Mechanisms of human erythrocyte clearance during human-to-porcine-liver xenoperfusion and the assessment of current pre-clinical models

Joshua Paul Waldman
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A Dissertation

Entitled:

Mechanisms of Human Erythrocyte Clearance During Human-to-Porcine-Liver Xenoperfusion and the Assessment of Current Pre-clinical Models

By:

Joshua Paul Waldman

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Biomedical Sciences

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December 2013
An Abstract of
Mechanisms of Human Erythrocyte Clearance During Human-to-Porcine-Liver Xenoperfusion and the Assessment of Current Pre-clinical Models

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Background: Xenotransplantation offers the potential to greatly expand the supply of organs available for human transplantation. It is thought that the most likely form of xenotransplantation to be utilized in the clinic in the near future will be the use of a porcine liver to treat patients in hepatic failure (FHF). Currently, patients in FHF do not have a temporary means of support while awaiting liver transplantation. A potential therapeutic approach for patients in FHF is the use of extracorporeal perfusion with porcine livers as a form of “liver dialysis”. During a 72 hour extracorporeal perfusion of porcine livers with human blood, porcine Kupffer cells (KC) bind to and phagocytose human red blood cells (hRBC) causing the hematocrit to decrease to 2.5% of the original value. Our laboratory has identified sialoadhesin (Sn) on the surface of porcine KC as the lectin responsible for binding N-Acetyleneuraminic acid (Neu5Ac) on the surface of the hRBC. Current food and drug administration regulations mandate that before xenotransplantation advances to the clinic, prior success in must have been shown in the nonhuman primate (NHP) model. However, it is well documented that humans are the only primate species which expresses Neu5Ac as the primary sugar on the surface of its
cells surface. In order for extracorporeal porcine liver perfusion to be used as a therapy for patients in liver failure, all forms of anti-human rejection must be resolved. We evaluated whether targeting Sn prevents the recognition and subsequent destruction of hRBCs seen during extracorporeal porcine liver xenoperfusion. Additionally, we evaluated the efficacy of NHP to serve as an effective pre-clinical model for extracorporeal porcine liver perfusion studies. Finally, we explored the possibility of using Brain Dead Research Subjects (BDRS) as a viable alternative for pre-clinical models of xenotransplantation.

Methods: In order to determine the effect of targeting Sn on preventing the destruction of human erythrocytes during an extracorporeal perfusion of porcine livers with human blood, a series of in vitro studies were performed wherein porcine macrophages were co-incubated with human erythrocytes in the presence of an anti-Sn monoclonal antibody (mAb). Next, a series of ex vivo porcine liver perfusions were performed using isolated packed hRBCs in the presence of either an anti-Sn mAb or an isotype control. In order to assess the ability of NHP to accurately reflect clinical liver xenoperfusion interactions, porcine macrophages were tested for ability to bind erythrocytes from various primate species, Human and NHP erythrocytes were co-incubated with porcine macrophages in an in vitro assay and binding was assessed using a colorimetric assay. Finally, public opinion on the use of BDRS as an alternative model to pre-clinical studies was assessed using a 17 question survey.

Results: We have shown that the pre-treatment of porcine macrophages with an anti-Sn monoclonal Ab significantly reduces their ability to bind human erythrocytes (p,<0.001). Furthermore, the addition of an anti-Sn monoclonal Ab to the perfusate on an
extracorporeal porcine liver xenoperfusion model significantly reduces the loss of hRBC over a 72 hour period (p,<0.01). Sustained liver function was demonstrated by continued bile production and other markers. Differences in the ability of porcine macrophages to bind human and NHP erythrocytes was shown (p,<0.001). Finally, our survey illustrated that 75% of the general public agreed to the use of BDRS in medical research with nearly 60% agreeing to the use of these subjects in medical research involving xenotransplantation research.

Conclusions: These studies suggest that the destruction of human erythrocytes in an extracorporeal porcine liver xenoperfusion model is, in part, mediated by Sn expressed on the surface of porcine KC. Furthermore, the in vitro study highlights the inability of NHP to serve as an adequate model for studying extracorporeal porcine liver xenoperfusion with human blood. Finally, data from the public survey illustrates that the majority of the public supports the use of BDRS in medical research, including studies of xenotransplantation.
This work is dedicated to my beautiful wife Erin, my beloved family members, including my mother and father, Paul and Carol Waldman, my Ph.D. advisor and friend, Michael Rees, and the numerous other individuals whose belief in me has provided me the strength and perseverance to achieve that which I once thought was impossible. The support of these individuals continues to provide me with the courage to follow my dreams.
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List of Abbreviations

Ab.............................Antibody
ALF..........................Acute Liver Failure
ALT..........................Alanine Aminotransferase
ASGR1.........................Asialoglycoprotein Receptor 1

BAL..........................Bronchoalveolar Lavage
BDRS........................Brain Dead Research Subject

CFSE..........................Carboxyfluorescein succinimidyl ester
CMP..........................Cystidine monophospho-

ECL.........................Enhanced Chemiluminescence
ECLP........................Extracorporeal Liver Perfusion
ECMO.........................Extracorporeal Membrane Oxygenation
ELISA........................Enzyme-linked immunosorbent assay

FHF..........................Fulminant Hepatic Failure

GPIb..........................Glycoprotein 1b
GTKO..........................alpha-1,3-galactosyltransferase

H&E..........................Hematoxylin and Eosin
HAR..........................Hyperacute Rejection
HCT..........................Hematocrit
hDAF..........................human Decay Accelerating Factor
hEGP..........................human Erythrocyte Glycoprotein
hGA..........................human Glycoporphin A
hRBC..........................human Red Blood Cell
HRP..........................Horseradish Peroxidase

IgG..........................Immunoglobulin G
IVC..........................Inferior vena cava

KC..........................Kupffer Cell
kDa..........................Kilodalton
LSECS......................Liver Sinusoid Endothelial Cells
mAb.......................Monoclonal Antibody
MAC-1 .....................Macrophage-1 Antigen

NANA .....................N-Acetylneuraminic acid
Neu5Ac  .................N-Acetylneuraminic acid
Neu5Gc ..................N-Glycolyneuraminic acid
NHP ......................Nonhuman Primate

OCT ......................Optimal Cutting Temperature

PAM .....................Porcine Alveolar Macrophage
PBS ......................Phosphate Buffered Saline
pMF ......................Porcine Macrophage
PRRSV ..................Porcine Reproductive and Respiratory Stress Virus
pSn ....................porcine Sialoadhedin

RBC .....................Red Blood Cell
RNA .....................Ribonucleic Acid

Siglec ....................Sialic acid-binding immunoglobulin-type lectin
SA .......................Sialic Acid
Sn  ......................Sialoadhesin

TF .........................Tissue Factor
TMB ......................Tetramethylbenzidine
TPN ......................Total Parenteral Nutrition

vWF .....................Von Willebrand Factor

WBC .....................White Blood Cell
WT .......................Wild Type
List of Symbols

\(\alpha\) .................................. Alpha
МФ  .......................................... Macrophage
Preface

Xenotransplantation offers the possibility of expanding the supply of organs to the more than 114,000 patients currently on the transplantation waiting list (1). The clinical practice of xenotransplantation is currently limited by several forms of rejections. Unlike the rejection seen in allotransplantation, which is driven largely through T-cells either in a direct or indirect activation pathway, organs transplanted across species barriers elicit a rejection response that involves the innate immune system. This thesis will describe a form of rejection that is mediated by macrophages of the innate immune system and that currently limits the use of porcine livers in serving as a bridge therapy for patients in need of liver transplantation.

Currently, there are more than 17,000 patients who are in need of liver transplantation and who find themselves on the transplantation waiting list. For those awaiting liver transplantation who have been diagnosed with acute liver failure (ALF), emergency liver transplantation serves as a life saving therapy. However, given the rapid progression of the disease, and the lack of organs available for transplantation, many of these patients will die before a suitable donor liver becomes available. One specific form of xenotransplantation that has the potential of helping patients in ALF is the use of a porcine liver in an extracorporeal liver perfusion (ECLP) device. It is thought that a porcine liver in a human-to-pig liver ECLP device could serve as a liver dialysis therapy,
acting to bridge patients diagnosed with ALF to liver transplantation or recovery (Figure 1-1) (1).

In this thesis, I will take the reader through the journey of how my collaborators and I came to understand the key mechanisms involved in mediating the loss of human erythrocytes perfused through a porcine liver, a graft-vs-host response that currently

Figure 1-1 Concept of Extracorporeal Liver Perfusion
limits the use of porcine liver ECLP in the clinic. I will also discuss other contributions that were made to the scientific and medical communities as a result of this research.
Chapter 1

Introduction to Extracorporeal liver perfusion*

(*A modified version of this chapter is in preparation for submission to the journal Xenotransplantation)

1.1 INTRODUCTION

Advancements in the treatment of end stage organ failure could be considered one of the greatest advancements in medicine to date. These types of treatments could be classified as temporary treatments as in cardiopulmonary bypass or as permanent treatments as in kidney dialysis. Further classification would go on to characterize treatments into either mechanistic as is illustrated with the previous two examples, or as biological, as is exemplified by the use of kidney transplantation to treat patients in end stage kidney failure. While treatments offering temporary support for cardiac, lung, renal, and pancreas have been discovered, hepatic failure remains unique in that we have failed to devise a temporary means of support.

As is often the case when attempts for novel therapeutics fail, it is not for a lack of effort, but rather, it is for a lack of understanding that these novel attempts fall short.
Development of a temporary liver support device exemplifies this. Although many scientists have diligently worked to develop such a device, the complexity of the liver has continued to serve as a daunting roadblock. It is no wonder attempts to develop a temporary means of support for the liver have failed despite the encouraging developments of such support for its neighboring organs. Contrary to the liver, organs such as the heart, lung, kidney and pancreas can be characterized using a simplistic description; the heart pumps, the kidney filters, the lungs are membranes and the pancreas produces insulin. However, a simplistic description of the liver is much harder to portray. The liver is a filter, a detoxification plant, and a synthetic factory, not to mention all of the other obvious yet unclassified functions for which it is responsible.

Our inability to develop a temporary support therapy for the liver could be in part due to our inability to accurately assess its function. While a creatinine clearance goes a long way towards classifying one’s renal function, clinicians have struggled to devise a test that accurately assesses the function of the liver. We have the ability to measure such things as, injury to hepatocytes, obstruction of the biliary secretion system, or a deficit in the components which are crucial in the coagulation system. However, attempts to identify markers that accurately predict metabolic potentials of the liver have remained unsuccessful. Given our inability to classify crucial functions of the liver, it is no wonder we have failed to develop a form of temporary liver support. If one cannot accurately assess function of the liver, how are they able to accurately assess whether or not they have successfully developed a device which is able to maintain hepatic function?
1.2 HISTORY OF ISOLATED LIVER PERFUSION

When Professor Ben Eiseman spoke to the assembled guests at the Royal College of Surgeons during his Moynihan Lecture in 1965, he stated,

“...it has always appeared to me rather naïve to think that failure of an organ so metabolically complex as the liver could be reversed by clearance of any single “toxin”. In this stage of electrophoretic discrimination, the blood needs not only a mere spot-remover but a thorough laundering. It would seem that only another liver, used either as a permanent graft or as a temporary support while the patient recovers from an acute metabolic insult, could thus be of clinical benefits...” (2)

Here, Eisenman expressed his strong view for the need of hepatic support using another functioning liver. As he seemed to predict, to date, both biological and mechanical perfusion devices have been designed, but have subsequently failed to decrease the mortality rate in patients with fulminant hepatic failure (3).

Although liver support began with the pioneering work from the Welch laboratory in 1958(4), a long line of investigators had previously advanced the study of isolated organ perfusions which helped Welch find success in the support of an animal in liver failure. The first artificial circulation was probably performed by Kay in 1828, in order to restore irritability of dying muscles (5). The first perfusion apparatus as well as the first device for perfusing blood under constant pressure from a reservoir was likely developed by Ludwig and Schmidt in the Ludwig laboratory in 1868 (5, 6). Using blood perfused under pressure, de Cyon is reported to have kept a frog’s heart beating for 48 hours (7).

The first study of extracorporeal liver perfusion dates back to 1855 when the conversion of glycogen to glucose by the liver was characterized by Claude Bernard by perfusing tap water through a liver (8-10). In his Comptes Rendus de l'Academie des
Sciences article, he described his perfusion technique in this way: “…the tube, filled with water was connected to the portal vein at one end and to the fountain of the laboratory of the Collège de France at the other end; perfusion was started with tap water!” As crude as the set-up may have been, Bernard is credited as the first to perfuse an isolated liver (11). With his work, which was done in the late 1800’s, de Cyon was the first to perfuse an isolated liver with blood. Using a technique with constant blood pressure perfusion, de Cyon was able to show that perfused livers still manufactured urea (5). It is interesting to note that by the turn of the 20th century, Brodie was already aware of many of the issues that continue to face current researchers. In his article written in 1903, he observed the following: 1) Xenoperfusions were inferior to alloperfusions and clotting of the blood seemed to be one of the problems associated with xenogeneic perfusions; 2) The Use of allogeneic blood was thus found to improve perfusion and he noted that citrated blood helped to improve both xenogeneic and allogeneic perfusion; 3) Minimizing ischemia improved extracorporeal organ function and decreased the amount of edema observed; 4) Artificial pumping of blood led to hemolysis and was improved by exposing the blood to less toxic substances such as glass rather than metal; 5) Oxygenating the blood by bubbling air through it led to a considerable amount of foaming which had to be removed in order to avoid embolism; 6) Pulsatile perfusion appeared to superior to non-pulsatile in terms of minimizing edema; 7) Various forms of heat exchangers were found to be adequate at maintaining normothermic temperatures; 8) Increased vascular resistance with decreased blood flow was often an omen of the end of a successful extracorporeal perfusion (12).
A pivotal point in the history of isolated organ perfusion happened by chance when the first person living in North America to win a Nobel Prize, Alexis Carrel, met Charles Augustus Lindbergh, the first person to fly the 33.5 hour transatlantic flight from Long Island to Le Bourget Field near Paris. During a visit to the Rockefeller Institute to see his sister-in-law who was there as a patient due to a heart disorder, Lindbergh learned that a necessary operation could not be done to help his sister-in-law because the organ could not be stopped long enough for the surgery to be performed. Upon further inquisition with Carrel, the attending physician, he went on to learn that the pumps that had been designed up to that point were not adequate at recirculating the patient’s blood as infection along with coagulation and hemolysis were observed due to the harsh surfaces and hammering valves (13, 14). Having been trained as an engineer, Lindbergh was not content knowing that it was because of a lack of design that patients were unable to undergo certain surgeries which relied on a machine to provide the functions of a beating heart. With his skills as an engineer and access to Carrel’s laboratory, Lindbergh was able to come up with sketches of a new design which Carrel passed on to Otto Hopf, an exceptional glass blower who had a workshop in the basement of the Rockefeller Institute. Given the limited technology of their time—that predated antibiotics—and the fact that they had to create their own perfusion apparatus from scratch, Carrel and Lindbergh, with this new design, were able to perfuse a cat’s thyroid gland for 18 days in an *ex vivo* fashion. Furthermore, they went on to successfully maintain the viability of a perfused organ for up to 18 days (13, 14).

One could argue that the advent of the cardiopulmonary bypass machine developed in the 1950s was necessary for the fostering of extracorporeal liver perfusion.
Or that the ability to produce heparin in mass quantities was essential for its success. As accurate as the aforementioned claims may be, it would be unreasonable not to identify Bauer et al as one of the founders of extracorporeal liver perfusion with the publication of their own control of circulation through the liver in 1932. In his studies, Bauer et al was able to show that the canine liver, when perfused, exhibited a sphincter like mechanism which was located near the caval orifices of the main hepatic veins (14). Interestingly enough, this vasomotor resistance to outflow was present in no species other than dogs. Although Bauer classified this phenomenon called “outflow” block in the 1930s it continued to cause considerable consternation to researchers in the 1960s studying isolated liver perfusion (2, 15, 16). It was not until Eiseman perfused 26 canine livers, that he gave up canine liver perfusion and switched to using porcine and calf livers (16).

