TREPROSTINIL FOR PROTECTION OF LIVER GRAFTS AGAINST ISCHEMIA AND REPERFUSION INJURY DURING ORTHOTOPIC LIVER TRANSPLANTATION - A TRANSLATIONAL STUDY

by

Nisanne S. Ghonem

Pharm.D., University of Rhode Island, 2004

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This dissertation was presented

by

Nisanne S. Ghonem

It was defended on

December 16, 2010

and approved by

Stephen C. Strom, Ph. D., Pathology

Donna B. Stolz, Ph.D., Cell Biology and Physiology

Wen Xie, M.D., Ph.D., Pharmaceutical Sciences

Michael Zemaitis, Ph.D., Pharmaceutical Sciences

Co-Advisor: Noriko Murase, M.D., Transplant Surgery

Major Advisor: Raman Venkataramanan, Ph.D., Pharmaceutical Sciences

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Nisanne S. Ghonem, Pharm.D.

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Orthotopic liver transplantation (OLT) is the only curative therapy for end-stage liver diseases. To overcome organ shortage, organs from extended criteria donors, which would ordinarily be discarded, are used sometimes. These organs provide additional grafts; however, they are more susceptible to ischemia-reperfusion (I/R) injury. I/R injury, an unavoidable process during OLT, is a major cause of liver graft non-function and failure, requiring urgent re-transplantation, which further depletes the scare organ pool. To date, no therapy is available to reduce or prevent I/R injury.

Prostaglandins (PG) have well characterized vasodilatory and anti-platelet aggregatory actions. Many PG analogues, including prostacyclin (PGI_2), have been evaluated for their ability to reduce hepatic I/R injury after OLT. Poor stability, intolerable side effects, and the inability to show a significant difference in primary endpoint have limited their clinical application so far. Treprostinil, a relatively new FDA-approved PGI_2 analogue, has a higher stability, potency, and longer elimination half-life than other PGI_2 analogues available.

The objectives of this dissertation were to examine the efficacy of treprostinil in protecting the liver graft against I/R injury during OLT. Proof of concept of treprostinil minimizing hepatic I/R injury was demonstrated in a rat OLT model. Further analysis showed

that I/R injury significantly down-regulated CYP2E1, CYP2C11, and CYP3A mRNA, protein expression, and activity, as well as the expression of several hepatic transporters in liver graft post-OLT. Treprostinil improved hepatic expression and activity of CYP450 enzymes and transporters. In particular, Bsep mRNA expression was restored to normal and Mrp2 and P-gp protein expression were up-regulated. *In vitro* studies confirmed that treprostinil does not inhibit or induce the metabolism of immunosuppressive medications. These findings support co-administration of treprostinil with cyclosporine A, tacrolimus, sirolimus, or mycophenolic acid to adult OLT patients without concern for any drug-drug interaction.

This is the first study to examine the efficacy of treprostinil for protection of liver grafts against I/R injury during OLT. A clinical study has been initiated to examine the safety and efficacy of perioperative treprostinil administration to adult OLT patients. Collectively, this work makes significant contributions to the field of liver transplantation and, potentially, solid organ transplantation.

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ABBREVIATIONS

AE	Adverse event
ANOVA	Analysis of variance
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
AUC	Area under the curve
cAMP	Cyclic adenosine monophosphate
CRF	Case report form
CsA	Cyclosporine A
CYP450	Cytochrome P450
CZN	Chlorzoxazone
6-OH CZN	6-hydroxychlorzoxazone
ECD	Extended criteria donor
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FDA	Food and Drug Administration
HPLC	High performance liquid chromatography

ICAM-1	Intracellular adhesion molecule-1
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-10	Interleukin-10
I/R	Ischemia-reperfusion
LC-MS/MS	Liquid chromatography-mass spectrometry
LPS	Lipopolysaccharide
LOQ	Limit of quantification
MDZ	Midazolam
1-OH MDZ	1-hydroxymidazolam
MPA	Mycophenolic acid
NF-κB	Nuclear Factor-KappaB
OLT	Orthotopic liver transplantation
PECAM	Platelet endothelial cell adhesion molecule
PG	Prostaglandin
PGI_2	Prostacyclin
PMN	Polymorphonuclear
RT-PCR	Reverse transcription polymerase chain reaction
SAE	Serious adverse event
SEC	Sinusoidal endothelial cell
SEM	Standard error of mean
Serpine1	Serpin peptidase inhibitor, member 1
SPE	Solid phase extraction

SRL	Sirolimus
t1/2:	Half-life
TAC	Tacrolimus
TNF-α	Tumor Necrosis Factor-α
TST	Testosterone
2α -OH TST	2α -hydroxytestosteron
6β -OH TST	6β -hydroxytestosterone
16α -OH TST	16α -hydroxytestosterone
UPLC-MS/MS	Ultra Performance Liquid chromatography-mass spectrometry
UPMC	University of Pittsburgh Medical Center
UW	University of Wisconsin
VCAM-1	Vascular adhesion molecule-1
VEGF-α	Vascular endothelial growth factor-α

1.0 INTRODUCTION

The liver is the largest internal organ in the body and it plays a vital role in maintaining the body's metabolic homeostasis. More specifically, the liver serves a multitude of functions, with essential roles in biosynthesis, metabolism, secretion, detoxification, excretion, and bile formation as part of normal physiology. The liver consists of parenchymal and nonparenchymal cells. Parenchymal cells, i.e. hepatocytes, constitute approximately 80% of the total cell number, and the nonparenchymal cells of the sinusoid include the sinusoidal endothelial cells (SECs), the Kupffer cell, stellate cell, and the Pit cell [1]. These cells are arranged in a matrix that facilitates their cooperative interaction and are capable of synthesizing, extracting, metabolizing, and eliminating a variety of molecules.

The liver, a highly vascularized organ with a high blood flow, contains different vasculatures, including the portal vein, the hepatic artery, the liver sinusoid, and the hepatic vein. This liver cellular matrix is perfused by blood at low pressure through uniquely structured capillary-size blood vessels, called sinusoids, which are supplied by a unique source of blood, consisting of both arterial, coming from the common hepatic artery, and venous, coming from the portal vein inflow. The portal vein flow has already circulated through the gut, pancreas, and spleen and is reduced in oxygen and pressure and is enriched in nutrients and toxins absorbed from the gut. The portal vein constitutes 75 – 80% of the liver blood flow and the hepatic artery constitutes the remaining 20 – 25% [2]. Arterial blood is well oxygenated at an elevated pressure, relative to portal venous blood. Venous and arterial blood mix together as they enter the sinusoids in the liver [2].

1.1 ORTHOTOPIC LIVER TRANSPLANTATION

Since the first liver transplantation was carried out by Thomas E. Starzl in 1963, techniques in surgery, immunosuppression, and patient management have improved, making orthotopic liver transplantation (OLT) a routine procedure. Currently, OLT is the only curative treatment available for patients with acute and chronic liver failure; however, donor shortage is a major factor limiting the number of organs available for transplantation. In 2009, approximately 6,300 liver transplantations were performed in the United States, while more than 17,000 patients were listed on the United Network for Organ Sharing waiting list [3]. The disparity between the number of organs available for liver transplantation and patients in need of livers as well as the number of patients who die while waiting for an available organ is shown in Figure 1.

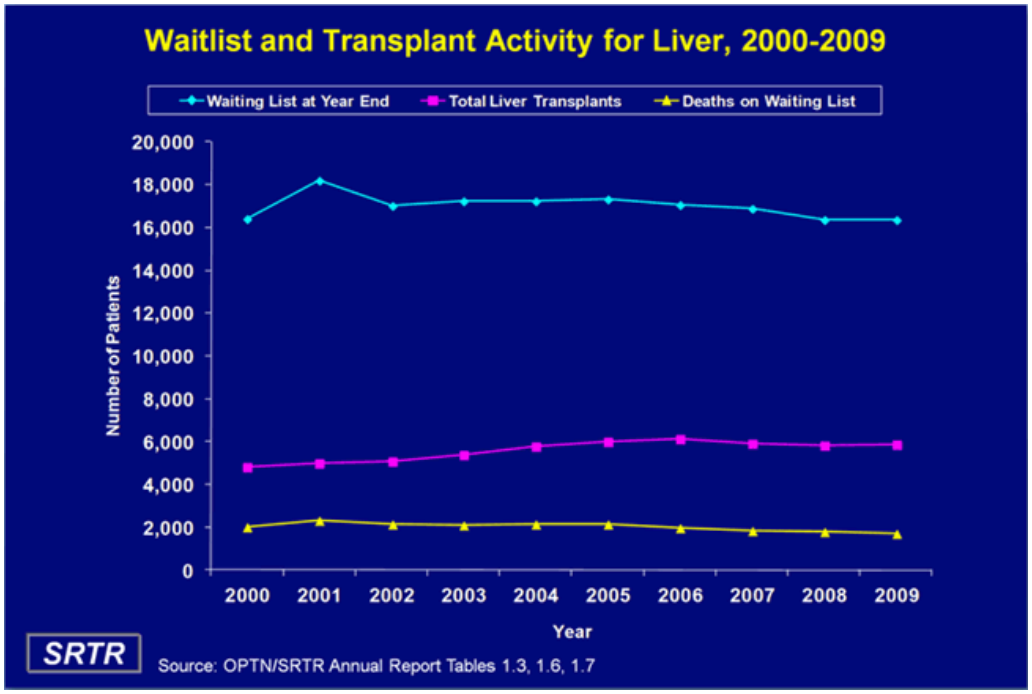


Figure 1: Number of patients on the waitlist for a liver transplantation in the USA (2000-2009)
Number of patients waiting for an organ (top line, circles); number of liver transplantations (middle line, squares); number of patient deaths while on the donor waiting list (bottom line, triangles). Reproduced from Organ Procurement and Transplantation Network (OPTN)/Scientific Registry of Transplant Recipients (SRTR) Annual Report Tables 1.3, 1.6, and 1.7.

The current shortage of cadaveric organ donors has forced the expansion of the donor pool and has led medical centers to accept marginal donors, i.e. extended criteria donors (ECD), including organs from older donors, non-heart beating donors, and grafts which have undergone prolonged cold storage in preservation solution [4]. Donor age greater than 70 years old has been associated with lower patient and graft survival [5] and an increased likelihood of steatosis [6]. Non-heart beating donors are confounded by prolonged warm ischemia before cold preservation, which is associated with a high risk of primary graft non-function [7]. The duration of cold ischemic storage has been associated with an increase in preservation damage resulting in sinusoidal endothelial cell damage and hypercoaguability, leading to a longer post-operative course and decreased graft survival [8].

In summary, while ECD organs provide the much needed additional grafts, they are also more susceptible to I/R injury, resulting in an increase in delayed or primary graft non-function, leading to prolonged hospitalization and, consequently, hepatic dysfunction and/or allograft failure [4, 9]. Thus, amelioration of I/R injury will improve the short- and long-term transplant outcomes; however, no treatment is currently available to prevent or minimize I/R injury.

1.1.1 Primary non-function

In liver transplantation, I/R injury is the main cause of both initial poor function and primary non-function of the allograft [4]. The incidence of primary non-function (PNF) of the transplanted liver, often as a result of I/R injury, is approximately 2 – 23% of OLT [8, 10]. There is no simple and clear definition of primary non-function (PNF) and it varies from center to center. Characteristic manifestations of PNF include failure of the newly implanted graft to make bile, very high and rapidly rising levels of serum aminotransferases, severe coagulopathy

which can progress to hypoglycemia, hepatic encephalopathy, and acute renal failure within the first 48 hours post-transplantation [11-13]. The causes of PNF can be broadly classified into four categories: donor-related factors (discussed above); procurement-related factors, i.e. I/R injury; host-related factors, i.e. hyperacute rejection, intercurrent diseases; and technical surgical factors, i.e. vascular occlusion or blood loss [14]. Primary non-function of the allograft leads to significant morbidity, necessitating re-transplantation [15], thereby further depleting the already limited donor organ pool. Any therapy to reduce the incidence of I/R injury would greatly increase the number of available organs for transplantation as well as the clinical outcomes in transplant patients.

1.2 ISCHEMIA AND REPERFUSION INJURY

Ischemia is defined as a state of no blood flow. Consequently, an interruption of blood supply prevents the delivery of oxygen and nutrients to the ischemic tissue. When the cause of ischemia is relieved, and molecular oxygen is reinstituted through the circulation, the reperfusion rescues the ischemic tissue but also enhances the injury caused during the ischemic period by oxidative stress and inflammatory-mediated reactions [16-18]. The pathophysiology of liver I/R injury includes direct cellular damage as a result of the ischemic insult as well as delayed graft dysfunction following reperfusion.

Clinically, hepatic I/R injury can occur in a setting of elective liver surgery, trauma, shock, and liver transplantation. The surgical procedure of liver transplantation inherently involves cold preservation (ischemia) and warm reperfusion of the transplanted graft, resulting in varying degrees of hepatic injury. Ischemia and reperfusion (I/R) injury, an unavoidable process

in liver transplantation, is a major cause of both initial poor function and primary allograft non-function, leading to organ dysfunction and early graft failure, carrying a high mortality rate of if patients are not re-transplanted immediately .

During liver transplantation, to a greater or lesser extent, the surgical procedure exposes a liver graft to three different types of ischemia: 1) in-situ warm ischemia, which occurs before organ procurement; 2) ex-situ cold ischemia, during graft preservation; and 3) in-situ rewarming ischemia, during engraftment [8]. Warm (37 ^{o}C) and cold (4 ^{o}C) ischemia share some common pathways of injury, yet there are important differences between the two and they each possess distinct processes and mechanisms of injury, which ultimately result in end-organ failure [19]. One of the biggest distinctions between the two types of ischemia are the targets of injury; hepatocytes are the primary targets of warm ischemia [20, 21], whereas sinusoidal endothelial cells [22-24] are predominantly injured during cold ischemia, and are primarily involved in the disruption of the microcirculation. Warm ischemia typically occurs as a result of hepatic trauma, including hypovolemic shock, hepatic resection, or inflow occlusion during liver surgery, or when the flow of blood has been temporarily stopped, yet the organ remains in the body. Alternatively, cold ischemia occurs exclusively during graft preservation when the liver graft is stored in cold preservation solution before transplantation.

At the time of organ harvest, the donor liver is perfused with and stored in University of Wisconsin (UW) preservation solution, where it remains ischemic until it is transplanted into the recipient. During cold ischemia, the organ is transiently cooled and deprived of oxygen, which initiates a cascade of cellular injuries. Subsequent injury occurs upon engraftment when the graft is subjected to warm reperfusion with normothermic blood, resulting in varying degrees of hepatic injury and graft dysfunction, or in worst cases, allograft non-function [25]. The actual

injury to the liver graft following ischemia is mainly detected post-reperfusion, once the oxygen supply, blood elements, and nutrients are restored and able to interact with hepatocytes and other cells present in the liver, leading to liver injury and impaired hepatic function [26, 27]. Ironically, while the reestablishment of blood flow to the liver represents a vital requirement for the recovery of cellular and organ function, reperfusion enhances ischemia-induced tissue and cellular damage further, potentially causing significant damage to the cellular architecture and function of the liver. Essentially, I/R injury begins as a disturbance in microcirculatory flow and is manifested by platelet, red blood cell, and polymorphonuclear (PMN) adhesion to endothelial cells causing sinusoidal congestion, followed by oxidative stress and pro-inflammatory response [18, 28, 29].

The liver is a highly aerobic, oxygen-dependent organ and I/R injury can affect all oxygen-dependent liver cells that require an uninterrupted blood supply. One of the first consequences of ischemia is tissue anoxia, which causes a drop in the cellular energy metabolism and enzyme function, resulting in adenosine triphosphate (ATP) depletion. Subsequent failure of the ATP-dependent plasma membrane pump (Na+/K+ ATPase) causes the inability to pump sodium out, intracellular sodium accumulation, and cellular edema [18, 30, 31]. Failure of the membrane ion pump also disrupts cellular homeostasis and causes an efflux of potassium, which activates voltage-dependent Ca^{2+} channels and leads to an influx of Ca^{2+}, thereby further disrupting cellular processes and functions [32]. Aerobic cells require mitochondrial oxidative phosphorylation for their energy supply and, as such, all aerobically metabolizing tissues and cells are potential targets of I/R injury.

Reactive oxygen species (ROS) and pro-inflammatory cytokines also play an important role in liver injury. A critical result of ischemia is activation of the Kupffer cells, also known as

the liver resident macrophages, which are one of the main sources of vascular reactive oxygen formation by xanthine oxidase during the initial reperfusion phase [33, 34], and significantly contribute to liver dysfunction and cell injury during reperfusion. In addition, Kupffer cells have the capacity to release a wide range of cytokines that critically determine the subsequent reactions of other immune cells and hepatocytes, as well as the degree of organ damage [35]. In the early stages of reperfusion, increased superoxide and other ROS derived from the activation of various sources play a crucial role in tissue damage. The accumulation of hypoxanthine during ischemia allows for a burst of superoxide and hydrogen peroxide production yielding hydroxyl radical when oxygen is reintroduced into the blood vessel, during reperfusion and causing impaired cellular functions [36]. The energy state at the time of reperfusion is an important indicator of cell and graft recovery.

Initially, all of these processes are reversible so that upon reintroduction of oxygen, cells can recover; however, if anoxia is prolonged further, irreversible cellular damage will occur. Secondary results of the energy and oxygen disturbance during the ischemic phase are reflected by the destruction of cellular and subcellular structures, increased membrane permeability during the reperfusion phase, which ultimately culminate in tissue structural alterations and lead to cellular dysfunction [29].

In summary, ischemic injury is a complex, multi-factorial pathophysiological process, in which cells undergo a series of metabolic, structural and functional damage. The process of I/R injury to the liver combines interrelated factors that produce a cascade of events, which can ultimately lead to graft failure. The extent of injury to the liver graft following reperfusion varies tremendously and largely depends on the duration of cold ischemia, and strongly predicts both the short- and long-term clinical outcome. [37]. Numerous efforts have been focused on

identifying an agent that is capable of interfering with and or reducing as many of these processes as possible with the goal of preventing I/R injury during OLT. Before discussing therapeutic approaches to prevent I/R injury, the importance of liver microcirculation and the involvement of platelets and cytokines in the development of I/R injury will be discussed in this chapter.

1.2.1 Liver microcirculation

Hepatic microcirculation is extremely important to maintain the physiology and function of the whole organism. Specifically, it supplies the liver tissue with oxygen and nutrients, serves as a gate for leukocyte entrance in hepatic inflammation, and is responsible for the clearance of toxins and foreign bodies from the bloodstream [38]. Hepatic circulation comprises a unique system of capillaries called sinusoids, which are lined by fenestrated endothelium with high permeability that allows maximum contact between hepatocytes and blood [39].

The liver sinusoid is a specific capillary network system where a variety of metabolic substances are exchanged between hepatic blood flow and hepatic parenchymal cells [40]. Within the sinusoid, the SEC accounts for approximately 70% of the cell population [17, 40]. Liver sinusoidal endothelial cells line the inner surface of the sinusoid to form a vital and dynamic structure that is essential for vascular homeostasis. The unique morphology of the liver SECs permits interactions between lymphocytes and hepatocytes [38]. The sinusoidal cells lining the endothelium are responsive to a wide variety of substances and, by contracting or swelling, they can selectively regulate the patency of the sinusoidal lumen, thereby altering the rate and distribution of blood flow [39].

The morphology of SECs is characterized by sieve-like plate structure clustering of the open fenestrae in their cytoplasm and no basement membrane along the space of Disse, shown in Figure 2. The SEC porosity and lack of an organized basement membrane are important for oxygen diffusion to hepatocytes. The fenestrae are dynamic structures that contract and dilate in response to alterations in sinusoidal blood flow and perfusion pressure, and act as a selective sieving carrier to control the extensive exchange of material between the blood and the liver cells, which contribute to the homeostatic control of the hepatic microcirculation [38].

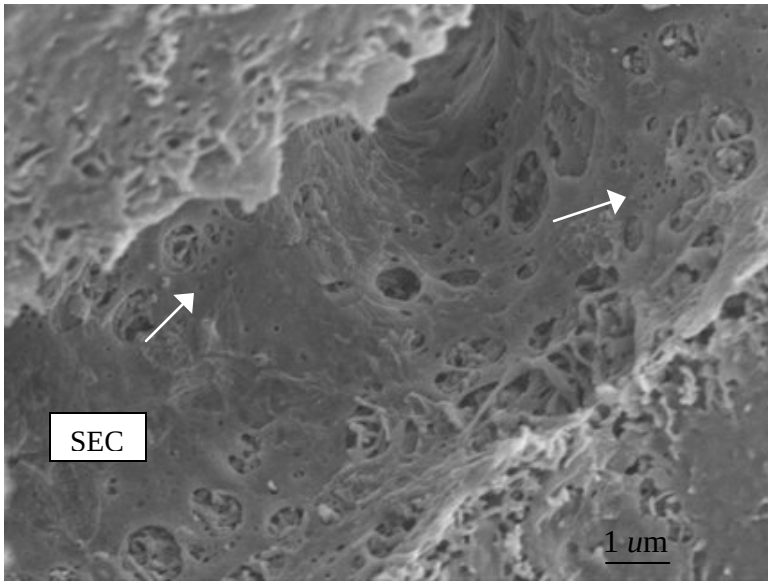


Figure 2: Lumen of rat hepatic sinusoid with the endothelial cell coating by SEM
SEC, sinusoidal endothelial cell; fenestrations (white, arrows). Original magnification x10,000.

The vascular endothelium-leukocyte interaction represents a central role in the pathogenesis of I/R-induced microvascular injury. Liver SECs are particularly vulnerable to cold ischemic injury [41] and during I/R injury, endothelial cells become activated to express an array of surface adhesion molecules, which primes the endothelium for further PMN interactions. This interaction is mediated by the expression of endothelial cell adhesion molecules, e.g. intracellular adhesion molecules (ICAM) and vascular adhesion molecules (VCAM), on the surfaces of both

vascular endothelial cells and leukocytes which subsequently produce several cell-damaging factors, to further promote hepatic injury [42]. The combination of Kupffer cell activation and SEC swelling with an increase in vasoconstrictors, e.g. endothelin and thromboxane (TxA2), and a decrease in vasodilators, e.g. prostacyclin, leads to sinusoidal narrowing. Upon reperfusion there is increase in neutrophil and platelet adhesion and aggregation in the sinusoids. The end result is a significant reduction of microcirculatory blood flow [31]. A deteriorated hepatic microcirculation and subsequent neutrophil emigration and increased vascular permeability are responsible in part for tissue injury [43].

Ischemic injury to the endothelium disrupts the delicate homeostasis in the microcirculation and promotes the attraction, activation, adhesion, and migration of polymorphonuclear neutrophils, causing local tissue destruction by release of proteases and oxygen free radicals. Eventually, damage to liver SECs leads to the loss of microvascular integrity and decreased blood flow. Increasing blood flow to the liver during reperfusion is essential for a good post-operative prognosis [17, 44].

1.2.2 Platelets

Platelets are well known for their important role in homeostasis in which the formation of a platelet aggregate is the first measure to seal a damaged blood vessel. Platelets are involved in multiple pathological processes, including inflammatory states and regeneration [45]. Platelets respond to both local and systemic inflammatory responses and are recruited to the liver where they adhere to activated sinusoidal endothelial cells in a liver exposed to cold or warm I/R injury [46-48]. Platelets are also attracted to the liver in response to inflammatory stimuli where they can translocate to enter the Space of Disse and attach to hepatocytes [45]. The mechanism by

which platelets induce organ damage is presumably by inducing apoptosis in SECs, a process which is facilitated by the presence of leukocytes and Kupffer cells [47, 49]. In addition, the extent of platelet adhesion to the liver endothelium has been shown to correlate with organ function in human liver transplantation recipients [50]. Therefore, inhibition of platelet aggregation is an important step in reducing hepatic I/R injury.

1.2.3 Cytokines

During hepatic I/R injury, which is characterized by an acute inflammatory response, several substances, including pro-inflammatory cytokines, are locally released to promote vasoconstriction, platelet aggregation, and leukocyte adherence. Pro-inflammatory cytokines are produced at the site of injury and are responsible for initiating and maintaining the inflammatory response, resulting in further organ injury [51]. The initial phase of I/R injury involves the release of ROS and the inflammatory cascade mediated by a variety of pro-inflammatory cytokines, including Tumor Necrosis Factor-α (TNF-α), Interleukin (IL)-1β, IL-6 by Kupffer cells [52]. In particular, TNF-α, IL-1β and -6, are known to mediate acute phase changes in hepatic protein synthesis at the transcriptional level, which contribute to disturbances in normal liver circulation [51, 53]. TNF-α is among the earliest cytokines activated [54] and is a central propagating factor [55]. TNF-α works primarily by stimulating many transcription factors, including nuclear factor-kappaB (NF-κB), which control and induce the gene expression of secondary inflammatory mediators, including IL-1, IL-6, chemokines, and vascular cell adhesion molecules [42]. TNF-α and IL-1β are also potent inducers of IL-6, which work together to up-regulate the expression of adhesion molecules, giving rise to increased leukocyte-sinusoidal

endothelial cell interactions, resulting in further tissue injury [56]. IL-6 is an important mediator of the hepatic acute-phase response during injury. Not only does IL-6 induce neutrophil activation, but it may also delay the phagocytic disposal of dysfunctional neutrophils, thereby prolonging the injurious effects of these cells [57]. Pro-inflammatory mediators work together with the expression of adhesion molecules, i.e. ICAM-1, VCAM-1, and P- and E-selectins, to further promote liver graft infiltration of neutrophils and further contribute to the progression of hepatic injury[42].

In summary, the primary targets of cold I/R injury are the liver SECs [58]. Damage to SECs leads to loss of microvascular integrity and decreased blood flow, while promoting the attraction, activation, adhesion, and migration of neutrophils to the endothelium. Platelet aggregation, local tissue destruction, up-regulation of inflammatory cytokines, and structural alterations in tissue leads to hepatocellular dysfunction [9, 29]. Each one of these factors represent a pharmacological target to reduce or prevent hepatic I/R injury.

1.3 EFFECTS OF INFLAMMATION ON DRUG DISPOSITION

Inflammation and pro-inflammatory cytokines are known to markedly impair hepatic detoxification pathways by suppressing the expression of several hepatic transporters and metabolic enzymes, thereby, altering drug disposition. Considering that the liver is the most important site of drug metabolism and clearance, inflammation-mediated changes in the activities or expression of drug metabolizing enzymes or hepatic transporters can have major implications when the capacity of the liver, such as the case during liver transplantation, to handle drugs is severely compromised, leading to altered hepatic clearance of drugs [59].

1.3.1 Drug Metabolism Pathways

The liver is the major organ responsible for the metabolism and clearance of endogenous and exogenous compounds in humans, and it expresses numerous drug-metabolizing enzymes, while some metabolism also occurs in the gut, lung, kidney, and brain.

The concentration of a drug in the blood (or plasma) is determined by the process of absorption, distribution, metabolism, and excretion. Metabolism can be broadly classified as either phase I (functionalization) or phase II (conjugation) reactions. Often, but not always, these two processes occur sequentially. Phase I reactions introduce a functional group or uncover a chemically reactive group on the parent compound, and the products may either be pharmacologically active or inactive and usually represent substrates for Phase II enzymes, which act to increase the polarity of a compound, through the process of conjugation, yielding a metabolite known as a "conjugate", that is more readily excreted. The process of converting a drug to a metabolite is often referred to as biotransformation and the four main categories of biotransformation include: oxidation, reduction, hydrolysis, and conjugation. The first three comprise Phase I, while the fourth comprises Phase II reactions.

1.3.1.1 Phase I Metabolism

Cytochrome P450 (CYP450) enzymes are a superfamily of heme-containing proteins having an iron protoporphyrin IX as the prosthetic group. Their name comes from the spectral absorbance peak of their carbon-monoxide-bound species which was determined to be at 450 nm [60].

Phase I metabolism is dominated by the microsomal mixed-function oxidase (MMFO) system which is involved in the metabolism of endogenous compounds (steroid hormones and fatty acids) as well as the biotransformation of xenobiotics. The CYP450-catalysed mixed-

function oxidation reaction is the most commonly studied drug metabolism reaction [61] and is carried out by incorporating one atom of molecular oxygen into the substrate while reducing the other atom of oxygen to water, with the corresponding enzymes being categorized as mono-oxygenases. Components of the system include cytochrome P450 (CYP450) and NADPH-CYP450 reductase, where CYP450 are the substrate- and oxygen-binding site of the enzyme system and the reductase serves as an electron carrier, transferring electrons from NADPH to the CYP450 complex [62]. As a result, xenobiotics can undergo hydroxylations, epoxidations, N-, S-, or O-dealkylations, deaminations, or N- or S-oxidations, and oxidative dehalogenations, sulphoxide formation, dehydrogenations, and deaminiation of mono-and diamines [63].

CYP450 isoforms are found in almost all living organisms and are widely distributed, but the largest concentration of CYP450s is located in the liver, predominantly in the membrane of the endoplasmic reticulum (ER). Analysis of the human genome has identified 57 human CYP450 enzymes involved in the metabolism of xenobiotics [64], which are comprised of four major families: CYP1, CYP2, CYP3, and CYP4 of which CYP3A4 is the most abundant and constitutes approximately 30% of the hepatic CYPs in human liver [65]. In rat, the CYP1, CYP2, and CYP3 families are largely involved in biotransformation of xenobiotics. Several CYP enzymes have orthologous forms in humans and rodents, although there are some differences in the expression and catalytic activities between human and rat CYP orthologs [66]. In rat liver, the mRNA of several members of the CYP3A subfamily, including CYP3A1/23, CYP3A2, CYP3A9, and CYP3A18 have been detected [67]. CYP2C11 and CYP2C12 are major and constitutlively expressed CYP450 gene products in male and female rat liver, respectively [68, 69]. In rat liver, CYP2E1 demonstrates approximately 80% amino acid homology with the human form of CYP2E1 [70].

1.3.1.2 Phase II Metabolism

Phase II conjugation leads to the formation of a covalent linkage between a functional group on the parent compound (or on a Phase I metabolite) with endogenously derived glucuronic acid, sulphate, glutathione, amino acids or acetate to produce a highly polar and water-soluble conjugate to that is more readily excreted in the urine or bile. The uridine diphosphate glucuronyltransferases (UGT) superfamily represents an important family of proteins that catalyze the transfer of glucuronic acid from UDP-glucuronic acid (UDPGA) to the substrate molecule, to enhance water solubility and excretion. The UGTs have been classified into two subfamilies, namely UGT1A and 2B, and are located in the ER, which has physiological effects in neutralization of reactive intermediates formed by the CYP450 enzyme system. Major Phase II reactions include glucuronidation, sulphation, acetlyation, and conjugation with glutathione or amino acids. The net effect is usually considered to be inactivation or detoxification.

1.3.2 Drug Transport System

The liver plays an important role in the enterohepatic circulation of bile acids, as well as the detoxification of endogenous and exogenous compounds through biotransformation and biliary excretion of these compounds. Hepatic drug elimination is a highly coordinated event, as drug disposition depends not only on the metabolism of a compound but also on the active uptake and efflux by specific transport proteins. Drug transporters are a class of membrane-bound proteins involved in the transport of numerous endogenous compounds as well as xenobiotics and their metabolites. Hepatic drug transporters serve a multitude of functions and play a critical role in the liver mediating drug uptake into hepatocytes and the translocation of compounds, such as bile acids, electrolytes, and xenobiotics, across biological membranes and elimination into bile.

Several lipophillic compounds move from the plasma into hepatic cytosol by simple or facilitated diffusion; however, numerous transport proteins are available on the sinusoidal (basolateral) membrane of the hepatocyte to mediate uptake of amphipathic and polar organic compounds. In addition, hepatic efflux transport proteins located on the apical (canalicular) side of the hepatocyte play an important role in the excretion of drugs and metabolites from the hepatic cytosol into the bile [71, 72]. Due to their wide tissue distribution and high level of expression in the liver, intestine, kidney, placenta, and blood-brain barrier, drug transporters of several gene families serve a major role in defining the disposition of many xenobiotics and impact the elimination of drugs by mediating the cellular uptake and export of compounds into and out of cells responsible for the degradation of compounds, and are categorized as uptake and efflux transporters, discussed below.

1.3.2.1 Uptake Transporters

A major function of the liver is the uptake of a large number of endogenous organic anionic compounds and xenobiotics from the circulation. Hepatic uptake of xenobiotics, waste products, and conjugated bile acid from the sinusoidal (basolateral) plasma membrane of the hepatocyte occurs by members of the superfamily of solute carriers (SLC), including the sodium-dependent taurocholate co-transporting polypeptide (NTCP, SLC10A) [73], the sodium-independent transport proteins including the organic anion transporter polypeptides (OATP, SLCO21A), and the organic cation transporter (OCT, SLC22A) families [74]. The substrate specificity for these transporters is broad and there is strong overlap between various members of the transporter families. The varied affinities of these transporters for different compounds in portal blood provide specific and redundant means for extracting bile salts and other compounds from the blood and excreting them from the hepatocytes [75].

1.3.2.2 Efflux Transporters

The bile canalicular membrane of the mammalian hepatocyte contains several primary active transporters which couple ATP hydrolysis to facilitate the transport of specific substrates into bile canaliculus [76, 77]. The largest family of hepatic drug transporters are comprised of the ATP-binding cassette "ABC" transporters [78], and are involved in the cellular efflux of xenobiotics and endogenous compounds from the hepatic cytosol into the bile.

The formation of bile serves two vital functions: 1) it is a major route for the elimination of drugs, toxins and waste products, and 2) it ensures the secretion of bile salts, which are crucial for lipid emulsification and subsequent lipid absorption in the intestine. Members of the ABC family of transporters include the multi-drug resistance protein (MDR1/P-glycoprotein, P-gp), multi-drug resistance associated protein (MRP) members 1-6. MRP2, encoded by the ABCC2 gene, is one of the most studied family members as it is involved in the biliary transport of glutathione and its conjugates. An important endogenous substrate for MRP2 is conjugated (glucuronidated) bilirubin. The hepatic canalicular membrane contains P-glycoprotein (P-gp), encoded for by the ABCB1 gene, responsible for cellular efflux of numerous clinically relevant therapeutic agents. P-gp is expressed at the apical epithelium of the liver, intestine, kidney and blood-brain barrier [79]. The human genome contains a single MDR1 (P-gp), whereas two orthologs of human MDR1 exist in rodents, denoted as mdr1a/b (Abcb1a/b). Other MDR gene family members include the liver canalicular bile salt export pump, BSEP/Bsep (or sister of P-gp, spgp) and the hepatic phospholipid transporter MDR3 (mdr2 in rodents). BSEP/Bsep pumps bile salts out of the hepatocyte, across the canalicular membrane and into bile and is the major transporter responsible for hepatic bile acid excretion and generation of bile flow. The location of the aforementioned transporters is illustrated in Figure 3.

The activity and expression of drug transporters can be regulated by various factors including environmental, genetic, oxidative stress and inflammation. The following section will provide an overview of the effect of inflammation on drug disposition.

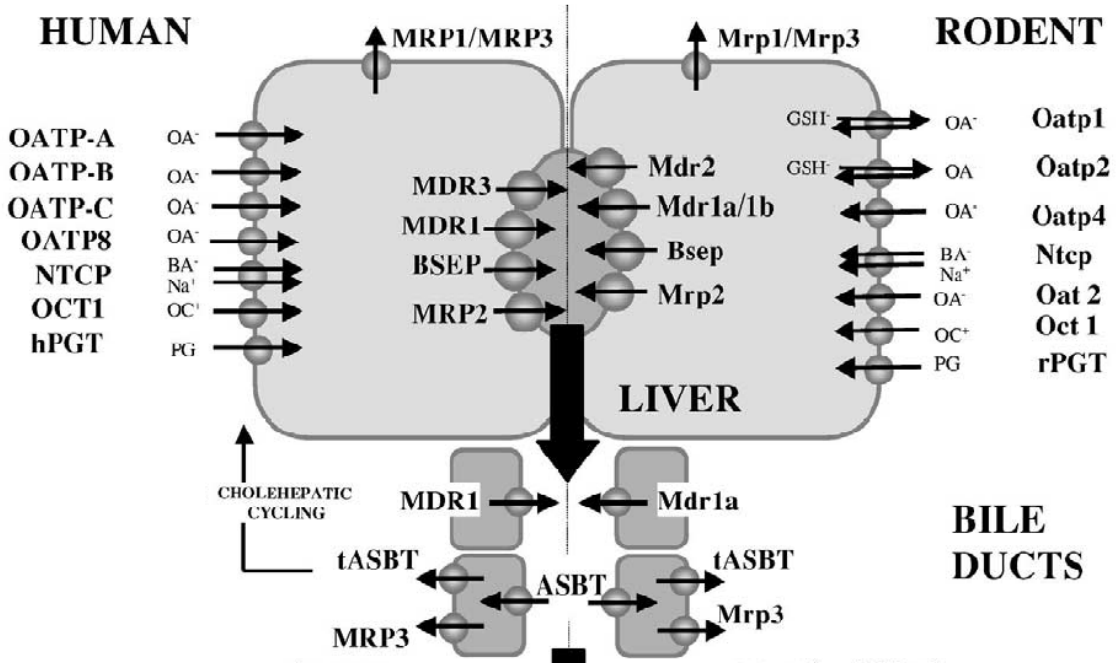


Figure 3: Hepatic drug transporters in human and rodent
Modified from Faber et. al [80]

1.3.3 Effect of Pro-inflammatory Cytokines on Drug Metabolism and Drug Transport

During the host response to inflammation, inflammatory mediators, including pro-inflammatory cytokines, have been associated with altered content, expression, and activity of CYP450 enzymes and drug transporters, consequently leading to alterations in the absorption, distribution, metabolism, and elimination of several drugs [81-85]. Usually, most CYP450s and drug transporters are down-regulated although a few may be refractory or actually up-regulated. The losses in drug metabolism and transport are channeled predominantly through the production of

cytokines that ultimately modify the expression and function of specific transcription factors, e.g. Nuclear Factor-Kappa B (NF-κB).

Acute inflammation of the liver typically begins with activation of the Kupffer cells, which initiates intracellular signaling cascades and culminates in cell activation. Cytokines and other pro-inflammatory mediators, e.g. ROS, are released and adhesion molecules for leukocytes are expressed on the plasma membrane of non-parenchymal cells. In particular, TNF-α is released from KCs early and plays a major role in precipitating downstream events.

Hepatobiliary transporters are important determinants of drug clearance; they regulate the access of drugs to the drug-metabolizing enzymes as well as control drug concentrations in the hepatocytes, and are essential for normal bile formation and efficient drug metabolism. Alterations in the functionality, protein, and mRNA expression of transporters occur to varying degrees by drugs, metabolites, oxidative stress, and cytokines. During the early post-transplant period, these effects can significantly alter drug disposition in this patient population, which has major implications when the capacity of the liver, and other organs, to handle drugs is severely compromised [81, 86-89].

In addition, CYP450 activity represents an important marker of liver function in graft post-transplantation and decreased levels can influence the clinical response and, in worse cases, precipitate hepatic dysfunction or lead to graft non-function and or failure. Several studies have indicated that drug metabolizing abilities post-transplantation are a reliable indicator of liver function *in vivo* [90-93]. In most cases, the decreased CYP activity is accompanied or preceded by decreases in hepatic levels of the corresponding P450 mRNAs and proteins (Morgan, 1997).

Hepatic I/R injury has been shown to lead to damaged hepatocytes and bile duct cells, resulting in altered biliary secretion of endogenous compounds and altered pharmacokinetics of

drugs in the recipients. Previous observations in liver transplant patients of a high RIA to HPLC ratio for cyclosporine A (RIA measuring parent and metabolite, while HPLC measuring the parent drug), indicated that formation of the metabolites was not altered but that biliary transport of the formed metabolites was, in grafts which exhibited early poor function post-OLT [94]. It was later shown that cyclosporine A is a P-gp substrate [95]. Similarly, ceftriaxone, a third generation cephalosporin, is excreted (approximately 40%) into the bile by the MRP2 protein and a lower clearance of this drug has been reported following OLT, suggesting hepatic dysfunction at the transporter level [96].

Hepatic I/R injury is frequently associated with cholestasis, where the bile flow is greatly diminished and bile constituents begin to accumulate in hepatocytes, which causes an increase in oxidative stress and inflammation, and subsequently leads to hepatotoxicity. Experimental studies have shown impaired hepatobiliary transport systems and the development of cholestasis as a result of down-regulation of canalicular transporters [97]. The result of reduced drug clearance, which accompanies inflammation and reduced blood flow, could be toxic or produce sub-therapeutic plasma drug concentrations. Therefore, an agent that is capable of suppressing the pro-inflammatory cascade following reperfusion, while improving hepatic metabolism and transport capacity, would significantly improve the liver graft function and improve patient outcomes post-OLT.

1.4 PHARMACOLOGICAL APPROACHES TO PREVENT LIVER I/R INJURY

Due to the many pathways and factors involved in the development and progression of I/R injury, many pharmacological approaches have been explored as therapies to minimize I/R-induced hepatic injury, including anti-inflammatory, antioxidant, and vasodilatory therapy.

Activation of Kupffer cells releases a variety of potentially harmful mediators, including TNF-α and ROS. Eliminating Kupffer cells with gadolinium chloride has been shown to improve SEC structure and reduce hepatic I/R injury [98]. The role of TNF-α in initiating hepatic I/R injury has also been well documented [55, 99, 100]. Approaches to block TNF-α signaling pathway upon reperfusion include TNF-α antibodies, pretreatment with pentoxifylline, a methylxanthine derivative, which prevents TNF-α synthesis and release in KCs [100], or the use of TNF-receptor 1 knockout mice [55, 99, 101].

Antioxidant therapy, e.g. Vitamin E, also known by its chemical name α-tocopherol, is an important antioxidant which works as a radical scavenger to inhibit ROS generation. A randomized clinical trial in 47 patients undergoing partial liver resection demonstrated that pre-operative administration of Vitamin E to the recipient reduced liver enzymes (ALT and AST) and decreased intensive care unit stays [102]. Treatment with a multivitamin containing two antioxidants- α-tocopherol and ascorbate, in patients undergoing major liver surgery failed to show a significant difference in lactate levels, which correlated with ischemic times post-operatively [103]. Allopurinol has also been tested for its inhibition of xanthine oxidase pathway, though no significant improvement in patient outcome was achieved [104].

