharmacokinetics of treprostinil was linear with increase in doses, from 1.25 up to 125 ng/kg/min, and the steady state

concentrations for a given dose was described by the following equation "Treprostinil Css (pg/mL) = 295.3 + (140.07 x treprostinil dose)".[125] The toxicity profile of treprostinil, delivered by continuous subcutaneous infusion, has been extensively evaluated. The studies conducted include the complete International Conference on Harmonization (ICH) battery of genetic toxicology studies; acute, single-dose, intravenous and oral toxicity studies in rats and mice; acute, single-dose, subcutaneous toxicity studies in rats and dogs; repeat dose, continuous subcutaneous infusion toxicity studies up to six months in duration in rats and dogs; and reproductive toxicity studies in rats (fertility, teratology and pre / postnatal) and rabbits (teratology). Side effects of treprostinil have been well characterized in extensive clinical studies and include subcutaneous infusion site pain, and events expected with excess prostacyclin and prostacyclin analogs, including flushing and headache.[127] A complete description of preclinical and clinical information is available in the treprostinil package insert that describes detailed information about treprostinil.[128]

Studies have been carried-out, by our group, to evaluate the efficacy of treprostinil as pharmacological agent to attenuate the hepatic I/R injury in orthotopic rat liver transplantation model.[129] Ghonem et al. (2011) have demonstrated that treating both donor and recipient rats with treprostinil results in decreased damage associated with 18 hours static cold preservation of the liver in UW solution. Treprostinil restored the expression and microsomal activity of Cyp2e1 and Cyp3a, respectively, that is decreased during cold preservation.[130] Furthermore, there was no drug interaction of treprostinil with primary immunosuppressant drugs that are normally used in liver transplant patients.[131] Treating donor rat with treprostinil also offered some protection against I/R injury.

Two patients diagnosed with PAH who were receiving treprostinil underwent liver transplantation at the University of Pittsburgh Medical Center (UPMC). The treprostinil infusion was established for 6 and 11 months before the LTx and the doses used at the time of transplantation were 36 and 45 ng/kg/min. Patients received treprostinil throughout the transplant procedure and following liver transplantation without any treprostinil-related side effects.[132] However, treprostinil has not previously been administered during liver transplant surgery as part of a formal clinical investigation. In a study in patients with portopulmonary hypertension with mild (4 subjects) or moderate (5 subjects) hepatic dysfunction, treprostinil at a subcutaneous dose of 10 ng/kg/min for 150 minutes demonstrated a maximum plasma level that was increased 2 and 4 folds, respectively. Furthermore, the total estimated exposure ($AUC_{0-\infty}$) were increased 3 and 5 folds, respectively, compared to healthy adults.[128] During surgery, liver transplant recipients experience an anhepatic period, that last for around 1 to 1.5 hours, when no hepatic metabolism of administered drugs will occur. Consequently, during this anhepatic period, plasma concentrations of treprostinil will be expected to increase significantly (likely by 5 folds' or more). PGI_2 has been safely administered intra-operatively at a maximum dose of 4 ng/kg/min during liver transplant surgery.[112] Experience with switching patients with pulmonary arterial hypertension from intravenous PGI_2 to treprostinil suggests that a two fold increase in the treprostinil dose will typically be required to obtain the same clinical response.[133-135] Given all of these preliminary observations, it is crucial to test its safety and protective effect on hepatic I/R in a clinical study.

1.9 GLOBAL HYPOTHESIS

Liver transplantation is the most effective treatment for patients with end-stage liver disease. Yet, cold preservation and warm reperfusion of the liver that are parts of the transplant procedure lead to harmful I/R injury that has been associated with higher primary non-function of the livers or early acute liver dysfunction. Use of extended criteria donation or marginal organs in order to save the life of the patients in the waiting list, further increases susceptibility to I/R injury of the liver grafts.

Several methods have been utilized to minimize the effect of I/R injury, surgically or pharmacologically. Many pharmacological agents being investigated target the etiology of the I/R injury. Prostaglandins and prostacyclins have been used in small and lager animal models as well as in humans over the past decades. PGE_1 and PGI_2 appear to prevent I/R associated damages to the liver grafts. These agents are not routinely used in clinical practice because of their limitations, such as lack of significant changes in the primary endpoints, because of their side effects, very short half-life and inherent chemical instability. Treprostinil, a prostacyclin analog, has the advantages of being chemically stable, with longer elimination half-life and being more potent. Treprostinil has been shown to be effective in rat OLT model. Treprostinil has been reported to be used safely in patients during liver transplantation procedure. **We hypothesize that vasodilation, anti-platelet aggregation and downregulation of proinflammatory cytokines by treprostinil will attenuate hepatic I/R injury of the liver grafts when treprostinil is supplemented in the UW solution during liver harvesting and liver preservation. We further hypothesize that administering treprostinil as a continuous infusion in liver transplant recipients after OLT procedure will be safe and will minimizes the I/R injury.**

Our hypothesis will be tested in this dissertation by demonstrating the effect of supplementing treprostinil into UW solution while the liver is preserved and infusing treprostinil while it is perfused in an isolated organ system. I/R injury will be measured by the release of hepatic injury biomarkers, changes in bile production and maintenance of liver function (Chapter 2.0). The effect of ischemia and reperfusion injury on the expression of primary hepatic drug transporters will be characterized and the effect of treprostinil on reducing the expression will be evaluated (Chapter 3.0). In addition, the ability of treprostinil in preserving or minimizing the I/R injury on a drug transporter (P-gp; Abcb1; Mdr1a) and a drug metabolizig enzyme (Cyp3a) in the intact liver will be evaluated using digoxin as a probe (Chapter 4.0). In order to test the second hypothesis, a translational clinical study was performed to evaluate the safety and preliminary efficacy of continuous infusion of treprostinil in liver transplanted patients (Chapter 5.0). The pharmacokinetics of treprostinil was also evaluated in liver transplant patients as part of phase I/II clinical trial (Chapter 6.0).

2.0 EFFECTS OF TREPROSTINIL ON LIVER ORGAN VIABILITY AFTER COLD ISCHEMIA AND WARM REPERFUSION INJURY

2.1 INTRODUCTION

The liver is an important organ in the human body with multiple synthetic and metabolic functions. Currently, orthotopic liver transplantation (OLT) is the most effective therapy for ESLD and acute liver failure. Liver is considered as the 2nd most transplanted solid organ in the US. Currently, there are more than 16,000 patients waiting for a liver transplantation.[136] Livers are sourced from either a living or deceased donors. In a recent report, the University of Pittsburgh Medical Center (UPMC) has reported that 88.7% of the liver allografts transplanted were from deceased donors.[137] Those grafts are normally subjected to ischemia and reperfusion (I/R) injury, during organ preservation, transportation and transplantation. I/R injury starts during cold preservation where oxygen and nutrient supplies are low or absent, a period known as cold ischemic phase. The damage is aggravated during warm reperfusion phase with organ re-oxygenation and increased nutrient utilization. I/R injury is a major leading cause of primary graft non-function (PNF) which is reported from 4% to 23% after OLT and requires immediate re-transplantation of the liver.[59] While I/R injury is an integral and unavoidable step in liver transplantation leading to liver graft damage, extended criteria donor livers are more susceptible to I/R injury because of the longer preservation times and higher risk factors associated with the donors. The pathophysiology and etiology of the I/R injury, which is considered as an antigen independent component of liver harvesting, has been extensively studied.[64]

Many elements play a crucial role in mediating the injury, which can be divided into multiple pathways: A) Microcirculatory interruption that results from an imbalance in nitric oxide (NO) and endothelin-1 (ET-1) levels, leading to vasoconstriction.[64, 69] This is the primary reason for the reduction in hepatic blood flow immediately after the liver transplantation. B) Pro-inflammatory cytokines release by activated Kupffer cells that is responsible in attracting

polymorphonuclear neutrophils, leading to stimulation of the macrophages and direct cellular damages.[76] It has been shown that increase in TNF-α and IL-1 levels promote the expression of intercellular adhesion molecule 1 (ICAM-1) and P-selectin that result in neutrophils recruitment.[76, 77, 138] C) Platelet-activating factor (PAF) was dramatically increased after the reperfusion. Additionally, this pattern of the PAF increase was shown to be associated with incidence of PNF.[63] D) Reactive oxygen species (ROS) are significant component of the I/R injury that are immediately released after reperfusion. Kupffer cell activation, mitochondrial stress and xanthine dehydrogenase and oxidase (XDH/XOD) are the main sources for ROS that damage the sinusoidal endothelial cells (SEC).[65, 74] Consequently, ROS activates several transduction pathways (i.e. nuclear factor $_{k}$B; NF-$_{k}$B) that results in SEC and hepatocytes necrosis and apoptosis and loss in the microvascular integrity and low blood flow in the transplanted organs.[47, 64, 85]

Our laboratory has shown that I/R injury is ameliorated in rats when both donors and recipients were treated with treprostinil, a potent synthetic prostacyclin (PGI_2) analog.[129] Also, treating only rat OLT recipients with treprostinil showed protection, but to a lesser extent. The protective effect has been associated with higher blood flow after rat OLT, as a result of vasodilation, decreased infiltrated neutrophils in to the liver tissues due to decreased expression of adhesion molecules and less circulating proinflammatory cytokines released from activated kupffer cells. In an ideal setting, it is important to introduce treprostinil before I/R injury starts to occur, but is not always practical to treat the donors before liver graft harvesting. Therefore, in this chapter, we aim to examine the effect of supplementing treprostinil in the preservation solution only or supplementing and adding it while the liver is reperfused in a IPRL. We hypothesize that vasodilation, anti-platelet aggregation and proinflammatory downregulation

activities of treprostinil will diminish the I/R injury associated effects. We evaluated this utilizing an isolated perfused rat liver (IPRL) system, which simulates liver graft in the recipient.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals

Treprostinil (Remodulin$^{(R)}$) 1 mg per mL in a 20 mL vial was courtesy provided by the manufacturer, United Therapeutics Corporation (Silver Spring, MD). CoStorSol^{TM} (University of Wisconsin; UW) cold organ preservation solution purchased from Preservation Solutions Inc. (Elkhorn, WI). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) assay kits were from Pointe Scientific, Inc. (Canton, MI). Sodium chloride, potassium chloride, calcium chloride, Magnesium sulfate, potassium phosphate, sodium taurocholate, sodium bicarbonate and glucose were of analytical grade and obtained from commercial sources.

2.2.2 Animals

Sprague Dawley (SD) rats were purchased from Charles River Laboratories, Inc. (Wilmington, Massachusetts). Those SD rats were adult male 10-12 weeks' old weighing 225-250 gm. Rats were kept in the animal facility and maintained under a 12 hours' light and dark cycle with free access to food and water. The procedures involving animals were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and were consistent with the Guide for

the Care and Use of Laboratory Animals (National Research Council, 2011, Washington, District of Columbia).

2.2.3 Study design

SD rats are the most common laboratory animals used to study the hepatic I/R injury.[81] Rats were randomly assigned to the following study groups: 1) Controls (no treprostinil): liver grafts were harvested then perfused in the IPRL for 2 hours without cold preservation. 2) Ischemia/Reperfusion group (no treprostinil used): liver grafts were harvested, cold (4^0C) preserved in UW solution for 24 hours followed by 2 hours of IPRL perfusion. 3) Treprostinil-1 (during preservation only): rat livers were harvested and preserved in UW solution supplemented with treprostinil (20 ng/mL) for 24 hours and flushed with treprostinil (20 ng/mL) added ringers lactate solution (~20 mL), then 2 hours IPRL perfused without treprostinil. 4) Treprostinil-2 (during preservation and perfusion): the livers in this group went through the similar procedures to group #3, but treprostinil (20 ng/mL) was added to the perfusion solution during IPRL perfusion.

In this study we evaluated two different IPRL systems: A) single pass perfusion and B) recirculation perfusion configurations. Initially, a single pass method was used to establish the model but it has some drawbacks that will be discussed below. We studied only the first two groups in this model. Then, recirculation model was established and used for the rest of the study.

2.2.4 Surgical procedure

Livers were harvested, preserved and perfused as previously described by Mehvar et al. (2002).[139-141] Rats were anesthetized using isoflurane and the depth of anesthesia was checked by toe pinch reflex. The abdominal cavity of the rat was opened with a V-shaped incision to expose the visceral organs. The bile duct was cannulated with PE10 tubing and the portal vein and external hepatic vein were cannulated with catheters for inlet and outlet of the perfusate, respectively. Livers were perfused with 30 mL UW solution (with or without treprostinil) and harvested. The isolated rat livers were preserved in UW solution (with or without treprostinil) for 24 hours at 4^0C. Then, livers were flushed with 25-30 mL cold ringers lactate solution to remove the UW solution completely and livers were transferred to organ chamber of the IPRL system. The perfusion was carried out for 120 minutes with Krebs–Henseleit bicarbonate buffer saturated with 95%/5% O_2/CO_2 and supplemented with sodium taurocholate (4.75 mg/L).[81, 142] The livers were perfused at 3 mL/min/g liver to maintain proper oxygen supply in the absence of the proteins and blood in the perfusate. Outlet perfusate samples were collected at 0, 15, 30, 45, 60, 80, 100 and 120 minutes. Additionally, cumulative bile samples were collected in pre-weighted centrifuge tubes at 30-minute intervals. After completing the perfusion, a portion of the liver was preserved in formalin for histological evaluation and the rest was flash frozen immediately in liquid nitrogen and stored at -80^0C for further analysis.

2.2.5 Determination of liver injury biomarkers

The hepatic injury biomarkers (aspartate aminotransferase; AST and alanine aminotransferase; ALT) in perfusate were measured in accordance to the manufacturer recommendations. Reagents

(R1 and R2) were reconstituted in 5:1 ratio. 96 well plate was used and loaded with 200 uL of the reconstituted reagent, then pre-warm at 37^0C for five minutes. Spectrophotometer was set zero at 340 nm. 10 uL of the sample matrix was added to the reagent mixture and incubated at 37^0C. After 1 minute, the plate was mixed and each cell absorbance at 340 nm was read and recorded. Absorbance readings were repeated every minute for four minutes. AST and ALT levels (International Unite/L) were calculated by multiplying mean absorbance difference/minute by factor 3376.2x, which is calculated by (210*1000)/(6.22*10*1). Where 1000 is the conversion factor of IU/mL to IU/L, 210 is the total reaction volume (uL), 6.22 is millimolar absorptivity of NADH, 10 is the sample volume (uL) and 1 is the light path in cm.

2.2.6 Other hepatic graft assessments

Assessment of the secretory function of the liver was determined by estimating the bile flow rate, which was calculated gravimetrically, as the difference in the weight of microcentrifuge tubes before and after 30 minutes of collection interval assuming of bile density to be 1.00 g/mL. Additionally, backpressure of the portal vein (inlet of the perfusate) was recorded throughout the perfusion duration. Wet liver weight was recorded at the end of the experiment and percentage of the total body weight (TBW) was calculated. Livers weighting 3-4% TBW were considered for further analysis.

2.2.7 Tissue histology staining

Collected liver graft tissues were fixed in 10% formalin in phosphate-buffered saline (PBS), embedded in paraffin. They were sliced into 6 um sections and stained by hematoxylin and eosin

(H&E) The necrotic areas were evaluated using morphometric analysis estimation for 3 randomly selected areas per H&E section.

2.2.8 Data Analysis

The Area Under the Perfusate Concentration-Time Curve (AUC) for ALT and AST was calculated using linear trapezoidal method. Levels at each time point and AUC of ALT and AST, bile flow rate (uL/gm/min) and the histological findings were compared between the groups. Overall, mean ± standard error of the mean (SEM) were calculated for all parameters. In single pass IPRL model, we applied student's t-test to compare between the two groups. However, in the recirculation model, the groups were compared using Analysis of Variance (ANOVA), followed by Dunnett's post hoc test for multiple comparisons in reference to the control group. P-value ≤ 0.05 were considered significant. Minimum of four rats for each group was selected to achieve adequate power with alpha = 0.05 and beta (power) = 0.8 considering 30% variability, based on previous experience, published results and assuming minimum variability of the model, and looking for 50% difference in the effect of treprostinil. We have utilized 31 rats as the total number of animals required for all of groups in this study.

2.3 RESULTS

2.3.1 Single Pass Model:

Figure 2-1 depicts the perfusate concentration-time courses of ALT and AST in the perfusate for experiments that lasted for 180 minutes. First, the levels of ALT in I/R injury group (Red triangles; also called positive controls) were substantially higher than control group (Blue circles; also referred as negative controls) and was seen to be time-dependent starting from 100 minutes onward, with around 6, 9, 3 and 2 fold increase at 100, 120, 150 and 180 minutes ($P < 0.05$), respectively. On the other hand, the difference in AST release was seen to be significant at 100 and 120 minutes only with 10 and 11 fold difference ($P < 0.05$), respectively. However, the effect of cold preservation on AST was not different in comparison to controls for the last hour. The area under the perfusate curve (AUC) for ALT and AST was significantly increased by 3 and 2.4 times ($P < 0.05$), respectively, in the I/R injury group, when compared to control group (Figure 2-2).

The bile flow rates for both positive and negative controls are calculated for a period of 30 minutes over 180 minutes of reperfusion in IPRL system (Figure 2-3). The bile flow rate in I/R injury group (0.54 uL/gm/min) started with 55% decline ($P < 0.05$) in comparison to negative controls (1.2 uL/gm/min). The bile flow rate in I/R injury group stayed significantly lower than controls for 90 minutes, but afterward the bile formation in negative controls dropped (0.42 uL/gm/min) and no significant difference was observed for the following 90 minutes between the groups.

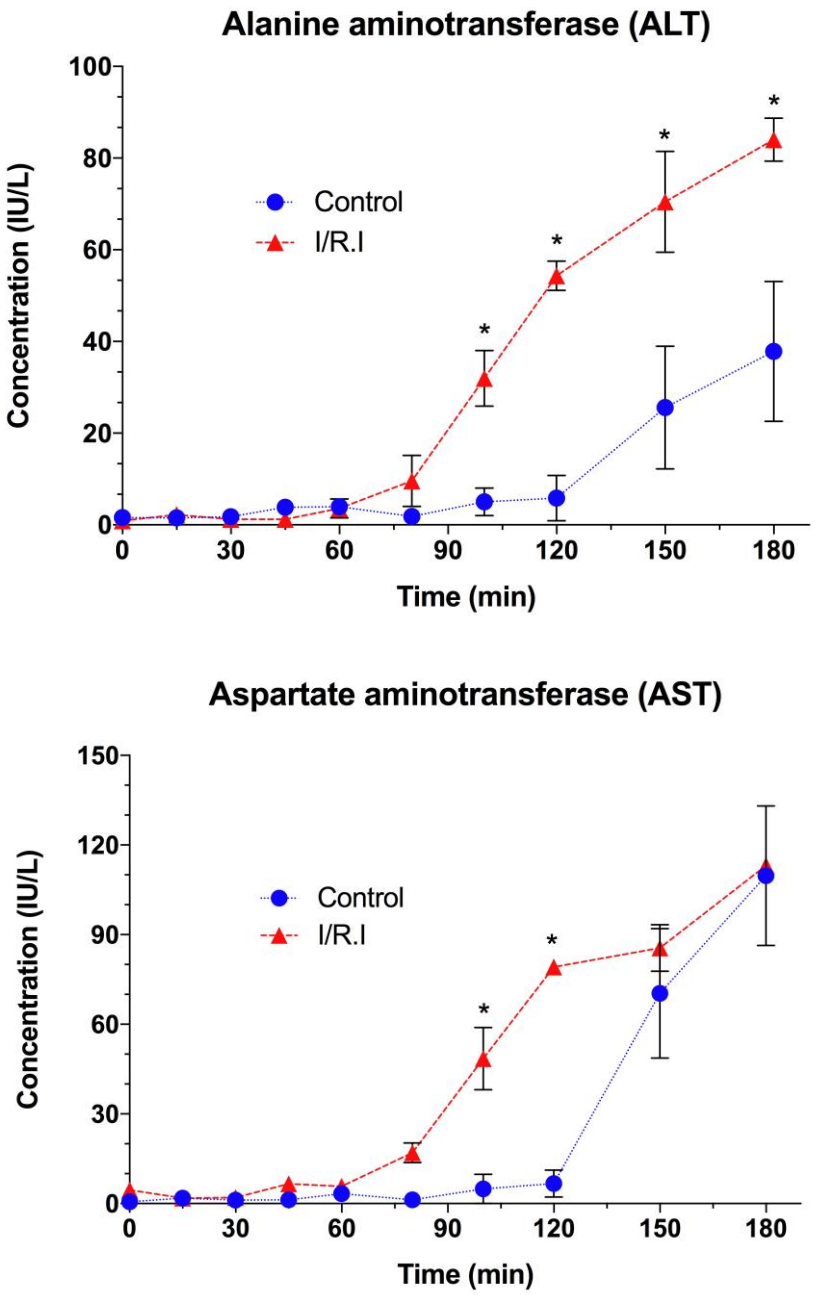


Figure 2-1: Perfusate concentration-time courses of ALT (top) and AST (bottom) in single pass IPRL.

Perfusate ALT levels over the 3 hours of reperfusion phase of the harvested rat liver grafts. Each time point is calculated and expressed as mean ± SEM. Each group composed of four rats (n = 4). * $p \leq 0.05$ compared to control.

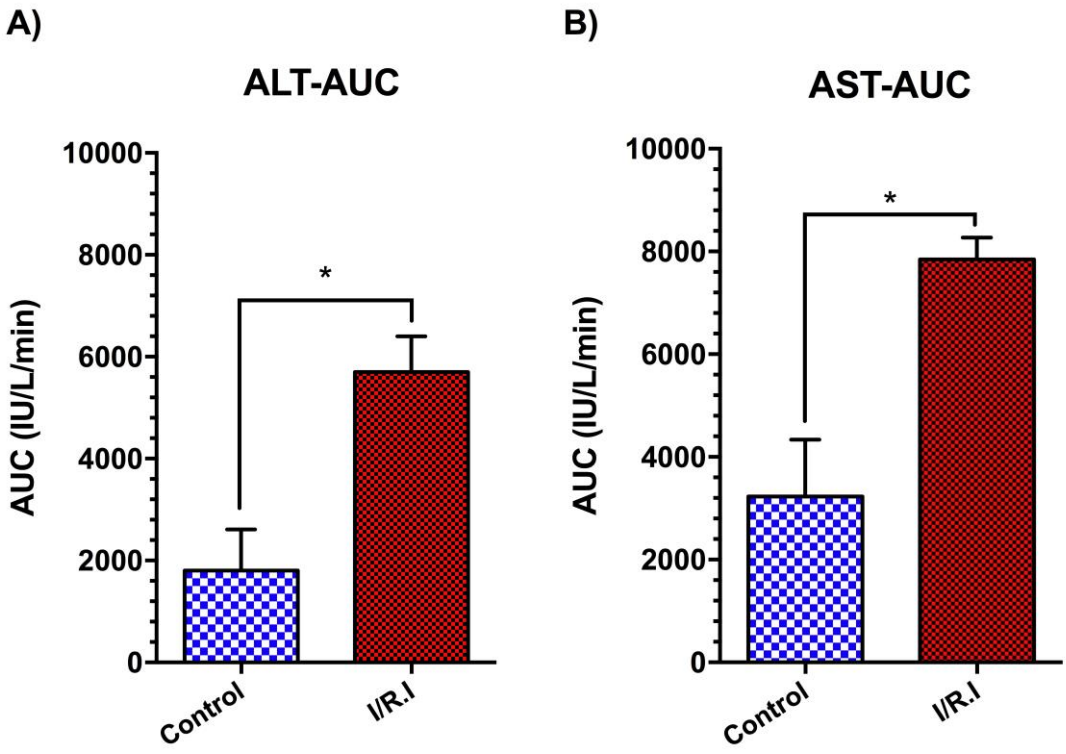


Figure 2-2: Area under the perfusate ALT and AST concentration vs. time curve for single pass IPRL.

Overall release of ALT (A) and AST (B) throughout 3 hours of reperfusion for control group vs ischemia and reperfusion injury group. Each bar is calculated from four rats in each group (n = 4), and results are expressed as mean ± SEM. *$p \leq 0.05$ compared to control.

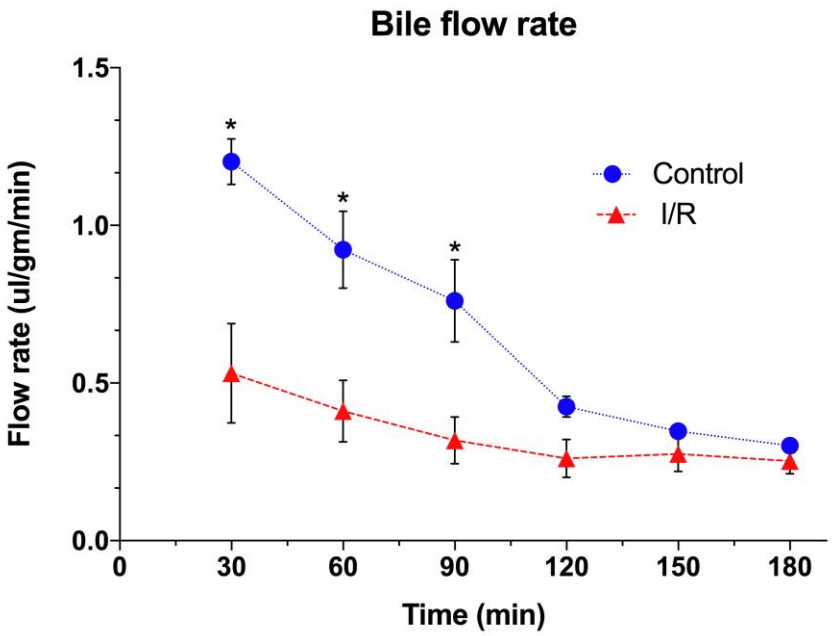


Figure 2-3: Bile flow rate for control and ischemia reperfusion groups in single pass configuration.

Bile formation rate for 30-minute duration normalized to liver graft weight. Each time point is expressed as mean ± SEM that is calculated from n = 4. *$p \leq 0.05$ compared to control.

2.3.2 Recirculation IPRL Model:

2.3.2.1 Hepatic injury biomarkers:

Figure 2-4 illustrates the time course of ALT (A; top) and AST (B; bottom) release throughout the 120 minutes reperfusion duration, where the levels of these hepatic injury markers were substantially ($P < 0.05$) increased after 24 hours cold (4^0C) preservation and warm reperfusion (Red upright triangles; IR.I group) at 80, 100, 120 minutes, when compared to livers that were reperfused in the IPRL system with no cold preservation (Blue circles; controls). However, the addition of 20 ng/mL of treprostinil in the UW solution only (Purple squares; T1) and during reperfusion (Green downward triangles; T2) diminished the cold ischemic and warm reperfusion effect that was seen in group IR.I. Furthermore, the hepatic ischemia marker, ALT (Figure 2-5A; top) was significantly increased from 835 IU/L/min in the negative controls group up to 3407 IU/L/min in the IR.I group, but stayed with no statistical difference to controls in both treprostinil groups T1 and T2 with values of 1443 and 1294 IU/L/min, respectively. AST (Figure 2-5B; bottom) was similar to ALT where cold preservation had significantly increased the AUC and treprostinil reversed this phenomenon.

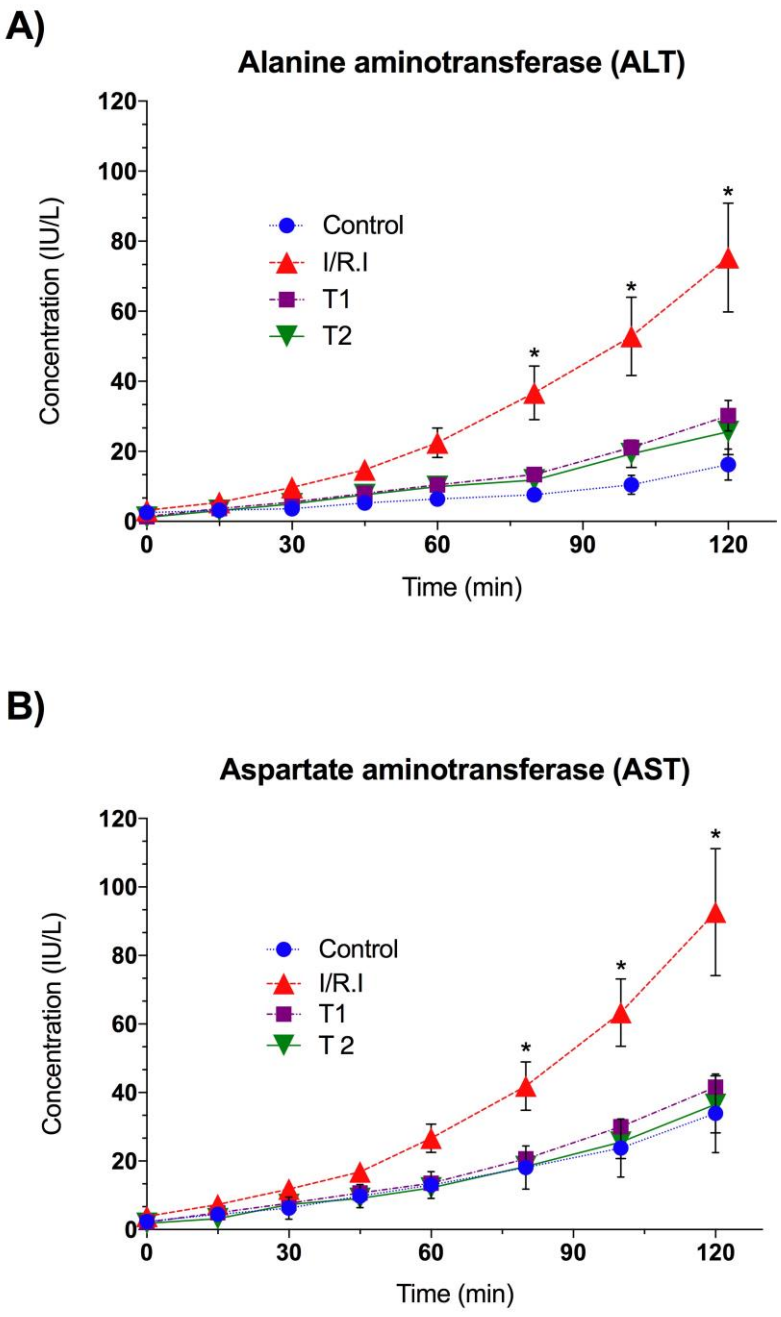


Figure 2-4: Effect of treprostinil on I/R injury based on aminotransferase perfusate levels in the recirculation perfusion configuration.

ALT (A) and AST (B) levels over 2 hours of reperfusion. Levels for controls (blue), ischemia/reperfusion injury group (I/R.I; red), treprostinil 1 (T1; purple; 20 ng/mL treprostinil supplemented in the UW only) and treprostinil 2 (T2; green; 20 ng/mL treprostinil added in the UW and perfusate). Results are expressed as mean ± SEM (n = 4-5/group). * $p \leq 0.05$ compared to control.

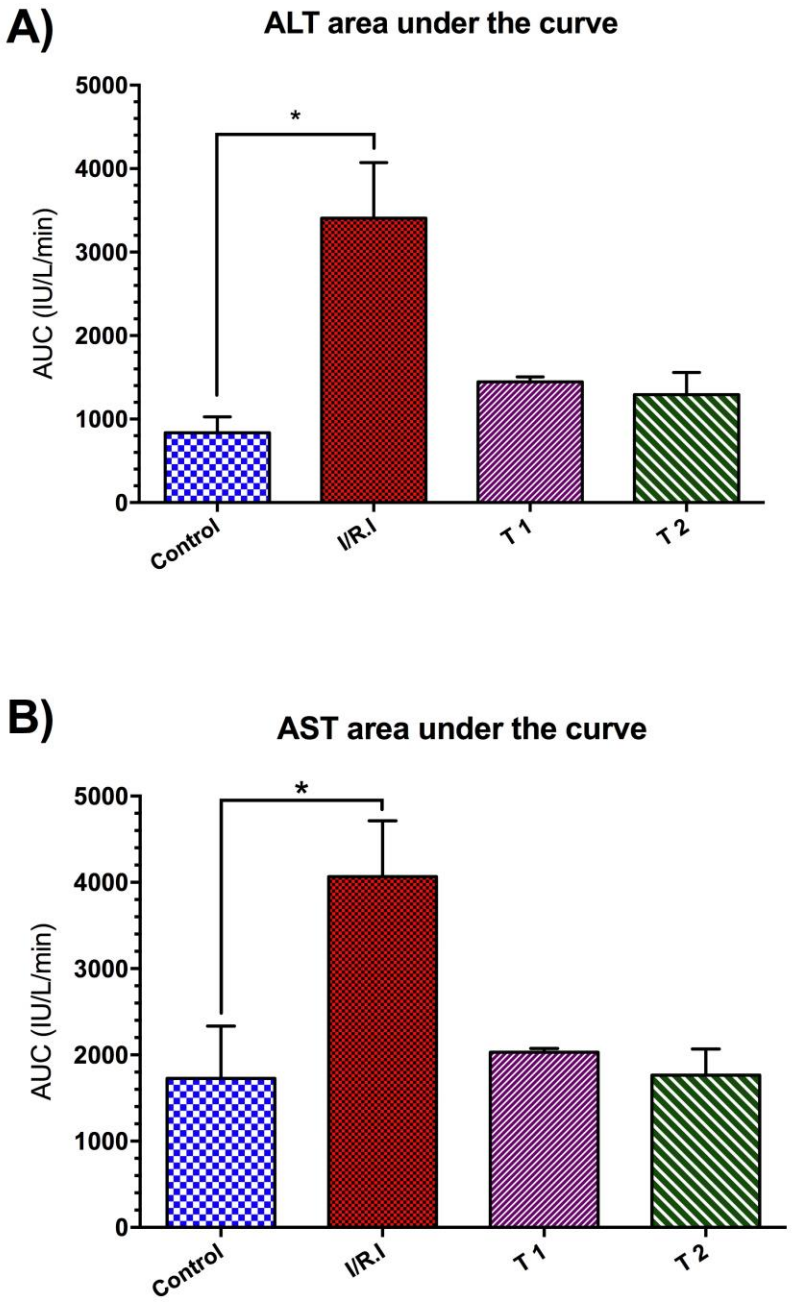


Figure 2-5: Hepatic transferases in perfusate during the recirculation perfusion in an IPRL.