Concurrent with the research and development of large animal isolated organ perfusion, investigators were using isolated rat liver perfusions to ascertain information regarding hepatic physiology. Trowell et al was the first to use the rat liver as a model for studying hepatic physiology. With a review of the literature, he was able to determine that the oxygen consumption of the rat liver was 2 cc O₂/g/hour. With this information and taking into account the solubility coefficient in saline, he was able to determine, based on the maximum delivery rate into the liver, that he would not be able to sustain a liver using standard perfusion practices. To overcome the limitations of O₂ delivery using the standard perfusion apparatus he resorted to a retrograde perfusion of the vena cava, noting that the saline flowed three times as fast in the reverse direction. By perfusing the liver in a retrograde fashion, he was able to supply the liver with enough oxygen to maintain a viable liver that allowed for the performance of biochemical studies (17). It
was through the use of this perfusion device that Trowell was able to maintain an extracorporeal liver long enough to study the production of urea (17). Although several others began using the isolated liver perfusion as a model to answer biological questions, it was not until Sir Hans Krebs used the utility of the isolated liver perfusion to study gluconeogenesis, that the technique became widely accepted by biochemists (18).

Being able to perfuse an isolated liver with a red blood cell free perfusate would have obvious implications. However, limitations in oxygen delivery have limited such hemoglobin free perfusions in livers that are less than 25-grams (10). Although the species that have been used in isolated liver perfusions include: humans, monkey, calf, sheep, pig, dog, cat, rabbit, guinea pig, hamster, rat, mouse, and frog, physiologist have generally chosen livers from small mammals. However, if one is trying to develop the use of an isolated liver perfusion device to serve as a therapy for patients in liver failure, it is obvious that larger mammals will have to become the liver donors in order to have sufficient mass to serve as an extracorporeal liver perfusion device. As the ability to perfuse the liver of a large mammal requires an ability to oxygenate the perfusate, it is appropriate to give a brief description of the pioneering efforts which led to our ability to oxygenate blood.

As many discoveries in biomedical sciences are spurred by one’s unrest, so too was the discovery of the cardiopulmonary by-pass machine. In 1931 as John (Jack) Heysham Gibbon (1903-1973) played in his mind the image of seeing a little girl die of a pulmonary embolism, he also began to construct in his mind an artificial device which would allow him to bypass the heart and lungs. By 1935 he had successfully developed a prototype which was able to maintain a cat for 30 minutes. After returning from serving
in World War II, Gibbons, like Carrel with Lindburgh, joined forces with an engineer. Thomas Watson, the chairman of IBM, along with 5 other engineers from IBM, who helped Gibbons develop an improved machine that was able to reduce the mortality rates in dogs from 80% to 10% (19). Years later and after several modifications to the extracorporeal oxygenator, on April 5, 1951, Clarence Dennis was the first to attempt to use complete cardiopulmonary bypass. Unfortunately the patient died of a massive air embolism. Two years later in May 1953, Gibbon was the first to successfully close an atrial septal defect using extracorporeal circulation and total cardiopulmonary bypass. Unfortunately, Gibbon was never able to replicate this cardiopulmonary bypass procedure and after the death of three subsequent patients, Gibbon never used this machine again (20).

As experience with extracorporeal corporeal circulation developed, it became clear that it had the potential to save lives. However, this technique, if performed over an extended period of time, could result in lethal complications. It was later discovered that the direct contact with gas resulted in damage to the erythrocytes. This discovery spurred research in the field of membrane oxygenators. By the early 1970s, several investigators demonstrated that extracorporeal circulation was possible for several days. In 1972, the first trial of extracorporeal membrane oxygenation (ECMO) was performed to treat a patient with ARDS. By 1975, the first prospective trials using ECMO had begun and in that same year, a newborn infant was successfully treated with this new technique (21).

Unfortunately, interest in extracorporeal liver perfusion had waned by the 1970s and these new developments in membrane oxygenation were not to be enjoyed by the field of hepatic support for nearly two decades. Furthermore, the first successful liver
transplant in 1967 by Starzl led to even less interest in the development of a temporary liver support as liver transplantation appeared to be the clear and obvious choice between the two. However, as organ transplantation became common practice, over time, it became clear that patients were dying—not due to a lack of a therapeutic option—but rather because there were not enough organs available to be transplanted. For this reason, interest in temporary hepatic support began to reemerge.

1.3 HISTORY OF EXTRACORPOREAL LIVER PERFUSION

There have been over 100 extracorporeal liver perfusions performed in an attempt to bridge patients in fulminant hepatic failure to liver transplantation. As is the case in most scientific research fields, the future is a product of those who paved the way in the past. Here, I will take you through a history of the key figures that have been instrumental in allowing scientists to bring the field of extracorporeal liver perfusion where it is today.

The first investigator to use the liver of one animal to support the life of another was James Otto in C. Stuart’s laboratory in 1958. As was reported in the journal, “Surgery”, Otto et al were able to successfully reduce the ammonia level in the arterial blood of a dog with meat intoxication syndrome using the liver of a donor dog as a hepatic support device (4). In as early as the 1960s, Eiseman tried perfusion human blood through a canine liver but was completely unsuccessful (2). It wasn’t until he switched to perfusing fresh rather than stored human blood, through porcine livers rather than canine livers, that he found success. Eiseman was the first to perfuse human blood through an isolated animal liver. While most livers were maintained for 6-10 hours, one
liver was reported to have been perfused for up to 30.5 hours (22). After perfusing well over 100 livers from a variety of species, and finding continued success with porcine livers, Eiseman was ready to attempt a very carefully conducted trial wherein he would perfuse a patient in liver failure with a pig liver in an attempt to reverse severe hepatic coma. The first patient was a 42 year-old man; it is reported that this man was run intermittently in parallel with the liver circuit for 70 minutes, gradually introducing his blood through the pig liver. Eventually, his blood was perfused through the pig liver for 15 minutes.

By the end of the perfusion therapy, he had improved from total unresponsiveness to sluggish movement upon painful stimulation. According to the Classification of Hepatic Encephalopathy, this would have corresponded to a decrease in hepatic encephalopathy from grade IV to grade III (23). Unfortunately, the patient died 3 days later. The main lesson learned from this experiment was that perfusion of human blood through an extracorporeal pig liver was feasible without harm to the patient (2). The second patient was perfused with a porcine liver for 1.5 hours and because the patient’s arterial blood was used, the oxygenator was able to be removed from the circuit. It was reported that this patient showed improvement after only 12 minutes of perfusion and was moving and reacting to painful stimuli when prior to the perfusion this patient was completely unresponsive. The third patient was perfused with a porcine liver for 2 hours and improved to the point of reacting to spoken commands after only 20 minutes of perfusion.

The fourth patient was perfused for 3 hours and for the first time a closed circuit was used. At this point, it became clear that the liver was producing “ascites” and that
the production of ascites was dependent on the pressure in the vena cava. The group decided that the ascites should be reintroduced back into the circuit. The fifth patient was perfused and for the first time the team perfused a patient with viral hepatitis rather than alcohol cirrhosis. The patient was a 17 year-old boy who had been in comatose for 14 days. His neurologic improvement was dramatic. After only 6 hours of perfusion, he is reported to have been talking and asking for water. He did well for 5 days and then, as the team was preparing for a second perfusion, he expired on the eighth day (2).

A sixth patient required two porcine liver perfusions. Given the potential risk of an antigenic response to the re-exposure to the second porcine liver, the group made preparations to use a calf liver if an antigenic response occurred. Fortunately, there was no antigenic response to the second liver. The patient responded well to the first liver. However, after having slipped back into coma, responded poorly to the second perfusion and expired two days later (2).

Up to this point, Eiseman’s team had been perfusing blood directly from the patient to the porcine liver via the portal vein and then back to the patient. Under these conditions, they noted that there was a sudden onset of hypotension upon the commencement of each perfusion. After further investigation, they determined that the observed onset of hypotension was due to nothing more than the loss of 500cc of blood from the patient, which was required to fill the porcine liver. This hypotension was overcome simply by transfusing 500cc of blood into the patient from a reservoir upon initiation of perfusion. The team was also able to determine the minimal flow that they felt was acceptable to maintain adequate liver function. They based these findings on oxygen consumption and determined that if only the portal vein were perfused, they
could maintain stable oxygen consumption with only 500cc/min for a 1Kg liver. By perfusing both the hepatic artery and the portal vein they could maintain the same amount of oxygen consumption with a flow of only 300cc/min. This minimization in the flow rate was considerable given that a lower flow rate resulted in less hemodynamic instability. The team used this strategy for the seventh patient (2).

This patient responded very well to perfusion. Upon being perfused, it is reported that this patient was awake, eating and appeared to be on his way to recovery. However, on the fourth day of recovery, he suffered massive bleeding from his esophageal varices. After operative varical ligation, he was perfused for a second and even a third time. All the while, antigenic response was never detected. Unfortunately, this patient ultimately succumbed to his liver disease (2).

In the eighth patient, temperature regulation proved to be a problem. To overcome this, a heat regulator was added to the circuit. Unfortunately, even though clearance of ammonia was achieved, this patient died. In all, 8 patients were treated using 11 porcine liver perfusions; with the longest surviving patient having survived for 12 days post perfusion, there were no long term survivors. Although longterm survival was never achieved, Eiseman’s studies opened the door to the possibility of temporary extracorporeal liver support (2).

Two and a half years after Eiseman et al had completed their studies, Watts et al were the first to successfully treat a patient with fulminate hepatic failure using extracorporeal porcine liver perfusion (24). Through a technique, which closely resembled that of Eiseman’s, this group reported the outcomes of three patients. The first two patients died, but the third patient survived and was discharged from the hospital 7
weeks later. She was a 22 year-old woman who was admitted to the hospital in October 1966 while in her 37th week of pregnancy. She was mildly jaundiced with nausea and vomiting. After a few days, she developed contractions and after 66 hours of difficult labor, she gave birth to a stillborn baby. During the period following delivery, she became progressively more jaundiced, incoherent and developed hypotension and oliguria. She was referred to the tertiary hospital in a coma, deeply jaundice, febrile, unconscious, and sluggishly responding to painful stimuli. She was intubated and treated with current intensive care therapy but did not respond. She was treated with porcine extracorporeal liver perfusion using blood obtained directly from her brachial artery, perfused through the hepatic artery and portal vein and returned to her cephalic vein. During the perfusion a drastic improvement was seen in her conscious state and she continued to improve for another day. A second perfusion was performed and again, drastic improvements were seen as the patient began to obey commands and to make purposeful movements. Three hours after extracorporeal perfusion through the porcine liver she began to speak and over the next week regained full consciousness.

While the woman above may have been reported as the first to be successfully treated by porcine liver xenoperfusion, it is possible that she was not the first to be successfully treated with extracorporeal perfusion. In May 1966, Sen and colleagues from India reported on four patients who they had treated with extracorporeal perfusion using human cadaveric livers (25). Using the femoral artery for outflow and the femoral vein for inflow, the patient’s blood was perfused for a maximum of 2 hours. Of the first four patients treated with the cadaveric livers, the longest lived for only 48 hours after perfusion. However, this group published an addendum to their first article which
highlighted the success of a fifth patient, which at the time of writing the paper was fully aware and doing well 10 days after perfusion. This patient was a 27 year-old male who had developed deep hepatic coma 60 hours after emergency portacaval shunt performed for recurrent severe hematemesis. He was perfused for 200 minutes and as described by Sen et al, he recovered from his coma during the perfusion.

The early perfusion experiments performed by Eiseman, Sen, and others were not exceptions. In addition to these early reports, there were over 64 patients treated by extracorporeal hepatic perfusion by the year 1973 (26). In 1966, Norman et al. from Mcdermott’s group in Boston treated five patients with 15 perfusions using porcine livers and had no survivors (27). In 1967, Darin and colleagues in Milwaukee treated 4 patients with 4 perfusions using human livers and had no survivors (28). Bertrand and his colleagues in Montpellier, France treated 10 patients with 15 porcine liver perfusion in 1968 and had 2 long-term survivors (28). Pirola, Ham and Elmslie reported their unsuccessful treatment of a patient in Sydney, Australia with two porcine liver perfusions (29). Although their patient died, this group made a very important contribution to the field of extracorporeal perfusion. They were able to modify the perfusion technique in such a way as to allow for the rate of blood flow to the extracorporeal liver to be independent of the rate of blood flow coming from the patient (30). This meant that it was possible to obtain high sustained blood flow to the liver while allowing the blood flow from the patient to remain at a low flow rate.

Condon was the first to begin looking at the liver’s ability to regenerate upon treatment using extracorporeal perfusion. In his 1970 article published in the American Journal of Surgery, Condon et al reported on 7 patients which he had treated with 14
bovine liver perfusions. Although long-term survival was not accomplished in any of the seven patients, Condon reported that while all patients went on to show biological improvements, only 4 of 7 patients showed marked neurological improvements and 2 patients recovered consciousness (31). Also, along with classifying the biological functions of the liver after performing the perfusion, Condon, et al were able to determine that the patient’s liver had not regenerated during the extracorporeal perfusion treatment (31). In 1971, Tygstrup’s group in Copenhagen, Denmark treated 5 patients with 5 porcine liver perfusions. However, all of them died within 10 days after perfusion (32). The first wave of clinical trials using extracorporeal liver perfusion in the treatment of fulminant hepatic failure ended in 1973 with the largest study to date. Abouna and colleagues, working out of 5 cities in 2 countries, reported their results summarizing the support of 10 patients over a 6 year period. In this study, Abouna performed 33 extracorporeal perfusions using livers from pigs, baboons, cows and human (33). In total, of the 10 patients treated with 33 perfusions, Abouna reported 2 survivors (33). A third patient was supported with 16 liver perfusions using livers from ten pigs, three baboons, one calf, one monkey, and one cadaveric human liver (34). Abouna noted that the longer the perfusion, the better the clinical response (35).

In the summer of 1969, many of the leaders in the field of hepatic support who were using extracorporeal porcine liver perfusion met at the Tenth Annual Meeting of the Society for Surgery of the Alimentary Tract to review their results and to determine what their future course of action should be. As can be seen in the discussion after the presentation of Condon’s paper at that meeting, the investigators in attendance had serious qualms about the role of extracorporeal porcine liver perfusion in treating acute
liver failure. Theodore Drapanas from Pittsburg asked three pertinent questions: 1) What are the metabolic errors we are trying to correct?; 2) Who are the candidates for the use of this technique?; and 3) What is the rate of regeneration of the diseased liver?

Drapanas went on to state:

“Therefore, I believe the concept of temporary support for a few days, waiting until regeneration occurs, is not valid and should be challenged. We need other modalities to carry such patients through for a longer period of time. It is our own belief that the use of heterologous perfusion should be discontinued until we have these answers.” (30)

He was followed at the microphone that day by Ben Eiseman who stated:

“We have given this technic (sic) an honest and thorough clinical trial, but the results have been disappointing. The extracorporeal pig liver functions, but unfortunately the livers in most of the patients in hepatic failure do not have the capacity to regenerate.” (30)

As one might expect following such a bleak review by the leaders in the field and the increasing success of orthotopic liver transplantation, interest for extracorporeal liver perfusion for the treatment of hepatic coma waned in the ensuing years. A few centers continued to perform extracorporeal liver perfusion using baboon and cadaveric human livers, but overall, interest in ex vivo liver perfusions, especially those using porcine livers, had waned. Due to improvements in veno-venous bypass and extracorporeal membrane oxygenation (ECMO), as well as the increasing number of people who might benefit from temporary hepatic support, a renewed interest in ex vivo support occurred in the early 1990s. One might not have expected that a co-evolution of orthotopic liver transplantation and extracorporeal perfusion could have occurred, however, a shortage in the number of livers available for transplantation helped to catalyze the resurgence of interest in extracorporeal liver perfusion in the treatment of fulminate hepatic failure.
Although orthotopic liver transplantation has proven itself to be successful with a 5-year survival rate of 85%, persistently high mortality rates seen in patients with diseases like acute liver failure has continued to motivate investigators to continue to use extracorporeal liver perfusion as a temporary form of hepatic support. By the end of the 1970’s, due to its relative success and feasibility, the pig had become the donor of choice for ECLP. However, several investigators continued to treat patients with cadaveric human livers.

Horslen et al, reported the latest clinical trial in which a cadaveric human liver was used to treat a patient in acute liver failure (36). In his study, Horslen et al performed 18 perfusions on 14 patients. Of the 14 patients treated, 8 of them were treated with cadaveric human liver perfusion and 8 were treated with porcine liver perfusion (two patients received treatment with both a cadaveric human and a porcine liver). The average time of perfusion in those treated with cadaveric human and those treated with porcine liver perfusion was 50.2 and 30.4 hours, respectively, with an overall survival rate of 64.28 percent. In this study, there was a tendency for patients to experience decreased arterial ammonia levels regardless of whether or not they were treated with cadaveric human or porcine livers. Additionally, patients treated experienced a decrease in bilirubin levels, although, patients treated with porcine livers experienced a gradual rise in the amount of circulating bilirubin beginning at 24 hours post perfusion. In general, Horslen reported that ECLP performed with porcine livers appeared to work as well as those performed with cadaveric human livers. In this article Horslen speaks to this end when he states,

“Although it seems likely that human livers should function better than pig livers for the purpose of hepatic support, it should be noted that the “quality” of the human livers used for ECLP made them
unsuitable for transplantation. Thus, it is difficult to assess the relative merits of a good pig liver against a poor human liver for purposes of ECLP” (36).