Agonists of endothelin (ET)-1 have been shown to cause contraction of isolated stellate cells in culture and to narrow the lumens of sinusoids in isolated perfused livers and well as intact cells [39], thereby, making ET-receptor antagonists targets for attenuation of

microcirculation disturbances [105]. To promote vasodilation, nitric oxide [106], carbon monoxide [107], and the prostaglandin (PG) class of drugs, including PGI_2 [17] have been tested. The focus of this dissertation is the use of treprostinil, a PGI_2 analogue, for protection of liver grafts against I/R injury in orthotopic liver transplantation.

A summary of the mechanisms involved in hepatic I/R injury and some of the therapeutic strategies are illustrated below, in Figure 4.

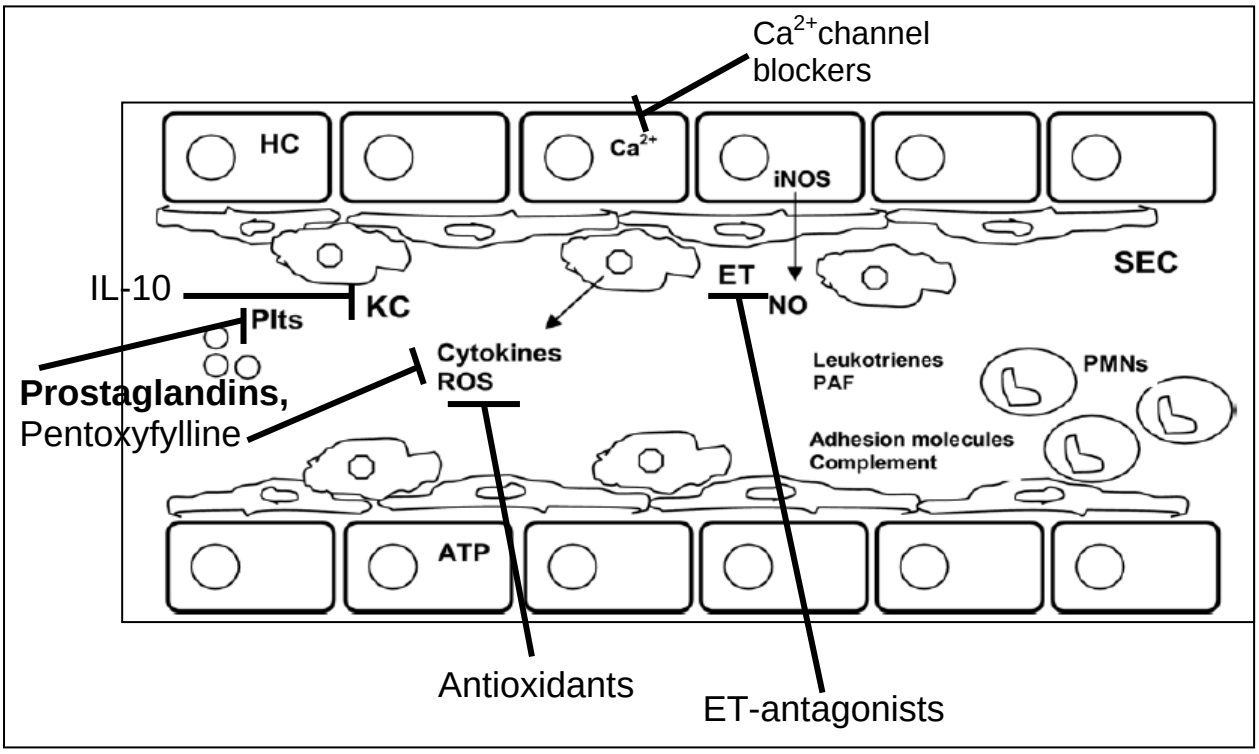


Figure 4: Targets of hepatic I/R injury
Ca2+: calcium; ET: endothelin; HC: hepatocytes; KC: Kupffer cell; PAF: platelet activating factor; Plts: platelets; PMNs: polymorphonuclear leukocytes; ROS: reactive oxygen species; SEC: sinusoidal endothelial cell; IL: interleukin; modified from Montalvo-Jave et al. [16].

1.5 PROSTAGLANDINS IN VASCULAR HOMEOSTASIS

Prostaglandins (PG) are a family of biologically active polyunsaturated fatty acids derived from arachidonic acid that possess a critical responsibility in maintaining vascular homeostasis of microcirculation. The first step in PG synthesis is the liberation of arachidonic acid from membrane-bound lipids via the enzymatic actions of Phospholipase A2 and subsequent enzymatic metabolism through the action of cyclooxygenase, shown in Figure 5. Prostaglandins contain a cyclopentane ring with two side chains, α and ω, and based on the ring modifications, they are classified into types A to I (PGA-PGI), then further classified by the number of double bonds (1, 2, or 3) in their side chains [108]. The enzyme responsible for PGI_2 synthesis is prostacyclin synthase which is localized to the endoplasmic reticulum in endothelial cells and to the nuclear and plasma membranes in smooth muscle cells [109, 110].

Classically, PGI_2 mediates its biological effects through binding to cell surface prostacyclin receptors (IP), which couple via the stimulatory G protein, leading to activation of adenylyl cyclase and an increase in intracellular cyclic adenosine monophosphate (cAMP) signaling, which acts as a second messenger to inhibit platelet aggregation, cell proliferation and inflammatory mediator release [109, 111, 112]. The IP receptor is located on a variety of cells, including platelets, vascular smooth muscle, and endothelial cells, where PGI_2 acts locally [113]. The increase in cAMP leads to activation of protein kinase A and the subsequent phosphorylation of specific target proteins in platelets, resulting in anti-platelet activity as well as relaxation of vascular smooth muscle [108]. At the endothelial level, prostacyclin exerts anti-inflammatory and anti-platelet activity and promotes an antithrombotic surface, which is required for proper function and maintenance of vascular integrity [114].

Prostacyclin is a potent endogenous inhibitor of platelet aggregation, which is reported to be 30-40 times more potent than PGE_1 [115] and is involved in the complex interactions between vessel wall, blood and platelet function. These actions provide protection against excessive vasoconstriction, platelet deposition, and cellular proliferation in the vessel wall [116]. Interestingly, prostacyclin is extremely unstable and its activity disappears within 15 seconds of boiling or within 10 minutes at 22 ^{o}C at neutral pH, and in blood at 37 ^{o}C it has a half-life of 2-3 minutes [117, 118]. Alkaline pH increases the stability of PGI_2 so that at pH 10.5 (25 ^{o}C) it has a half-life of 100 hours, whereas at 4 ^{o}C, the half-life is reduced to 14.5 minutes [119]. Several analogues with improved stability have been developed, and are described in Section 1.5.1.

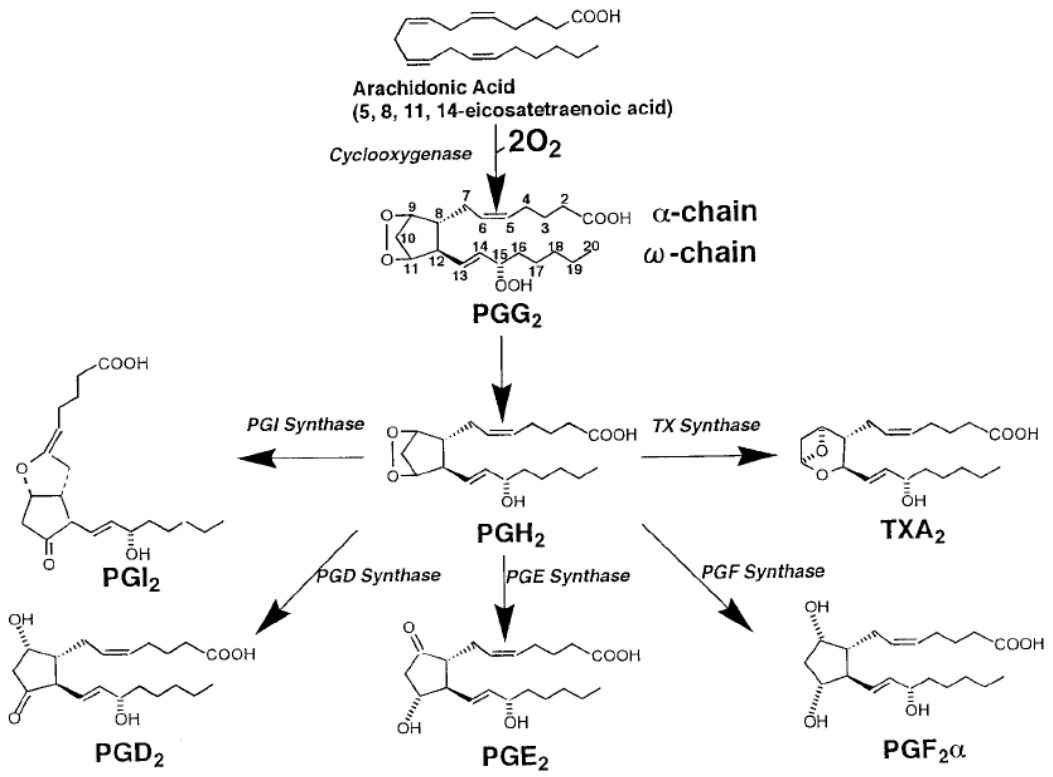


Figure 5: Biosynthetic pathway of prostaglandins
PG: Prostaglandin; TxA_2: thromboxane. Modified from Narumiya et al. [108]

1.5.1 Prostacyclin Analogues

Several prostacyclin analogues have received FDA approval for their use in treatment of pulmonary arterial hypertension (PAH). PAH is a rare disease characterized by a progressive elevation of pulmonary vascular resistance and pulmonary artery pressure, which often leads to right ventricular failure, ultimately resulting in death [120]. While the principle pharmacological effects of all prostanoids are similar due to their actions on the IP receptor, there are notable differences in the pharmacokinetics and metabolism, with a wide range in half-lives. Also, the modes of application of PGI_2 analogues vary from continuous intravenous infusion of epoprostenol to inhaled application of iloprost, to oral administration of beraprost, and subcutaneous infusion of treprostinil. In addition, the doses vary, ranging from pg/kg/min to ng/kg/min. As such, there is no set dose and each analogue is titrated to response.

1.5.1.1 Epoprostenol

Prostacyclin has been stabilized as a pharmaceutical preparation (epoprostenol) by freeze-drying and reconstitution in an alkaline glycine buffer [121]. Intravenous (IV) epoprostenol (Flolan®, GlaxoSmithKline, Durham, NC) is approved by the US Food and Drug Administration (FDA) for the treatment of PAH associated with the scleroderma spectrum of disease, and the structure is shown in Figure 6A [122].

At neutral blood pH, epoprostenol is rapidly and spontaneously hydrolyzed to 6-keto-PGF1α [119] and enzymatically metabolized to 6, 15-diketo-13, 14-dihydro-PGF1α [122]. Neither metabolite has the same degree of biological activity as the parent compound. Although this agent is an effective therapy for PAH, the administration of epoprostenol is complex. Epoprostenol requires daily reconstitution under sterile conditions and ice packs to be changed

every 12 hours unless the cassette is changed every 8 hr making it a cumbersome and inconvenient treatment option in this patient population. Adverse effects include headaches, jaw pain, nausea, diarrhea, hypotension, and leg pain [123]. Long-term epoprostenol infusion is associated with problems due to its short half-life (2-3 minutes), which requires continuous IV infusion through a permanent catheter into a large central vein with an ambulatory infusion pump with all the associated risks which range from local infections, thromboembolic events, or life-threatening sepsis [116]. Abrupt discontinuation of the infusion, i.e. catheter displacement or pump malfunction, can lead to episodes of worsening PAH and hemodynamic decompensation. Any interruptions in administration, could be life-threatening, in addition to its significant systemic side effects [113]. Due to these limitations, additional PGI_2 analogues have been developed.

1.5.1.2 Iloprost

Iloprost (Ventavis®, Actelion Pharmaceuticals, San Francisco, CA) is an inhaled synthetic prostacyclin analogue, that produces potent pulmonary vasodilation and inhibits platelet aggregation [124]. In the U.S., iloprost has been approved by the FDA for oral inhalation using the I-neb® AAD® (Adaptive Aerosol Delivery) System or Prodose® AAD® Systems and the chemical structure is shown in Figure 6B. In Europe, iloprost has been approved for use with two compressed air nebulizers with AAD® Systems (Halolite and Prodose) as well as with two ultrasonic nebulizers Ventaneb® and I-Neb®. The half-life of iloprost is approximately 20-30 minutes and the bioavailability after inhalation has not been determined [124]. Administration of this analogue is not ideal; patients are required to inhale 6-12 doses per day, which still may not be sufficient to cover a 24 hr cycle [123]. Adverse effects include flushing, headache, and

cough. The long-term efficacy of iloprost is still under investigation and need to be addressed by large clinical trials.

1.5.1.3 Beraprost

Beraprost is a synthetic prostacyclin analogue that has been developed as an orally active agent. Beraprost acts by binding to prostacyclin membrane receptors to inhibit the release of intracellular calcium, which causes relaxation of the smooth muscle cells and vasodilation [125]. The half-life is between 30 – 45 minutes [126] and the chemical structure is shown in Figure 6C. Adverse effects include jaw pain, headache, flushing, diarrhea, and palpitations. A randomized, placebo-controlled trial was performed in the US and while the drug showed some benefit after 3 and 6 months of treatment, no benefit was seen at 9 or 12 months [125]. Based on these data, beraprost has not been approved in the US.

1.5.1.4 Treprostinil

Treprostinil is a chemically stable PGI_2 analogue, shown in Figure 6D. In 2002, the US FDA approved treprostinil (Remodulin®, United Therapeutics, Durham, NC) for treatment of PAH. Treprostinil has an elimination half-life of 3-4 hours and it is rapidly and completely absorbed after subcutaneous (SC) administration with an absolute bioavailability of 100% and steady-state plasma concentrations are reached after approximately 10 hours [113]. Treprostinil is stable in sterile water or 0.9% sodium chloride at room temperature, which allows for IV or SC infusion without the need for ice packs. Also, its solubility at physiologic pH enables SC delivery, thereby avoiding the potential complications of the epoprostenol IV delivery system.

For patients with PAH, treprostinil provides an alternative to previous PGI_2 analogues and has favorable pharmacokinetic and stability characteristics. The longer stability in the

delivery system (up to 48 hours at room temperature) and a longer half-life increases the feasibility of rescue upon unintended interruptions or pump malfunctions, as well as the ease of dosage preparation. Treprostinil has many advantages over other PGI_2 analogues, including a 3-fold longer half-life and 6-fold increase in cAMP response than other PGI_2 analogues [127], which make treprostinil an attractive candidate for protection of the liver graft against I/R injury during OLT.

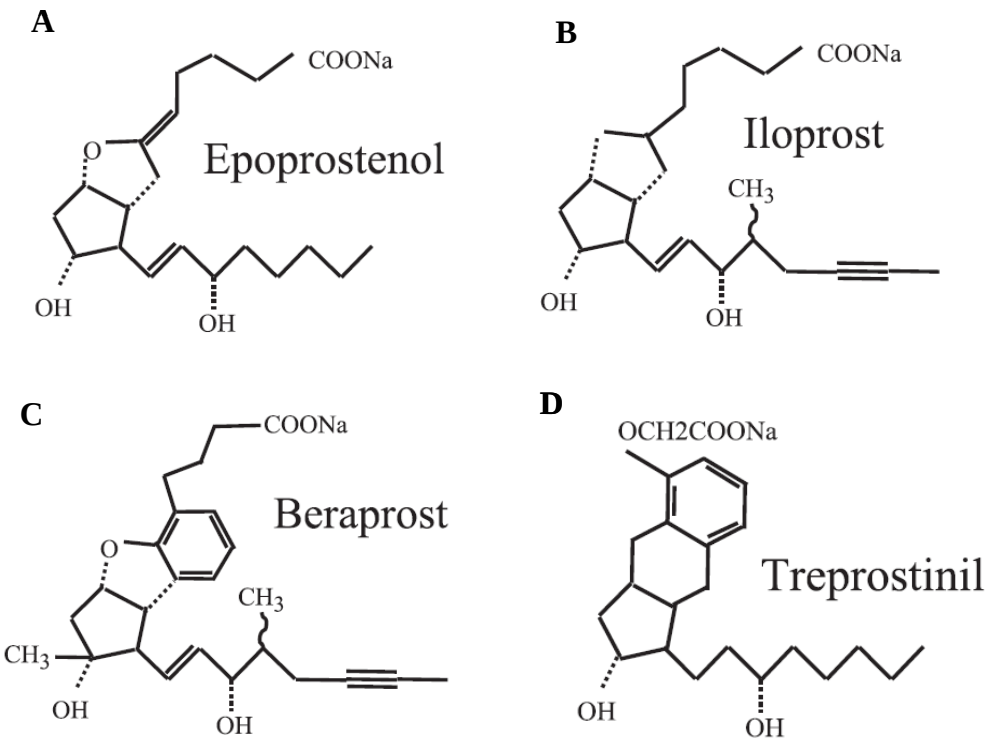


Figure 6: Chemical structures of prostacyclin analogues
A) Epoprostenol, B) Iloprost, C) Beraprost, and D) Treprostinil. Modified from Olschewski et al. [128]

During liver transplantation, improving microcirculation through the liver graft during reperfusion and inhibiting platelet aggregation and pro-inflammatory cytokines are essential for a good post-operative prognosis [129, 130]. PGI_2 is mainly synthesized in endothelial cells and

regulates various physiological processes occurring at the interface between the blood and endothelium [13], which may serve to protect the liver graft against I/R injury during OLT by maintaining hepatic blood flow, counteracting the activity of vasoconstrictors and platelet aggregation as well as the release of pro-inflammatory cytokines. Despite extensive research, previous PG analogues tested for protection of liver grafts against I/R injury in OLT have been limited due to intolerable doses or failure to meet primary endpoints and, to date, have not successfully made their way into the clinic. Treprostinil has the potential to restore therapeutic levels of PGI_2 in liver SECs, thereby maintain vascular homeostasis, improve hepatic blood flow through the sinusoids, reduce platelet aggregation and inflammatory cytokines, and, ultimately, serve as a therapeutic option to protect liver grafts against I/R injury during orthotopic liver transplantation.

1.6 CLINICAL EXPERIENCE WITH PROSTACYCLIN ANALOGUES IN LIVER TRANSPLANTATION

Numerous strategies have been investigated as options to improve the ischemic tolerance of the liver and to minimize I/R injury in patients undergoing OLT, and the prostaglandin class of drugs is one such strategy [131-134]. Animal and human studies have shown a decrease in the ratio of PGI_2 and TXA_2 in I/R injury [135, 136] suggesting a potential therapeutic benefit of this class of drugs. Analogues of PGE_1 and PGI_2 have been examined for their ability to protect the liver from I/R-induced injury due to their role in maintaining hepatic blood flow via dilation of the arterial and vascular bed, by inhibiting platelet aggregation [137], cell adhesion molecules, neutrophil activation, the generation of reactive oxygen species, leukocyte activation, migration,

and adherence, and inhibit the production and expression of pro-inflammatory cytokines [13, 17]. Considering the many factors involved in I/R injury and the role of PGs in maintaining vascular and cellular homeostasis, as well as the primary target of I/R injury being the liver SECs [138, 139], PGI_2 has a particular relevance in the setting of hepatic I/R injury associated with OLT.

Since the late 1980s, PG analogues, i.e. PGE_1 and PGI_2, have been tested for their ability to reduce I/R-induced liver injury in several animal models [131-133, 137, 140] and in clinical liver transplantation [10, 141-149]. A summary of clinical trials investigating the use of PG analogues to reduce I/R injury in OLT is provided in Table 1. Early studies using intravenous PGE_1 were promising in minimizing primary liver graft non-function; however the clinical application of PGE_1 and PGI_2 has been limited by their inherent instability and very short half-life, thus requiring intolerable doses in addition to not showing a significant difference in patient outcomes compared to placebo or historical control. In order to successfully treat an OLT patient with PGI_2, it is crucial to understand the shortcomings of previous trials and identify an agent capable of overcoming previous limitations.

Grieg et al. studied 16 patients with PNF. Six patients were listed for re-transplantation and ten patients were started on an infusion of PGE_1 within 4-34 hours post-transplantation and continued for 4-7 days [10]. The untreated group had a 33% survival rate whereas the PGE_1 treated group had a 90% survival rate. Secondary findings of a significant decrease in peak serum aminotransferases and normalization of clotting factors were also reported. The authors concluded that PGE_1 reduced hepatocellular necrosis and improved liver function but recommended larger placebo controlled studies to confirm these findings.

In 1992 -1993, UPMC reported inferior results in liver recipients with positive cytotoxic crossmatch that occurs in about 20% of liver transplant cases, i.e., the presence in the recipient of preexisting antidonor cytotoxic antibodies [150]. Combined treatment with intravenous PGE_1 and high doses of prednisone reduced the number of adverse outcomes [149]. A large number of patients who were crossmatch-negative also were treated in this study, and PGE_1 treatment conferred important benefits in these recipients and it also significantly improved kidney function in liver recipients [147]. This finding was later confirmed in an extensive study in which patients treated with steroids and PGE_1 had only a single case of primary liver graft non-function (1 in 174 cases) compared to an incidence of 5.9% in historical controls who were treated with steroids only, though some patients did not tolerate PGE_1 [148]. That same year, in 1995, Henley et al. reported results from a double-blind, randomized placebo-controlled, single center trial of continuous infusion of PGE_1 started during the anhepatic phase in 172 patients undergoing orthotopic liver transplantation. Although the trial failed to show an effect of treatment on patient and graft survival, the study's primary endpoint, the study did show significantly shorter post-transplant ICU and hospitalization stays, reduced needs for renal support, and less need for surgical intervention other than re-transplantation in the active treatment group [141].

Results from a subsequent randomized, double-blinded, multicenter trial of PGE_1 infusion immediately following restoration of portal and arterial flow [142] were similar to those reported by Henley [141] and Takaya [147-149], although this study failed to demonstrate a difference between the two groups in the primary endpoint- reduction of PNF, peak serum aminotransferase and alkaline phosphatase levels were lower, and bile volumes were higher in the PGE_1 group.

Also, infusion of PGE_1 ameliorated post-transplant renal dysfunction, and a reduction in ICU days (PGE_1: 4.0 ± 3.6 vs. placebo: 10.5 ± 17.1, $P<0.01$).

One small placebo controlled study in patients evaluated the effect of a seven day infusion of PGI_2 [144] immediately following reperfusion on hepatic dysfunction by measuring hepatic-splanchnic oxygenation and serum aminotransferase. One year survival was 100% in placebo- and PGI_2-treated groups; AST levels were lower in the PGI_2 group, compared to placebo. The study also demonstrated an improved hepatic-splanchnic oxygenation assessed by hepatic venous oxygen saturation ($SvhO_2$) levels at 24 and 48 hours post-OLT, suggesting that treatment with PGI_2 improves early microvascular blood flow by augmenting hepatic-splanchnic oxygenation.

The previous studies have focused on treating the transplant recipient post-reperfusion of the liver graft. Knowing that reperfusion exacerbates cold ischemic injury, PGI_2 treatment commenced prior to reperfusion in the recipient may provide the most beneficial therapy for improving graft function. A randomized trial treated the donor during liver retrieval of 106 patients undergoing OLT with a 500 μg bolus of epoprostenol (PGI_2) or no drug as control immediately before cold perfusion [143]. Significant reduction of peak levels of transaminases in the PGI_2 group was observed, although neither group experienced PNF, and no differences between number of hospitalization days in the PGI_2 and control group were noted.

In summary, the prostaglandin-class of drugs, including prostacyclin and its analogues, could represent an important advancement toward the goal of reducing transplant related morbidity, mortality and associated costs by providing these benefits. Additionally, the reduction in serum creatinine and reduced need for post operative dialysis observed in some studies has implications in protecting the kidneys from the nephrotoxic affects of the immunosuppressant

agents, especially during the early post-operative period. Routine use of PGE_1 and PGI_2, however, has been limited by its instability, short half life, and failure to show primary endpoint. Hepatic I/R injury remains a significant limitation in clinical liver transplantation and the need for therapy to reduce I/R injury is imminent, however, no therapy is currently available. Due to its pharmacological properties, treprostinil has the potential to ameliorate I/R injury in human liver transplantation.

Table 1: Summary of previous clinical studies using PGI_2 analogues to prevent I/R injury in OLT

Author	Year	PG analogue	RTC	Infusion timing		Subjects PG/control	Safety	Outcomes
				Pre	Post			
Greig [10]	1989	Alprostadil (PGE_1)	No		X	10/6	Not reported	Graft and patient survival: 80 and 90% in PGE_1 vs. 17 and 33% in placebo, respectively.
Takaya [149]	1992	Alprostadil	Yes		X	14	Not reported	Superior renal function; graft protection with PGE_1
Takaya [147]	1993	PGE_1	Yes	X	X	41	Not reported	Improved renal function with PGE_1
Takaya [148]	1995	Alprostadil	No	X	X	174/304	1 death, 1 PNF, 1 hepatic artery thrombosis, 1 excessive bleed intra-op in PGE_1	PNF: 1.1 vs. 5.9% in PGE_1 vs. historical control, respectively.
Henley [141]	1995	Alprostadil	Yes	X		78/82	2 patients withdrawn by attending without meeting PNF criteria	Reduced ICU and hospital stay, renal support; Improved graft and patient survival; renal function with PGE_1
Klein [142]	1996	Alprostadil	Yes		X	58/60	10 patients discontinued (7 placebo, 3 PGE_1); death, liver failure, other	Lower incidence of renal dysfunction and shorter ICU days with PGE_1.
Neumann [145]	1999	Epoprostenol (PGI_2)	Yes		X	15/15	1 excluded for bleeding, not related to PGI_2 infusion	Improved ΔSO_2[1]; Initial poor function in 2 control, 0 PGI_2; 1 PGI_2 re-transplant
Klein [143]	1999	Epoprostenol	Yes	X		53/53	Hypotension requiring catecholamine (30 PGI_2, 26 control)	Donor with PGI_2; Significant ALT and AST reductions (peak and AUC)

RTC: Randomized controlled Trial; Infusion time in relation to transplantation; [1]ΔSO_2: a measure of hepatic-splachnic oxygenation

1.7 HYPOTHESIS AND STUDY AIMS

The process of I/R injury to the liver graft during OLT combines interrelated factors that produce a cascade of events, which can ultimately lead to hepatic graft failure. Due to the multiple factors that contribute to hepatic I/R injury, conventional approaches that target one of these factors have not succeeded in solving this problem. Considering the many factors involved in I/R injury, including increasing blood flow to the liver and inhibition of platelet aggregation and pro-inflammatory cytokines, and the role of PGI_2 in maintaining cellular homeostasis, an agent, such as treprostinil, capable of combating the multiple factors involved in the development of I/R injury would have a particular relevance in the setting of I/R injury in OLT. Such an agent would be a tremendous advancement to the field of liver transplantation and, perhaps, in solid organ transplantation.

The ultimate goal for the use of treprostinil in liver transplantation is to protect the liver grafts against I/R-associated hepatic injury in adult patients undergoing OLT, and to increase the number of suitable grafts available for transplantation and patients who successfully recover from OLT. The first step to fulfill this goal is to examine the hypothesis that treprostinil, based on the pharmacological properties, will protect the liver graft against I/R injury during rat OLT. This hypothesis is based on the ability of treprostinil, a prostacyclin analogue, to inhibit pro-inflammatory cytokine expression, increase blood flow to the liver, and preserve homeostasis in the liver graft during transplantation, primarily by preserving liver SEC structure during cold storage and post-OLT. We further hypothesized that I/R injury, as an inflammatory response, following OLT would significantly down-regulate the expression and activity of CYP450 drug metabolizing enzymes and drug transporters and that treprostinil would improve the metabolic and functional capacity of the liver graft post-transplantation.

To examine these hypotheses, it was first necessary to determine whether or not treprostinil was able to protect the liver graft against I/R injury in a rat OLT model, and this is discussed in Chapter 2. The effects of I/R injury and treprostinil on 1) CYP450-mediated metabolism and 2) mRNA and protein expression of hepatic drug transporters in liver graft tissue post-OLT, has been examined in Chapter 3 and Chapter 4, respectively. *In vitro* studies to determine whether or not treprostinil could be safely co-administered without concern for a drug-drug interaction studies between treprostinil and cyclosporine A, tacrolimus, sirolimus, and mycophenolic acid has been carried out in Chapter 5. The conclusions and future research recommendations are discussed in Chapter 6. Lastly, a Phase I/II clinical study in adult OLT patients has been initiated to examine the preliminary safety, efficacy, and pharmacokinetics of a two-day peri-operative course of treprostinil in adult patients undergoing OLT, and the protocol is presented in Appendix A.

2.0 TREPROSTINIL AMELIORATES ISCHEMIA-REPERFUSION INJURY IN RAT ORTHOTOPIC LIVER TRANSPLANTATION[*]

[*]N. Ghonem, J. Yoshida, D.B. Stolz, A. Humar, T.E. Starzl, N. Murase, and R. Venkataramanan. Treprostinil, a Prostacyclin Analogue, Ameliorates Ischemia-Reperfusion Injury in Rat Orthotopic Liver Transplantation. Submitted to *American Journal of Transplantation*, October 2010.

2.1 INTRODUCTION

Liver transplantation is the only therapy available for end-stage liver diseases; however, donor shortage is a major factor limiting the number of organs available for liver transplantation. The current shortage of deceased donors has forced the expansion of the donor pool and has led centers to accept ECDs, i.e. older, non-heart beating, high steatosis, and those with prolonged ischemia. These organs provide the much-needed additional grafts; however, they are more susceptible to I/R injury, which is an unavoidable process during liver transplantation and is a major cause of initial liver graft dysfunction [9, 29]. The need for therapy to reduce I/R injury in liver transplantation is imminent, unfortunately no treatment is available.

Since the late 1980s, PG analogues, i.e. PGE_1 and PGI_2, have been explored as a potential therapy to reduce I/R injury in several animal models [131-133, 137, 140] and in clinical liver transplantation [10, 141-149], primarily due to their vasodilatory and platelet anti-aggregatory effects. Some studies have shown PG therapy to be useful for prevention of liver injury following transplantation; however the clinical utility of PGE_1 and PGI_2 is limited due to their inherent instability, intolerable side effects, a very short half-life, and the inability to show a significant difference in primary endpoint. Treprostinil sodium, a relatively new PGI_2 analogue, is FDA-approved (Remodulin®) for the treatment of pulmonary arterial hypertension. Advantages of treprostinil include a longer elimination half-life and increased potency (three- and six-fold, respectively) as well as its stability at room temperature and neutral pH [127]. These advantages enable lower doses and correspondingly lower side effects, to achieve therapeutic efficacy. Treprostinil has the potential to minimize I/R-associated hepatic injury in liver transplantation due to its cytoprotective effects, including its ability to preserve cellular homeostasis and microcirculation within the vasculature. Use of an agent, such as treprostinil, in

the clinic may ultimately increase the number of suitable grafts available for transplantation, and improve overall patient outcome. To the best of our knowledge, no PGI_2 analogue has ever been tested for its ability to prevent I/R injury in a rat OLT model. Therefore, the objective of this study was to examine the efficacy of treprostinil in protection of the liver grafts against I/R injury during rat OLT.

2.2 MATERIALS AND METHODS

2.2.1 Animals

All procedures were performed according to the guidelines of the National Research Council's Guide for the Humane Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. Male Lewis rats weighing 200 - 300 g (Harlan Sprague Dawley, Inc, Indianapolis, IN) were maintained in a laminar-flow, specific-pathogen–free atmosphere at the University of Pittsburgh with a standard diet and water supplied ad libitum.

2.2.2 Orthotopic Liver Transplantation Model

The basic techniques of liver harvesting and OLT without hepatic arterial reconstruction were performed as previously described [151]. Briefly, rats were anesthetized with isoflurane inhalation and a midline incision in the abdominal cavity was made and the liver graft was excised and immediately flushed with cold UW solution, stored in UW solution at 4 ^{o}C for 18

hours, and orthotopically transplanted into recipients. All surgeries were performed by the same surgeon.

2.2.3 Treprostinil Administration

Treprostinil (1 mg/ml) and placebo (sodium chloride, metacresol, sodium citrate, water for injection) were provided by United Therapeutics, Inc. (Durham, NC). Treprostinil (100 ng/kg/min) or placebo was administered to donor and recipient animals subcutaneously via an Alzet® osmotic pump (Durect Corp., Cupertino, CA). The surgeon was blinded to treatment.

2.2.4 Experimental Design

Donor animals received treprostinil or placebo 24 hours before hepatectomy and the corresponding recipient animal received the same treatment. Recipients were sacrificed at 1, 3, 6, 24, and 48 hours post-transplantation to examine the early events after I/R injury. In additional sets of experiments, a group of recipients were treated with treprostinil or placebo (same dose) for 24 hours before surgery and until the time of sacrifice. Only liver enzymes levels were measured in this additional group.

2.2.5 Post-operative Care

Recipients were kept under a heating lamp for approximately 2 hours and were given regular food and water ad libitum. The general condition of the rats was checked three times daily.

2.2.6 Liver Enzymes Levels

Blood was collected at 6, 24, and 48 hours post-transplantation. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured by standard enzymatic methods in the clinical laboratory at UPMC.

2.2.7 Histopathology

Liver graft tissues were fixed in 10% buffered formalin, embedded in paraffin, cut into 6 um sections, and stained with hematoxylin and eosin (H&E). The percentage of necrotic area was estimated by the morphometric analysis of five randomly selected low-power fields (40x) per H&E section. Neutrophils were stained with naphthol AS-D chloroacetate esterase-staining kit (Sigma Diagnostics, St. Louis, MO). Positively stained cells were counted in five high-power fields (200x) per section.

2.2.8 RNA Extraction and Real Time RT-PCR Analysis

Total RNA was extracted from liver tissue (50 – 100 mg) using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. RNA concentration was determined by UV absorbance at 260/280 nm (μQuant Microplate 25 Spectrophotometer) and RNA integrity was checked by 0.5% agarose gel electrophoresis stained with ethidium bromide. Two micrograms of total RNA from each sample was used to generate first-strand cDNA by use of the First Strand cDNA synthesis kit (Promega, Madison, WI). A reaction mixture containing 200 U monkey myeloblastosis virus reverse transcription reaction (MMLV, Promega, Madison,

WI)-Reverse transcriptase, 1 mM dNTPs and 25 U RNasein (Promega) was added to the previous mixture and incubated at 37 °C for 60 minutes. DNase-I treated total RNA from each sample was mixed with 0.5 μg of Random Hexamers (Promega) heated to 70 °C for 5 minutes then cooled to 4 °C. Hepatic mRNA levels were measured by SYBR® Green PCR Master mix using primers purchased from Super Array Biosciences (Frederick, MD), listed in Table 2. Samples were analyzed in triplicate and relative gene expression was measured using the comparative C_T method, using GAPDH as internal control.

Table 2: Real-Time PCR assay IDs for genes detected by SYBR® green gene expression assays

Gene Symbol	Gene Name	RefSeq Accession #
TNF-α	Tumor Necrosis Factor (TNF superfamily, member 2)	NM_000594.2
IL-1β	Interleukin 1, beta	NM_000576.2
IL-6	Interleukin 6	NM_000600.3
IL-10	Interleukin 10	NM_000572.2
IFN-γ	Interferon, gamma	NM_000619.2
Serpine1	Serpin peptidase inhibitor, clade E, member 1	NM_012620
Pecam1	Platelet endothelial cell adhesion molecule 1	NM_031591
ICAM-1	Intracellular cell adhesion molecule 1	NM_012967
VCAM-1	Vascular cell adhesion molecule 1	NM_012889
VEGF-α	Vascular endothelial growth factor A	NM_031836
P-Selectin	P-Selectin	NM_013114
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_017008.3

2.2.9 Electron Microscopy

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analysis were performed on 2.5% gluteraldehyde in PBS perfusion-fixed liver, as previously described [152]. After labeling, tissue was dehydrated through graded-ethanol (30-100%), critical point dried (Emscope, CPD 750, Ashford, Kent, UK), and overcoated with carbon (108Carbon/A Coater, Watford, UK). Tissues were visualized on a JEM-6335F SEM and a JEM 1210 TEM (JEOL, Peabody, MA).

2.2.10 Hepatic Tissue Blood Flow

A Laser-Doppler flow meter probe (ALF21N; Advance, Tokyo, Japan) was placed on the surface of the medial, left, and right hepatic lobe to measure hepatic-tissue blood flow in liver graft before and after transplantation. Measurements were repeated five times and recorded by the surgeon without knowledge of the treatment groups.

2.2.11 Protein Estimation

The protein concentration was determined according to the procedure of Bradford [153], using bovine serum albumin (BSA) as a standard. The concentration was calculated based on the standard curve of known bovine serum albumin (BSA) concentration (0.025 – 0.4 mg/ml).

2.2.12 Hepatic Levels of Cyclic AMP

Cyclic adenosine monophosphate (cAMP) levels were measured using an enzyme-linked immunosorbent assay (ELISA; R & D Systems, Frederick, MD) according to the manufacturer's instructions. Liver tissue was homogenized and assayed in triplicate. The optical density was calculated against a standard curve to determine the concentration of cAMP.

2.2.13 Hepatic Levels of Adenine Nucleotides

Liver samples were immediately frozen in liquid nitrogen and stored at –80 ^{o}C until the extraction procedure. The frozen tissue was weighed (approximately 0.1 gm) and homogenized with a Polytron homogenizer (Brinkmann Inc., Westbury, NY) in 1.0 mL of ice-cold 6 % perchloric acid containing 0.77 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged for 10 minutes at (4 ^{o}C) 10,000*g* (Beckham J25.15 Rotor) and the pH of supernatant was adjusted to 5-7 with 69% K_2CO3 solution. Following centrifugation (15 minutes at 10,000*g*, 4 ^{o}C), the concentration of adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP), hypoxanthine, xanthine, inosine, and adenosine were measured by high performance liquid chromatography (HPLC) with a Waters HPLC 2695 Alliance, Photodiode Array Detector, monitored at 254 nm (Waters, Inc., Milford, MA). Reverse-phase column (E. Merk, Darmstadt, Germany; LiChrospher® 100 RP-18 (5 um), 4 x 250 mm) was used with a precolumn (Waters; RCSS Guard-PAK) at 27^{o}C. The mobile phase consisted of A, 0.15 M ammonium dihydrogen phosphate buffer, pH 5-7 and B, acetonitrile and methanol (50:50) containing 1% triethanolamine. The concentration of ATP, ADP, and AMP, hypoxanthine, xanthine, inosine, and adenosine were calculated from a standard

curve constructed at the same time by means of standard powder (>99% pure) of ATP, ADP, and AMP, hypoxanthine, xanthine, inosine, and adenosine dissolved in the appropriate solution for each experiment. Total adenine nucleotides (TAN) = ATP + ADP + AMP.

2.2.14 Treprostinil Plasma Concentration

The plasma concentration of treprostinil was measured by an ultra performance liquid chromatographic system equipped with a triple quadrupole tandem mass spectrometer (AB/MDS Sciex API-5000) detector operated in negative TurboIonSpray® mode. Treprostinil-d4 was used as the internal standard. Separation of treprostinil from extracted matrix materials was performed using a Waters BEH C18 (2.1 x 100 mm, 1.7 μm) column (Waters, Milford, MA) operated at 65 °C. The gradient mobile phase system consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile at a flow rate of 0.775 mL/min. The C.V. was less than 2 % for this assay.

2.2.15 Statistical Analysis

Data are represented as the mean ± standard error of mean (SEM). Comparisons between the groups were performed using Student's *t* test or one-way ANOVA with Tukey's post-hoc test using Prism software version 4.0 (GraphPad, San Diego, CA). Significance was defined as *P*-value < 0.05.

2.3 RESULTS

2.3.1 Clinical Course and Assessment of Tolerability

Following vascular anastomoses, there was no excessive bleeding in the treprostinil-treated group, relative to placebo. Bile formation was immediate upon reperfusion of the liver graft in the treprostinil-treated group. Treprostinil-treated animals functioned normally and appeared to recover sooner after surgery than the placebo-treated animals, which appeared weaker throughout the post-OLT period. No difference in body weight was noted between the two treatment groups.

2.3.2 Hepatic I/R Injury

In the placebo-treated group, serum ALT and AST levels reached a peak of 2810 ± 202 and 4445 ± 951 IU/L, respectively, at 24 hours post-transplantation (Figure 7A and 7B). Donor plus recipient treatment with treprostinil significantly reduced serum ALT and AST levels to 807 ± 140 and 1231 ± 112 IU/L, respectively. In the recipient only placebo-treated group, serum ALT and AST levels reached a peak of 2519 ± 239 and 5822 ± 222 IU/L, respectively at 24 hours post-reperfusion. Liver injury was also reduced in the recipient only treprostinil-treatment group, albeit to a lesser extent then donor plus recipient treatment, reaching values of 1367 ± 306 and 1469 ± 296 IU/L, respectively, shown in Figure 8A and 8B.