Area under the perfusate ALT (A) and AST (B) concentration-time curve (AUC; IU/L/min) for controls (blue), ischemia/reperfusion injury group (I/R.I; red), treprostinil 1 (T1; purple; 20 ng/mL treprostinil supplemented in the UW only) and treprostinil 2 (T2; green; 20 ng/mL treprostinil added in the UW and perfusate).Results are expressed as mean ± SEM (n = 4-5/group). * $p \leq 0.05$ compared to controls.

2.3.2.2 Biliary flow rates and hepatic microcirculatory integrity:

The bile flow rates over every 30 minutes period for all the groups are shown in Figure 2-6 A (top). There were no statistical differences between all groups in terms of bile flow rates. Similarly, the total bile formation over 120 minutes (Figure 2-6 B; bottom) for all livers were not statistically different; however, the addition of treprostinil (20 ng/mL) while in preservation and in reperfusion significantly increased the bile flow by 44.7% compared to controls (Green triangles; T2). Overall, prolonged treprostinil exposure increased the total volume of the bile formed in those livers. Figure 2-7 shows the average difference in portal vein backpressure throughout 120 minutes of IPRL reperfusion. For control livers, this did not change with time. No statistical difference was observed for the other groups. H&E stained liver tissues after 2 hours of IPRL perfusion showed a minor effect on the control livers, which was not preserved or treated with treprostinil. Those livers were pallor at zone 3 with minimal hepatocytolysis. However, 24 hours' cold preservation showed increased pallor area and hepatocytolysis. These morphological changes were not prevented or decreased by treprostinil treatment.

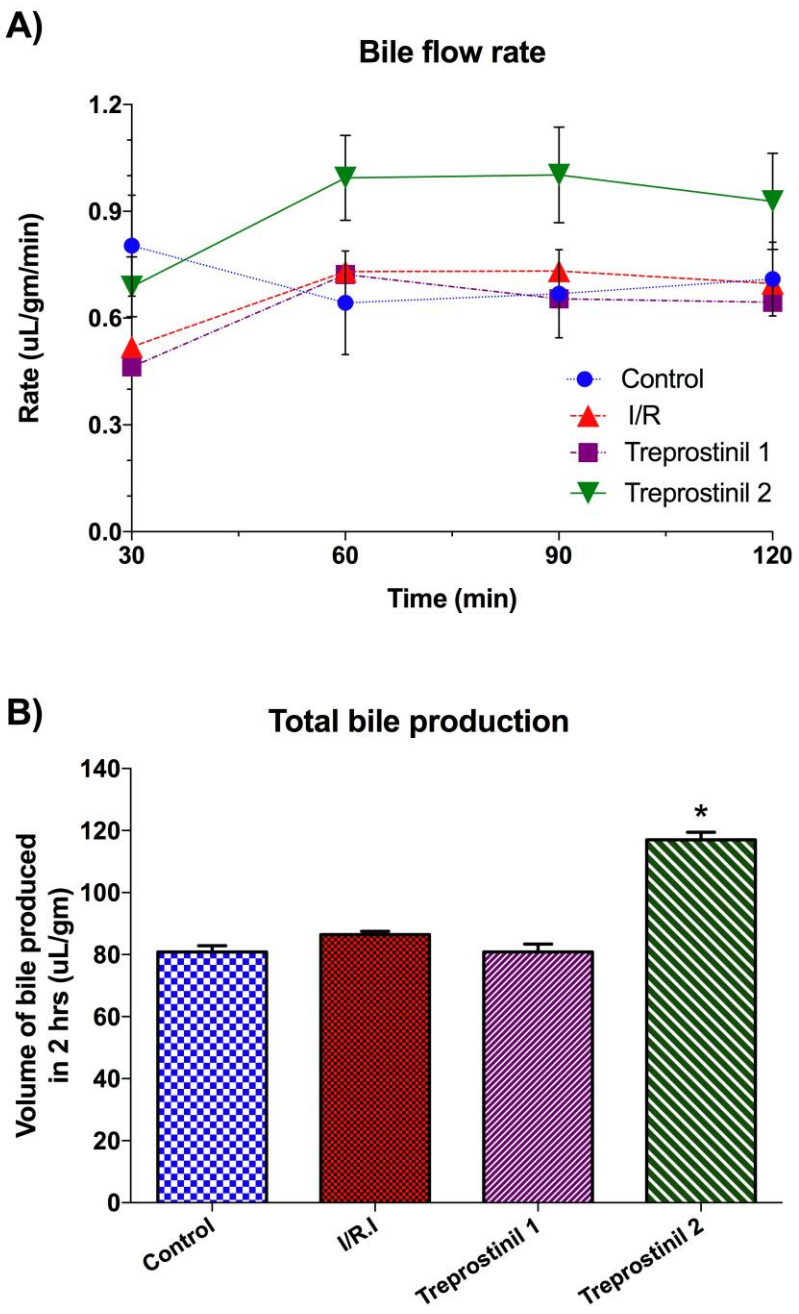


Figure 2-6: Effect of treprostinil on bile production after ischemia and reperfusion injury.

Bile production in the liver grafts was evaluated; A) 30 minutes' interval bile formation rate calculated and normalized to liver weight. B) shows the total bile formation during 120 minutes reperfusion on the IPRL system (uL/gm/120min) for controls (blue), ischemia/reperfusion injury group (I/R.I; red), Treprostinil 1 (purple; 20 ng/mL treprostinil supplemented in the UW only) and treprostinil 2 (green; 20 ng/mL treprostinil added in the UW and perfusate). Results are expressed as mean ± SEM (n = 4-5/group). *$p \leq 0.05$ compared to controls.

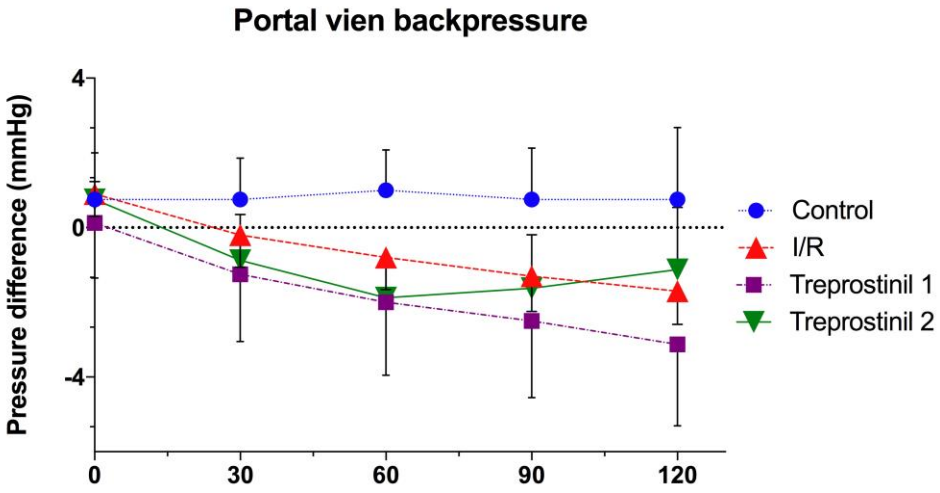


Figure 2-7: Effect of treprostinil and ischemia/reperfusion injury on portal vein backpressure.

This figure shows the difference in portal vein backpressure for 120 minutes of reperfusion. Controls (blue), ischemia/reperfusion injury group (I/R; red), treprostinil 1 (purple; 20 ng/mL treprostinil supplemented in the UW only) and treprostinil 2 (green; 20 ng/mL treprostinil added in the UW and perfusate). Results are expressed as mean ± SEM (n = 4-5/group).

2.4 DISCUSSION

Among all the experimental animal models, rats have been often recommended as the most suitable species to study hepatic I/R injury.[143] Furthermore, IPRL machine is a well-established *ex-vivo* model to study several pathophysiological and toxicological conditions, especially in I/R injury.[81, 142] The use of IPRL system comes with advantages and disadvantages when compared to other models such as rat orthotropic liver transplantation (OLT). However, for the purpose of the current study, the IPRL system was established in our laboratory. It has been shown that the use of albumin in the IPRL system significantly increased the levels of TNF-α, IL-6 and nitric oxide, which overlaps with the underlying effects of I/R injury.[139, 144] Therefore, a physiological buffer (Krebs–Henseleit bicarbonate buffer without albumin) was used in our experiments.[83]

Two IPRL configurations were tested to establish a reliable and reproducible rat hepatic I/R injury model. First, we examined a single pass model, which basically means that the perfusate passes through the liver once, and the perfusate can either be collected for further analysis or goes to waste. In this experiment, levels of the hepatic biochemical markers (ALT and AST) were significantly increased in the positive controls (I/R injury) group that was dependent on the duration of reperfusion. However, after 2 hours of perfusion, AST release promptly increased in the control group up to I/R group levels. Additionally, the bile flow rate in the livers that were stored for 24 hours' was significantly reduced at the early time points, and the control livers biliary function also rapidly declined. Other investigators also emphasized that single pass model is more preferable to carry out pharmacokinetic studies; such as to identify the extraction ratio and hepatic clearance of a molecule of interest, but may not be suitable for other studies.[141, 145] Therefore, we concluded that this model will not be ideal to investigate the protective effect of treprostinil in I/R injury. Our observations led us to optimize the second

model, where the recirculation configuration was adjusted to continue for 2 hours only. Additionally, this model mimics the physiological situation in liver transplantation, where the perfusate (~180 mL) continuously recirculates through the liver. As expected, our findings have shown that the recirculation mode of IPRL system can be successfully established and the results are comparable to observations by other investigators, where an increase in the AUC of ALT and AST by 4.5 and 3 folds, respectively, were reported.[146] This model truly simulates the conditions where both cold ischemia and warm reperfusion injury occur, instead of using only complete or partial warm ischemia models.[147] Thus, the recirculation IPRL manner was used to further investigate our study intervention (treprostinil).

The time of introduction of the pharmacological intervention to minimize I/R injury has previously been examined. Several investigators have shown some benefits in treating the donors with prostaglandins before harvesting the liver.[104, 117] In a pig OLT study, the investigators found that 75% of pigs transplanted with non-heart beating donors with 6 hours of cold ischemia developed biliary track damages that was significantly diminished (12.5%) by only treating the livers with 400 mg/kg of L-arginine during the cold preservation.[118] Previously, treating donors and recipients with treprostinil in rat OLT model has been shown to reduce transaminases, TNF-α and INF-γ suppression and increase in the hepatic blood flow.[129] In the current study, our results showed that supplementing treprostinil into the UW solution only decreases the release of aminotransferases in to the perfusate. Furthermore, for the first time here we report such a promising observation of supplementing treprostinil into the UW solution only, with a significant attenuation the cold I/R injury in an IPRL system. This approach is very suitable and practical for the clinical situation, where there is no need to treat the liver donors to get the most impact of the pharmacological agent.

The addition of treprostinil (20 ng/mL) while the liver is being perfused was to test the possibility of maximizing the protective effect of only supplementing treprostinil in the UW solution. One complication that is occasionally seen after OLTs is cholangitis, which is an inflammation of the bile ducts that leads to decreased bile formation. A study in an arterial liver deprivation as a model of ischemia in rats showed that bile flow was reduced by 40%, from 100 to 60 ul/min/kg.[148] In fact, in that study they demonstrated that number of bile duct sections were identical between the groups, which might suggest that drug transporters expression and activity may be affected in that model. Another preclinical rat study also showed significant reduction in bile flow after I/R injury.[146] In this report, we have shown that adding treprostinil during preservation and reperfusion sustains and increases bile production, which might suggest a protective role in reducing cholangitis incidence in the clinical situation. Previous studies have shown that methylprednisolone decreased the release of TNF-α into the IPRL system, but the bile formation was still decreased.[140, 149] Our findings has shown that prolonged exposure of treprostinil has significantly enhanced bile production without introducing any shortcomings.

Prostaglandin's and prostacyclin's have long been used for considerable time experimentally and clinically to minimize the effects of ischemia and sepsis.[101-103, 150] It is well-documented that PGI_2 has anti-platelet aggregation activity and inhibits leukocyte endothelial cell adhesion expression.[151] The current study has shown the beneficial use of treprostinil when added to UW solution. However, using an *in-vivo* transplant model may be important as recently published by Veres et al. (2015).[152] In conclusion, our study has shown that recirculation model of IPRL machine is suitable system to study hepatic cold ischemia and reperfusion injury. Furthermore, treprostinil in this model attenuates the effect of I/R injury when incorporated in the organ preservation solution.

3.0 EFFECTS OF ADDITON OF TREPROSTINIL IN THE PRESERVATION SOLUTION ON HEPATIC DRUG TRANSPORTER EXPRESSION AFTER COLD ISCHEMIA AND WARM REPRFUSION INURY USING IPRL

3.1 INTRODUCTION

The liver is a vital organ in the human body that is responsible for clearing xenobiotics and toxins from the body. This process involves conversion of molecules to more polar products to facilitate their elimination from the body, via phase I and II drug metabolizing enzymes, and/or active transport by phase III processes.[1] Phase III consists of drug transporters that play a major role in transferring exogenous and endogenous molecules into and out of several organs and cells. Many physiological factors and disease states can affect drug transporter expression and activity resulting in changes in the therapeutic effect due to the alterations in the pharmacokinetics of some medications. Examples include but not limited to: age, gender, pregnancy, organ disease, solid organ transplantation, medications, and genetic polymorphism.[27, 28, 30, 33, 56, 153]

Liver transplantation is the therapeutic option for several end stage liver diseases. Hepatic ischemia and reperfusion (I/R) injury is a common shortcoming that occurs when livers are harvested and preserved in cold University of Wisconsin (UW) solution. In the deceased donor liver transplant (DDLT) population, the transplanted liver grafts are more susceptible to cold and warm I/R injury, which is associated with vasoconstriction, increased release of pro-inflammatory cytokines and activation of the platelet activating factors (PAF).[64] I/R injury is associated with poor clinical outcome known as primary graft non-function (PNF) and resultant biliary complications.[59]

Several studies have shown that proinflammatory gene expression is altered and is associated with cellular damage during I/R injury. A study by Dr. Nolin's group has shown that systemic exposure of orally given fexofenadine, a nonspecific substrate for hepatic and intestinal drug transporters, was significantly increased in chronic kidney disease patients, when compared

to healthy subjects. They found a direct association between accumulation of pro-inflammatory cytokines (e.g. interleukin-6, tumor necrosis factor (TNF), interlukin-1β) and reduction in the levels of mRNA, protein, and activity.[154, 155] In another study they have shown that uremic serum incubated primary hepatocytes and enterocytes have altered the expression of P-glycoprotein (P-gp) and organic anion-transporting polypeptides (Oatp), which is in line with fexofenadine pharmacokinetic alteration.[156] It also has been shown that chronic hepatic graft rejection leads to downregulation of the expression of CYP3A4 in the intestine that was associated with an increase in the pro-inflammatory cytokines (COX2, IL-2, IL-6, IL-8, IL-10, and TNF-α).[58] In contrast, P-gp expression was increased in the intestine, which explained the higher need for immunosuppressant (cyclosporine) in this patient population. Patients undergoing DDLT have a higher potential for risk of I/R injury due to changes in pharmacokinetics of medications as they receive a large number of drugs, which are known to be either substrates or inhibitors of P-gp (ABCB1; MDR1), i.e. cyclosporine, tacrolimus, and mycophenolic acids.[53]

Understanding effect of hepatic I/R injury effect on drug transporters is important to optimize drug therapy. Phase III pathway, drug transporters, is classified into two main super families; ATP-binding cassette (ABC) and solute carrier (SLC) transporters. ABC transporters are dependent on the energy (ATP) consumption to actively uptake or efflux the drug from one side of the cell membrane to another; whereas, SLCs facilitate the passage of certain solutes (e.g., sugars, amino acids) across the membrane and actively transport other solutes against their electrochemical gradients by coupling the process with other solute or ion. Conceptually, uptake transporters help in transferring molecules into the cells, and efflux transporters pump them outside the cell. In the liver, the main uptake transporters are Na^+-taurocholate co-transporting polypeptide (Ntcp; Slc10a1), organic cation transporter 1 (Oct1; Slc22a1), organic anion

transporter 2 (Oat2; Slc22a7) and organic anion-transporting polypeptides (Oatp1a1, Oatp1a4, Oatpab2; Slco1a1, Slco1a4, Slco1b2, respectively). The hepatic efflux transporters are P-glycoprotein also referenced as multi drug resistance protein 1 (P-gp; Mdr1; Abcb1), bile salt export pump (Bsep; Abcb11), multidrug resistance-associated protein 2 (Mrp2; Abcc2) and breast cancer resistance protein (Bcrp; Abcg2). Those drug transporters are highly expressed and have been shown to be important clinically.[22-24, 157]

Earlier studies by our group have shown that treating both donors and recipients with treprostinil partially alleviated I/R injury on Abcb11 (Bsep) and upregulated Abcc2 (Mrp2) and Abcb1 (Mdr1; P-gp). In an earlier chapter we have shown that adding treprostinil while liver graft harvesting only and during reperfusion processes led to minimized injury after cold ischemia and warm reperfusion in an isolated perfused rat liver (IPRL) system. We hypothesize that adding treprostinil to the organ preservation solution (UW) will minimize the associated I/R injury effects on primary drug transporters, due to it vasodilation, cytoprotective and that proinflammatory downregulation by treprostinil will diminish the I/R injury associated effects on transplantation.

Our goal in this study was to utilize IPRL system to characterize the differential expression of major hepatic uptake (Slc22a7/Oat2, Slc22a1/Oct1, Slc10a1/Ntcp, Slco1a1/Oatp1a1, Slco1a4/Oatp1a4 and Slco1b2/Oatp1b2) and efflux (Abcb1/Mdr1a, Abcc2/Mrp2, Abcb11/Bsep and Abcg2/Bcrp) drug transporters after I/R injury and document the protective effect of supplementing treprostinil in the UW solution and during reperfusion against that injury.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals

Treprostinil (Remodulin(R)) 1 mg per mL in a 20 mL vial was provided by the manufacturer, United Therapeutics Corporation (Silver Spring, MD). CoStorSol™ (University of Wisconsin; UW) cold organ preservation solution purchased from Preservation Solutions Inc. (Elkhorn, WI). Sodium chloride, potassium chloride, calcium chloride, magnesium sulfate, potassium phosphate, sodium taurocholate, sodium bicarbonate and glucose were of analytical grade and obtained from commercial sources. QIAshredder and RNeasy Mini kits were purchased from QIAGEN (Hilden, Germany). iScript™ Reverse Transcription Supermix for RT-qPCR was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). TaqMan primers for drug transporters and housekeeping genes were purchased from Life Technologies (Carlsbad, CA). E-Gel(R) EX agarose gel (4%) catalog # G401004 and lot # H24125 manufactured by invitrogen (Carlsbad, CA) and purchased from Thermo Fisher Scientific Inc. (Waltham, MA). 10 bp DNA Ladder catalog # 10821015 and lot # 1755729 manufactured by invitrogen (Carlsbad, CA) and purchased from Thermo Fisher Scientific Inc. (Waltham, MA). E-Gel® iBase™ and E-Gel® Safe Imager™ Combo Kit catalog # G6465 by Thermo Fisher Scientific Inc. (Waltham, MA).

3.2.2 Animals

Sprague Dawley (SD) rats were purchased from Charles River Laboratories, Inc. (Wilmington, Massachusetts). SD rats were adult male 10-12 weeks' old weighing 225-250 gm. Rats were kept in the animal facility and were maintained under a 12 hours' light and dark cycle with free access

to food and water. The procedures involving animals were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and were consistent with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011, Washington, District of Columbia).

3.2.3 Study design

SD rats were randomly assigned to the following study groups: 1) Controls (no treprostinil): liver grafts were harvested then perfused in the IPRL without cold preservation. 2) Ischemia/Reperfusion group (no treprostinil): liver grafts were harvested, cold (4^{o}C) preserved in University of Wisconsin (UW) solution for 24 hours followed by IPRL perfusion. 3) Treprostinil 1 (during preservation only): rat livers were harvested and preserved in UW solution with treprostinil (20 ng/ml) for 24 hours and flushed with treprostinil (20 ng/ml) added ringers lactate solution (20 ml), then IPRL perfused without treprostinil. 4) Treprostinil 2 (during preservation and perfusion): the livers in this group went through a similar procedure to group #3, but treprostinil (20 ng/ml) was added to the perfusion solution during IPRL perfusion. 5) Blank (no treprostinil): liver grafts were harvested and preserved immediately without cold preservation or warm perfusion. The recirculation model was used for the purpose of this study.

3.2.4 Surgical procedure

Livers were harvested, preserved and perfused as previously described by Chimalakonda et al. [140, 141]. Rats were anesthetized using isoflurane and depth of anesthesia was checked by toe pinch reflex. The abdominal cavity of the rat was opened with a V-shaped incision to expose the

visceral organs. The bile duct was cannulated with PE10 tubing and the portal vein and external hepatic vein were cannulated with catheters for inlet and outlet of the perfusate, respectively. Livers were perfused with 30 ml UW solution (with or without treprostinil addition) and harvested. The isolated livers were preserved in UW solution (with or without treprostinil addition) for 24 hours at 4^{o}C. Then, livers were flushed with 20 - 30 ml cold ringers lactate solution to remove the UW solution completely and transferred to organ chamber in the IPRL system. The perfusion was carried out for 120 minutes with Krebs–Henseleit bicarbonate buffer saturated with 95%/5% O_2/CO_2 and supplemented with sodium taurocholate (4.75 mg/L).[81, 142] The livers were perfused at 3 mL/min/g liver to maintain proper oxygen supply in the absence of the proteins and blood in the perfusate. After completing the perfusion, a portion of the liver was flash frozen immediately in liquid nitrogen and stored at -80^{0}C for mRNA expression analysis.

3.2.5 mRNA isolation and purification

The extraction was performed as per the instructions of the manufacturer and described briefly as follow: on ice, 30 mg or less of liver tissue was transferred to homogenizing microcentrifuge tube. Instantly, RNeasy RLT buffer (600 uL) was added to the sample and tissue disrupted and homogenized by clean a conventional rotor–stator homogenizer. Lysate was transferred to QIAshredder column placed into 2 mL tube centrifuged at maximum speed for 2 minutes then another 3 minutes after removing the column. Only supernatant was pipetted and mixed with 600uL 70% ethanol. 700uL (then 500uL) of the mixture was transferred to RNeasy spin column and centrifuged for 15 seconds at 12,000 rpm. This was followed by RNeasy column cleaning steps; 1) 700µL with RW1 buffer (15 seconds at 12,000 rpm), 2) 500µL RPE (15 seconds at

12,000 rpm) and 3) 500µL RPE (2 minutes at 12,000 rpm). mRNA was eluted with 80uL of RNase-free distilled water (1 minute at 12,000 rpm) and kept in -80^{O}C for further analysis.

3.2.6 Quantification and assessment mRNA impurity and integrity

Sample mRNA yield and impurity assessment was performed using NanoDrop 2000c Spectrophotometer. Onto the spectrophotometer pedestal, 2 uL of RNase free water was loaded to blank the instrument; then 2 uL of sample was added to measure the RNA concentrations and levels of impurities presence in the sample. Agilent RNA 6000 nano kit and Agilent 2100 Bioanalyzer instrument were used to test the integrity of the mRNA samples. In brief, 9 uL premixed gel-dey mixture and 5 uL of RNA marker were added to the appropriate wells then ladder and samples were added to their corresponding wells. Chip was vortexed for 1 minute at 2400 rpm and samples read in 2100 bioanalyzer immediately. Each sample was assigned an RNA Integrity Number (RIN).[158, 159]

3.2.7 cDNA preparation and real time qPCR running

iScript™ Reverse Transcription Supermix for RT-qPCR was used to generate cDNA from mRNA samples. We mixed 1 ug of total RNA with 4 uL of iScript RT supermix, then adjusted the volume to 20 uL with RNase free water. Then, incubated the mix in the following thermal cycles; 5 minutes at 25^{O}C for priming; 30 minutes at 42^{O}C to reverse transcriptase; then 5 minutes at 85^{O}C to inactivate the transcriptase enzyme. Yielded cDNA samples were diluted with 80 uL RNase free water. Real-time PCR reaction was run in 96 well plate that contained 20 uL of the reaction mix, 1 uL of TaqMan primer, 10 uL of TaqMan master mix, 4 uL of cDNA

and 5 uL of RNase-free water. Plates were covered and centrifuged at 3000 rpm for 3 minutes and temp 4^{O}C. Applied Biosystems® 7500 Real-Time PCR System was used to amplify and detect targeted genes. Initially, uracil DNA glycosylase (UDG) incubation was performed at 50^{O}C for 2 minutes, then AmpliTaq Gold® enzyme activation at 95^{O}C for 10 minutes, followed by 40 cycles of 95^{O}C for 15 seconds and 60^{O}C for 1 minute to denature the DNA strands and extend them, respectively. All genes were run in the same plate for each sample to minimize sample variability.

3.2.8 Primers efficiency and specificity

A pooled cDNA sample was prepared from all experiments. Then, serial dilutions of the pooled sample were made to test the efficiency of each used primer. We have used four fold dilutions for primers that have a cycle threshold (Ct) of around 24 and two folds' dilutions for primers with Ct around 30. Each dilution level was run in triplicates and all dilutions were run in the same plate for each primer to minimize variability. We ran all RT-qPCR plates similar to steps described in section 3.2.7.

Samples from the amplified RT-qPCR wells were run on 4% agarose gel to identify and confirm the primer identities by size. In each well, 5 uL of RT-qPCR amplified DNA was mixed with 15 uL of deionized water, for a total volume of 20 uL. For the 10 bp DNA ladder, 2 uL of 1.0 ug/uL was mixed with 18 uL of deionized water. Samples and ladder were loaded into the wells of pre-casted 4% E-Gel EX, agarose gel. Empty wells were loaded with 20 uL deionized water. Gels were placed on iBase™ then were run the electrophoresis for approximately 12 minutes. Bands were visualized and photographed by the E-Gel® Safe Imager™.

3.2.9 Data analysis

Standard curves for each primer was generated to assess the efficiency. A standard curve was generated using a two or four folds' dilutions of a template amplified on real-time qPCR system. Each dilution was assayed in triplicates. Standard curve with the Ct plotted against the log of the starting quantity of a template for each dilution. The amplification efficiency (E) is calculated from the slope of the standard curve using the following formula:

$$\text{Equation 3-1: } E = 10^{-1/\text{slope}}$$

We have converted the amplification efficiency (E) into a percentage efficiency (E%) using the formula:

$$\text{Equation 3-2: } E\% = (E - 1) \times 100$$

The relative levels of mRNA expression of transporters were normalized with the copy number of β–actin. The relative levels of mRNA fold changes of all genes were quantified using the $2^{-\Delta\Delta Ct}$ method.[160] Groups were compared to controls utilizing analysis of variance (ANOVA) then applied Bonferroni post-hoc test. A minimum of 4 rats in each group was used and each RT-qPCR run were carried out in triplicates. All data were expressed as mean ± standard error of the mean (SEM).

3.3 RESULTS

3.3.1 mRNA sample quality assessment

All mRNA samples were thoroughly assessed by several methods to assure that all real-time quantitative polymerase chain reaction (RT-qPCR) runs are appropriate and reproducible. NanoDrop was used to quantify the sample mRNA concentrations and to measure its purity. The mean ± standard deviation of the RNA concentrations in the 20 samples was 1.12 ± 0.19 ug/uL (range 0.79 - 1.48 ug/uL), please see Table 3-1 for more details. Additionally, the 260/280 nm ratio 2.05 ± 0.02 and 260/230 nm ratio 2.08 ± 0.17 showed that the samples are pure, where generally ratios of ~1.8 and 2.2 is considered acceptable. The integrity of mRNA was tested by using electrophoresis, where 18S and 28S fractions are separated and quantified by Agilent 2100 Bioanalyzer. Figure 3-1 shows the results of electrophoresis run for all samples and Figure 3-2 shows a representative electropherograms for highest and lowest integrity samples. An algorithm was used to calculate the RNA Integrity Number (RIN) from the information gathered for each sample electropherograms. Our samples RIN values were between 7 and 9.6 which indicates a very good integrity of RNA, where RIN values could vary from 0 (very poor) up to 10 (excellent quality). All of the details found in Table 3-2.

Table 3-1. Shows the concentration and impurity RNA extracted results from NanoDrop. Absorbance at 260 (A260) is used to calculate the RNA concentration in the sample. The purity and contamination of the RNA sample are evaluated by the ratios 260/280 and 260/230, respectively.

ID	*Sample*	*Concentration (ug/uL)*	*Purity (260/280)*	*Contamination (260/230)*
1	Blank	1.25	2.03	2.24
2	Blank	1.18	2.02	2.20
3	Blank	0.79	2.03	1.95
4	Control	1.04	2.05	1.90
5	Control	1.20	2.05	2.26
6	Control	1.17	2.05	2.19
7	I/R injury	1.15	2.05	2.18
8	I/R injury	0.95	2.07	1.97
9	I/R injury	0.81	2.09	1.81
10	Treprostinil 1	1.28	2.06	1.90
11	Treprostinil 1	1.02	2.07	1.81
12	Treprostinil 2	0.99	2.07	2.16
13	Treprostinil 2	1.45	2.04	2.20
14	Treprostinil 2	1.21	2.05	1.95
15	Control	1.48	2.05	2.19
16	I/R injury	0.94	2.03	2.29
17	Treprostinil 1	1.24	2.07	1.77
18	Treprostinil 1	1.33	2.06	2.19
19	Treprostinil 2	0.98	2.03	2.23
20	I/R injury	0.95	2.04	2.22

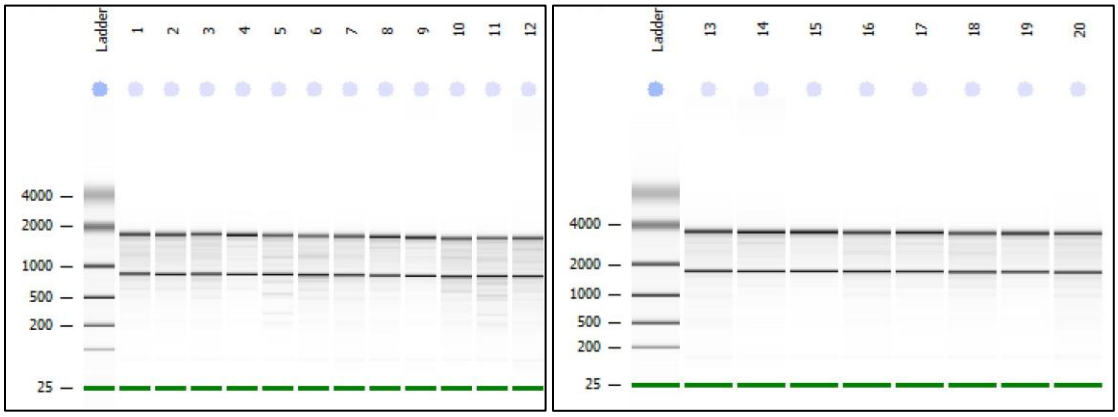


Figure 3-1: Electrophoresis of the RNA samples.

This figure shows the separation of nucleic acid fragments, 18S and 28S, based on their size as they are driven through it electrophoretically.