... nonetheless, this study helped to define the validity of using porcine livers in ECLP treatments as an alternative to the use of cadaveric human livers, which are increasingly scarce. Studies like those conducted by Horslen et al, as well as others, helped to pave the way for the use of porcine livers in ECLP.

Shortly after Horslen et al performed the studies described above, Levy et al. treated 2 patients in liver failure with ECLP using genetically-modified porcine livers in an attempt to bridge patients to allograft transplantation (37). In this study, both of the patients were successfully bridged to transplantation and continued to survive at the time that this article was published. Similar to Levy, Tector et al., attempted to bridge patients in liver failure to transplantation using porcine liver perfusion (38). Tector et al. treated two patients and in both cases, these patients were successfully bridged to allotransplantation.

In total, 151 patients have been treated with extracorporeal porcine liver perfusion. Of those patients treated, 51% experienced improved neurologic function, 56% experienced decreased ammonia levels, and 75% had decreased bilirubin levels. The overall survival rate, which includes both those bridged to transplantation and those that experience liver recovery, was 22.5%. Table 1 illustrates the experience gained from 151 extracorporeal porcine liver perfusions. Although the survival rate of patients treated with extracorporeal porcine liver perfusion appears to offer only modest improvement in mortality rates, it is important to note that the patients treated in these studies were determined to be the sickest of the sick and it was determined that death was the likely outcome for these individuals if a suitable allograft liver did not become available.
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Table 1: Published Reports of Clinical Extracorporeal Porcine Liver Perfusion
The field of porcine liver xenoperfusion has progressed towards clinical application in large part due to the clinical studies highlighted in this chapter. Although these results suggest that extracorporeal porcine liver perfusion has the potential to serve as an effective means of bridging FHF patients to allograft transplantation, it is evident that improvements to the therapy are needed in order for it to become a routine part of liver support therapy. In the next chapter, I will discuss the immunological barriers which have previously served as road blocks to the application of porcine liver xenotransplantation and xenoperfusion. Additionally, I will spend the later part of the chapter highlighting the immunological barriers which continue to stand in the way of using extracorporeal porcine liver xenoperfusion in the clinic.
Chapter 2

Immunological and Physiological Barriers to the Use of Porcine Livers in Extracorporeal Liver Perfusion

(*a modified version of this chapter has been published in the journal Expert Reviews in Clinical Immunology)


2.1 INTRODUCTION

Organ transplantation is currently the most effective therapy for treating patients in end stage organ failure. However, a shortage of organs from both living and nonliving donors limits the use of this therapy and thus leaves patients with minimal options. Currently, there or over 117,000 people waiting for an organ in the US; of those people,
almost 16,000 people are waiting for a liver. At the present time, patients in need of either a kidney or a heart are capable of being supported until a donor organ becomes available through the use of kidney dialysis and ventricular assist respectively. This is not the case for patients in need of a liver. Unfortunately, there are no current treatments capable of replacing the function of the liver that allows a patient to wait an extended period of time for a liver to become available. Thus, patients in end stage liver failure awaiting transplantation, particularly those in fulminant hepatic failure, are especially vulnerable to mortality.

Xenotransplantation offers the prospects of greatly expanding the supply of organs available for patients in end stage organ failure. Patients in end stage liver failure, especially those diagnosed with FHF, could be greatly benefited by the use of a xenogeneic liver in either orthotopic transplantation or in an extracorporeal liver support device. Between the years of 1995-2011, 38,760 end stage liver failure patients were removed from the UNOS waiting list because they either became too ill or died while waiting for a liver to become available. Pigs are considered to be the best candidates as xenogeneic liver donors for human xenotransplantation or xenoperfusion remedies due to several factors: i) the physiology and size of pig organs are comparable to humans; ii) the pig’s ability to breed rapidly; iii) their ability to be genetically engineered; iv) the relatively low risk of disease and microorganism transmission to humans; v) the ethical acceptance of the use of pigs for the purpose of organ donors.

Though pigs seem to be the most suitable donor animal for clinical xenotransplantation, various immunological and physiological barriers have stood in the way of the utilization of their organs in human transplantation or “bridging therapies.”
Although genetic modifications have been made in pigs in an attempt to mitigate the most immediate barriers, new barriers, previously undetectable, have been discovered. This chapter will serve as an overview of the barriers. While some of these barriers have been overcome, this chapter will highlight challenges that remain in the quest to make extracorporeal porcine liver perfusion a clinical reality.

2.2 HYPERACUTE REJECTION

Hyperacute rejection (HAR) is the most immediate form of rejection seen when wild type porcine organs are used in xenotransplantation studies in either humans or nonhuman primates (NHP). HAR is characterized by complement deposition on the surface of the vasculature of the porcine organs. The deposition of complement is driven by the classical pathway and is the result of natural antibodies produced in both humans and NHP binding to antigens expressed on the pig vasculature endothelium. HAR is characterized by hemorrhage, edema and thrombosis of small vessels. The main antigen responsible for the onset of HAR in the pig-to-human combination is the carbohydrate epitope, galactose α-1,3-galactose (Gal), which is generated through the activity of galactose-α-1,3-glactosyltransferase (39-41). Various human and NHP studies were performed in order to better understand the effect of natural anti-Gal antibodies on mediating HAR. In a study conducted by Powelson et al., anti-Gal antibodies were absorbed prior to the transplantation of a porcine graft into a NHP using either a Gal affinity column or a pig organ (42). However, graft survival did not extend past 3 days suggesting the possibility of non-Gal antibodies capable of initiating HAR.
In an attempt to mitigate the affect of HAR on graft survival during porcine liver xenotransplantation and xenoperfusion, various studies were performed using pig livers derived from pigs transgenic for human CD55 (hDAF) (decay-accelerating factor, a complement regulatory protein). First, Remirez et al conducted a series of studies wherein hDAF transgenic pig liver were transplanted into NHP recipients (43). In this study, two baboons were transplanted with hDAF porcine liver grafts and survived for 4 and 8 days respectively. In both of these cases, the presence of HAR was not recorded. However, thrombocytopenia was noted shortly after cross-circulation of the liver with baboon vasculature (43). Additionally, this group illustrated the absence of HAR using livers derived from polytransgenic pigs expressing hDAF along with CD59 and H-transferase. Although the HAR was eliminated, none of the baboons survived for more than 24 hours (44). Rees et al utilized livers from hDAF transgenic pigs in order to determine whether the expression of hDAF could prolong graft function in an extracorporeal perfusion model of extracorporeal porcine liver xenoperfusion. Previously, Rees et al illustrated hepatic function for 72 hours in livers that were maintained extracorporeally with human blood. This group went on to show increased function in livers derived from pigs transgenic for hDAF in comparison to livers derived from WT pigs (45). Using this extracorporeal model, Rees showed that the porcine livers consumed human platelets and red blood cells with hDAF pig livers consuming more hRBCs than livers derived from WT pigs (45).

In an attempt to further understand the role of Gal in mediating HAR in porcine liver xenotransplantation studies, both in human and NHP, α-1,3-galactotrasferase gene-knockout (GTKO) pigs were generated (46). Since the generation of GTKO pigs, studies
have been done in primates to illustrate the absence of HAR upon xenografting of a GTKO pig liver primates. Ekser et al. performed a series of studies wherein baboons were transplanted with livers derived from GTKO pigs that expressed CD46 (GTKO.CD46) (a complement inhibitor protein) in the presence of a clinically relevant immunosuppressive regimen (47-49). In these studies, this group saw no HAR, cellular rejection, or humoral rejection. As illustrated by immunohistochemistry, there was little deposition of IgM, and an absence of IgG, C3, C4d or C5b-9. Additionally, there was very little T and B cell infiltration. In these studies, recipient survival was limited to between 4 and 7 days. Thrombocytopenia (severe reduction in the number of circulating platelets) was reported as early as one hour post transplantation. Confocal and electron microscopy showed platelet aggregation in the sinusoids of the liver confirming the presence of thrombocytopenia.

2.3 THROMBOCYTOPENIA IN PORCINE LIVER XENOGRIFTING

It is well known that platelets play a significant role in facilitating the survival of allografts as they are known to influence interactions with monocytes/macrophages and T cells with endothelial cells of vascularized grafts (50). Based off of studies mentioned previously, as well as others performed, both in vivo and ex vivo, it is clear that platelet consumption continues to act as a barrier to the use of porcine livers in the clinic.

The onset of thrombocytopenia has severely limited the clinical use of porcine livers in both the transplantation and extracorporeal perfusion of these livers. In order to better understand the kinetics of platelets in the xenotransplantation of discordant pig
livers, various studies have been performed utilizing both in vivo pig-to-nonhuman primate models, as well as in vitro and ex vivo pig-to-human models.

2.3.1 IN VIVO STUDIES

A series of studies performed at the University of Pittsburg looked at platelet loss in baboons transplanted with livers derived from GTKO.hCD46 pig. As expected, this group noticed severe platelet reductions only minutes after transplantation was initiated. Additionally, by performing daily flow cytometry analysis, this group showed the presence of platelet-white blood cell (WBC) aggregates in recipients of orthotopic liver xenografts from GTKO.hCD46 pigs (48, 51). Although mean platelet volume remained stable throughout the experiment, platelet count fell significantly the first hour after transplantation. Thus, the development of profound thrombocytopenia was likely associated with aggregation of platelets and of platelets with WBC-subtypes, especially with monocytes. Post-transplant biopsies at 2 hours demonstrated platelet and fibrin deposition by immunofluorescence and electron microscopy. This group concluded that the early disappearance of platelets from the circulation was in part due to platelet-platelet and platelet-WBC aggregation peripherally, resulting in measurement of falsely low platelet counts. However, the presence of aggregated platelets in the blood and in the liver graft, and possibly in the native lungs, prevented normal platelet function, resulting in spontaneous hemorrhages (51). Platelet aggregation was seen in the liver sinusoids together with PBMCs and fibrin, as shown by confocal and electron microscopy (48, 49, 52). This group then performed a series of studies aimed at understanding the role of platelet activation in mediating the platelet-to-platelet and platelet-to-WBC aggregation
possibly responsible for the onset of thrombocytopenia. They demonstrated the presence of tissue factor within only 2 hours of xenotransplantation and furthermore, the presence of microparticles positive for TF suggesting that platelets are activated shortly after cross circulation of the xenograft.

2.3.2 EX VIVO STUDIES

The presence of thrombocytopenia seen in porcine liver xenotransplantation has also been seen in ex vivo models wherein human blood is perfused through porcine livers being maintained extracorporeally. Rees et al. first illustrated that porcine livers perfused with whole human blood consume nearly all of the human platelets (45). In a study aimed at determining the efficacy of using a porcine liver to bridge a patient in liver failure to transplantation, Rees et al. showed that after only 15 minutes of extracorporeal perfusion, the platelet count fell to <30,000/mm$^3$ (45). Other groups have since illustrated the onset of thrombocytopenia in ex vivo perfusion models and have went on to performed studies aimed at elucidating the mechanisms involved in mediating the loss of platelets.

Using GTKO.hCD46 pig livers in an ex vivo perfusion of baboons, Burdorf et al performed a series of studies aimed at delaying the onset of thrombocytopenia by administering anti-Glycoprotein 1 beta (GPIb) antibody to the baboon and desmopressin to the donor pig in order to obstruct the GPIb-von Willebrand Factor (vWF) pathway (53). In this study, Burdorf et al. delayed the onset of thrombocytopenia in an pig liver-to-baboon xenoperfusion for up to 3 hours (53). This study suggests a potential benefit of using livers from pigs whose GPIb-vWF axis has been targeted by genetic engineering.
Cantu et al. addressed this concept with a relevant study wherein lungs derived from vWF deficient donor pigs were transplanted into baboons (54). In this study, Cantu et al. demonstrated prolonged graph survival in xenografts that were deficient in vWF.

Burlak et al performed a series of ex vivo perfusion experiments aimed at trying to better understand the fate of the platelets upon perfusion through the liver. In this study, Burlak et al. isolated and stained human platelets with CFSE. The stained platelets were then re-suspended and perfused through an isolated porcine liver. This study showed that after only 15 minutes of perfusion, 93% of platelets had been removed from the perfusion circuit (55). Furthermore, by performing liver biopsies throughout the perfusion, this group illustrated that liver sinusoid endothelial cells, Kupffer cells, and hepatocytes were all involved in the removal of platelets from the perfusion circuit (55). Platelet phagocytosis appeared to occur in the absence of platelet activation (based on the lack of TF present in the perfusate) and was not dependent on antibody opsonization (55).

2.3.3 IN VITRO STUDIES

This group then performed as series of in vitro studies aimed at determining the mechanism by which porcine endothelial cells phagocytize human platelets. Paris et al. investigated the role of asialoglycoprotein receptor-1 (ASGR1) in mediating the phagocytosis of human platelets. ASGR1 has previously been shown to play a role in the clearance of chilled platelets, a limitation to the transfusion of platelets for clinical purposes (56). In order to test the role of ASGR1 in the removal of xenogeneic platelets by porcine liver sinusoid endothelial cells (LSEC), human platelets were then co-incubated with isolated LSECs derived from wild type (WT) and GTKO.hCD55 pigs
(57). In doing so, Paris et al. first illustrated that ASGR1 on the surface of the platelet was co-localized with the platelet phagosome. Next, using an anti-ASGR1 antibody, this group was able to inhibit platelet phagocytosis in a dose dependent fashion, suggesting that involvement of ASGR1 in the phagocytosis of human platelets by porcine LSECs. To further clarify the role of ASGR1, this group transfected WT and GTKO.hCD55 derived isolated LSECs with antisense RNA of ASGR1 to knock down ASGR1 expression. After showing a decrease in the amount of ASGR1 expressed, this group showed a 21% and 31% decrease in platelet phagocytosis in vitro by WT and GTKO.hCD55 pig LSECs, respectively (57).

Based off the evidence produced by Burlak et al. suggesting that porcine Kupffer cells (KC) were involved in mediating platelet phagocytosis, Chihara et al. performed a series of experiments aimed at better understand the mechanism used by KC to phagocytose human platelets. This group performed studies wherein human platelets were co-incubated with isolated porcine KCs in the presence of an anti-CD18 antibody, and found that CD18 co-localized with platelets upon phagocytosis (58). Furthermore, Chihara et al. showed a 43-73% and 18-55% reduction in platelet phagocytosis in isolated KCs derived from WT and GTKO.hCD55 pigs, respectively, upon treatment with anti-CD18 antibody (58). In order to more fully elucidate the role of CD11b/CD18 (MAC-1), Chihara then performed a series of studies wherein isolated porcine KCs were transfected with CD18 antisense RNA in order to knockdown CD18 expression. After showing that antisense RNA decreased the expression of CD18 by 24% and 11% in WT and GTKO.hCD55 derived KCs, respectively, this group showed that platelet binding and
phagocytosis was reduced by 32% and 14% in WT and GTKO.hCD55 pig KCs, respectively (58).

2.4 CONSUMPTION OF HUMAN ERYTHROCYTES BY PORCINE LIVERS

As noted above, it is thought that patients in liver failure would benefit from a “bridge therapy” which utilized an extracorporeal porcine liver in order to support liver function in the patient. Previous reports suggest that some patients diagnosed with FHF need to be transplanted within 24-36 hours in order to avoid rapid clinical deterioration that would render them unsuitable recipients for liver transplantation (59). In an attempt to develop an *ex vivo* liver assist therapy that utilized porcine livers in an extracorporeal liver perfusion device, Butler et al. performed a series of extracorporeal porcine liver perfusion studies aimed at prolonging porcine liver function in the *ex vivo* model (60). This group illustrated maintained liver function for up to 72 hours in a pig blood-to-pig liver alloperfusion as was reported by normal physiologic levels of pH, normal protein synthesis, and normal hemodynamic parameters throughout the perfusion. Normal histology was observed at the end of the perfusion with no major architectural changes (60). Next, Rees et al. used this system to determine whether porcine livers could be maintained in the extracorporeal liver perfusion (ECLP) model when human blood was perfused through the porcine liver (45). In this study, Rees et al. perfused isolated human blood through WT pig livers and livers derived from pigs transgenic for human decay accelerating factor (hDAF) (a protein involved in the complement regulatory system). Rees et al. showed that the function of the porcine liver could be maintained throughout the xenoperfusion and that hDAF pig livers functioned better throughout the 72 hour
perfusion as compared to WT livers (45). In addition to platelet loss as noted previously in this review, this group also illustrated for the first time, that human erythrocytes are destroyed by porcine livers and are removed from the circuit of an ECLP when human blood is perfused through porcine a liver. At the end of a 72 hour perfusion, the hematocrit remaining in the circuit was 20.4% and 2.5% for livers derived from WT and hDAF donor pigs, respectively (Figure 2-1) (45).