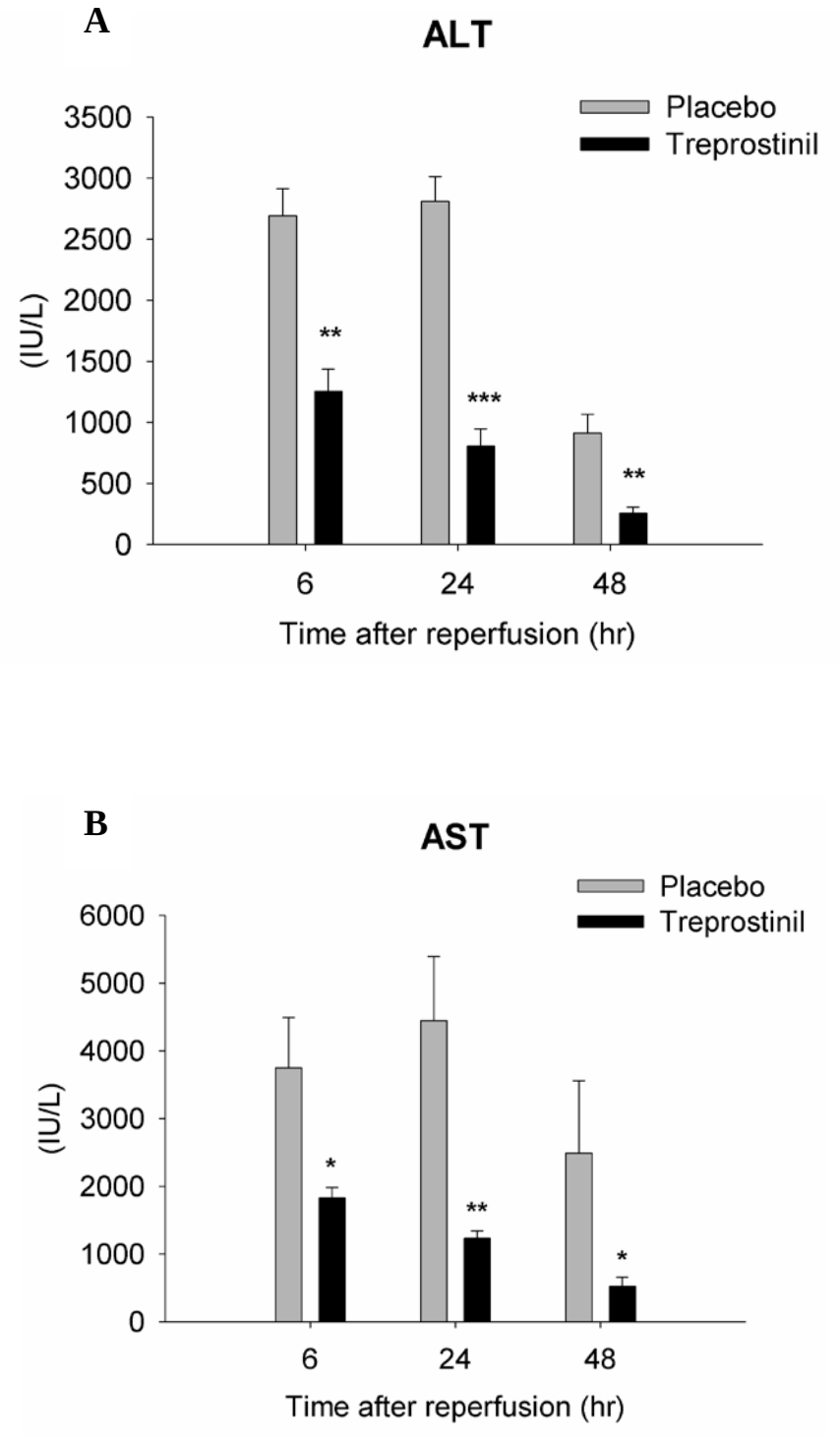


Figure 7: Hepatic injury in donor + recipient groups

Serum ALT (A) and AST (B) levels at 6, 24, and 48 hrs post-reperfusion. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. placebo (n=3-4/group).

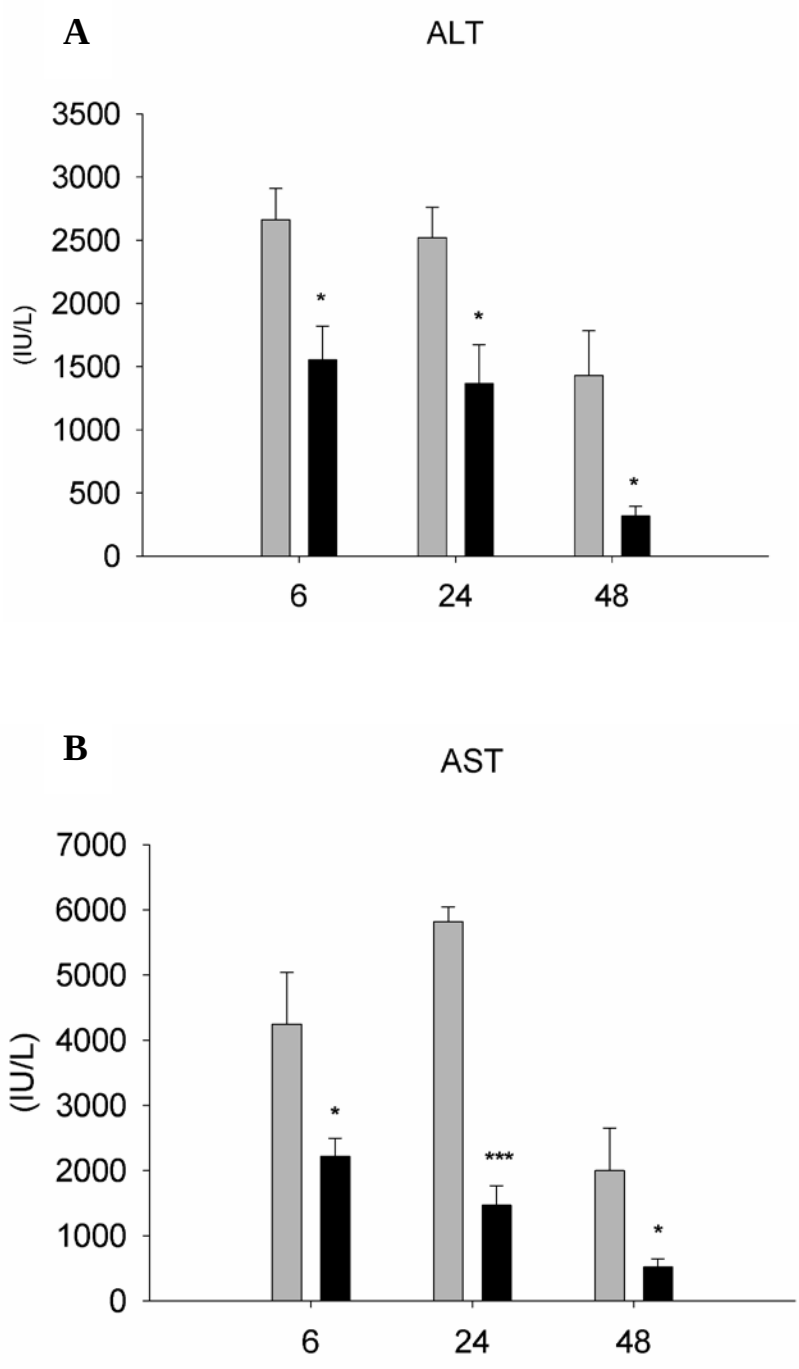


Figure 8: Hepatic injury in recipient only groups

Serum (A) ALT and (B) AST levels at 6, 24, and 48 hrs after reperfusion. *$P < 0.05$, ***$P < 0.001$ vs. placebo (n=3-4/group).

At 48 hours post-transplantation, the necrotic area in the treprostinil-treated group (0.8 ± 0.03%) was significantly reduced, compared to placebo (41.7 ± 10.0%), shown in Figure 9. Placebo-treated grafts showed massive necrosis and severe congestion (Figure 10A), which was attenuated by treprostinil administration, shown in Figure 10B. These results indicate that treprostinil-treated rats experienced a relatively low degree of hepatic injury, compared to placebo-treated rats.

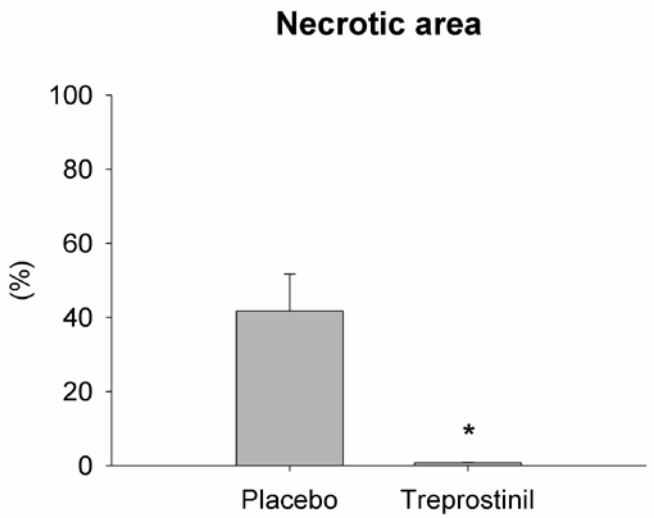


Figure 9: Percentage of necrotic area in liver grafts

Comparison of necrotic area (%) in placebo- and treprostinil-treated animals at 48 hours post-transplantation. **P*<0.05 vs. placebo (n=3-5/group).

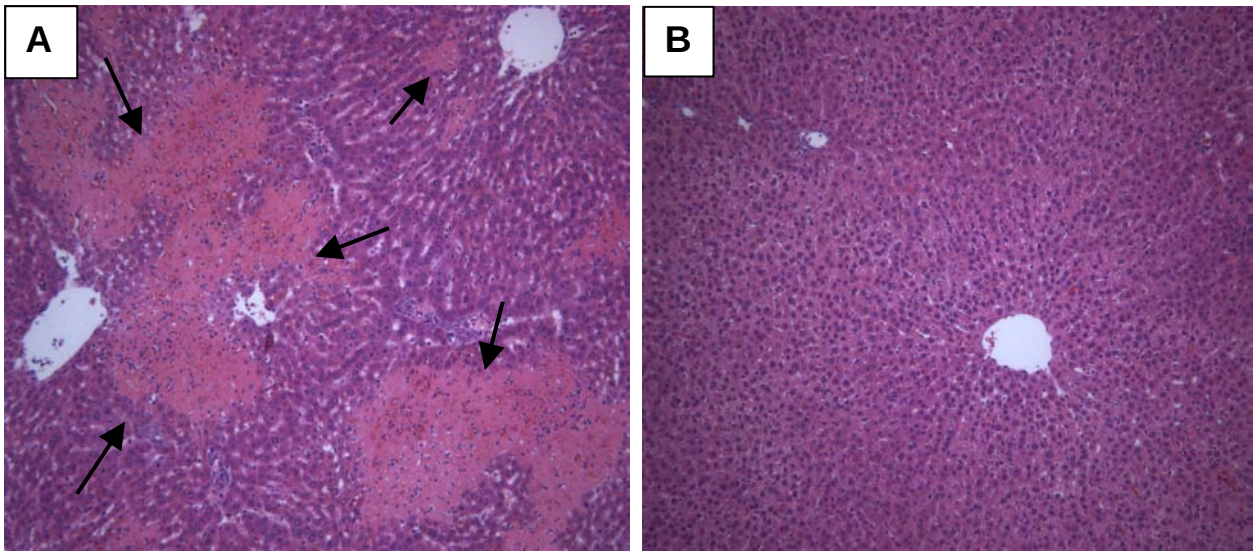


Figure 10: Representative histopathological images of liver grafts
Necrotic area (arrows) in (A) placebo- and (B) treprostinil-treated animals at 48 hours post-reperfusion. H&E-stained, original x40.

2.3.3 Neutrophil Accumulation in Hepatic Tissue

Neutrophil extravasation and accumulation contribute to the progression of I/R injury in liver [154]. The number of infiltrating neutrophils into the hepatic sinusoids rapidly increased in the liver grafts of placebo-treated animals, shown in Figure 11. In contrast, treprostinil administration reduced the number of neutrophils at 1, 3, and 48 hours after reperfusion, compared to placebo. At 48 hours post-transplantation, neutrophils homogenously infiltrated the hepatic sinusoids in placebo-treated animals (Figure 12A), whereas treatment with treprostinil significantly reduced neutrophil infiltration, shown in Figure 12B.

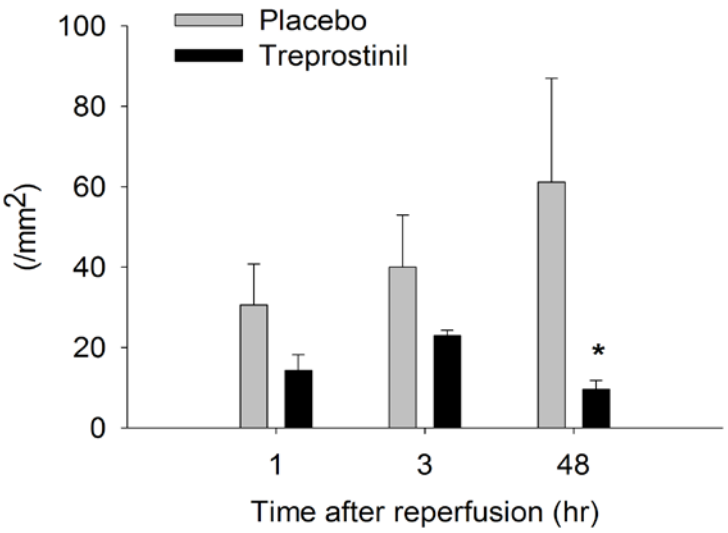


Figure 11: Neutrophils in liver graft
Comparison of neutrophil infiltration in placebo- and treprostinil-treated group at 1, 3, and 48 hrs post-reperfusion. Values are expressed as the number of cells per field of 1 mm^2. *$P < 0.05$ vs. placebo (n=3-4/group).

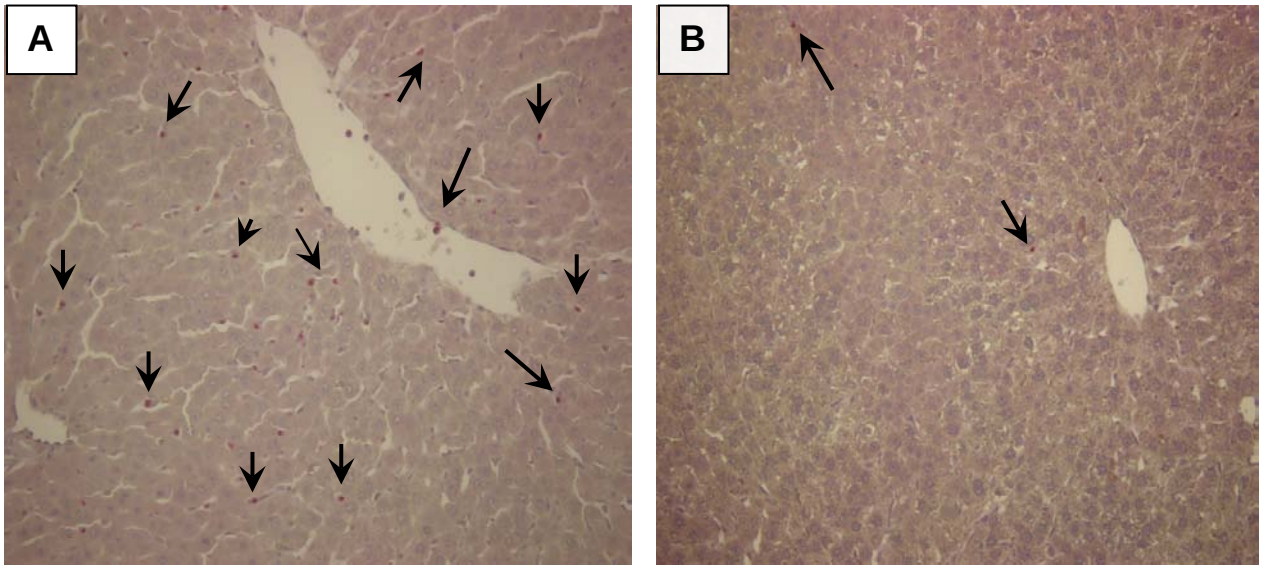
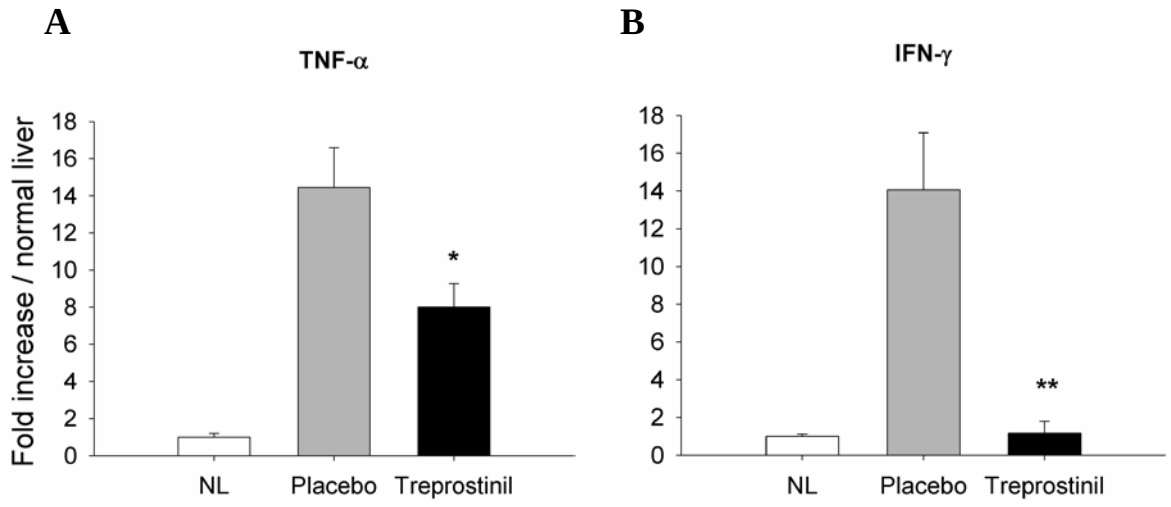


Figure 12: Neutrophil infiltration
Neutrophil infiltration (arrows) in (A) placebo- and (B) treprostinil-treated animals; original x200.

2.3.4 Cytokine Response

The mRNA levels of pro-inflammatory cytokines peaked at one hour post-transplantation. Specifically, the mRNA expression of TNF-α, and IFN- γ were significantly increased in placebo group, which were suppressed by treprostinil administration (Figures 13A and 13B, respectively). Tissue mRNA levels of IL-6 (Figure 13C) were lower also in the treprostinil-treated group vs. placebo. The increase in IL-1β mRNA expression in placebo- and treprostinil-treated group was minimal (Figure 13D). This effect may be attributed to the circulation half-life of IL-1 of approximately 6 minutes [54], making its detection less likely than other cytokines. IL-10 has been shown to exert anti-inflammatory effects by inhibiting the activity of IkB kinase complex to prevent NF-kB translocation [155]. Treprostinil increased IL-10 mRNA levels, compared to placebo (Figure 13E). Together, a reduction of pro-inflammatory cytokines and an increase in IL-10 expression is likely to have significantly contributed to the protective effect of treprostinil.



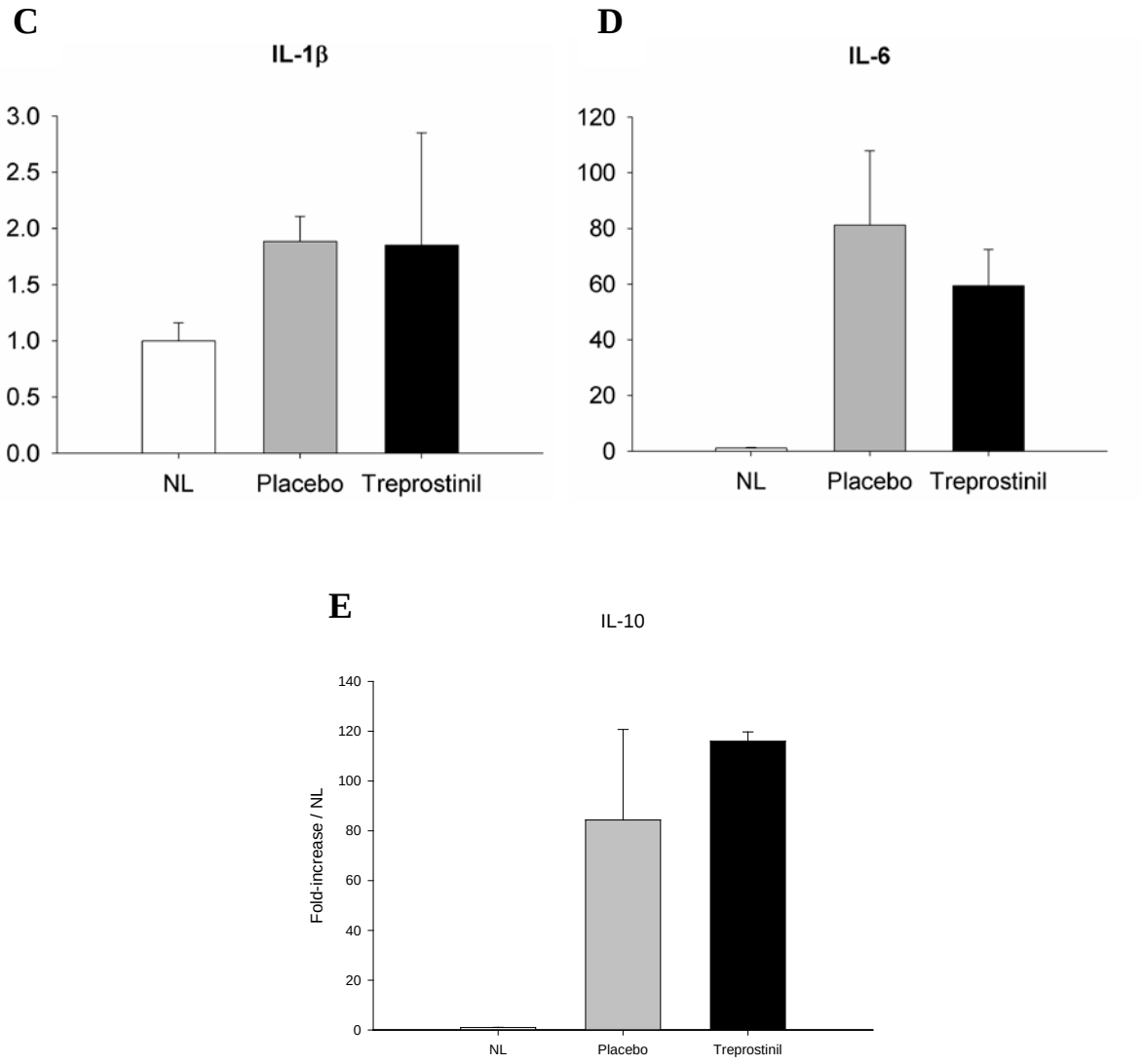
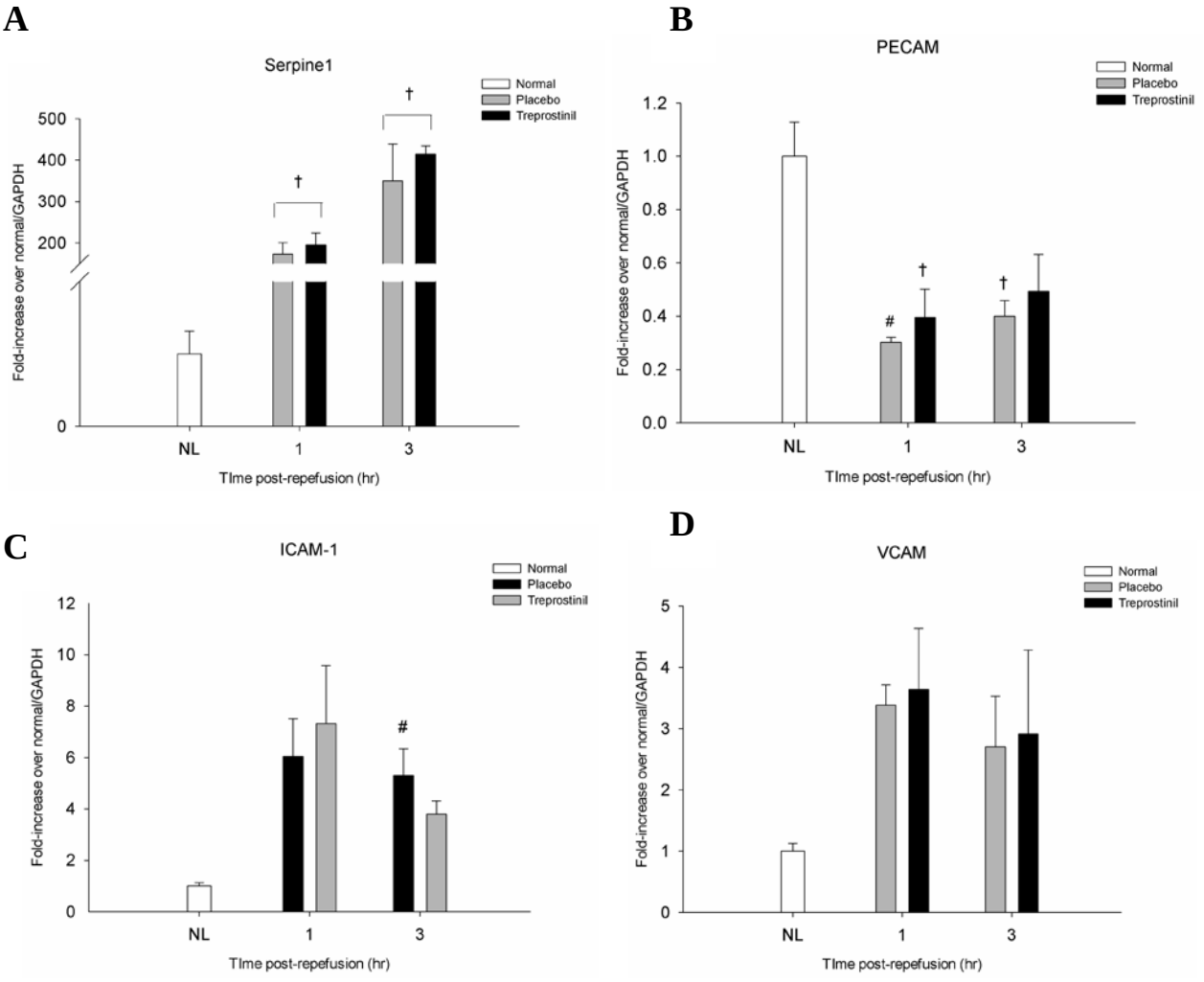


Figure 13: Peak mRNA expression of cytokines
(A) TNF-α, (B) IFN-γ, C) IL-1β, and D) IL-6 in liver graft at 1 hr; (E) IL-10 in liver graft at 3 hrs post-OLT. *P < 0.05, **P < 0.01 vs. placebo (n = 3).

Adhesion molecules and selectins promote leukocyte adhesion and migration, and thrombosis, which contribute to the progression of hepatic I/R injury [156]. To determine if treprostinil reduced cellular infiltration via blockade of these molecules, the mRNA expression of Serpine1, Pecam, ICAM-1, VCAM-1, P-selectin, and VEGF-α were examined at 1 and 3 hr post-OLT, shown in Figures 14A-F. No remarkable differences in serpine1, PECAM, VCAM-1, VEGF-α, or P-Selectin expression between the treprostinil and placebo-treated group were observed,

which indicate that treprostinil protected the liver graft against I/R injury independent of these pathways. Notable findings in the treprostinil-treated group are reduced ICAM-1 mRNA levels at 3 hr post-OLT compared to placebo-treated group, with no significant difference between normal liver, shown in Figure 14C. ICAM-1 is known to play an important role in the adhesion and infiltration of leukocytes in the vascular lining and parenchyma [157]. The results suggest that reduced ICAM-1 levels in the treprostinil-treated group participated in the reduction of hepatic injury in liver grafts.



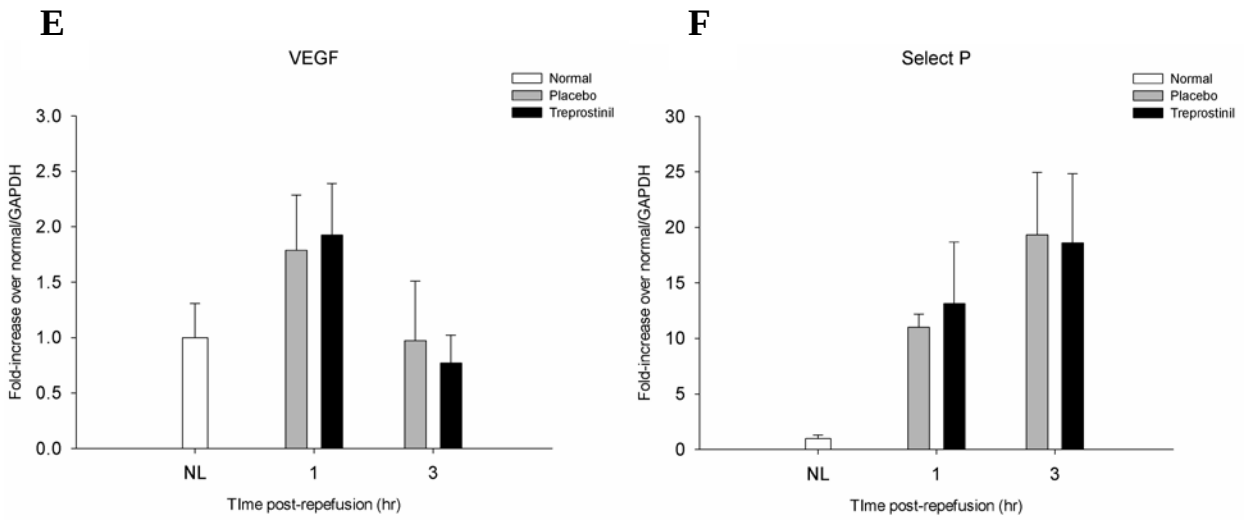


Figure 14: Hepatic mRNA expression of adhesion molecules
A) Serpine1, B) Pecam, C) ICAM-1, D) VCAM-1, E) VEGF, and F) P-selectin in liver graft at 1 and 3 hr post-OLT. #$P < 0.05$, †$P < 0.01$ vs. normal liver (n = 3).

2.3.5 Liver Sinusoidal Endothelial Cells

Having previously shown that the 18-hour cold ischemic storage induces significant SEC damage with many retracted cells [41], we conducted SEM analysis to investigate SEC ultra structural changes during the restorative period after OLT. Figure 15A shows normal liver with intact SECs and typical fenestrations (arrows). In contrast, at 1 hour post-reperfusion, placebo-treated grafts showed a significant retraction of SECs (Figure 15B). Furthermore, at 3-6 hours post-OLT, large areas of destroyed structural SEC lining with platelet infiltration within the sinusoidal surface was visible. Stolz et al. [41] have shown that SECs recover from I/R-induced SEC denudation in approximately 24 -48 hours post-reperfusion. Interestingly, as early as 1-3 hours post-reperfusion, treprostinil-treated group showed preserved SECs (Figure 15C). More impressive is that by 6 hours post-reperfusion, the treprostinil-treated group had detectable segments of intact liver SECs with show typical fenestration, similar to that of control.

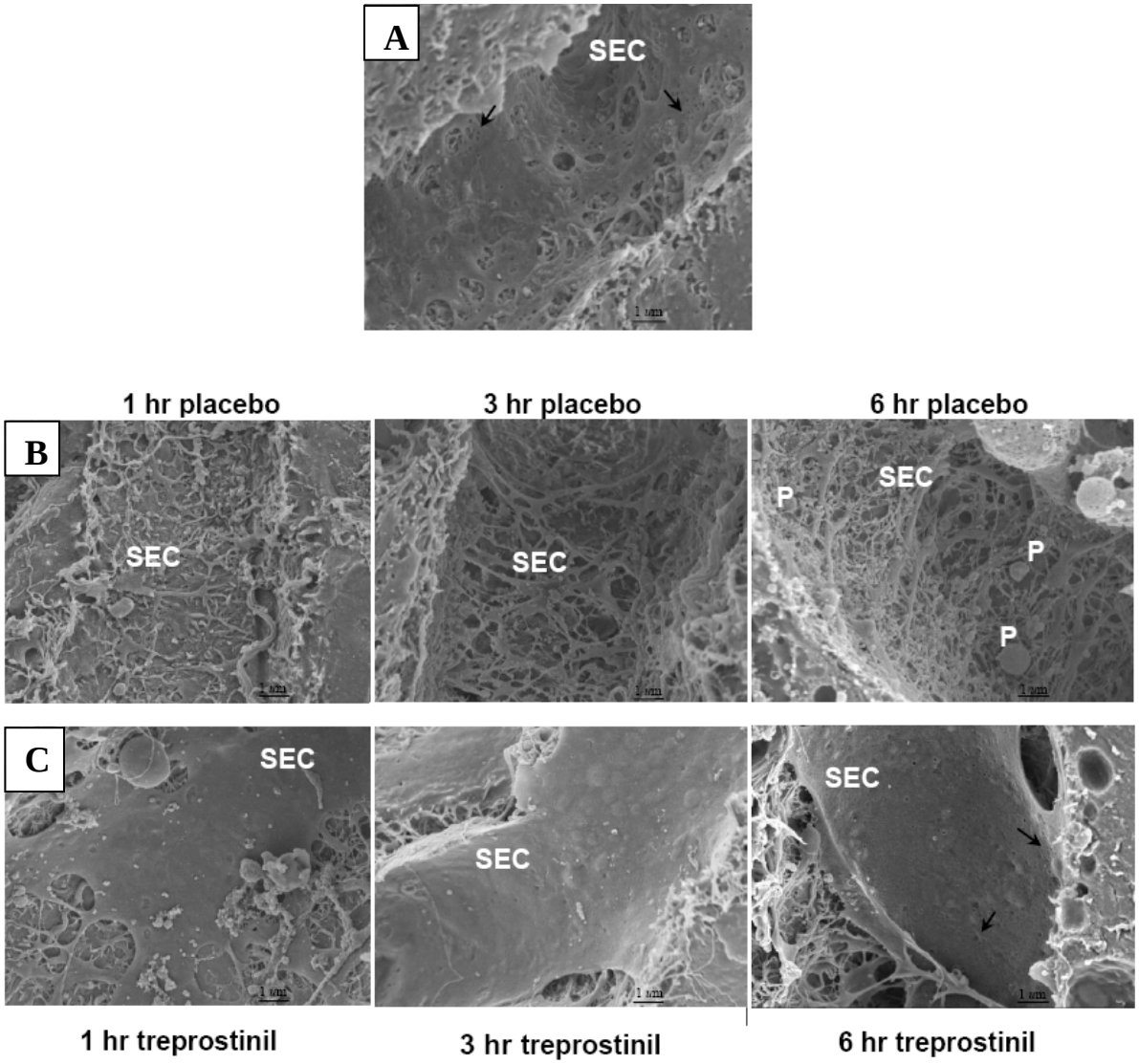


Figure 15: Rat liver SEC analysis by SEM

(A) Normal liver showing typical fenestration (arrows), (B) placebo- and (C) treprostinil-treated animals at 1, 3, and 6 hours post-OLT. Data are representative of 3 separate animals. SEC, sinusoidal endothelial cell; P, platelet aggregation.

Additional morphological and intracellular detail was acquired using TEM analysis of liver graft early post-transplantation. Normal liver with typical fenestration and close association with hepatocyte microvilli and space of Disse, shown in Figure 16A. In placebo-treated animals, at 1 hour post-reperfusion (Figure 16B), the sinusoidal surface of the placebo group was completely devoid of SECs, the space of Disse was greatly reduced, while macrophages and red blood cells infiltrated the sinusoidal space. At 3-6 hours post-reperfusion, the placebo group developed sinusoidal congestion, endothelial cell detachment, increased platelet deposition, and hepatocytes showed vacuolization. Alternatively, treprostinil administration significantly alleviated these structural abnormalities. Figure 16C shows that as early as 1 hour post-transplantation, liver graft from the treprostinil-treated group had preserved SEC fenestration and sinusoidal congestion was minimized. Further, at 3 hours post-OLT, the restored proximity of SECs to hepatocyte microvilli and the space of Disse were visible, and a lack of platelet aggregation was noted. By 6 hours post-reperfusion, the structure of SECs resembled that of a normal liver sinusoid. These findings indicate that treprostinil preserved liver graft SEC structure and inhibited platelet, red blood cell, and macrophage infiltration into the sinusoid, thereby avoided hindrance of blood flow through the liver microvasculature in the early post-OLT period. These findings indicate that treatment with treprostinil restored SEC structure similar to that of a normal rat as early as 6 hours post-OLT.

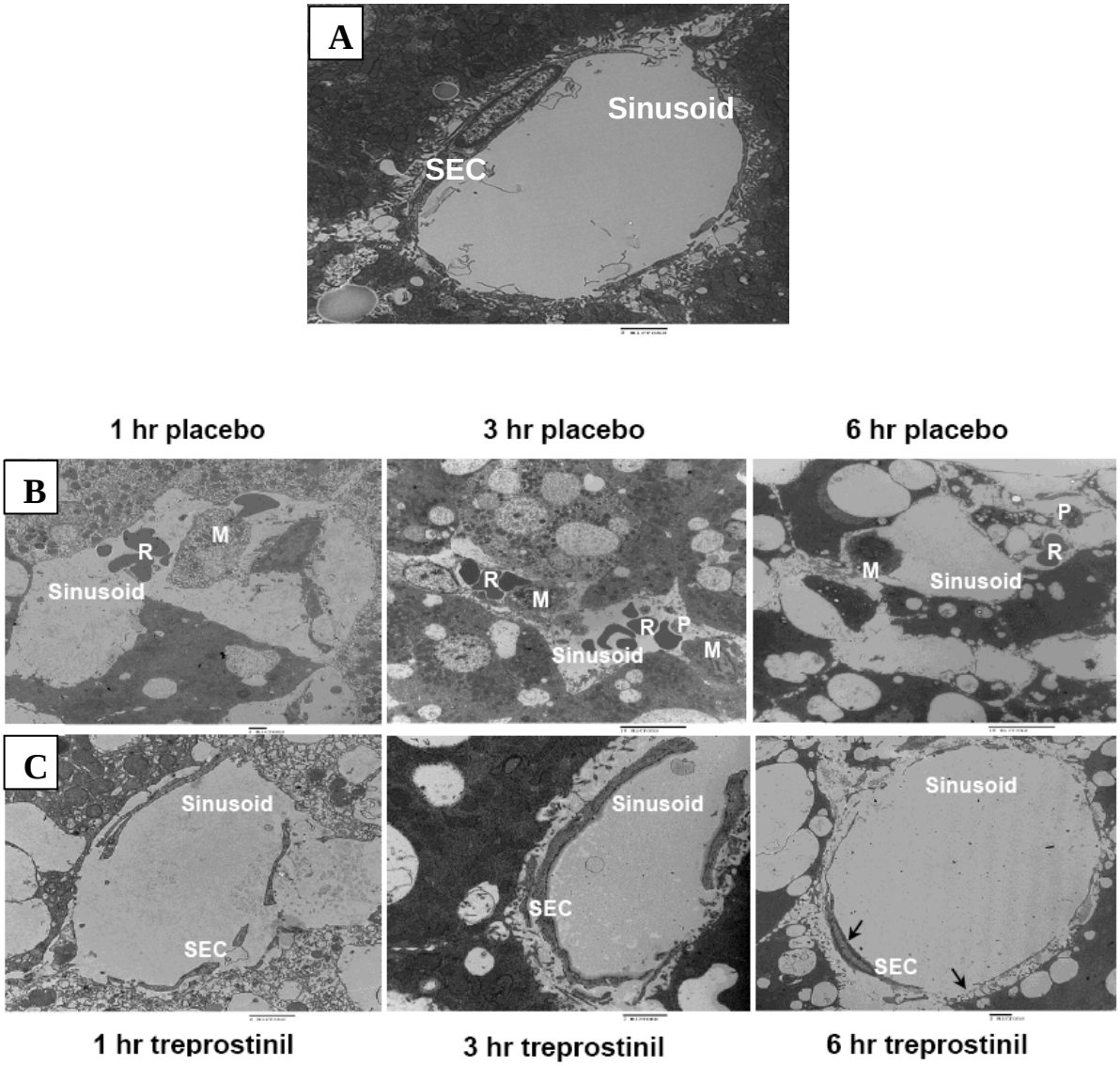


Figure 16: Rat Liver SEC analysis by TEM

(A) normal, (B) placebo- and (C) treprostinil-treated liver grafts at 1, 3, and 6 hours post-OLT**.** Data are representative of 3 separate animals. SEC, sinusoidal endothelial cell; M, macrophage; P, platelet aggregation; R, red blood cells.

2.3.6 Hepatic Tissue Blood Flow

Hepatic tissue blood flow decreased to 56% of the pre-OLT levels and further decreased to 51% of the pre-ischemic levels at 3 hours post-reperfusion, in the placebo-treated group, shown in Figure 17. Administration of treprostinil significantly increased pre-OLT hepatic tissue blood flow in donor graft to 150% of control. At time zero, immediately post-reperfusion, hepatic tissue blood flow only dropped to ~80% of control in treprostinil-treated grafts. Continuous treatment with treprostinil maintained approximately 70% of control blood flow at 3 hours post-transplantation. These results suggest that treprostinil preserved microcirculation through an increase in liver graft blood flow post-reperfusion.

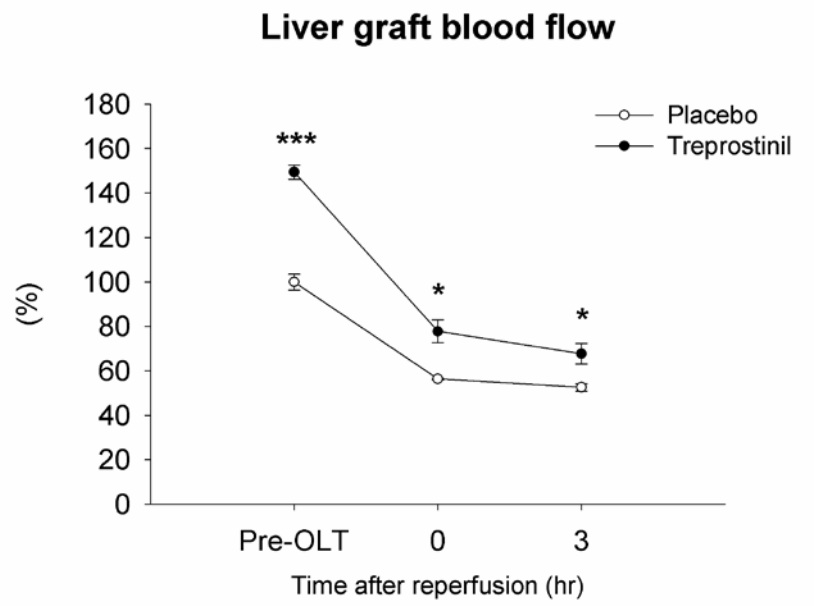


Figure 17: Hepatic tissue blood flow in placebo- and treprostinil-treated animals
Blood flow measured by Laser Dopper Flow-Meter immediately before donor graft harvest (Pre-OLT), immediately after reperfusion (time zero), and at 3 hours post-transplantation. Results are expressed as a percentage of the pre-OLT level in control group. *$P < 0.05$, ***$P < 0.001$ vs. placebo (n = 3-6).