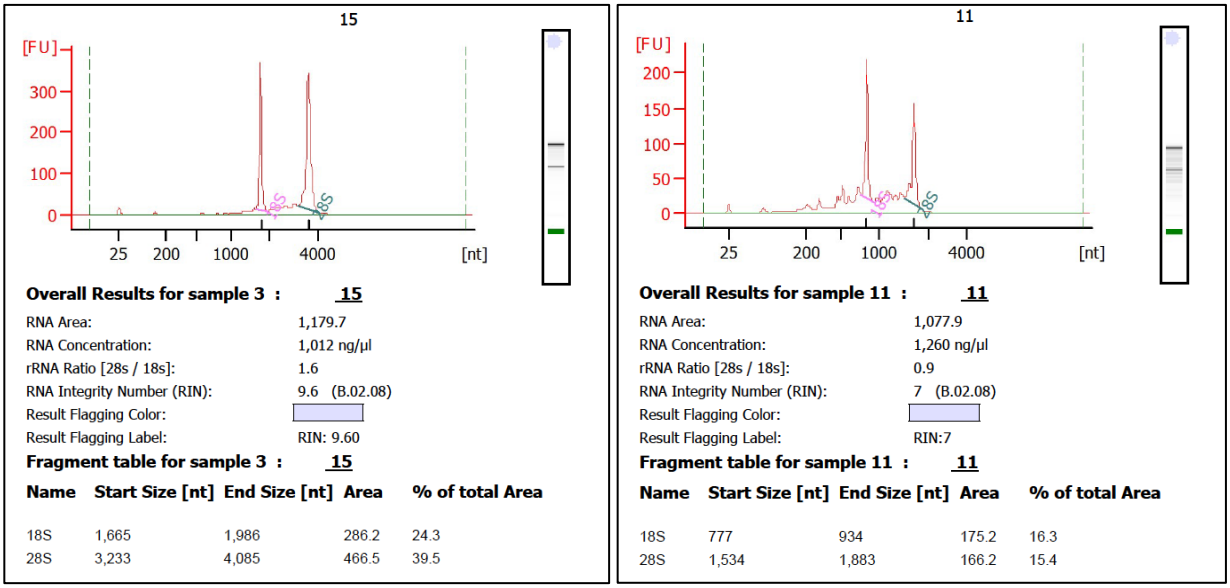


Figure 3-2: Details of two representative electropherograms for highest and least integrity (RIN) samples.

Box # 15 shows the details for the analysis of sample with RIN = 9.6 and box #11 shows sample with RIN = 7.0.

Table 3-2. List of RNA Integrity Number (RIN) score for all samples used. All samples used for RT-qPCR was analyzed and the highest RIN means most the least degradation of the RNA.

Sample ID	*Sample name*	*RIN*	*Sample ID*	*Sample name*	*RIN*
1	Blank	8.9	*11*	Treprostinil 1	7
2	Blank	8.6	*12*	Treprostinil 2	7.5
3	Blank	8	*13*	Treprostinil 2	8.5
4	Control	9.3	*14*	Treprostinil 2	9.1
5	Control	8.1	*15*	Control	9.6
6	Control	7.7	*16*	I/R injury	8.8
7	I/R injury	8.1	*17*	Treprostinil 1	9.1
8	I/R injury	8.6	*18*	Treprostinil 1	8.5
9	I/R injury	9.3	*19*	Treprostinil 2	9.3
10	Treprostinil 1	8	*20*	I/R injury	8

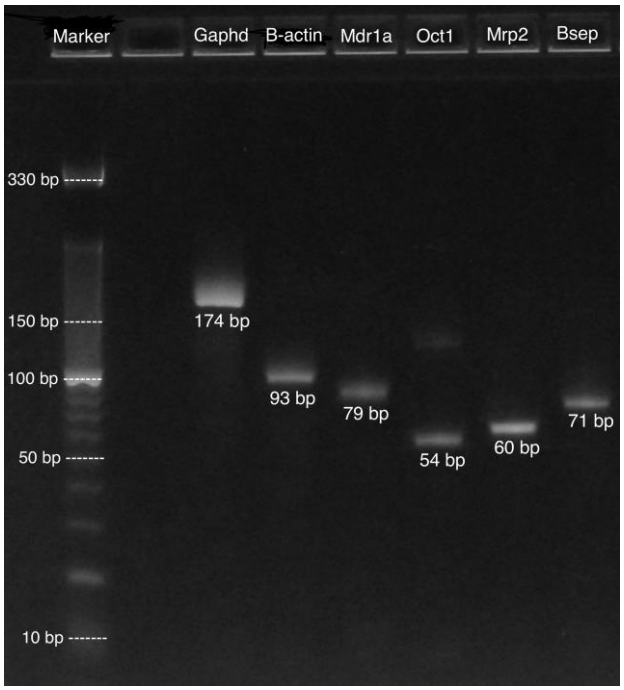
3.3.2 Primers efficiency

TaqMan primers were validated in the lab for the used ten hepatic drug transporters and two housekeeping genes. Table 3-3 lists the names, symbols, amplicon sizes and efficiencies of the gene primers. All genes were verified by running 4% agarose gels electrophoresis for the RT-qPCR products to ensure the specificity and size of each amplicon. Additionally, gels were visually examined for any extra bands in each well to make sure there is no contamination in the amplification of each primer (Figure 3-3). Standard curves to quantify the primer efficiency values were generated by plotting cycle threshold (Ct) versus the log value of the serial concentrations. The slope was used to calculate the efficiency using the equations described in the methodology. The slope and coefficient of determination (R^2) values are shown in all of the twelve standard curves in both Figure 3-4 and Figure 3-5. The percentage efficiency values for the primers used were between 90 and 100%, except for Bcrp (Abcg2; 84.3%) and Gapdh (84.7%). Our results meet or exceed the guideline of minimum information for publication of quantitative real-time PCR experiments (MIQE).

Table 3-3. List of gene names, symbol, amplicon size and amplification efficiency of primers used. Efficiency is calculated by serial dilutions (below details). All other information is supplied with TaqMan primers.

Gene	*Gene symbol*	*Assay ID*	*Amplicon size*	*Efficiency*
Glyceraldehyde-3-Phosphate Dehydrogenase	Gapdh	Rn01775763_g1	174	84.7%
β-actin	LOC681152	Rn01424440_s1	93	92.7%
Multi-drug resistance 1a (Mdr1a)	Abcba1	Rn01639253_m1	79	93.8%
Multidrug resistance-associated protein 2 (Mrp2)	Abcc2	Rn00563231_m1	60	95.5%
Breast cancer resistance protein (Bcrp)	Abcg2	Rn00710585_m1	94	84.3%
Bile salt export pump (Bsep)	Abcb11	Rn01515444_m1	71	90.8%
Na^+-taurocholate co-transporting polypeptide (Ntcp)	Slc10a1	Rn00566894_m1	63	90.6%
Organic cation transporter 1 (Oct1)	Slc22a1	Rn00562250_m1	54	92.5%
Organic anion transporter 2 (Oat2)	Slc22a7	Rn00585513_m1	54	95.9%
Organic anion-transporting polypeptide 1a1 (Oatp1a1)	Slco1a1	Rn01463125_m1	93	99.8%
Organic anion-transporting polypeptide 1a4 (Oatp1a4)	Slco1a4	Rn00756233_m1	134	94.5%
Organic anion-transporting polypeptide 1b2 (Oatp1b2)	Slco1b2	Rn01492635_m1	79	90.1%

A)



B)

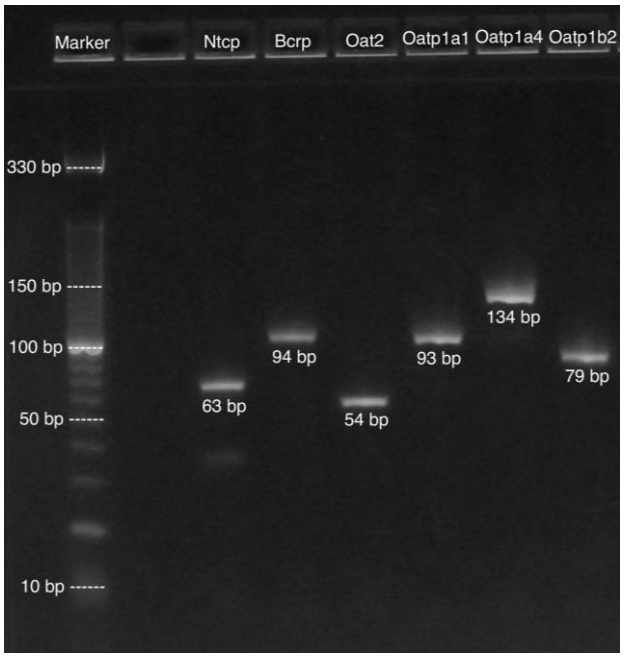


Figure 3-3: 4% Agarose gel runs for primers after RT-qPCR runs.

A) Includes runs for 10 bp ladder, Gapdh, β-actin, Abcb1/Mdr1a, Slc22a1/Oct1, Abcc2/Mrp2 and Abcb11/Bsep; B) Includes runs for 10 bp ladder, Slc10a1/Ntcp, Abcg2/Bcrp, Slc22a7/Oat2, Slco1a1/Oatp1a1, Slco1a4/Oatp1a4 and Slco1b2/Oatp1b2.

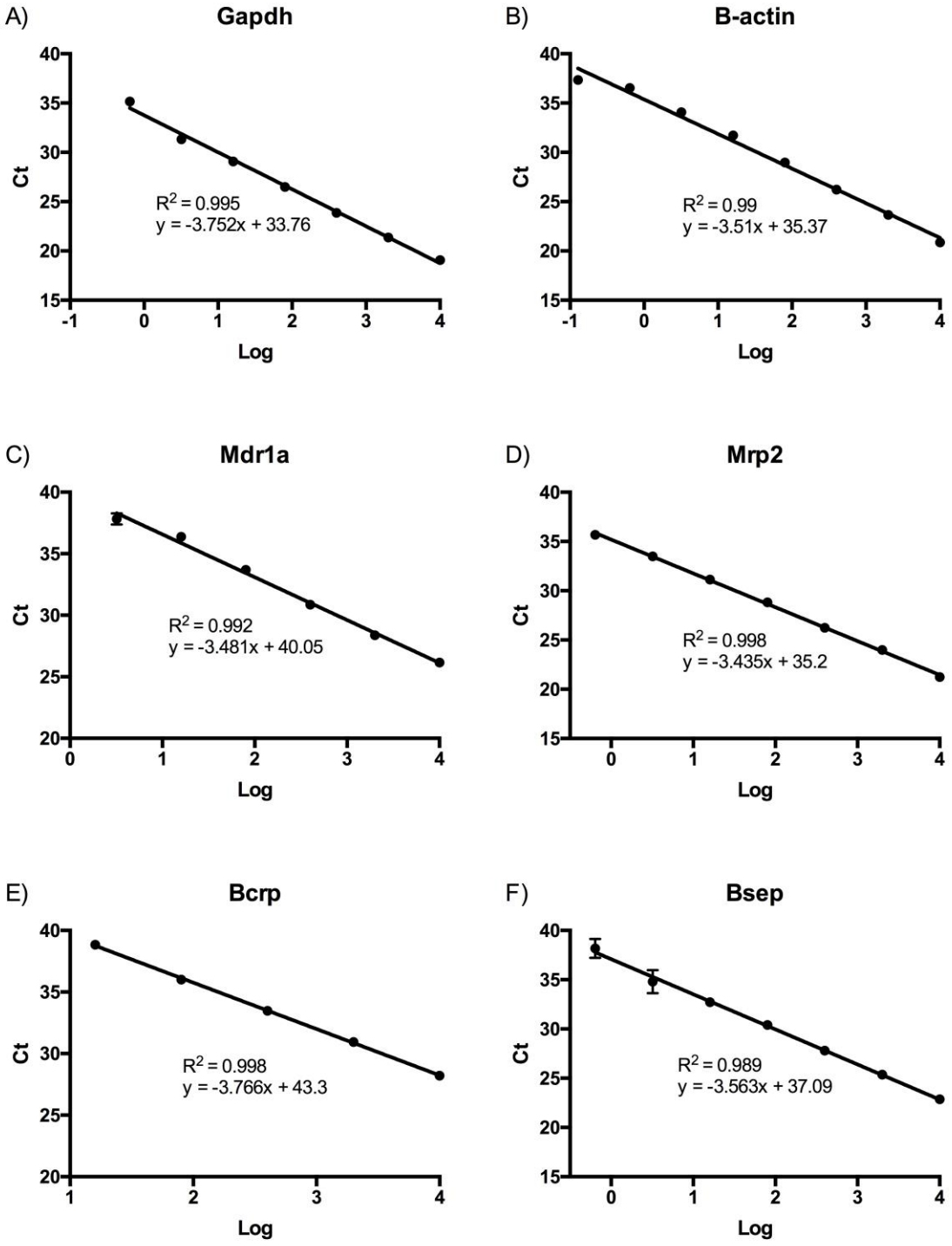


Figure 3-4: TaqMan primers efficiency curves for housekeeping genes (A-B) and efflux transporters (C-F).

Standard curve was generated using several serial dilutions of a template amplified in real-time qPCR system. Standard curves with the cycle thresholds (Ct) plotted against the log concentration of the serial dilutions. Equation for the regression line and the R^2 value are shown in each graph. Each dilution point was assayed in triplicates.

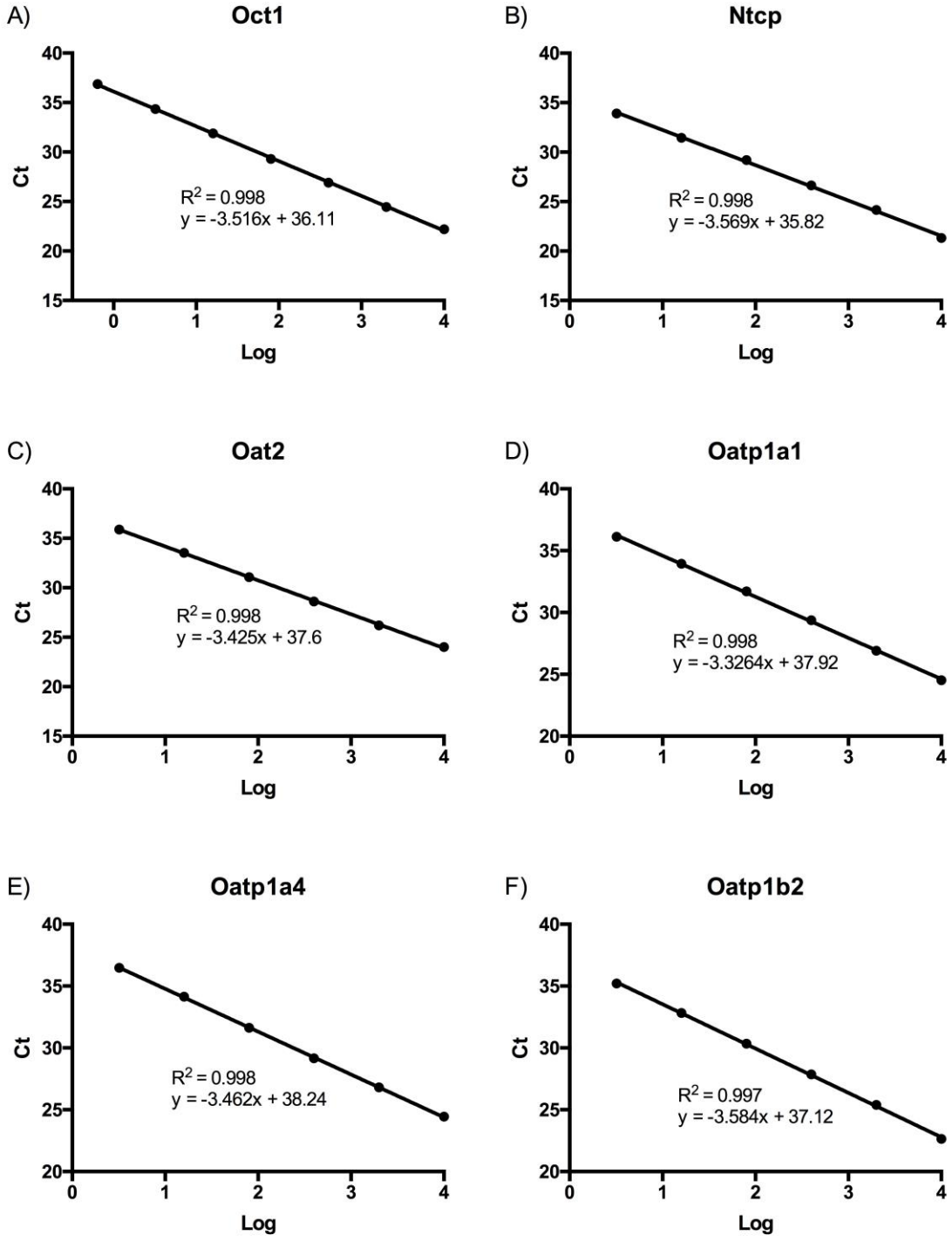


Figure 3-5: TaqMan primers efficiency curves for six uptake transporters (A-F).

Standard curve was generated using several serial dilutions of a template amplified in real-time qPCR system. Standard curves with the cycle thresholds (Ct) plotted against the log concentration of the serial dilutions. Equation for the regression line and the R^2 value are shown in each graph. Each dilution point was assayed in triplicates.

3.3.3 Effect of I/R injury and treprostinil on uptake transporters

The effect of twenty-four hours cold preservation and two hours of warm IPRL perfusion on primary uptake drug transporter gene expressions were measured using RT-qPCR (Figure 3-6). First of all, 2 hours of IPRL warm perfusion (control group) of harvested livers significantly downregulates the expression of Slc10a1/Ntcp, Slc22a1/Oct1, Slc22a7/Oat2 and Slco1b2/Oatp1b2 by at least ~40%, when compared to blank group, where the liver was collected and frozen with no ischemia or perfusion. However, Slco1a1/Oatp1a1 and Slco1a4/Oatp1a4 expression did not change after warm reperfusion.

The effect of twenty-four hours' cold ischemia followed by two hours of warm reperfusion was evaluated in group I/R.I. These livers showed that cold ischemia and warm reperfusion significantly increases the mRNA expression of Slc10a1/Ntcp, Slc22a1/Oct1 and Slc22a7/Oat2 by 1.42, 1.76 and 1.47 folds relative to controls (warm perfusion). However, all measured organic anion-transporting polypeptide 1a1, 1a4 and 1b2 in the liver grafts were not affected by I/R injury.

Adding 20 ng/mL of treprostinil in to the UW solution during liver preservation the I/R injury mediated effect on Slc10a1/Ntcp and Slc22a1/Oct1 was diminished. However, Slc22a7/Oat2 was upregulated after the cold ischemia and continued to be upregulated after treatment with treprostinil. Other transporters (Oatp1a1, 1a4 and 2b1) were not affected in our experiments by either cold ischemia or treprostinil treatment. Further supplementation of treprostinil showed similar results to the group of livers that were preserved with treprostinil.

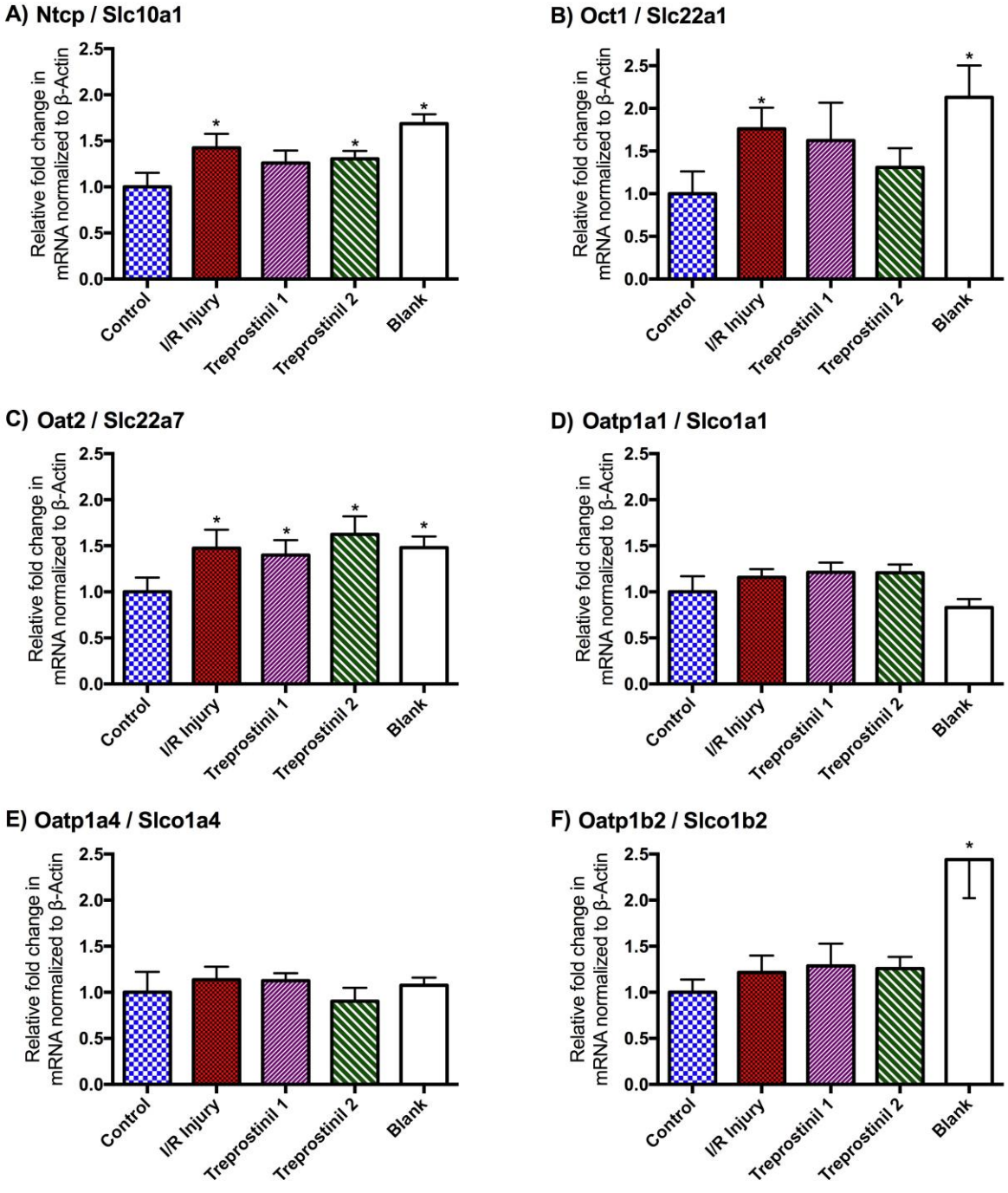


Figure 3-6: Effect of cold ischemia, warm reperfusion and treprostinil treatment on expression of uptake transporters.

Expressions of Slc10a1, Slc22a1, Slc22a7, Slco1a1, Slco1a4 and Slco1b2 were measured by RT-qPCR and relatively normalized to the mRNA expression of β-actin. n equals 4 in each group and each one was assayed in triplicates. Results are expressed as mean ± SEM. * $p \leq 0.05$ compared to control.

3.3.4 Effect of I/R injury and treprostinil on rat liver canalicular membrane transporters

The effect of warm reperfusion, cold I/R injury and treatment with treprostinil on the expression of four efflux drug transporters (shown in Figure 3-7). Multidrug resistance protein 1a (Mdr1a; P-gp; *Abcb1*) was not affected by 2 hours of warm perfusion in the IPRL when compared to blank tissues. However, the cold I/R injury significantly decreased the mRNA levels by 46.7% when compared to controls but this effect was attenuated when 20 ng/mL of treprostinil was added to the UW solution. The gene expression of *Abcb1*; Mdr1a was similar to normal tissues when treprostinil was supplemented in the UW solution and during the reperfusion.

Multidrug resistance-associated protein 2 (Mrp2) and bile salt export pump (Bsep) were downregulated by approximately 67% and 44%, respectively, as an effect of warm perfusion in the IPRL system and continued at the same levels throughout other experimental conditions, cold preservation and treatments with treprostinil. On the other hand, Bsep was significantly upregulated when treprostinil (20ng/mL) was incorporated into the preservation solution only. The expression of breast cancer resistance protein (Bcrp; Abcg2), was not changed after 2 hours of warm perfusion and 24 hours of cold ischemia. However, treating the harvested livers with 20 ng/mL of treprostinil significantly increase the expression of Abcg2 (Bcrp) by 56%.

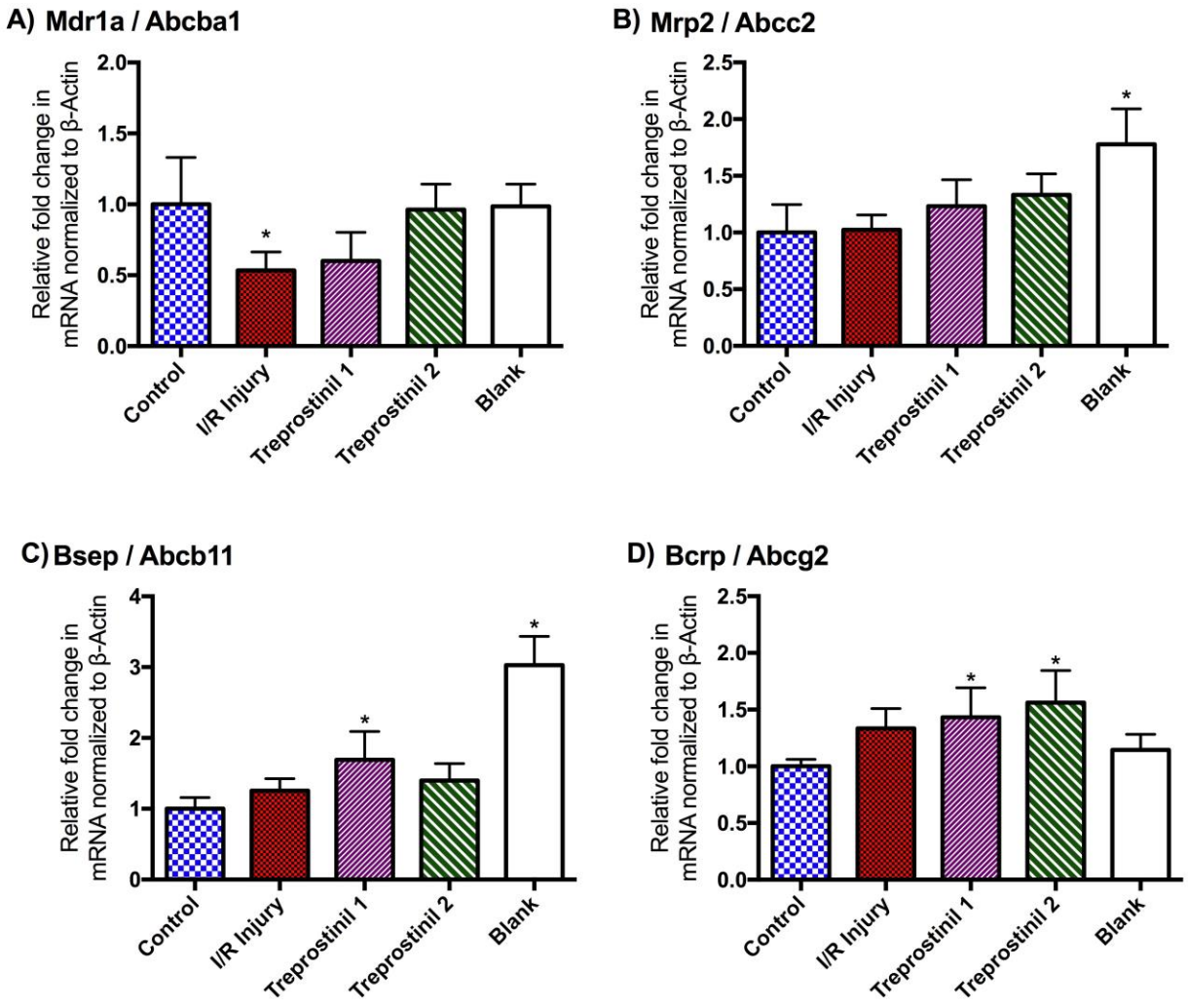


Figure 3-7: Effect of 24 hours of cold preservation, 2 hours of warm reperfusion and treprostinil treatment on expression of efflux transporters.

Expressions of (Abcb1, Abcc2, Abcb11 and Abcg2 were measured by RT-qPCR and relatively normalized to the mRNA expression of β-actin. n equals 4 in each group and each one was assayed in triplicates. Results are expressed as mean ± SEM. *$p \leq 0.05$ compared to control.

3.4 DISCUSSION

Liver injury is triggered by various causes such as trauma, infections, physical and chemical stress and more importantly here the ischemia and reperfusion injury. Hepatic injury is associated with increase in pro-inflammatory cytokines, leading to higher morbidity and mortality of the patients due to multiple organ dysfunction.[161-163] Local insult to the liver by static cold preservation and warm reperfusion contributes to the hypoxic damage to the liver followed by severe oxidative damage during the reperfusion phase, that activates inflammatory pathways leading higher reactive oxygen species in the organ.[161] I/R injury is known to alter phase I, II and III drug metabolizing enzymes. However, the magnitude of injury is depend on models used, ischemia duration (short versus long), time of reperfusion and specific enzymatic pathways.[147, 149, 164]

Previous studies have shown that ischemia decreases the mRNA levels of Slc10a1/Ntcp, Slco1a1/Oatp1a1, Slco1a4/Oatp1a4, Abcb11/Bsep, and Abcc2/Mrp2. Expression of these drug transporters were significantly decreased followed 24 hours of arterial ischemia.[148, 165, 166] In contrast, other study demonstrated that Mrp2 was upregulated acutely after 60 mins of local ischemia by clamping portal vein, hepatic artery and bile ducts.[167] Fouassier *et. al.* (2007) showed that gene expression of drug transporters and transcriptional factors, such as hepatic nuclear factor (HNF4α), retinoid X receptor (RXRα), and farnesoid X receptor (FXR), were reduced in rat hepatocytes subjected to hypoxia. However, cystic fibrosis transmembrane conductance regulator (Cftr) gene, which is important transporter for cholangiocytes, was induced by approximately four times in the ischemia rats compared to controls.[148] Furthermore, activation of the those nuclear receptors by some medications, such as phenobarbital and rifampicin, is used to treat hepatobiliary drug transporters in cholestasis.[168] Another study

showed that 12 hours cold preservation suppresses the levels of mRNA drug transporters and proteins more after 1 hour cold preservation prior rat orthotopic liver transplantation (OLT). This effect was more pronounced on day one post OLT compared to day 3 and 7.[169] Inflammation, particularly sepsis, is known to downregulates the hepatic drug transporters in both clinical setting and in preclinical studies in rats.[170, 171] Kupffer cells activation, by partial hepatic ischemia, triggers alteration in the cytokines levels and mRNA expression of hepatic transporters. These effects were inhibited by rats pretreated with gadolinium chloride, due to its ability to deplete kupffer cells.[165]

In our study, we have documented that prolonged cold ischemia and acute warm reperfusion induced the gene expression for some solute carrier transporters in the apical sinusoidal membranes. The mRNA expression of the three basolateral uptake drug transporters Slc10a1/Ntcp, Slc22a1/Oct1 and Slc22a7/Oat2 after 24 hours' cold static preservation and warm reperfusion was statistically increased in our study. However, P-gp transporter expression was decreased after 24 hours of cold ischemia and after 2 hours' reperfusion. This phenomena was in line with the reduced expression and activity of the P-gp after reperfusion of livers that were subjected to either 60 min of partial ischemia, endotoxins (lipopolysaccharide (LPS)) and IL-6.[145, 172, 173] Furthermore, Thorling et. al. (2014) found that P-gp (Mdr1a; Abcb1) was significantly downregulated after prolonged I/R injury.[164] Around 10% - 40% of liver transplant recipients develops biliary complications that are associated with up to 15% mortality rate. Zhu et. al. (2012) have shown that at least 20 minutes of warm ischemia in rats significantly injured the biliary ducts, which was associated to a higher apoptosis index.[174] As a result of the alteration in the gene, protein and activity of drug transporters which night have a direct impact on poor therapeutic treatment and higher incidence of side effects.