**Figure 2-1 Hematocrit During Extracorporeal Porcine Liver Perfusion**

Transgenic, hDAF transgenic porcine livers perfused with human blood (n=5); Non-transgenic, wild-type porcine livers perfused with human blood (n=5); Alloperfusion, wild-type porcine livers perfused with allogeneic porcine blood (n=5); Human blood/No liver, human blood perfused through the circuit in the absence of liver (n=1); Hematocrit is expressed as a percent of the starting Hematocrit (transgenic vs. nontransgenic, p,NS; alloperfusion vs. nontransgenic, p,<0.012; alloperfusion vs. transgenic, p,<0.004
This group used electron microscopy to determine that erythrocyte loss was mediated by KC (Figure 2-2).

The loss of erythrocytes was significantly greater in xenoperfusion experiments as compared to experiments conducted with pig blood (45). The loss of erythrocytes from

![Image of electron microscopy of porcine liver perfused for 72 hr with human blood.](image)

**Figure 2-2** Electron microscopy of porcine liver perfused for 72 hr with human blood.

a. Transmission electron micrograph of a porcine Kupffer cell (arrow) demonstrating erythrocytes (RBC) bound on the cell surface, RBC located intracellularly, and evidence of RBC fragments within Kupffer cell lysosome. (Magnification, 5000x).  
b. Scanning electron micrograph showing the fenestrated liver sinusoidal endothelium with attached Kupffer cell (arrow) binding multiple erythrocytes. (Magnification, 1200x)
the perfusion circuit of a human blood-to-pig liver xenoperfusion appeared to decrease liver function over time as was determined by a significant decrease in bicarbonate production and an inability to maintain normal potassium levels in those livers perfused with human blood as opposed to pig blood (45). Furthermore, there was a tendency for livers perfused with pig blood to have higher amounts of bile production over the 72 hours perfusion as compared to pig livers perfused with human blood (45).

Next, this group set out to determine the mechanism of human erythrocyte destruction by porcine livers. It was initially hypothesized that antibody opsonization and complement activity were likely responsible for hemolysis and ultimately the decrease in hematocrit throughout the ex vivo of the perfusion. However, upon performing studies wherein the presence of anti-human antibodies were detected throughout a 72 hour xenoperfusion using flow cytometry, this group was unable to confirm the presence of anti-human erythrocyte antibodies in the perfusate (61). These data likely rule out the possibility of complement mediated lysis of human erythrocytes through the classical pathway. Though this data failed to suggest that anti-human erythrocyte antibodies present in the perfusate were responsible for the lysis of human erythrocytes, histological analysis suggested that the KC were binding to and phagocytosing the erythrocytes causing erythrocyte destruction and a subsequent decay in the hematocrit throughout the xenoperfusion (62). Based on the fact that this group was unable to identify the presence of anti-human erythrocyte antibody in the perfusate, it appeared that the KC mediated binding of human erythrocytes was independent of Fc mediated binding (61). This group then performed a set of in vitro experiments aimed at understanding the role of classical pathway mediated complement destruction of human
erythrocytes perfused through a porcine liver. In this study, human erythrocytes were incubated with porcine plasma or human plasma collected from xenoperfusion. Erythrocytes were then incubated with or without opsonizing antibody and the amount of CH50 binding to erythrocyte surface was quantified. In every case, CH50 binding was minimal as compared to the amount of binding shown when pig and sheep erythrocytes were incubated with human plasma and plasma from human xenoperfusion (61).

Armed with data suggesting a lack of complement mediated lysis, and data showing a lack of antibody opsonization, Rees et al. set out to determine whether porcine KC binding to human erythrocytes was mediated by an innate receptor on the surface of the KC binding to its ligand on the surface of the erythrocyte. In order to test for the presence of an “innate xenogeneic receptor” capable of binding human erythrocytes in the absence of antibody opsonization, this group performed a series of in vitro assays wherein erythrocyte resetting by porcine KC was quantified either in the absence of antibody, in the presence of opsonizing porcine antibody, or in the presence of either normal pig serum, or fetal calf serum (63). In every case, there was no difference in the amount of KC mediated erythrocyte binding when comparing erythrocytes in the absence of antibody with erythrocytes in the presence of either serum or opsonizing antibody (63). This data further verifies that erythrocyte binding to porcine KC is independent of antibody opsonization and is likely the result of an innate cellular receptor.

Next, in order to determine the receptor on the surface of the porcine KC responsible for binding human erythrocytes, Burlak et al. performed a series of in vitro experiments wherein it was illustrated that KCs were capable of binding human Glycophorin A (hGA) (64). This group then performed a series of erythrocyte resetting
assays and showed that erythrocyte binding by porcine KC was inhibited after pretreatment of porcine KCs with human erythrocyte glycoprotein (hEGP) in a dose-dependant fashion (64). Erythrocyte binding was nearly ablated after pretreatment of KCs with hEGP at a concentration of 0.5mg/ml (64). These studies suggest that hGA found on hEGP expressed on the surface of the human erythrocyte is the ligand being bound by the receptor on the surface of the porcine KC. Furthermore, this suggested that the receptor on the KC was a lectin (a receptor protein known to bind sugars). Knowing that glycoproteins are made up of various terminating sialic acid (SA) molecules, Burlak et al. set out to determine which SA was serving as the sugar involved in mediating the recognition of human erythrocytes by porcine KCs. Using the in vitro resetting assay, Burlak et al. tested the ability of known hGA terminating sugars to inhibit the recognition of human erythrocytes by porcine KCs. Of all the monosaccharides tested in the resetting assay, N-Acetylneuraminic acid (NANA, or Neu5Ac) was the only monosaccharide capable of inhibiting erythrocyte binding (65). Furthermore, this group utilized sialidase treatment to remove Neu5Ac from the surface of the erythrocytes to illustrated that desialation inhibited KC binding to human erythrocytes (65). These studies identified that erythrocyte binding by porcine KC was mediated by Neu5Ac on the surface of the human erythrocyte being bound by an unknown lectin on the surface of the porcine KC.

Knowing the ligand involved in mediating the binding of human erythrocytes, the Rees laboratory set out to determine the lectin involved in mediating the binding of porcine KC to human erythrocytes. Previous studies have illustrated macrophage mediated erythrocyte resetting, but never in the pig-to-human pattern (66, 67). Clues to the question of which lectin was involved in mediating the recognition of human
erythrocytes by porcine KC came from a surprising source. The virology field, specifically laboratories interested in how Porcine Reproductive and Respiratory Stress Virus (PRRSV) infects swine, performed many of the initial experiments that would lead to the later discovery of how porcine macrophages bind human erythrocytes.

2.5 PORCINE REPRODUCTIVE AND RESPIRATORY STRESS VIRUS AND ITS IMPLICATIONS OF XENOGENEIC CELLULAR RECOGNITION PATHWAYS

Porcine Reproductive and Respiratory Stress Virus is a small enveloped positive-stranded RNA virus and is considered the most costly disease causing virus in the swine industry. It infects millions of pigs each year and costs the swine industry more than 5.5 million dollars annually (68). Porcine Reproductive and Respiratory Stress Virus, like other viruses, has been shown to contain SA residues on the surface of the virion and similar to other commonly known viruses, expresses neuraminidase which likely allows it to release from the host cell during replication. Porcine Reproductive and Respiratory Stress Virus has been shown to infect swine through the respiratory pathway via infecting porcine alveolar macrophages (PAM) (69, 70). The mechanism of viral entry into the host’s alveolar macrophages has been well studied by several groups, and in macrophages, viral entry is expected to involve several receptors on the macrophages surface. Viral entry into the macrophage is known to initially involve binding of the virus to heparan sulphate. Subsequently, the virus undergoes a more stable binding interaction with the macrophage by binding to porcine sialoadhesin (pSn), upon which time it is internalized into an endosomal compartment where a low pH can be used by the
virus to facilitate viral replication (71-73). Sialoadhesin, or Sialic Acid-Ig-Like-Lectin-1 (Siglic-1) is characterized by its large extracellular domains made up of 17 Ig-like domains and by a small intracellular domain (Figure 2-3).

![Figure 2-3 Schematic diagram showing the protein structure and characteristic feature of sialoadhesin.](image)
The extracellular region of sialoadhesin consists of 17 Ig-like domains. The intracellular region consists of a short cytoplasmic tail that lacks immunoreceptor tyrosine-based inhibitory motifs (ITIM) and ITIM-like motifs that function in the recruitment of signaling molecules.

In studies using utilizing monoclonal antibodies (mAbs) directed towards pSn, Delputte and Nauwynck et al. were able to reduce the amount of virus binding to PAM, suggesting that the virus-to-macrophage binding step is mediated by pSn on the surface of the alveolar macrophages binding to SA on the surface of the virion (74). Taking into consideration the work by Burlak et al. showing that the porcine macrophages utilize SA to bind human erythrocytes, and the findings from the virology field showing that pSn is necessary for the interaction of porcine macrophages and PRRSV, Brock et al. set out to
determine whether pSn is an essential player in the xenogeneic recognition of human erythrocytes by porcine macrophages.

2.6 PORCINE SIALODHESIN: A XENOGENEIC RECEPTOR ESSENTIAL FOR THE RECOGNITION OF HUMAN ERYTHROCYTES BY PORCINE MACROPHAGES

In order to understand the role of pSn in mediating human erythrocyte binding, Brock et al. performed a series of *in vitro* studies wherein PAM and KC were co-incubated with human erythrocytes and, like Burlak and Rees et al., found that the cultured porcine macrophages readily bound human erythrocytes. Next, Brock et al. performed a series of experiments wherein PAM and KC were pre-treated with increasing amounts of anti-pSn mAb and subsequently co-incubated with human erythrocytes. Brock et al. found that pre-treatment of porcine macrophages with anti-Sn mAb blocked the ability of porcine macrophages to bind human erythrocytes (75). Additionally, in this study, Brock et al. illustrated that expression of pSn in Chinese hamster ovarian (CHO) cells facilitated binding of CHO cells to human erythrocytes (75). These data illustrate that pSn is not only involved in mediating macrophage binding to virus, but that pSn expressed on the surface of the porcine macrophage facilitates macrophage binding to human erythrocytes as well. A model depicted the hypothesized mechanism of interaction in shown in Figure 2-4.
2.6 SUMMARY

Researchers have overcome many of the obstacles that once limited the use of a porcine ECLP device in serving as a bridge therapy for patients in need of liver transplantation. In addition to overcoming some of the obstacles limiting porcine ECLP, research in this field has provided insight into other areas of xenotransplantation. For example, this research has helped to identify other potential barriers to organ xenotransplantation. Additionally, this research has helped to develop porcine genetic manipulation techniques that have the potential to be utilized for clinical and agricultural purposes. Though several barriers to the use of porcine ECLP have been overcome, this form of therapy is still limited by several forms of graft-vs-host responses elicited by the porcine liver towards components of human blood. In the remaining chapters, I will present data that has helped to elucidate the cause of erythrocyte loss when human blood is perfused through a porcine liver as in the "liver dialysis therapy" extracorporeal.
porcine liver perfusion. Additionally, I will discuss the impact that research on human erythrocyte consumption by porcine livers has had on our understanding of the use of nonhuman primates in porcine liver xenotransplantation. Lastly, giving the potential inadequacies of nonhuman primates to serve as preclinical models, I will introduce an alternative model to the use of nonhuman primates in pre-clinical studies of ECLP and will discuss public survey data aimed at assessing public perception of the use of this alternative model and the ethical dilemmas that could arise therein.
Chapter 3

Blocking Porcine Sialoadhesin Improves extracorporeal porcine liver xenoperfusion*

(*a modified version of this chapter has been published in the journal Xenotransplantation)


3.1 ABSTRACT

Background: Patients in fulminant hepatic failure (FHF) currently do not have a temporary means of support while awaiting liver transplantation. A potential therapeutic approach for patients in FHF is the use of extracorporeal perfusion with porcine livers as a form of “liver dialysis”. During a 72 hour extracorporeal perfusion of porcine livers with human blood, porcine Kupffer cells (KC) bind to and phagocytose human red blood cells (hRBC) causing the hematocrit to decrease to 2.5% of the original value. Our laboratory has identified porcine sialoadhesin (pSn) on the surface of porcine KC as the
lectin responsible for binding N-Acetylneuraminic acid (Neu5Ac) on the surface of the hRBC. In order for extracorporeal porcine liver perfusion to be used as a therapy for patients in liver failure, all forms of anti-human rejection must be resolved. We evaluated whether targeting pSn prevents the recognition and subsequent destruction of hRBCs seen during extracorporeal porcine liver xenoperfusion.

Methods: In vitro experiments were performed wherein cultured porcine macrophages were pretreated with the anti-pSn monoclonal antibody (mAb), 1F1. Porcine macrophages were subsequently assayed for their ability to bind hRBC. Next, ex vivo experiments were performed wherein six wild type pig livers were perfused with isolated hRBCs for 72 hours. In one group, three livers were treated with 1F1. As a control group, three livers were treated with an antibody of the same isotype but with no known specificity for pSn.

Results: We have shown that treatment of cultured porcine macrophages with 10µg/ml of 1F1 significantly reduces the ability of the porcine macrophages to bind hRBC (p,<0.001). Additionally, we have shown that the addition of 1F1 to an extracorporeal porcine liver xenoperfusion model significantly reduces the loss of hRBC over a 72 hour period (p,<0.01). Sustained liver function was demonstrated by continued bile production and other markers.

Conclusions: This study suggests that the destruction of human erythrocytes in an extracorporeal porcine liver xenoperfusion model is, in part, mediated by pSn expressed on the surface of porcine KC. Furthermore, this data illustrates that the addition of anti-pSn antibody to the circulation of a pig-to-human xenoperfusion may inhibit the
destruction of human erythrocytes by porcine KC. Future studies should utilize livers derived from pSn deficient pigs.

3.2 INTRODUCTION

Xenotransplantation provides the hope for greatly expanding the supply of organs available for patients awaiting organ transplantation. A potential application within the field of xenotransplantation is the use of a porcine liver in an extracorporeal support device to serve as a bridge therapy for patients in FHF. Initially the clinical application of ECLP was limited by complement-mediated rejection (76-78). However, the development of GTKO pigs that express hDAF have helped to mitigate the effects of this form of rejection (79, 80). Despite modifications to overcome complement-mediated rejection, additional obstacles involving the direct recognition and destruction of xenogeneic cells exist and limit the clinical application of ECLP. Previously, we and others have shown that porcine livers perfused with human blood mount several forms of graft versus host “reactions”. Within only 15 minutes of perfusion, porcine livers consume as many as $10^{11}$ human platelets (55, 81). Additionally, our laboratory has shown that porcine livers perfused with whole human blood consume the equivalent of 2 units of human erythrocytes over a 72-hour period (82). The consumption of human platelets and erythrocytes by the porcine liver decreases the ability of the liver to maintain metabolic parameters in the extracorporeal perfusion system (81). Before extracorporeal porcine liver perfusion can be realized as a viable treatment for patients in FHF, these graft-vs.-host “responses” must be addressed.

Our laboratory has identified the involvement of porcine KC in mediating the clearance of hRBC by porcine livers in an extracorporeal perfusion model (83). Previous
studies demonstrated that the loss of hRBCs was not due to antibody-mediated destruction and did not involve complement opsonization of the xenogeneic erythrocytes (84). Rather, the clearance of hRBCs resulted from a direct recognition of hRBCs by porcine macrophages (83). Burlak et al identified Neu5Ac on the surface of the human erythrocyte as the sugar involved in mediating the interaction between human erythrocytes and the porcine macrophages (65). Brock et al. subsequently identified pSn expressed on the surface of the porcine KC as the lectin responsible for binding Neu5Ac on the surface of the hRBC (75).