2.3.7 Tissue Concentration of Cyclic Adenosine Monophosphate

To confirm that treprostinil acts as an agonist at cell surface prostanoid receptors (IP), cAMP levels were measured in hepatic tissue (Figure 18). There was no difference in cAMP levels in the placebo-treated group compared to normal liver at all time points measured. Alternatively, in the treprostinil-treated group, cAMP levels significantly increased to 353.2 ± 9.7 and 363.1 ± 23.9 pmol/gm at 1 and 3 hours post-OLT, respectively, compared to normal (178.4 ± 36.7 pmol/gm). By 48 hours post-transplantation, the amount of cAMP increased to 438.2 ± 11.8 pmol/gm in the treprostinil-treated group, compared to 166.4 ± 66.7 pmol/gm in the placebo-treated group. These results confirmed that treprostinil is a potent stimulator of the IP receptor.

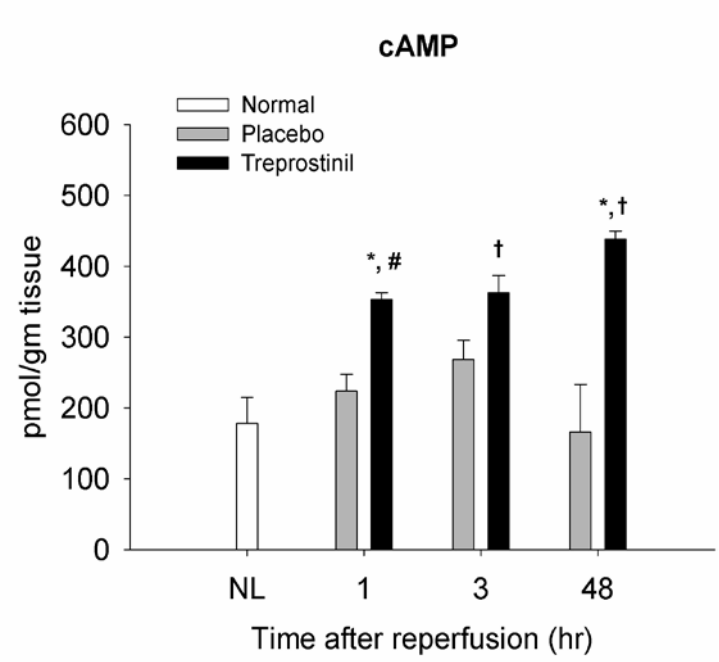


Figure 18: Hepatic tissue levels of cAMP
Measured by ELISA. *$P < 0.05$ vs. placebo; #$P < 0.05$, †$P < 0.01$ vs. normal liver (n = 3-6).

2.3.8 Tissue Concentration of Adenosine Nucleotides

The concentration of ATP soon after the onset of reperfusion of ischemic liver has been reported to be a good predictor of hepatic function [158]. Therefore, we examined whether an improvement of hepatic function after reperfusion by treprostinil was a result of a preserved energy metabolism. Figures 19A-D shows the changes in hepatic tissue levels of adenine nucleotides after reperfusion. The normal value of ATP in hepatic tissue was 6.8 ± 0.3 nmol/mg, and is shown in Figure 19A. In the placebo-treated group, the post-ischemic tissue content of ATP was significantly reduced early post-OLT, an effect which lasted at all time points thereafter, until sacrifice at 48 hr post-OLT. Cold storage caused an 80% reduction in ATP at 1 hour post-reperfusion and levels only recovered to 30% (2.1 ± 0.3 nmol/mg) of normal, which resulted in a less than 50% recovery of total adenine nucleotides (10.7 ± 2.3 nmol/mg) at 48 hours post-transplantation, compared to normal (22.5 ± 1.5 nmol/mg), shown in Figure 19D. While the re-synthesis of ATP remained suppressed in the placebo group, ATP levels significantly increased in the treprostinil-treated group, reaching nearly 80% (5.4 ± 1.6 nmol/mg) of normal by 48 hours post-reperfusion. Furthermore, at 1 hour post-reperfusion, total adenine nucleotide levels in the treprostinil-treated group had fully recovered to 100% of normal values (26.5 ± 6.2 vs. 22.5 ± 1.5 nmol/mg, respectively). These results suggest that treprostinil restored energy metabolism in the liver graft early post-OLT.

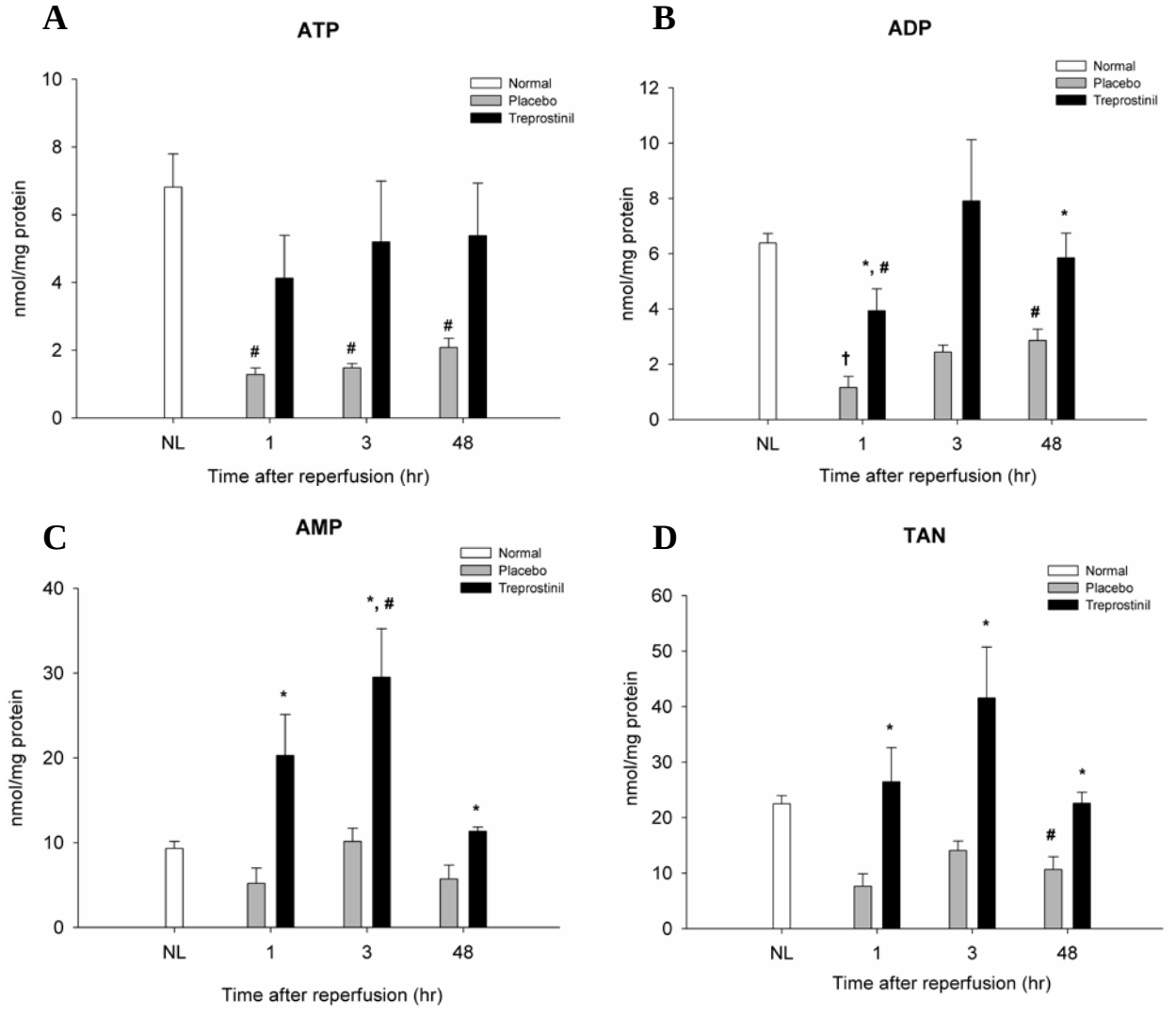


Figure 19: Hepatic tissue levels of adenine nucleotides
(A) ATP, (B) ADP, (C) AMP, and (D) TAN at 1, 3, and 48 hrs post-OLT. *P < 0.05 vs. placebo; #P < 0.05, †P < 0.01 vs. normal liver, (n = 3). TAN, total adenine nucleotide

Accumulation of hypoxanthine is converted to xanthine by xanthine oxidase, which is accompanied by free radical production and capable of cell injury [159]. The increase in hepatic tissue levels of hypoxanthine (Figure 20A) and corresponding rise in xanthine (Figure 20B) in both the placebo- and treprostinil-treated groups suggested that treprostinil protected hepatic grafts from I/R injury independent of the xanthine oxidase pathway. Alternatively, the preserved

hepatic tissue levels of adenosine (Figure 20C) and increased levels of inosine (Figure 20D) in the treprostinil-treated groups is likely to have contributed to the resynthesis of ATP, which is dependent on available total adenine nucleotides as salvageable precursors for AMP synthesis after cold storage [32].

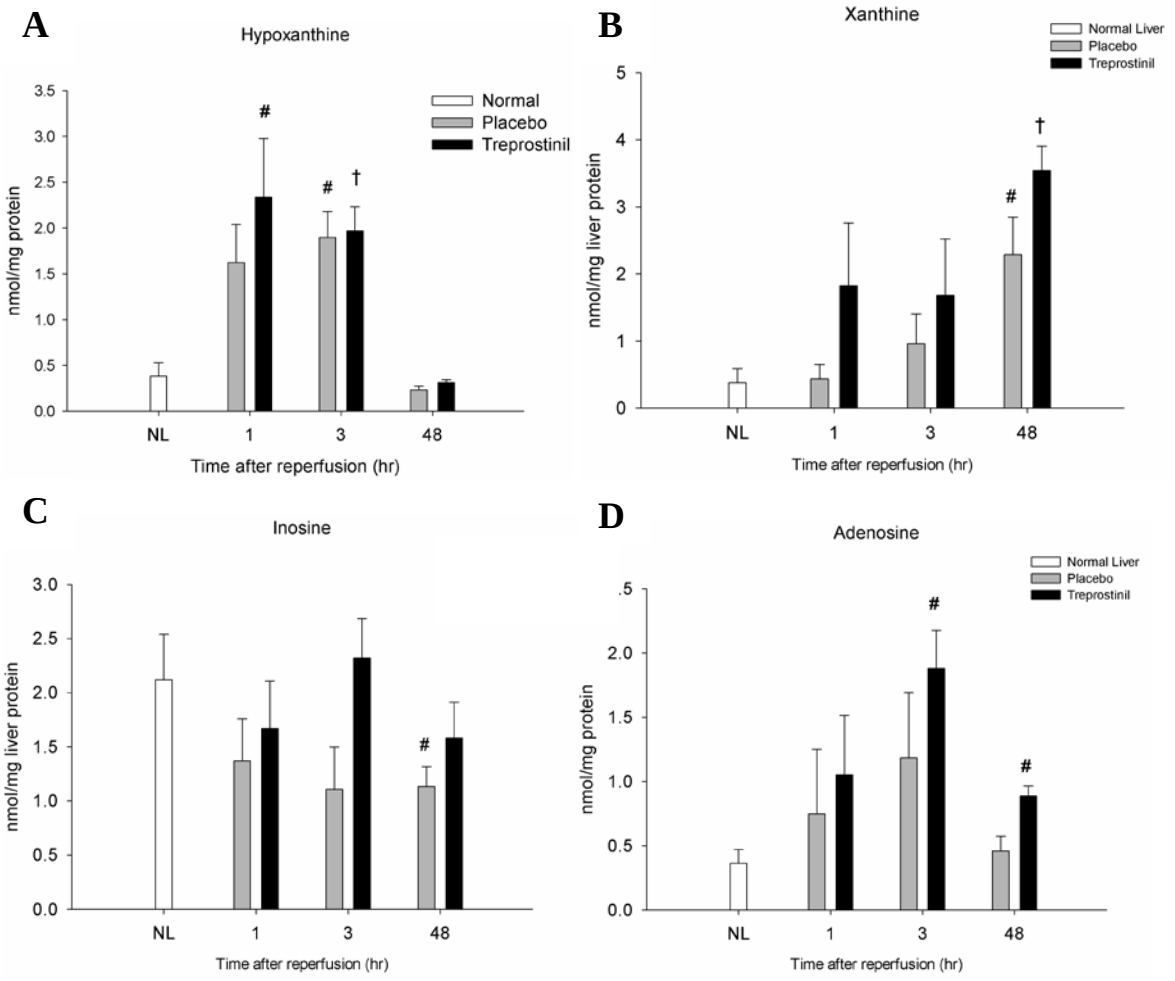


Figure 20: Hepatic tissue levels of purines
(A) hypoxanthine, (B) xanthine, (C) inosine, and (D) adenosine in liver grafts at 1, 3, and 48 hrs post-OLT. #$P < 0.05$, †$P < 0.01$ vs. normal liver (n=3)

2.3.9 Treprostinil Plasma Concentration

Based on therapeutic responses of treprostinil and corresponding plasma concentrations, a plasma concentration between 5-10 ng/ml was targeted for this study (Figure 21). To achieve this concentration, the dose of treprostinil (100 ng/kg/min) was selected, which is within the range of tolerated doses in previous animal studies (Personal communication - Mike Wade, PhD, United Therapeutics, Inc.). To reach steady-state plasma concentration at the time of hepatectomy and transplantation, it was necessary to begin treprostinil administration approximately 18 hrs prior to surgery. Plasma concentrations in the placebo-treated group and normal rat plasma were below the limit of quantification (< 0.025 ng/mL).

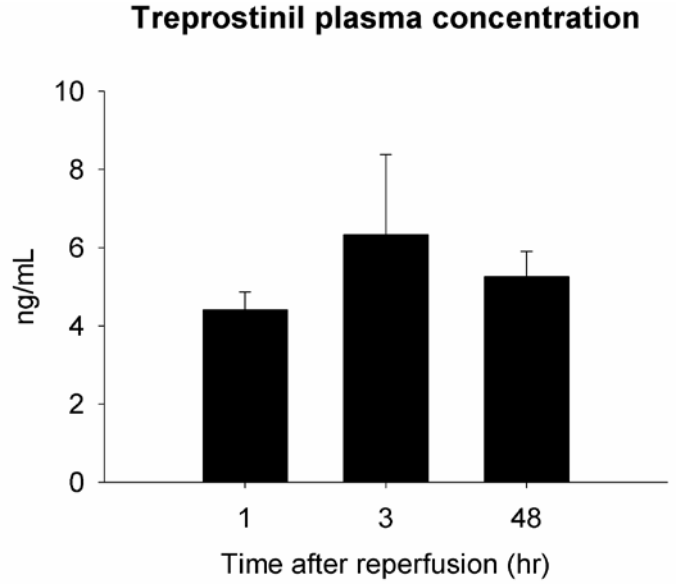


Figure 21: Treprostinil plasma concentration
Plasma samples from treprostinil-treated animals at 1, 3, and 48 hours post-OLT (n = 3).

2.4 DISCUSSION

Previous clinical studies using intravenous PGE_1 were promising in minimizing ischemic injury, but the sample size was too small [10]. In the early 1990s, Takaya and colleagues [147, 149] demonstrated that PGE_1 protected the liver graft from antibody-mediated rejection in crossmatch positive recipients comparable to negative cross matches and it also significantly improved kidney function in liver recipients. Later, in 1995, Henley et al. [141] initiated PGE_1 treatment during the anhepatic phase in patients undergoing OLT, but there was no significant effect on the primary end-points of patient or graft survival. However, the study showed significantly shorter intensive care unit stays and hospitalizations post-transplantation, reduced needs for renal support and less need for surgical intervention other than re-transplantation in the treatment group. The same year, Takaya et al. [148] reported successful treatment of adult liver recipients for PNF with postoperative PGE_1 therapy. Despite improved 1-year graft survival and a lower incidence of PNF in the PGE_1 group vs. historical control, some patients did not tolerate PGE_1. Subsequently, Neumann et al. [144] showed that epoprostenol (PGI_2) improved early microvascular blood flow and that it could be safely administered to adult patients post-operatively; however the primary endpoint of this study was not met.

Prostaglandins (PGE_1 and PGI_2) play a critical role in maintaining vascular homeostasis of microcirculation, which contributes to its wide range of protective effects against I/R-induced liver injury [2, 17, 138]. Stable PGI_2 analogues have been shown to produce vasodilation of pulmonary and systemic arterial vascular beds and inhibition of platelet aggregation [127]. In addition, PGI_2 analogues have been reported to maintain blood flow, inhibit local vascular thrombosis, and decrease leukocyte activation by inhibiting TNF-α production and other pro-

inflammatory cytokines, neutrophil activation and adhesion to the vascular endothelium, and counteract the activity of vasoconstrictors and platelet aggregation [17, 139].

In this study we evaluated the effect of treprostinil, a commercially available PGI_2 analogue, for protection of liver grafts against I/R injury during OLT. Advantages of treprostinil over other PGE_1 and PGI_2 analogues include its increased stability, as well as longer elimination half-life, and increased potency (three- and six-fold, respectively) [127]. The current study provided evidence for the multiple factors involved in I/R injury and for the protective effect of treprostinil, which included morphological evidence of a preserved SEC structure, preserved energy stores, in addition to the class-wide effects of PGs. Treprostinil has the potential to minimize I/R injury during clinical OLT, and ultimately increase the number of suitable grafts available for transplantation and improve overall patient outcomes.

To a greater or lesser extent, the surgical procedure of human liver transplantation exposes the liver graft to three different types of ischemia, warm ischemia- before organ procurement; cold ischemia- during graft preservation; and rewarming ischemia- during graft implantation [8]. Most experimental models in the rodent employ a technique of clamping the hepatic artery and or the portal vein to induce hepatic I/R injury. While this procedure occludes hepatic blood flow and induces ischemic damage to the liver, it does not reflect the ischemic injury which occurs during graft preservation, when liver grafts are stored in cold UW preservation solution, before implantation into the recipient, i.e. in clinical OLT. The primary targets of cold I/R injury are the liver SECs, whereas hepatocytes are the main targets in warm ischemia [58]. Damage to SECs leads to loss of microvascular integrity, decreased blood flow, and an accumulation of neutrophils in the liver allograft. Further platelet aggregation, local

tissue destruction, up-regulation of inflammatory cytokines, and structural alterations in tissue leads to hepatocellular dysfunction [9, 29]. Several mechanisms of ischemic injury share a common pathway, however, there are important differences between the warm and cold ischemia model, most notably being the targets of cellular injury, which require the OLT model to fully characterize I/R injury during clinical OLT.

SECs have a crucial role in the overall homeostasis with the microvasculature, accounting for approximately 70% of the cell population within the liver sinusoid [40] and destruction of these cells during I/R injury significantly augments liver graft injury post-transplantation [29]. Ischemic injury to the endothelium disrupts the delicate homeostasis in the microcirculation [29], leading to prominent intra-sinusoidal coagulation, which promotes neutrophil activation and adhesion, platelet aggregation, resulting in a reduced hepatic blood flow and impeding hepatic microcirculation. Treprostinil-treated animals exhibited an almost completely preserved SEC lining, complete with fenestration resembling that of normal, as early as six hours post-reperfusion, indicating the effects of treprostinil on leukocyte adherence is part of its favorable interactions between leukocytes and endothelial cells. A result of this interaction is the prevention of damage to SECs, which includes attenuation of swelling of sinusoidal lining cells and hepatocytes to avoid hindrance of blood flow through the liver microvasculature during reperfusion. Improved preservation of the endothelial cell lining, along with a reduced release of pro-inflammatory cytokines, will avoid accumulation and activation of granulocytes, thereby limiting local concentration of deleterious cytotoxic oxygen free radicals. Increasing blood flow to the liver during reperfusion and inhibition of platelet aggregation and pro-inflammatory cytokines are essential for a good post-operative prognosis, and treprostinil appears to have accomplished these goals.

The initial ischemic injury is a result of tissue deprivation of oxygen, which disturbs the intracellular energy metabolism and enzyme function, resulting in a depletion of adenine nucleotides leading to cellular edema [160]. Secondary results of the oxygen disturbance and progressive degradation of energy metabolism are reflected by the destruction of tissue and cellular structures [29]. Cyclic AMP, an important intracellular second messenger in many cell types, is reported to produce an efflux of ischemia-induced accumulated intracellular calcium, which prevents ATP depletion, stabilizes the hepatocellular membrane, and preserves intracellular adenine nucleotides [161]. The reduction of intracellular calcium results in vasodilation [161]. Addition of the membrane permeable cAMP analogue, dibutyryl-cAMP, to preservation solution resulted in significantly enhanced metabolic activity and secretion function demonstrated by cumulative bile production of reperfused liver following 24 hr cold graft storage [162]. Traditionally, PGI_2 and its analogues exert their biological effects by binding to cell surface IP receptors, which couple via the stimulatory G protein to stimulate adenylyl cyclase and activate intracellular cAMP [111] to act as a second messenger of PGI_2 on vascular smooth muscle, platelets, endothelial cells, and neutrophils [161]. The concentration of cAMP depends on the balance between its synthesis and degradation in the cytoplasm; this molecule is formed by ATP in the reaction catalyzed by adenylyl cyclase, and catabolized by cyclic nucleotide phosphodiesterase [163]. Thus, homeostasis within the vasculature is achieved by maintaining the PGI_2/TxA2 balance, where each substance has opposing effects on cAMP [109], thereby regulating various physiological processes occurring at the interface between the blood and endothelium.

Loss of SEC viability, as determined morphologically, was completed after 24 hours or longer of ischemic storage [164]. While cold storage times for human transplantation varies

widely, animal studies focus on storage times of up 18 hrs, with some extended to 24 hrs or more. We employed a rat model with 18 hr of cold ischemic storage to induce significant hepatic injury. Considering the many factors involved in I/R injury and the role of PGI_2 in maintaining cellular homeostasis, treprostinil has a particular relevance in the setting of I/R injury in OLT. Treprostinil therapy could be administered to patients undergoing OLT as a clinical strategy against hepatic I/R injury because of its simple method of application prior to, during the surgery, and the early post-transplant period. After demonstrating efficacy in the current treatment model (donor plus recipient), additional animals were included to examine the efficacy of treprostinil in a treatment model (recipient only) which more closely resembles that of the clinical situation. Results from the recipient only treatment group showed significantly reduced hepatocellular injury, supporting the clinical utility of this agent. Recently, Sakai et al. demonstrated that treprostinil could be safely administered at doses >100 ng/kg/min to adult patients with pulmonary arterial hypertension, who were not therapeutically controlled by other PGI_2 analogues. This conversion enabled two of the patients to successfully undergo OLT [165]. No hemodynamic issues were observed during surgery, indicating safety of treprostinil administration during OLT.

In conclusion, the process of I/R injury to the liver during OLT combines interrelated factors that produce a cascade of events, which can ultimately lead to hepatic graft failure. I/R injury remains a significant limitation in clinical liver transplantation. The significance of this study is in demonstrating that treprostinil is an effective approach to ameliorate hepatic I/R injury associated with rat OLT. In addition, the results of this study strongly support clinical investigation of treprostinil as a potential therapy for the protection of liver grafts against I/R injury during OLT. This finding is an important advancement to the field of liver transplantation

and, potentially, to the field of solid organ transplantation. Amelioration of hepatic graft injury with treprostinil may improve both short- and long-term transplant outcomes.

3.0 EFFECT OF ISCHEMIA-REPERFUSION INJURY ON DRUG METABOLISM DURING RAT ORTHOTOPIC LIVER TRANSPLANTATION*

*N. Ghonem, J. Yoshida, N. Murase, S.C. Strom, and R. Venkataramanan. Ischemia-reperfusion decreases CYP450 Metabolism during Rat Orthotopic Liver Transplantation. Submitted to *Drug Metabolism and Disposition*, January 2011.

3.1 INTRODUCTION

Ischemia-reperfusion injury, an inflammatory disease-state manifested during OLT, significantly contributes to the impaired function of the transplanted liver graft. Inflammatory mediators, including pro-inflammatory cytokines, have been shown to reduce the metabolism of drugs primarily by the down-regulation of cytochrome P450 (CYP450) enzymes expression and or activity [85]. An alteration in drug metabolism as a result of inflammation or infection has major implications when the capacity of the liver, such as the case during liver transplantation, and other organs to handle drugs is severely compromised. Decreased catalytic activities of hepatic CYP450 enzymes can cause dose-dependent drug toxicity associated with impaired *in vivo* drug clearance [85, 166, 167]. The resulting outcomes of reduced drug clearance, which accompanies inflammation and states of reduced blood flow, could be toxic or sub-therapeutic plasma drug concentrations [168].

The CYP450 enzyme system reflects the liver's ability to metabolize drugs [169] and several studies have indicated that CYP activity is an important indicator of liver graft function post-transplantation [90-93]. Experimental animal studies have shown that live bacterial, viral, and parasitic infections are each capable of down-regulating the activities and or expression of CYP450 enzymes in the liver during inflammation [85, 166, 170]. Several models of inflammation, i.e. partial hepatectomy or LPS-stimulation, have also been used to examine the effect of inflammation on different subsets of hepatic P450s *in vivo* [85, 171]. Limited data exist on the direct effect(s) of hepatic I/R injury on CYP450 expression and activity in an OLT model, following graft storage in cold UW solution. Therefore, to more closely examine the hepatic

injury which occurs during clinical liver transplantation, we performed this study to examine the effect of I/R injury on the CYP450 mRNA and protein expression and activity on the major rat hepatic CYP450 enzymes and the impact of treatment with treprostinil to prevent I/R injury in a rat OLT model.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals

Chlorzoxazone (CZN), 6-hydroxychlorzoxazone (6-OH CZN), nicotinamide adenine dinucleotide phosphate-oxidase (NADPH), and testosterone (TST) were purchased from Sigma (St. Louis, MO). 2α-, 6β-, and 16α-hydroxytestosterone (2α-, 6β-, and 16α-OH TST, respectively) were purchased from Steraloids (Newport, RI). Midazolam (MDZ) and 1-hydroxymidazolam (1-OH MDZ) were purchased from Toronto Research Chemistry (Ontario, CA). Methanol and water [high-performance liquid chromatography (HPLC) grade] were purchased from Thermo Fisher Scientific (Waltham, MA). All other chemicals used were of HPLC grade or the highest purity available.

3.2.2 Animals

All procedures were performed according to the guidelines of the National Research Council's Guide for the Humane Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. Male Lewis rats weighing 200

- 300 g (Harlan Sprague Dawley, Inc, Indianapolis, IN) were maintained in a laminar-flow, specific-pathogen–free atmosphere at the University of Pittsburgh with a standard diet and water supplied ad libitum.

3.2.3 Orthotopic Liver Transplantation

The basic techniques of liver harvesting and OLT without hepatic arterial reconstruction were performed as previously described [151]. Briefly, rats were anesthetized with isoflurane inhalation and a midline incision was made in the abdominal cavity and the donor liver was excised and immediately flushed with cold UW solution, stored in UW solution at 4 ^{o}C for 18 hours, and orthotopically transplanted into recipients. All surgeries were performed by the same surgeon.

3.2.4 Treprostinil Administration

Treprostinil (1 mg/ml) and placebo (sodium chloride, metacresol, sodium citrate, water for injection) were provided by United Therapeutics, Inc. (Durham, NC). Treprostinil (100 ng/kg/min) or placebo was administered to donor and recipient animals subcutaneously via an Alzet® osmotic pump (Durect Corp., Cupertino, CA). The surgeon was blinded to treatment.

3.2.5 Experimental Design

Donor animals received placebo or treprostinil (100 ng/kg/min) for 24 hours before hepatectomy and corresponding recipient animals received placebo or treprostinil for 24 hours before

transplantation and until the time of sacrifice, to ensure steady-state concentrations. Recipients were sacrificed at 1, 3, 6, and 48 hours post-transplantation.

3.2.6 RNA Extraction and Real Time RT-PCR Analysis

Total RNA was extracted from liver tissue (50 – 100 mg) using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. RNA concentration was determined by UV absorbance at 260/280 nm (μQuant Microplate 25 Spectrophotometer) and RNA integrity was checked by 0.5% agarose gel electrophoresis stained with ethidium bromide. Two micrograms of total RNA from each sample was used to generate first-strand cDNA by use of the First Strand cDNA synthesis kit (Promega, Madison, WI). A reaction mixture containing 200 U monkey myeloblastosis virus reverse transcription reaction (MMLV, Promega, Madison, WI)-Reverse transcriptase, 1 mM dNTPs and 25 U RNasein (Promega) was added to the previous mixture and incubated at 37 °C for 60 minutes. DNase-I treated total RNA from each sample was mixed with 0.5 μg of Random Hexamers (Promega) heated to 70 °C for 5 minutes then cooled to 4 °C. Hepatic mRNA levels were measured with the TaqMan® system using primers purchased from Applied Biosystems, listed in Table 3. Samples were analyzed in triplicate and relative gene expression was measured using the comparative C_T method, with GAPDH as internal control.

Table 3: Real-Time PCR assay IDs for genes detected by TaqMan® gene expression assays

Gene Symbol	Gene Name	RefSeq Accession #
CYP3A1/3A23	Cytochrome P450, family 3, subfamily a, polypeptide 1/23	NM_013105.2
CYP3A2	cytochrome P450, family 3, subfamily a, polypeptide 2	NM_153312.2
CYP3A18	cytochrome P450, family 3, subfamily a, polypeptide 18	NM_145782.1
CYP2E1	cytochrome P450, family 2, subfamily e, polypeptide 1	NM_031543.1
CYP2C7	cytochrome P450, family 2, subfamily c, polypeptide 7	NM_017158.1
CYP2C11	cytochrome P450, family 2, subfamily c, polypeptide 11	NM_019184.2
CYP2D3	cytochrome P450, family 2, subfamily d, polypeptide 3	NM_173093.1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_017008.3

3.2.7 Preparation of Liver Microsomes

Snap-frozen slices of liver were used to prepare microsomes by a standard differential centrifugation procedure with minor modifications [172]. Briefly, liver pieces were homogenized with 3 volumes of a homogenization buffer (50 mM Tris-HCl buffer, 1.0% KCl, and 1 mM EDTA, pH 7.4) using an electrical homogenizer (Polytron, Brinkmann Instruments, Westbury, NY). The crude homogenate was centrifuged (Optima XL-100K ultracentrifuge, Beckman Instruments, Palo Alto, CA) at 10,000*g* for 20 minutes (4 °C). The supernatant was further centrifuged at 105,000*g* for 65 min at 4 °C. The microsomes were reconstituted using a manual glass homogenizer (Wheaton, Millville, NJ) with 50 mM Tris-HCl buffer (pH 7.4) containing 20% glycerol. Aliquots were immediately stored at -80 °C until used. The protein content of microsomes was determined by the Bradford method [153] using BSA as standard.

3.2.8 Western Blot Analysis of Microsomal P450 Protein Expression

Protein levels of CYP2E1, 2C11, and 3A2 in rat liver microsomes were measured by western blot analysis. Microsomal protein (25 ug) was separated by SDS-polyacrylamide gel electrophoresis (10% NuPAGE, Invitrogen, Carlsbad, CA). The proteins were transferred to a PVDF membrane, briefly incubated in Ponceau S (Sigma-Aldrich) to ensure equal protein load on membrane and complete transfer, then blocked overnight with TBST containing 5% Non Fat Dry Milk (Bio-Rad). After washing in TBST, membranes were probed with polyclonal rabbit anti-rat CYP2E1, CYPC11, or CYP3A2 antibodies (1:10,000; 1:4,000, and 1:5000, respectively; Abcam, Cambridge, MA). Next, the membranes were washed and probed with a secondary monoclonal goat anti-rabbit IgG antibody coupled to horseradish peroxidase (1:20,000; Abcam). Immunodetection was performed using an ECL detection kit (Thermo Scientific, Rockford, IL). The density of the protein bands were quantified using ImageJ software 1.40 (National Institutes of Health, Bethesda, MD). Values were normalized to GAPDH (1:30,000; Abcam).

3.2.9 Microsomal Incubations

Conditions for each substrate were optimized by varying the time of incubation, the protein concentration, and substrate concentration such that each reaction took place in the linear working range. The incubation included microsomes, $MgCl_2$ (10 mM), and phosphate buffer (0.1 mM), pH=7.4. The samples were pre-incubated in a shaking water bath for 5 min at 37 °C before addition of NADPH (1 mM) to initiate the reaction. All reactions were terminated upon addition of ice-cold methanol. Following termination, samples were centrifuged at 3,000 *rpm* for

10 minutes at 4 ^{o}C and samples were analyzed immediately. Normal liver represent samples from healthy rats not subjected to OLT.

3.2.10 Chlorzoxazone Assay

The formation of 6-OH CZN from CZN was used to measure CYP2E1 activity. Microsomal incubations contained 0.75 mg/ml microsomal protein and 200 uM of CZN. Samples were incubated for 30 minutes at 37 ^{o}C in a shaking water bath. The concentration of 6-OH CZN was measured using an Alliance HPLC system (Waters 2695, Milford, MA) with a Photodiode Array detector (Waters 2998) set at 297 nm. The mobile phase consisted of acetonitrile: 0.25% acetate (18:82) pH 3.8 at a flow rate of 1.2 ml/min and 6-OH CZN was separated using a Symmetry® C18 (4.6 x 250 mm, 5 um) column (Waters). The formation rate was calculated from a standard curve of known concentration of 6-OH CZN (0.15 – 10 ug/ml). The C.V. was less than 10% for this assay.

3.2.11 Testosterone Assay

The main male-specific isoform of cytochrome P-450 is 2C11 which gives a high yield of oxidized testosterone in positions 2α- and 16α- [173, 174]. Therefore, the formation of 2α- and 16α-OH TST from TST was used to measure CYP2C11 activity and the formation of 6β-OH TST was used to measure CYP3A activity. Microsomal incubations consisted of 0.5 mg/ml microsomal protein and 150 uM TST, final volume 0.25 mL. Samples were incubated for 20 minutes at 37 ^{o}C in a shaking water bath. The concentrations of 2α-, 6β-, and 16α-OH TST were measured by HPLC-UV. Compounds were separated using a LiChrospher® 100 C18 (4.6 x 250

mm, 5 μm) column (Merck, Gibbstown, NJ). The mobile phase consisted of 60% methanol in water. The HPLC system consisted of an autosampler (Waters 717, Milford, MA) and solvent delivery system (Waters 501) attached to a UV detector (Waters 486), set at 242 nm. The 2α-, 6β-, and 16α-hydroxylation activities of TST were calculated from a standard curve of known concentration (0.2 -10 ug/ml). The C.V. was less than 10% for this assay.

3.2.12 Midazolam Assay

Midazolam is predominantly metabolized to 1-OH MDZ by CYP3A1 and CYP3A2 in rats and thus it can be used as a biomarker of CYP3A activity *in vivo* [174]. Microsomal incubations contained 0.375 mg/ml microsomal protein and 0.3 uM of MDZ. The samples were incubated for 20 minutes in a shaking water bath at 37 ^{o}C. The mobile phase consisted of (A) 2 mM ammonium acetate with 0.1 % formic acid in 5% methanol and (B) 100% methanol at a flow rate of 0.3 ml/min. MDZ and 1-OH MDZ were separated using a Symmetry® C18 (2.1 x 50 mm, 3.5 um) column (Waters) with a Symmetry® (2.1 x 10 mm, 3.5 um) guard column (Waters). The Alliance HPLC (Waters 2695, Milford, MA) was attached to a Quatromicro™ mass spectrometer (Waters), operated in positive electrospray ionization. The selected reaction monitoring transitions of m/z 326.05 → 291.05 (collision energy 28 eV, cone voltage 55) for MDZ; m/z 341.87→ 323.97 (collision energy 20 eV, cone voltage 37) for 1 -OH MDZ; m/z 331.09 → 295.97 (collision energy 28 eV, cone voltage 55) for deuterated midazolam were monitored. Parameters were optimized to obtain the highest [M+H] ion abundance and were as follows: source temperature, 100 °C; capillary voltage, 0.8 kV; desolvation temperature, 500 °C; cone gas flow, 50 L/hr; desolvation gas flow, 50 L/hr. Analytical data were analyzed using Masslynx software version 4.1 (Waters). The formation rate of 1-OH MDZ was calculated by

use of a standard curve of known concentration of 1-OH MDZ (1.25 – 25 ng/ml). The C.V. was less than 5% for this assay.

3.2.13 Statistical Analysis

Data are represented as the mean ± SEM. One-way ANOVA was performed to determine the difference between groups, followed by Bonferroni post-hoc analyses, using Prism software v4.0 (GraphPad, San Diego, CA). Differences were considered significant at a *P*-value < 0.05.

3.3 RESULTS

3.3.1 Hepatic I/R Injury

In Chapter 2, we demonstrated that treprostinil significantly reduced serum ALT and AST levels compared to the placebo-treated groups (Figures 7 and 8). Corresponding areas under the curve (AUC) from 0 to 48 hours post-reperfusion of serum ALT and AST were calculated by non-compartmental analysis using WinNonlin® software (Pharsight, Mountain View, CA). In both the donor + recipient (D+R) and recipient only (R) treatment groups, administration of treprostinil resulted in significantly lower AUC values, compared to the placebo-treated group, listed in Table 4 and 5, respectively. The results indicate that treprostinil-treated rats experienced significantly less hepatic injury than placebo-treated rats in both treatment groups.

Table 4: $AUC_{0\text{ -48 hrs post-reperfusion}}$ serum ALT and AST in donor + recipient treatment group
D+R, donor + recipient treatment;*$P < 0.05$, ***$P < 0.001$ vs. placebo (n=3-4).

Group (Treatment)	$AUC_{0\text{-48 hr post-OLT}}$ (IU*hr/L)	
	ALT	**AST**
Placebo (D+R)	106,650 ± 7,888	188,470 ± 40,540
Treprostinil (D+R)	32,650 ± 2,479***	52,246 ± 5,380*

Table 5: $AUC_{0\text{ -48 hrs post-reperfusion}}$ serum ALT and AST in recipient only treatment group
R, recipient only treatment; **$P < 0.01$, ***$P < 0.001$ vs. placebo (n=3-4).

Group (Treatment)	$AUC_{0\text{-48 hr post-OLT}}$ (IU*hr/L)	
	ALT	**AST**
Placebo (R)	102,165 ± 2,811	197,281 ± 13,710
Treprostinil (R)	51,339 ± 10,366**	63,779 ± 2,325***

3.3.2 CYP450 mRNA Expression

The effect of I/R injury on the mRNA expression of CYP enzymes in hepatic tissue at 6 and 48 hours post-OLT was examined by real time RT-PCR. At 6 hr post-OLT, differences between the placebo- and treprostinil-treated groups were observed and data are shown in Figure 22. In the placebo-treated group, hepatic mRNA expression of CYP2E1 was reduced to 18% of normal. In contrast, treprostinil improved mRNA expression by two-fold, to 38% of normal. CYP2C11 was reduced to 20% of normal compared to 40% in treprostinil-treated group. CYP3A2 expression was reduced to 15% of normal, and treprostinil improved it to 27% of normal. In addition,

mRNA expression of CYP3A1/23, 3A18, and 2C7 were also reduced in the placebo group to 37, 27, and 26%, respectively. Treprostinil administration improved the mRNA expression of these CYP enzymes to 81, 45, and 42%, respectively. No difference in CYP2D3 mRNA expression was observed; levels were reduced to 62% in both the placebo- and treprostinil-treated groups.

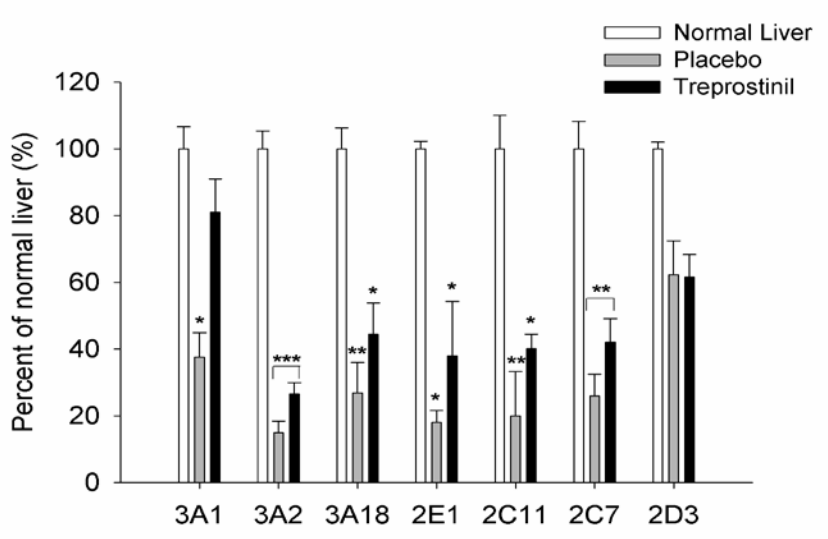


Figure 22: Hepatic mRNA expression of CYP450 enzymes at 6 hours post-OLT
P* < 0.05, ***P* < 0.01, * *P* <0.001 vs. normal liver (n=3).

The mRNA expression of CYP450 enzymes at 48 hours post-OLT is shown in Figure 23. The suppression of hepatic mRNA expression of CYP2E1 in the placebo-treated group slightly improved to 29% of normal, whereas the levels in the treprostinil-treated group further improved to 49% of normal. Interestingly, the mRNA expression CYP2C11 in both the placebo-treated group and treprostinil-treated group remained at approximately 30% of normal. No change in CYP3A2 mRNA expression was observed and levels remained at 15 and 22% of normal in placebo and treprostinil-treated animals, respectively. Alternatively, the hepatic mRNA

expression of CYP3A1/23 continued to decline to 18% of normal in the placebo-treated group while expression was almost doubled in the treprostinil-treated group to 30% of normal.