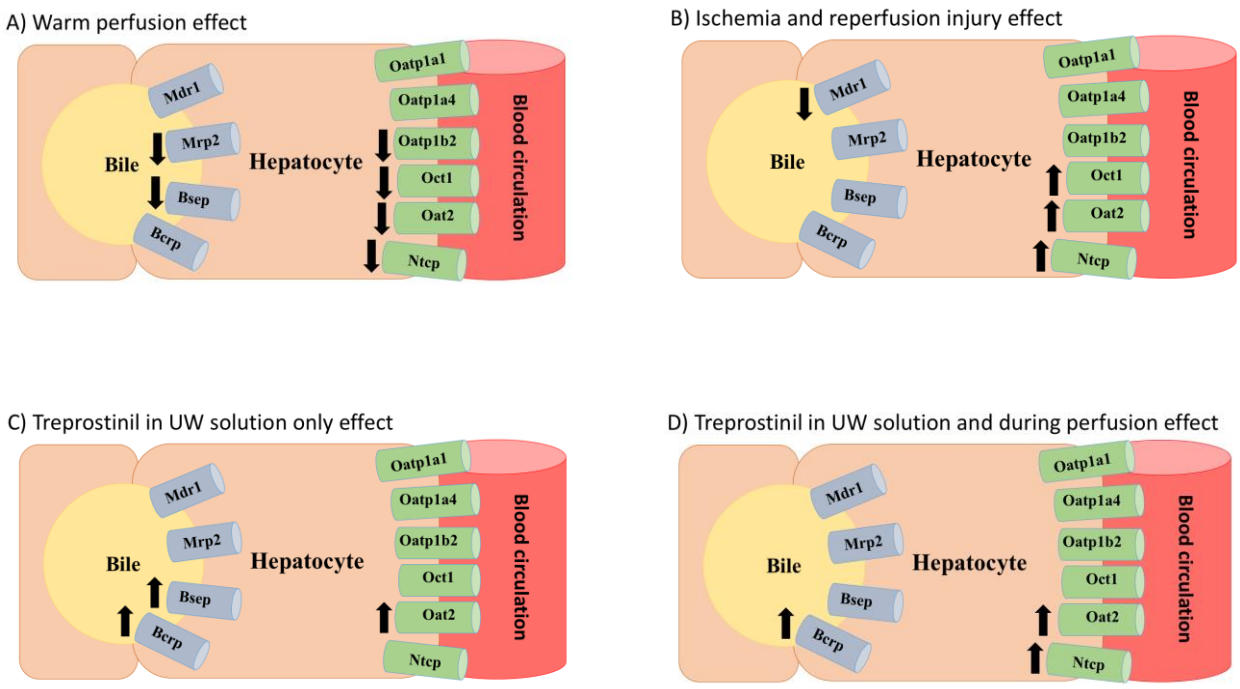


Figure 3-8: Effect of warm perfusion, I/R injury, treprostinil supplement in the UW solution and treprostinil in the UW solution and during perfusion of drug transporters.

A) shows the effect of 2 hours warm perfusion in comparison to blank liver tissues. B) indicates the effect of 24 hrs cold preservation and 2 hrs of warm perfusion compared to only warm perfusion. C) and D) shows the differential expressions of drug transporters when treprostinil was used in the UW solution and during perfusion, respectively, relative to 2 hrs of warm perfusion only.

Our study drug (treprostinil) a synthetic stable prostacyclin (PGI_2), abolished the response that was initiated after the cold ischemia and perfusion injury for almost all of the affected parameters. Additionally, it prevented the upregulation of the uptake transporters (both sodium taurocholate co-transporting polypeptide and organic cation transporter-1). This protective effect was increased when the exposure to treprostinil was prolonged, since one group was only treated during preservation and the other group was treated with treprostinil in the preservation fluid and in the reperfusion medium. Similarly, the effect on apical efflux transporter (P-gp; Mdr1a; Abcb1a) was significantly minimized by treprostinil and it was brought to normal levels when treprostinil was supplemented in the preservation solution and during perfusion. Our findings are in line with the results previously reported by Ghonem (2010), where Abcb11 (Bsep) expression was restored after treating donor and recipient rats using OLT model. Furthermore, Mrp2 and P-gp were significantly up-regulated.[131] Our findings and Ghonem studies were accompanied with proof of reduced I/R injury, as measured by ALT and AST levels.[131] Also, we have showed previously that treprostinil increases the rate of bile formation.

In conclusion, the present study has demonstrated the effect of cold ischemia and warm perfusion on the expression of basolateral (SLC; uptake) and apical (ABC; efflux) hepatic drug transporters in an isolated rat liver perfusion system. The transcriptions of important drug transporter genes were significantly alerted in both direction, increased and decreased. However, this alteration was prevented when treprostinil was added to preservation fluids or reperfusion medium. These findings suggest that improved function of liver grafts can be achieved by adding treprostinil in UW solution when livers from deceased donors and more importantly extended criteria donors harvested.

4.0 EFFECTS OF TREPROSTINIL ON DIGOXIN DISPOSITION IN COLD HEPATIC ISCHEMIA AND REPERFUSION MODEL

4.1 INTRODUCTION

Liver transplantation is the only treatment of choice for patients with compromised and functionally failed livers. However, hepatic ischemia and reperfusion (I/R) injury, which is a phenomenon in liver transplantation and liver resection, can lead to primary graft nonfunction.[64] Primary graft dysfunction consists of two phases: first, ischemia characterized by oxygen and energy deprivation followed by re-establishment of the blood flow to the liver that aggravates the damages that were initiated earlier.[69] Numerous experimental liver transplantation and warm ischemic models have shown that drug metabolizing enzymes, drug transporters and bile flow are significantly altered. However, morphological examination of the bile ducts, in some of these studies, showed no difference when normal and ischemic livers are compared.[146, 148, 175] These studies suggested that observed changes could be due to the differential expression and/or activities of drug transporters.

I/R injury has been comprehensively investigated to alter the hepatic metabolic activity. Investigators have shown alterations in drug disposition after I/R injury in microsomal systems, hepatocytes and/or isolated livers.[141, 145, 146, 176-178] We recently have shown that I/R injury affected the gene expression of drug transporters in an isolated perfused rat liver system (see Chapter 3.0). Our results showed that P-gp drug transporter was significantly downregulated after cold preservation and warm reperfusion. Furthermore, we have shown that treatment of livers with treprostinil maintained the mRNA levels similar to normal livers. However, it has been documented that functional changes in the activity of drug transporters do not always correlate with the changes in their mRNA and/or protein levels.[179]

Certain drugs can be used as probes to characterize uptake and efflux drug transporters and metabolizing enzymes activities *in-vitro, ex-vivo* and *in-vivo*.[180] A cocktail approach also has

been utilized in rats to evaluate CYP activities.[181] Thorling et al. (2014) have demonstrated that one hour localized hepatic ischemia significantly decreased rat *in-vivo* biliary excretion of Rhodamine-123, a P-glycoprotein (P-gp) substrate, and its protein expression.[164] In another study, multidrug resistance-associated protein-3 (Mrp3) drug transporter content in rat livers was significantly decreased after I/R injury that was associated with significant accumulation of fluorescein, a substrate of organic anion transporting polypeptide (Oatp; Slco), Abcc2/Mrp2 and Abcc3/Mrp3 transporters, in the hepatocytes.[167] Statins, rhodamine-123, indocyanine green and others were used to specifically understand the ability of the liver to clear drugs and associate this with their respective transporters.[182-184] Digoxin is a well-known probe for Abcb1 (Mdr1; P-gp) and Slco (Oatp) drug transporters and often recommended for phenotyping these drug transporters.[22] Benet and colleagues have demonstrated that rats, unlike human, can extensively metabolize digoxin to digoxigenin bisdigitoxoside (Dg2) by Cyp3a.[185] They also have illustrated the feasibility of using digoxin to characterize the activity of Slco (Oatp) and Abcb1 (Mdr1; P-gp).[186] We hypothesize that treprostinil, a stable synthetic PGI_2, will minimize the effect of cold preservation and warm reperfusion on digoxin disposition due to its ability to vasodilate and downregulate proinflammatory cytokines.

The aim of the present study was to characterize the effect I/R injury on the activity of Abcb1 (P-gp), Slco (Oatp) and Cyp3a simultaneously using digoxin as substrate and quinidine as Abcb1 (P-gp) inhibitor in an IPRL system. We also documented the protective effect of treprostinil on metabolic capacity of livers after I/R injury. Changes in the activities were monitored by measuring the concentrations and exposure of digoxin and its metabolite "digoxigenin bisdigitoxoside" (Dg2) in the perfusate using a UPLC-MS/MS system.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals

Digoxin (CAS № 20830-75-5) and quinidine (CAS № 56-54-2) were purchased from Tocris Bioscience (Bristol, United Kingdom). Digoxigenin bisdigitoxoside (metabolite; CAS № 5297-05-2) and digoxin-d3 (Internal Standard; CAS № 127299-95-0) were purchased from Toronto Research Chemicals, (Toronto, Ontario, Canada). ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 µm, 100Å) (part № 186003539) and ACQUITY UPLC HSS T3 VanGuard Pre-column, (5 mm × 2.1 mm, 1.8 µm, 100Å) (part № 186003976) were procured from Waters Corporation (Milford, MA, USA). Treprostinil (Remodulin$^{(R)}$) 1 mg per mL in a 20 mL vial was provided by the manufacturer, United Therapeutics Corporation (Silver Spring, MD). CoStorSol^{TM} (University of Wisconsin; UW) cold organ preservation solution purchased from Preservation Solutions Inc. (Elkhorn, WI). Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and Lactate dehydrogenase (LDH) assay kits were purchased from Pointe Scientific, Inc. (Canton, MI). All the solvents were of MS grade and were obtained from Fisher Scientific (Pittsburgh, PA, USA). Sodium chloride, potassium chloride, calcium chloride, Magnesium sulfate, potassium phosphate, sodium taurocholate, sodium bicarbonate and glucose were of analytical grade and obtained from commercial sources.

4.2.2 Animals

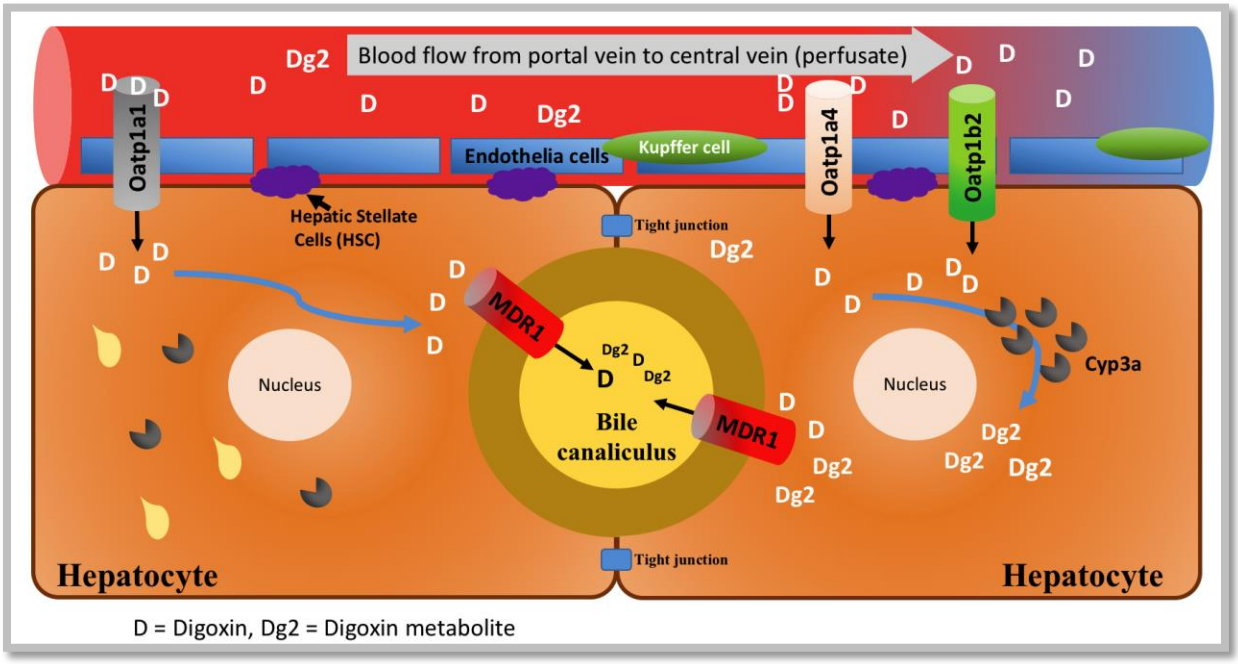
Sprague Dawley (SD) rats were purchased from Charles River Laboratories, Inc. (Wilmington, Massachusetts). Those SD rats were adult male 10-12 weeks' old weighing 225-250 gm. Rats

were kept in the animal facility and maintained under a 12 hours' light and dark cycle with free access to food and water. The procedures involving animals were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and were consistent with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011, Washington, DC).

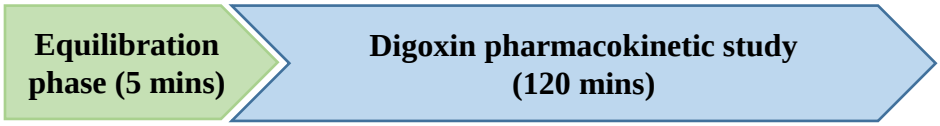
4.2.3 Study design

SD rats are the most common laboratory animals used to study the hepatic I/R injury and IPRL is preferable model to study drug disposition.[81, 187] Rats were randomly assigned to the following study groups: 1) Controls (no treprostinil): liver grafts were harvested then perfused in the IPRL for 2 hours without cold preservation. 2) Ischemia/Reperfusion group (no treprostinil used): liver grafts were harvested, cold (4^0C) preserved in UW solution followed by 2 hours of IPRL perfusion. 3) Treprostinil (during preservation and perfusion): rat livers were harvested and preserved in UW solution supplemented with treprostinil (20 ng/mL) and flushed with treprostinil (20 ng/mL) added ringers lactate solution (~20 mL), then perfused for 2 hours IPRL with treprostinil (20 ng/mL). The other three groups 4) controls + quinidine, 5) I/R injury + quinidine and 6) treprostinil + quinidine were exactly the same, but quinidine was injected over 5 minutes into the perfusate after liver stabilization. The IPRL system was configured in recirculation manner for the purpose of this study. 28 μg of digoxin was added as bolus dose into the perfusate (180 mL) at time zero to achieve 155.6 ng/mL as initial concentration in the total perfusate (Figure 4-1).

A)



B)



C)

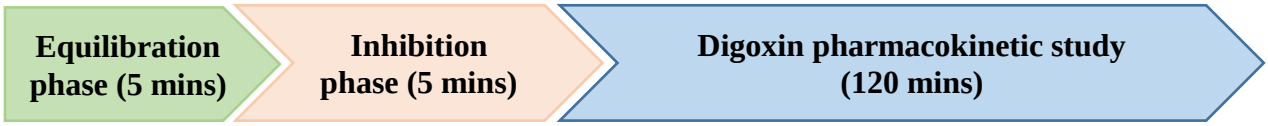


Figure 4-1: Schematic explanation of digoxin pharmacokinetic study.

A) Illustrates the fate of digoxin in the liver up-taken by Oatp drug transporters; effluxed into the biliary canaliculus by Abcb1 (Mdr1; P-gp), while some of digoxin is metabolized by Cyp3a to Dg2 that will be excreted into the bile. B) and C) demonstrate the timing and order of each phase of digoxin pharmacokinetic with and without quinidine, respectively.

4.2.4 Surgical procedure

Livers were harvested, preserved and perfused or perfused immediately as previously described by Mehvar et al. (2002).[139-141] Rats were anesthetized using isoflurane and depth of anesthesia was checked by toe pinch reflex. The abdominal cavity of the rat was opened with a V-shaped incision to expose the visceral organs. The bile duct was cannulated with PE10 tubing and the portal vein and external hepatic vein were cannulated with catheters for inlet and outlet of the perfusate, respectively. Livers were perfused with 30 mL UW solution (with or without treprostinil) and harvested. The isolated rat livers were preserved in UW solution (with or without treprostinil) for 24 hours at 4^0C. At the end of the preservation time period, the livers were flushed with 25-30 mL cold ringers lactate solution to remove the UW solution completely and transferred to organ chamber of the IPRL system. The perfusion was carried out for 120 minutes with Krebs–Henseleit bicarbonate buffer saturated with 95%/5% O_2/CO_2 and supplemented with sodium taurocholate (4.75 mg/L).[81, 142] The livers were perfused at 3 mL/min/g liver to maintain proper oxygen supply in the absence of proteins and blood in the perfusate. Periodic outlet perfusate samples were collected at 0, 5, 10, 15, 20, 30, 40, 50, 60, 80, 100 and 120 minutes. Additionally, cumulative bile samples were collected in pre-weighted centrifuge tubes at 30-minute intervals. At the end of the perfusion, a portion of the liver was preserved in formalin for histological evaluation and the rest was flash frozen immediately in liquid nitrogen and stored at -80^0C for further analysis.

4.2.5 Determination of liver injury biomarkers

The standardized hepatic injury biomarkers (AST, ALT and LDH) levels in perfusate were measured in accordance to the manufacturer recommendations. Reagents (R1 and R2) were reconstituted in 5:1 ratio. A ninety-six well plate was loaded with 200 uL of the reconstituted reagent, then pre-warmed at 37^0C for five minutes. Spectrophotometer was set to zero at 340 nm. Ten uL of the sample matrix was added into the reagent mixture and incubated at 37^0C. After 1 minute, the plate was mixed and each cell absorbance at 340 nm was read and recorded. Absorbance readings were repeated every minute for four minutes. AST and ALT levels (International Unite/L) were calculated by multiplying mean absorbance difference/minute by factor 3376.2x, which is calculated by (210*1000)/(6.22*10*1). Where 1000 is the conversion factor of IU/mL to IU/L, 210 is the total reaction volume (uL), 6.22 is millimolar absorptivity of NADH, 10 is the sample volume (uL) and 1 is the light path in cm.

4.2.6 Assay of digoxin, metabolite (Dg2) and quinidine

4.2.6.1 Preparation of standards and quality control samples

Stock solutions were prepared at 1 mg/mL. Digoxin, digoxigenin bisdigitoxoside and digoxin-d3 were solubilized in methanol and quinidine was dissolved in DMSO. Stock solutions were kept at -20^oC in the dark. Working solutions were prepared on the days of the assay and were diluted in 50% methanol yielded s concertation of 0.1 µg/mL. Standards for the calibration curve were prepared by serial dilution from the working solution by spiking in Krebs–Henseleit bicarbonate buffer. The concentrations of calibrators used in the assay for digoxin and quinidine are 0.3, 1, 5, 10, 20, 30, and 100 ng/mL equivalent to 5.625, 18.75, 93.75, 187.5, 375, 562.5 and 1875 pg

injected into the column, respectively. Similar to the calibration standards, but in a separate process, the quality control samples (QCs) were prepared. The concentrations of low (QC-L), medium (QC-M) and high (QC-H) quality controls were 2, 20 and 80 ng/mL (37.5, 375 and 1500 pg on column), respectively. For the internal standard (digoxin-d3), the stock solution was diluted serially in 50% methanol to achieve a final concentration of 100 ng/mL to be used in the standard curve, quality controls and samples preparations. Since the metabolite (Dg2) was expected to be lower in concentration, the standard concentrations of the metabolite for the calibration curve were are 0.1, 0.3, 1, 5, 10, 20 and 30 ng/mL that were equivalent to 1.875, 5.625, 18.75, 93.75, 187.5, 375 and 562.5 pg injected onto the column, respectively. The concentrations of quality controls were 0.2, 2, 10 and 20 ng/mL (3.75, 37.5, 187.5 and 375 pg on column), respectively.

4.2.6.2 Sample processing

All calibration standards, quality controls, and perfusate samples were thawed at room temperature. In a microcentrifuge tubes, 200 µL of sample, 1000 µL of acetonitrile and 10 µL of internal standard were mixed. The mixture was centrifuged at 19000 g. Supernatants were transferred into glass culture tubes and dried at room temperature under gentle air. Then, the residue was reconstituted in 80 µL of 30% acetonitrile. 7.5 µL of the reconstituted solution was injected onto the LC–MS/MS system.

4.2.6.3 Chromatographic and mass spectrometer conditions

An Acquity ultra performance liquid chromatography H-class was used (Waters Corporation, Milford, MA). Analytes were separated using Acquity UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 µm, 100Å) with Acquity UPLC HSS T3 VanGuard Pre-column, (5 mm × 2.1 mm, 1.8

µm, 100Å). The column temperature was maintained at 30^0C and the auto sampler temperature was set at 8^0C. Mobile phases, delivered at a flow rate of 0.3 ml/min, consisted of (A) 0.1% acetic acid and 2 mM ammonium acetate, 5% acetonitrile in H_2O and (B) 0.1% acetic acid and 2 mM ammonium acetate in 95% acetonitrile: 5% H_2O. A gradient mobile phase system was used at 0.3 mL/min, initial 0.5 minutes the "A" and "B" mixture were 70:30, respectively. Linearly mixture "A" was decreased to 10% during 3.5 minutes. The column recalibrated by returning the condition of 70% mobile "A" in 0.1 minute and held till 6 minutes, which was the total run time. Retention time for digoxin, Dg2, digoxin-d3 (I.S.) and quinidine were 2.94, 2.48, 2.93 and 1.08 minutes, respectively.

Analytes detection was performed using Waters® Xevo TQS tandem mass spectrometer operating in the positive electrospray ionization mode (Waters, Milford, MA). Highest ion abundance was used to optimize the instrument parameters and were as follows: desolvation temperature 500^0C, electrospray capillary voltage 3.2 kV, cone voltage 0V, collision energy 20 eV, source offset 50V, desolvation gas flow 1000 L/hr and cone gas flow 150 L/hr. Digoxin, Dg2, digoxin-d3 and quinidine were detected in multiple reaction monitoring (MRM) mode with a dwell time of 0.125 second/channel. MRM transitions were m/z 781.4 > 97.0 for digoxin, 651.3 > 97.0 for Dg2, 784.4 > 654.4 for digoxin-d3 and 325.4 > 80 for quinidine. Collision gas flow was maintained at 0.15 mL/min. Resolution was 2.7 (low mass) and 14.88 (high mass) for both MS1 and MS2. Analytical data was acquired and analyzed using TargetLynx™ software (Waters, Milford, MA).

4.2.7 Data Analysis

The Area Under the perfusate concentration-time Curve (AUC) for ALT, AST and LDH were used as indicators of liver injury. The AUC was calculated using linear trapezoidal method. Overall the AUC of digoxin and Dg2 in the perfusate is used as markers for P-gp and Cyp3a activities, respectively. Quinidine inhibition study was used to assess the role of P-gp. Overall, mean ± standard error of the mean (SEM) were calculated for all parameters. The groups were compared using Analysis of Variance (ANOVA) followed by Tukey's multiple comparisons test to identify the differences between the groups. A P-value ≤ 0.05 was considered significant. We used a total of 18 rats for all of groups used in this study.

4.3 RESULTS

4.3.1 Liver injury biomarkers

Control group serves as reference to I/R injury and treprostinil groups. Figure 4-2, A, B and C, depict the perfusate concentration-time courses for ALT, AST and LDH, respectively. The amount of hepatic injury markers released were seen to increase with time of perfusion in all of the three groups. Our results indicate that 2 hours of warm reperfusion in the IPRL system resulted in AUCs (mean ± SEM) for ALT, AST and HDL in the perfusate equal to 1402 ± 398.2, 2521 ± 393.2 and 6644 ± 2768 IU/L/min, respectively.

Twenty-four hours of cold ischemia with 2 hours of warm perfusion significantly increased the release of ALT, AST and LDH in the perfusate. The total release of these enzymes in the I/R injury group (Red column) was significantly higher by 4, 2.5 and 3.4 fold ($P < 0.05$), respectively, in comparison to controls (Blue column). However, the addition of 20 ng/mL treprostinil in the preservation and perfusion mediums (Green column; also known as treatment group) attenuated the effect of cold I/R injury and results were not different from normal livers.

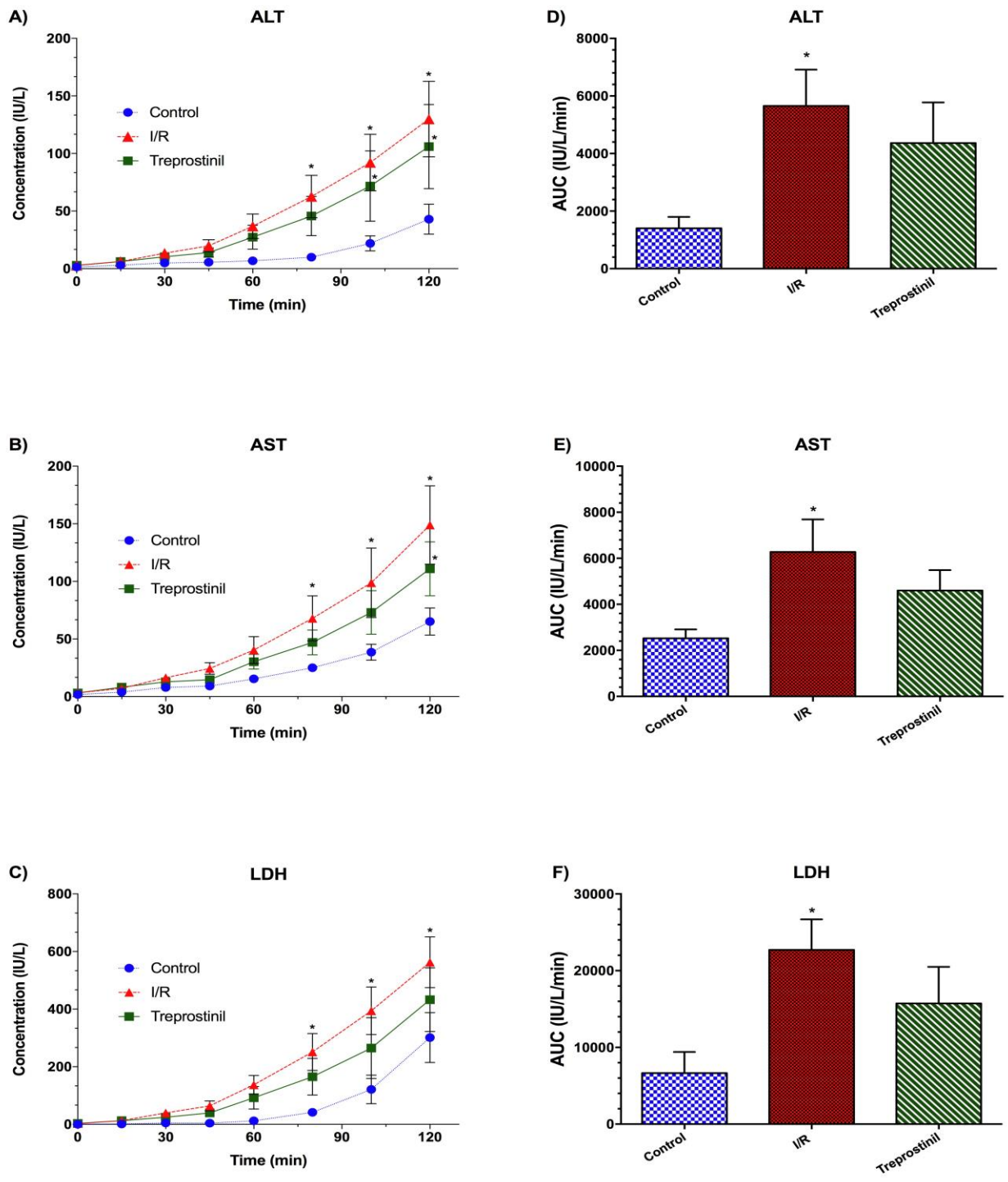


Figure 4-2: Concentration-time courses and AUCs of ALT, AST and LDH in the perfusate.

Perfusate ALT, AST and LDH levels (A, B and C) and AUCs (D, E and F) throughout the 2 hours of reperfusion in the IPRL system. Each time point is calculated and expressed as mean ± SEM. Each group composed of three rats (n = 3). *$p \leq 0.05$ compared to control.

4.3.2 Digoxin and metabolite (Dg2) pharmacokinetics results

The accuracy and precision for digoxin and metabolites assays were evaluated at three level concentrations 2, 20 and 80 ng/mL for digoxin and 0.2, 1 and 20 ng/mL for metabolite by utilizing the quality control samples. The calculated values for the three QC levels using the generated equation of the linear regression from the calibration curves that were run. The average accuracy for digoxin and metabolite were 2.68 and 15.5% or less, respectively. Also, the assays were reproducible where all calculated precisions at each concentration for both assays were less than 12%.

Figure 4-3: A) illustrates the levels of digoxin in the perfusate for the three groups after adding 28μg of digoxin in the perfusate reservoir (around 180 mL). In the control group, digoxin levels were lower than the other two groups and this continued over the period 120 minutes' of perfusion. The AUC of digoxin was significantly higher in the I/R injury livers compared to controls by 3.5 times, with an increase from 3182 ± 341 to 11129 ± 1047 ng/mL/min ($P \leq 0.05$)(Figure 4-3:B). However, treprostinil treatment during cold preservation and warm reperfusion decreased the AUC of digoxin significantly by 44% in comparison to I/R group to 6353 ± 355 ng/mL/min ($P \leq 0.05$), but we still observed significant difference when compared to controls.

Six-hundred microgram of quinidine was infused over the initial 5 minutes of the experiment to inhibit P-gp, then 28 μg of digoxin was added to the perfusate reservoir at the end of this phase. Figure 4-3:D demonstrates that digoxin AUC of control and treatment groups increased after inhibiting P-g. The digoxin AUCs increased from 3182 ± 341 to 5755 ± 842 and from 6353 ± 355 to 17891 ± 1070 ng/mL/min, in control and treprostinil groups, respectively,

which indicates 1.8 and 2.8 folds increase. However, the AUC in I/R injury group remained similar 11129 ± 1047 vs. 11854 ± 1612 ng/mL/min, with or without quinidine.

On the other hand, the concentration of major digoxin metabolite (Dg2) gradually increased in all of the groups during the experiment; however, Dg2 levels in the control perfusate started to decline after 60 minutes of digoxin dosing (Figure 4-4). The Dg2 amount formed in the I/R injury was not significantly increased when compared to controls. However, treprostinil significantly increased the Dg2 AUC when compared to controls, 2775 ± 296 vs. 379 ± 17 ng/mL/min ($P \leq 0.05$), respectively. The P-gp inhibition study has shown a similar increase on Dg2 production in the control group, where Dg2 AUC increased from 379 ± 17 to 529 ± 80 ng/mL/min. However, the I/R injury group have remained comparable with and without quinidine, 1891 ± 727 vs. 2099 ± 467 ng/mL/min.

The ratio of metabolite AUC over parent AUC for the three groups were calculated and presented as mean ± SD in Figure 4-5. The ratios of Dg2 to digoxin AUCs in control and I/R injury groups were not different; however, treprostinil treatment during preservation and reperfusion increased the ratio to 0.45 ± 0.19 in comparison to control and I/R injury, 0.13 ± 0.05 and 0.15 ± 0.13, respectively ($P < 0.05$). The addition of quinidine to the experiment introduced no changes in control and I/R injury but decreased the ratio for the treprostinil group, which was not different than the other two groups.

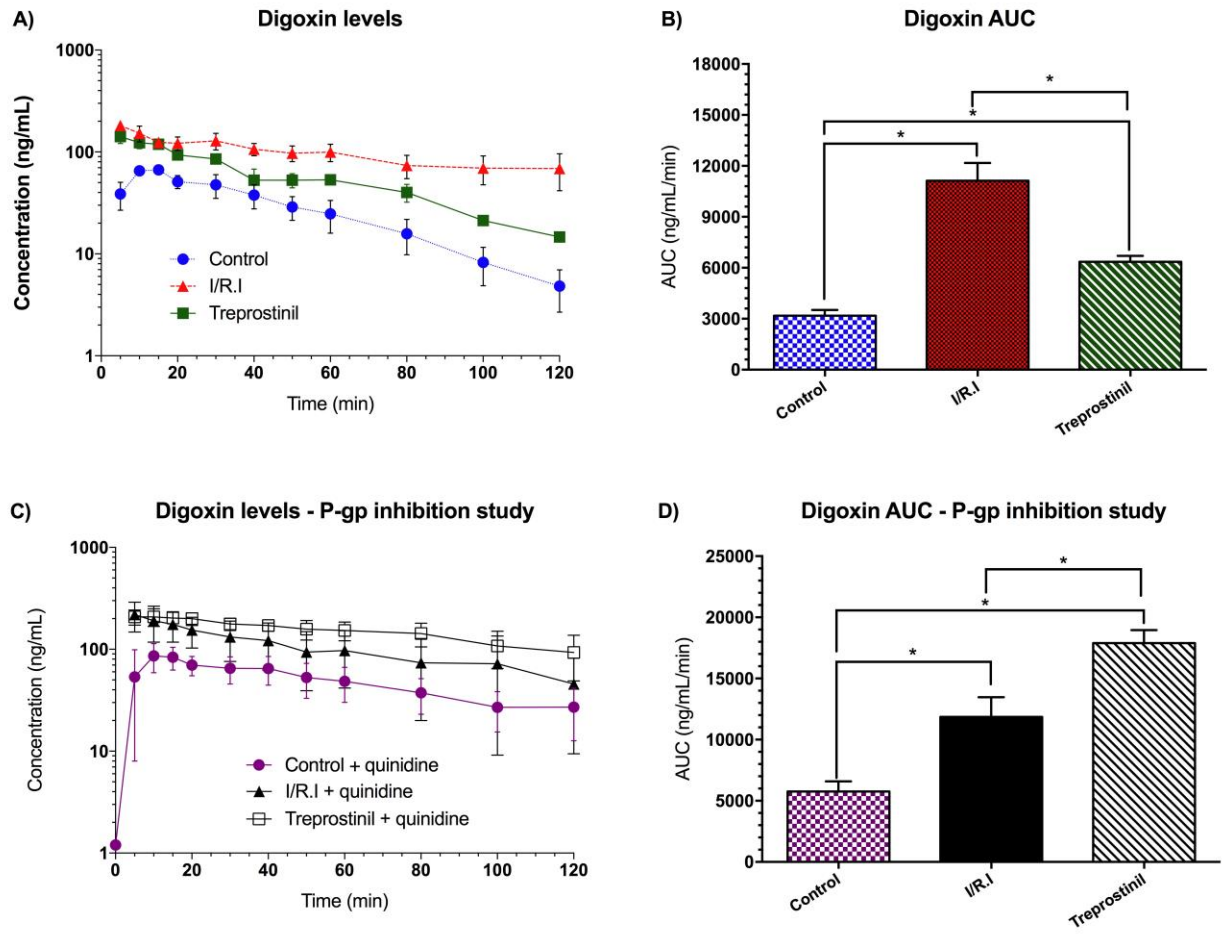


Figure 4-3: Effect of I/R injury and treprostinil on digoxin levels in perfusate in the absence and presence of quinidine.