Sialoadhesin, a highly conserved protein expressed in mammals, was originally identified as a sheep erythrocyte receptor and is known to bind the Neu5Ac form of sialic acid (85-88). Additionally, sialoadhesin has been shown to play a role in various immune functions including antigen presentation and internalization of viruses like porcine reproductive and respiratory syndrome virus and human immunodeficiency virus (89-91). Sialoadhesin has also been suggested to play a role in immune cell regulation by facilitating the maintenance of spleen and lymphoid morphology (92). Given that anti-pSn mAb prevents the in vitro recognition of the hRBCs by porcine macrophages (75), we wished to test if this anti-pSn mAb would prevent the loss of human erythrocytes during extracorporeal porcine liver xenoperfusion. We observed that addition of anti-pSn mAb during extracorporeal porcine liver xenoperfusion reduced hRBC clearance and maintained hepatic function over a 72-hour period.
3.3 METHODS AND MATERIALS

3.3.1 IN VIVO ERYTHROCYTE BINDING ASSAY

The ability of porcine macrophages to bind human erythrocytes in the presence of a mouse IgG<sub>2a</sub>-anti-pSn mAb, 1F1, previously described by Revilla et al. (93), or an IgG<sub>2a</sub> isotype control, was analyzed using an in vitro binding assay. Porcine macrophages isolated from the lung as described by Wensvoort et al, were cultured for three days and then seeded into 96-well round bottom plates at 3x10<sup>4</sup> cells per well (94). Porcine alveolar and Kupffer cell macrophages were used interchangeably for in vitro experiments as previously demonstrated by Brock et al (75). Cells were then treated with 1F1 mAb or an isotype control Ab for 1 hour after which the RPMI-1640 media (Sigma-Aldrich, St. Louis, MO) was removed and human erythrocytes were added. 1F1/isotype control mAb and hRBCs were diluted with RPMI at concentrations of 1 and 10µg/ml of 1F1 or isotype control and 0.1% packed hRBCs. Macrophages were co-incubated with erythrocytes for 2 hours upon which time wells were washed with RPMI to remove unbound erythrocytes. Cells were then fixed with 100% methanol and bound hRBCs were quantified using the tetramethylbenzidine (TMB) reaction. Plates were reacted using the TMB reaction and then quantified using a spectrophotometer at the 450nm wave length. Data were calculated as percent binding, relative to non-treated porcine macrophages co-incubated with human erythrocytes.

3.3.2 DETERMINING AMOUNT OF 1F1 NEEDED IN EX VIVO PERFUSION

In vitro and ex vivo techniques were utilized in order to determine the concentration of 1F1 mAb needed to block pSn in the ex vivo perfusion model. As
described above, we performed a series of *in vitro* sighting assays wherein cultured porcine macrophages were incubated with the 1F1 blocking antibody in increasing concentrations and subsequently exposed to human erythrocytes. To calculate the amount of mAb needing to block all pSn molecules expressed in the liver, we determined the amount of mAb needed to block erythrocyte binding of one macrophage. Based on our *in vitro* data where 100 µl of a 10µg/ml solution of 1F1 mAb saturated the pSn receptors of 3x10^4 porcine macrophages, we determined that 0.03ng of 1F1 mAb was needed to block the erythrocyte binding of one macrophage. Using the estimate of Bouwens et al., which estimated 4.1x10^7 to 1x10^8 KC in 100 grams of rat liver (95), we calculated the expected number of KC in a 1200g porcine liver as being 4.9x10^8 and 1.2x10^9 KC. Taken together with the amount of mAb needed to block erythrocyte binding of one macrophage, we estimated that 14-30 mg of the 1F1 mAb would achieve complete saturation of all pSn sites.

In order to account for the kinetics of 1F1 mAb in the *ex vivo* perfusion model, given flow, time, possible binding and internalization, and the de novo expression of new pSn, we performed a single sighting experiment to determine the calculated amount of mAb needed to block sialoadhesin expressed in the *ex vivo* porcine liver. We aimed to achieve a concentration of approximately 10µg/ml, consistent with our *in vitro* inhibition data. Using the liver perfusion method by Butler et al and Rees et al. (60, 82), a porcine liver was perfused with porcine blood and 1F1 mAb was added to the perfusate every hour in 5mg increments starting with 0mg at time zero and finishing with 40mg at 8 hours of perfusion. Serum samples and liver biopsies were collected every half hour prior to 1F1 mAb injection, flash frozen, and later analyzed for 1F1 mAb serum
concentration using an enzyme-linked immunosorbant assay (ELISA) and also for 1F1 mAb binding in the liver by immunohistochemistry.

3.3.3 BLOOD AND LIVER DONORS

The methods used in this study have been published previously and are described here briefly (82). Human packed red blood cells were donated by the United Kingdom National Health Service blood bank. Blood was from group O patients and was leukocyte depleted prior to use. Blood was fully heparinized immediately prior to use, calcium levels were corrected by the addition of calcium gluconate. Large white pigs (50-60 kg) were used in all experiments. All animals were treated in accordance with the Animals (Scientific Procedures) Act, 1986.

3.3.4 REMOVAL AND PREPERATION OF PORCINE LIVER

Liver donors were intubated and ventilated. Anesthesia was maintained with inhalative isoflurane. The liver was mobilized until connected only by its vascular attachments. After systemic heparinization, a cannula was placed into the distal aorta and 3 liters of cold Soltran (Baxter Healthcare, Deerfield, IL) commenced after aortic cross clamping. Following hepatectomy, the infrahapatic inferior vena cava (IVC), portal vein, and the hepatic artery were cannulated. The bile duct was cannulated and bile output was measured during perfusion. The final 500 ml of cold Soltran infused into the liver contained 12 mg of 1F1 or isotype control mAb and cold ischemic time was approximately 2 hours before initiating perfusion.
3.3.5 EXTRACORPOREAL LIVER PERFUSION

Six separate normothermic extracorporeal liver perfusions, 3 with an anti-Sn mAb (1F1) and 3 with the isotype control. The liver was perfused according to the method of Butler et al. using a prototype of the OrganOx perfusion device (82, 96). In short, the perfusion circuit, consisting of a blood reservoir, an oxygenator, a heating element, and a centrifugal pump, was primed with crystalloid solution (stereofundin®, B.Braun, Taguig City, Philippines) upon which time packed leukocyte depleted red blood cells were added until hematocrit reached normal physiologic range. At this time pH, p$_{a}$CO$_2$, p$_{a}$O$_2$ and Ca$^{2+}$ were adjusted to normal physiologic range. Before connection of the liver to the perfusion circuit, the preservation fluid was flushed from the liver using 1L of crystalloid infusion solution at room temperature. The liver was then connected to the primed perfusion apparatus. Prostacyclin, heparin, and total parenteral nutrition (TPN) were infused throughout the perfusion. Additionally, glucose and insulin were given in order to maintain normal glucose levels. Cefotaxime was added at the beginning of the perfusion and every 24 hours thereafter. Bile acids were replaced with taurocholic acid (New Zealand Pharmaceuticals, NZ) at 140 mg/hour.

Consistent with our data from the ex vivo sighting experiment, we administered either the 1F1 or the isotype control with the goal of maintaining a concentration of 10µg/ml throughout the duration of the perfusion. In order to achieve the desired concentration of antibody and block pSn prior to exposure of the pig livers to hRBC, we chose the following method to administer 1F1 mAb and the isotype control Ab during porcine liver xenoperfusion: 1) 12 mg of antibody was infused into the liver immediately following procurement and left in the preservation solution in the liver for approximately
two hours; 2) 12 mg of antibody was infused into the human blood perfusate prior to connection to the pig liver; 3) 12 mg of antibody was infused after 24 hours and again after 48 hours of perfusion. In this way, we expected to achieve saturation of pSn receptors prior to exposure of the pig livers to hRBC as well as allowing for replacement of phagocytosed 1F1 mAb and blockage of new pSn synthesized and expressed over the course of the perfusion with subsequent bolus administration. Perfusion was electively stopped at 72 hours.

Discussions with pharmaceutical company scientists with expertise in determining appropriate doses of mAb for clinical trial determined this approach was reasonable given the time and budgetary constraints of these *ex vivo* perfusion experiments that cost over $5,000/perfusion.

### 3.3.6 COLLECTION OF BLOOD AND TISSUE SAMPLES

Blood samples were obtained immediately prior to cross circulation of liver (t=-1), immediately following cross circulation (t=0), and every 2 hours thereafter for the remainder of the perfusion. Blood gas analysis was performed immediately. Blood samples were later analyzed for full blood count, urea, electrolytes, creatinine, and biochemical liver function tests. Plasma was collected, flash frozen liquid nitrogen and stored for later analysis.

### 3.3.7 MICROSCOPY

Biopsies were harvested from the periphery of the right lobe at time of back table cannulation (t=-2hrs), at 24hrs, 48hrs and preserved in either 10% formalin or flash
frozen in optimal cutting temperature (OCT) media (Electron Microscopy Sciences, Hatfield, PA). Complete liver dissection was performed at the termination of perfusion and samples were prepared for both immunohistochemical analysis and Hematoxylin and Eosin staining. Determining co-localization of mouse antibody with sinusoidal walls was performed by labeling frozen sections fixed with 4% paraformaldehyde and blocked with 50% odyssey blocking buffer (LI-COR, Lincoln, NB) in phosphate-buffered saline (PBS) with 1µg/ml goat anti-porcine CD31 antibody (R&D Systems, Minneapolis, MN). Goat antibody was detected with DyLight 549 labeled rabbit anti-goat IgG (H&L) antibody (0.2µg/ml) (Jackson ImmunoResearch Laboratories, West Grove, PA). Goat antibody was detected with DyLight 549 labeled rabbit anti-goat IgG (H&L) antibody (0.2µg/ml) (Jackson ImmunoResearch Laboratories, West Grove, PA). In order to identify 1F1 mAb or the isotype control Ab, tissue sections were labeled with DyLight 649 labeled Donkey anti-mouse IgG antibody (0.2µg/ml) (Jackson ImmunoResearch Laboratories, West Grove, PA). Tissue sections were washed using blocking buffer containing 0.1% tween 20. Nuclei were stained using DAPI (Life Technologies, Carlsbad, CA) (1:5000 in PBS) for 1 min.

3.3.8 FLOW CYTOMETRY

Blood was collected from healthy individuals and red blood cells, leukocytes, and lymphocytes were isolated. Porcine macrophages were isolated according to Wensvoot et al (94) and used as a positive control. Isolated cells were suspended in blocking buffer (phosphate-buffered saline (PBS) containing 20% horse serum) and incubated on ice for 30 minutes in PBS and then stained for 30 minutes at 4°C with 1F1 or the isotype control at a concentration 0.9µg/ml diluted in blocking buffer. Cells were washed and then stained with 0.1µg/ml ALEXAFLOUR-488 conjugated goat anti-mouse IgG secondary
(Invitrogen, Carlsbad, CA) at 4°C for 30 minutes in order to detect primary antibody binding. Individual cell populations were analyzed for 1F1 or isotype control binding via flow cytometry.

**3.3.9 WESTERN BLOT ANALYSIS**

To determine 1F1 mAb reactivity with pSn, porcine macrophage homogenates were prepared and protein was separated on 6% SDS-PAGE gel. Protein was transferred to a nitrocellulose membrane, blocked with blocking buffer (PBS containing 0.5% nonfat dried milk). Membranes were then incubated with 1µg/ml of 1F1 mAb diluted in blocking buffer. As a loading control, membranes were probed with 0.2µg/ml mouse anti-human α-tubulin (Santa Cruz, Santa Cruz, CA). Primary antibody binding was detected using horseradish peroxidase (HRP) conjugated goat-anti-mouse IgG secondary antibody (Southern biotech, Birmingham, AL) at a concentration of 0.25µg/ml in blocking buffer for 2 hours at room temperature. Membranes were developed using an ECL western blot analysis kit (GE Healthcare, Waukesha, WI). To detect soluble pSn in the perfusion circuit, serum samples collected from perfusions were first diluted 1:100 in PBS and separated via electrophoresis. Protein was transferred to a nitrocellulose membrane. Membranes were blocked with blocking buffer for 2 hours at room temperature. Membranes were then probed using 1F1 mAb at a concentration of 1µg/ml of 1F1 mAb in blocking buffer at 4°C overnight. 1F1 mAb binding was detected as described above.
3.3.10 ELISA

High protein binding 96-well plates (Corning Incorporated, Corning, NY) were incubated with 10µg/ml of goat anti-mouse IgG2a antibody (Jackson Immunoresearch Laboratories, West Grove, PA) in coating buffer for 4 hours at room temperature and then blocked overnight with blocking buffer (PBS containing 1% BSA, 1% casein and 0.5% lysine) at 4°C. After washing, capture antibody was incubated with plasma serially diluted in ELISA diluent for 45 minutes at room temperature. Captured 1F1 mAb, or the isotype control Ab, was detected with HRP conjugated goat anti-mouse IgG antibody (Jackson Immunoresearch Laboratories, West Grove, PA) at 1:5000 in ELISA diluent by incubation for 45 min at room temperature. Plates were developed using OPD solution (dH2O containing o-phenylenediamine) and were analyzed with a spectrophotometer.

High protein binding 96-well plates (Corning Incorporated, Corning, NY) were incubated with 10µg/ml of goat anti-mouse IgG2a antibody (Jackson Immunoresearch Laboratories, West Grove, PA) in coating buffer for 4 hours at room temperature and then blocked overnight with blocking buffer (PBS containing 1% BSA, 1% casein and 0.5% lysine) at 4°C. After washing, capture antibody was incubated with plasma serially diluted in ELISA diluent for 45 minutes at room temperature. Captured 1F1, or the isotype control, was detected with HRP conjugated goat anti-mouse IgG antibody (Jackson Immunoresearch Laboratories, West Grove, PA) at 1:5000 in ELISA diluent by incubation for 45 min at room temperature. Plates were developed using OPD solution (dH2O containing o-phenylenediamine) and were analyzed with a spectrophotometer.
3.3.11 STATISTICAL ANALYSIS

Data were analyzed with SPSS Statistics 17.0 using Student’s $t$-tests and repeated measures ANOVA. p-Values of $\leq 0.05$ were considered statistically significant.