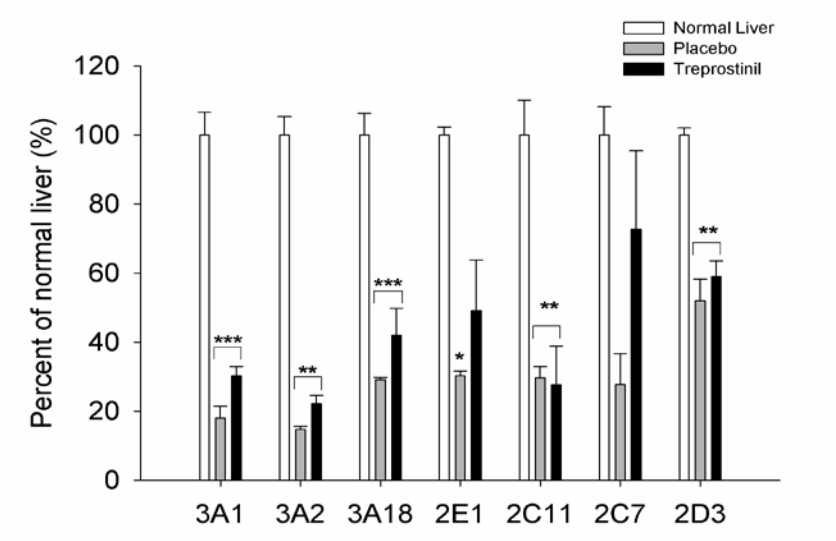


Figure 23: Hepatic mRNA expression of CYP450 enzymes at 48 hours post-OLT
*$P < 0.05$, **$P < 0.01$, *** $P < 0.001$ vs. normal liver (n=3).

3.3.3 CYP450 Protein Expression

The effect of I/R injury on the protein expression of CYP2E1, CYP2C11, and CYP3A2 in liver graft at 48 hr post-OLT was examined using western blot analysis. The protein levels of all three CYP450 enzymes were significantly decreased in the placebo-treated group, compared to normal liver. Shown in Figure 24, the protein expression of CYP2E1 was reduced to 63% of normal in placebo and treprostinil restored expression to 96% of normal. The protein expression of CYP2C11 was significantly reduced to 58% of normal levels, and treprostinil significantly improved expression to 67% of normal (Figure 25). In this model system, CYP3A2 was the enzyme most significantly down-regulated by I/R injury, with protein levels reduced to 27% of normal in the placebo group, shown in Figure 26. Treprostinil significantly improved CYP3A2 protein expression by more than two-fold of placebo, to 62% of normal. The results indicate that

the impact of I/R injury on CYP450 protein expression persisted at least up to 48 hr post-OLT and treatment with treprostinil attenuated this injury and significantly improved the protein expression of CYP2E1, 2C11, and CYP3A2 in liver graft following OLT.

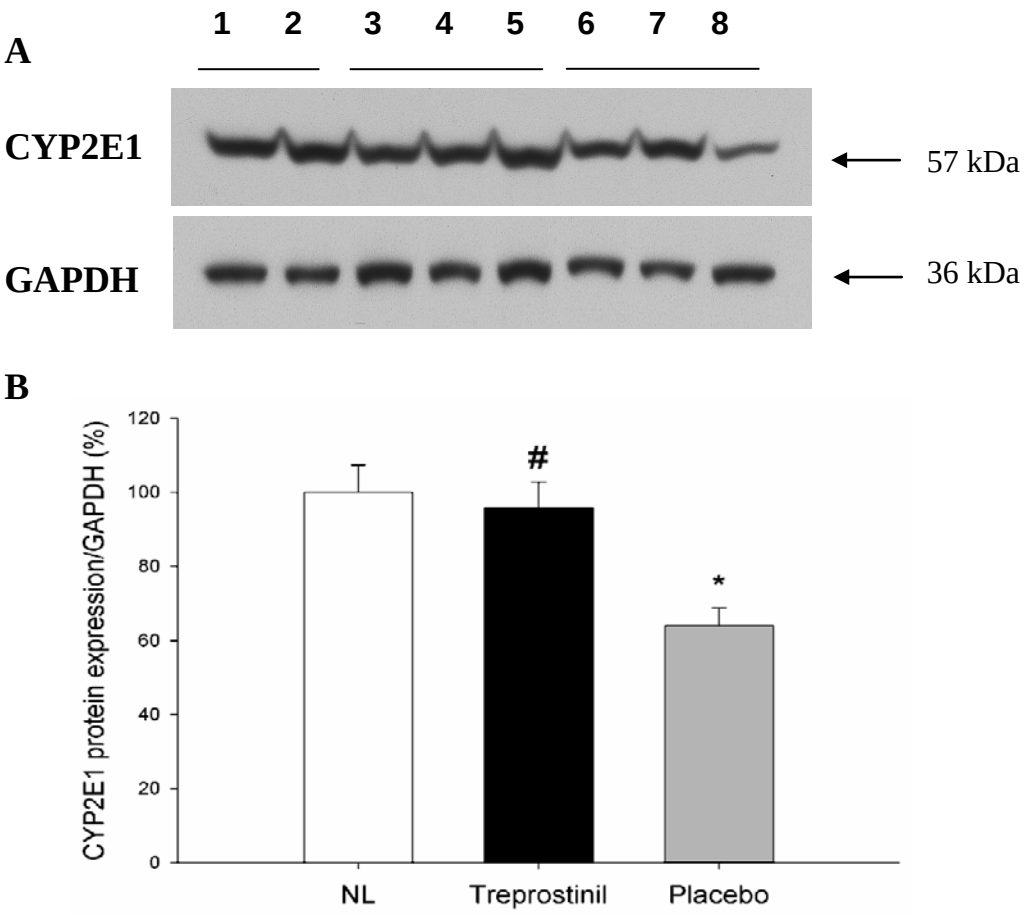


Figure 24: Hepatic microsomal CYP2E1 protein

A) Western blot analysis of normal (lanes 1-2), treprostinil-treated (3-5), and placebo-treated (6-8) animals at 48 hr post-OLT. (B) Data are expressed as a percentage of normal liver, normalized to GAPDH expression; **P* < 0.05 vs. normal liver; #*P* < 0.05 vs. placebo.

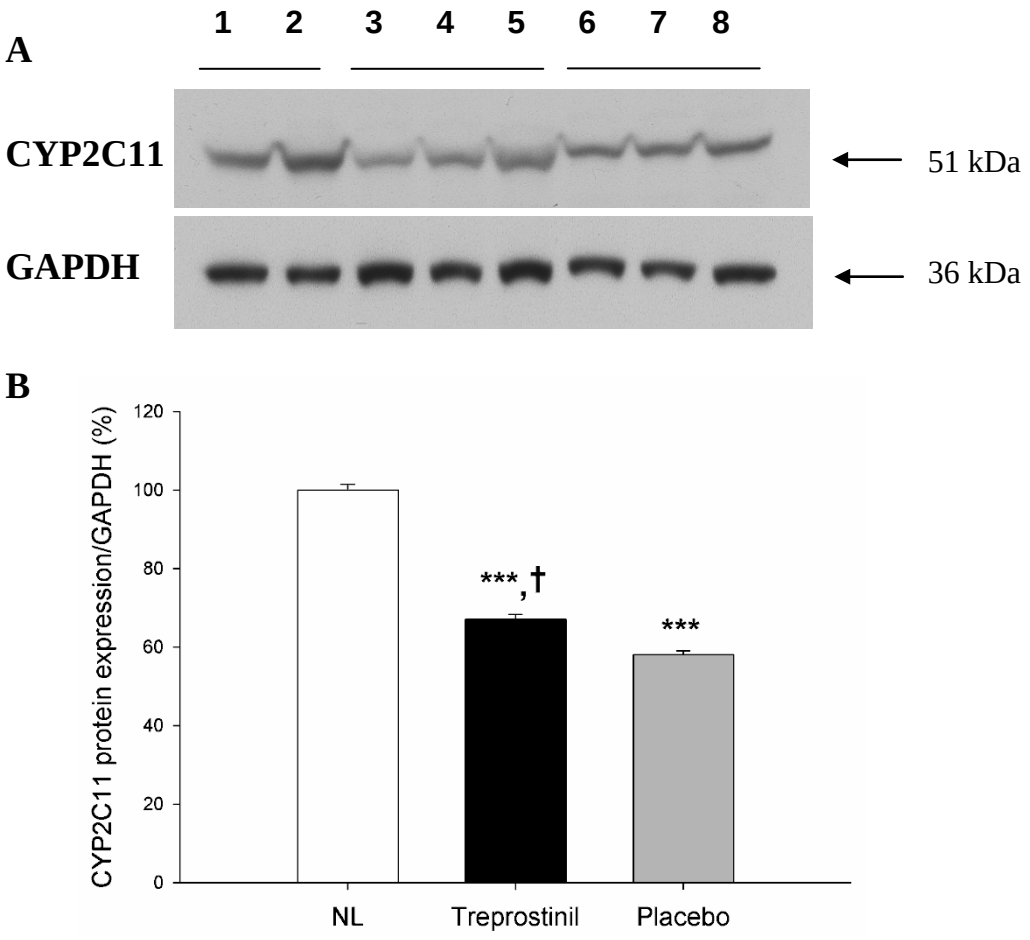


Figure 25: Hepatic microsomal CYP2C11 protein

(A) Western blot analysis of normal (lanes 1-2), treprostinil-treated (3-5), and placebo-treated (6-8) animals at 48 hr post-OLT. (B) Data are expressed as a percentage of normal liver, normalized to GAPDH expression; ***$P < 0.001$ vs. NL; †$P < 0.01$ vs. placebo.

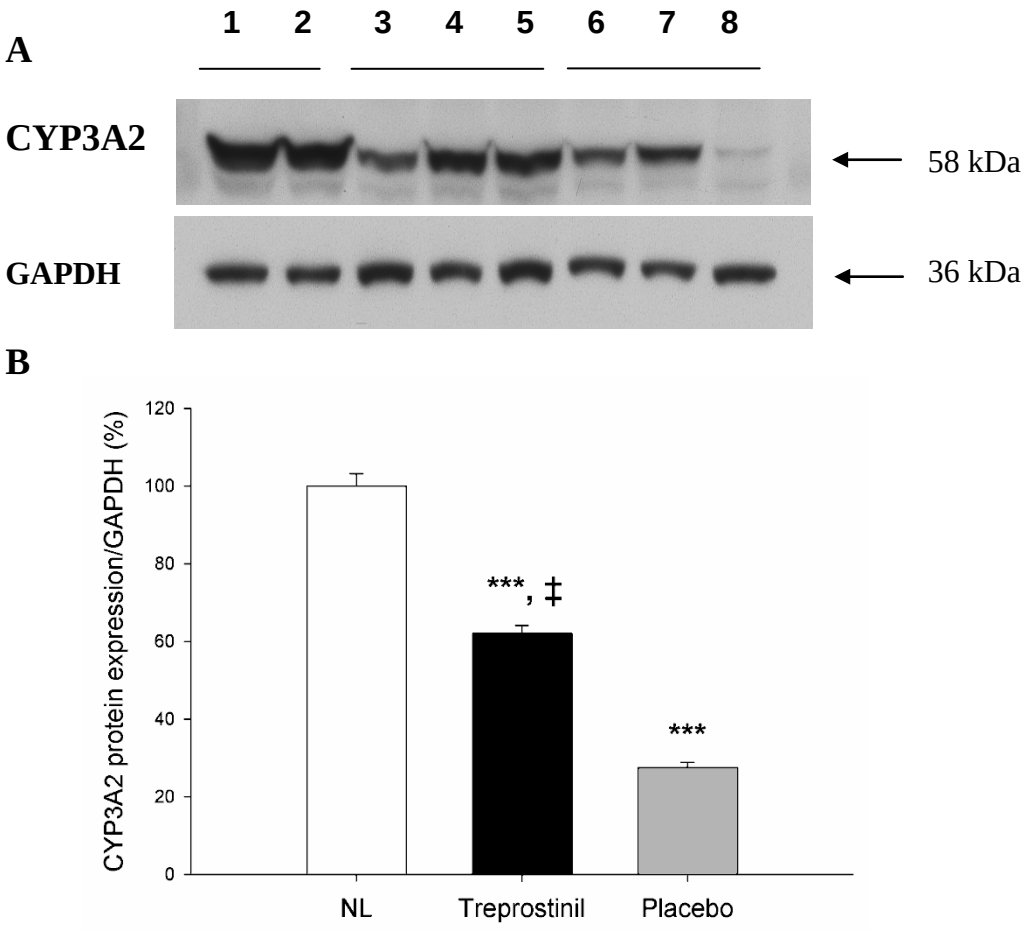


Figure 26: Hepatic microsomal CYP3A2 protein
(A) Western blot analysis of normal (lanes 1-2), treprostinil-treated (3-5), and placebo-treated (6-8) animals at 48 hr post-OLT. (B) Data are expressed as a percentage of normal liver, normalized to GAPDH expression; ****P* < 0.001 vs. normal liver; ‡*P* < 0.001 vs. placebo.

3.3.4 CYP450 Enzyme Activity in Liver Graft Post-OLT

The impact of I/R injury on the drug metabolizing activity of CYP2E1, CYP2C11, and CYP3A was examined at 1, 3, and 48 hr post-OLT in rat liver microsomes. The enzymatic activity of CYP2E1 was determined using chlorzoxazone as a substrate. The formation rate of 6-hydroxychlorzoxazone in normal rat liver was 0.59 ± 0.01 umol/min/mg, shown in Figure 27. In the placebo group, no significant changes in CYP2E1 activity occurred early post-OLT, but at 48

hours post-transplantation, CYP2E1 activity in the placebo group was reduced to 35% (0.21 ± 0.05 umol/min/mg) of normal. In contrast, treprostinil significantly improved CYP2E1 activity at all time points, and at 48 hr post-OLT, CYP2E1 activity was increased by more than two-fold that of placebo, to 73% of normal (0.43 ± 0. 11 umol/min/mg).

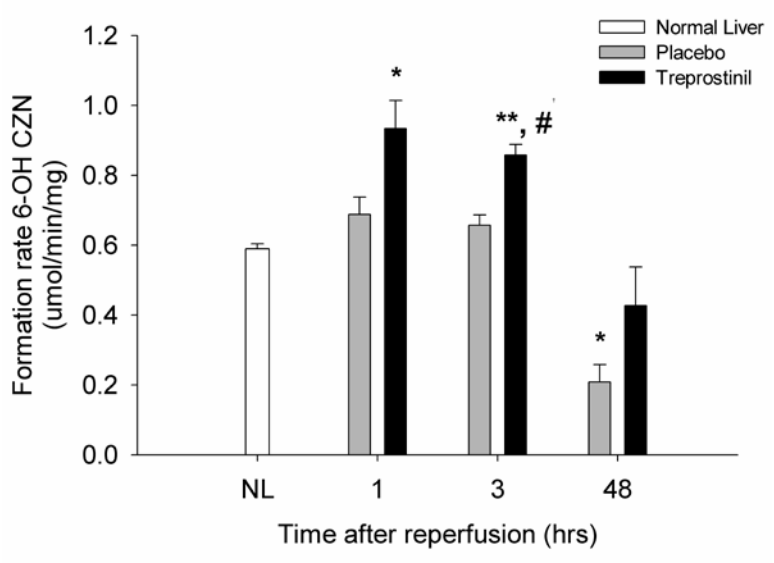


Figure 27: Hepatic CYP2E1 activity
Formation rate of 6-OH CZN in rat liver microsomes from normal, placebo-treated, and treprostinil-treated group at 1, 3, and 48 hr post-OLT; *P < 0.05, **P < 0.01 vs. normal liver; #P <0.05 vs. placebo.

The major component of microsomal CYPs in male rat liver is CYP2C11 [175]. The enzymatic activity of CYP2C11 was determined using two substrates: 2α- and 16α-hydroxytestosterone, for which the formation rates in normal liver were 100.4 ± 15.9 and 190.7 ± 31.9 nmol/min/mg (Figure 28A and 28B), respectively. While no significant changes were observed at 1 and 3 hr post-OLT, the hydroxylation of testosterone markedly decreased in the placebo group to 16% (16.2 ± 7.1 nmol/min/mg) and 25% (47.9 ± 14.6 nmol/min/mg) of normal, respectively, at 48 hr post-OLT. In contrast, treprostinil improved CYP2C11 activity to 56% and 40% of normal (55.8 ± 14.4 and 75.4 ± 28.3 nmol/min/mg, respectively) at 48 hr post-OLT.

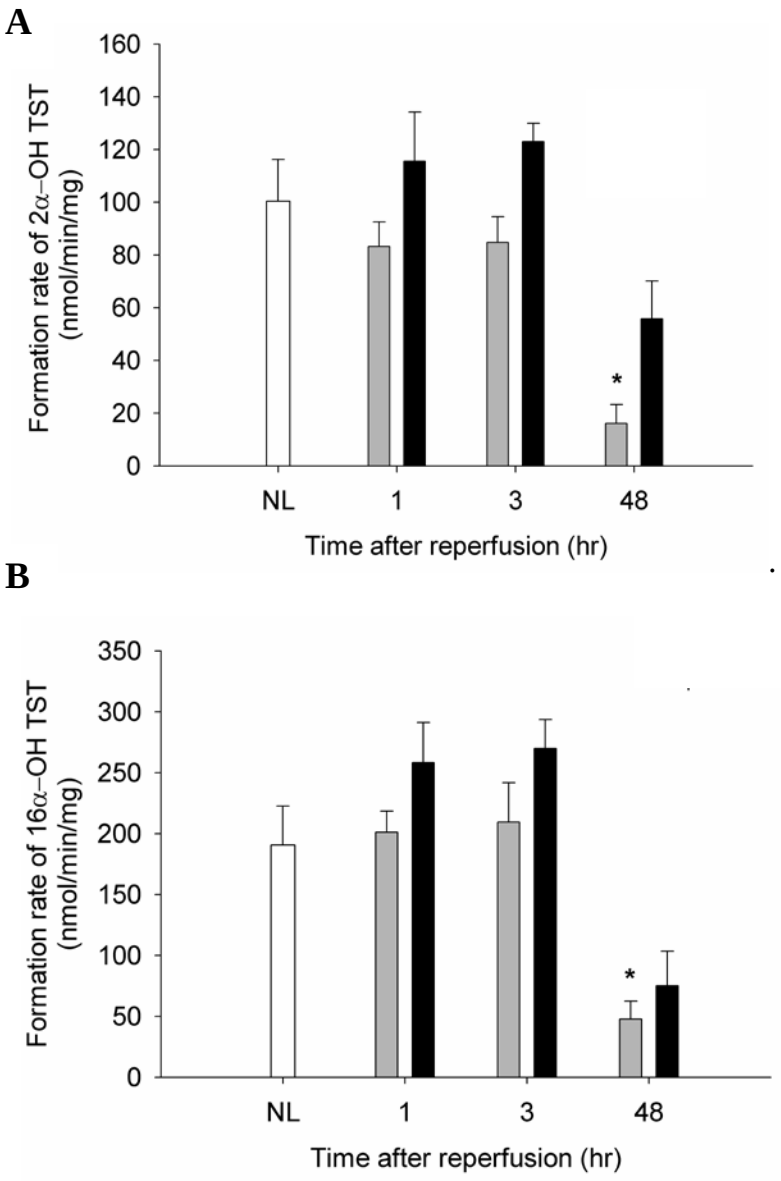


Figure 28: Hepatic CYP2C11 activity

Formation rate of (A) 2α-hydroxytestosterone and (B) 16α-hydroxytestosterone in rat liver microsomes from normal, placebo-treated, and treprostinil-treated group at 1, 3, and 48 hr post-OLT; **P* < 0.05 vs. normal liver.

The activity of CYP3A was determined using two substrates, 6β-OH TST and 1-OH MDZ, shown in Figures 29A and 29B. The normal formation rate of 6β-OH TST was 94.5 ± 18.4 nmol/min/mg, shown in Figure 29A. Initially, at 1 and 3 hour post-OLT, the formation rate of 6β-OH TST in both the placebo (202 ± 32.3 and 181.3 ± 48.0 nmol/min/mg, respectively) and the treprostinil-treated group (179 ± 15.7 and 195.7 ± 25.7 nmol/min/mg, respectively) slightly increased, which could be attributed to substrate specificity. By 48 hr post-OLT, the activity in the placebo group significantly declined to 30% of normal (28.7 ± 7.7 nmol/min/mg). Alternatively, the treprostinil-treated group experienced less injury than the placebo group and preserved activity to 64% of normal (60.2 ± 12.6 pmol/min/mg).

The normal formation rate of 1-OH MDZ was 5.5 ± 0.2 pmol/min/mg (Figure 29B). At 1 and 3 hours post-OLT, the formation rate of 1-OH MDZ in the placebo group decreased to 49% and 62% of normal (2.67 ± 0.17 pmol/min/mg and 3.4 ± 0.29 pmol/min/mg, respectively). Similar results were observed in the treprostinil-treated group; at 1 hr post-OLT, the formation rate of 1-OH MDZ had decreased to 49% with a slight improvement to 69% of normal at 3 hr post-OLT (2.7 ± 0.2 and 3.8 ± 0.3 pmol/min/mg, respectively). At 48 hr post-OLT, however, the formation rate of 1-OH MDZ in the placebo group further decreased to 18% of normal (1.0 ± 0.4 nmol/min/mg), whereas the activity in the treprostinil-treated group was maintained at 54% of normal (3.0 ± 0.5 nmol/min/mg). The data indicate that I/R injury significantly reduced CYP450 activity and that treprostinil significantly improved CYP450 activity in the liver graft post-OLT.

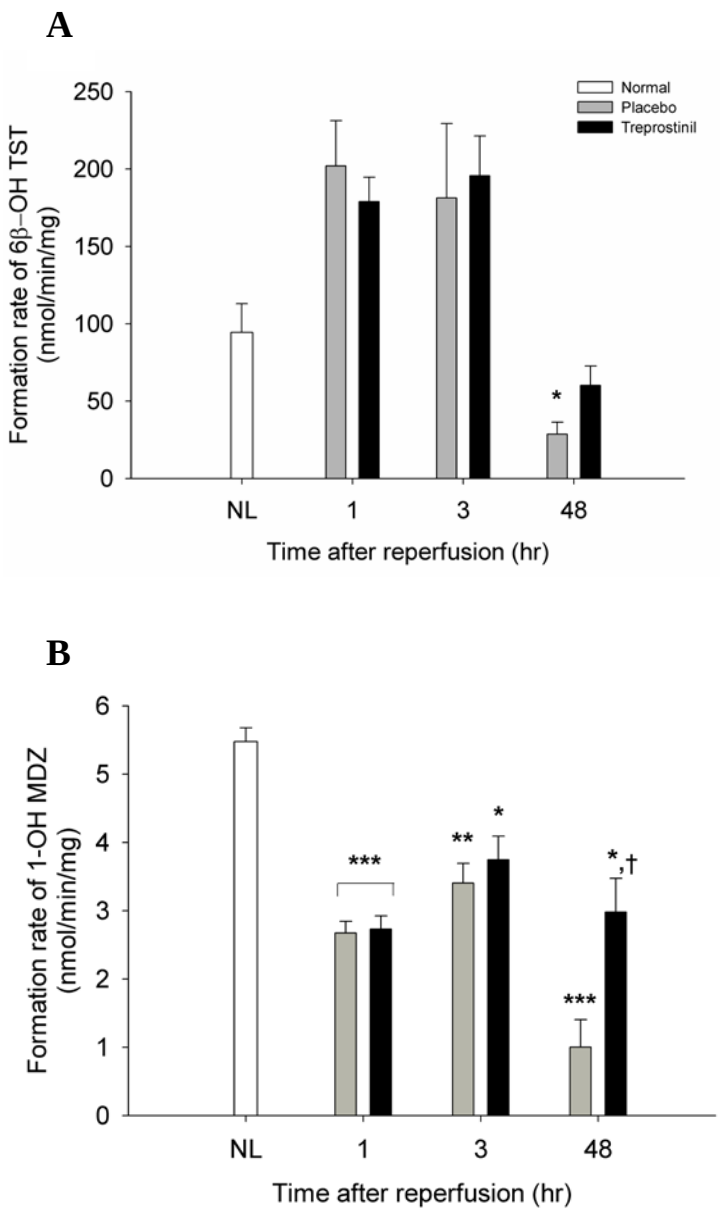


Figure 29: Hepatic CYP3A2 activity

Formation rates of (A) 6β-hydroxytestosterone and (B) 1-hydroxymidazolam in rat liver microsomes of normal, placebo-treated, and treprostinil-treated group at 1, 3, and 48 hr post-OLT; **P* < 0.05, ***P* < 0.01, *** *P* < 0.001 vs. normal liver; †*P* < 0.05 vs. placebo.

3.4 DISCUSSION

During the host response to inflammation, inflammatory mediators, including release of pro-inflammatory cytokines, have been associated with altered content, expression, and activity of CYP450 enzymes, consequently leading to alterations in the metabolism and elimination of certain drugs [85]. Several studies have demonstrated that the catalytic activities of many hepatic CYP450 enzymes in experimental models of liver inflammation or infection and in man are down-regulated, which can cause dose-dependent drug toxicity associated with impaired *in vivo* drug clearance [166, 167]. In most cases, the decreased activity is accompanied or preceded by decreased hepatic levels of the corresponding CYP450 mRNA and protein expression [85]. The losses in drug metabolism are predominantly mediated through the production of pro-inflammatory cytokines, which ultimately modify the expression and function of specific transcription factors. There is evidence for both transcriptional and post-transcriptional down-regulation of CYP450 mRNA by inflammatory stimuli [176]. Other proposed mechanisms that apply to specific P450s involve post-translational steps including enzyme modification and increased degradation [170].

Administration of LPS is a classic model of bacterial sepsis, perhaps the best characterized model to investigate CYP450 down-regulation by inflammation, although different concentrations of LPS and cytokines administered *in vivo* or *in vitro* can have enzyme-selective effects on CYP450 expression [85, 177]. In addition, different models of inflammation or infection can result in different rates of drug clearance and or reduced microsomal metabolism of drugs [178]. The down-regulation of CYP2C11 following treatment with bacterial LPS,

turpentine, or by other inflammatory responses has been shown to primarily occur via decreased mRNA expression, which is followed by a similar decrease in its protein levels [85, 179, 180]. Additional studies have shown that the CYP2C11 promoter contains a binding site for NF-kB, and that mutation of the promoter to inhibit NF-kB binding also prevented suppression of CYP2C11 transcription by either Il-1 or LPS [175]. Alternatively, CYP2E1 has been shown to be most affected by inflammation at the protein level [181, 182], through post-translational mechanisms, i.e. protein stabilization, to prevent degradation [70, 183-185].

Central to the mechanism of I/R-associated liver injury is the activation of the pro-inflammatory cascade resulting in formation of pro-inflammatory cytokines. Serum and hepatic mRNA levels of TNF-α, IL-6, and ICAM-1 were significantly up-regulated early post-OLT following 18 hr cold liver graft storage [107, 186]. Prostacyclin analogues have been shown to inhibit leukocyte activation by inhibiting TNF-α production, neutrophil activation and adhesion to endothelial cells [44, 139]. In Chapter 2, we demonstrated that treprostinil reduced significantly elevated hepatic tissue mRNA levels of TNF-α, IFN-γ, IL-6, and ICAM-1 early post-OLT, as well as increased hepatic blood flow before and immediately post-OLT. In addition, treprostinil has been shown to inhibit the mRNA expression of multiple cytokines including IL-6, TNF-α and IL-1β by blocking the translocation of NF-kB *in vitro* [187].

In a model of cold graft storage followed by reperfusion using a recirculating method, Izuishi et al. [169] examined the effects of prolonged cold graft storage on CYP content, protein and activity. Significant changes were only observed after 48 hours of cold storage, which does not translate to the clinical setting. Alternatively, in a rat model of partial (70%) ischemia, 1 hour of warm ischemia followed by 3 hours reperfusion resulted in no significant changes in CYP2E1 or CYP2C11 protein, whereas CYP2E1, CYP2C11, and CYP3A2 activity decreased by

17, 34, and 30% [188-190]. Thirdly, in a porcine model of warm ischemia, after 6 hours of partial hepatic occlusion, the activities of CYP2C, CYP2E1, and CYP3A were decreased to 62, 62, and 31%, respectively; however, CYP3A4 protein expression remained unchanged [191]. It is difficult to translate the results from a model of warm I/R injury or a liver graft reperfused *ex vivo* to the clinical setting, as it involves cold ischemia and warm reperfusion, thereby invoking different cellular injuries and, consequently, different patterns in host response. While there are a plethora of studies demonstrating the effects of inflammation and infection on CYP enzyme regulation, there is a paucity of data that directly examine the effect(s) of I/R injury during OLT, which more accurately represents hepatic CYP450 regulation during clinical liver transplantation. Therefore, to more accurately characterize the direct effects of I/R injury during OLT on hepatic CYP450 expression and activity, the current study was performed. We hypothesized that I/R injury, an inflammatory disease-state manifested during OLT, would significantly impair CYP mRNA, protein, and activity in the liver graft, and that treprostinil would improve the expression and activity of CYP450 isoforms post-transplantation by inhibiting the inflammatory response and improving hepatic tissue blood flow.

The finding that CYP2E1 protein expression was reduced to 62% of normal at 48 hr post-OLT in the placebo group, and that treprostinil administration preserved CYP2E1 protein expression to 96% of normal (Figure 25), supports the hypothesis that CYP2E1 protein is stabilized, and suggests that treprostinil might interact with different sites on the CYP2E1 protein to stabilize or prevent degradation. Treprostinil improved the protein expression and activity of all CYP450 enzymes examined. The patterns of mRNA and protein expression and corresponding CYP450 activity support the idea that different inflammatory mediators regulate P450 expression at different levels and are enzyme-specific. The discrepancy between our

results and those discussed above are most likely due to the difference in I/R models and the CYP450 substrates selected. Inhibition of pro-inflammatory cytokines is most likely one of the major mechanisms responsible for improved hepatic CYP450 expression and activity in the treprostinil group. Furthermore, activity of CYP450 enzymes are an important indicator of liver graft function *in vivo* [90-93]. Decreased levels can influence the clinical response and, in worse cases, precipitate hepatic dysfunction or lead to graft failure. The results presented herein indicate a discrpenacy between protein expression and CYP450 activity, which could possibly be the result of an accumulation of non-functional protein.

The need to ameliorate I/R injury in liver transplantation is imminent; however, no treatment is currently available. An agent that is capable of suppressing the inflammatory response as well as improving hepatic blood flow would greatly improve hepatic function in clinical liver transplantation. Treprostinil has the potential to serve as a therapeutic option to protect the liver graft against I/R injury in OLT and to greatly improve CYP450 function post-OLT.

In conclusion, the results from the current study demonstrated that the activity of the major rat CYP450 enzymes were significantly reduced, secondary to reduced CYP450 protein and mRNA expression in rat liver graft post-reperfusion. Treprostinil administration significantly reduced hepatic I/R injury and improved the CYP450 expression and function in rat liver graft tissue post-OLT.

4.0 EFFECT OF ISCHEMIA-REPERFUSION INJURY ON HEPATIC DRUG TRANSPORTERS DURING RAT ORTHOTOPIC LIVER TRANSPLANTATION*

*N. Ghonem, J. Yoshida, N. Murase, S.C. Strom, and R. Venkataramanan. Changes in the Expression of Hepatic Drug Transporters during Rat Orthotopic Liver Transplantation. Submitted to *Journal of Pharmacology and Experimental Therapeutics*, January 2011.

4.1 INTRODUCTION

The liver plays an important role in the detoxification of endogenous and exogenous compounds through biotransformation as well as enterohepatic circulation of bile acids and biliary excretion of these compounds. Numerous endogenous compounds and xenobiotics are transported across membranes during the process of absorption, distribution, and clearance by transporters that are expressed in various organs.

Hepatic transporters contribute to the translocation of substances across biological membranes and play a critical role in the body's defense mechanism by aiding in the disposition and elimination of a variety of physiological substrates, metabolic products, and xenobiotics, to prevent the accumulation of potentially harmful compounds. Hepatic transporters also play a significant role in the overall pharmacokinetics of various drugs. Alterations in the expression of hepatic drug transporters as a result of inflammation or infection have been reported [81-84, 192]. In rodents, treatment with endotoxin, or its LPS component released from gram negative bacteria, can translocate across the intestinal mucosa into the circulation and has been shown to result in a pronounced alteration in the expression of hepatic transporters at the basolateral and canalicular membrane, including Ntcp (Slc10a1), Oatp (Slc1a1 and Slc1a2), and Oct (Slc22a1), as well as the ABC transporters Mdr1a/P-glycoprotein (Abcb1a), Bsep (Abcb11), and Mrp2 (Abcb2) [86, 193], which correlated to elevated levels of inflammatory cytokines, including TNF-α, IL-1β, -6, and IFN-γ [194].

Pro-inflammatory cytokines, e.g. TNF-α, IL-1β, and ICAM, are increased in the context of I/R injury associated with rat OLT [107, 186]. Limited data are available on the effect(s) of I/R injury on the expression of hepatic transporters in a relevant animal OLT model. To better understand and characterize the effects of I/R injury on hepatic drug transport proteins during

liver transplantation, it is important to have a model that simulates the clinical conditions, including cold ischemic injury followed by warm reperfusion injury. The purpose of this study was to examine the effects of I/R injury and evaluate the protective effect of treprostinil on the hepatic expression of uptake and efflux drug transporters in a clinically relevant rat OLT model.

4.2 MATERIALS AND METHODS

4.2.1 Animals

All procedures were performed according to the guidelines of the National Research Council's Guide for the Humane Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. Male Lewis rats weighing 200 - 300 g (Harlan Sprague Dawley, Inc, Indianapolis, IN) were maintained in a laminar-flow, specific-pathogen–free atmosphere at the University of Pittsburgh with a standard diet and water supplied ad libitum.

4.2.2 Orthotopic Liver Transplantation

The basic techniques of liver harvesting and OLT without hepatic arterial reconstruction were performed as previously described [151]. Briefly, rats were anesthetized with isoflurane inhalation and a midline incision was made in the abdominal cavity and the donor liver was excised and immediately flushed with cold UW solution, stored in UW solution at 4 ^{o}C for 18

hours, and orthotopically transplanted into recipients. All surgeries were performed by the same surgeon.

4.2.3 Treprostinil Administration

Treprostinil (1 mg/ml) and placebo (sodium chloride, metacresol, sodium citrate, water for injection) were provided by United Therapeutics, Inc. (Durham, NC). Treprostinil (100 ng/kg/min) or placebo was administered to donor and recipient animals subcutaneously via an Alzet® osmotic pump (Durect Corp., Cupertino, CA). The surgeon was blinded to treatment.

4.2.4 Experimental Design

Donor animals received placebo or treprostinil (100 ng/kg/min) for 24 hours before hepatectomy and corresponding recipient animals received placebo or treprostinil for 24 hours before transplantation and until the time of sacrifice, to ensure steady-state concentrations. Recipients were sacrificed at 1, 3, 6, and 48 hours post-transplantation.

4.2.5 RNA Extraction and Real Time RT-PCR Analysis

Total RNA was extracted from liver tissue (50 – 100 mg) using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. RNA concentration was determined by UV absorbance at 260/280 nm (μQuant Microplate 25 Spectrophotometer) and RNA integrity was checked by 0.5% agarose gel electrophoresis stained with ethidium bromide. Two micrograms of total RNA from each sample was used to generate first-strand cDNA by use of

the First Strand cDNA synthesis kit (Promega, Madison, WI). A reaction mixture containing 200 U monkey myeloblastosis virus reverse transcription reaction (MMLV, Promega, Madison, WI)-Reverse transcriptase, 1 mM dNTPs and 25 U RNasein (Promega) was added to the previous mixture and incubated at 37 °C for 60 minutes. DNase-I treated total RNA from each sample was mixed with 0.5 μg of Random Hexamers (Promega) heated to 70 °C for 5 minutes then cooled to 4 °C. Real-time RT-PCR was performed with the SYBR® Green system using primers purchased from Super Array Biosciences (Frederick, MD), listed in Table 6. Samples were analyzed in triplicate and relative gene expression was measured using the comparative C_T method, with GAPDH as internal control.

Table 6: Real-time PCR assay IDs for genes detected by SYBR® gene expression assays

Gene Symbol	Gene Name	RefSeq Accession#
Slc10a1	Solute carrier family 10 (sodium/bile acid cotransporter family), member 1	NM_017047.1
Slco1a1	Solute carrier organic anion transporter family, member 1a1	NM_017111.1
Slco1a4	Solute carrier organic anion transporter family, member 1a4	NM_131906.1
Slc22a1	Solute carrier family 22 (organic cation transporter), member 1	NM_012697.1
Abcb1	ATP-binding cassette, sub-family B (Mdr), member 1	NM_133401.1
Abcb4	ATP-binding cassette, sub-family B (Mdr), member 2	NM_012690.1
Abcc2	ATP-binding cassette, sub-family C (Mrp), member 2	NM_012833.1
Abcc3	ATP-binding cassette, sub-family C (Mrp), member 3	NM_080581.1
Abcb11	ATP-binding cassette, sub-family B (Mdr), member 11	NM_031760.1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_017008.3

4.2.6 Liver Membrane Isolation

Snap-frozen slices of liver were used to isolate total liver membranes by a standard differential centrifugation procedure with minor modifications [172]. Briefly, liver pieces were homogenized with 3 volumes of a homogenization buffer (50 mM Tris-HCl buffer, 1.0% KCl, and 1 mM EDTA, pH 7.4) using an electrical homogenizer (Polytron, Brinkmann Instruments, Westbury, NY). The crude homogenate was centrifuged (Optima XL-100K ultracentrifuge, Beckman Instruments, Palo Alto, CA) at 10,000*g* for 20 minutes at 4 °C. The supernatant was further centrifuged at 105,000*g* for 65 min at 4 °C. Membrane pellets were resuspended using a manual glass homogenizer (Wheaton, Millville, NJ) with 50 mM Tris-HCl buffer (pH 7.4) containing 20% glycerol. Aliquots were immediately stored at -80 °C until used. The protein content of microsomes was determined by the Bradford method [153] using BSA as standard.

4.2.7 Western Blot Analysis

Protein levels of Mrp2 and P-gp in rat liver membranes were measured by western immunoblotting. Liver protein (25 ug) was separated by SDS-polyacrylamide gel electrophoresis (10% NuPAGE, Invitrogen, Carlsbad, CA). The proteins were transferred to a PVDF membrane, briefly incubated in Ponceau S (Sigma-Aldrich) to ensure equal protein load on membrane and complete transfer, then blocked for 1-2 hours in TBST containing 5% Non Fat Dry Milk (Bio-Rad). After appropriate washings, membranes were probed overnight with monoclonal mouse anti-rat C-219 (P-gp) and M2 III-6 (Mrp2) antibodies (1:400; Abcam, Cambridge, MA). Next, the membranes were washed and probed with a secondary monoclonal rabbit anti-mouse IgG antibody coupled to horseradish peroxidase (1:20,000; Abcam). Immunodetection was

performed using an ECL detection kit (Thermo Scientific, Rockford, IL). The density of the protein bands were quantified using ImageJ software 1.40 (National Institutes of Health, Bethesda, MD). Values were normalized to GAPDH (1:30,000; Abcam) and results are expressed as percentage of normal liver.

4.2.8 Serum Bilirubin

Blood was collected at 1, 3, 6, 24, and 48 hrs post-OLT and serum bilirubin levels were measured by standard enzymatic methods in the clinical laboratory at UPMC (Pittsburgh, PA).

4.2.9 Statistical Analysis

Data are presented as the mean ± SEM. Comparisons between the groups were performed by one-way ANOVA with Tukey post-hoc test using GraphPad Prism software Version 4.0 (San Diego, CA). Differences were considered significant at a P-value < 0.05.

4.3 RESULTS

4.3.1 Hepatic Drug Transporter mRNA Expression

The effect of I/R injury on the mRNA expression of hepatic drug transporters in rat liver graft tissue was studied at 1, 3, 6, and 48 hrs post-OLT. The mRNA levels of Oatp1a1, Oatp1a4, Ntcp, and Oct1 were significantly decreased post-OLT, shown in Figures 30A-D, respectively. Specifically, at 48 hr post-OLT, the mRNA levels in the placebo-treated group had decreased to

57 ± 14.3%, 8.2 ± 1.2%, 43.1 ± 1.5%, and 35.9 ± 5.0% of normal (Figure 30A-D), respectively. While no improvement in Oatp1a1 mRNA expression group was observed (50.3 ± 5.2% of normal), treprostinil improved mRNA expression of Oatp1a4, Ntcp, and Oct1 to 24.1 ± 1.6%, 56.9 ± 6.9%, and 42.1 ± 5.1% of normal, respectively.