A) and B) show the perfusate levels and AUCs of digoxin, respectively, during the 2 hours' perfusion after adding 28 μg of digoxin into the perfusate (180 mL) for the three study groups. C) and D) presenting the effect of P-gp inhibition by quinidine of the levels and AUCs of digoxin, respectively. Each time point is calculated and expressed as mean ± SEM. Each group composed of three rats (n = 3). *$p \leq 0.05$ compared to control, otherwise noted in the figure.

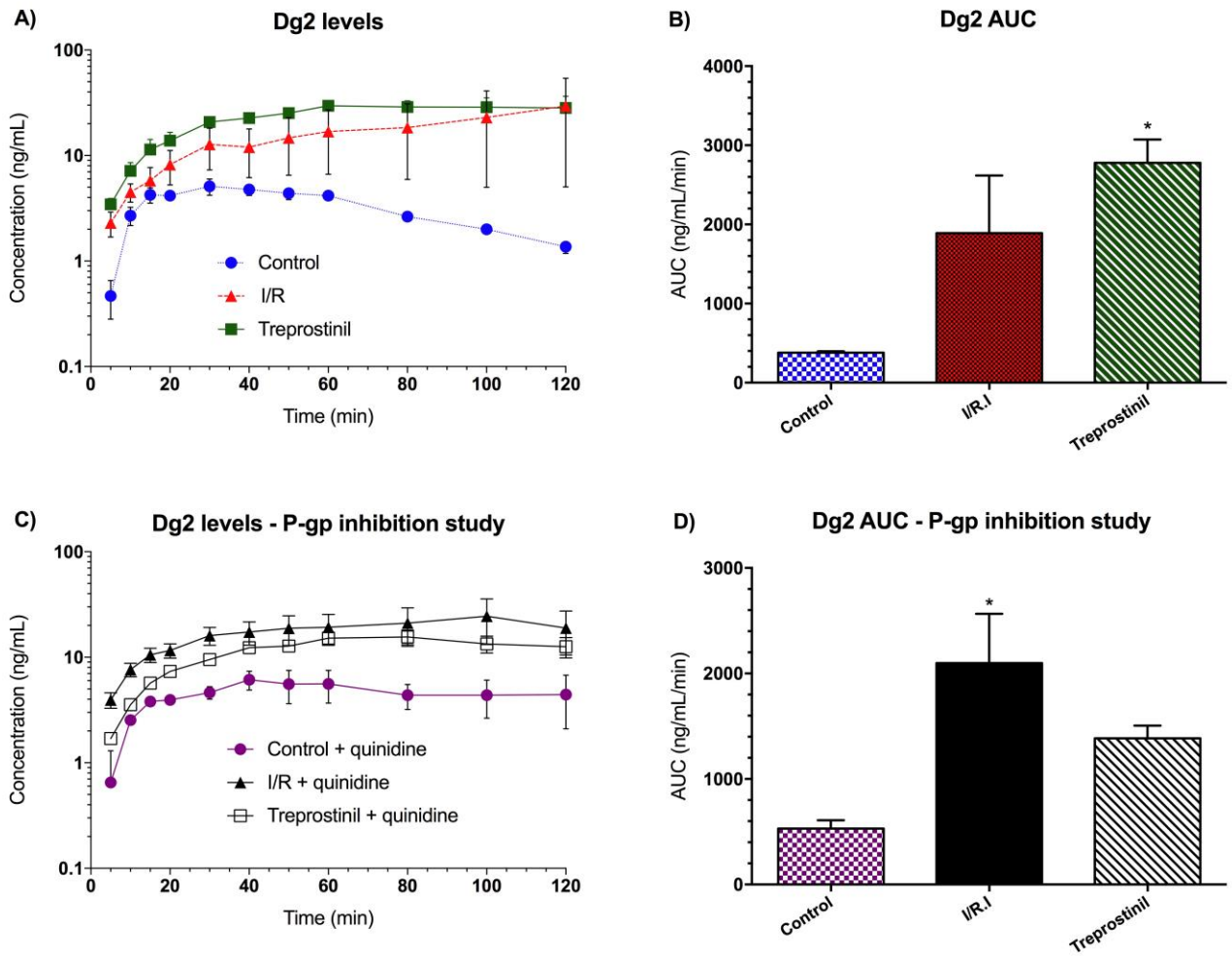


Figure 4-4: Effect of I/R injury and treprostinil on Dg2 levels in the absence and presence of quinidine.

A) and B) are showing the perfusate levels and AUCs of Dg2, respectively, during the 2 hours' perfusion after adding 28 μg of digoxin into the perfusate (180 mL) for the three study groups. C) and D) presenting the effect of P-gp inhibition by quinidine on the concentrations and AUCs of Dg2, respectively. Each time point is calculated and expressed as mean ± SEM. Each group composed of three rats (n = 3). *$p \leq 0.05$ compared to control, otherwise noted in the figure.

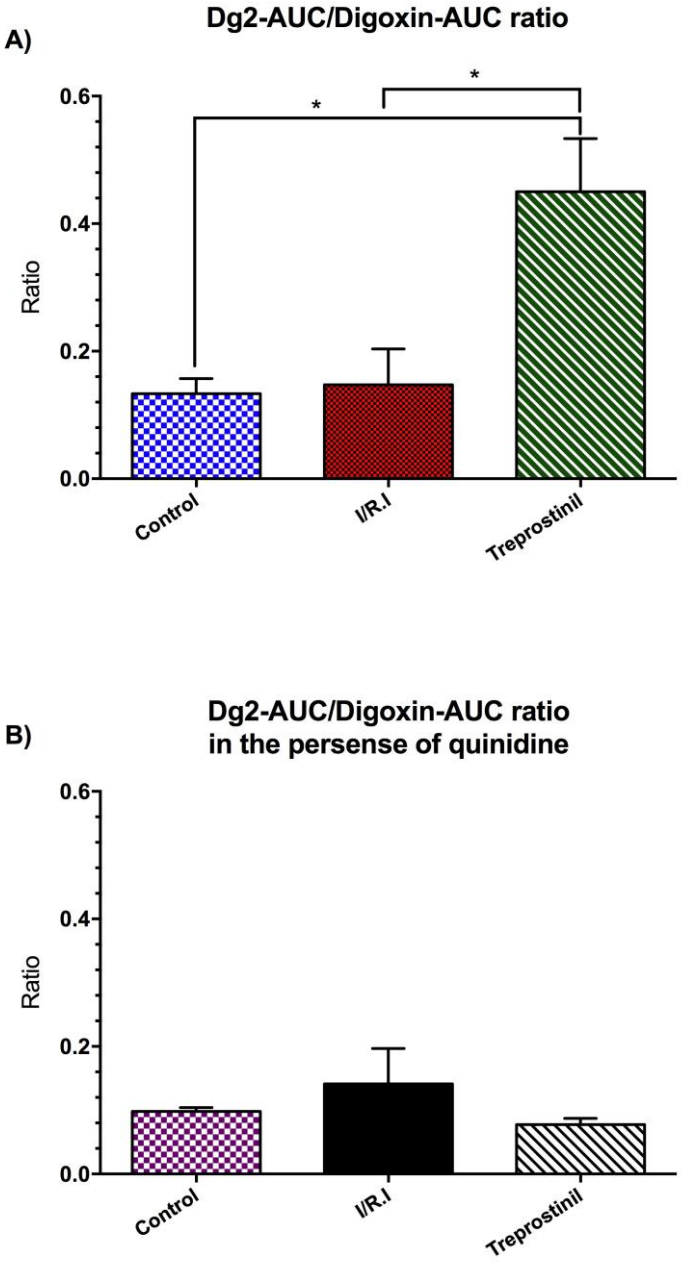


Figure 4-5: Ratio of metabolite-AUD/parent-AUC.

This is the calculated ratios of Dg2-AUC/Digoxin-AUC in controls, I/R injury and treprostinil treated liver groups A) in the absence and B) in the presence of quinidine. Each time point is calculated and expressed as mean ± SEM. Each group composed of three rats (n = 3). *$p \leq 0.05$ in comparison to the noted group in the figure.

4.4 DISCUSSION

The aim of the present study is to document the protective capacity of treprostinil treatment on the metabolic and drug transport functions of liver using digoxin disposition in a rat hepatic ischemia model. Most of the critical effects of I/R injury happen in the early phase after establishing the reperfusion through portal vein and hepatic artery.[147] In this study we showed that 24 hours of cold ischemia and 2 hours of warm reperfusion significantly increased the digoxin AUC in perfusate when compared to control livers using IPRL system. This could be explained by a reduction in the activity of either hepatic up-take (Slco; Oatp) and efflux (Abcb1; Mdr1; P-gp) transporters and the drug metabolizing enzyme Cyp3a, since they are the main hepatic clearance routes for digoxin in rats.[188] In chapter 3.0 , we have shown that P-gp mRNA expression was significantly decreased by an average of 46.7% but supplementing preservation solution with treprostinil, a stable PGI_2, significantly maintained the mRNA expression to levels similar to normal livers. We, here, show the same pattern in terms of P-gp activity, where 24 hours' cold preservation results in lower clearance of digoxin. Treprostinil mediated enhancement in the P-gp expression translated into an actual decrease in the digoxin AUC value indicating that the P-gp activity was increased compared to I/R. Hepatic I/R injury is well recognized to deplete energy (ATP) storage.[122] Ghonem et. al. (2011) showed that 24 hours cold preservation significantly decreased ATP hepatic content and treprostinil attenuated this effect.[129] Other investigators have shown that lower Rhodamine 123 clearance correlated with less ATP in the liver.[178] This correlation demonstrated that partial hepatic ischemia resulted in less ATP that is needed for P-gp drug transporter activity. Additionally, other report have shown that lower P-gp protein content was detected after prolonged I/R injury.[164] The present work

showed that higher activity can result from higher mRNA recovery of P-gp in the treated livers that ultimately increased the disposition of digoxin.

This interplay between P-gp and Cyp3a enzyme and the overlapping in expression and substrate pattern, i.e. digoxin, tacrolimus, erythromycin, etc, have been investigated.[189, 190] In our study, digoxin metabolite (Dg2) concentrations were quickly increased in the perfusate, where at time zero there was no Dg2 detected in any of the experiments. This indicates that formation of the metabolite is very rapid and consistent with previously reported study where it was found that digoxin metabolism to Dg2 in rat microsomal system was the fastest pathways by at least 20 times compared to all other metabolic products.[188] AUCs of Dg2 in both control and I/R injury were not different; however, the concentration of Dg2 in control group decreased after 60 minutes. An explanation of this reduction is either hepatic tissue accumulation or higher bile efflux activity, which is expected. On the other hand, the levels of Dg2 in the treatment group was more than 7 folds higher than controls. In fact, metabolite-AUC to parent-AUC ratio clarify that Cyp3a in treprostinil treated lives are more active than I/R, since the ratio will take into account of bulk digoxin available in the hepatocytes to be biotransformed by Cyp3a. Hughes et al. (2010) has illustrated that Dg2 metabolite was excreted by P-gp and the magnitude of Dg2 phase III related clearance was similar to digoxin in a Caco-2 sub clone (CLEFF) over-expressing P-gp with and without using several P-gp inhibitors.[191] This confirm that the much lesser levels of Dg2 in the control livers because of the higher P-gp activity, being healthier livers, where the other groups have continued to accumulate the metabolites in the perfusate.

Quinidine has been shown to be potent inhibitor to P-gp drug transporter *in-vitro* and *in-vivo*.[189, 192] A study on P-gp overexpressed cell line have calculated the IC_{50} value to be approximately 10 μM (3.3 μg/mL) of quinidine while using digoxin as the substrate.[193] Liang et

al. (2014) have showed that inhibiting P-gp in an *in-situ* liver perfusion increased the exposure of periplocin, which is structurally similar to digoxin and transported by Slco (Oatp) and Abcb1 (Mdr1; P-gp).[194] They also demonstrated that inhibiting uptake transporters (Oatps) by rifampicin further increases the exposure. The total biliary excretion of periplocin was significantly decreased with both inhibitors by one third and two third with verapamil and rifampicin, respectively. The relationship between digoxin and quinidine is also clinically proven where at least 50% digoxin dose reduction is recommended, due to higher digoxin blood concentrations, in almost 90% of patients receiving quinidine as concomitant medication.[191, 195] In the current study, pretreating livers with quinidine doubled the total exposer of digoxin in control livers and increased the metabolite AUC by ~37%, which is explained by longer retention time of digoxin in the hepatocytes, as reported previously.[185] The ratio of digoxin in mice treated with quinidine versus untreated (digoxin concentration + quinidine / digoxin concentration - quinidine) increased by 1.7, 1.7, 2 and 2 in plasma, brain, liver and small intestine, which is consistent with our report.[193] Furthermore, quinidine increased the digoxin-AUCs but the metabolite to parent ratios were not different between all groups. The resulted alteration in the uptake transporter activity could happen due to the efflux transporter inhibition. The main limitation in the current study was the lack of quinidine effect on the I/R injury group (no significant increase of relative digoxin AUC), which might be due to already malfunction of P-gp in this group.

In conclusion, this study has demonstrated that cold ischemia and warm reperfusion decreases both phase I and III activities, where we showed that digoxin exposure increased and less metabolite formed using *ex-vivo* rat perfused liver model. Furthermore, a key finding was that treprostinil protects not only the expression of Abcb1 (Mdr1; P-gp) but also preserved the

functionality of Abcb1 (P-gp), Slco (Oatp) and Cyp3a when digoxin was used as a probe substrate. Treprostinil is a very promising entity to be incorporated in the UW solution because of its protective effects.

5.0 SAFETY AND PRELIMINARY EFFICACY EVALUATION OF CONTINUOUS I.V. INFUSION OF TREPROSTINIL (REMODULIN®) IN ORTHOTOPIC LIVER TRANSPLANT PATIENTS

5.1 INTRODUCTION

Liver grafts are not immune to damages caused by many clinical conditions. Hepatitis and abuse of certain toxic substances, such as alcohol, may lead to end-stage liver disease (ESLD) and/or eventually will lead to liver failure. In 1965, Thomas E. Starzl performed the first successful liver transplantation (LTx).[36, 38, 39] Since then, LTx is considered the only cure for patients with ESLD. Surgical procedures and standard of care used in organ transplantation have significantly improved overtime. Currently, there are more than 14,000 patients who are listed in the active waiting list for LTx and less than 50% are expected to get new livers.[42, 43] Usually livers are sourced from: deceased donors (DD) or living donors (LD) that are distributed into > 95% and < 5%, respectively.[43] Majority of those grafts are normally subjected to cold ischemia and subsequent warm reperfusion. Both ischemia and reperfusion (I/R) can cause significant damage to the cellular architecture and function of the liver. The overall response that occurs upon reperfusion is a primary cause of liver injury and is directly related to the duration of ischemia. Hepatic injury post LTx is apparent with a rapid rise of bilirubin and aminotransferase levels within the first 24 hours following LTx.[122] There is strong evidence to support that I/R injury is a major leading cause of primary graft non-function (PNF) and the underlying cause for early organ dysfunction, seen in 10% of patients.[59-64] Livers from extended criteria donors are more susceptible to I/R injury and the extent of the resulting graft destruction strongly predicts both the short- and long-term clinical outcomes.

The use of Prostaglandin (PG) and prostacyclin (PGI_2) analogs could improve the clinical outcome by decreasing morbidity and mortality associated with liver transplantation. Many experimental and clinical studies have demonstrated that prostaglandins in general help in protecting the livers, specifically against I/R injury.[76, 92] The mechanisms of actions include

enhancing the microcirculatory blood flow by suppressing thromboxane A2 and increasing PGI_2, platelet aggregation inhibition, decreased sinusoidal endothelial cells (SECs) apoptosis, and downregulation of proinflammatory cytokines by decreasing the activation and infiltration of leukocytes.[100] The odds ratio for the PNF of the allografts in a compiled analysis of nine studies that included 488 patients randomized into PG versus controls was 0.55 (95% CI 0.23 to 1.33). Furthermore, PG significantly decrease the risk of acute kidney failure that required dialysis with an odds ratio of 0.37, suggesting prevention of calcineurin inhibitors induced nephrotoxicity.[101] A randomized-placebo controlled study that included 160 subjects showed that PGE_1 significantly reduced the hospital and ICU stays by 20% and 40%, respectively.[109] Most recently, Barthel et al. (2012) have shown that one week of continuous infusion of iloprost decreased the PNF incidences from 20% in controls to 5% in the iloprost treated group ($P = 0.087$) and improved allograft synthetic function.[115] Many other studies have examined the protective properties of prostaglandins in liver grafts but the major drawbacks were not reaching significance for the primary aim or challenge in using PG as standard of care in LTx patients due to its chemical instability, short half-life and high cost.

Our group, at the University of Pittsburgh Medical Center (UPMC), have some experience in using treprostinil, a stable synthetic PGI_2 analog, in LTx patients. Two patients who were diagnosed with pulmonary arterial hypertension (PAH) who went through LTx have been using treprostinil infusion for 6 and 11 months before they got a LTx. Patients received 36 and 45 ng/kg/min, as prescribed for their PAH, during the liver transplant procedure and afterward in the ICU without any treprostinil-related problems.[132] It has been documented that maximum plasma levels of treprostinil were elevated by 2 or 4 folds in patients, with mild (n=4) or moderate (n=5) hepatic dysfunction, respectively, who received subcutaneous dose of 10

ng/kg/min for 150 minutes for portopulmonary hypertension treatment.[125] Furthermore, the $AUC_{0-\infty}$ of treprostinil was increased 3 and 5 folds, respectively, compared to healthy adults.[128] Therefore, during the anhepatic period, plasma concentrations of treprostinil will be expected to rise significantly by nearly five times. PGE_1 has been safely administered intra-operatively at a maximum dose of 1 ug/kg/min during liver transplant surgery.[112] Clinical experience indicates that switching from IV epoprostenol to SC treprostinil infusion can be done at 1:1 ratio.[133] However, two fold increase in treprostinil dose will typically be required to obtain similar clinical response as IV PGI_2.[135] There has been no systematic clinical study of evaluating treprostinil infusion during and after the liver transplantation as part of a standard of care. We hypothesized that patients who undergo orthotropic liver transplantation can be safely infused with IV treprostinil perioperatively and the infusion can be sustained after transferring the patient to the ICU. Due to the vasodilation, platelet aggregation inhibition and proinflammatory cytokines down-regulation activities of treprostinil, we predicted a protective effect on the liver graft and enhanced graft and patient survival.

The aim of this prospective, single center, open labeled pilot study was to investigate the safety of continuous IV infusion of treprostinil and document the preliminary efficacy on I/R injury by reducing the graft dysfunction during the first seven days' post-transplantation. Three dose levels have been investigated in a 3 plus 3 dose escalating model.

5.2 METHODOLGY

5.2.1 Study design

This study was a prospective pilot single center, open-label, dose-escalation phase I/II, in transplant patients, with outpatient follow-up for up to 180 days. A signed informed consent (below), approved by the University of Pittsburgh Institutional Review Board (IRB), was obtained from all subjects before any study related procedure was initiated. Treprostinil was administered as a continuous infusion at a dose of 5 ng/kg/min for 2 days (in the initial 3 patients) and 2.5 and 5 ng/kg/min for a period of approximately 120 hours. Figure 5-1 shows the study design. Treprostinil infusion started once the patient was hemodynamically stable and continued for up to 48 or 120 hours. During infusion, hemodynamic parameters and blood samples were collected and indocyanine green plasma disappearance rate (ICG-PDR) was evaluated on day 2 and 5 post-LTx. On day 6 and 7 post-transplantation, serial blood samples were collected from the patients and functional assessment were also performed. The subjects were followed up for approximately 180 days' post-transplantation to document graft and subject survivals. Figure 5-2 describes the enrollment of subjects in the study and the dose escalation procedure.

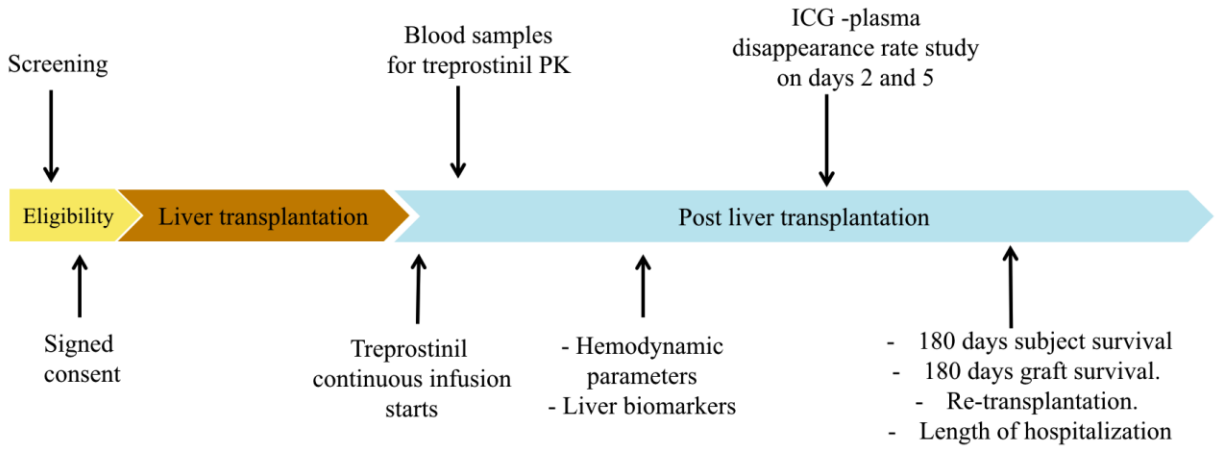


Figure 5-1: Study design flowchart.

The study starts with actively screening for new subjects then approaching them and describing the benefits and risks that are related to the study and obtaining the signed informed consent from the subject. Initially, treprostinil infusion was initiated after induction of general anesthesia. Subsequently, treprostinil infusion starts once the patient was hemodynamically stable and continued for up to 120 hours. During infusion, hemodynamic parameters and blood samples were collected and indocyanine green plasma disappearance rate (ICG-PDR) study at day 2 and 5 were performed. Subjects were followed up for approximately 180 days post-transplantation to document graft and subject survivals.

5.2.2 Subjects eligibility criteria

Subjects between 18 and 65 years of age who were approved candidates for deceased donor liver transplantation at the Thomas E. Starzl Transplantation Institute, UPMC were eligible to participate in the study. Eligible subjects included those receiving liver transplant; liver with < 40% macrosteatosis, if biopsy results were available; livers with necrosis score of < 10; cold ischemia time approximately > 5 hours, but less than 12 hours. Subjects were excluded if participating in any other investigational study; if undergoing a living donor liver transplant or re-transplantation because of a failed transplant within the previous 180 days; if undergoing multi-organ transplantation; if receiving organs from hepatitis C positive donors; if they have a fulminant hepatic failure (FHF); if the model for end-stage liver disease (MELD) score was $\geq$ 40; if they currently received prostanoid therapy to treat portopulmonary hypertension; if they had any significant cardiovascular disease; if they were on renal replacement therapy; if they received methylene blue; and if they are allergic to ICG or iodine.

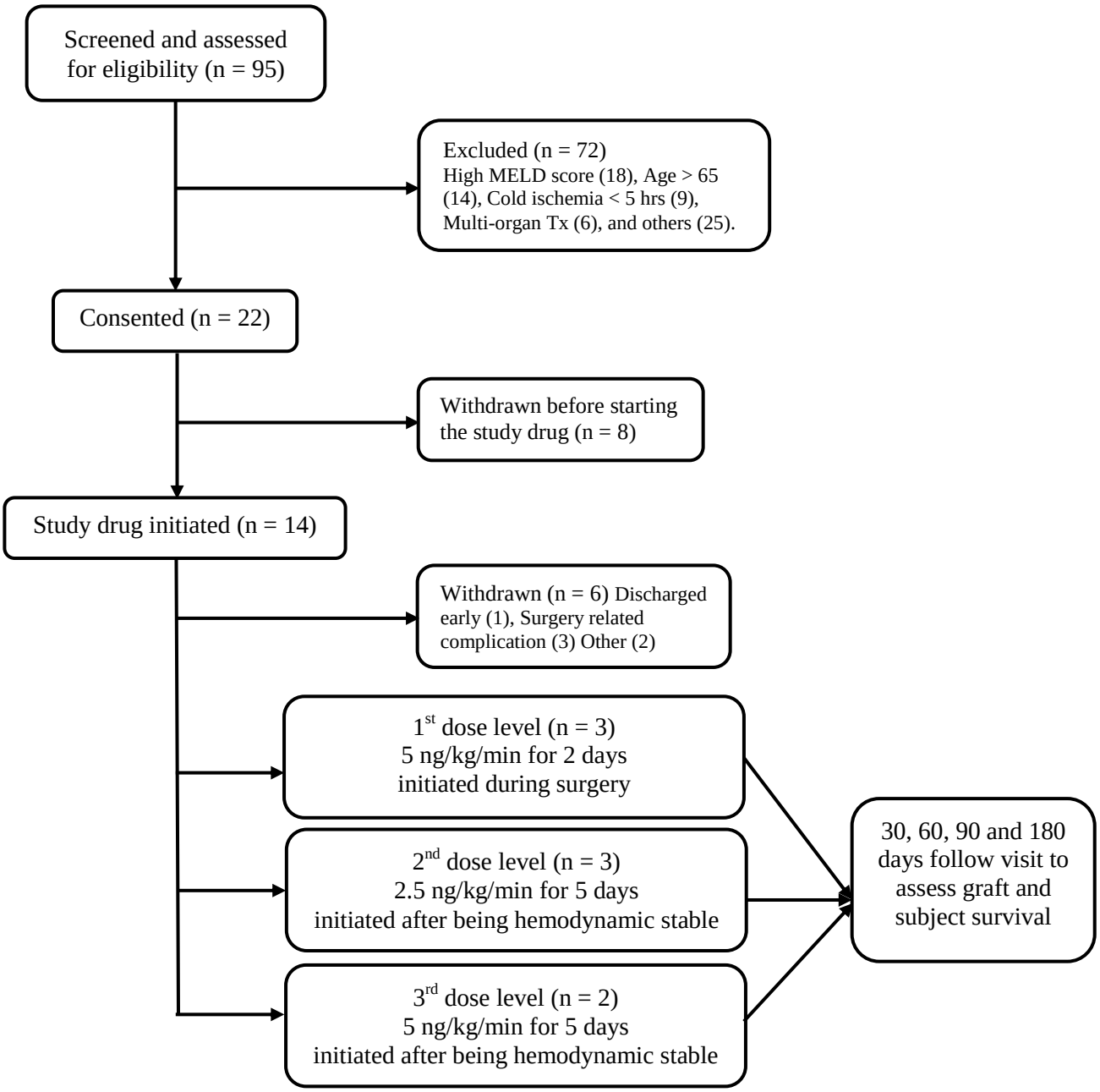


Figure 5-2: Study enrollment flowchart of the Remodulin study.

This flowchart describes the patient enrollment in Remodulin clinical study at Montefiore hospital-UPMC. The flowchart shows number of subjects screened and assessed for eligibility (n = 95). Subjects consented (n = 22) were evaluated through-out the study and were enrolled to three dose levels in a 3 + 3 dose escalating phase I design. Subjects were followed for up to 180 days post-transplantation.

5.2.3 Study drug and dose escalating regimen

FDA approved treprostinil (20 mg / 20 mL, Remodulin®, United Therapeutics, Inc.) was used for the study. All treprostinil cassettes were prepared by the investigation drug services (IDS) office in UPMC. Treprostinil was administered intravenously commencing after the patient is stable following the placement of new liver graft, and for approximately up to 48 or 120 hours, unless hemodynamic changes or tolerability require discontinuation of treprostinil. The route of administration was continuous intravenous (I.V.) infusion using an appropriate infusion pump into a dedicated central line or peripherally inserted central line. Treprostinil dose escalation following a standard 3 + 3 phase I design. At the beginning, 3 patients were enrolled at dose level of 5 ng/kg/min for 2 days'. Then, a major modification of the study procedure, where the infusion initiation was postponed to start at the ICU once the patient is hemodynamically stable. The next 3 patients were enrolled at dose level of 2.5 ng/kg/min for a duration of 5 days. Since no patient experienced a dose-limiting toxicity (DLT), another 3 patients completed 5 ng/kg/min for 5 days.

5.2.4 Study objectives

5.2.4.1 Primary endpoints:

The primary endpoints of this study was to evaluate the safety and preliminary efficacy of a two days' perioperative or five days' post-operative course of treprostinil (Remodulin®) infusion. The safety profile was assessed in terms of tolerability of the drug, hemodynamic parameters, and need for inotropes. All adverse events were monitored and evaluated by licensed physician. Hemodynamic measurements were recorded for up to a week after transplantation, whenever

available. These parameters include, mean pulmonary arterial pressure (mPAP, mmHg), cardiac output (CO, L/min), cardiac index (CI, l/min/m2), heart rate (HR, bm), systolic blood pressure (SBP, mmHg) and diastolic blood pressure (DBP, mmHg). Also, serum bilirubin concentrations were assessed for seven days after transplantation as the primary preliminary efficacy.

5.2.4.2 Secondary endpoints:

The secondary endpoints were assessed based on biochemical end points, clinical endpoints, and ICG-PDR study at day 2 and 5 post-transplantation. The biochemical analysis includes; alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the first seven days after transplant. Also, serum creatinine (SCr) levels in the first seven days following transplant were collected to assess the renal function. Prothrombin time (PT) was used as blood coagulation biomarker. Clinically, primary allograft non-function, which is defined as patient death or re-transplantation within 30 days due to liver failure, was monitored. Duration of time spent on ventilator during the initial hospitalization days and in the intensive care unit (ICU) were documented. All subjects and graft survivals were continued to be followed up for up to study day 180.

5.2.5 Data analysis

Shapiro-Wilk test was applied on our study data. Based on the normality test, a Mann-Whitney non-parametric test was used to compare between treprostinil (n = 10) and controls (patients who signed informed consent, got liver transplantation and did not receive treprostinil (n=5)). The effect of intervention on the hemodynamic parameters was evaluated by fitting a linear regression of the detected change (compared to time zero) in each parameter (HR, SBP, DBP,

mPAP, CO and CI) through-out the infusion course. The Area Under the plasma concentration-time Curve (AUC) for ALT, AST, total bilirubin and serum creatinine were calculated using linear trapezoidal method.

5.3 RESULTS

5.3.1 Patient recruitment and baseline demographics

Ninety-five patients who were selected to receive liver transplantation between December 2012 and July 2016 at UPMC were screened. Twenty-two subjects signed the informed consent form (ICF). The primary reason for failure to consent patients was higher MELD score than our inclusion criteria (24.7%). More details are shown in Table 5-1. Out of the twenty-two consented subjects, eight have completed three dose levels (5 ng/kg/min for 2 days (n=3) and 2.5 (n=3) and 5 (n=2) ng/kg/min for 5 days). Six subjects received treprostinil for limited time and did not complete the full treatment course. Of those six, one was discharged early (completed only four days); one had treprostinil infusion interrupted in the floor but without any reason related to the study, for a complete day during the five days of treatment; one was terminated because the central IV access was removed after approximately thirty-two hours of infusion and three were terminated after less than six hours from the study because of surgery complications that were clarified not to be study drug related complications. The remaining seven patients who signed the ICF and did not get any treprostinil, four because they continued to be hemodynamically unstable for more than twenty-four hours after the OLT procedure; one was not eligible for the study because of the short cold ischemia duration; two were not eligible for the OLT in the

UPMC and one subject passed away during the LTx procedure. Table 5-2 summarizes the status of all consented patients.