3.4 RESULTS

3.4.1 SIALOADHESIN IS EXPRESSED IN LIVER AND LUNG TISSUE

First, we reasoned that if pSn was involved in mediating the binding of human erythrocytes in an extracorporeal porcine liver perfusion, pSn should be abundantly expressed in the liver. In order to determine whether pSn was expressed in the liver and lung, tissue was collected, snap frozen, and pSn expression was determined using a fluorescently labeled antibody known to be specific for pSn (Figure 3-1 (red)). Samples were also labeled with CD31 in order to identify endothelial cells (Figure 3-1 (green)). We found that both porcine liver and lung tissue expressed pSn. Further, we found that there was a propensity for pSn and CD31 to be co-localized (Figure 3-1 (yellow)). This was not surprising given the expected proximity of resident macrophages to the CD31 expressing endothelial cells of the vascular wall.
Figure 3-1 Sialoadhesin expression in porcine liver and lung. Confocal microscopy analysis of sialoadhesin expression was detected in the liver by staining fresh frozen biopsies taken from the liver and lung. Samples were stained with a fluorescently labeled mouse anti-porcine Sn (CD169) primary antibody (Red) and goat anti-CD31 (green). Nuclei were stained with DAPI (Blue). Images were taken at two magnifications.
3.4.2 1F1 INHIBITS pSN MEDIATED BINDING OF HUMAN ERYTHROCYTES

IN VITRO

Next, we set out to determine whether porcine macrophage mediated human erythrocyte binding could be inhibited using an anti-pSn mAb as previously described by Brock et al. (75). The mAb 1F1 was used based on its known specificity for the sialic acid binding region of pSn as previously described by the Dominguez laboratory (B. Alvarez, Z. Escalona, J. Dominguez, unpublished data). In this study, isolated porcine macrophages were stained with 1F1 or the isotype control in order to verify the ability of 1F1 to bind porcine macrophages (Figure 3-2 a. i). Western blot analysis showed a single band of about 185KDa, the expected size of pSn (Figure 3-2 a. ii). An in vitro erythrocyte binding assay was performed to determine the ability of 1F1 to inhibit rosette formation. We found that 1µg/ml of 1F1 inhibited rosette formation by nearly 60% of that seen when hRBCs are exposed to untreated macrophages (p,<0.01), while 10µg/ml of 1F1 blocked rosette formation by 95% (p,<0.001), levels comparable to that of porcine erythrocyte binding (Figure 3-2b). Microscopic images of porcine macrophages pretreated with either 10µg/ml of 1F1 or the isotype and subsequently co-incubated with human erythrocytes verified quantitative analysis of rosette formation (Figure 3-2c).
Figure 3-2  Porcine macrophage mediated binding of human erythrocytes is inhibited by the anti-pSn mAb, 1F1.  A: Flow cytometry demonstrated substantial binding of 1F1 mAb to porcine macrophages (pMΦ) as compared with the isotype control (i) (n=3). Immunoblotting confirmed 1F1specificity for pSn (ii).  B: Erythrocyte rosetting by porcine macrophages.  Human erythrocytes were incubated with porcine macrophages previously treated with either 1F1 mAb (Black) or isotype control Ab (Dark Grey) at a concentration of either 1µg/ml or10µg/ml. In addition, erythrocytes were incubated with pMΦ left untreated (Light Grey). Compared with the isotype control, pretreatment of pMΦ with 1F1 led to significant inhibition of human erythrocyte rosette formation (1µg/ml = p, <0.01 and 10µg/ml = p, <0.001) (n=3). Treatment of porcine macrophages with isotype control antibody did not significantly decrease human erythrocyte binding as compared to untreated pMΦ (1µg/ml = p,NS and 10µg/ml = p,NS) (n=3). For comparison, porcine macrophage binding to porcine erythrocytes was determined (White). Error bars represent standard deviation.  C: Phase contrast micrographs of porcine macrophages co-incubated with human erythrocytes. Porcine macrophages were either treated with 10µg/ml isotype (Left) or 10µg/ml 1F1 mAb (Right).
3.4.3 DETERMINING AMOUNT OF 1F1 mAb REQUIRED TO BLOCK pSN DURING EXTRACORPOREAL LIVER PERFUSION

In a single ECLP sighting experiment, we aimed to achieve a concentration of 10µg/ml of 1F1 in the perfusate consistent with the concentration producing 95% inhibition of porcine KC binding of the human RBC in vitro (Figure 3-2B). As described in the section, “Determining the amount of 1F1 needed in the ex vivo perfusion”, we performed an ex vivo sighting experiment wherein an increasing amount of 1F1 was added to the perfusate of an extracorporeal porcine liver perfusion in increments of 5mg. As seen in Figure 3-3A, upon adding 25mg of antibody, the concentration of 1F1 in the perfusate had reached approximately 10µg/ml (Figure 3-2A). Fluorescence microscopy images of the liver taken at times following injection of 1F1 suggests that 1F1 saturation of pSn binding sites on Kupffer cells in the liver was achieved after 25mg of 1F1 had been added to the perfusate (Figure 3-3B). Taken together, we determined that a concentration of approximately 10µg/ml of 1F1 mAb would result in consistent inhibition of pSn and that this amount could be achieved by the infusion of approximately 25 mg of 1F1 mAb into the perfusion apparatus (Figure 3-2B and Figure 3-3).
Figure 3-3 Kinetics of 1F1 in the ex vivo liver perfusion model. 1F1 serum concentration and binding in the liver were assessed in an ex vivo liver perfusion model. Porcine livers were perfused with increasing amounts of 1F1 delivered every hour administered in 5 mg boluses. A: During the perfusion, serum samples were collected and serum 1F1 concentration was analyzed with ELIZA (Actual 1F1 concentration). Expected 1F1 serum concentration is shown as calculated 1F1 concentration. B: Punch biopsies of the liver were collected throughout the perfusion and analyzed for the presence of 1F1. Samples were stained with a fluorescently labeled goat anti-mouse IgG2a secondary (Red). Nuclei were stained with DAPI (Blue).
3.4.4 NORMAL HEMODYNAMIC AND SYNTHETIC FUNCTION IN LIVERS TREATED WITH 1F1

In order to determine the effect of the addition of 1F1 on liver function, hemodynamic and synthetic parameters were assessed. In all of the perfusions, portal flow and pressure, as well as, IVC flow and pressure, remained constant and were not significantly different (Figure 3-4A). Additionally, continuous production of albumin, urea and bile were demonstrated in the 1F1 and isotype treated groups with no differences observed (Figure 3-4B).

Finally, we examined alanine transaminase (ALT) levels and found that in both the 1F1 treated and isotype treated, terminal ALT levels were within the normal range for the pig and were not significantly different (Figure 3-4B).
Figure 3-4 Normal hemodynamic and synthetic function in livers treated with 1F1. 
A: Hemodynamic parameters were measured throughout perfusion. Portal vein and inferior vena cava (IVC) (total) flow rate were measured during perfusion of 1F1 (Black) and isotype (Grey) (p,NS and p,NS respectively). Additionally, portal and IVC pressure were measured throughout the 72 hour perfusion (p,NS and p,NS respectively). (N=3) B: Synthetic function of 1F1 treated (Black) and isotype treated (Grey) livers was assessed throughout the perfusion by measuring albumin (p,NS), urea (p,NS), alanine transaminase levels (p,NS) and bile production (p,NS). (N=3)
3.4.5 THE ADDITION OF 1F1 IMPROVES METABOLIC FUNCTION OF PORCINE LIVERS PERFUSED WITH HUMAN ERYTHROCYTES

In order to investigate the metabolic effect of blocking pSn, and presumably reducing erythrocyte destruction, on xenogeneic liver perfusion, metabolic function was assessed by measuring pH, bicarbonate, and base excess throughout the perfusion of 1F1 and isotype treated livers. Although progressive acidosis was observed in both 1F1 and isotype control treated livers beginning at 48 hours, acid-base function was markedly impaired in the isotype treated group as compared to those livers treated with 1F1 (p=.003) (Figure 3-5A). Additionally, oxygen consumption (ml/min) by each liver was measured throughout the perfusion using the Fick principle (Figure 3-5b).
Figure 3-5  Addition of 1F1 prolongs metabolic function. Metabolic function was measured throughout the perfusion of livers treated with 1F1 (Black) and the isotype (Grey). Base Excess A: and oxygen consumption B: were measured. Significance differences were seen beginning at 48 hours for base excess and 54 hours for oxygen consumption (p<0.003 and p<0.003, respectively). (n=3)
A consistent volume of oxygen was consumed throughout perfusion in those livers treated with 1F1. However, continuous decrease in oxygen consumption over time was seen in those livers treated with the isotype antibody. The progressive decrease in oxygen consumption seen in isotype treated livers resulted in a significant difference in oxygen consumed by isotype treated livers as compared to those liver treated with 1F1 beginning at 54 hours of perfusion (p,0.003) (Figure 3-5b).

3.4.6 KUPFER CELL MEDIATED ERYTHROCYTE DESTRUCTION IS REDUCED BY 1F1 mAb

In order to determine the effect of 1F1 mAb on Kupffer cell-mediated destruction of hRBCs by porcine livers, perfusate samples were obtained throughout the perfusion and analyzed for the presence of intact red blood cells present (hematocrit). As shown in Figure 5A, there was a time dependent decrease of the hematocrit in livers treated with both the isotype control Ab and 1F1 mAb (p,<0.001 and p,<0.001, respectively). Analysis of the 1F1 mAb versus isotype control Ab treated livers revealed a greater loss of hematocrit in the perfusion of those livers treated with the isotype control Ab as compared to livers treated with 1F1 mAb (p,0.01) (Figure 3-6A).
Figure 3-6  Kupffer cell mediated erythrocyte binding and partial rescue of hematocrit with 1F1 antibody. A: The percentage of intact erythrocytes (hematocrit) remaining in the circuit was assessed throughout perfusion. Hematocrit was measured during perfusion of livers treated with 1F1 (Black) or the isotype (Grey) via blood gas analysis (p<0.01). (n=3) B: Mouse Ab concentrations in the serum of livers treated with 1F1 (Black) and the isotype control (Grey) were analyzed using ELISA (p<0.01). (n=3) C: (page 65) Hemotoxylin and Eosin staining was performed on tissue collected at the end of the perfusion from livers treated with 1F1 and the isotype control.
C. 1F1    Isotype Control

1F1 1  IC 1
1F1 2  IC 2
1F1 3  IC 3
Levels of circulating mouse Ab in the serum of livers treated with anti-pSn antibody or an isotype control Ab were determined by an enzyme-linked immunosorbant assay. We found that circulating isotype control Ab levels reached a concentration of 15.95µg/ml whereas the 1F1 mAb concentration reached a maximum of only 5.14µg/ml (Figure 3-6B). H&E analysis was performed showing the presence of erythrocyte binding within the sinusoids of livers treated with 1F1 mAb and the isotype control Ab (Figure 5C). Black arrows represent erythrocyte rosetting.

3.4.6 1F1 CO-LOCALIZES TO THE LIVER

In order to determine if decreased erythrocyte destruction was the result of 1F1 binding to pSn in the liver, we biopsied livers throughout the perfusion and subsequently performed confocal microscopy to evaluate the amount of mouse antibody binding. We found that livers treated with the 1F1 mAb had a considerable amount of mouse IgG deposition, whereas those treated with the isotype control mAb did not (Figure 3.7a).
**Figure 3-7** 1F1 localizes in the liver. A: Confocal analysis of 1F1 binding in the liver. The presence of mouse Ab, either 1F1 (Top Row) or isotype (Bottom Row), was detected in the liver by staining fresh frozen biopsies taken at indicated times. Samples were stained with a fluorescently labeled goat anti-mouse IgG2a secondary (Yellow). Endothelial cells were labeled for CD31 (magenta). Nuclei were stained with DAPI (Blue). B: Lack of 1F1 binding to cells in perfusate. Human: erythrocytes, platelets, lymphocytes, and granulocytes were stained with 1F1 (Red) or the isotype (Black) and analyzed by flow cytometry. Porcine alveolar macrophages were used as a positive control. (n=3) C: 1F1 does not bind soluble pSN in perfusate. Western blot analysis was performed on serum collected during the perfusion at the times indicated and probed using 1F1. Porcine macrophage homogenates were used as a positive control.
These data suggest that inhibition of erythrocyte destruction was the result of 1F1 binding in the liver and that the difference observed in the concentration of 1F1 and the isotype Ab found in the perfusion circuit was the result of 1F1 being bound in the liver. Next we determined whether the liver was the only place where 1F1 was being sequestered. We reasoned that other potential sites for 1F1 sequestration in this model were limited primarily to cells in the perfusate and free floating solubilized pSn in the serum. In order to test if 1F1 was being bound by cells in the perfusate, we analyzed the ability of 1F1 to bind human erythrocytes, platelets, lymphocytes, and granulocytes; 1F1 binding was not noticeably different than that of the isotype control (Figure 3-7b). Finally, in order to determine if soluble pSn was present in the serum of the perfusate and sequestering 1F1, we performed western blot analyses on serum samples isolated from the circuit throughout the perfusion. 1F1 was used as the primary Ab in this study. We saw no detectable pSn using this method (Figure 3-7c). These data suggest that 1F1 sequestration was largely the result of 1F1 binding in the liver.

3.5 DISCUSSION

In this study, we demonstrated the ability of an anti-pSn mAb to inhibit the destruction of human erythrocytes perfused through a porcine liver during extracorporeal porcine liver xenoperfusion. These data highlight the role of pSn in mediating the destruction of human erythrocytes by porcine Kupffer cells in the liver. Furthermore, we demonstrated the maintenance of liver function in an extracorporeal xenoperfused liver for a 72 hour duration, with improved function observed in those livers treated with the anti-pSn mAb 1F1.
The use of a porcine liver in an extracorporeal support device for patients in FHF has the potential of serving as either a bridge to allo-transplantation or to recovery, thereby avoiding the need for transplantation. However, clinical application is limited by several forms of graft-vs-host reactions directed toward components of human blood (82). Initial experiments demonstrated that both wild type pig livers and human decay accelerating factor transgenic pig livers perfused with human blood consumed nearly all of the human platelets within minutes of initiating perfusion and over three days of perfusion destroyed the majority of human erythrocytes, with transgenic livers causing greater erythrocyte loss than wild type (81). Since these initial experiments, a mechanism underlying the recognition of human erythrocytes by porcine Kupffer cells has been revealed. Human erythrocyte removal by porcine Kupffer Cells appears to be mediated through a mechanism involving surface expressed sialoadhesin binding to N-Acetyleneuraminic acid on the surface of human erythrocytes (65, 75). Experiments aimed at assessing the ability of the 1F1 antibody to inhibit porcine macrophage binding of hRBC in vitro, suggest that pSn is involved in the recognition of hRBCs by porcine macrophages (see Figure 3.1). Consistent with this observation, the ex vivo results of the current study confirm that pSn plays a role in mediating human erythrocyte loss when hRBCs are perfused through a porcine liver (see Figure 3.5). The 1F1 antibody bound in the liver and significantly reduced the amount of erythrocyte destruction in this ex vivo xenoperfusion model. However, the presence of 1F1 was not as effective at reducing erythrocyte loss as what might have been expected from the in vitro data. The inability to completely prevent human erythrocyte loss in the ex vivo model could have resulted for several reasons. It is possible that the incomplete blockage of erythrocyte destruction
was the result of inadequate concentrations of 1F1 in the perfusion circuit. It is worth
noting that the lowest rates of erythrocyte loss throughout the perfusion occurred at times
immediately following injections of 1F1; times during the perfusion when mAb
concentrations were highest (see Figure 3.4A and 3.4B). Prior in vitro work from our
laboratory suggested that a concentration of 10µg/ml of 1F1 resulted in 95% inhibiting of
human erythrocyte binding by porcine macrophages (see Figure 3.1). As seen in Figure
3.2, an ex vivo sighting experiment performed in our laboratory suggested that the
addition of 25mg of 1F1 would result in a circulating concentration near 10µg/ml. It was
expected that an additional 12mg of Ab added at times 24hrs and again 48hrs after
commencement would counteract a decrease in circulating 1F1 resulting from pSn
binding and internalization by porcine KCs over the course of the perfusion. Despite
efforts to maintain 10µg/ml of 1F1 in the perfusate, ELISA assays performed on
perfusate serum collected throughout the present study illustrated that 1F1 Ab
concentrations reached a maximum of 5.14µg/ml while the concentration of the isotype
control mAb reached concentrations of 15.9µg/ml. Thus, it is possible that insufficient
1F1 antibody was present to achieve maximal inhibition. Further studies could be
performed to determine a more optimal dose of 1F1, but as our purpose was to determine
whether pSn inhibition would reduce hRBC destruction, these experiments have served
their purpose. Rather than further experiments to refine the optimal dose of 1F1, work is
in progress to produce a sialoadhesin knock out pig as a definitive strategy.

In addition to the possibility of inadequate dosing of 1F1, the discrepancy
between in vitro and ex vivo inhibition may be explained by antibody-mediated receptor
endocytosis triggered by antibody binding to pSn removing antibody from the circulation
(93, 97). Alternatively, it is possible that 1F1 binding to circulating cells or proteins altered the circulating concentration of 1F1. However, flow cytometric analysis of cells expected to be found in the perfusate showed no binding of 1F1 and western blot analysis of serum isolated from the perfusate revealed no appreciable amount of pSn.

Data from this study may also be evidence that additional recognition pathways are involved in the recognition and destruction of human erythrocytes during extracorporeal porcine liver perfusion. Chihara et al. have illustrated that the consumption of human platelets by porcine KC is mediated, in part, by CD18 on the surface of the KC (58). CD18 is known to have a carbohydrate binding site for N-acetyl glucosamine β1-4 N-acetyl glucosamine (βGlcNac) oligosaccharides (98). Recently, Paris et al. has shown that the expression level of βGlcNac is higher on the surface of human platelets as compared to the expression level on porcine platelets (99). It is possible that CD18 expressed on the surface of the KC are involved in mediating the destruction of human erythrocytes. It is also possible that human erythrocyte destruction is mediated in part by receptors known to play active roles in immune signaling. For instance, CD47 and SIRPα are known to serve as markers of self and are said to serve as a “don’t eat me” signal in the case that autologous cells come into contact with “self” phagocytic cell (100, 101). “Self” CD47 causes phosphorylation of SIRPα and blockade of phagocytosis (102). However, in the case of interactions across the species barrier as in the setting of xenotransplantation, SIRPα is not phosphorylated, and thus, phagocytosis is permitted to proceed (100). It is likely that CD47 and SIRPα are involved in mediating the anti-erythrocyte response described above. Given the *in vitro* data from our laboratory showing 1F1 treatment of cultured macrophages reduces the amount of human
erythrocyte binding to levels comparable to that of autologous erythrocyte binding, we propose that CD47/SIRPα acts as a regulator downstream from the initial pSn-mediated binding.