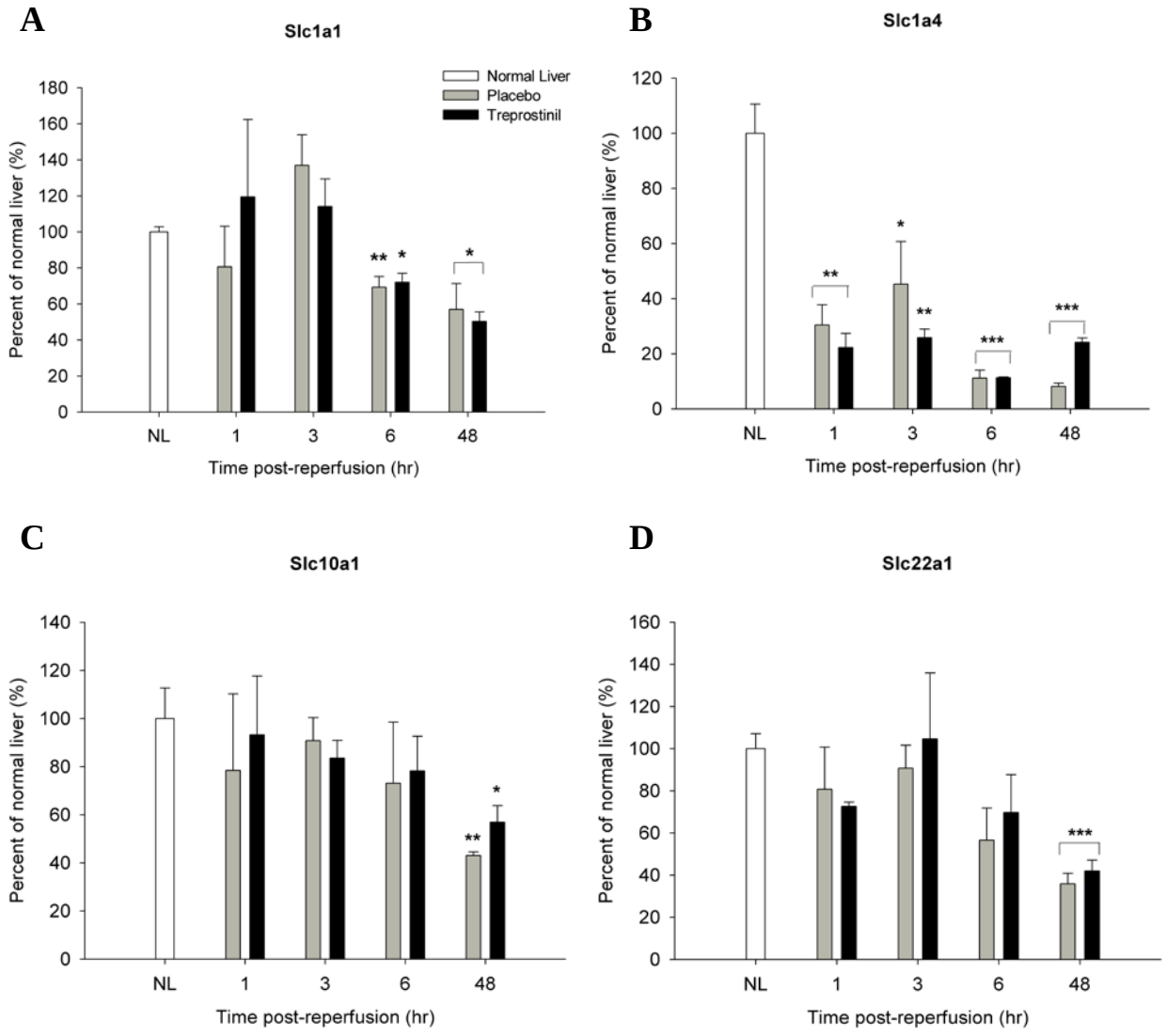


Figure 30: Hepatic mRNA expression of hepatic uptake transporters
(A) Oatp1a1/Slc1a1, (B) Oatp1a/Slc1a4, (C) Ntcp/Slc10a1, and (D) Oct1/Slc22a1 in liver graft tissue at 1, 3, 6, and 48 hr post-OLT. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. normal (n=3).

The mRNA levels of hepatic efflux transporters were also down-regulated in liver grafts post-OLT. At 6 hr post-OLT, the mRNA expression of P-gp (Mdr1a) was significantly reduced to 14 ± 5.2% of normal, whereas treprostinil significantly up-regulated P-gp expression to 144 ± 40.1% of normal at 3 hr post-OLT and improved expression to almost three-fold that of placebo (40 ± 14.3%) at 6 hrs post-OLT, shown in Figure 31A. At 48 hrs post-OLT, no improvement in the placebo group was observed (16 ± 7% of normal), whereas treprostinil improved levels to approximately two-fold of placebo (30 ± 7.3%). At 6 hr post-OLT, Mdr2 (Abcb4) and Mrp2 (Abcc2) levels declined to 31 ± 6.4% and 10 ± 3.1% of normal, respectively, in the placebo-treated group. In contrast, treprostinil improved Mdr2 and Mrp2 to 50 ± 7.6% and 20 ± 5.5% of normal (Figure 31B and 31C), respectively. Mrp3 (Abcc3) expression gradually increased in the placebo-treated group to a peak of 77 ± 8.5% of normal at 48 hr post-OLT, where treprostinil restored Mrp3 expression to normal by 6 hr post-OLT (Figure 31D). In the placebo-treated group, Bsep (Abcb11) levels reached a low of 27 ± 15.2% of normal at 48 hr post-OLT. Treprostinil preserved Bsep mRNA expression similar to normal throughout the post-operative study period, shown in Figure 31E. The results indicate that hepatic drug transporters are significantly altered post-OLT and suggest that treprostinil may be involved in transcriptional regulation or stabilization of some of these transporter proteins.

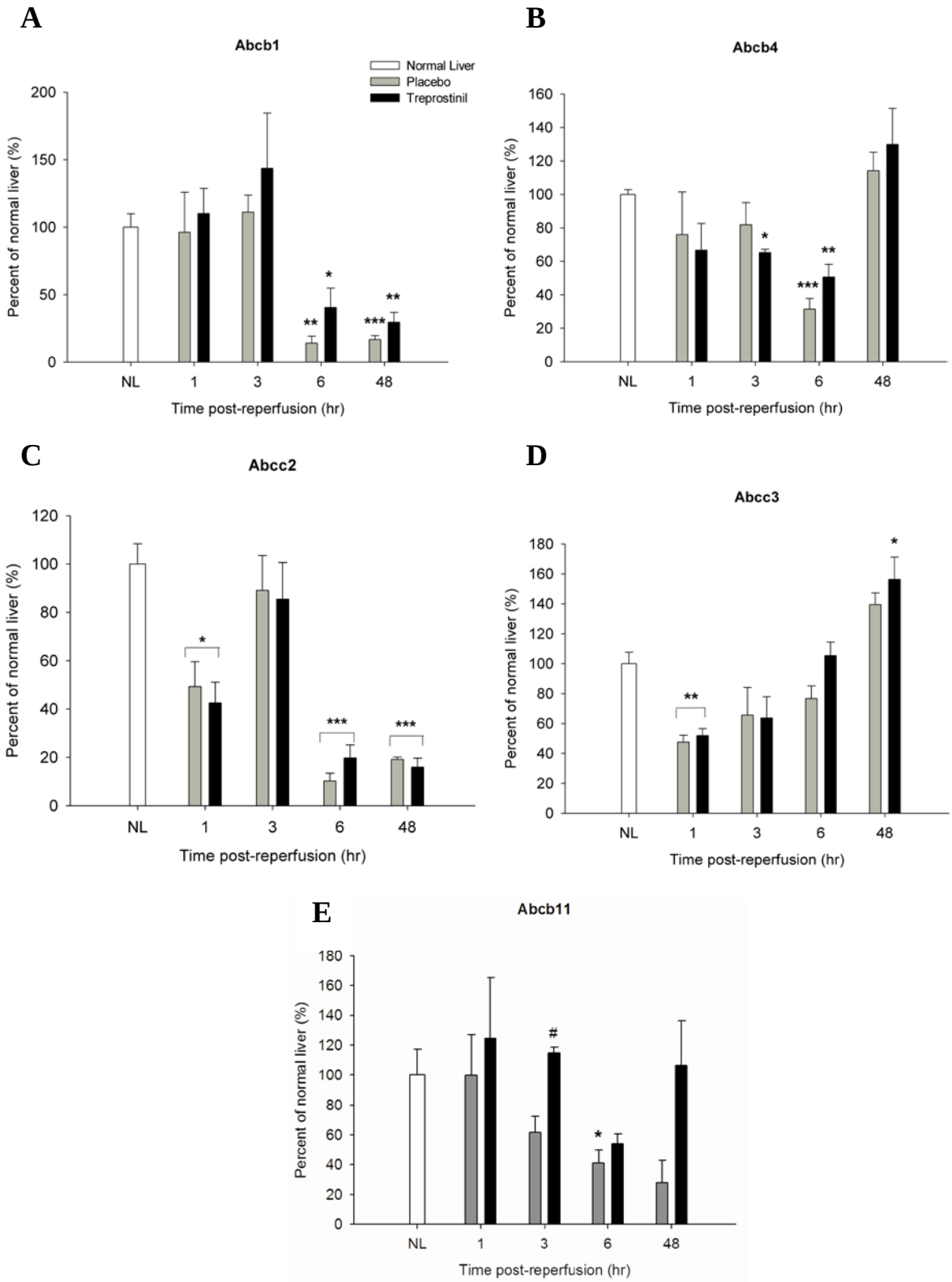


Figure 31: Hepatic mRNA expression of efflux transporters

(A) P-gp/Mdr1a, (B) Mdr2/Abcb4, (C) Mrp2/Abcc2, (D) Mrp3/AbcC3, and (E) Bsep/AbcC11 at 1, 3, 6, and 48 hr post-OLT. Results are expressed as a percentage of NL, **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. normal; #*P* < 0.05 vs. placebo (n=3).

4.3.2 Mrp2 and P-gp Protein Expression in Liver Graft Post-OLT

To determine whether the changes in mRNA also occurred at the protein level, hepatic microsomal expression of Mrp2 and P-gp protein at 48 hr post-OLT were studied since this time point demonstrated the most significant changes in mRNA expression in the liver grafts of placebo- and treprostinil-treated animals. In the placebo-treated group, the expression of Mrp2 and P-gp were 144.1 ± 13.3% and 124.5 ± 3.7% of normal, respectively, shown in Figure 32 and 33. Interestingly, in contrast to mRNA down-regulation, in the treprostinil-treated group, Mrp2 and P-gp protein was up-regulated to 179 ± 7.6% and 159 ± 5.4% of normal, respectively. Also, the appearance of Mrp2 protein bands as doublets (190 and 200 kDa) was observed. The results indicate a discrepancy exists between Mrp2 and P-gp mRNA and protein levels in liver graft as a result of I/R injury post-OLT.

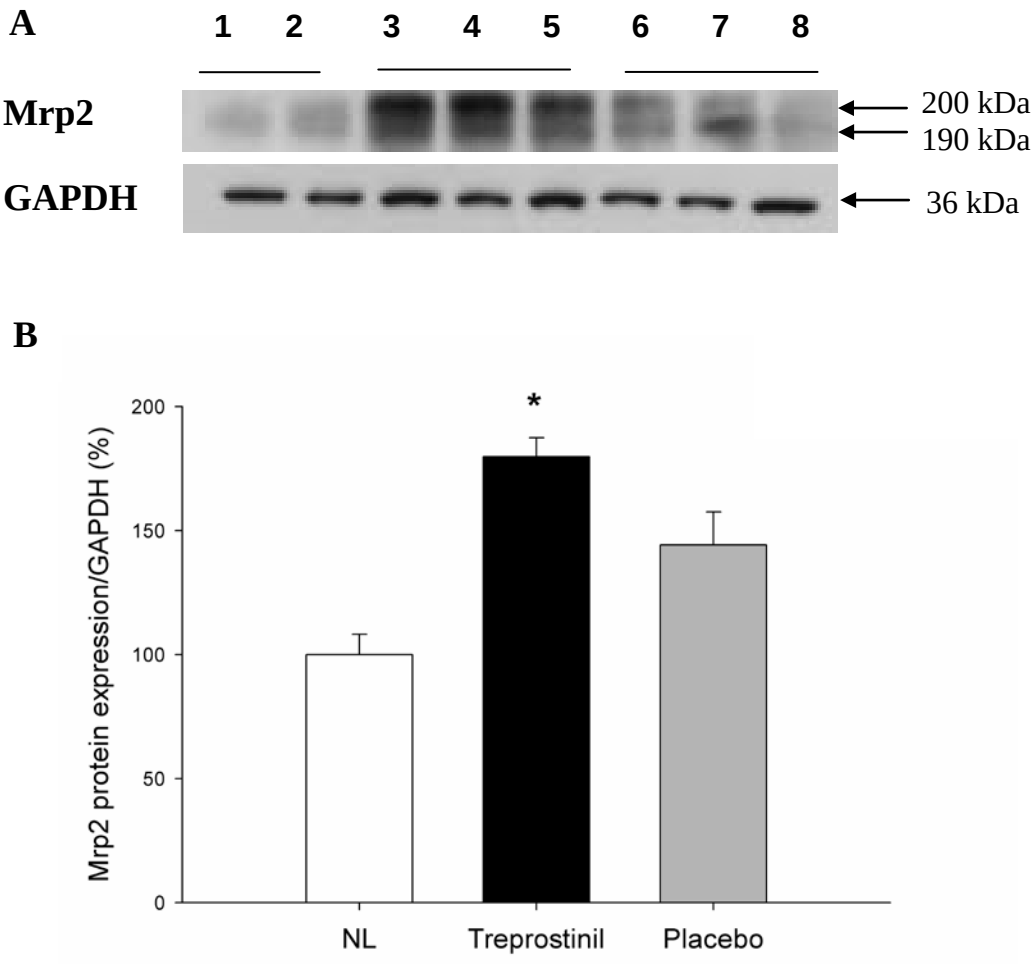


Figure 32: Hepatic microsomal Mrp2 protein expression

(A) Western blot analysis of normal (lanes 1-2), treprostinil-treated (3-5), and placebo-treated (6-8) animals at 48 hr post-OLT. (B) Data are expressed as a percentage of normal liver, normalized to GAPDH expression; **P* < 0.05 vs. normal.

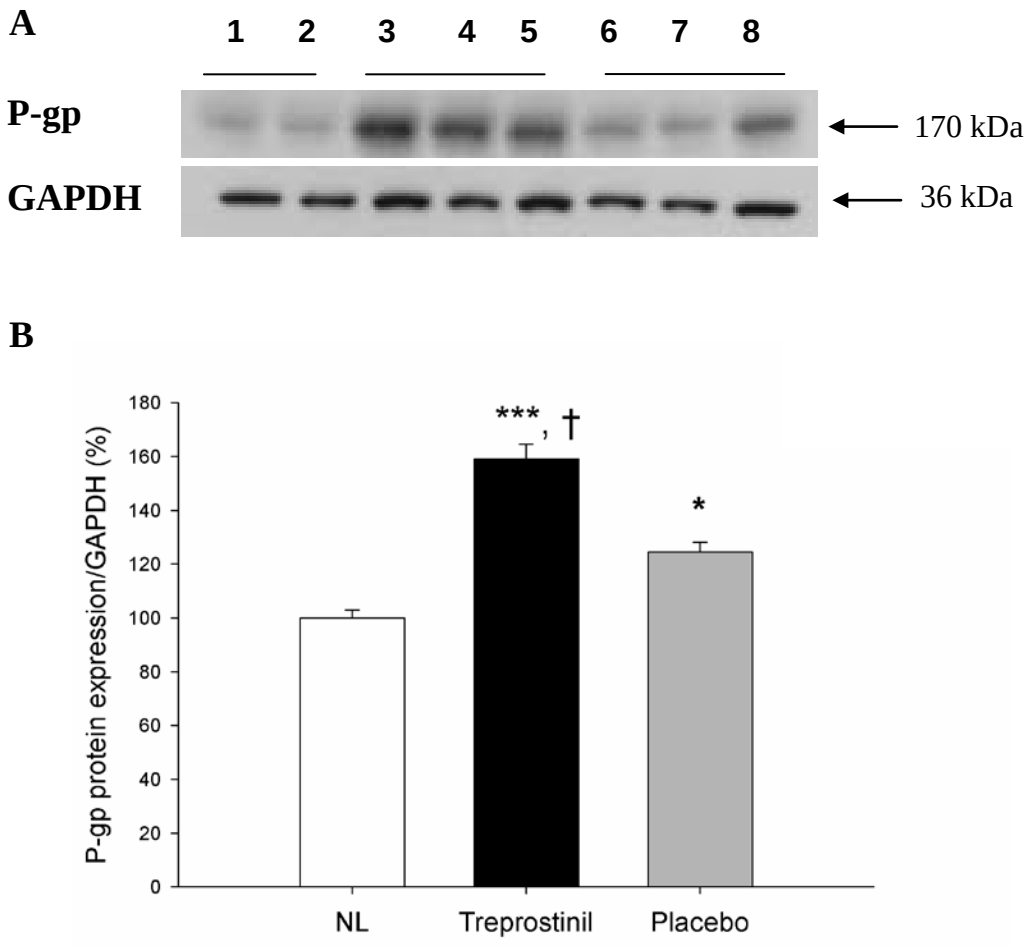


Figure 33: Hepatic microsomal P-gp protein expression

(A) Western blot analysis of normal (lanes 1-2), treprostinil-treated (3-5), and placebo-treated (6-8) animals at 48 hr post-OLT. (B) Data are expressed as a percentage of normal liver, normalized to GAPDH expression; **P* < 0.05, ****P* < 0.001 vs. normal; †*P* < 0.01 vs. placebo.

4.3.3 Serum Bilirubin

Bilirubin is rapidly and selectively taken up into the liver [195, 196], biotransformed upon conjugation and secreted into bile across the canalicular membrane of hepatocytes by Mrp2 [197, 198]. Serving as a marker of hepatic function, serum bilirubin concentrations were measured at 1, 3, 6, 24, and 48 hrs post-reperfusion in placebo- and treprostinil-treated groups. Normal rats not subjected to OLT served as a baseline value of 0.17 ± 0.06 mg/dl, shown in Figure 34. In the placebo, total serum bilirubin peaked at 3-hr post-OLT (0.38 ± 0.11 mg/dl) and gradually returned to baseline by 24 hrs post-OLT. Alternatively, treprostinil-treated animals had a lower peak at 1 hr post-OLT (0.30 ± 0.10 mg/dl) and returned to baseline by 3 hr post-OLT. These results indicate that treprostinil maintained biliary excretion of bilirubin early post-OLT, which suggests that treprostinil preserved the hepatobiliary transport processes early post-OLT.

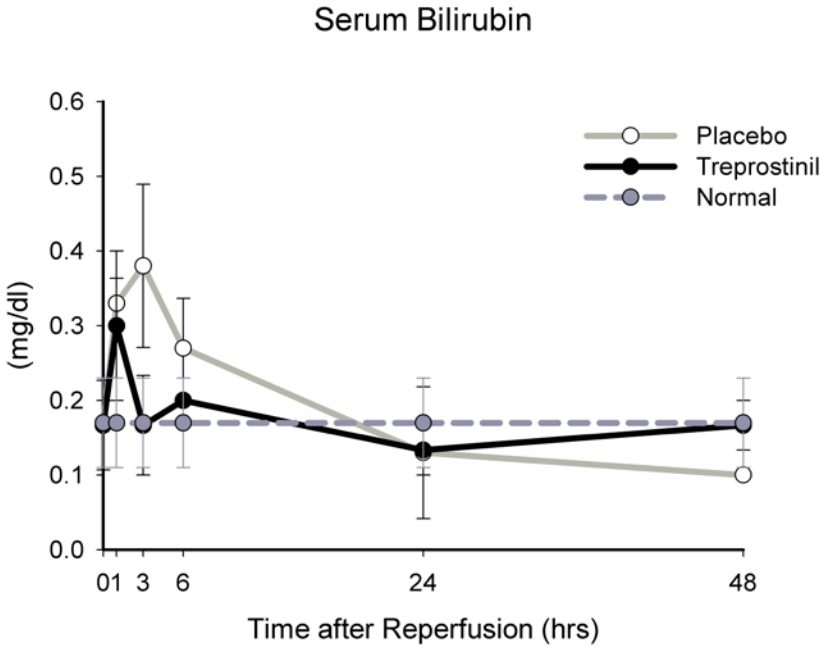


Figure 34: Serum bilirubin in placebo- and treprostinil-treated group, compared to normal liver (n=3).

4.4 DISCUSSION

During liver transplantation, I/R injury often leads to damaged hepatocytes and bile duct cells, resulting in altered biliary secretion of endogenous compounds and altered pharmacokinetics of drugs in the recipients as a consequence of down-regulated hepatic drug transport expression. While the role of hepatic transporters continues to evolve, evidence of their role in drug disposition after liver transplantation was observed several years ago. Initial observations of a high RIA to HPLC ratio for cyclosporine A (RIA measuring parent and metabolite, while HPLC measuring the parent drug), indicated that formation of the metabolites was not altered but that biliary transport of the formed metabolites was, in grafts which exhibited early poor function post-OLT [94]. It was later shown that cyclosporine A is a P-gp substrate [95]. Similarly, ceftriaxone, a third generation antibiotic, is excreted (approximately 40%) into the bile by the MRP2 protein and a lower clearance of this drug has been reported following OLT, suggesting hepatic dysfunction at the transporter level [96]. Thirdly, the plasma chlorzoxazone metabolic ratio (metabolite/parent) in liver transplant recipients was significantly elevated post-transplantation compared to healthy controls [199]. At the time, the results were attributed to an increase in CYP2E1 activity via the induction response by cytokines; however, the multiple mechanisms involved in drug disposition including transport proteins are now recognized. Several of the immunosuppressive medications that transplant patients take to prevent rejection are substrates for various hepatic transport proteins and consequently alterations in protein expression could further complicate transplantation outcomes.

The hepatic excretion of a large variety of endogenous and exogenous compounds from hepatocytes into bile is an ATP-dependent process, which is performed primarily by members of

the ATP-binding cassette (ABC) protein superfamily, including the Mdr and Mrp subfamilies [72]. In Chapter 2, we demonstrated that hepatic tissue levels of ATP were significantly reduced in liver grafts post-reperfusion and that administration of treprostinil restored ATP levels in liver grafts soon after reperfusion and lasted throughout the post-operative period studied. Extending this finding, we hypothesized that liver graft activity of hepatic uptake and efflux drug transporters would be down-regulated after OLT, secondary to reduced tissue ATP. Given that pro-inflammatory cytokine expression is increased after OLT, we hypothesized that expression of hepatic transporters would be decreased post-OLT. Since treprostinil increased hepatic concentration of ATP and decreased the mRNA expression of pro-inflammatory cytokines, we expected treprostinil to minimize the loss of expression and activity of these hepatic transporters.

Mrp2 is a 190 kDa glycoprotein located in the canalicular membrane of the hepatocytes and several lines of evidence have shown that Mrp2 protein is under post-transcriptional regulation, in addition to classical translational regulation [200]. Regulation of Mrp2/MRP2 function occurs by at least three distinct levels, including endocytic retrieval from the canalicular membrane of the hepatocyte, transcriptional, and translational regulation [201]. Glycosylation is critical for normal health and development, as it is an important step for proper protein folding, stabilization, localization, and function of newly synthesized proteins and it is also a common post-translational modification of membrane proteins [202]. Some glycoproteins require glycosylation for their trafficking from the ER to the apical membrane of the hepatocyte. In particular, Bsep is reported to require at least two of its four N-linked glycans for proper protein stability, intracellular trafficking and functional activity [203].

P-gp (Mdr1a/b), a 170 kDa glycoprotein, is present at low levels along the canalicular membrane in normal rodent liver [72, 204]. While over-expression of P-gp has been shown to

confer resistance against a broad variety of natural product drugs [205], up-regulation of these proteins during oxidative stress has also been shown to serve as a protective mechanism to preserve hepatic efflux as a mechanism to reduce hepatic accumulation of bile salts and down-stream consequences [192]. The expression of transport proteins is highly variable and subject to complex transcriptional regulation, predominately regulated by nuclear hormone receptors. Nuclear receptor regulation of hepatic transporter expression by Farnesoid X receptor and Pregnane X receptor have been shown to be involved in the regulation of bile acid levels and Bsep expression [206]. This particular area of research requires further investigation.

Following tissue injury, e.g. LPS, the acute phase reaction lasts approximately 24 hrs [207] so it was not unusual that Mrp2 and P-gp protein expression in the placebo-treated group had returned to normal by 48 hr post-OLT (Figure 32 and 33, respectively). Interestingly, Mrp2 and P-gp protein expression in the treprostinil-treated groups rebounded at 48 hr post-OLT. Further, immunodetection of a second Mrp2 band at 200 kDa in the treprostinil-treated group is indicative of post-translational modification by protein glycosylation, which is consistent with previous findings [207, 208]. The discrepancy between mRNA and protein expression of Mrp2 suggests that treprostinil mediates post-transcriptional and translational regulation of certain transporters. Similar discrepancies between the mRNA and protein expression of Mrp2 [209, 210] and BSEP [207] have been described, which supports the theory that different ABC transporters are mediated by post-transcriptional and translational regulation *in vitro* and *in vivo* as well as in different species.

The C219 antibody recognizes an epitope on all classes of P-gp, including Mdr1, Mdr2, and Bsep [211]. The up-regulation of P-gp protein expression in the treprostinil-treated group may be a cumulative effect of significantly increased P-gp and Mdr2 and preserved Bsep mRNA

expression (Figures 31A, 31B, and 31E, respectively). Administration of dibutyryl cAMP has been shown to stimulate bile flow [212] and influence sorting of Mrp2 to the apical membrane [213]. In Chapter two, we showed that treprostinil administration markedly up-regulated liver graft cAMP levels early post-OLT throughout the post-operative period. Taken together, the additive effects of preserved tissue ATP content and Bsep mRNA expression in addition to up-regulated hepatic mRNA levels of P-gp, tissue cAMP and Mrp2 protein are likely to have contributed to improved hepatic transport of serum bilirubin early post-OLT. Further detailed investigation is needed to reveal the mediators that regulate hepatic drug transporter expression in liver graft post-OLT.

An integral component of I/R-associated liver injury during OLT is the activation of the pro-inflammatory cascade, resulting in the production of pro-inflammatory cytokines. These cytokines are likely to be involved in the decreased mRNA expression of uptake and efflux hepatic transporters. Inflammation has been linked to reduced Mrp2 mRNA expression and increased Mrp3 protein levels, to compensate for diminished Mrp2 transport capacity in response to inflammation as a protective mechanism to reduce hepatic accumulation of bile salts and the down-stream hepatotoxicity [214, 215]. It is important to note that different bacterial strains of LPS as well as different causative inflammatory conditions can elicit different responses in cytokine release and consequently different patterns of gene expression [216]. Considering the liver is the most important site of drug metabolism and clearance, inflammatory-mediated changes in the expression of hepatic transporters can have major implications when the capacity of the liver, such as the case during liver transplantation, and other organs to handle drugs is severely compromised. Most animal studies of hepatic I/R injury utilize partial ischemia, i.e. 70% occlusion [51, 217], or ligation of the hepatic artery [218]. A major limitation of these

models is the induction of warm ischemia, which does not reflect cold ischemic injury that takes place prior to transplantation, during graft storage, and, therefore, does not fully represent the effects of I/R injury on hepatic drug transport processes associated with clinical OLT.

Treprostinil has been shown to inhibit the secretion and gene expression of many pro-inflammatory cytokines by blocking the translocation of NF-kB *in vitro* [187]. Again, in Chapter two we showed that treprostinil reduced significantly increased mRNA levels of TNF-α, IFN-γ, IL-6, and ICAM-1 in liver graft post-OLT, and increased IL-10 mRNA early post-reperfusion. Taken together, the preserved ATP content, reduced pro-inflammatory cytokine and increased IL-10 mRNA, as well as increased cAMP levels in liver graft are likely to account for the improved response in hepatic drug transport processes in the treprostinil-treated group.

In summary, this study showed that I/R injury lead to impaired hepatobiliary functions and altered the expression of hepatic uptake and efflux transporters in liver grafts after rat OLT and these effects were partially alleviated by treprostinil administration. To the best of our knowledge, this is the first study to directly examine the effects of I/R injury on the mRNA and protein expression of the major hepatic transporters in a rat OLT model.

5.0 EVALUATION OF THE DRUG-DRUG INTERACTION POTENTIAL BETWEEN TREPROSTINIL AND IMMUNOSUPPRESSIVE MEDICATIONS*

*N. Ghonem, S. Zhang, A. Sharma, S.C. Strom, R. Venkataramanan. Evaluation of the Drug-Drug Interaction Potential between Treprostinil and Immunosuppressive Medications *In Vitro*. Submitted to *Drug Metabolism and Disposition,* December 2010.

5.1 INTRODUCTION

Orthotopic liver transplantation (OLT) is the only curative therapy available for patients with various end-stage liver diseases. The surgical procedure subjects the transplanted graft to varying periods of cold ischemia and warm reperfusion, which inevitably results in varying degrees of hepatic injury and dysfunction [25]. I/R injury is an unavoidable process in OLT and it is the major cause of both initial poor function and PNF of the liver, with a high mortality rate, if patients are not re-transplanted immediately. The need to prevent I/R injury is imperative; however, no therapy is commercially available.

In Chapter two, it was demonstrated that treprostinil protected liver grafts against I/R injury during rat OLT and treprostinil is now being examined for its safety and efficacy in ameliorating I/R injury in adult patients undergoing OLT. Treprostinil, a stable analogue of prostacyclin (PGI_2), is approved by the US Food and Drug Administration for the treatment of pulmonary arterial hypertension (Remodulin®). Treprostinil is substantially metabolized by the liver, but the precise enzymes responsible are unknown [113]. Results of *in vitro* cytochrome P450 studies performed in expressed enzymes show that treprostinil does not inhibit CYP1A2, 2C9, 2C19, 2D6, 2E1, or 3A [113], however, whether or not treprostinil induces these enzymes has not been evaluated.

Immunosuppressive drugs such as cyclosporine, tacrolimus, and sirolimus predominantly undergo hepatic metabolism via cytochrome P450 (CYP) 3A4 [219]. MMF, an ester prodrug of mycophenolic acid (MPA) [220] predominantly undergoes hepatic glucuronidation via UDP-glucuronosyltransferase (UGT) 1A9 to its glucuronide metabolite (MPAG) and, to a lesser extent, by UGT2B7 to its non-active MPA-acyl-glucuronide metabolite (AcMPAG) [221]. In addition to immunosuppressants, most solid organ transplant recipients are prescribed additional

medications to treat transplant-associated conditions, which occur either secondary to transplantation or are underlying conditions, including but not limited to osteoporosis, hypertension, bacterial and or fungal infections, ulcers, high cholesterol, and depression, i.e. antihypertensive, antibacterial and antifungal agents, anti-ulcer, cholesterol-lowering agents, and antidepressant medications [222, 223]. Several of these agents are also metabolized by CYP3A and UGTs, therefore, each of these medications have the potential to interact with treprostinil. Furthermore, literature reports have shown that some prostaglandin analogues, including PGI_2, can alter the clearance and or half-life of certain immunosuppressive agents [224, 225]. The risk for a DDI must be thoroughly investigated whenever a new drug is added to a regimen in a transplant patient since most of the immunosuppressive drugs have a narrow therapeutic index. In transplant recipients, supra- or sub-therapeutic blood/plasma concentrations can increase the risk of organ rejection, or lead to infection or drug-specific side effects, respectively.

The objective of this study was to examine *in vitro* the DDI potential of treprostinil when co-administered with CsA, TAC, SRL, or MPA. While treprostinil is not a new molecular entity *per se*, the potential use of treprostinil in OLT would be a new indication and, as such, in addition to providing essential data regarding the potential for a DDI, this study also complies with the FDA guidelines, which requires *in vitro* DDI studies be performed prior to a drug entering a clinical trial [226].

5.2 MATERIALS AND METHODS

5.2.1 Chemicals

Treprostinil was supplied by United Therapeutics (Research Triangle Park, NC), Cyclosporine A was purchased from USP Pharmacopeia (Rockville, MD). Tacrolimus (TAC) was supplied by Fujisawa (Osaka, Japan), Sirolimus was purchased from LC Laboratories (Woburn, MA) and 32-desmethoxyrapamycin was a gift from the University of Pittsburgh Medical Center, Pittsburgh, PA. Dimethyl sulfoxide (DMSO), mycophenolic acid (MPA), nicotinamide adenine dinucleotide phosphate-oxidase (NADPH), uridine 5'diphospho-glucuronic acid (UDPGA), Brij 57, rifampicin (RIF), ketoconazole (KTZ), and ascomycin were purchased from Sigma (St. Louis, MO). Mycophenolic acid glucuronide (MPAG) was a generous gift from Professor Leslie Shaw (University of Pennsylvania, Philadelphia, PA). Bovine serum albumin (BSA) was purchased from Fluka (Buchs, Switzerland, 98% pure). Hepatocyte maintenance medium (HMM) and medium supplements, dexamethasone and insulin, were obtained from BioWhittaker (Walkersville, MD). Penicillin G/streptomycin was purchased from Gibco Laboratories (Grand Island, NY). Falcon 6-well culture plates were purchased from Becton Labware (Franklin Lakes, NJ). Reagents for real-time RT-PCR were purchased from Promega (Madison, WI). Forward and reverse primers were purchased from Applied Biosystems (Foster City, CA). Methanol and water [high-performance liquid chromatography (HPLC) grade] were purchased from Thermo Fisher Scientific (Waltham, MA). All other chemicals used were of HPLC grade or the highest purity available.

5.2.2 Evaluation of CsA, TAC, SRL, and MPA inhibition in microsomes

5.2.2.1 Microsome Preparation

Microsomes were prepared from five human liver lobes by a standard differential centrifugation procedure with minor modifications [172]. Briefly, liver pieces were homogenized with 3 volumes of a homogenization buffer (50 mM Tris-HCl buffer, 1.0% KCl, and 1 mM EDTA, pH 7.4) using an electrical homogenizer (Polytron, Brinkmann Instruments, Westbury, NY). The crude homogenate was centrifuged (Optima XL-100K ultracentrifuge, Beckman Instruments, Palo Alto, CA) at 10,000*g* for 20 minutes at 4 °C. The supernatant was further centrifuged at 105,000*g* for 65 min at 4 °C to sediment the microsomes. The microsomes were reconstituted using a manual glass homogenizer (Wheaton, Millville, NJ) in twice their weight of with 50 mM Tris-HCl buffer (pH 7.4) containing 20% glycerol. Aliquots were immediately stored at -80 °C until used. The protein concentration was determined by Lowry's method [227] using BSA as standard.

5.2.2.2 Microsome Incubations

Optimal conditions for microsomal incubations with CsA, TAC, SRL, and MPA were determined by performing separate studies for the time of incubation (0-120 minutes) and protein concentration (0–2 mg/ml). To work in the linear range, the time selected for CsA, TAC, and MPAG was 30 minutes and for SRL 15 minutes; the protein concentration selected for CSA and SRL, TAC, and MPA was 0.3, 0.2, and 0.4 mg/ml, respectively. The microsomal incubation included CsA, TAC, SRL, and MPA, pooled and mixed together from five different human livers, $MgCl_2$ (10 mM), and phosphate buffer (0.1 mM), pH=7.4. The incubation was allowed to pre-equilibrate in a shaking water bath for approximately 5 minutes at 37 °C. Each drug

equilibrated in a shaking water bath under the experimental conditions that were determined to be linear at 37 ^{o}C in the absence or presence of treprostinil. For CsA, TAC, and SRL microsomal reactions, NADPH (1 mM) was added to initiate the reactions. For MPA microsomal reaction, the incubation also included Brij 58 (0.1 mg/mg protein) and UDPGA (1 mM) to initiate reaction. The concentration of CsA (5 ug/ml), TAC (200 ng/ml), SRL (200 ng/ml), MPA (10 ug/ml), and treprostinil (10, 50, and 100 ng/ml) were selected based on clinically relevant blood concentrations. All reactions were terminated upon addition of ice-cold methanol. Following termination, CsA and 32-desmethoxyrapamycin were added as internal standard for TAC and SRL, respectively. Samples were then centrifuged at 3,000 *rpm* for 10 minutes at 4 ^{o}C and the supernatant was subjected to solid phase extraction (SPE). Under experimental conditions described above, control samples represented regular metabolism, omission of co-factor served as negative control. Co-incubation with ketoconazole (KTZ, 0.5 uM) served as positive control.

5.2.3 Solid Phase Extraction (SPE)

An Oasis HLB C18 cartridge (Waters, Milford, MA) was pre-equilibrated with 1 ml of HPLC grade methanol followed by 1 ml of HPLC grade water. The supernatant was passed through the column and washed with 40% methanol. CsA, TAC, and SRL were individually eluted from the sample preparation column with dichloromethane. The organic phase was transferred to a new tube and the liquid evaporated to dryness under air. Samples were reconstituted with mobile phase and analyzed immediately.

5.2.4 Evaluation of CsA, TAC, SRL, and MPA Induction in Human Hepatocytes

5.2.4.1 Preparation of Human Hepatocytes

Hepatocytes were prepared from human liver samples obtained from the Liver Tissue Cell Distribution System, from the Hepatocyte Transplantation Laboratory at the University of Pittsburgh (Pittsburgh, PA). Donors of human liver tissue had no history of liver disease, but the liver was not used for transplantation or the patient underwent liver resection for different pathologies. Informed consent was obtained from all patients for the use of liver tissue for research purposes. Hepatocytes were prepared by a three-step collagenase perfusion technique [228]. Cell viability was determined by the trypan blue exclusion method and ranged from 71 to 92%. Briefly, equal volumes of trypan blue (0.4%) and cell suspension were mixed and a portion of this suspension was then placed on a hemocytometer. The cells were observed under a light microscopy and the numbers of live and dead cells (stained blue), were counted in two fields. Concentration of cells (number of cells / ml) was determined using the following formula: Live cells in two fields x 10,000 = # of cells/ml. Cells were diluted to final volume of 1 x 10^6 cells/mL. Hepatocytes were plated on Falcon 6-well culture plates at a density of 1.5 x 10^6 cells, previously coated with rat tail collagen, and maintained in Hepatocyte Maintenance Medium (HMM; Lonza Walkerville, Inc.) supplemented with 0.1 μM insulin, 0.1 μM dexamethasone, 0.05% streptomycin, 0.05% penicillin, 0.05% amphotericin B and 10% bovine calf serum. After cells attached for 4 to 6 h, medium was replaced with serum-free medium containing all of the supplements described above (HMM+). Cells were maintained in culture at 37 °C in an atmosphere containing 5% CO_2 and 95% air. After 24 h in culture, unattached cells were removed by gentle agitation and the medium was changed every 24 hours.

5.2.4.2 Hepatocyte Incubations

After allowing the cells to acclimate for 48 hr after plating, cells were treated with HMM+ containing rifampicin (RIF 10 uM), phenobarbital (PB 2 mM), or treprostinil (10, 50, and 100 ng/ml), all dissolved in DMSO, except for PB (water) for 96 hrs. The final concentration of DMSO (vehicle control) in culture medium was 0.1%. The cells were observed daily under a phase microscope to monitor attachment and cell morphology. At the end of the incubation period, cells were washed with HMM devoid of insulin, dexamethasone, antibiotics and amphotericin B. Following this wash period, media containing CsA (10 ug/ml), TAC (500 ng/ml), SRL (500 ng/ml), or MPA (10 ug/ml) was applied to the cells and sampled for the time of incubation. At the end of treatment, hepatocytes and the media were collected into 1.5 mL Eppendorf tubes. The samples were sonicated and centrifuged at 3,000*rpm* for 5 minutes at 4 ^{o}C. Cyclosporine D, ascomycin, 32-desmethoxyrapamycin were added as internal standard for CsA, TAC, and SRL. Following centrifugation, SPE was applied to the supernatant. Time zero reflects the time at which no metabolism took place and is referred to as the original concentration. Control samples reflect regular metabolism when hepatocytes were pretreated with vehicle control. Co-treatment with KTZ served as negative control. Rifampicin served as positive control for CYP3A4- and phenobarbital served as positive control for CYP3A4-, UGT1A9- and UGT2B7-mediated metabolism. To account for donor variability, experiments were performed in hepatocytes from at least three different donors. Characteristics of human liver donors are listed in Table 8.

Table 7: Donor information for human livers used to prepare primary culture of human hepatocytes

Donor	Sex	Age (yrs)	Medical History	Viability (%)
HH997	M	43	Brain Death	73
HH1117	F	68	Brain Death	82
HH1234	M	56	Anoxia	87
HH1286	M	50	ICH	90
HH1336	M	54	ICH	77
HH1426	F	23	Anoxia	92
HH1432	F	72	MCC	77
HH1434	F	71	MCC	88
HH1454	F	42	Breast adenocarcinoma	80
HH1456	F	50	MCC	80
HH1458	M	43	ICH	88
HH1460	F	46	MCC	85
HH1464	F	70	HCC, cirrhosis	75
HH1466	F	68	CC	82
HH1467	F	52	MCC	89
HH1469	F	46	Anoxia	88
HH1492	F	45	MCC	86
HH1511	F	67	MCC	71
HH1582	F	25	Encephalopathy	81
HH1602	M	40	Head trauma	81
HH1606	M	28	MCC	74

M, male; F, female; MCC: Metastatic colon cancer; ICH: Intracranial hemorrhage.

5.2.4.3 Evaluation of the hepatocyte mitochondrial activity

The MTT assay was performed according to manufacturer's guidelines (Invitrogen, Carlsbad, CA). Cells were treated with DMSO, RIF (10 uM), PB (2 mM), or treprostinil (10, 50, and 100 ng/ml), After 72 hours in culture, cells were incubated with the tetrazolium salt [(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole), MTT] dissolved in culture

media for approximately 30 minutes, washed in blank HMM, and treated with isopropyl alcohol. The conversion of MTT into aqueous, soluble, formazan by metabolically active cells was determined by measuring the absorbance at 490 nm.

5.2.5 Analytical methodology

5.2.5.1 Microsomal incubations

CsA, TAC, and SRL were individually analyzed on an HPLC system that consisted of an autosampler (Waters 717, Milford, MA) and a solvent delivery system (Waters 600E), attached to a UV detector (Waters 486), set at 214 nm (for CsA and TAC) and 278 nm (for SRL). The mobile phase consisted of 68% (CsA and SRL) and 60% acetonitrile (TAC) in water. MPA and MPAG were analyzed on an Alliance HPLC system (Waters 2695, Milford, MA) attached to a photodiode array detector (PDA, Waters 2998) set at 254 nm was used. The mobile phase consisted of 76% acetonitrile in water containing 0.05% phosphoric acid. Each compound was separated individually using a Symmetry® C18 (4.6 x 250 mm, 5um) column (Waters). The concentration of each compound was determined from a linear standard curve of the known concentrations for CsA (0.5 – 5 ug/ml), TAC (0.2 –5 ug/ml), SRL (0.2 – 5 ug/ml), and MPAG (0.675 – 10 ug/ml). The C.V was less than 10% per assay.

5.2.5.2 Hepatocyte incubations

CsA and SRL were quantified using the HPLC system described above. Following SPE, samples for CsA and SRL were reconstituted with mobile phase consisting of 68% acetonitrile and 85% methanol in water, respectively. CsA, CYD, SRL, and 32-desmethoxyrapamycin were separated using a Symmetry® C18 (4.6 x 250 mm, 5 um) column (Waters). The concentration

of each compound was determined from a linear standard curve of the known concentrations for CsA (0.5 – 10 ug/ml) and SRL (0.5 – 15 ug/ml).