All patients who received treprostinil were Caucasian males aged between 50 and 63 years (median = 57) and their median weight was 92 kg. The indications for LTx were 60% of the patients have hepatocellular carcinoma (HCC) and/or hepatic virus C (HCV). Liver grafts transplanted had a median 362 (335-672) minutes' cold ischemia and 33.5 (28-42) minutes of warm ischemia. On the other hand, patients who signed ICF and got liver transplantation but did not receive treprostinil were considered as controls. 80% were male with median age and weight were 60.5 years (43-63) and 80.25 kg (70-139), respectively. Time of cold and warm ischemia were 364.5 (282-531) and 38 (32-44) minutes. All demographics were not different between the groups and summarized in Table 5-3.

Table 5-1: List of reasons for patients who were excluded before consenting.

	Reason	*Times of occurrence*	*Percentage (%)*
1.	High MELD	18	24.7%
2.	Age > 65 years	14	19.2%
3.	Cold ischemia < 5 hours	9	12.3%
4.	Multiple organ transplant	6	8.2%
5.	Conflicting clinical study	4	5.5%
6.	Failed LTx within 180 days	3	4.1%
7.	Not eligible for LTx	3	4.1%
8.	Cardiovascular history	2	2.7%
9.	HCV donor	2	2.7%
10.	Fulminat hepatic failure	2	2.7%
11.	Split liver	2	2.7%
12.	Refused study	2	2.7%
13.	Haemophilia - bleeding risk	1	1.4%
14.	HIV positive	1	1.4%
15.	Allergic to iodine	1	1.4%
16.	International patient	1	1.4%
17.	Staff insufficiency	1	1.4%
18.	Needed proxy consent	1	1.4%

Table 5-2: Status of consented patients.

	Patient status	*Number*
Patients completed the study		8
A	Completed 5 ng/kg/min for 2 days	3
B	Completed 2.5 ng/kg/min for 5 days	3
C	Completed 5 ng/kg/min for 5 days	2
Patients discharged earlier		1
A	Completed 2.5 ng/kg/min for 4 days only	1
Patients withdrawn from the study		13
A	Hemodynamically instable **did not initiate** the infusion	4
B	Hemodynamically instable **after** starting the infusion (not related to study intervention)	3
C	Patient died in the OR, before receiving treprostinil	1
D	Completed 4 days only (5 ng/kg/min)	1
D	Central line removed after ~ 32 hours before completing the study	1
E	Cold ischemia < 5 hours	1
F	Macrosteatosis > 40%	1
G	Metastasis	1

Table 5-3: Patient demographics and baseline characteristics.

	Treprostinil (n= 10)	*Control (n =5)*
Gender		
Male	100%	80%
Female	0%	20%
Race		
White	100%	100%
Others	0%	0%
Age	57 (50-63)	60.5 (43-63)
Body weight	92 (63-136)	80.25 (70-139)
LTx indications		
HCC	60%	40%
HCV	60%	40%
Alcohol cirrhosis	40%	60%
Cold ischemia time (minutes)	362 (335-672)	364.5 (282-531)
Warm ischemia time (minutes)	33.5 (28-42)	38 (32-44)

5.3.2 Safety assessment

Cardiovascular parameters in all patients who received treprostinil were within the normal range of healthy volunteers. We have fitted regression line to detect any significant changes in these parameters compared to time zero, when the treprostinil was started. Heart rates were within the normal range when the infusion of treprostinil was started and continued to get lower within time. The reduction in HR slopes between treprostinil infused patients and controls were comparable. At the end of the study, all patients' HRs were within normal range (Figure 5-3). In both groups, the systolic arterial blood pressures were not changed over the seven days, but the DBP showed a significant increase over time. Even with the detected increase in the DBP, almost all subjects (treprostinil and controls) started and ended being within the normal values. Other main hemodynamic parameters were not affected as seen in Figure 5-4. Most of our patients have relatively high mean pulmonary arterial pressure, cardiac output and cardiac index at the start of treprostinil administration. CO was the only factor that had significantly reduced over time in patients received treprostinil. But when those values were normalized to the body surface area (m^2), the reduction was neglected as seen in CI.

Adverse events (AEs) and complications in critically ill patients are not surprising but proper documentation is important to document any safety concerns that are related to our intervention. All AEs have been evaluated by licensed physician investigators and were found to be not related to the study drug. One participant had an event of vomiting twice in one night on day 2 after transplantation. Another subject was admitted to the hospital two weeks following OLT for right sided pleuritic chest pain with no cough that was identified by chest x-ray as residual effusions. Incisional hernia was reported in one of the subjects in the 6 months visit after OLT. None of the AEs were related to treprostinil treatment.

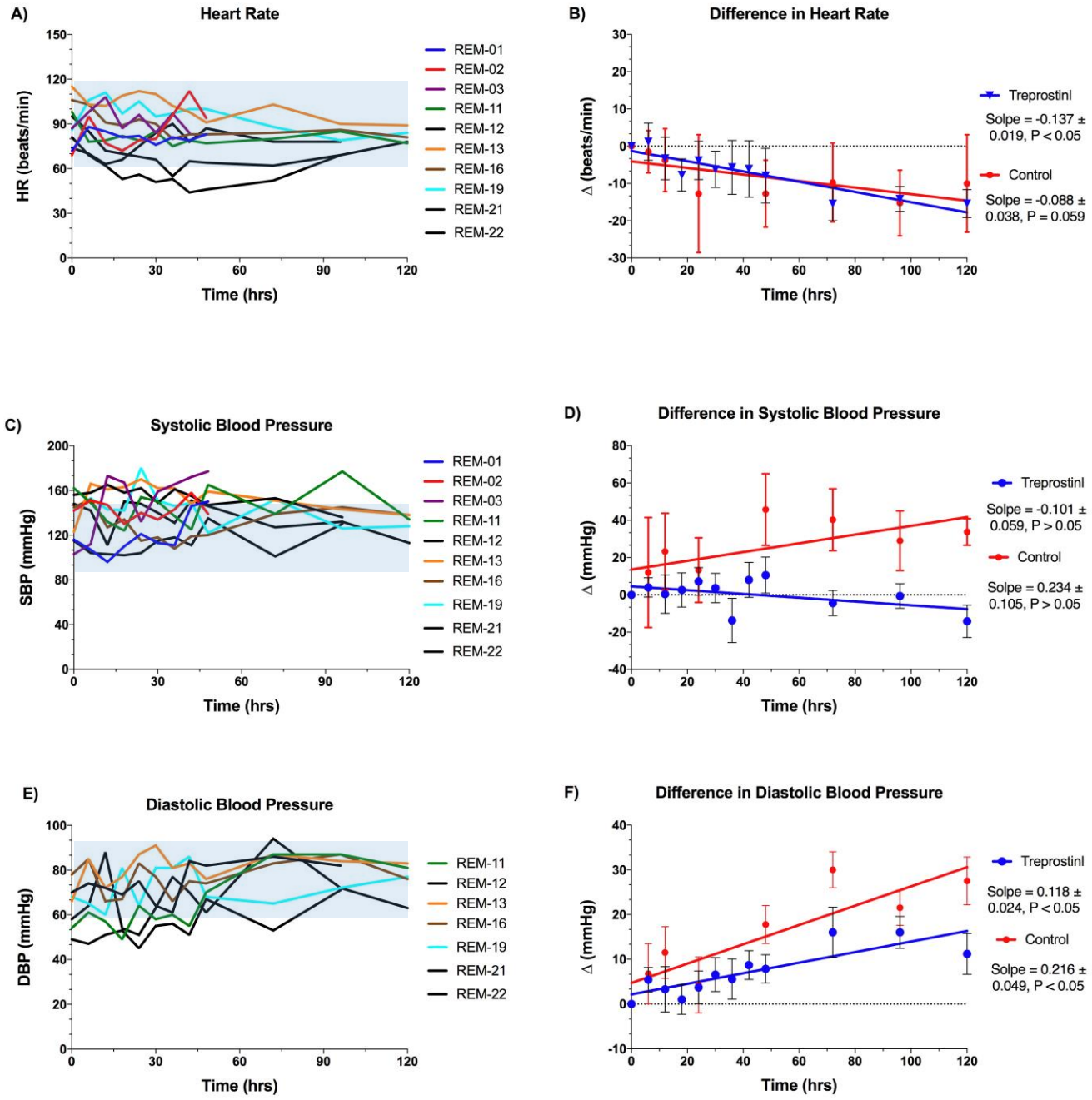


Figure 5-3: Heart rate and arterial blood pressure parameters for patients received treprostinil.

A, C and E depicts the individual heart rate, systolic and diastolic blood pressures over the 120 hours from the time of infusion start, where the blue shaded areas represent the normal range for healthy subjects. B, D and F show the fitted linear regression (with slopes and significance) of the difference of each time point from time zero in HR, SBP and DBP, respectively, for treprostinil (blue) and controls (red). Data is expressed as mean ± SD (N = 14).

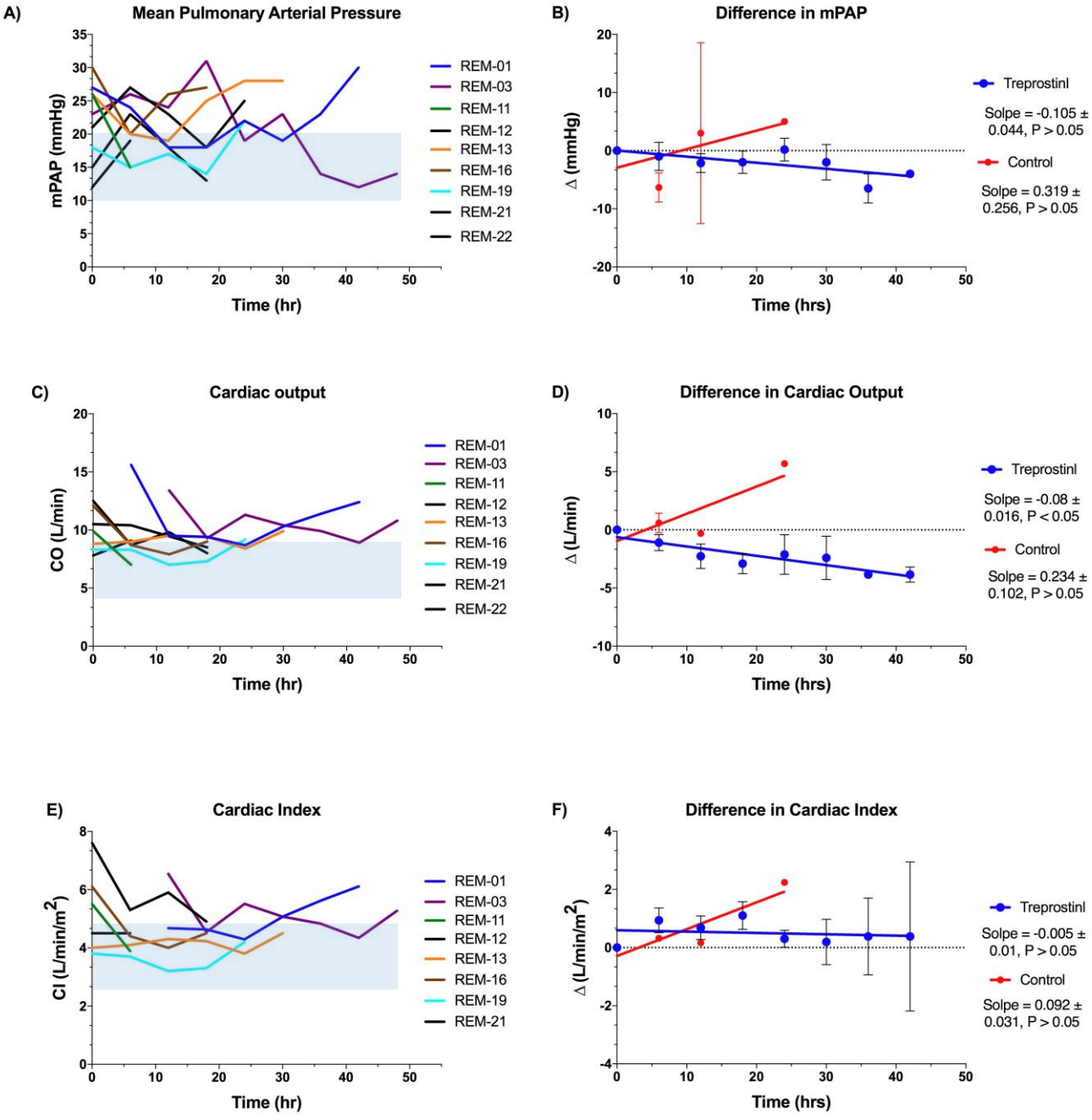


Figure 5-4: Hemodynamic parameters for patients received treprostinil infusion.

A, C and E depicts the individual mean pulmonary arterial pressure, cardiac output and cardiac index over two days followed liver transplantation, where the blue shaded areas represent the normal ranges. B, D and F show the fitted linear regression (with slopes and significance) of the difference of each time point from time zero in mPAP, CO and CI, respectively, for treprostinil (blue) and controls (red). Data is expressed as mean ± SD (N = 14).

5.3.3 Liver function assessment and clinical outcomes

Figure 5-5 demonstrates that ALT and AST levels following the LTx were at the maximum concentration at time zero, 684 vs. 513 and 1515 vs. 1166 IU/L (medians) respectively, in patients received treprostinil vs. controls. These values are expected in our patient population since they just recovered from liver transplantation. Levels were recovered to normal ranges more rapidly in treprostinil group. The total bilirubin concentration was around 3 mg/dL at the start and reached normal levels (less than 1.5 mg/dL) at day three; whereas median total bilirubin in controls continued to be high over the first week. AUCs for ALT, AST, T.Bilirubin and SCr were calculated using trapezoidal method and none of the showed difference between the study and control groups.

The median serum creatinine concentration was sustained between 1 – 1.5 mg/dL which indicate normal renal function. Coagulation factors are important to evaluate the synthetic function of transplanted grafts. Platelet counts were low during the study but those levels are comparable to controls. The prothrombin times were prolonged in the beginning, for both group similarly, and recovered to normal during the first week. Hepatobiliary capacity for all patients were evaluated using indocyanine green plasma disappearance (ICG-PDR) test. The median values were 21 and 19.9 %/min at at day 2 and 5 post-LTx, respectively. There were no primary graft nonfunctional events documented. We followed the patients for up-to 180 days post-OLT and all grafts and subjects were survived in the treprostinil group, but the survival rate in controls was 80%. Treprostinil infusion significantly reduced the need of needed of ventilator usage when compared to controls, 1 vs. 2.5 days ($p < 0.05$), respectively. In addition, the length of stay in the ICU and hospital were 3 vs. 4 and 8 vs. 15 days for treprostinil patients vs. controls, respectively, but was not significant.

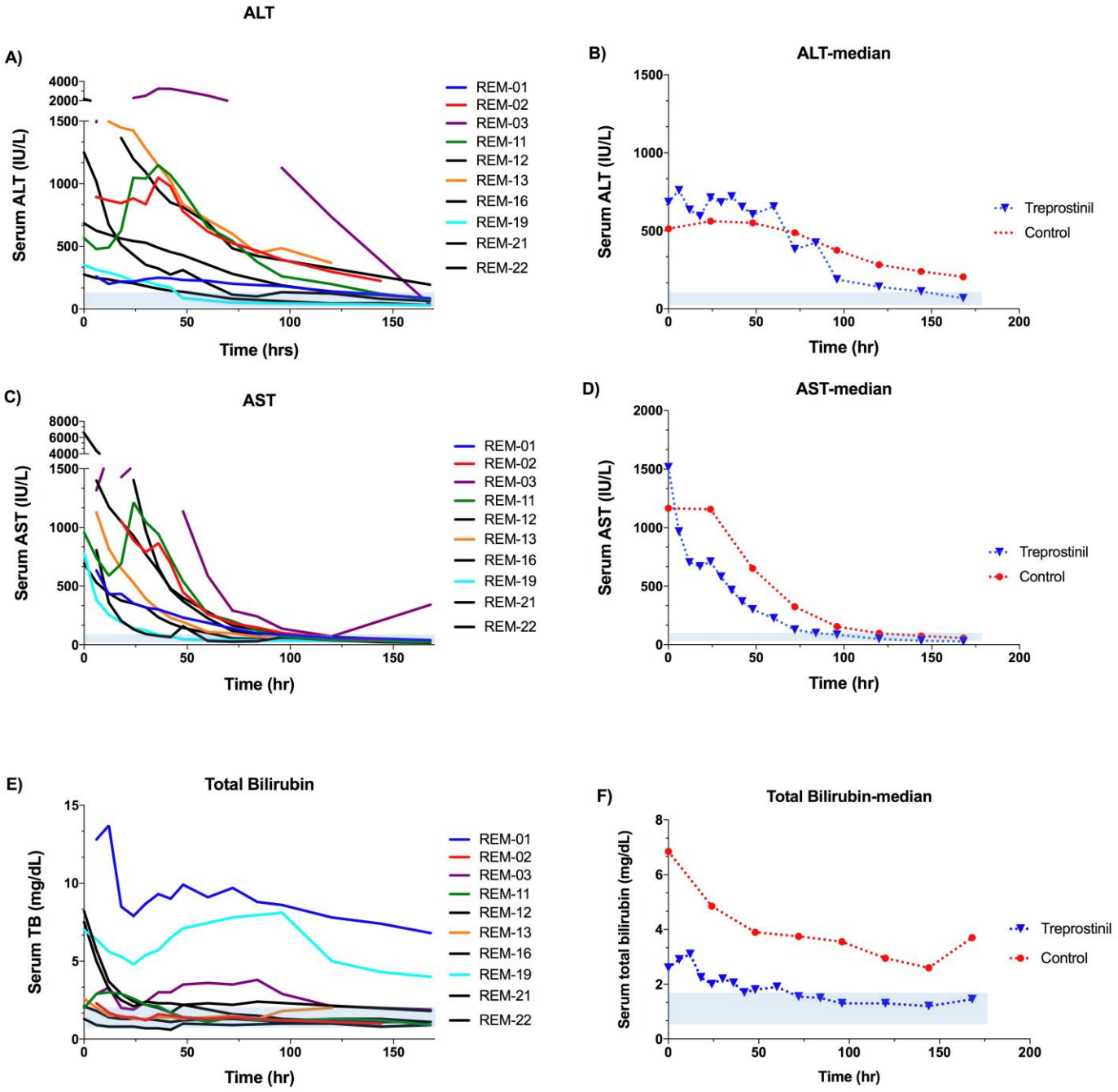


Figure 5-5: Hepatic injury markers during the first week of treprostinil infusion initiation.

A, C and E show the individual ALT, AST and total bilirubin levels during the first week. B, D and F depict the median values of those tests, respectively, for treprostinil infused patients (blue) and controls (red). The blue shaded areas represent the normal range for healthy subjects. Data is expressed as medians (N = 14).

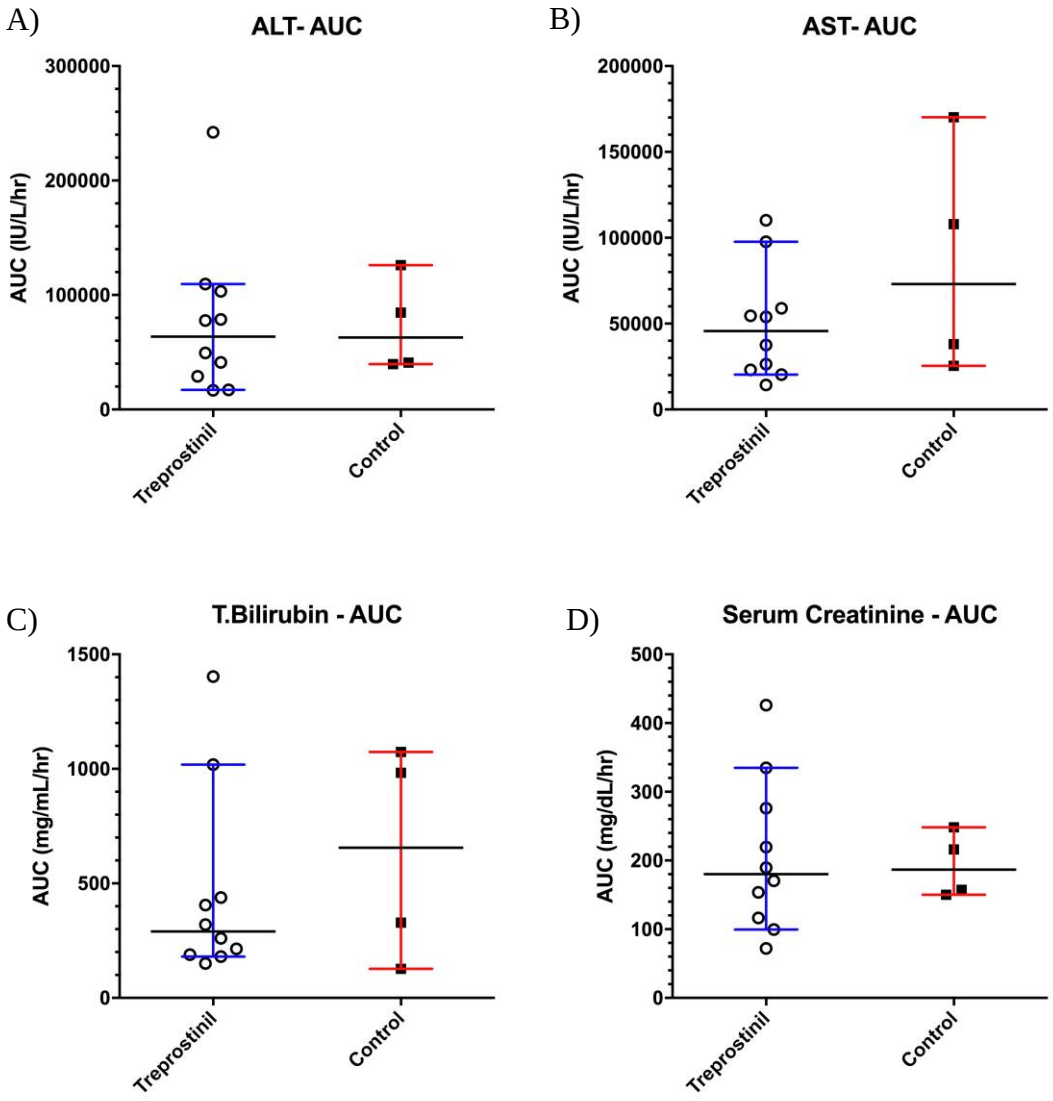


Figure 5-6: Area under the concentration curves (AUC) for liver and kidney biomarkers for 7 days post-LTx. A, B, C and D show the median (95% CI) for the calculated individual AUCs for ALT, AST, total bilirubin and SCr during the first week. Patients received treprostinil plotted in blue and controls in red. Data is expressed as median with 95% confidence intervals (N = 14).

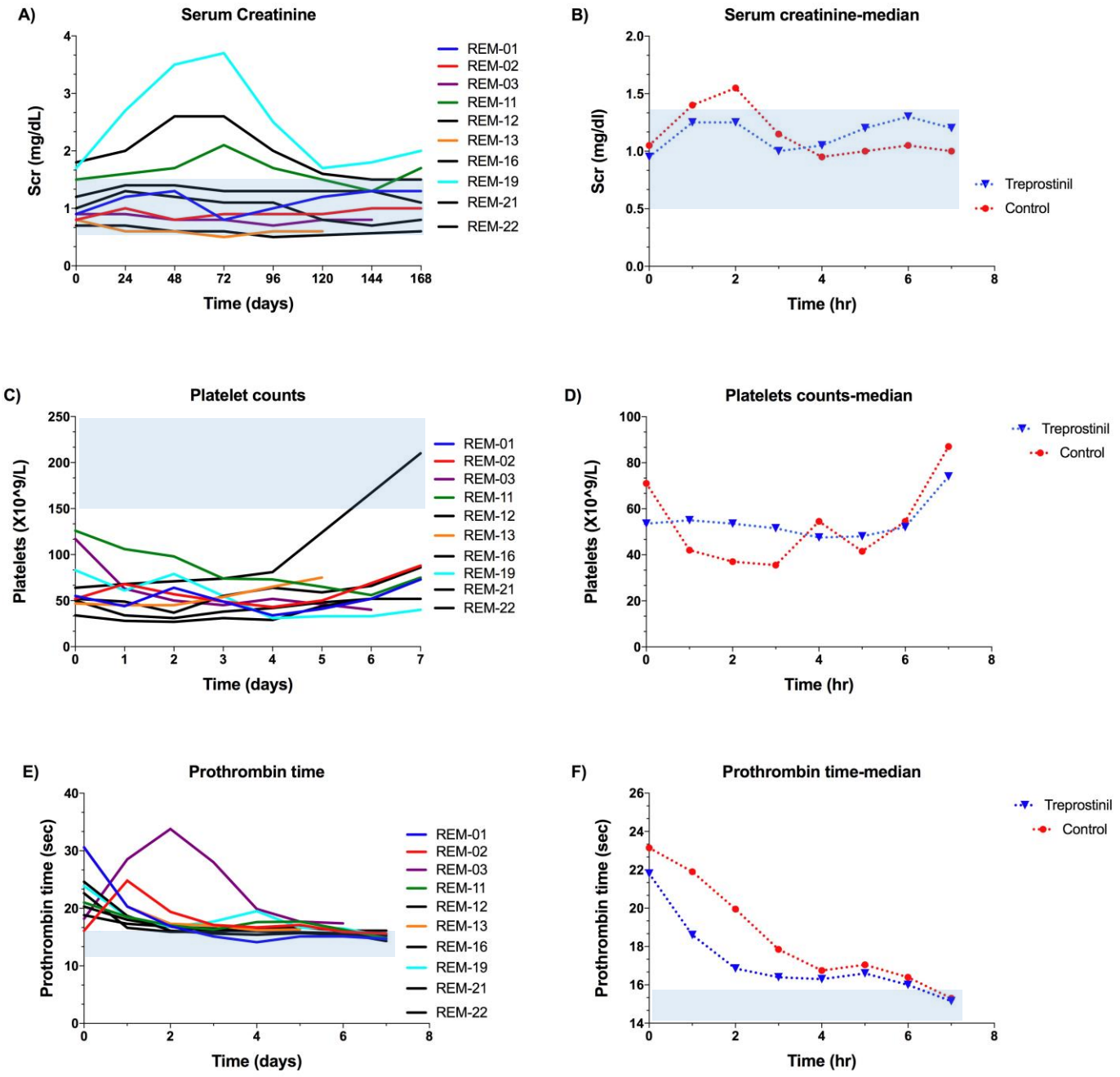


Figure 5-7: Renal injury marker and blood coagulation markers.

A, C and E demonstrate the individual values during the first week for serum creatinine, platelet counts and prothrombin time and, B, D and F, are the median values of those tests, respectively, for treprostinil group (blue) and controls (red). The blue shaded areas represent the normal range for healthy subjects. Data is expressed as medians (N = 14).

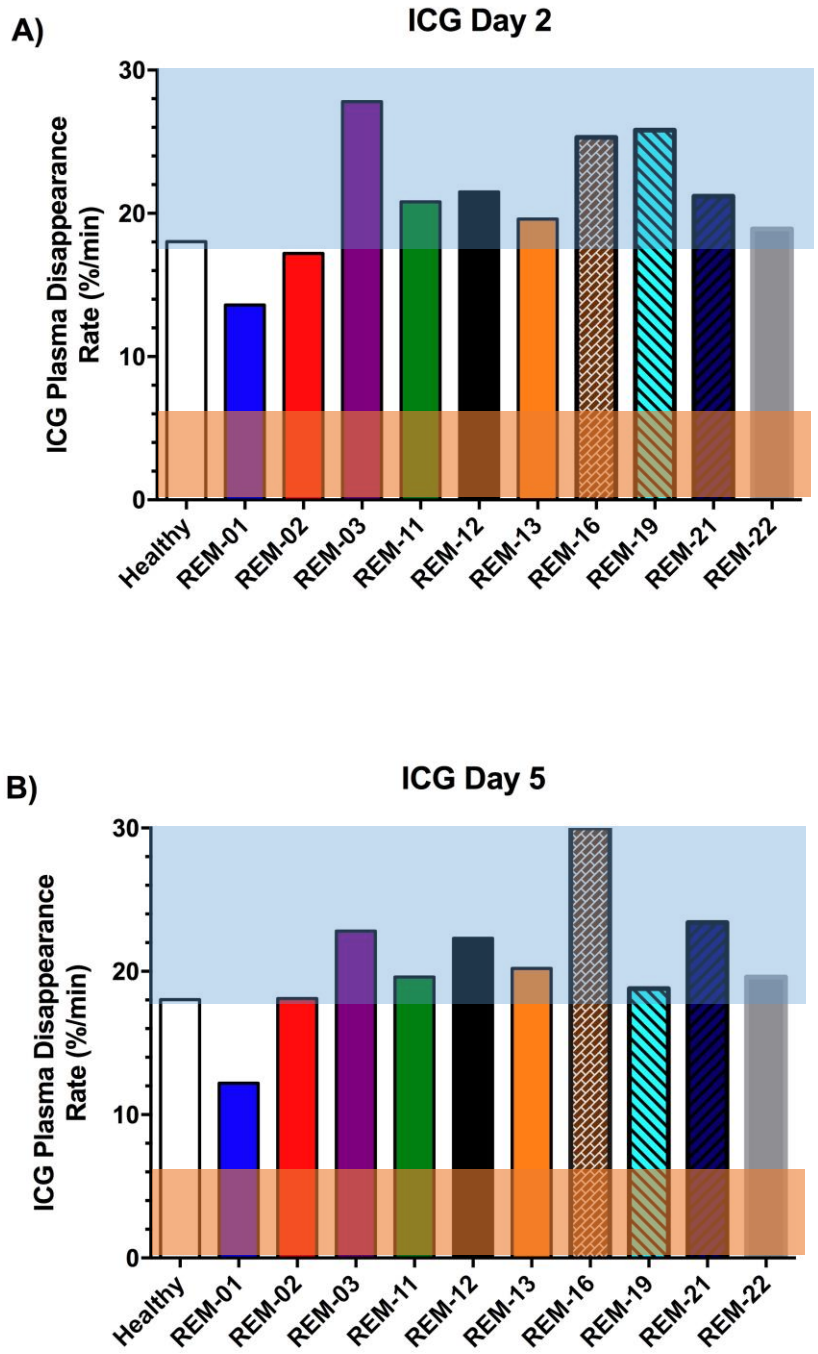


Figure 5-8: Hepatobiliary excretory functional assessment via ICG-PDR tests.

A and B illustrate the individual results of indocyanine green-plasma disappearance (ICG-PDR) at day 2 and day 5, respectively. The blue shaded areas represent the normal range for healthy subjects and the red shaded areas represent the range of values for patients with compromised liver grafts.

Table 5-4: Summary of clinical outcomes.

	Treprostinil	*Control*
Indocyanine green – plasma disappearance rate (ICG-PDR)		
At day 2	*21 (13.6-27.8)*	-
At day 5	*19.9 (12.2-30)*	-
Duration of stays (days)		
On ventilator	1 (1-2) *	2.5 (1-3)
ICU	3 (2-4)	4 (2-12)
Hospital (total)	8 (6-26)	15 (7-58)
Survival rates		
Liver graft (180 days)	100%	80%
Subject (180 days)	100%	80%
Primary graft nonfunctional (PNF)	0%	20%

*P < 0.05 compared to control group

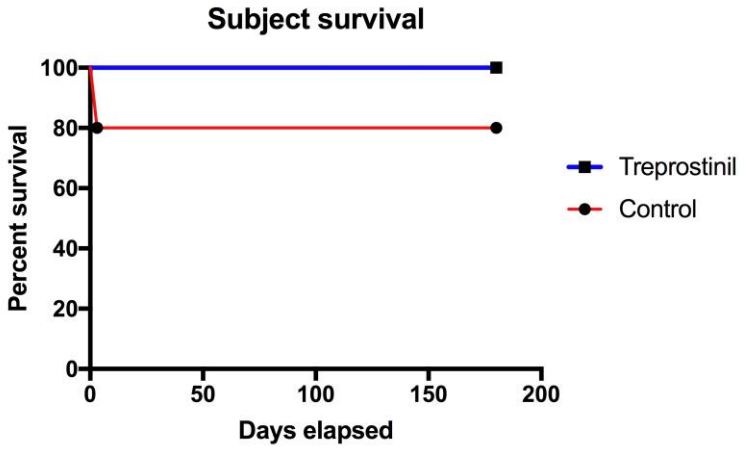


Figure 5-9: Kaplan-Meier curve for 180 days post liver transplantation.

The graph shows the results of subjects survival (treprostinil; blue and controls; red) follow-up for everyday for the first week, every month for 3 months then at 180 days post LTx.