The present study illustrates the involvement of pSn in recognition events leading to the destruction of human erythrocytes during extracorporeal porcine liver perfusion. Although the addition of an anti-pSn mAb did not fully rescue erythrocytes from destruction, the partial blockade of erythrocyte destruction seen in livers treated with anti-pSn mAb suggest that refinements in preventing this recognition pathway will lead to improvement in this potential therapeutic approach. Future studies utilizing livers derived from pSn-deficient pigs would clarify the relative importance of pSn recognition and enable identification of alternative receptor-ligand interactions that may be involved.

3.6 ACKNOWLEDGEMENTS

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Chapter 4

A Human Specific Mutation Limits Nonhuman Primate Efficacy in Preclinical Xenotransplantation Studies

(*a modified version of this chapter has been submitted for peer review in the journal Transplantation)

4.1 ABSTRACT

Background: Patients in fulminant hepatic failure (FHF) currently do not have a temporary means of support while awaiting liver transplantation. A potential therapeutic approach for these patients is the use of extracorporeal liver perfusion (ECLP) with a porcine liver to serve as a form of “liver dialysis”. Previously, our laboratory has shown that during a 72 hour extracorporeal perfusion with human blood, porcine Kupffer cells (KC) bind to and phagocytose human red blood cells (hRBCs) causing the hematocrit to fall to 2.5% of the original value. Furthermore, we have identified N-Acetylated neuraminic acid (Neu5Ac) on the surface of the hRBC as the ligand being bound and sialoadhesin on
the surface of the porcine KC as the lectin receptor mediating erythrocyte removal. The FDA has stated that in order for ECLP with a porcine liver to advance as a potential therapy for patients in FHF, data from preclinical studies in nonhuman primates (NHP) must first demonstrate the its efficacy. Given that no primate other than humans express Neu5Ac to the exclusion of Neu5Gc, we evaluated whether porcine ECLP of NHP would provide adequate evaluation of the loss of erythrocytes that might be expected in a clinical trial.

Methods: We evaluated the ability of porcine macrophages to recognize erythrocytes from various NHPs including those classically used in pre-clinical xenotransplantation studies.

Results: The ability of porcine macrophages to recognize hRBCs is significantly greater than their ability to recognize RBCs from all NHPs tested (p, <0.001).

Conclusions: This study suggests that the mechanism of erythrocyte loss seen in pig liver/human blood xenoperfusion in not replicated by nonhuman primate erythrocytes. Therefore, NHP may not be an adequate model for studying extracorporeal porcine liver xenoperfusion.
4.2 INTRODUCTION

Despite modern techniques utilized to expand the allograft donor pool, patients in end stage liver failure are faced with high mortality rates while awaiting liver transplantation. Especially susceptible, are those patients diagnosed with FHF. With the rapid progression from the onset of symptoms to hepatic encephalopathy, the morality rate of patients diagnosed with FHF remains unacceptably high. A potential remedy for patients diagnosed with FHF is the use of a porcine liver in an ECLP devise. ECLP with a porcine liver has the potential to serve as a “bridge therapy” resulting in increased patient survival. Several anti-human responses have been identified which currently limiting the use of this therapy. Previously, it was shown that during a 72-hour perfusion with isolated human blood, porcine livers consume the equivalent of two units of erythrocyte (103). Furthermore, Rees et al. has shown that this anti-human-erythrocyte reaction is mediated by the porcine KC and is independent of complement activation or antibody opsonization (83, 84). Subsequently, our laboratory has shown that the ability of porcine KC to recognize human erythrocytes is mediated by pSn on the surface of KC binding to sialic acid on the surface of human erythrocytes (104, 105). Sialic acids are N- or O- linked neuraminic acids consisting of nine-carbon molecules and are often found as the terminal sugar of many oligosaccharides (106). Although more than 32 natural glycoforms of sialic acid exist (107), two main forms are found in mammals, Neu5Ac and N-glycolynneuraminic acid (Neu5Gc). These two glycoforms are different from one another by only a single hydroxyl group (Figure 4-1).
The difference between Neu5Ac and Neu5Gc is brought on by the activity of cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMP-Neu5Ac hydroxylase), an enzyme encoded by the CMAH gene that acts to hydroxylate the Neu5Ac precursor and give rise to Neu5Gc. This subtle distinction between Neu5Ac and Neu5Gc brings to light a rare genotypic distinction between humans and our nearest common relative, the great ape. Due to an Alu-mediated deletion in the human genome,
humans are unable to express CMP-Neu5Ac-hydroxylase and thus express the Neu5Ac form of sialic acid to the exclusion of Neu5Gc (108-111). On the contrary, great apes, as well as all other mammals excluding humans, have retained the ability to hydroxylate the Neu5Ac precursor. As a result, the majority of the sialic acid expressed on their cell surfaces is expressed as the Neu5Gc form (112). Given that human erythrocyte recognition by porcine KC is mediated by Neu5Ac expression on the surface of human erythrocytes, we assessed the ability of porcine macrophages to recognize erythrocytes from various nonhuman primates not known to express the majority of their sialic acid in the Neu5Ac form.

4.3 METHODS AND MATERIALS

4.3.1 TISSUE SAMPLES

All animal experiments were approved by the University of Toledo Institutional Animal Care and Use Committee and completed in the Department of Laboratory Animal Research at the University of Toledo Medical Center, Toledo, OH. Porcine macrophages were obtained via bronchoalveolar lavage (BAL) according to Brock et al. and Wensvoort et al. (75, 94). In short, porcine lungs were carefully excised and lavaged with approximately 1500 ml (2 X 750 ml flushes) of cold 0.9% saline (Baxter Healthcare, Deerfield, IL). Lungs were massaged in order to facilitate the release of resident alveolar macrophages. All animals were treated in accordance with the Institute for Laboratory Animal Research (ILAR) publication, The Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, Washington, D.C., 1996) and the Animal Welfare Act. Under sterile conditions, the procedure was done quickly in
order to preserve cell viability. The BAL fluid was then aliquoted into 50 ml conical tubes and centrifuged at 200 x g for 5 min. The supernatants were decanted and cells were suspended in Roswell Park Memorial Institute Medium (RPMI). Cells were then layered onto lymphocyte separation media (Mediatech, Manasses, VA) and spun at 500 x g for 40 min, at which time mononuclear cells were extracted from the mononuclear cell monolayer. Cells were then washed in Hanks Balanced Salt Solution (HBSS, Life Technologies, Carlsbad, CA) and re-suspended at a concentration of 10X10⁶ in chilled RPMI 1640 (Mediatech) supplemented with 10% FBS (Life Technologies), 1% penicillin/streptomycin (100 U/ml, 100 lg/ml, Life Technologies), and 5% dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO). Samples remained frozen in liquid nitrogen until use. They were removed and placed into culture for 4 days before being utilized in experiments.

Nonhuman primate whole blood samples were obtained from the Toledo Zoo (Toledo, OH), the Southwest National Primate Research Center (San Antonio, TX), or the Yerkes National Primate Research Center (Atlanta, GA) through a protocol approved by the University of Toledo Institutional Animal Care and Use Committee. Type O blood was used for human blood experiments. Written informed consent was obtained for all human volunteers under a University of Toledo Institutional Review Board (IRB) approved protocol. Human and nonhuman primate whole blood was collected into a lithium-heparin tube and mixed (Thermo Fisher Scientific, Waltham, MA). The whole blood was washed twice with HBSS. A 0.1% red blood cell solution to be applied to cultured macrophages was prepared with RPMI.
4.3.2 FLOW CYTOMETRY

Isolated cells were suspended in 100 µl of blocking buffer (phosphate-buffered saline, PBS, containing 20% horse serum) and incubated on ice for 30 min at 4°C. Primary antibodies included pSn mAb, 1F1(93), and the isotype control monoclonal antibody at a concentration of 0.9µg/ml. Cells were washed with HBSS and then stained with 0.1µg/ml ALEXA FLUOR-488® conjugated goat anti-mouse IgG secondary antibody (Life Technologies). Samples were washed twice between treatments with blocking buffer. Samples were analyzed on a Cytometrics FC 500 flow cytometer (Beckman Coulter, Brea, CA). Data were analyzed using FlowJo, version7.6.4.

4.3.3 ROSETTING ASSAY

Binding: Determination of porcine macrophage recognition of xenogeneic erythrocytes was performed using an erythrocyte rosetting assay as previously described (83). In brief, after 4 days in culture, macrophages were removed from culture flasks and seeded into a 96-well plate at 3X10⁴ cells per well in RPMI. After 2 hours of incubation, RPMI was replaced and macrophages were incubated with a 0.1% erythrocyte solution. After 1 hour of erythrocyte/macrophage co-incubation, wells were washed with RPMI in order to remove unbound erythrocytes. Wells were then allowed to dry and cells were subsequently fixed with cold methanol.

Quantification: After xenogeneic erythrocyte binding, rosettes were quantified using a TMB reaction in which erythrocyte binding is measured based on the presence of oxygen via oxidation and subsequent color change(113). Here, 100 µl of solution A and B (R & D systems, Minneapolis, MN) was added to each well and allowed to incubate in the dark
for 20 minutes. Following incubation, the reaction was stopped with 2M sulfuric acid and
the plate was quantified with a spectrophotometer at a wave length of 450 nm. Data were
calculated as percent human erythrocyte binding.

Microscopy: In order to verify quantitative data, rosettes were visualized using bright
field microscopy. Porcine macrophages were seeded into a 24-well plate and cultured as
previously discussed. After co-incubation, unbound erythrocytes were removed and cells
were fixed with 4% paraformaldehyde and then visualized by bright field microscopy
using an Olympus FSX100 microscope (Olympus, Tokyo, Japan).

4.3.4 STATISTICAL ANALYSIS

Significant differences between groups were determined using a student’s t-test.

A p-value of <0.05 was considered to be significant.

4.4 RESULTS

4.4.1 ISOLATION OF SIALOADHESIN EXPRESSING PORCINE
MACROPHAGES

In order to assess the susceptibility of xenogeneic erythrocytes to porcine
macrophages through a Sn-Neu5Ac mechanism, we first determined the percentage of
pSn expressing cells isolated via BAL (Figure 4-2). Lavage of adult swine lungs
according to Wensvoort et al. (94), resulted in high yields of pSn expressing.
Figure 4-2 Flow cytometry analysis of porcine mononuclear cells isolated by BAL. Porcine cells were obtained via bronco-alveolar lavage and then separated by centrifugation in lymphocyte separation media. Mononuclear cells were then extracted from the mononuclear cell monolayer and either frozen for later use or plated on polystyrene tissue culture plates. After three days in culture, cells were analyzed for sialoadhesin expression by flow cytometric analysis. Number in quadrant represents percentage of total cell count.

4.4.2 PORCINE MACROPHAGES BIND HUMAN ERYTHROCYTES BUT NOT ERYTHROCYTES FROM HUMAN’S NEAREST COMMON ANCESTOR

Previously, we have shown that the recognition of human erythrocytes by porcine macrophages is mediated by pSn on the surface of the porcine macrophage binding to the Neu5Ac form of sialic acid expressed on the surface of the human RBC. Furthermore, we have illustrated that removal of Neu5Ac from the surface of the human erythrocyte
decreases its’ susceptibility to porcine macrophages. Given that humans are the only primate known to express the majority of their sialic acid in the Neu5Ac form, we assessed whether retained CMP-Neu5Ac-hydroxylase activity and expression of Neu5Gc in erythrocytes from the chimpanzee, a primate considered to be a “most recent common ancestor” to humans, were bound less frequently by porcine macrophages as compared to human erythrocytes. To this end, cultured porcine macrophages were co-incubated with erythrocytes from either humans or chimpanzees and subsequently assessed for their relative ability to recognize and bind xenogeneic RBCs. The binding of porcine macrophages to chimpanzee erythrocytes was significantly less as compared to the binding of porcine macrophages to human erythrocytes (p,< 0.001, Figure 4-3A). Light microscopy confirmed this observation (Figure 4-3B).
Figure 4-3 Porcine macrophages fail to bind chimpanzee erythrocytes. (A) The ability of porcine macrophages to recognize pig, human and chimpanzee erythrocytes was quantified using a TMB rosetting assay (p,<0.001, N=3). Binding was quantified as percent human erythrocyte binding. (B) In order to verify quantitative rosetting data, human erythrocytes (left) and chimpanzee erythrocytes (right) were co-incubated with porcine macrophages. After washing to remove any unbound erythrocytes, bright field images were taken.
4.4.3 PORCINE MACROPHAGES FAIL TO BIND ERYTHROCYTES FROM COMMONLY USED NONHUMAN PRIMATE PRE-CLINICAL MODELS

Baboons are a commonly used NHP model used in pre-clinical studies of liver transplantation across discordant barriers. Given the results from the chimpanzee study, we set out to determine whether erythrocytes from the baboon would be recognized and bound by porcine macrophage as baboons are considered to be even more genetically dissimilar from humans than is the chimpanzee. To this end, a rosetting assay was performed in order to compare the relative erythrocyte susceptibility between human and baboon erythrocytes. We found that porcine macrophage bound baboon erythrocytes significantly less than human erythrocytes (p,<0.001, Figure 4-4A). Phase contrast microscopy images supported the quantitative data (Figure 4-4B).
Figure 4-4 Porcine macrophages fail to bind baboon erythrocytes. (A) The ability of porcine macrophages to recognize pig, human and baboon erythrocytes was quantified using a TMB rosetting assay ($p<0.001$). (N=5) Binding was quantified as percent human erythrocyte binding. (B) In order to verify quantitative rosetting data, human erythrocytes (left) and baboon erythrocytes (right) were co-incubated with porcine macrophages. After washing to remove any unbound erythrocytes, phase contrast microscopy was used to visualize rosettes.
Next, we determined whether porcine macrophage bound erythrocytes from Cynomolgus monkeys and Rhesus macaques, two NHP species commonly used in medical research, and found that macrophage binding to Cynomolgus monkey or Rhesus macaque erythrocytes were bound significantly less than human erythrocytes (p,<0.001, N=3, Figure 4.5)

![Figure 4-5 Porcine macrophages fail to bind erythrocytes from primates commonly used preclinical model.](image)

The ability of porcine macrophages to recognize erythrocytes from human and Cynomolgus monkeys (A) (p,<0.001) and Rhesus macaques (B) was quantified using a TMB rosetting assay as compared to human erythrocyte binding (p,<0.001). (N=3)
4.4.4 PORCINE MACROPHAGES FAIL TO BIND NONHUMAN PRIMATE ERYTHROCYTES

Given that porcine macrophages bound erythrocytes from both chimpanzees, and other nonhuman primates commonly used in medical research, significantly less than human erythrocytes, we tested whether this phenomena was common among a panel of nonhuman primates from two broad super families, including Hominoidea and Cercopithecoida. We performed the in vitro rosetting assay using erythrocytes from 5 addition nonhuman primates, including: Gorilla, Gibbon, De Brazza, Colobus, and Langur. In every species tested, porcine macrophages bound nonhuman primate erythrocytes significantly less than human erythrocytes (p,<0.001, Figure 4-6).

Figure 4.6 Susceptibility of various NHP erythrocytes to porcine macrophages. The ability of porcine macrophages to recognize erythrocytes from various nonhuman primates was quantified using a TMB rosetting assay. Experiments were done as one offs (n=1) and binding was quantified as percent human erythrocyte binding (Human vs. NHP, p,<0.001).
4.5 DISCUSSION

Extracorporeal porcine liver xenoperfusion for the treatment of fulminant hepatic failure has been suggested as a reasonable first step in the clinical evaluation of xenotransplantation. This approach has been proposed for multiple reasons; 1) the shortage of human livers available for transplantation; 2) the high mortality rate associated with fulminant hepatic failure; 3) the lack of a temporary support therapy for patients in hepatic failure; 4) exposure to the porcine organ will be limited to a few days rather than permanent implantation; and 5) long-term immunosuppression may be avoided if the patient in “bridged” to recovery.