The concentration of TAC was determined using the Acquity® Ultra Performance Acquity Liquid Chromatography (Waters) system with Thermo Finnigan TSQ® Quantum Ultra triple quadrupole mass spectrometer (Thermo Scientific), operated in positive electrospray ionization mode with unit resolutions at both Q1 and Q3 set at 0.70 full width at half maximum. The selected reaction monitoring (SRM) transitions of m/z 821.4→ 768.3 (collision energy 10 eV, scan time 0.01 s) for TAC and m/z 809.4→ 756.0 (collision energy 19 eV, scan time 0.20 s) for ascomycin were monitored. Parameters were optimized to obtain the highest [M+H] ion abundance and were as follows: capillary temperature, 360 °C; spray voltage, 3000 V. Sheath gas, auxiliary gas, and ion sweep gas pressures were set at 43, 37, and 0, respectively. Collision gas pressure was set at 1.0 mTorr. TAC and ascomycin were separated using an Acquity® UPLC BEH C18 1.7 um (2.1 μm x 100 mm) column (Waters). Following SPE, samples were reconstituted with mobile phase consisting of 60 % acetonitrile in water. The standard curve was linear from 0.15 – 5 ug/ml. The C.V was less than 5% for this assay.

The concentration of MPA and MPAG were detected using the HPLC system described above. The mobile phase consisted of (A) 61% NaAc-HA in acetonitrile, pH 4.4, (B) methanol, (C) 5% methanol in water, and (D) acetonitrile. MPA and MPAG were separated using a Symmetry® C18 (4.6 x 250 mm, 5um) column (Waters) set at 25 ^{o}C. The standard curve was linear from 0.675 – 10 ug/ml.

5.2.6 CYP3A4, UGT1A9, and UGT2B7 mRNA expression

5.2.6.1 RNA Isolation and Quantitation

Total RNA was extracted from primary culture human hepatocytes using TRIzol reagent (Invitrogen, Carlsbad, CA) as described in Chapter 2. Real-time RT-PCR was performed with the TaqMan® system and conditions designated by Assays on Demand, Gene Expression Products (Applied Biosystems, Forster City, CA). The primers were purchased from Applied Biosystems, and are listed in Table 9. The mRNA expression levels were calculated based on the threshold cycles using the Applied Biosystems sequence detection system software, version 2.0 (Applied Biosystems). Samples were analyzed in triplicate and relative gene expression was measured using the comparative C_T method, using CYC as internal control.

Table 8: Real-time PCR Assay IDs for genes detected by TaqMan® gene expression

Gene Symbol	Gene Name	RefSeq Accession #
CYP3A4	cytochrome P450, family 3, subfamily A, polypeptide 4	NM_017460.3
UGT1A9	UDP glucuronosyltransferase 1 family, polypeptide A9	NM_021027.2
UGT2B7	UDP glucuronosyltransferase 2 family, polypeptide B7	NM_001074.2
CYC	peptidylprolyl isomerase A (cyclophilin A)	NM_021130.3

5.2.7 Statistical Analysis

The data are presented as the mean ± SEM. One-way ANOVA followed by group comparisons using Dunnette's multiple comparison was performed using Prism software v4.0 (GraphPad, San Diego, CA). Significance was determined when the *P*-value < 0.05.

5.3 RESULTS

5.3.1 Enzyme Inhibition Potential of Treprostinil

Pooled human liver microsomes were used to evaluate the inhibitory potential of treprostinil on the metabolism of CsA, TAC, SRL, and MPA. The formation of hydroxycyclosporine (MI) was used as a marker of CsA metabolism (Figure 34A). Co-incubation with KTZ significantly inhibited 64% of MI formation. In contrast, co-incubation with all three concentrations of treprostinil did not inhibit MI formation, compared to control. The loss of tacrolimus was used as a marker of metabolism, shown in Figure 34B. In control samples, 31% of TAC was metabolized, compared to 7% when co-incubated with KTZ. Co-incubation of TAC with each of the three concentrations of treprostinil did not change TAC metabolism (32, 34, and 32% of original, respectively). The loss of sirolimus was used as a marker of metabolism, shown in 34C. In control samples, 43% of SRL was metabolized, whereas only 5% of SRL was metabolized when co-incubated with KTZ. Co-incubation with treprostinil (10, 50, and 100 ng/ml) resulted in 45, 35, and 36% of SRL metabolism, respectively. The formation of mycophenolic acid glucuronide (MPAG) was used as a marker of MPA metabolism, shown in Figure 34D. Compared to control, co-incubation with all three concentrations of treprostinil did not inhibit MPAG formation. The results indicate that co-incubation with treprostinil in pooled human liver microsomes did not inhibit the metabolism of CsA, TAC, SRL, or MPAG.

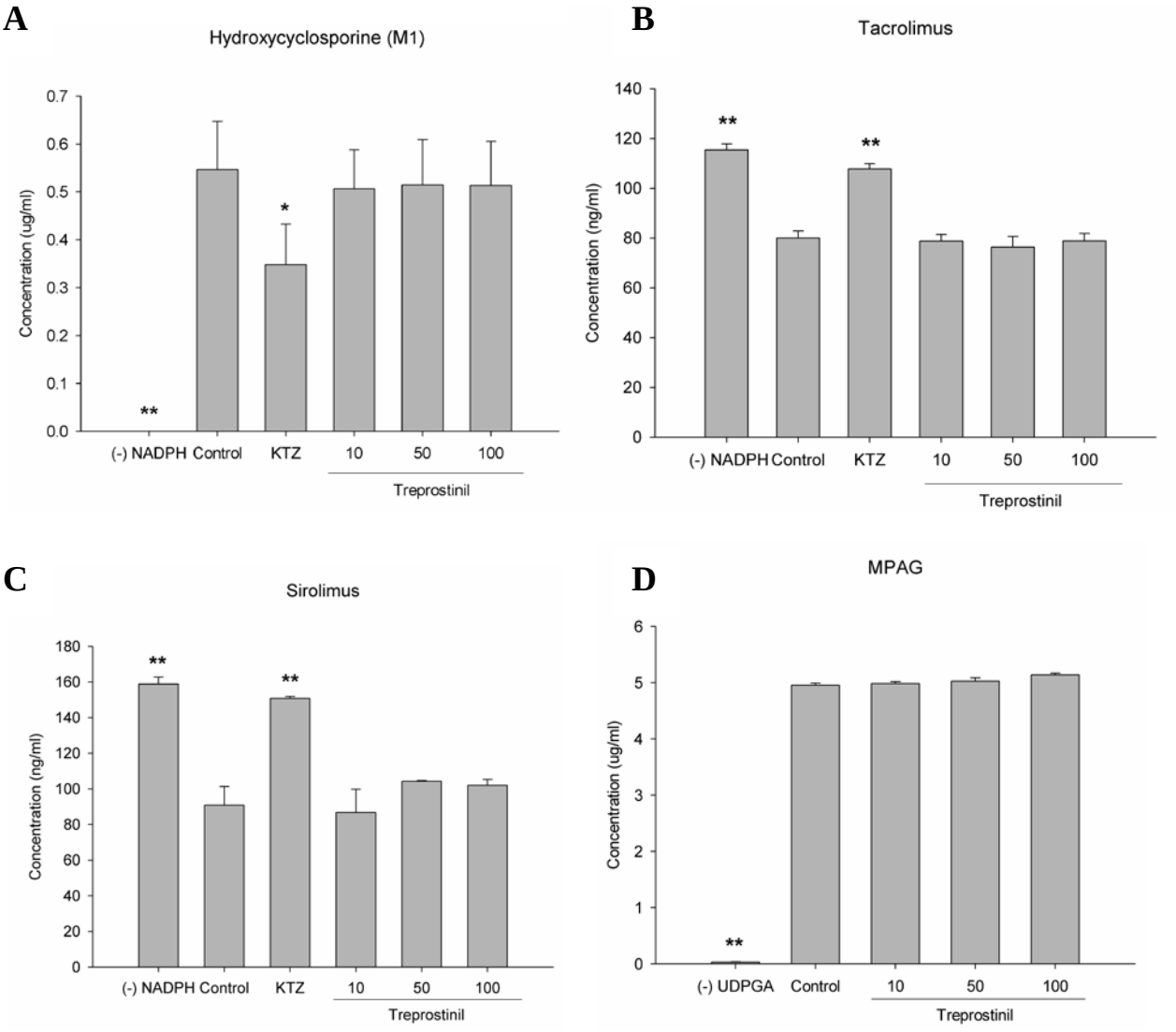


Figure 35: Inhibitory potential of treprostinil

Pooled liver microsomes (n=5) co-incubated with treprostinil (10, 50, and 100 ng/ml) and (A) CsA, (B) TAC, (C) SRL, and (D) MPAG; (-) NADPH served as negative control for CsA, TAC, or SRL; (-) UDPGA served as negative control for MPA. Control samples represent regular metabolism (samples not co-incubated with treprostinil). *P < 0.05 and **P < 0.01 vs. control.

5.3.2 Evaluation of Cytotoxicity

Hepatocytes prepared from four donors (HH1516, HH1582, HH1601, and HH1602) were treated with DMSO, RIF (10 uM), PB (2 mM), and treprostinil (10, 50, and 100 ng/ml) for 72 hr to determine the effect of treprostinil on hepatocyte mitochondrial activity, using the MTT assay. Our lab has previously shown that the concentration of DMSO (0.1%) used for primary culture of human hepatocyte experiments does not alter cellular activity compared to cells treated with HMM+. Cellular activity with each of the three concentrations of treprostinil was similar to those treated with DMSO, RIF, and PB (Figure 35), which indicated that pre-treatment with treprostinil did not alter cellular activity, compared to DMSO-treated cells.

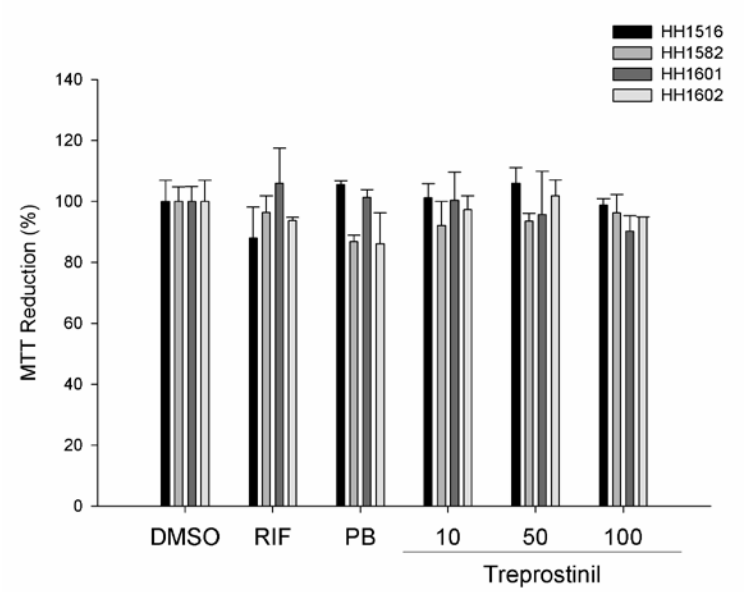


Figure 36: MTT assay
Rifampicin (RIF 10 uM), phenobarbital (PB, 2 mM), or treprostinil (10, 50, and 100 ng/ml) for 72 hr.

5.3.3 Enzyme Induction Potential of Treprostinil

Primary cultured human hepatocytes were used to examine the induction potential of treprostinil on the metabolism of CsA, TAC, SRL, and MPA. Original samples represent the baseline concentration of the immunosuppressant in the system. Control samples represent regular metabolism of the immunosuppressant after pretreatment with DMSO. Loss of CsA was used as a marker of metabolism, shown in Figure 36A. Under the experimental conditions, 38, 39, and 61% CsA was metabolized in HH1457, 1464, and 1448, respectively. Co-treatment with KTZ significantly reduced CsA metabolism to 20, 7, and 12%, respectively. Pretreatment with RIF significantly increased CsA metabolism to 56, 60, and 76%, respectively. Pretreatment with treprostinil did not change CsA metabolism.

A decrease in TAC concentration was used as a marker of metabolism, shown in Figure 36B. In control samples, 19, 69, and 45% of TAC was metabolized in HH1454, HH1492, and HH1511, respectively. Co-treatment with KTZ significantly reduced TAC metabolism to -0.5, 11, and 6%, respectively. Pretreatment with RIF significantly increased TAC metabolism to 67, 85, and 89%, respectively. Pre-treatment with treprostinil did not increase TAC metabolism, compared to control. A decrease in SRL concentration served as a marker of metabolism, shown in Figure 36C. In control samples, 54, 67, and 65% of SRL was metabolized in HH1426, HH1432, and HH1434, respectively. Co-treatment with KTZ significantly reduced the SRL metabolism to 17, 41, and 8%, respectively. Pretreatment with RIF increased SRL metabolism to 82, 83, and 76%, respectively. Compared to control, pre-treatment with treprostinil did not increase SRL metabolism. The formation of MPAG served as a marker of metabolism (Figure 36D). Pretreatment with PB increased MPA metabolism by 163, 234, and 168% in HH1458, HH1461, and HH1466, respectively. Pre-treatment with treprostinil did not increase MPAG

formation, compared to control. Collectively, the results indicate that pretreatment in primary culture of human hepatocytes with all three concentrations of treprostinil did not inhibit or induce the metabolism of CsA, TAC, SRL, or MPA and is therefore, unlikely to alter the metabolism of these drugs when co-administered.

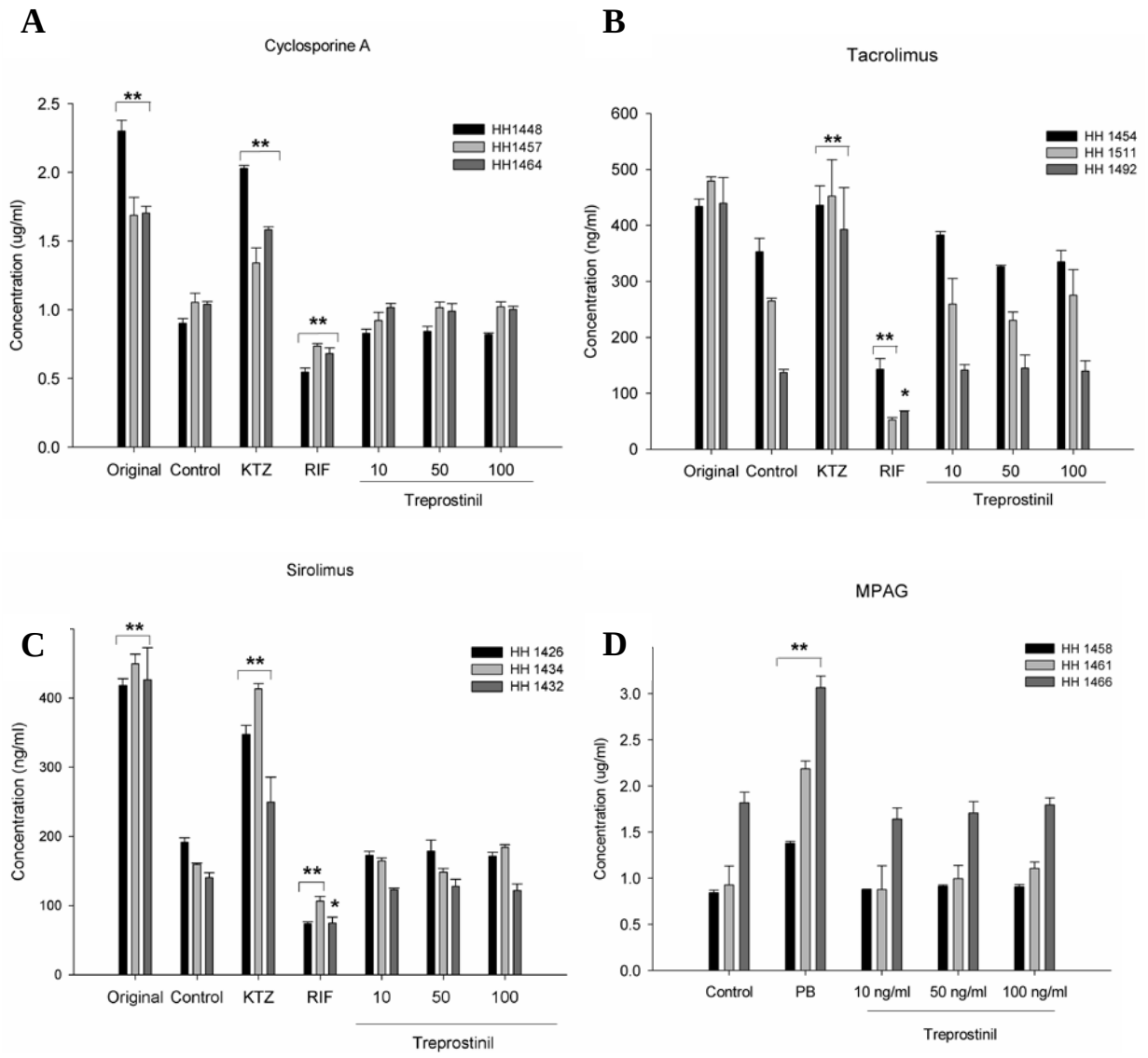


Figure 37: Induction potential of treprostinil

Primary culture of human hepatocytes pretreated with DMSO (control), RIF (10 uM), PB (2 mM), or treprostinil (10, 50, and 100 ng/ml) for 72 hours before treatment with (A) CYA, (B) TAC, (C) SRL, or (D) MPA. Original represents baseline concentration. Control represents regular metabolism of the immunosuppressive agent for the indicated time without treprostinil pre-treatment. *$P < 0.05$, ** $P < 0.01$ vs. control (n=3).

5.3.4 Effect of Treprostinil on mRNA expression

To determine whether or not treprostinil altered the mRNA expression of CYP3A4, UGT 1A9, or UGT2B7, real-time PCR analysis was performed. Pretreatment with RIF significantly induced mRNA expression of CYP3A4 by 9.2 ± 0.9, 13.3 ± 0.5, and 33.6 ± 5.9-fold in HH1464, HH1466, and HH1467, respectively (Figure 37A), compared to DMSO-treated cells. Likewise, PB treatment induced CYP3A4 mRNA expression by 5.4 ± 0.3, 17.1 ± 2.8, and 29.7 ± 2.1-fold, respectively. Treatment with treprostinil (10, 50, and 100 ng/ml) had no effect on CYP3A4 mRNA expression, compared to DMSO-treated cells and positive controls. Treatment with PB induced UGT1A9 mRNA expression by 5.2 ± 0.3, 2.8 ± 0.2, and 4.8 ± 0.6-fold in HH1464, HH1466, and HH1467, respectively, compared to DMSO-treated cells, shown in Figure 37B. Pretreatment with treprostinil (10, 50, and 100 ng/ml) did not significantly increase UGT1A9 mRNA expression, relative to positive control. Similarly, PB induced UGT2B7 mRNA expression by 3.5 ± 0.5, 1.9 ± 0.1, and 2.0 ± 0.2-fold in HH1464, HH146, and HH1467, respectively (Figure 37C). Treatment with treprostinil (10, 50, and 100 ng/ml) did not significantly increase UGT2B7 mRNA expression, relative to the positive control. The results demonstrated that treprostinil is not expected to alter the clearance of CsA, SRL, TAC, or MPAG.

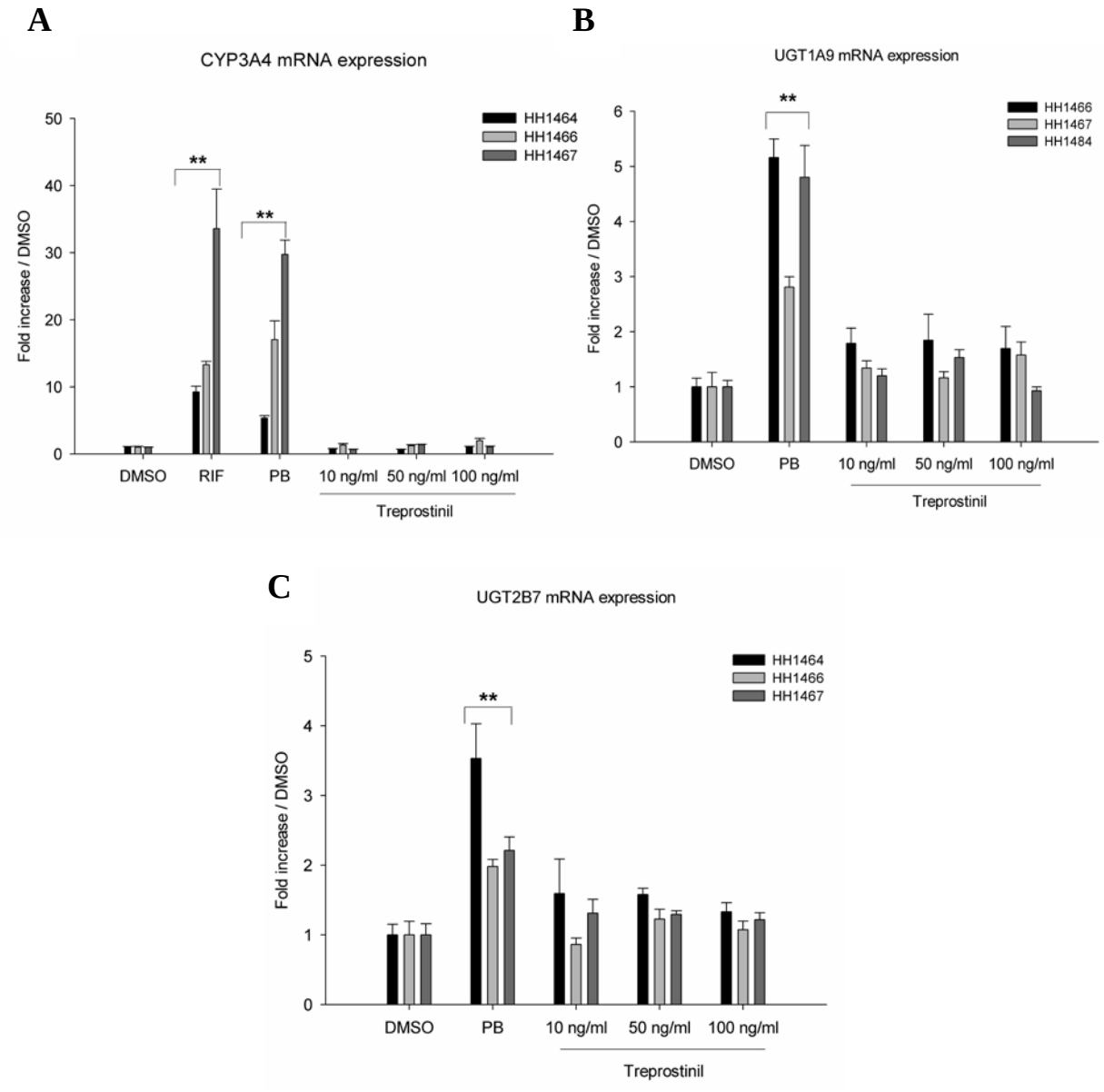


Figure 38: mRNA expression in hepatocytes

Hepatocytes from three donors (HH1464, HH1466, and HH1467) were pre-treated with DMSO (vehicle control), Rif (10 uM), PB (2 mM), or treprostinil (10, 50, and 100 ng/ml) for 72 hours to measure mRNA levels of (A) CYP3A4, (B) UGT1A9, and (C) UGT2B7 * P <0.05 and ** P <0.01 vs. control.

5.4 DISCUSSION

In Chapter two, it has been shown that treprostinil is effective in protecting the liver graft against I/R injury during rat OLT. The ultimate goal of treprostinil therapy in orthotopic liver transplant recipients is to ameliorate ischemia-reperfusion injury. Liver transplant patients who are likely to receive treprostinil are maintained on immunosuppressant therapy to prevent graft rejection and several other drugs that are metabolized by the liver. It is important to assess *in vitro* inhibition and induction potential of treprostinil in liver transplant patients in order to confirm that it can be safely administered in combination with immunosuppressive medications. Previous reports have shown that some prostaglandin analogues altered the clearance or half-life of certain immunosuppressive agents [224, 225]. Therefore, we performed this study to address the question of whether or not there is potential for a drug-drug interaction between treprostinil and the four most commonly administered immunosuppressant medications, including cyclosporine A, tacrolimus, sirolimus, and mycophenolic acid. According to the FDA guidelines for drug-drug interaction studies, the agent under investigation must increase the metabolism of the second drug comparatively to a positive control or induce the metabolism by greater than 40% in order to be classified as a causative agent of a drug-drug interaction [226]. Current industrial practices to assess drug induction of CYP450 enzymes by examining a change in the area under the plasma concentration curve (AUC), maximum concentration, or half-life.

Drug interactions involving the CYP450 isoforms are generally of two types, namely: enzyme inhibition or enzyme induction [229]. The two most common mechanisms by which enzyme induction occurs include stabilization of the mRNA or enzyme and increased gene transcription, mediated by nuclear receptors [230]. In the present study, the inhibition and induction potential of treprostinil in human liver microsomes and hepatocytes co-incubated with

CsA, TAC, SRL, CsA, and MPA were evaluated at the level of enzyme activity and gene transcription. The results demonstrated that in pooled human liver microsomes all three clinically relevant concentrations of treprostinil tested did not inhibit metabolism of the immunosuppressive agents tested. Likewise, in primary cultures of human hepatocytes we showed that all three clinically relevant concentrations treprostinil did not induce the metabolism of CsA, TAC, SRL, or MPA. Collectively, the results from this study demonstrate that clinically relevant concentrations of treprostinil are unlikely to alter the clearance of CsA, TAC, SRL, or MPA when administered concomitantly. Some CYP and UGT isoforms are subject to induction by xenobiotics via activation of nuclear hormone receptors, with a consequent result of decreased exposure of the affected compound leading to therapeutic failure. Real-time RT-PCR results confirmed that treprostinil had no induction potential on CYP3A4 mRNA expression. The ligand-activated nuclear hormone receptor, peroxisome proliferator-activated receptors (PPAR)-α is a common regulator of the gene expression of UGT1A9 [231] and UGT2B7 [221] and various PGI_2 analogues, including treprostinil, have been reported to be ligands for the different PPARs isoforms [232]. As a PGI_2 analogue, we investigated the effects of treprostinil on mRNA expression of UGT1A9 and UGT2B7, as these two UGT isoforms have been reported to be the dominate UGT isoforms responsible for the metabolism of MPA to its active and inactive metabolites, respectively [221, 233, 234]. Interestingly, RT-PCR results indicated that in one case of hepatocytes (Figure 377B, HH1466), treatment with treprostinil resulted in a slight increase in UGT1A9 mRNA expression but not of UGT2B7, compared to the positive control and vehicle control. However, the increase was minor and was less than 40% of the positive control. Therefore, according to regulatory guidelines, there is no concern for a DDI via this pathway. The observed effects are most likely attributed to the inherent inter-individual

variation among human hepatocytes. The UGT1A9 and 2B7 isoforms are both reported to have genetic polymorphisms [235], which may partially explain the observed inter-individual variations. Since administration of treprostinil is expected to be acute, i.e. during the transplantation procedure and up to 48 hours post-transplantation, and *in vitro* formation of MPAG is not increased greater than 40%, no DDI is expected between treprostinil and the immunosuppressive agents tested.

Taken together, the results demonstrate that treprostinil is unlikely to alter the metabolism of the four most widely used immunosuppressant medications when co-administered, thus supporting continued investigation with treprostinil for its targeted indication in orthotopic liver transplantation.

6.0 CONCLUSIONS AND FUTURE RESEARCH

6.1 DISCUSSION AND SUMMARY

Orthotopic liver transplantation is the only curative therapy for patients with end-stage liver diseases; however, there is a tremendous shortage of organs available for transplantation. This shortage has prompted the use of what would otherwise be discarded organs, i.e. extended criteria donors (ECDs), in efforts to increase the donor pool. Although ECDs provide additional grafts, they are more susceptible to cold ischemia and reperfusion injury. The process of I/R injury to the liver graft combines interrelated factors that produce a cascade of events, which can ultimately lead to hepatic graft failure. Ischemia-reperfusion injury remains a significant limitation in clinical liver transplantation. Despite extensive research, no therapeutic approach is available to alleviate I/R injury during OLT.

Of the various pharmacological agents that have been explored to minimize I/R injury, the prostaglandin class of drugs has been evaluated to the greatest extent. Prostacyclin, an endogenous metabolite of arachidonic acid, has a critical role in maintaining cellular homeostasis, largely due to its vasodilatory and anti-platelet aggregatory properties. Because the half-life of prostacyclin is very short (2-3 minutes), several analogues have been developed with extended half-lives. Considering the many factors involved in I/R injury and the role of PGI_2 in maintaining cellular homeostasis, PGI_2 analogues have been evaluated to reduce I/R injury

associated with OLT, however, no attempts to date have successfully made their way to the clinic. As such, extensive efforts have continued to identify an approach to minimize I/R injury associated with OLT. Treprostinil sodium, a recently FDA-approved PGI_2 analogue (Remodulin®), possesses potent pulmonary and systemic and vasodilatory and platelet anti-aggregatory effects [236] and has a higher potency and the longest elimination half-life than other PGI_2 analogues currently commercially available [127]. These advantages of treprostinil make it an attractive candidate for protection of the liver graft against I/R injury associated with OLT. This dissertation examined the hypothesis that treprostinil would protect the liver graft against I/R injury during OLT. This is the first study in the field of I/R injury during OLT to investigate treprostinil as a therapeutic approach to protect the liver graft against I/R injury in OLT. Also, this dissertation provides a deeper understanding of the metabolic changes in the liver graft during the post-operative period, and the widespread protective effects of treprostinil. The work presented herein has generated several key and novel findings, summarized below.

1. Treprostinil minimizes hepatic I/R injury to the liver graft during OLT.

To examine our hypothesis, the first step was to perform proof of concept studies in an animal OLT model following cold graft storage. The initial evidence that treprostinil reduced hepatic injury post-transplantation was noted by a drastic reduction in serum aminotransferases at 6, 24, and 48 hrs post-reperfusion. These findings warranted additional studies be carried out to further investigate the extent of protection conferred by treprostinil. These studies examined the degree of I/R-induced hepatic damage by neutrophil infiltration, necrosis, pro-inflammatory cytokines, energy status, and SEC structure. The results demonstrated that administration of treprostinil to

donor and recipient animals prior to hepatectomy and transplantation, respectively, significantly reduced neutrophil infiltration and hepatic necrosis, as well as hepatic mRNA levels of pro-inflammatory cytokines early post-reperfusion. In addition, cold storage of liver grafts resulted in a significant reduction in adenosine nucleotide levels in the liver graft of placebo-treated animals, compared to normal liver. In contrast, treprostinil restored ATP levels in liver grafts similar to normal following reperfusion. Furthermore, structural analysis by electron microscopy revealed the finding that treprostinil preserved the sinusoidal endothelial cell lining and reduced platelet deposition very early post-transplantation compared to placebo. Hepatic tissue blood flow was significantly compromised in the placebo-treated group, whereas treprostinil maintained blood-flow to near normal values. To answer the question of whether or not treatment administered to the recipient alone would yield protective effects, additional groups of recipients only were treated with treprostinil prior to transplantation and until the time of sacrifice. The significant reduction in serum ALT and AST levels post-OLT in the recipient only treatment group compared to placebo-treated group further confirmed treprostinil as a viable approach to protect liver grafts against I/R injury post-reperfusion.

For more than two decades, PG analogues have been studied for their ability to reduce I/R injury after liver transplantation; however stability issues, side effects, and the inability to show significant difference in primary endpoint have limited its clinical application. This is the first study to demonstrate the efficacy of treprostinil in an animal OTL model and to elucidate the protective effects of this particular PGI_2 analogue on liver grafts against I/R injury following OLT. The findings from Chapter 2 support continued investigation of treprostinil as a pharmacological agent to protect the liver graft against I/R injury during clinical OLT.

In the clinical study, treprostinil will be administered intravenously to the recipient commencing after induction of anesthesia for the transplant surgery, and will continue throughout the transplant procedure and for approximately 48 hours after completion of the transplantation surgery until termination of the study drug infusion, unless hemodynamic changes or tolerability require dose reduction or discontinuation of treprostinil. Treprostinil dosing will follow a standard 3 + 3 Phase 1 dose-escalation study design. Three patients will be enrolled at the first dose of 5 ng/kg/min. If 0/3 patients experience a dose limiting toxicity (DLT), the next 3 patients will be started at 7.5 ng/kg/min, and this procedure will be followed with 10, 12.5, and 15 ng/kg/min. If 1/3 patients experience a DLT then 3 more patients will be added at the 5 ng/kg/min dose. If 2/6 patients experience DLT at 5 ng/kg/min, the dose will be decreased to 2.5 ng/kg/min, and a maximum of six patients will be treated at this dose level. If 2/6 patients experience DLT at 2.5 ng/kg/min then the trial arm will be discontinued because of excessive toxicity. If only 1/6 patients experience DLT at 5 ng/kg/min, then dose will be escalated to 7.5 ng/kg/min, and dose escalation will be done by 2.5 ng/kg/min. If <1 out of six patients experience DLT at 2.5 ng/kg/min, then 2.5 ng/kg/min will be the maximum tolerated dose for the expanded study. Treprostinil undergoes extensive hepatic metabolism [113] and in patients with renal insufficiency, the AUC was increased 3-5-fold [236]. Since there will be brief periods where patients undergoing liver transplantation are anhepatic, a maximal dose that will be used is 15 ng/kg/min using the above scheme. Also, the dose of treprostinil may be temporarily reduced or stopped at any time during the transplant procedure if, in the opinion of the investigator, the subject experiences intolerable side effects (e.g. low systemic blood pressure or other clinically significant changes in hemodynamics) that may be attributable to study drug.

Upon completion of the 48-hour infusion, administration of treprostinil will be terminated. An additional aim of the study is to determine the target dose for use in a larger clinical study.

2. Treprostinil minimizes I/R-mediated changes in the expression and activity of major rat CYP450 enzymes.

An integral component of I/R injury is activation of the pro-inflammatory cascade resulting in the production of pro-inflammatory cytokines, i.e. TNF-α, IL-1β, and IL-6. Inflammation is known to markedly impair hepatic detoxification pathways, which can alter the disposition of certain drugs. In fact, changes in drug disposition have been linked to alterations in the expression of hepatic drug metabolizing enzymes and drug transporters as a result of inflammation or infection [81-84, 192]. Therefore, we hypothesized that I/R injury, an inflammatory disease-state manifested during OLT, would significantly impair metabolic functions of the liver graft, and that treprostinil would alleviate the impaired drug metabolism post-transplantation by inhibiting the inflammatory response and improving hepatic tissue blood flow. Continuing with the donor plus recipient treatment model, in Chapter 3 we examined the effects of I/R injury and protection of liver grafts against I/R injury by treprostinil on the expression and activity of CYP450 enzymes post-OLT. Results showed a significant decrease in the mRNA expression of all CYP isoforms tested in the placebo-treated group with parallel reductions in protein expression and microsomal activity post-OLT, compared to normal liver. In contrast, administration of treprostinil improved the mRNA expression of CYP2C11, 2E1, and 3A1/A23, 3A2, and 3A18, compared to placebo and restored CYP2E1 protein expression and activity to normal. Treprostinil also significantly improved protein expression and hepatic

activity of CYP2C11 and 3A, compared to the placebo-treated group. These findings highlight the impact of I/R injury on CYP450-mediated drug metabolism in the liver graft post-OLT. These data also show the impact of treprostinil and the extent of liver graft protection on several of the major rat CYP450 isoforms post-transplantation.

3. Treprostinil reduces I/R-mediated changes in the mRNA and protein expression of major drug transporters in the rat.

Hepatic drug transporters are important determinants of the clearance of endogenous compounds and xenobiotics and their expression is variable and subject to complex regulation by drugs, metabolites, oxidative stress and pro-inflammatory cytokines. Consequences of impaired hepatic function include altered pharmacokinetics of drugs. Extending the analysis further, we studied the effects of I/R injury and protection by treprostinil on the expression of uptake and efflux transporters in liver grafts post-OLT. Results from Chapter 4 showed that administration of treprostinil significantly reduced peak serum bilirubin levels post-OLT, compared to placebo, and restored values to normal by 3 hours post-OLT. In addition to confirming the protective effect of treprostinil on hepatic function post-OLT, these results highlight the particular improvement on hepatic transport processes. In the placebo-treated group, the mRNA expression Oatp1a1, Oatp1a4, Ntcp, Oct1, Mdr1a (P-gp), Mdr2, Mrp2, and Bsep in liver graft were significantly reduced compared to normal expression post-reperfusion. Treatment with treprostinil improved mRNA expression of several transporters as well as up-regulated Mrp2 and P-gp protein expression. Earlier reports indicated that activation of the IP receptor led to glycosylation [140]. Knowing that treprostinil activates the IP receptor, these unexpected findings of up-regulated Mrp2 and P-gp, in addition, to doublet bands of Mrp2 detected, indicate

additional mechanism(s) by which this prostacyclin analogue stabilized CYP and transporter protein expression. Conclusions that can be drawn from this study are that I/R injury associated with OLT significantly down-regulated the expression of several hepatic transporters and treprostinil improved hepatic transport processes in the liver graft post-OLT.

4. Treprostinil does not directly alter the metabolism of four most commonly used immunosuppressive medications when co-administered.

Success of solid organ transplantation requires the use of immunosuppressive medications to prevent organ rejection in the recipient. These immunosuppressive agents, including tacrolimus, sirolimus, cyclosporine A, and mycophenolate mofetil, have a narrow therapeutic index and fluctuations in the blood concentration of these agents could precipitate allograft rejection or organ toxicity. In Chapter 5, we examined the potential for a drug-drug interaction between treprostinil and cyclosporine A, tacrolimus, sirolimus, and mycophenolic acid *in vitro*. The results indicated that treprostinil does not inhibit or induce the metabolism of these drugs, nor does it alter the mRNA expression of CYP3A4, UGT1A9, or 2B7. These results lead to the conclusion that treprostinil is unlikely to directly alter the clearance of these immunosuppressive medications, when co-administered. In addition to being clinically relevant, these studies comply with the FDA requirements for a new drug approval.

In conclusion, the significance of this research is the identification of treprostinil, a commercially available PGI_2 analogue, as a viable approach to protect the liver graft against I/R injury associated with OLT. This finding is an important advancement to the field of liver transplantation and, potentially, to the field of solid organ transplantation. Amelioration of hepatic graft injury with treprostinil will likely improve both short- and long-term transplant

outcomes. This is the first study to demonstrate that treprostinil protected the liver graft against I/R injury associated with OLT. Treprostinil has the potential to serve as a therapeutic option to protect liver graft against I/R injury in patients undergoing OLT. The results of this work support continuation with the investigation of a clinical Phase I study to examine the efficacy of treprostinil in protecting the liver grafts against I/R injury in human OLT.

6.2 FUTURE RESEARCH RECOMMENDATIONS

While treatment of donor plus recipient offers the greatest liver graft protection, it is often not feasible to treat both the donor and the recipient in the clinical setting. Therefore, to optimize the treatment regimen for human transplantation, the next step is to characterize the extent of protection by treprostinil in two additional treatment models: 1) recipient only treatment and 2) storage only treatment, and compare the results to those conferred in the current donor plus recipient model. We initiated studies with recipient only treatment model to determine the benefit of treprostinil as a more clinically relevant treatment model. Results confirmed that recipient only treatment with treprostinil significantly reduced serum ALT and AST values, validating this approach to protect the liver graft against I/R injury in clinical OLT.

In terms of liver graft protection against I/R injury, the work presented herein demonstrates the several ways by which treprostinil protects the liver graft following OLT. The first aim that we set out was to examine was proof of concept. Specific mechanistic pathways remain to be examined in future studies, as outlined below.

Methodology and Optimization

- The ideal treatment model would be to use treprostinil *ex vivo* added to the liver graft during preservation in UW solution, prior to transplantation. This approach is the simplest and least cumbersome during the clinical transplantation procedure. To examine this treatment model, it is first necessary to optimize the concentration of treprostinil applied to the UW solution.

- Lactated Ringers (LR) solution is used to flush the UW preservation solution out of the graft immediately prior to engraftment. Addition of treprostinil to LR represents an additional means of enhancing liver graft protection; however, the compatibility of treprostinil in LR solution has not been established. Treprostinil is compatible with normal saline or water for injection. Whether or not this step would yield additional protection has yet to be determined.

- The ultimate goal for the use of treprostinil in adult orthotopic liver transplantation is protect the liver graft against I/R-associated injury, thereby improve patient and graft survival, as well as increase the number of suitable grafts for transplantation and patients who successfully recover from OLT. To achieve this goal, survival data are essential to make functional conclusions with respect to graft protection offered by treprostinil.

Mechanistic Experiments

- Previous reports have shown that treprostinil inhibits the release of pro-inflammatory cytokines by inhibiting NF-$_K$B translocation *in vitro* [187]. An important mechanism of hepatic I/R injury is activation of NF-$_K$B and treprostinil's inhibitory effect on this transcription factor is very important and should be examined.

- In the current donor plus recipient treatment model, hepatic tissue levels of pro-inflammatory cytokines were measured at the mRNA level. Plasma cytokine concentrations should be measured to correlate levels with biochemical results.

Metabolism and Transporter Experiments

- Another important finding resulted from studying the impact of I/R injury on the hepatic expression and activities of the major rat CYP450 Phase I enzymes. Extensions of this study would be to examine the effects of I/R injury and treprostinil on Phase II enzymes, as this pathway is also important for metabolism.

- To better characterize the time course of the effects of I/R injury and the protection offered by treprostinil on hepatic metabolism and transport processes, additional time point post-OLT, i.e. 12 hr and 24 hrs, should be examined. Also, studies in rat and human hepatocytes are important to compare activity and expression levels between species.

- Functional assessment of hepatic transporters in an isolated liver perfused system to further study the consequences of I/R injury and treatment with treprostinil on the function of these transporters and regulation of cellular homeostasis, i.e. bile acid transport and bile flow are important. The current treatment model did not allow for studying bile flow, though this function was greatly improved in the treprostinil-treated group (Personal observations, confirmed by Dr. Yoshida). To better understand the implication of up-regulated Mrp2 and P-gp protein expression, the effect of I/R injury and treprostinil on Mrp2 and P-gp function in liver graft post-OLT is worth examining.