5.4 DISCUSSION

This is the first clinical study to systematically investigate the safety and feasibility of five days of continuous IV infusion of treprostinil, a stable synthetic PGI_2, in liver transplant patients in order to attenuate the ischemia and reperfusion mediated organ damage. Our results reveal that there are no safety concerns related directly or indirectly to treprostinil. A total of 95 subjects were screened and eight patients received the entire course of planned treprostinil infusion. These patients were distributed into three dose levels and chronologically escalated in terms of total amount of treprostinil, where 3, 3 and 2 participants were included in 5 ng/kg/min for two days, 2.5 and 5 ng/kg/min for five days, respectively.

Cold static organ storage is the golden standard of allograft preservation but comes with certain limitations. Extended criteria or marginal livers are often not used because they are more susceptible to I/R injury.[196] Report indicated that more than 26% of the livers that are retrieved from donors after cardiac death (DCD) are not transplanted.[197] Many different approaches have been utilized to enhance the clinical outcomes and overcome the complications, such as PNF. A new era in developing more competitive techniques in the methods of organ preservation is the use of machine perfusion. Recently, Ravikumar et al. (2016) published the first report on utilizing the normothermic machine perfusion in human liver transplantation. They concluded that only peak AST in the first week was significantly reduced and the other parameters were similar to cold static preservation group.[198] Other investigators also have developed cell-free oxygen carrier solution that complement the use of liver perfusion machine under subnormothermic conditions.[199] Several logistics and technical issues still limit practical use of these machines. However, one of the most suitable and easy to be generalized to other transplant

centers is the addition of pharmacological agents in the UW solution to improve LTx graft outcomes.

This phase I/II study that was initiated at the UPMC was to assess the safety and preliminary efficacy of treprostinil in liver transplant patients. Our results show that using treprostinil in liver transplant patients after they are hemodynamically stabilized is generally safe. All patients received their respective doses and continued to be hemodynamically stable over the study period. Systemic and pulmonary arterial pressure parameters were closely monitored and were not affected by the administration of treprostinil. No participants experienced any significant complications that required the use of inotropes at any time. In the current study, we have demonstrated that our primary end points were achieved, since mean pulmonary arterial pressure, systemic blood pressure and cardiac index values stayed within normal values. In patients with PAH, targeting a reduction in the mPAP is essential and is considered the only predictor of patient's survival, which was not altered in our LTx patients at the dosing regimen used.[200]

Various methods have been employed to assess the liver functions, either by static or dynamic tests. We have shown that the transaminases concentrations were rapidly dropping and reached the normal levels within the first week. Previously, rodent OLT study and *ex-vivo* isolated liver perfused system have shown that treprostinil significantly reduced the peak and AUC of ALT and AST, which was accompanied with less necrosis.[129] Those proteins are released from hepatocytes and are used as surrogate markers of the preservation injury.[201] Moreover, the excretory function of the liver grafts recovered in three days as measured by total bilirubin (<1.5 mg/dL); whereas, values of more than 10 mg/dL were associated and used as predictor of initial poor function.[202] In fact, the levels in control group were higher than

treprostinil treated group. An improved coagulation parameter, as measured by prothrombin time, brings an insight of the synthetic capabilities of the transplanted allografts. There was no alteration in the prothrombin time test when treprostinil was used and results are comparable to liver transplant patients without treatment. In the same way, the renal function post-transplantation for patients received the intervention were maintained and not different from the control group. Cochrane review analysis supports that PGs significantly minimized the risk of acute kidney failure.[101]

Indocyanine green, an inert and water-soluble dye, was used to measure the hepatic dynamic function. ICG-plasma disappearance rate (PDR) parameter denotes the clearance of the grafts, where 97% of the dose is excreted unchanged into the bile after uptake by transporters such as SLCO1B3 (OATP1B3) and SLC10A1 (NTCP) then effluxed via ABCC2 (MRP2), ABCB4 (MDR3) and ABCB1 (MDR1; P-gp) with no enterohepatic recirculation.[201, 203] Other reports have shown that ICG-PDR ranged between < 8 and > 20 %/min, where 8 %/min or less predicted death with a sensitivity and specificity of 81 and 70 %, respectively.[202, 204, 205] Using MELD score and early ICG-PDR test were proposed as a tool of risk assessment and outcome prediction.[206] The PDR test results at 2 and 5 post-operative days showed that almost all of the grafts cleared the ICG as efficiently as normal healthy subjects. These findings indicate recovery of hepatic excretory function, energy and microcirculatory blood flow.

Another secondary endpoint was to prevent any primary graft nonfunction (PNF), where the incidence can reach up to 23%.[59] All grafts survived through the study period of 180 days. We had 100 vs. 80% survival rate in treprostinil versus controls groups, respectively. Two other patients have excellent functioning grafts after two years, where received treprostinil before, during and after OLT procedure.[132] Additionally, a retrospective study reported that using

continuous subcutaneous treprostinil infusion (ranged 19 - 53 ng/kg/min) in five moderate to severe portopulmonary hypertension diagnosed patients underwent OLT and all have survived on an average for 30 months of follow-up.[200] Barthel et al. (2012) have administered iloprost, PGI_2 analog, immediately after transferring liver transplanted patients to the ICU in order to minimize the PDR but they have documented that six months mortality (12.5%; n=40) was not different than controls.[115] It has been documented higher mortality and 20% lower 1-, 3-, and 5-years survival rates in patients after retransplantation.[46]

The impact of treprostinil on the need for supportive care, intensive care unit and total hospital stays was also evaluated. Treprostinil treated patients needed ventilation support for shorter time with a median of one day; however, control subjects needed significantly longer duration of a median of 2.5 days. The length of stay in both ICU and hospital was only 3 vs. 4 and 8 vs. 15 days for treprostinil and controls, respectively, which might indicate some of the beneficial effects of treprostinil. In fact, one patient discharged before completing the five days of infusion of treprostinil. This was in line with a study that showed PGE_1 significantly reduced the hospital and ICU stays by 20% and 40%, respectively.[109] However, there have been other studies documenting longer length of stays in ICU (6-12 days) and hospitalization (27-32 days) (median-mean) in patients received iloprost.[115, 206]

In general, prostacyclin's are well-known for their vasodilation and antiplatelet aggregation activities. Moreover, treprostinil was 10 fold more potent than other stable PGI_2 analogs.[207] The TRUST study group has shown that using treprostinil in PAH patients significantly decreased the levels of some cytokines, such as matrix metalloproteinase-9 (MMP-9) and angiopoietin-2 (Ang-2).[208] Furthermore, we have demonstrated in several other preclinical studies the beneficial effects on attenuating I/R injury, when treprostinil was supplemented into

WU preservation solution and administered after liver transplantation. Those protective effects were associated with lower hepatic injury, down-regulated cytokine expression, and improved microcirculatory blood flow integrity and metabolic functions.

In conclusion, this clinical trial indicates the safety and feasibility of using treprostinil in liver transplant patients. We anticipate a promising future for implementing treprostinil treatment in a larger phase III clinical study to document its efficacy in minimizing cold ischemia and warm reperfusion injury.

6.0 PHARMACOKINETIC CHARACTERIZATION OF TREPROSTINIL ORTHOTOPIC LIVER TRANSPLANT PATIENTS

6.2 INTRODUCTION

For liver transplantation, the grafts are retrieved mainly from deceased donors, transported in ice cold University of Wisconsin (UW) preservation solution for durations up to 12 hours and subsequently transplanted. These grafts are susceptible to cold ischemia and warm reperfusion injury. This injury is associated with early poor dysfunction, primary graft nonfunctional and morbidity and mortality of the liver recipients. Many experimental and clinical studies have shown beneficial effect of using PG and PGI_2 to attenuate the ischemia and reperfusion associated damages.[101] Treprostinil has demonstrated a protective effect in several preclinical rat studies (chapters 2.0 , 3.0 and 4.0).[129-131] Treprostinil (Remodulin®; United Therapeutics, Inc.), a stable long-acting synthetic prostacyclin (PGI_2), has been approved in the United States and several other countries for pulmonary arterial hypertension (PAH) with New York Heart Association Class II-IV symptoms. It has a potent pulmonary and systemic vasodilatory activities, inhibitory action against platelet aggregations and cytokine release.[126, 207] Treprostinil has more favorable pharmacokinetic properties than any other prostaglandins and PGI_2 analogs, where half-life ($T_{1/2}$) is about 3 to 4 hours compared to 30 minutes of the longest PGI_2 analog.[124, 209] The injectable formulation with concentrations as low as 4 μg/mL can be stored at ambient temperature for up to 48 hours due its chemical stability.[128]

Treprostinil doses in typical PAH patients starts from 1.25 ng/kg/min and escalated every week based on the clinical response. However, liver transplant population is known to have compromised metabolic functions due to several mechanisms; hepatic blood flow disruption, high concentrations of proinflammatory cytokines in the circulation, altered plasma protein concentrations and decreased bile formation.[49, 55] Treprostinil is primarily metabolized in the liver by CYP2C8 and CYP2C9. Co-administration of gemfibrozil (CYP2C8 inhibitor) and

rifampin (CYP inducer) have increased and decreased treprostinil exposer respectively.[128] The manufacturer (United Therapeutics, Inc.) also recommend to start with 50% of the regular dose (0.625 ng/kg/min) in patients with mild to moderate hepatic insufficiency. Therefore, understanding the pharmacokinetics of treprostinil immediately after liver transplantation is crucial for future dosing purposes.

The purpose of this study was to evaluate the pharmacokinetic parameters of three infusion rates (5 ng/kg/min for 48 hours and 2.5 and 5 ng/kg/min for 120 hours) of treprostinil in hemodynamically stable liver transplant recipients using a partially validated UPLC-MS/MS assay for treprostinil.

6.3 METHODOLOGY

6.3.1 Participants

Nine adult patients who went through orthotopic liver transplantation received a continuous intravenous infusion of treprostinil (Remodulin® Injection, United Therapeutics Corporation, Research Triangle Park, NC). Three different dose levels were given in a 3 consecutive patients, first, second and third group of three patients received 5 ng/kg/min for two days, 2.5 ng/kg/min for five days and 5 ng/kg/min for five days, respectively. All patients received standard immunosuppression protocol for adult liver transplant patients at the University of Pittsburgh Medical Center (UPMC). The clinical study protocol and informed consent (Appendix A below) were approved by the University of Pittsburgh Institutional Review Board. All patients were agreed and signed the approved written informed consent.

6.3.2 Specimen Collection

Multiple blood samples were drawn at time of treprostinil infusion initiation (time 0) and approximately 2, 4, 6, 12, 18, 24, 30, 36, 42, 48, 72, 96 and 120 hours during the continues infusion and approximately 0.5, 1, 2, 4, 6, 8, 12 and 24 hours post infusion termination. All samples (3 mL) were collected from either central or peripheral lines in K_2EDTA coated tubes. Blood samples were processed immediately or initially kept at 4°C for maximum 6 hours till plasma separation by centrifugation. Samples were aliquoted and frozen at -80°C until analysis.

6.3.3 Assay

6.3.3.1 Chemicals and materials

Treprostinil (Remodulin® Injection, United Therapeutics Corporation, Research Triangle Park, NC) 1 mg per mL in a 20 mL vial was provided by the manufacturer, United Therapeutics Corporation (Silver Spring, MD). Pure treprostinil (CAS № 81846-19-7) and 6-keto Prostaglandin $F_{1\alpha}$-d_4 (CAS № 82414-64-0) were purchased from Cayman chemical (Ann Arbor, MI). Oasis HLB 1 mL (30 mg) extraction cartridges and Acquity UPLC® BEH C18 column (100 mm × 2.1 mm, 1.7 µm, 130Å) (part № 186002352) was procured from Waters Corporation (Milford, MA, USA). All the solvents were of MS grade and were obtained from Fisher Scientific (Pittsburgh, PA, USA).

6.3.3.2 Preparation of standards and quality control samples

Stock solutions of treprostinil and 6-keto Prostaglandin $F_{1\alpha}$-d_4 were prepared at 1 mg/mL in methanol and methyl acetate, respectively. Stock solutions were kept at −20°C in the dark.

Working solutions were prepared on the assay days and were diluted in 50% methanol for to produce 0.1 µg/mL. Standards for the calibration curve were prepared by serial dilution of the working solution by spiking into blank plasma. The concentrations of calibrators used in the assay are 10, 50, 100, 500, 1000, 2500 and 5000 pg/mL equivalent to 0.19, 0.94, 1.88, 9.38, 18.75, 46.88 and 93.75 pg injected onto the column, respectively. Similar to the calibration standards, but in a separate process, the quality controls (QCs) were prepared. The concentrations of low (QC-L), medium (QC-M) and high (QC-H) quality controls were 25, 2500 and 4500 pg/mL (0.47, 46.88 and 84.38 pg on column), respectively. For the internal standard (6-keto Prostaglandin $F_{1\alpha}$-d_4), the stock solution was diluted serially in 50% methanol to achieve final concentration of 100 ng/mL to be used with standard curve, quality controls and plasma samples preparations.

6.3.3.3 Sample processing

All calibration standards, quality controls, and plasma samples were thawed at room temperature. In a centrifuge tube, 200 µL of sample, 1800 µL of water and 20 µL of internal standard were mixed. Samples were passed through via Oasis HLB 1 mL (30 mg) extraction cartridges, after conditioning the cartridges with 1 mL methanol and 1 mL water and followed by then washing step of the cartridge with 1 mL of 5% methanol. Analytes were eluted with 1 mL of 0.1% acetic acid in methanol. The eluent was completely evaporated under air at room temperature and the residue was reconstituted in 80 µL of 50% methanol. 7.5 µL of the reconstituted solution was injected into the LC–MS/MS system.

6.3.3.4 Chromatographic and mass spectrometer conditions

Acquity ultra performance liquid chromatography H-class used was used (Waters Corporation, MA, USA). Analytes were separated using UPLC® BEH C18 column (100 mm × 2.1 mm, 1.7 µm, 130Å). The column temperature was maintained at 55°C and the auto sampler temperature was set at 4 °C. Mobile phases, delivered at a flow rate of 0.5 ml/min, consisted of (A) 0.005% acetic acid, 5% acetonitrile in deionized water and (B) 0.005% acetic acid in acetonitrile. A gradient mobile phase system was used at 0.5 mL/min, initial 0.4 minutes the A and B mixture were 65:35, respectively. Linearly mixture A was decreased to 30% during 3.6 minutes then to 5% by 4.5 minutes and continued till 4.8 minutes. The column recalibrated by returning the condition of 65% mobile A in 0.1 minute and held till 6.4 minutes, which was the total run time.

Analytes detection was performed using a TSQ Quantum Ultra from Thermo Fisher Scientific (San Jose, CA). This is a triple quadrupole mass spectrometer coupled with heated electrospray ionization source (HESI) operated in negative selective reaction monitoring (SRM) mode with unit resolutions at both Q1 and Q3 set at 0.70 full width at half maximum. Treprostinil and I.S. were quantitated by SRM analysis by monitoring their respective m/z transitions. The m/z transitions for treprostinil and I.S. (6-Keto Prostaglandin F1 alpha-d4) were 389.3 → 331.2 and 373.2 → 249.2, respectively. Retention time for I.S. was 0.9 minutes and treprostinil was 2.65 minutes. Highest [M-H]- ion abundance was used to optimize the instrument parameters and were as follows: vaporizer temperature 325°C, capillary temperature 365 °C, spray voltage -3.5 kV, sheath gas pressure 60 psi, aux gas pressure 34 psi, tube lens offset 82, skimmer offset 0, collision energy 25V and Q2 gas pressure (Argon) 1.5 mTorr. Scan time was set at 0.01 second. Analytical data was acquired and analyzed using Xcalibur software version 2.0.6 (Thermo Finnigan, San Jose, CA).

6.3.3.5 Calibration curve and lower limit of quantitation (LLOQ)

Standard curves were prepared as described above and were injected to the analytical system. The lower limit of quantitation was identified by achieving a signal-to-noise (S/N) ratio of at least 5:1. The response for each sample was calculated by dividing the area of treprostinil peak by the area of 6-keto Prostaglandin $F_{1\alpha}$-d_4 (I.S.) peak. Standard curves were constructed by plotting the analyte-to-I.S. response ratio versus the nominal concentration of treprostinil in each sample. A linear regression was fitted with 1/X weighting, without forcing the line through the origin. The deviations of the back calculated concentrations from the nominal concentrations, expressed as percentage of the nominal concentration, reflected the assay performance over the concentration range.

6.3.3.6 Accuracy and precision

The accuracy and precision of the developed method were determined by analyzing the three levels of quality controls. QC-L, QC-M, and QC-H samples were run together with an independently prepared calibration curve for three days; followed-up with, fourth day of running triplicates for each calibration standards and QC samples. Accuracy, or relative standard error (RE%), was calculated as follow:

Equation 6-1: Accuracy (or RE%) = (calculated value (*E*) - true value (*T*))/ *T* x 100%

The precision was expressed as the relative standard deviation (RSD%) of the mean concentrations and calculated as shown in Equation 6-2. Accuracy and precision values of ≤ ±15% of the nominal value were considered acceptable. Intraday and interday assay values were

assessed by replicate analysis of specimen aliquots on a single day or successive days, respectively.

Equation 6-2: Precision (or RSD%) = (standard deviation (SD) / Mean (M)) x 100%

6.3.3.7 Extraction recovery and matrix effects

The extraction recovery (ER) efficiency of treprostinil sample processing was determined at for all quality control levels in quadruplicates (n=4) by comparing the absolute responses (areas) of samples spiked before extraction to those spiked after blank plasma extraction (Equation 6-3). Extraction recovery samples were processed using of the solid phase extraction and determined by UPLC-MS/MS, as described above.

Equation 6-3: ER = Response of spiked samples **before** extraction / Response of spiked samples **after** extraction

Matrix effect (ME) on the analytical assay of plasma specimens was defined as the relative ratio of the absolute signal of spiked blank plasma samples after the extraction procedure, as described above, to the absolute signal of the same concentration in neat preparation, which is reconstituted in 50:50v methanol:H_2O. The ME was calculated as seen in Equation 6-4 for all three quality control levels and represented as mean (M) with coefficient of variation (CV%) (n=4) as precision indicator. Values of matrix effect less than or more than 1 were considered as ion suppression or ion enhancer effects, respectively.

Equation 6-4: ME = Response of spiked samples **after** extraction / Response of neat samples

6.3.4 Pharmacokinetic analyses

All pharmacokinetic analyses were based on the plasma treprostinil concentrations using the analytical procedure described above. Phoenix WinNonlin 6.4 was used to perform the pharmacokinetic evaluation.[210] A non-compartment model was utilized to determine the pharmacokinetic parameters for treprostinil. A separate pharmacokinetic analyses for each patient plasma concentration-time profile were carried out and the maximum plasma concentration at steady state ($C_{ss,max}$), the time to $C_{ss,max}$ (T_{max}), average steady state concentration ($C_{ss.avg}$) and the terminal elimination rate constant (k_{el}) were obtained. The area under plasma concentration versus time curve (AUC) was determined using the trapezoidal method, from the time of treprostinil infusion initiation to infinity ($AUC_{0-\infty}$). Among patients with missing data, the k value was calculated from at least 3 data points in the terminal phase. The clearance (CL_{ss}) and apparent of distribution (Vz) were calculated by total Dose/ $AUC_{0-\infty}$ and clearance/k_{el}, respectively.[211]

6.3.5 Statistical analysis

GraphPad Prism software, version 6.0h (GraphPad Software, La Jolla, CA) was used to conduct the statistical analyses and plot the graphs. To test the statistical significance in each pharmacokinetic parameter between the three groups, Mann-Whitney nonparametric test was applied and Dunn's multiple comparisons test was used as post-hoc test to identify the different

group. Data is represented as median and interquartile range (IQR) and $p \leq 0.05$ was considered statistically significant.

6.4 RESULTS

6.4.1 Method of analysis partial validation

6.4.1.1 Calibration curve, linearity and lower limit of quantitation

Several standard curves were run in the analytical system and the lower limit of quantitation (LLOQ) was identified as 10 pg/mL with a signal-to-noise ratio of more than 5 and coefficient of variation (CV%) of less than 20%. Human blank plasma samples were run and no endogenous molecule peaks that interfere with treprostinil or I.S. were identified within the specific retention time of both. The final concentrations range of calibration standards was 10 to 5000 pg/mL with regression equation "y = -0.0067 + 0.0037x" and a proven linearity after using of weighting factor 1/x that showed best option to provide best fit with correlation coefficient of determination (R^2) = 0.9981 and was visually inspected. Figure 6-1 shows the representative curve of the sample concentrations vs. treprostinil-to-I.S. signal ratio. All quality controls (L, M, and H) fell within 15% deviation of back calculated amounts from nominal spiked amounts for all levels and the correlation coefficients (R^2) for all curves were > 0.99.

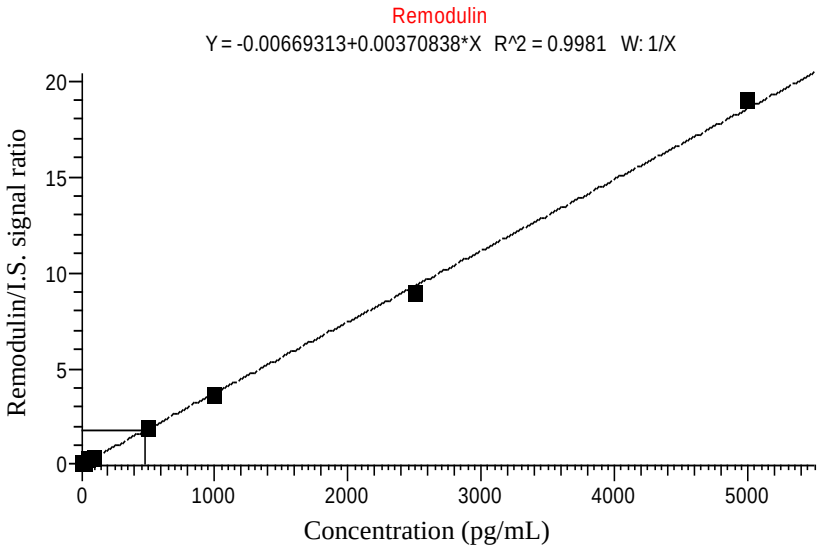


Figure 6-1: Representative standard curve run on the UPLC-MS/MS instrument.

6.4.1.2 Intra-day and inter-day validation

The intra-day and inter-day accuracy and precision for treprostinil assay were evaluated at three level concentrations 25, 2500 and 4500 pg/mL by utilizing the quality control samples within four validation runs. The intra-day reproducibility was determined using the calculated concentrations using triplicates at each concentration within a single day. On the other hand, the inter-day variability was determined using triplicate runs of each concentration at four separate days. Table 6-1 shows the calculated values for the three QC levels using the generated equation of the linear regression from the calibration curves that were run in the same day. All RSD% values were less than 15% which indicate reproducible assay results.

Table 6-1: Intra-day and inter-day accuracy and precision for treprostinil (Remodulin®) using blank human plasma samples.

Concentration (pg/mL)	Intra-day assay (n=3)			Inter-day assay (n=4)		
	Mean ± SD	RE (%)	RSD (%)	Mean ± SD	RE (%)	RSD (%)
Low QC (25)	25.4 ± 1.6	1.6	6.2	26.9 ± 2.2	7.8	8.3
Medium QC (2500)	2319.6 ± 85.0	-7.2	3.7	2732.7 ± 278.8	9.3	10.2
High QC (4500)	4224.0 ± 89.9	-6.1	2.1	4634.8 ± 492.3	3.0	10.6

6.4.1.3 Extraction efficiency and matrix effect

Extraction recovery efficiency at all three quality control levels, 25, 2500 and 4500 pg/mL, were 87.9, 76.8 and 80.2%, respectively. The overall value for treprostinil recovery was 81.6 ± 7.3%. The reproducibility was good with a RSD% of less than 15% (Table 6-2). The matrix of our samples, which was blank human plasma, enhanced the ion product of treprostinil. The signal of spiked samples after processing the plasma in solid phase extraction was higher than the area of neat samples of the same concentrations. The mean and SD of the matrix effect on treprostinil ionization was 1.19 with a RSD equal to 8.1%, which shows a good precision and reliability.

Table 6-2: Extraction recovery efficiency and matrix effect in spiked blank human plasma samples for treprostinil (Remodulin®).

Concentration (pg/mL)	Extraction Recovery (n=4)		Matrix Effect (n=4)	
	Efficiency (%)	RSD (%)	Mean	RSD (%)
Low QC (25)	87.9	9.8	1.10	9.2
Medium QC (2500)	76.8	2.8	1.28	1.2
High QC (4500)	80.2	6.9	1.20	1.8
Overall	81.6	8.9	1.19	8.1

6.4.2 Treprostinil pharmacokinetics

Collected patient blood samples in tri-potassium ethylenediaminetetraacetic acid (K_3EDTA) were processed immediately after blood collection and aliquoted into 3 microcentrifuge tubes after separating the plasma by centrifugation. All plasma samples were stored at -80^{o}C till UPLC-MS/MS analysis. Plasma treprostinil concentrations (median (IQR)) versus time for the three dose groups were plotted. Figure 6-2A illustrates the 5 ng/kg/min profile for 48 hrs and followed by the terminal slope up to 56 hours. Data from participants who received 120 hours continuous infusion both 2.5 (green) and 5 (red) ng/kg/min are plotted in Figure 6-2B. The steady state clearance values were 2.98, 6.02 and 5.22 mL/min/kg for the three groups 5 ng/kg/min for 2 days, 2.5 and 5 ng/kg/min for 5 days, respectively. The exposure that was measured by estimating the area under the plasma curve median values were 91, 50.3 and 115.4 hrs*ng/mL. The steady state plasma clearance values were 2.98, 6.02 and 5.22 mL/min/kg. The median treprostinil half-live ($t_{1/2}$) was calculated from the terminal elimination rate constant were 1.47, 0.87 and 0.88 hours, respectively. The maximum concentrations were reached at the end of the infusion for the first group with 3.07 ng/mL, whereas; the other two groups reached 0.82 and 1.47 ng/mL after 4 and 48 hours, respectively. All pharmacokinetic parameters were calculated using non-compartmental analysis in WinNonlin and summarized in Table 6-3.

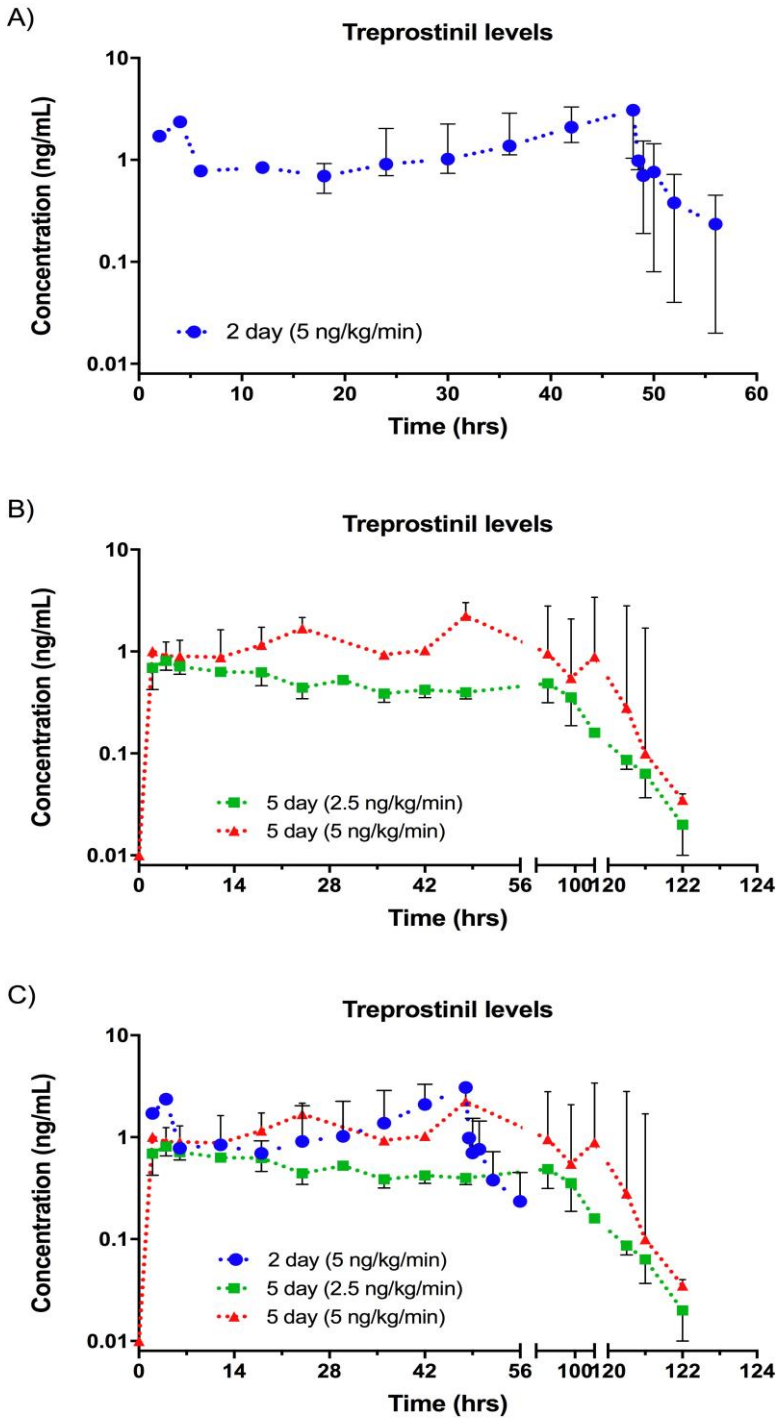


Figure 6-2: Plasma treprostinil concentrations versus time for all three dose levels.

A) First group of patients who received 5ng/kg/min for 2 days. B) includes the second and third group of patients for the doses 2.5 and 5 ng/kg/min for 120 hours of continues infusion of treprostinil. C) shows the overlap of all patients in one graphs.

Table 6-3: Pharmacokinetic parameters for treprostinil in liver transplant patients.

	5 ng/kg/min for 48 hrs	2.5 ng/kg/min for 120 hrs	5 ng/kg/min for 120 hrs	Normal volunteers 10 ng/kg/min for 72 hrs
k_{el} (1/hrs**)**	0.47 (1.29)	0.80 (0.58)	0.79 (0.83)	0.24 (0.12)
$T_{1/2}$ (hrs**)**	1.5 (3.3)	0.9 (0.6)	0.9 (0.5)	4.4 (4)
T_{max} (hrs**)**	42 (46)	4 (6)	48 (46)	36 (27)
C_{max} (ng/mL**)**	3.07 (1.59)	0.82 (0.32)	1.47 (2.40)	1.82 (0.94)
$Cavg_{ss}$ (ng/mL**)**	1.68 (1.26)	0.42 (0.27)	0.96 (2.03)	1.09 (0.23)
$AUC_{0-\infty}$ (hrs*ng/mL**)**	91 (61.2)	50.3 (32.4)	115.4 (249)	77.3 (12.6)
Cl_{ss} (mL/min/kg**)**	2.98 (4.28)	6.02 (3.22)	5.22 (10.9)	9.56 (1.59)
V_z (mL/kg**)**	691.2 (922.7)	435.5 (323.1)	205.8 (806.5)	3650 (3220)

Data represented in the table is the calculated median (IQR) and as mean (SD) for healthy volunteers.

6.6 DISCUSSION

This was the first study to evaluate the pharmacokinetics of treprostinil in liver transplant recipients. First, we developed a sensitive, accurate and reproducible UPLC-MS/MS analytical method for treprostinil in plasma. The assay was partially validated in accordance with the recommendations in the bioanalytical method validation guidance by Food and Drug Administration (FDA).[212, 213] The linearity of the assay was good for the range of concentrations 10 pg/mL to 5 ng/mL with an acceptable coefficient of variations for both LLOQ and other concentrations. The intra-day and inter-day variances were less than 15% while the extraction recovery was at least 76.8%. Adding acetic acid into the eluent composition that was used in the solid phase extraction enhanced both the signal peak and minimized the matrix effect for the analyte and the internal standard. All participants' plasma samples were analyzed using the validated assay.

The pharmacokinetic parameters after infusion of treprostinil were determined in this population. Our results showed that the clearance of treprostinil lower by a range of 40 to 70% when compared to the average values in normal healthy subjects (9.56 mL/min/kg).[124] The clearance was seen to increase over time in the second group (2.5 ng/kg/min for 5 days), which indicate the recovery of liver function. Since treprostinil is extensively metabolized (> 95%) by CYP2C8 and to a lesser extent by CYP2C9, the overall effect on clearance early post-transplant can be explained by mainly reduction in hepatic intrinsic clearance and/or blood flow. Also, the noted high variation within groups could be due to the polymorphism that has been documented in CYP2C8. Another pharmacokinetic study in hepatic impaired patients concluded that the clearance decreases with the increase in the severity of impairment.[214] As consequence of reduction in clearance, the total exposure ($AUC_{0-\infty}$) and $Cavg_{ss}$ of treprostinil were increased by

factors of 2 - 3 relative to healthy subjects. Interestingly, the estimated half-life of our patients were shorter than healthy subjects, which was expected to be relatively longer if not similar. We have estimated the half-life to be 1.47 and 0.87 hours for patients receiving the study drug for 48 hours and 120 hours; whereas, normal volunteers received 10 ng/kg/min for 72 hours exhibited a $t_{1/2}$ equal to 4.41 hours. However, it is noteworthy that in a clinical study of 150 minutes of treprostinil IV continues infusion in healthy volunteer's half-life of 0.87 hrs has been reported for treprostinil.

The median C_{max} for patients who were started with treprostinil infusion perioperatively (5 ng/kg/min) was 3.07 ng/mL, which was six times higher than the dose normalized C_{max} in two clinical trials of short (150 mins) and long (72 hours) term I.V. infusions in health subjects. Our patient population was expected to have an increase in the plasma concentrations due to the reduced overall clearance as a consequence of initial lower metabolic functions due to the interrupted blood flow, complication of surgery, and presence of inflammatory milieu. In a similar fashion, there was 1.6, 4 and 4.8 folds increase in C_{max} for subjects with mild, moderate and severe hepatic insufficiency, respectively.[125] In patients who received treprostinil for 120 hours (2.5 and 5 ng/kg/min), the C_{max} increased proportionally from 0.82 to 1.47 ng/mL with a dose increase. This is consistent with the previous findings where McSwain et. al. (2008) showed that the pharmacokinetics was linear over a 100 fold dose range, starting from 1.25 up to 125 ng/kg/min.[128, 215] The mean time to reach the peak plasma concentrations (T_{max}) was 36.4 hours in healthy volunteers, whereas our results showed that the patients needed relatively longer times (42 hours) in most of the patients. Due to the small number of patients, the data showed a large variation but overall the T_{max} prolongation could be due to the normal delay of the grafts recovery or presence of polymorphism in CYP2C8.

This is the first study to systematically characterize the pharmacokinetic parameters of treprostinil in liver transplanted patient population. The achieved plasma levels were relatively proportional to the given doses but higher than health volunteers, which was expected. Finally, our study shows that treprostinil clearance is decreased in liver transplant patients.

7.0 CONCLUSION AND FUTURE DIRECTIONS

7.1 SUMMARY AND CONCLUSIONS

Hepatic ischemia and reperfusion (I/R) injury is a phenomenon that accompanies liver transplantation, which is the only acceptable therapy for patient with end-stage live disease (ESLD). I/R injury has been extensively investigated and has been associated with early poor graft function and primary graft non-function (PNF). The pathophysiology and etiology of the I/R injury is considered an antigen independent component of liver harvesting effect. Prostaglandins (PGs) and prostacyclin (PGI_2) analogs have been tested for decades on their beneficial effect in improving the clinical outcomes; however, there were shortcomings such as their chemical instability, short elimination half-life and high costs. Previously, our lab has shown that treprostinil attenuate the I/R injury in rat orthotropic liver transplantation model when donors and recipients or only recipients were treated. Furthermore, treprostinil minimizes the effect of I/R injury on the *in-vitro* activity of some drug metabolizing enzymes and expression of select drug transporters. The primary evaluation was restricted to treatment of both donors and recipient rats, and the metabolic effect was tested in an *in-vitro* microsomal system.

The current dissertation work was A) to evaluate the effect of supplementing treprostinil in the organ preservation solution on attenuating hepatic I/R injury in a preclinical animal studies; and B) demonstrate the safety and preliminary efficacy of infusing treprostinil in liver transplanted patients. First chapter, included a background and introduction to the dissertation work. In the second chapter, we have established an I/R injury model using an isolated perfused rat liver (IPRL) system and illustrated the beneficial effect of incorporating treprostinil to prevent I/R injury. Two models of perfusate circulation, single pass and recirculation, were performed on the IPRL system using Krebs–Henseleit buffer that was supplemented with

taurocholate, 95% O_2 and 5% CO_2. The recirculation model was more appropriate because the magnitude of injury was higher after 24 hours of cold preservation when compared to controls. This mimicked the physiological situation in the animals and humans. Supplementing treprostinil in the UW organ preservation solution significantly minimized the injury seen. Treprostinil also increased the bile formation when added during the 2 hours of reperfusion.

In the third chapter, we demonstrated the effect of warm perfusion, cold ischemia and reperfusion and I/R in the presence of treprostinil on the expression of major drug transporters. Several drug transporters were significantly downregulated after 2 hours of IPRL perfusion when compared to fresh livers that were harvested are kept in -80^{o}C. Further alteration on the mRNA expression of drug transporters due to I/R injury was attenuated by treprostinil.

In the fourth chapter, the effect of cold ischemia and treprostinil treatment on the disposition of digoxin in a whole intact liver using the *ex-vivo* perfused liver apparatus was examined. This study was performed by administering digoxin into the established recirculation IPRL system, as single bolus dose. For the purpose of this study, a UPLC-MS/MS method to assay digoxin and metabolite (Dg2) simultaneously was developed and validated. Our results showed that AUC of digoxin was significantly increased after cold ischemia and reperfusion compared to controls; however, treprostinil treatment significantly minimized that effect. The metabolic capacity of treprostinil treated livers were higher than I/R injury group as characterized by the significant increase in Dg2 AUC and the ratio of Dg2/digoxin.

In the fifth chapter, a prospective and open label clinical study was discussed to document the safety of infusing treprostinil in liver transplanted patients. Patients eligible for liver transplant in the UPMC were approached and enrolled, whenever eligible and signed the informed consent. Treprostinil could be safely infused preoperatively but the initiation was

postponed till transfer to the ICU, to assess its effects on the hemodynamic parameters. We have shown that doses up to 5 ng/kg/min was tolerated well, with no adverse effects that are related to the drug. Indocyanine green-plasma disappearance studies were conducted on day 2 and 5 and almost all patients have showed a plasma disappearance rate that is similar to normal health volunteers. The time needed for ventilation assistance, ICU and hospital stays were lower than liver transplanted patients not received treprostinil. Other hepatic, coagulation and kidney function markers were comparable to patients not receiving treprostinil.

In the sixth chapter, the pharmacokinetic of treprostinil in liver transplant patients was characterized. A UPLC-MS/MS assay was developed and validated to measure treprostinil in the plasma samples. A serial plasma samples were collected over the duration of treprostinil infusion and 24 hours post-infusion termination. We found that the clearance of treprostinil was decreased by at least 40% and the plasma maximum concentrations were ~6 times higher than subjects with normal livers. The half-life of treprostinil was found to be around 1-2 hours which is longer than any other prostaglandin and prostacyclin analogs. The same trend was seen in patients with hepatic dysfunction and the magnitude is proportional to the severity. In normal patient's elimination half-life was comparable for short-term infusion which is ~1 hour. Overall, incorporating treprostinil into the organ preservation solutions is a promising technique and the clinical study shows the feasibility of using treprostinil in OLT patients.

7.2 LIMITATIONS

7.2.1 Preclinical studies limitations:

- In the current preclinical study, we used a buffer for the perfusion medium (buffer krebs-henseleit) that was used in the isolated perfused liver system and had no added circulating proteins, oxygen carriers or blood products. However, this was intentional to minimize any risk factors, such as increasing the circulating proinflammatory cytokines as documented previously. The histopathology results showed a huge variability that could be overcome using the *in-vivo* rat liver transplant model.
- Our work focused on the effect of I/R injury on the hepatic drug transporters but did not evaluate the activities of all the phase I and II metabolizing enzymes, which might be worth looking on how those genes are altered in the IPRL experiments. Furthermore, our work focused on the gene expression of the transporters but not their regulators (i.e. nuclear receptors) and effectors (i.e. proinflammatory cytokines).
- The preclinical activity study focused on using only digoxin to characterize Slco (Oatp), Abcb1a (P-gp) and Cyp3a in the IPRL system. However, we have not measure the ATP levels in the liver tissue that could reveal a broader understanding of our findings. A cocktail approach to evaluate various drug metabolizing enzymes will provide more comprehensive data. The use of *in-vivo* model could perhaps show more practical observations.

7.2.2 Clinical study limitations:

- The clinical study had small number of patients. Normally, the number of patients who get liver transplants are limited at a given clinical center. To increase number of subjects could be achieved by collaborating with other transplant centers.
- The clinical study showed large variability in some of the findings, which can be explained by the small number of studied participants and normal difference between human beings. Also, each case had an added risk factors form the donors and retrieved grafts that increases the variabilities.
- The used data for the control group were for patients who consented for the study but did not receive the treprostinil infusion, which might not be considered the most appropriate because they were not eligible for treprostinil infusion for most of the time of their hemodynamic instability. Ideally, our results can be compared to patients who received liver transplantation for the same period at UPMC. This will minimize the existing variability due to the low number of patients but keep the other factors similar, such as the liver transplant team and the standard of care post-surgery.

7.3 FUTURE DIRECTIONS

- Blood like products or animal blood diluted with K-H buffer can be used in future experiments because it will simulate the *in-vivo* conditions and might give more insights into the protective response to the I/R injury, since many key factors in the blood can significantly

contribute to I/R injury. Also, carrying rat *in-vivo* studies could be another approach to be used.

- The use of livers from bigger animal model, such as pig, in a machine perfusion similar to the isolated rat liver study that we used in the IPRL experiment. This could result with more accurate findings and increase the confidence of transition of the animal results to humans because they are more comparable in the physiology and genetic buildup.
- Steatotic livers are known to be more susceptible to cold preservation and warm reperfusion injury. Our results do show worth value in evaluating the treprostinil treatment while preserving steatotic livers that will be used for the transplantation. This will increase number of grafts available because liver steatosis is considered one major reason for not being used for transplantation.
- In the near future, our group planning to measure the circulating proinflammatory cytokines and evaluate the synthetic function of the transplanted livers by determining the levels of factor V, albumin and alpha 1-acid glycoprotein. Also, liver tissue biopsies are available to investigate the histopathology and measure the expression for several targets.
- Continuing the current clinical study to include the next doses is recommended, since the doses that were tested so far show no safety concern to the patients and there were promising efficacious trend in the intervention group that treated with treprostinil compared to the current control group. A larger multicenter phase II/III clinical study to evaluate the efficacy of treprostinil infusion in liver transplant patients is highly recommended to overcome the problem of low rate of recruitment.
- The preclinical study findings show that incorporating treprostinil into the organ preservation solution is promising in decreasing the I/R injury. Implementing this approach might be

beneficial in the clinical situation. A new human study protocol will be initiated to document the safety and efficacy of adding treprostinil in the composition of the organ preservation solution.

APPENDIX A

CLINICAL TRIAL INFORMED CONSENT FORM

CONSENT TO ACT AS PARTICIPANT IN A RESEARCH STUDY

TITLE: An Evaluation of the Safety and Preliminary Efficacy of Perioperative Treprostinil in Preventing Ischemia and Reperfusion Injury in Adult Orthotopic Liver Transplant Recipients

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SOURCE OF SUPPORT: United Therapeutics Corporation (Partial)

Thomas Starzl Transplantation Institute

Who is being asked to take part in this research study?

You are being invited to take part in this research study because you are a liver transplant candidate and will receive a liver transplant. Female and male liver transplant patients, between the ages of 18 and 65 years of age are being asked to participate in this clinical study. This study will take place at the University of Pittsburgh Medical Center, Pittsburgh, PA, and will include approximately 30 patients.

Why is this research being done?

The liver is subjected to low temperatures during transportation from the person who donates the liver (the donor) to the person who receives the liver (the recipient). When the liver is put inside the recipient it is warmed up to normal body temperature. Sometimes during these steps the liver cells may undergo damage and may not function well. If this happens patients may have to stay in the hospital for a longer period of time so that the liver will eventually become better or in certain cases the patient may need a second liver transplant. There are no medical treatments approved by the U.S. Food and Drug Administration (FDA) to prevent such problems.

Treprostinil is a drug that is approved by the FDA (Remodulin®) for the treatment of a disease called pulmonary arterial hypertension, or PAH. PAH is a condition where there is high pressure in the blood vessels that supply the lungs. Treprostinil works by widening the blood vessels and decreasing pressure in the blood vessels. In addition to this property Remodulin also prevents blood components from sticking together. Drugs like Treprostinil can also protect cells from the kinds of injury described above. By its several methods of action, Remodulin improves the oxygen supply to the liver and is expected to minimize damage due to lack of sufficient oxygen. Treprostinil has been given to more than 2,000 patients with PAH and has been shown to be safe and effective. Treprostinil has also been given safely to patients with a form of PAH called Porto-Pulmonary Hypertension, who had some degree of liver problems. Treprostinil has not been studied before in patients undergoing liver transplant surgery as part of a formal clinical investigation. Results from a recent animal study proved that Treprostinil is effective in reducing liver injury during liver transplantation.

The purpose of this research study is to evaluate the safety and preliminary efficacy of Treprostinil (study drug). In addition we will also obtain blood samples to evaluate the time course of drug level in your body (pharmacokinetic samples). We will also test to see whether or not Treprostinil decreases damage to liver cells and decreases the length of stay in the hospital.

How will the study be done?

If you decide to participate in this study, you will undergo a screening visit, a baseline visit that is on the day of transplantation, a study treatment phase that will start in the operating room and

last for 5 days, and follow up phase that will last up to 180 days after you receive the new liver. We will be collecting information on your medical and surgical history from your hospital, surgical and clinic records during your participation in this study.

Procedures

Before any study-related tests and procedures are performed, you will be asked to read and sign this consent document. The following tests and procedures will be performed to determine if you qualify to take part in this study:

Screening Visit

The Screening Visit will occur after you sign the informed consent following your selection as a candidate for liver transplantation. This can occur the same day as the Baseline visit. To determine if you meet the criteria for participation in this study, the doctor will review and collect information about your medical history including but not limited to your age, gender, weight, height, medical history, as well as clinical laboratory test results indicative of your liver and kidney function will be collected for the study. You will also have a physical examination and your vital signs will be taken. Additional blood tests if needed may be collected to further check your liver and kidney function. If you meet all the study participation conditions, you are eligible to enter the "Baseline visit".

Baseline Visit

The baseline visit occurs the day you enter the hospital for the liver transplant surgery. At this time, informed consent will be confirmed by you and your doctor. Your doctor will make a final decision if you are still eligible to enter the study. The routine pre-operative examinations and test will be conducted including a physical exam, medical history update, and blood tests to evaluate your liver and kidney function. A serum pregnancy test will be performed in women of child bearing potential as part of your routine workup for the liver transplant surgery. Once the Baseline assessments are complete, you will enter the Study Treatment Phase.

Treatment Phase

The treatment phase will begin once the infusion of Treprostinil (Study Drug) is started. This will occur after the new liver is in place and the surgeon feels that you are stable, the study drug will be started. Treprostinil will be given through a central line (a tube placed into a large blood vessel) or peripherally inserted central catheter (usually a longer tube inserted in a vein in your arm that will reach the larger vessel) that will only be used for Treprostinil. No other medications (drugs) can be given in this line.

During your surgery while you are still asleep your doctor may take 2 liver biopsies (a small piece) of your new liver. One may be taken while the surgeons are preparing your new liver and the other will be taken approximately 60 minutes following the restoration of blood flow to your newly transplanted liver. This will be used to assess substances that indicate injury to your new liver.

These biopsy samples will be stored for up to ten years under the control of the investigators and be used to assess various markers of liver injury.

At approximately once every 6 hours during the first two days, at least once every 12 hrs on days 3-4, and at least once on days 5, 6 and 7 after the start of the study drug infusion additional blood samples may be used for the assessment of substances in the blood that indicate injury to your new liver. These samples will be obtained from the samples you have already had drawn to check your liver and kidney function.

You will begin receiving the study drug (Treprostinil) when the new liver is in place and the surgeon feels that you are stable and will continue for a total of approximately 5 days (120 hours). At approximately 120 hours the infusion of the study drug will be terminated. You will be in the hospital and will be closely watched by members of your medical team and the research team for any problems during this entire time.

After the transplant, in addition to the routine blood sampling, additional blood samples will be taken at specific time points to measure how well your liver and kidney are working. A maximum of 24mL of blood may be taken for these samples. Medical information that is part of the routine care of liver transplant surgery will also be collected and includes but is not limited to blood tests to evaluate your liver and kidney function, length of the liver transplant surgery, any signs or symptoms of liver injury, time admitted to the intensive care unit, time spent needing a machine to assist you with breathing (ventilator), physical examinations, vital signs, surgical history.
Additional vital signs including heart rate and blood pressure may be collected every 6 hours for as long as you are receiving the study drug.

Throughout the study, you will be asked to report any unusual problems that you experience, regardless of whether or not you feel they are related to, or caused by, the study medication. It is very important for you to discuss any difficulties or side effects with your doctor. If you have any significant side effects or problems, you should quickly contact your doctor. Your doctor will then decide if you should receive other treatment.

Pharmacokinetic (PK) Samples
Up to twenty two blood samples may be obtained just prior to, during, and/or after study drug administration to evaluate the study drug (Treprostinil) levels in your blood. The sampling will be done prior to initiation of the study drug therapy, and at approximately 2, 4, 6, 12, 18, 24, 30, 36, 42, 48, 72, 96 and 120 hrs during therapy and approximately 0.5, 1, 2, 4, 6, 8, 12 and 24 hr post study drug termination. A maximum of 4 tbsp (57ml) of blood will be collected for all of the PK samples except for 72, 96 and 120 hr samples that are collected as part of the samples to check the liver and kidney function. These samples will be labeled with the numerical code assigned to the subject in order to correlate with clinical data obtained during the study and only the study doctors will know to whom the sample belongs.
These samples will be processed in our local laboratory, located at 718 Salk Hall, 3501 Terrace St Pittsburgh, PA 15261.

Follow-Up Phase
The Follow-Up Phase will begin after termination of study drug infusion and completion of all Treatment Phase assessments, and continue for 180 days after your liver transplant surgery. During the follow-up phase we will collect medical information obtained from the routine care

that you receive after your liver transplant surgery. This includes but is not limited to blood tests that measure the function of your liver and kidney, physical examinations, if you are still in the hospital or intensive care unit following the original surgery, vital signs, current medications, total days spent in the hospital after your liver transplant surgery as well as total days spent in the intensive care unit.

During the study, a test called the indocyanine green (ICG) clearance test will be performed to find out how well your liver works. The ICG test will be performed a total of 2 times throughout the entire study: Once on Day 2 and again on day 5 after your surgery. During the ICG clearance test, a special dye, called indocyanine green (ICG) will be injected intravenously through a small tube already inserted into your blood vessel. ICG elimination in blood will be monitored using monitor connected to a sensor placed on the index finger, which will be recorded continuously from 0 minutes to 15 minutes after administration. Additionally, a total of five blood samples (3ml each for a total of less than 3 teaspoons) will be collected prior to the administration of ICG and at approximately 5, 7, 10, and 15 minutes after administration of ICG from a tube already inserted into your blood vessels. In the rare case that no tubes are available in your body to withdraw blood a small tube will be inserted into a blood vessel. These blood concentrations will be used to calculate ICG clearance in the blood. The entire test takes about 30 minutes.

In addition to routine blood sampling additional blood samples may be collected to evaluate your liver and kidney function on days 3, 4, 5, 6 and 7 after your liver transplant. The total volume of the additional blood sample will not be more than one tablespoon. The total amount of blood obtained for research purposes is approximately 9 tablespoons (123mL).

We will also be collecting data on your survival, your liver status and information on if you have been re-transplanted on days 30, 90, and 180 days after transplantation.

Blood for Future Research

Additional blood samples or any other biological material already collected during your research visits may also be stored and used for future testing related to the study drug (Treprostinil). The samples will be stored for a maximum of ten years in the Clinical Pharmacokinetics Laboratory at the University of Pittsburgh under the direct supervision of Co- Investigator Dr. Raman Venkataramanan. Only members of the research team or laboratory personnel conducting the laboratory tests will have access to the samples. Samples will be labeled with the numerical code assigned to the subject in order to correlate with clinical data obtained during the study and only the study doctors will know to whom the sample belongs. You will not be notified about pending results on these tests as they have no bearing on your medical management. In the event that you should withdraw your consent for this study, the Clinical Pharmacokinetics Laboratory will destroy the samples. These samples will not be used for any genetic testing.

What are the possible risks, side effects, and discomforts of this research study?

There may be certain risks associated with participation in this study. These may include risks due to the administration of Treprostinil (all of which are not known at this time), risk of liver biopsy, risk associated with blood sampling for measuring Treprostinil levels.

As with any investigational drug there may be adverse events that are currently unknown and it is possible that certain of these unknown risks could be permanent, serious and life threatening.

Risks of Treprostinil: Common risks of Treprostinil may include, but are not limited to, flushing of the skin, headache, nausea, vomiting, diarrhea, and jaw pain. If these symptoms develop and are intolerable, the dose of the study drug may be reduced or stopped until they disappear.

Likely (>25%):	Headache; diarrhea;
Common (10-25%):	Nausea, vomiting, rash, itchiness, jaw pain, flushing (increase in diameter of blood vessels), leg or foot pain
Infrequent (1-10%):	Dizziness, edema, skin reaction, line infection, pain and bruising at the infusion insertion site.
Rare:	Allergic reaction, Decreased blood pressure

The study drug (Treprostinil) will be delivered using a tube placed into a large vein called a central venous catheter. This route of delivery can cause pain and bruising at the insertion site and there is an increase risk of blood stream infections (BSI).
Treprostinil is broken down in the body by the liver. In subjects with liver problems, blood levels of Treprostinil may be higher than normal. Treprostinil may cause your blood pressure to decrease during the surgery. Your blood pressure and vital signs will be watched very carefully during your surgery and the dose of study drug could be reduced or stopped if there are problems. However, in spite of these precautions the study drug may increase the risk of problems resulting from low blood pressure. The medical team may stop the study medication without your agreement based on medical information available to them.

There is the possibility of a severe allergic reaction in which you may have difficulty breathing, become itchy, develop a rash, nausea, fever, or other possible symptoms. Such reactions can be life threatening. You will be monitored closely by health care professional at the University of Pittsburgh Medical Center for any signs and symptoms of an allergic reaction.

Risks of Reproduction: Being a part of this study while pregnant or breastfeeding may expose the unborn child or nursing infant to risks known and unknown. Therefore, pregnant and nursing women will not be included in this study. If you are a woman of childbearing potential, a serum pregnancy test will be done during baseline visit as part of your routine work- up for liver transplant surgery. It must be negative before you can enter this study. While receiving study drug, and for a period of 30 days after that you must agree to use two appropriate methods of birth control. Medically acceptable birth control methods include: (1) surgical sterilization, (2) approved hormonal contraceptives (such as birth control pills or Lupron Depot), (3) barrier methods (such as a condom or diaphragm) used with a spermicide, or (4) an intrauterine device (IUD).

You should not take part in this study if you plan to become pregnant with in a month after transplant surgery, are currently pregnant, or you are currently breast feeding. You must notify your doctor if you suspect you have become pregnant while participating in this study.

Risks associated with obtaining a blood sample:
Common risks (occurs in 10-25% or 10 to 25 out of 100 people) include pain, bleeding, slight swelling and bruising at the puncture site.

Infrequent (rare) risks (occur in 1-10% of 1 to 10 out of 100 people) may include infection at the site of where the blood was drawn or fainting may occur after you have had your blood drawn.

Risk of a liver biopsy at the time of your surgery:
The main risk of a liver biopsy is bleeding. This is usually minimal (1-10% or 1 to 10 out of 100 people) and your surgeon can treat this at the time of surgery with a small stitch or cautery (an instrument that uses heat to stop bleeding). Since this is done while you are asleep, the biopsy will not cause discomfort.

Risks with the ICG clearance test:
Most common risks included pain or bruising at the injection site. Rarely, allergic reaction to indocyanine green (ICG) dye has occurred. The indocyanine green (ICG) dye contains sodium iodide. Individuals who are allergic or have had a reaction in the past to sodium iodide or iodine will not receive ICG dye. You must notify your doctor if you suspect you may be allergic to sodium iodide or iodine, or if you have had a reaction to ICG dye in the past.
Symptoms of an allergic reaction may include itchy rash, fast heart beat, low blood pressure, and trouble breathing.

UNFORESEEN RISKS
Since the study drug is investigational when taken alone or in combination with other medications, there may be other risks that are unknown.

What are possible benefits from taking part in this study?
There is no guarantee that you will receive any benefit from participating in this study. However, it is hoped that this drug will protect your liver and your stay in the hospital following liver transplant surgery will be less and you will spend less time in the intensive care unit. Your participation may also help others in the future by what the doctors learn from your involvement in this study.

What treatment or procedures are available if I decide not to take part in this research study?
If you decide not to take part in this research study, you will undergo normal procedures associated with the liver transplantation surgery.

If I agree to take part in this research study, will I be told of any new risks that may be found during the course of the study?
You will be promptly notified if, during the conduct of this research study, any new information develops which may cause you to change your mind about continuing to participate in this study.

Will my insurance provider or I be charged for the costs of any procedures performed as part of this research study?
All costs and tests done to treat you before and after your liver transplant should be covered by your medical insurance. These are tests that would normally be performed in patients undergoing

liver transplant surgery.

Some of the services you will receive during this time are "research only services" that are being done only because you are in the study. These services will be paid for by the study and will not be billed to your health insurance company or you. Examples are the administration/infusion of the study drug (Treprostinil), biopsy (small piece taken) of your new liver while you are still asleep in surgery, blood samples obtained to evaluate the study drug (Treprostinil) levels in your blood, and any additional blood samples taken to measure how well your liver and kidney are working.

Some of the services you will receive during this study are considered to be "routine clinical services" that you would have even if you were not in the study. Examples are the actual liver transplant, surgery, hospitalization and all associated care. These services will be billed to your health insurance company or you, if you do not have health insurance.

You will be responsible for paying any deductibles, co-payments or co-insurance that are a normal part of your health insurance plan. If you have the Medicare Advantage Plan you could be billed as if you were a Fee-for Service patient. You may also be responsible for the total cost of the transplant under a 3rd party Medicare plan. You may want to get more detailed information about what "routine clinical services" your health insurance is likely to pay for. You may want to talk to a member of the study staff and/or a UPMC financial counselor to get more information.

Will I be paid if I take part in this research study?
You will not receive any payment for taking part in this clinical study.

Who will pay if I am injured as a result of taking part in this study?
University of Pittsburgh investigators and their associates who provide services at UPMC recognize the importance of your voluntary participation in their research studies. These individuals and their staffs will make every reasonable effort to minimize, control and treat any injuries that may arise as a result of this research. If you believe that you are injured as the result of the research procedures being performed, please contact the Principal Investigator or one of the investigators listed on the first page of this form.

Emergency medical treatment for injuries solely and directly related to your participation in this research study will be provided to you by the hospitals of UPMC. Your insurance provider may be billed for the costs of this emergency treatment, but none of those costs will be charged directly to you. If your research-related injury requires medical care beyond this emergency treatment, you will be responsible for the costs of this follow-up care. At this time, there is no plan for any additional financial compensation.

Who will know about my participation in this research study?
Any information about you obtained from this research will be kept as confidential (private) as possible. All records related to your involvement in this research study will be stored in a locked file cabinet. Your identity on these records will be indicated by a case number rather than by your name, and the information linking these case numbers with your identity will be kept

separate from the research records. You will not be identified by name in any publication of the research results.

Will this research study involve the use or disclosure of my identifiable medical information?
This research study will involve the recording of current and/or future identifiable medical information from your hospital and/or other (e.g., physician office) records. This research study will result in identifiable information that will be placed into your medical records held at UPMC Presbyterian and Montefiore. Records of your participation in this study will be held confidential except as disclosure is required by law or as described in this informed consent document (under "Confidentiality" or "Authorization to Use and Disclose Protected Health Information"). The study doctor, the sponsor or persons working on behalf of the sponsor, and under certain circumstances, the United States Food and Drug Administration (FDA) will be able to inspect and copy confidential study-related records which identify you by name. Therefore, absolute confidentiality cannot be guaranteed. If the results of this study are published or presented at meetings, you will not be identified.

Who will have access to identifiable information related to my participation in this research study?
In addition to the investigators listed on the first page of this authorization (consent) form and their research staff, the following individuals will or may have access to identifiable information (which may include your identifiable medical information) related to your participation in this research study:

Authorized representatives of the University of Pittsburgh Research Conduct and Compliance Office may review your identifiable research information (which may include your identifiable medical information) for the purpose of monitoring the appropriate conduct of this research study.

Authorized representatives from the Food and Drug Administration may review and or obtain your identifiable (which may include your identifiable medical information) related to your participation in this research study for the purposes of monitoring the accuracy and completeness of the research data. While the U.S. Food and Drug Administration understands the importance of maintaining the confidentiality of your identifiable research and medical information, the UPMC and University of Pittsburgh cannot guarantee the confidentiality of this information after it has been obtained by the U. S. Food and Drug Administration.

Authorized representatives of UPMC hospitals or other affiliated health care providers may have access to identifiable information (which may include your identifiable medical information) related to your participation in this research study for the purpose of (1) fulfilling orders, made by the investigators, for hospital and health care services (e.g., laboratory tests, diagnostic procedures) associated with research study participation; (2) addressing correct payment for tests and procedures ordered by the investigators; and/or (3) for internal hospital operations (i.e. quality assurance).

In unusual cases, the investigators may be required to release identifiable information

(which may include your identifiable medical information) related to your participation in this research study in response to an order from a court of law. If the investigators learn that you or someone with whom you are involved is in serious danger or potential harm, they will need to inform, as required by Pennsylvania law, the appropriate agencies.

For how long will the investigators be permitted to use and disclose identifiable information related to my participation in this research study?
The investigators may continue to use and disclose, for the purposes described above, identifiable information (which may include your identifiable medical information) related to your participation in this research study for a minimum of 7 years and for as long (indefinite) as it may take to complete this research study.

May I have access to my medical information that results from my participation in this research study?
In accordance with UPMC Notices of Privacy Practices document that you have been given, you are permitted access to information (including information resulting from your participation in this research study) contained within your medical records filed with your health care provider.

A description of this clinical trial will be available on http://www.ClinicalTrials.gov as required by US Law. This website will not identify you. At most the Web site will include a summary of the results. You can search this site at any time.

Is my participation in this research study voluntary?
Your participation in this research study, to include the use and disclosure of your identifiable information for the purposes described above, is completely voluntary. (Note, however, that if you do not provide your consent for the use and disclosure of your identifiable information for the purposes described above, you will not be allowed to participate in the research study.) Whether or not you provide your consent for participation in this research study will have on effect on your current and future care at a University or Pittsburgh or UPMC hospital or affiliated health care provider or your current or future relationship with a health care insurance provider.

Your doctor may be an investigator in this research study, and as an investigator, is interested both in your medical care and in the conduct of this research. Before entering this study or at any time during the research, you may discuss your care with another doctor who is in no way associated with this research project. You are not under any obligation to participate in any research study offered by your doctor.

May I withdraw, at a future date, my consent for participation in this research study?
You may withdraw, at any time, your consent for participation in this research study, to include the use and disclosure of your identifiable information for the purposes described above. (Note, however, that if you withdraw your consent for the use and disclosure of your identifiable medical record information for the purposes described above, you will also be withdrawn, in general, from further participation in this research study.) Any identifiable research or medical information recorded for, or resulting from, your participation in this research study prior to the date that you formally withdrew your consent may continue to be used and disclosed by the investigators for the purposes described above.

To formally withdraw your consent for participation in this research study you should provide a written and dated notice of this decision to the principal investigator of this research study at the address listed on the first page of this form.

If you decide to withdraw form study participation after you have received the study drug, you should participate in described monitoring follow-up procedures directed at evaluating the safety of the study drug.

If I agree to take part in this research study, can I be removed from the study without my consent?

It is possible that you may be removed from the research study by the researchers if, for example, your pregnancy test proves to be positive. You may be removed from the study if you experience unexpected side effects and in the opinion of the investigators that it is in your best interest. The study may also be stopped by the investigators or the sponsor if it felt that it is in the best interest of the patients.

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VOLUNTARY CONSENT

All of the above has been explained to me and all of my current questions have been answered. I understand that I am encouraged to ask questions about any aspect of this research study during the course of this study, and that such future questions will be answered by the researchers listed on the first page of this form.

Any questions which I have about my rights as a research participant will be answered by the Human Subject Protection Advocate of the IRB Office, University of Pittsburgh (1-866-212-2668). By signing this form I consent to participate in this research study and provide my authorization to share my medical records with the research team. A copy of this consent form will be given to me.

_____ _____

Participant's Signature Date/Time

CERTIFICATION of INFORMED CONSENT

I certify that I have explained the nature and purpose of this research study to the above-named individual(s), and I have discussed the potential benefits and possible risks of study participation. Any questions the individual(s) have about this study have been answered, and we will always be available to address future questions as they arise.
I further certify that no research component of this protocol was begun until after this consent form was signed.

_____ _____

Printed Name of Person Obtaining Consent Role in Research Study

_____ _____

Signature of Person Obtaining Consent Date/Time

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