- It would also be interesting to determine whether or not the protein expression of other ABC transporters is preserved in treprostinil-treated group. For instance, the mRNA expression of Bsep was maintained similar to normal in the treprostinil-treated group post-OLT. Finally, the implications of the transporter findings raise new questions about the potential use of treprostinil for other hepatic disease-states and it is attractive to speculate that treprostinil could have addition applications, e.g. cholestasis, which is characterized by impaired Mrp2 function.

APPENDIX A

A.1 IRB PROTOCOL

PRINCIPAL INVESTIGATOR:

Abhinav Humar, M.D.
Professor of Surgery
Montefiore Hospital North 725, 3459 Fifth Avenue, Pittsburgh, PA 15213
Phone: 412-692-4553, Fax: 412-692-4180

CO-INVESTIGATORS:

University of Pittsburgh

Department of Pharmaceutical Sciences

1. Raman Venkataramanan, Ph.D; F.C.P.
Professor of Pharmaceutical Sciences and Pathology
718 Salk Hall,
3501 Terrace Street, Pittsburgh, PA 15261
Phone: 412-648-8547

2. Nisanne Ghonem, Pharm.D., Ph.D.
731 Salk Hall, 3501 Terrace Street, Pittsburgh, PA 15261
Phone: 412-648-2377

Department of Pathology

Anthony Demetris, Ph.D.
E737 UPMC-Montefiore,
3459 Fifth Avenue, Pittsburgh, PA 15213
Phone 412-647-7646

Thomas Starzl Transplantation Institute Liver Surgeons

Paulo Fontes, M.D, Mark L. Sturdevent, M.D., Ruy J. Cruz, M.D., Roberto C. Lopez, M.D., and Raymond Planinsic M.D.(Liver Transplant Anesthesia Team)
Montefiore University Hospital,
3459 Fifth Avenue Pittsburgh, PA 15213
Phone: 412-692-4553

Starzl Transplantation Institute

Clinical Research Manager: Sheila Fedorek, RN CCRC

Research Coordinators: Laurie Hope, R.N., Stephanie Kikla, R.N. and Leslie Mitrik, B.S.

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

A.1.1 Study Rationale

The hypothesis of this study is that treprostinil can be safely administered perioperatively to adult patients undergoing OLT, and will ameliorate or prevent I/R-mediated dysfunction of the liver graft and thereby reduce morbidity, leading to shorter hospital stays as compared to historical controls.

Treprostinil, as a prostanoid, is expected to facilitate restoration of the blood supply to the revascularized graft and provide the well-characterized protective effects of this class of compounds in liver transplant patients. Treprostinil has the advantage of having a longer

elimination half-life and increased potency than other prostanoids previously tested in this patient population.

A.2 STUDY OBJECTIVES

A.2.1 Primary Objective:

To evaluate the safety, pharmacokinetics and preliminary efficacy of a two-day peri-operative course of treprostinil in preventing ischemia-reperfusion of the liver graft post-OLT.

A.3 CLINICAL ASSESSMENTS

A.3.1 Primary Safety Assessment

The primary safety assessments include the following hemodynamic measurements in the operating room (OR) and in the intensive care unit (ICU):

- Mean pulmonary arterial pressure (mPAP, mmHg)
- Transpulmonary gradient (tPG, mmHg)
- Pulmonary capillary wedge pressure (CPWD, mmHg)
- Cardiac output (CO, L/min)
- Cardiac Index (CI, l/min/m2)
- Left ventricular ejection fraction (LVEF, %)

In addition, heart rate (HR, beats per minute) and Systolic blood pressure (SBP, mmHg) will be measured/collected every 6 hrs. The need for ionotropes will be noted for 7 days.

A.3.2 Pharmacokinetic Assessments

Pharmacokinetic assessments will also be carried out during the study period by collecting multiple blood samples. To measure treprostinil plasma concentration, up to eighteen 3-mL blood samples may be obtained in EDTA-coated tubes just prior to, during, and/or after study drug administration The sampling will be done prior to initiation of study drug therapy, at approximately 2, 4, 6, 12, 18, 24, 30, 36, 42, 48 hrs during therapy and approximately 0.5, 1, 2, 4, 6, 8 and 12 hrs after therapy. Samples will be analyzed using a validated UPLC-MS-MS assay. Various pharmacokinetic parameters will be calculated as per standard methodology, including clearance and half-life.

A.3.3 Preliminary Efficacy Assessment:

The primary efficacy assessment will be determined by serum bilirubin concentration (peak and AUC) measured during the first seven days after transplantation; the secondary efficacy assessments will be determined by several biochemical end points in the first seven days after transplantation, including:

- Biochemical end points: Serum ALT and AST levels in the first seven days after transplant (Peak and AUC); Post-transplant renal function, as assessed by serum creatinine levels in the first seven days following transplant (Peak and AUC); Blood biomarkers of ischemia reperfusion injury; INR.
- Clinical end point: Primary allograft non-function defined as patient death or re-transplantation within 30 days due to liver failure; Graft survival at day 30, 90 and 180; Subject survival at day 30, 90, and 180.

- Intra-operative blood product usage;

- Biopsy: Post perfusion liver biopsy – histology; biomarkers; Liver biopsy is a safe and important diagnostic tool for liver disease. Microscopic examination of a biopsy specimen can reveal disease-specific patterns. Histological examination of hepatic architecture can more accurately stage the disease or estimate the extent of damage.

  The biopsy results will be classified as follows:
  0 = no evidence of reperfusion injury

  1 = mild reperfusion injury

  2 = moderate reperfusion injury

  3 = severe reperfusion injury.

When possible, the following will also be obtained for subsequent analysis as markers of I/R injury and subjects will be followed up to study day 180.

- Duration of time (days) spent in the ICU during the initial hospitalization.
- Graft up-regulation of pro-inflammatory cytokines (TNF-α, IL-6); chemokines (IL-8)
- Ultra-structural analysis and immunohistochemistry (CD31)
- Intra-operative blood product usage
- Total costs for initial transplant hospitalization

A.3.4 Number of Subjects

The total enrollment will be up to 30 patient-subjects.

A.3.5 Estimated Study Duration

The estimated study duration will be approximately 3 years.

A.4 EXPERIMENTAL PLAN

A.4.1 Study Design

This is a single center, open-label, dose-escalation Phase I study of treprostinil in subjects who are undergoing orthotopic liver transplantation for end stage hepatic disease at the Thomas E. Starzl Transplantation Institute at the University of Pittsburgh Medical Center, Pittsburgh, PA. The study will evaluate the safety and preliminary efficacy of treprostinil in adult OLT patients. An appropriately signed informed consent form will be obtained for each study subject once transplant candidacy is established and prior to any study-related procedures. Informed consent will be confirmed at baseline and the subject will be asked if they wish to proceed with the study or wish to withdraw prior to any baseline assessments. Approximately 30 subjects who have signed informed consent and who continue to meet entry criteria will be enrolled during pre-transplantation procedures. Treprostinil dosing will follow a standard 3 + 3 phase 1 design. Three patients will be enrolled at the first dose level of 5 ng/kg/min. If 0/3 patients experience a dose-limiting toxicity (DLT*) then the next 3 patients will be started at 7.5 ng/kg/min. If 1/3 patients experience a DLT then 3 more patients will be added at the 5 ng/kg/min dose. If 2/6 patients experience DLT at 5 ng/kg/min, the dose will be decreased to 2.5 ng/kg/min, and a maximum of six patients will be treated at this dose level. If 2/6 patients experience DLT at 2.5 ng/kg/min then the trial arm will be discontinued because of excessive toxicity. If <1 out of six patients experience DLT at 2.5 ng/kg/min, then 2.5 ng/kg/min will be the maximum tolerated dose for the expanded study. If only 1/6 patients experience DLT at 5 ng/kg/min, then dose will be escalated to 7.5 ng/kg/min, and dose escalation will be done by 2.5 ng/kg/min. The maximal dose that will be used is 15 ng/kg/min, using the above scheme. The dose of treprostinil may be temporarily

reduced or stopped at any time during the transplant procedure if, in the opinion of the investigator, the subject experiences intolerable side effects (e.g. low systemic blood pressure or other clinically significant changes in hemodynamics) that may be attributable to study drug. However, every effort will be made to maintain the study drug dose at the target dose or maximum tolerated dose to provide the best chance of a protective effect at the moment of reperfusion of the donor organ. At the completion of the 48-hour infusion, administration of treprostinil will be terminated. The Follow-up phase will begin after the completion of study drug infusion. Follow-up phase study assessments will occur at Study Days 3-7, 30, 90, and 180.

*DLT is defined as any of the following:

1. Volume and vasopressor refractory hypotension (norepinephrine or epinephrine > 0.5 ug/kg/min, dopamine > 10 ug/kg/min, and/or vasopressin > 4 U/hr), for which no other reasonable cause(s) can be found and promptly treated.
2. Sustained (> 4 minutes) hypotension defined as systolic pressure of < 80 mmHg that is not responsive to usual interventions for a liver transplant patient, such as fluid bolus and the use of vasopressors.
3. Persistent, uncontrolled and clinically significant hemorrhage.
4. Vomiting non-responsive to medical intervention such as use of ondansetron, prochlorperazine, promethazine and with no other obvious mechanical causes such as bowel obstruction and gastric ileus.
5. Seizure

A.4.2 Schedule of Time and Events

The time and events schedule for the study is presented in Table 10.

Table 9: Overall Time and Events Schedule for the Study

Study Phase	**Screening**	**Baseline**	**Treatment**	**Follow Up**				
Study Day	-180 to 0	0	1-2	3	4-7	30	90	180
Informed Consent/Medical History	X	X						
Physical Examination/Vital Signs		X	X	X				X
MELD Score	X	X						
Recipient Demographics/Indication for Transplant	X	X						
Cadaver Donor Demographics		X						
Cold Ischemia Time (hr)			X					
Donor liver biopsy (Back Table biopsy)		X						
Cytotoxic Crossmatch		X						
Clinical Laboratories[1]	X	X	X	X	X	X	X	X
Intra-operative Liver Biopsy			X					
Intra-operative blood usage			X					
Total bilirubin (mg/dl)/AST/ALT[2]	X	X	X-------	------	------	------	------	X
International Normalized Ratio (INR)	X	X	X-------	------	------	------	------	X
Study Drug Infusion			X------X					
Graft Survival			X--------	------	----	---------	---------	-----X
Subject Survival			X--------	------	----	---------	---------	-----X
Retransplantation			X--------	------	----	---------	---------	------X
Initial Hospitalization (days)			X--------	------	----	---------	---------	-----X
Time in Intensive Care Unit (days)			X--------	------	----	---------	---------	------X
treprostinil Plasma Level Sample(s)[3]		X	X------X	-------X				
Concomitant Medications			X-------	------	-------X			
Adverse Events			X------	---	------X			
Heart rate (HR, bpm)		X	X------	-------	-------X			
Systolic blood pressure (SBP, mmHg)		X	X-------	-------	------X			
Left ventricular ejection fraction (LVEF,%)		X	X					
Cardiac Index (CI, l/min/m^2)		X	X					
Cardiac output (CO, L/min)		X	X					
Pulmonary capillary wedge pressure (CPWD, mmHg)			X					
Transpulmonary gradient (tPG, mmHg)		X	X					
Need for ionotropes			X	-------	------X			

1. Clinical laboratories include all liver function tests carried out as part of the standard of care of the liver transplant patients and include ALT, AST, Alkaline phosphatase , gamma glutamyl transpeptidase, bilirubin, Prothrombin time , Partial Thromboplastin time, International Normalized Ratio, serum creatinine and BUN.
2. Additional blood samples may be taken so that bilirubin, AST and ALT data are collected at least 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 6 and 7.

3. Up to eighteen 3-mL blood samples may be obtained in EDTA tubes just prior to, during, and/or after study drug administration to evaluate treprostinil plasma levels. The sampling will be done prior to initiation of study drug therapy, at approximately 2, 4, 6, 12, 18, 24, 30, 36, 42, 48 hrs during therapy and approximately 0.5, 1, 2, 4, 6, 8 and 12 hr.

A.5 SUBJECT ELIGIBILITY CRITERIA

Inclusion and exclusion criteria will be assessed during the Screening and Baseline phases prior to starting study drug.

A.5.1 Inclusion Criteria

Subjects must:

1. Have signed appropriate informed consent.
2. Be between 18 years and 65 years of age.
3. Have been accepted as a liver transplant candidate at the UPMC.
4. Be receiving a cadaver donor liver transplant, including a donor liver with less than 40% macrosteatosis; receiving a donor liver with necrosis score of greater than 10; those receiving livers with cold ischemia time greater than 6 hours, but less than 12 hours.
5. Be treated in accordance with the standard of care protocol(s) currently in effect for liver transplant recipients at the UPMC, including immunosuppression and other elements of pre- and post-operative care.

A.5.2 Exclusion Criteria

Subjects must not:

1. Be receiving a living donor liver transplant.
2. Be receiving a donor liver with a cold ischemia time less than 6 hours or greater than 12 hours.
3. Be receiving a donor liver with macrosteatosis greater than 40%.
4. Be receiving any investigational drug (a drug other than treprostinil administered under an IND) or participating in any other investigational study, with the exception of alemtuzamab (Campath).
5. Be receiving any prostanoid to treat portopulmonary hypertension.
6. Have had a failed liver transplant within the previous 180 days.
7. Be undergoing multi-organ transplantation (transplantation of organs other than liver at the same time as the liver transplantation procedure).
8. Have fulminant hepatic failure
9. MELD score of > 35
10. Hepatitis C positive donor liver
11. On ionotropes at the time of the study
12. On renal replacement therapy at the time of study
13. Be receiving any non-standard immunosuppression protocol or other non-standard treatment that could affect interpretation of the study results.

14. Those currently receiving treatment for portapulmonary hypertension.

15. Those with significant cardiovascular disease.

16. Have any known hypersensitivity to prostaglandins, prostacyclin or treprostinil.

17. If female, be pregnant or nursing (as confirmed by urine pregnancy test at Baseline).

18. HIV positive

A.5.3 Concomitant Medications

Therapy with investigational agents will be prohibited throughout this study. No alteration in the use of immunosuppression or other standard of care drugs or anesthetics at the center will be required. Analgesics including narcotics may be used during this study if needed to treat pain. No clinically important drug interactions with treprostinil have been reported [236].

A.5.4 DRUGS AND DOSING

A.5.5 Drug Dosage, Administration, and Schedule

A single strength of commercially available, FDA-approved, treprostinil (1.0 mg/mL, Remodulin®, United Therapeutics Inc.) will be provided in 20-mL multi-dose vials. Study drug will be administered intravenously (IV) through a dedicated central venous line or peripherally inserted central catheter only (PICC).

Briefly, treprostinil will be diluted in sterile saline and administered through a dedicated line using a pump capable of accurate delivery at the selected infusion rates (normally in the range of 1-2 ml/hour). A PICC may be placed if an appropriate central line is not available. IV treprostinil may not be administered peripherally except for very short periods (a few hours) because of the possibility of thrombophlebitis.

Assistance for this study also will be available from several staff members at UPMC who are familiar with administration of treprostinil because of its use in portopulmonary hypertension subjects undergoing liver transplantation.

Treprostinil, at the pre-determined dose level, will be administered intravenously commencing after induction of anesthesia for the transplant surgery and continued throughout the transplantation procedure and for approximately 48 hrs after the transplantation surgery, unless hemodynamic changes or tolerability requires discontinuation of dosing. If the prescribed dose is not well tolerated, the dose may be reduced and the subject maintained at the maximal tolerated dose, based primarily on hemodynamics, which will be carefully monitored throughout the surgery and during the remainder of the treatment period. If necessary, treprostinil administration may be completely terminated. Note, in this regard, that gradual termination of dosing is recommended in the treprostinil package insert for PAH patients because of the possibility of acute worsening of PAH symptoms. However, rebound applies to acute decompensation in PAH patients who had been on long-term treatment; which would not apply to the patient population being studied under this clinical protocol. Blood samples to determine treprostinil plasma levels may be obtained prior to and/or during surgery from all subjects. It must be understood that treprostinil blood level data will not be available in time to help with dosing decisions for an individual subject. Once study drug infusion has been terminated, the

subject will be monitored for at least 24 hours to ensure there are no untoward effects, e.g. changes in vital signs.

A.5.6 Compliance with Dosing

Because subjects will be hospitalized during the entire Treatment Phase, no special compliance assessments will be conducted.

Adverse events related to treprostinil administration are summarized below, in Table 11.

Table 10: Expected Events Attributable to Treprostinil

Abdominal cramping	Nausea
Backache	Leg pain
Chest pain	Pallor
Diarrhea	Pre-syncope / Syncope
Dyspnea	Premature ventricular contractions
Jaw pain	Restlessness
Fatigue	Sweating
Flushing	Warmness
Headache	Vomiting
Hypotension	Hypoxia

A.5.7 Storage and Handling of Study Drug

Treprostinil will be stored in accordance with manufacturer instructions at room temperature of 15 to 30 °C (59 to 86 °F), and will not be frozen or exposed to heat. The Investigational Drug Service (IDS) at the University of Pittsburgh Medical Center will maintain a log sheet of all study drug as it is received and used during the study. Treprostinil will not be used beyond the expiration date assigned by the manufacturer.

A.6 EXPERIMENTAL PROCEDURES

A.6.1 Screening Phase

The study population will be recruited from all adult, age 18-65 years, subjects who meet routine candidacy criteria at the center to undergo liver transplantation and otherwise meet study entry criteria. Eligible subjects will be given the opportunity to sign informed consent for the study as soon as transplant candidacy is confirmed, which usually occurs weeks or months prior to confirmation that an appropriate donor liver has been procured. Obtainment of informed consent prior to donor liver procurement will be implemented, because subjects may only have a relatively brief time in which to make decisions between notification of procurement, hospitalization, and the transplant procedure. Screening assessments may be conducted after informed consent has been obtained and prior to the Baseline Phase.

Screening activities include:

- Informed consent
- Medical history
- MELD score
- Clinical laboratories
- Recipient demographics (including indication for transplant)

A.6.2 Baseline Phase

The baseline phase activities will occur after donor organ procurement and hospitalization of the subject prior to the transplantation surgery. At this time, informed consent will be confirmed and the subject will be asked if they wish to proceed with the study or wish to withdraw. Baseline activities include the following:

- Medical history (if updated from Screening)
- Physical examination / vital signs
- MELD score
- Clinical laboratories
- Recipient demographics and indication for transplant (if updated from Screening)
- Cadaver donor demographic
- Cytotoxic cross match

A.6.3 Treatment Phase

The Treatment Phase will begin at the initiation of treprostinil after induction of anesthesia for the transplant surgery. The Treatment Phase will continue throughout the surgery, and until termination of the study drug infusion (approximately 48 hrs post-transplantation) and Day 2 assessments are completed.

The first clinical laboratory specimen acquired immediately following the completion of the transplant surgery will be considered the postoperative Day 0 sample. Clinical laboratory samples during the Treatment Phase Day 1 and 2 will be drawn at the institution's routine laboratory collection times. Table 3 describes the schedule for collecting serum bilirubin, ALT and AST levels. The various endpoint assessments including, survival, retransplantation, hospitalization times, etc. are continuous assessments that will be obtained. Study drug will be infused for approximately 48 hours following completion of the transplant surgery.

Treatment Phase activities include:

- Physical examination / vital signs
- Clinical laboratories
- Whenever possible, an intra-operative post reperfusion liver biopsy for histopathology
- Cold ischemia time (hr)
- Subject survival
- Graft survival
- Retransplantation

- Initial hospitalization (days)
- Time (days) in ICU
- Study drug infusion
- Pharmacokinetic samples
- Adverse events

A.6.4 Follow-up Phase

The Follow-Up Phase will begin after termination of study drug infusion and completion of all Treatment Phase assessments, and continue until Study Day 180. The various endpoint assessments (survival, retransplantation, hospitalization times, etc.) will be continuous assessments and obtained for entry on the case report form (CRF) from routine documentation at the center. Transplant recipients may require extended hospitalizations at the transplant center or elsewhere during the recovery period, or they may be released within days of the transplant surgery in the absence of complications. Duration of initial hospitalization, ICU stay, and graft and subject survival will be recorded on the CRF based on routine transplant center documentation (e.g. discharge summaries).

Study-specific laboratory assessments required for Days 3-7 will be collected starting at the institution's first routine morning laboratory collection time.

A.7 STUDY TERMINATION

A.7.1 Subject Discontinuation

A subject may voluntarily withdraw or be withdrawn from the study and/or study drug administration by the investigator or treating sub investigators at any time for reasons including, but not limited to, the following:

- The subject wishes to withdraw from further participation.
- A serious or life-threatening AE occurs or the investigator considers that it is necessary to discontinue study drug to protect the safety of the subject.
- The investigator elects to discontinue the study
- Changes in personnel or facilities adversely affect performance of the study
- The reviewing IRB requires termination of the study for safety or compliance reasons.

In the event that a subject discontinues study drug prematurely due to an AE, the subject will be followed until either the investigator determines that the AE has resolved, it is no longer considered clinically significant, or the subject is lost to further follow-up. If a subject discontinues study drug prematurely for any reason, the subject will be encouraged to remain in the study and attend the remaining scheduled study assessments.

A.8 ADVERSE EVENT REPORTING

A.8.1 Adverse Event

An adverse event (AE) is any untoward medical experience occurring to a subject during a clinical trial whether or not it is related to the study drug. An AE may include a current illness, injury, or any other concomitant impairment of the subject's health, as well as abnormal laboratory findings if deemed to have clinical significance. An AE may also include worsening of an existing symptom or condition or post-treatment events that occur as a result of protocol-mandated procedures.

A.8.2 Serious Adverse Event

Serious adverse event (SAE) is an AE occurring at any dose that results in any of the following:

- Death
- A life-threatening AE
- Inpatient hospitalization or prolongation of existing hospitalization
- A persistent or significant disability / incapacity
- A congenital anomaly / birth defect

In addition, important medical events that may not result in a fatal outcome, be life-threatening, or require hospitalization may be considered serious when, based upon appropriate medical judgment, they may jeopardize the subject and require medical / surgical intervention to prevent

one of the outcomes listed above. Examples of such medical events include allergic bronchospasm requiring intensive treatment in an emergency room or at home, blood dyscrasias or convulsions that do not result in hospitalization, or the development of drug dependency or drug abuse.

Life-threatening means that the subject was, in the view of the sponsor-investigator, at immediate risk of death from the event as it occurred. It does not mean that the event, had it occurred in a more severe form, might have caused death.

A.8.3 Expected Adverse Event

AEs associated with liver transplant surgery outcome include:

All study subjects will be liver transplant recipients who may be critically ill from underlying liver disease and/or associated conditions prior to transplant surgery, and who will be recovering from the transplant procedure afterward. According to the "Consent to Adult Liver Transplant" used at UPMC, the risk of some type of complication (major or minor) from liver transplant surgery is 45 to 55%, and the death rate from surgery is 4%. Subjects are likely to be in an ICU with or without ventilator support after the surgery, and to be hospitalized for periods ranging from days to months. Expected non-serious and serious AEs in liver transplant recipients include a long list of intra-operative complications and sequelae from major surgery and transplant, including bleeding, major infection, renal failure with or without hemodialysis, cardiovascular problems, reactions to or side effects from immunosuppressive drugs, acute rejection of the donor liver, and many other issues.

Events that are normally observed in liver transplant recipients are listed in Table 12. All AEs will be captured on the CRF.

Any event that occurs under circumstances in which it is considered possible that study drug may have caused or contributed to the event MUST be reported as an AE, rather than as a normal liver transplant event, because of the possible relationship to study drug. Known adverse events related to treprostinil are in the package insert [236].

AEs known to be associated with treprostinil therapy: an expected AE for treprostinil is defined as any AE that is defined in terms of nature, severity, and frequency in the current Investigators' Brochure (United Therapeutics, Inc.). These findings should be listed in the CRF as AEs.

Table 11: Expected Event is Liver Transplant Patients

Category of Event	Events
Blood disorders	Hemorrhage / coagulopathy / thrombosis Exposure to communicable disease and other risks of blood / blood products as listed on liver transplant consent form Thrombocytopenia Leukopenia Anemia Disseminated intravascular coagulopathy (DIC)
Cardiac and vascular disorders	Hypotension
Gastrointestinal disorders	Ascites
Hepatobiliary disorders	Jaundice Hepatic failure Portal vein thrombosis Bile duct stenosis Hepatic artery thrombosis Hepatic artery stenosis Hepatoportal venous flow-hyperperfusion syndrome
Immune system disorders	Liver transplant rejection Immunosuppression
Infections	Sepsis Septicemia Lower respiratory tract infection Peritonitis Urinary tract infection Pneumonia Bacteremias Wound infection
Injury, poisoning and procedural disorders	Post procedural bile leak Splenic injury Injury to structures in the abdomen Damage to nerves due to contact or positioning during surgery burns (e.g. from cauterization or other electrical equipment) Scarring
Nervous system & Psychiatric disorders	Confusional state / agitation / encephalopathy Cerebrovascular accident
Renal disorders	Acute renal failure Electrolyte disorders
Respiratory disorders	Acute respiratory failure
Skin disorders	Decubitus ulcer [pressure ulcer]

A.8.4 Documentation of Adverse Events

An AE or SAE occurring during the study, and which is felt to be possibly or likely related to treprostinil administration, must be documented in the subject's source documents and on the appropriate CRF page. Information relating to the AE such as onset and cessation date and times, intensity, seriousness, relationship to study drug, and outcome is also to be documented in the CRF. Where possible, AEs should be recorded using standard medical terminology. If several signs or symptoms are clearly related to a medically defined diagnosis or syndrome, the diagnosis or syndrome should be recorded on the CRF page, not the individual signs and symptoms.

All AEs must be followed until resolution (or return to normal baseline values), or until they are judged by the investigator to no longer be clinically significant, or for at least 4 weeks if the AE extends beyond the Day 180 assessments.

All treprostinil-related SAEs should be followed until resolution, death, or the subject is lost to follow up or up to Day 180 assessments if the SAE is still continuing. Supplemental measurements and/or evaluations may be necessary to fully investigate the nature and/or causality of an AE or SAE. This may include additional laboratory tests, diagnostic procedures, or consultation with other healthcare professionals. CRF pages should be updated with any new or additional information as appropriate.

A.8.5 Reporting Responsibilities of the Investigator

In accordance with guidelines established by the University of Pittsburgh Institutional Review Board (IRB), the sponsor-investigator will promptly notify the IRB of all serious and unexpected adverse events felt to be related or possibly related to the study drug.

A.8.6 Safety Reports

In accordance with FDA regulations, the sponsor-investigator will notify the FDA, other competent authorities, and the sub-investigators of any AE that is considered to be reasonably or possibly attributable to study drug and is both serious and unexpected.

A.9 STATISTICS

A.9.1 Data Collection and Retrieval

Results of all assessments will be collected in an excel spread sheet for each subject enrolled in the study.

A.9.2 Primary Safety and Preliminary Efficacy Endpoint

This is a phase I/II study and by nature is descriptive. It follows a typical phase 1 protocol.

A.10 SAFETY ANALYSIS

The safety of treprostinil will be evaluated by analyses of AEs and clinical laboratory parameters. They will be summarized according to intensity, seriousness and causality. For all safety endpoints, tabular summaries will be provided. Secondary endpoints will be calculated and/or analyzed based on data routinely obtained by the Transplant Institute.

Pharmacokinetic parameters such as clearance, volume of distribution, half-life, terminal disposition rate constant will be calculated.

Preliminary Efficacy: Peak and AUC values for bilirubin, AST and ALT will be calculated. The hospital costs, and total hospitalization days, where relevant will also be collected. The data collected in this study will be compared to historical data as preliminary estimate of efficacy.

A.10.1 Interim Analyses

No interim efficacy analysis is planned.

A.10.2 Data Monitoring Committee

The protocol will be submitted to the Institutional Review Board (IRB) at the University of Pittsburgh. The Starzl Transplantation Institute PRC/DSMB will serve as the data and safety monitoring committee. The data collected from each subject will be reviewed by the investigators on a patient to patient basis.

A.11 PACKAGING AND FORMULATION

A.11.1 Study Drug Content

FDA-approved treprostinil (1 mg/mL) will be obtained from United Therapeutics, Inc. who will supply the study drug in 20-mL multiple-entry vials

A.11.2 Study Drug Storage and Handling

The treprostinil will be stored securely in a controlled-access area at room temperature of 15 to 30 °C (59 to 86 °F). It will not be frozen or exposed to heat.

A.11.3 Study Drug Accountability

The sponsor-investigator is responsible for study drug accountability and reconciliation overall and on a per subject basis. Drug accountability records will be maintained during the study and these records will include: the amount of study drug received from the manufacturer for this study, the amount dispensed to each subject, and the amount of unused drug. During the Treatment Phase site personnel should assess drug dispensed, drug returned, and dosing information to confirm drug accountability and compliance.

A.11.4 Study Documentation and Storage

Study records will be retained in accordance with FDA and IRB requirements.

A.12 REGULATORY AND ETHICAL OBLIGATION

A.12.1 Regulatory Requirements

The investigator will obtain the required FDA and ethics committee approval to conduct the study. During the conduct of the study an Annual Report will be compiled by the sponsor-investigator for submission to the FDA, as required. Any additional local reporting requirements as specified by the IRB or other institutional authorities will also be fulfilled during the conduct of the study.

A.12.2 Informed Consent Requirements

Before a subject is enrolled in the study, the investigator or their designated sub-investigator(s) must explain the purpose and nature of the study, including potential benefits and risks and all study procedures to the subject. The subject must sign and date an IRB-approved informed consent form prior to the conduct of any study-related activities. A copy of the signed consent form will be given to the subject and the original will be retained in the study site's records.

A.12.3 Institutional Review Board

Prior to study initiation the investigator will obtain approval for the study from the University of Pittsburgh IRB. This IRB operates in accordance with the FDA regulations at 21 CFR Parts 50 and 56. If, during the study, it is necessary to amend either the protocol or the informed consent form, the investigator is responsible for obtaining IRB approval of these amended documents

prior to implementation. A written summary of the study will be provided by the investigator to the IRB following study completion or termination according to the IRB standard procedures. Additional updates will also be provided in accordance with the IRB standard procedures.

A.12.4 Subject Confidentiality

Every effort will be made to keep medical information confidential. The FDA and the IRB may inspect the medical records of any subject involved in this study. The investigator may release the subject's case records to the IRB or the FDA or appropriate local regulatory agencies for purposes of checking the accuracy of the data and/or regulatory compliance. A number will be assigned to all subjects and any report published will not identify the subjects' names.

A.13 PROTOCOL AMENDMENTS AND STUDY TERMINATION

Protocol Amendments will be submitted prospectively to the FDA for any change to the protocol that significantly affects the safety of the subjects. Other changes to the protocol will be submitted as a Protocol Amendment at the time of requisite Annual Reports to the IND application. All changes to the protocol must be prospectively approved by the University of Pittsburgh IRB. No deviations from the IRB-approved protocol are permitted, except as necessary to protect the safety of individual research subjects. Such deviations from the protocol will be promptly reported to the IRB. A final report will be submitted to the IRB and FDA at the time of study termination.

APPENDIX B

B.1 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

PRINCIPAL INVESTIGATOR: Dr. Abhinav Humar, Professor of Surgery

Montefoire Hospital North 725, 3459 Fifth Avenue, Pittsburgh, PA 15213

Phone: 412-692-4553, Fax # 412-692-4180

Co-Principal Investigator: Raman Venkataramanan, Ph.D; F.C.P.;

Professor of Pharmaceutical Sciences and Pathology

718 Salk Hall, University; of Pittsburgh School of Pharmacy

3501 Terrace Street, Pittsburgh, PA 15261

Phone: 412-648-8547 Fax: 412-383-7436

Co-investigators: Thomas Starzl Transplantation Institute Liver Surgeons:

Paulo Fontes, M.D, Mark Sturdevent, M.D., Ruy J. Cruz, M.D., Mark L. Sturdevant, M.D., Roberto C. Lopez, M.D., and Raymond Planinsic M.D.(Liver Transplant Anesthesia Team)
Montefiore University Hospital,
3459 Fifth Avenue Pittsburgh, PA 15213
Phone: 412-692-4553

Co-investogators: Pathology

Anthony Demetris, E737 UPMC-Montefiore, 3459 Fifth Avenue, Pittsburgh, PA 15213

Phone 412-647-7646

Co-investigators: School of Pharmacy
Nisanne Ghonem, PharmD, PhD: 731 Salk Hall, 3501 Terrace Street, Pittsburgh, PA

Phone: 412-648-2377

Starzl Transplantation Institute:

Tracy Grogan, Unit Director: UPMC Montefiore South 555, 200 Lothrop Steer, Pittsburgh, PA

15213. Phone: 412-647-8560

Clinical Research Manager: Sheila Fedorek, RN CCRC

Research Coordinators: Laurie Hope, R.N., Stephenie Kikla, R.N. and Leslie Mitrik, B.S.

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Transplantation Institute

Why is this research being done?

The liver is subjected to low temperature during transportation from the person who donates the liver (the donor) to the person who gets it (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 transplantation. There are no medical treatments approved by the U.S. Food and Drug Administration (FDA) to prevent such problems. This purpose of this research study is to find out whether a drug called Treprostinil is useful in preventing 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 by preventing blood components from sticking together. Drugs like Treprostinil can also protect cells from the kinds of injury described above. 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. At UPMC, two patients with end stage liver disease have received Treprostinil (36 and 45 ng/kg/min) during liver transplant, continuing throughout the transplant procedure and afterward in the intensive care unit without any treprostinil-related problems. However, 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. In this study we will test to see whether or not Treprostinil decreases damage to liver cells and decreases the length of stay in the hospital.

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 transplantation. 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.

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 2 days, and follow up phase that will last up to day 7 after you receive the new liver. On post-op days 30, 90 and 180 we will only be collecting information on your survival and liver status, information if you have been retransplanted, initial hospitalization and time in the intensive care unit. This information will be obtained from your hospital and clinic records.

Screening Visit

The Screening Phase can occur up to 180 days before your liver transplant surgery following your selection as a candidate for liver transplantation. To determine if you meet the criteria for participation in this study, the doctor will review and collect information about your medical history. You will have a physical examination and your vital signs will be taken. Blood tests will be done that are part of the clinic's standard screening for liver transplant surgery and information (age, gender, weight, height, medical history, clinical laboratory test results indicative of your liver and kidney function) will be collected for the study from these tests. If you meet all the study participation conditions and sign the informed consent, you can enter the "Baseline visit".

Baseline Visit

The baseline visit occurs the day you enter the hospital for the liver transplant surgery. During this time, your doctor will make a final decision if you can 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 urine pregnancy test will be performed in women of child bearing potential. If you meet all the study entry conditions, you will be enrolled in the study and will receive one of the doses selected by your doctor. Once the Baseline assessments are complete, you will enter the Study Treatment Phase.

Treatment Phase

You will begin receiving Treprostinil at the time you receive medications that put you to sleep and prepare you for the surgery. Treprostinil will be given through a central line (a tube placed into a large blood vessel in your chest) 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 medication (drug) can be given in this line.

You will continue to receive study drug (Treprostinil) during your surgery and for 2 days (48 hours) after your surgery and then the study drug will be stopped. You will be in the hospital and will be closely watched by members of your medical team for any problems during this entire time. On the first day after the transplant, in addition to the routine blood sampling, two additional blood samples will be taken to measure the certain liver enzymes such as AST, ALT, that tell us how your liver is working.

Medical information that is part of the routine care of liver transplant surgery will also be collected and includes 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) and information about the donor liver such as age, gender and weight. You may also be asked questions to find out whether you had any unusual problems or symptoms that may be related to the administration of study drug. Blood samples or any other biological material (optional liver biopsy) already collected may also be used for assessment of substances in the blood that indicate injury to the new liver. They will not be used for any genetic testing.

Follow-Up Phase

The Follow-Up Phase visits for the study will be done during the routine follow-up care that you receive after liver transplant surgery. Results from blood tests that measure the function of your liver and kidney and physical examinations that are done on day 3 and 7 will be collected.

In addition, on post-op days 30, 90 and 180 we will only be collecting information on the condition of your new liver and if you needed another transplant, if you are still in the hospital or intensive care unit following the original surgery, and are you alive and well at these time points. This information will be obtained from your hospital and clinic records. Information regarding amount of time you may have spent needing a breathing machine will also be collected.

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.

If you decide to participate in the study, your medical records will be reviewed for demographic information (age, gender, and race), lab results (done as part of your routine post transplant care), medication information, and information about the results of testing and procedures that are preformed during the transplant follow-up period for days 1 through 7, as described above.

Additional Assessments That May Be Conducted During the Treatment and Follow-up Phases

Pharmacokinetic (PK) Samples

Up to eighteen 3 mL of blood samples will be collected. The times the blood samples may be taken include before the start of study drug, during surgery, and after the study drug is stopped (48 hours after the start of infusion), and up to 12 hours after the study drug was stopped. Theses blood samples will not be used for any other 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 side effects of Treprostinil, all of which are not known at this time, the risks associated with a line used for giving you the drug and risk associated with blood sampling for measuring Treprostinil levels.

As with any investigational drug there may be adverse events or side effects 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 side effects of Treprostinil may include, but are not limited to, flushing of the skin, headache, nausea, vomiting, diarrhea, and jaw pain. If these side effects develop and are intolerable, the dose of the study drug may be reduced or stopped until the side effects 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,
Rare:	Decreased blood pressure

Risks of Intravenous infusion of treprostinil: The study drug may be delivered using a tube placed into a large vein in the chest 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 problem, blood levels of Treprostinil may be higher than normal. Treprostinil has not been studied in patients with severe liver failure, although it has been administered safely to such patients at the University of Pittsburgh Medical Center and elsewhere. In one small study, Treprostinil blood levels were found to be 2-4 times higher in patients with some degree of liver failure. Infusion of Treprostinil or drugs similar to Treprostinil occasionally has been done during liver transplant surgery without causing any serious problems. However, because there is period when the diseased liver has been removed and the new liver has not started to work, there is a time during the surgery when Treprostinil blood levels may increase five times or more. This could 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 without your agreement based on medical information available to them.

Treprostinil has not been shown to cause cancer or affect fertility or mating performance in rats at a dose that is 60 times the highest dose used in this study. In pregnant rats a dose that is 120 times the maximum dose to be used in this study did not have any evidence of harm to the fetus. Because animal studies are not always predictive of what might happen in humans, pregnant subjects should not use Treprostinil.

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 urine pregnancy test will be done during baseline visit. 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 of blood sampling: The risks associated with blood sampling are minimal as the subjects will already have a catheter inserted for other blood sampling. In rare cases when a catheter is not already in, a small tube will be inserted in the arm vein for blood collection.

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. No other routine treatment will be withheld.

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 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. United Therapeutics Corporation will cover the costs associated with the following procedures and tests carried out for research purposes: study drug cost, pump for infusion, and Treprostinil blood level analysis

University of Pittsburgh Thomas Starzl Transplantation Institute will cover the costs associated with the following procedures and tests carried out for research purposes:

- Drug administration
- Post reperfusion liver biopsy (optional)
- Additional ALT/AST on day 1 and 2
- Study Day 7 procedures, in the event a patient is to be discharged before day 7.
- Treprostinil pharmacokinetic sampling will be performed by research technician hired to perform this.

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 coast 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.

No compensation will be provided by United Therapeutics Corporation. This includes no financial support for lost wages, disability, pain or discomfort.

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 researchers and their associates who provide services at University of Pittsburgh Medical Center (UPMC) recognize the importance of your voluntary participation in their research studies. These individuals and their staffs will make reasonable efforts to minimize, control, and treat any injuries that may arise as a result of this research. If you believe that you are injured as a result of the research procedures being performed, please contact immediately the Principal Investigator or one of the co-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 UPMC. It is possible that UPMC may bill your insurance provider for the costs of this emergency treatment, but none of these 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 unless otherwise specifically stated below. If you are physically injured by the study drug and you have followed the directions of the study personnel, United Therapeutics Corporation will cover the medical expenses necessary to treat the injury. United Therapeutics Corporation will provide no additional financial compensation. There is no plan for monetary compensation. You do not, however, waive any legal rights by signing this form.

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 unless you sign a separate consent form giving your permission (release).

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. The information that will be recorded will be limited to information concerning demographics (age, gender, and race) and concurrent conditions and medications you are receiving.

This research study will result in identifiable information that will be placed into your medical records held at UPMC Presbyterian and Montefiore.

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 of the United Therapeutics Corporation may review your identifiable research information (which may include your identifiable medical information) related to your participation in this research study for the purpose of monitoring the accuracy and completeness of the research data and for performing required scientific analyses for the research data. While the study sponsor 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 study sponsor. The investigators involved in the conduct of this research study may receive funding

form the sponsor to perform the research procedures and to provide the sponsor with identifiable research and medical information related to your participation in the 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?

All the blood samples collected from you will be labeled using an identification number without your name. They will be stored in the laboratory of the researchers until all the data is obtained from these samples. 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 5 years and for as long (indefinite) as it may take to complete this research study.

The blood samples collected in this study will be kept for an indefinite time period until a complete report of the study has been published. The sample with out the identification may be utilized in future studies by the investigators. These samples will not be shared with any secondary investigators not listed on the current 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

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 agree to participate in this research study. A copy of this consent form will be given to me.

☐ By signing this form, I agree to participate in the additional Pharmacokinetic Sampling

_____ _____

Participant's Signature Date

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."

_____ _____

Printed Name of Person Obtaining Consent Role in Research Study

_____ _____

Signature of Person Obtaining Consent Date

ONLY WHEN APPLICABLE FOR PROXY CONSENT:

The patient is unable to consent because:

I therefore, consent to participation for the patient

_____ _____

Signature Date

_____ _____

Legal Representative Relationship to Subject

_____ _____

Witness Signature Date

VERIFICATION OF EXPLANATION

I certify that I have carefully explained the purpose and nature of this research to _____ in appropriate language. He/She has had an opportunity to discuss it with me in detail. I have answered all of his/she provided affirmative agreement (i.e., assent)to participate in this research.

_____ _____

Printed Name of Person Obtaining Consent Role in Research Study

_____ _____

Signature of Person Obtaining Consent Date

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