In this study, we assessed the ability of porcine macrophage to bind human and nonhuman primate erythrocytes in an attempt to model the macrophage mediate graft-vs.-host response seen when porcine livers are perfused with xenogeneic blood. Here, we were able to replicate in vitro human erythrocyte binding data previously described by our laboratory (75, 104, 105). Although porcine macrophage readily bound human erythrocytes, macrophage binding to nonhuman primate erythrocytes was significantly reduced as compared to human. Given that the ability of porcine macrophage to bind human erythrocytes is dependent on mechanisms involving the expression of the Neu5Ac form of SA on the surface of the RBC (105), and that nonhuman primates express the majority of their SA in the Neu5Gc form (112), we suggest that the varying degree of binding between human and nonhuman primate erythrocytes by porcine macrophage likely results from the variation in the form of SA expressed on the surface of xenogeneic cells.
Although pre-clinical animal studies sometimes fail to accurately predict human responses (114), current FDA requirements for xenotransplantation to advance to clinical trial include evidence for efficacy of the therapy using nonhuman primates in a pre-clinical setting. The data in this study provide evidence from nine different nonhuman primate species, including the three species most commonly used in pre-clinical studies, suggesting that nonhuman primates will not recapitulate the graft-vs.-host response seen when human blood is perfused through a porcine liver. In addition, Lutz et al. recently produced double knockout pigs deficient in both Neu5Gc and galactose α-1,3-galactose (Gal α-1,3-Gal), and demonstrated a significant reduction in the human anti-pig immune response to cells from these animals as compared with cells derived from Gal α-1,3-Gal single knockouts (115). Data in the current study, along with data published by Lutz et al., suggest that, as a result of retained CMAH activity and Neu5Gc expression, nonhuman primates will fail as a model to evaluate the graft-vs.-host response seen when human blood is perfused through a porcine liver as in ECLP; and will fail to illustrate the benefit of eliminating Neu5Ac in the host-vs.-graft response seen in pig-to-human whole organ xenotransplantation. We propose that the efficacy of extracorporeal porcine liver perfusion and transplantation of porcine organs from Neu5Gc/Gal α-1,3-Gal double knockouts be evaluated in brain dead patients determined not to be suitable candidates for organ donation, rather than in nonhuman primates, as a precondition for clinical trial (116).
Chapter 5

The Use of Brain Dead Subjects in Xenotransplantation Research: An assessment of Public Opinion

5.1 ABSTRACT

Background: While the field of xenotransplantation has benefited enormously from the use of animal models, previous studies have demonstrated limitations in moving directly from animal studies to clinical trials. As a compromise approach, some have proposed the use of brain dead patients, whose organs have been determined to be unsuitable for transplantation, as research subjects for trials of xenotransplantation. We refer to such patients as brain dead research subjects (BDRS). Experiments involving xenotransplantation into BDRS have two major ethical implications: 1) Using brain dead human subjects in research; and 2) Transplanting animal organs into brain dead humans. Experiments involving BDRS should not proceed until public opinion is assessed to determine what types of practices are considered morally acceptable and the point where
ethical principles are perceived as violated. In this survey research study, we attempted to ascertain the type of experiments that the general public will support, and determine the extent where this support ends.

Methods: A questionnaire designed to gauge demographic information and assess opinion on the use of BDRS in xenotransplantation research was administered to 426 people at a public venue in a Midwestern U.S. city.

Results: Demographic distribution of respondents were: female (62%), Caucasian (74%), College degree (39%), Christian (74%), Registered organ donor (65%), Income 20-60K/year (48%). 75% agreed that it is “appropriate to conduct medical research on these brain dead patients.” 60% agreed it was appropriate to use BDRS in experiments that “would involve transplanting animal organs into the human body.” Further correlation with demographic information is available.

Conclusions: In the population studied, we found an overall positive attitude towards the use of BDRS in medical research and the use of these subjects in xenotransplantation studies. These data suggest that further exploration of the possibility of using BDRS to study xenotransplantation is warranted.

5.2 INTRODUCTION

Many fields of medicine, including xenotransplantation, have benefited greatly from the use of nonhuman primates (NHP) in preclinical models. Though NHP have allowed researches to predict outcomes of many experimental therapies prior to administration in patients, there are certain instances where such models have failed to predict clinical outcomes. Previously, our laboratory has published data illustrating that the use of a porcine liver in an extracorporeal support therapy for patients in fulminant
hepatic failure is limited by a graph-vs-host response involving the consumption of human erythrocytes by porcine livers (62). Furthermore, we have shown that this graft-vs-host response elicited towards the human erythrocytes is mediated by the expression of N-acetylneuraminic acid (Neu5Ac) on the surface of the human erythrocyte (75).

Neu5Ac is the precursor form of N-glycolyneuraminic acid (Neu5Gc) and as a result of an Alu-mediated deletion in the CMAH (the gene responsible for Neu5Ac to Neu5Gc), has been shown to be the principal form of sialic acid (SA) expressed on the surface of human cells (109, 112). On the contrary, NHP have retained function of the CMAH gene and subsequently express the majority of their SA in the Neu5Gc form (109). In a series of in vitro studies highlighted in chapter 4, we determined that the difference in SA expression between humans and NHP limits the ability of porcine macrophages to bind erythrocytes derived from NHP, as compared to human, and will likely limit the ability of NHP to serve as preclinical models for studying extracorporeal porcine liver xenoperfusion. In response to this data, and data published by Lutz et al. (115), we began to explore alternative preclinical models better suited for predicted clinical efficacy of extracorporeal porcine liver perfusion. One such model is the use of brain dead patients whose organs have been determined unsuitable of donations. Brain dead subjects could offer researchers great insight into clinical outcomes of experimental therapies without jeopardizing the lives of sick patients whose health would already be severely compromised.

We are not the first group to propose the use of BDRS in medical research. Several groups have previously utilized BDRS in medical experimentation in order to better predict clinical outcomes without putting living patients at risk. Arap et al.
recently performed a study aimed at mapping the human vasculature with respect to peptide specificity. In this study, Arap et al. perfused a BDRS with a library of small peptides and later performed a complete autopsy on the subject to determine the tissue specificity of these peptides (117). This study yielded great results that will likely contribute to the development of drugs capable of targeting specific tissue.

Public concern with studies utilizing BDRS in medical research is expected. For this reason, research institutions conducting research on BDRS have established specialized review committees consisting of physicians, scientists, medical ethicists, and legal experts in order to review studies utilizing BDRS for their scientific merit and impact on society (118-120). Additionally, various peer reviewed articles have been published in order to help assess the ethical implications of conducting research that utilizes BDRS (120-124).

Although institutions utilizing BDRS have implemented vigorous review boards aimed at assessing the appropriateness of BDRS in medical research, the medical community would benefit from understanding the public view of performing such studies. To this end, we conducted a public survey study aimed as assessing public opinion on the use of BDRS in medical research.

5.3 METHODS AND MATERIALS

5.3.1 SAMPLE POPULATION

A questionnaire was administered over a three day period in a public venue in Toledo, OH, a midsize Midwestern city located in the United States. In total, 426 subjects completed the survey.
In order to determine whether educating the public would have a positive outcome on the response, a separate group of subjects were asked to complete the questionnaire before and after a symposium aimed at educating participants on topics relating to brain death and on the current status of xenotransplantation. In total, 61 subjects completed the survey administered at the symposium.

5.3.2 SURVEY

This survey consisted of 17 questions (appendix); subjects completed the survey in approximately 5 minutes. The survey began with a brief definition of the law that currently defines brain death. The survey consisted of three sections. First, the subject’s were asked to respond to a set of questions designed to gauge whether they agree with the law defining brain death as death and whether they believe that brain death accurately defines death. Next, subjects were asked a series of questions aimed at determining to what extent they support the use of brain dead subjects in medical research. Finally, demographical data were attained in order to allow for analysis of the variables that influenced the outcomes in parts two and three. Less than 1% of subjects who agreed to complete the questionnaire failed to do so.

*The complete survey can be found in the Appendix of this thesis.

5.3.3 STATISTICAL ANALYSIS

Data were converted to a database and then analyzed using SPSS 17.0 statistical package (SPSS inc., Chicago, IL, USA). A descriptive statistical analysis was performed.
In order to determine the variables that influenced the outcomes, student’s $t$-test and chi squared analysis were performed.

5.4 RESULTS

5.4.1 DEMOGRAPHICS

The following demographic data were obtained from respondents: Age, Gender, Race, Education, Marital Status, Religion, Income, Number of Kids, and Organ Donor status. Demographic data obtained in this study were comparable to national and statewide demographics with the exception of Gender, Education, and Organ Donor. Sixty-one point five percent of the respondents were female while the percentage of females nationally, state wide and in Toledo is 50.8, 51.2, and 51.6 percent, respectively (Figure 5-1).
Respondents in this study were more educated than the national, state, and Toledo average. Thirty-nine percent of respondents in this study had a college degree while the national, statewide and Toledo average is 35.7, 32.4, and 26.1 percent, respectively (Figure 5.1). Lastly, we found that 65.2% of respondents in this study were organ donors while the state wide average is only 52% (Figure 5.1).
5.4.2 PUBLIC OPINION ON THE LAW DEFINING BRAIN DEATH AND THE USE OF BDRS IN MEDICAL RESEARCH

The degree to which the general public agrees with the law that states that brain death defines an individual as being “dead” could greatly impact whether they support the idea of conducting medical research on brain dead subjects. In order to determine whether respondents in this study agreed with the law that defines death as being brain dead, subjects were asked to read a short description of the current law that defines brain death and were then asked to answer the following question. “Do you agree with the law stating that brain death accurately define death?” Here we found that 62% of the respondents agreed with the law defining brain death as death (Figure 5.2).
Figure 5-2 Public opinion of the law stating that brain death defines death.
Sixty-two point three percent of respondents agreed that the law on brain death accurately defines death. Various demographic data were obtained and assessed for their ability to influence the way the subjects responded. p-Values for variables significant, or marginal significant, are shown in bold.
In order to determine the demographic variables that influenced the subject’s response, a chi squared analysis was conducted. We found that five demographic variables influenced responses. These include: Race, Education, Number of Kids, Income, and organ donation status (Figure 5-2).

Next, we wanted to determine whether the public would support the use of BDRS in medical research. In order to assess whether the public supports the use of BDRS in medical research, we asked the question, “Is it appropriate to conduct medical research on brain dead patients (who have previously consented to the use of their organs)?” We found that 75% of respondents thought that is acceptable to conduct research on brain dead subjects (Figure 5-3).

| Variables That Influence Public Opinion Towards the Use of the Recently Deceased in Medical Research |
|------------------------------------------------|------------------------------------------------|------------------------------------------------|-----------------|-----------------|
| Variables                                         | Agree, n = 300 (75%) | Disagree, n = 71 (17.4%) | Undecided, n = 31 (7.6%) | P value |
| Mean age (40 ± 14 years)                          |                     |                         |                          |          |
| Education                                         |                     |                         |                          |          |
| No high school diploma (n = 18)                   | 13 (72%)            | 2 (11%)                 | 3 (17%)                 | 0.059   |
| High school diploma (n = 97)                      | 65 (67%)            | 20 (21%)                | 12 (12%)                |          |
| Some college (n = 123)                            | 98 (80%)            | 98 (80%)                | 9 (7%)                  |          |
| Technical school certificate (n = 13)             | 11 (65%)            | 0 (0%)                  | 2 (15%)                 |          |
| College degree (n = 118)                          | 84 (71%)            | 28 (24%)                | 6 (5%)                  |          |
| Graduate school degree (n = 44)                   | 37 (84%)            | 6 (14%)                 | 1 (2%)                  |          |
| Number of Kids                                    |                     |                         |                          |          |
| 0 (n = 89)                                        | 59 (66%)            | 25 (28%)                | 5 (6%)                  | 0.013   |
| 1 or 2 (n = 188)                                  | 144 (77%)           | 23 (12%)                | 21 (11%)                |          |
| 3 (n = 81)                                        | 64 (79%)            | 13 (16%)                | 4 (5%)                  |          |
| 4 (n = 35)                                        | 26 (74%)            | 7 (20%)                 | 2 (6%)                  |          |
| 5 (n = 10)                                        | 6 (60%)             | 3 (30%)                 | 1 (10%)                 |          |
| 6 (n = 8)                                         | 6 (75%)             | 2 (25%)                 | 0 (0%)                  |          |
| 7 or more (n = 4)                                 | 2 (50%)             | 0 (0%)                  | 2 (50%)                 |          |
| Organ Donor                                       |                     |                         |                          |          |
| Yes (n = 271)                                     | 215 (70%)           | 37 (14%)                | 19 (7%)                 | 0.028   |
| No (n = 145)                                      | 94 (65%)            | 35 (24%)                | 16 (11%)                |          |
| D/K (n = 1)                                       | 1 (100%)            | 0 (0%)                  | 0 (0%)                  |          |
| Income                                            |                     |                         |                          |          |
| Less than or equal to 20,000 (n = 85)             | 51 (60%)            | 23 (27%)                | 11 (13%)                | 0.051   |
| 20,001 - 40,000 (n = 105)                         | 79 (75%)            | 16 (15%)                | 10 (9.5%)               |          |
| 40,001 - 60,000 (n = 88)                          | 72 (52%)            | 11 (13%)                | 8 (6%)                  |          |
| 60,001 - 80,000 (n = 57)                          | 49 (70%)            | 12 (21%)                | 5 (9%)                  |          |
| 80,001 - 100,000 (n = 39)                         | 34 (87%)            | 3 (8%)                  | 2 (5%)                  |          |
| More than 100,000 (n = 32)                        | 27 (84%)            | 4 (13%)                 | 1 (3%)                  |          |

**Figure 5-3 Public opinion on the use of brain dead subjects in medical research.** Participants were asked whether they think it is appropriate to use brain dead patients in medical research. P-Values for variables significant, or marginal significant, are shown.
or no time limit (Indefinitely). We found that there was a strong correlation between the public’s approval rate of the use of BDRS in medical research and the length of time that the bodies would be used ($R^2=0.9937$, Figure 5-4).

![Graph showing the percentage of respondents agreeing with the use of BDRS for different lengths of time.](image)

**Figure 5-4 Length of time appropriate to conduct research on brain dead research subjects.**

Participants who agree to the use of brain dead research subjects in medical research were asked how long it was appropriate to conduct research on BDRS. Approval of the use of BRDS is correlated to the length of time required to perform the research ($R^2=0.9937$).

Only those who answered, “Agree” to the question, “Is it appropriate to conduct medical research on brain dead patients (who have previously consented to the use of their organs)?” were accounted for in this analysis. Furthermore, we found that while 86.7% of those who agree with the use of BDRS in medical research thought it was appropriate to conduct medical research on these subjects for a time period of hours, only 24.2% of these respondents thought it was appropriate to conduct research on the brain dead subjects for an indefinite amount of time (Figure 5-4).
5.4.4 THE TYPE OF MEDICAL RESEARCH CONDUCTED INFLUENCES

PUBLIC OPINION ON THE USE OF BDRS

Support for the use of BDRS could vary based on the type of biomedical research being conducted. We determine whether public support of the use of BDRS in medical research was influenced by the type of research being proposed. Survey takers were asked whether they approved the use of brain dead subjects in a series of research scenarios of increasing invasiveness. These scenarios are listed in Figure 5.5.

1. It is appropriate to use the bodies of brain dead subjects in experiments that determine the effects of substances we are commonly exposed to, such as the sun (UV rays), skin creams, and deodorants. This would not require entering the body or puncturing the skin.

   Agree ☐  Disagree ☐  No opinion ☐

2. It is appropriate to use the bodies of brain dead subjects in experiments to help researchers develop better treatments. This would involve entering the body or puncturing the skin to inject drugs, bacteria, cancer cells, or viruses.

   Agree ☐  Disagree ☐  No opinion ☐

3. It is appropriate to use the bodies of brain dead subjects in experiments to help researchers develop alternative transplant methods. This would involve transplanting animal organs into the human body.

   Agree ☐  Disagree ☐  No opinion ☐

4. It is appropriate to use the bodies of brain dead subjects in experiments to help researchers learn about arm, leg, and face transplants. This would involve physical alteration of body parts.

   Agree ☐  Disagree ☐  No opinion ☐

*Figure 5-5 Research scenarios from research questionnaire aimed at assessing what types of medical research the public thinks is acceptable for the utilization of brain dead research subjects.*
In this analysis, we found that the population studies was generally supportive of each scenario presented (Figure 5-6).

While there was very little difference in the support of using BDRS in less invasive procedures (“This would not require entering the body or puncturing skin”, and “This would involve entering the body of puncturing the skin”) (71.9 and 73.6 percent, respectively, Figure 5-6), studies involving transplantation of animal organs and studies requiring physical alterations to the body had noticeably lower support (59.1 and 66.7 percent, respectively, Figure 5-6).

![Figure 5-6 Public support for the use of brain dead research subjects in various type of medical research.](chart)
5.4.5 PUBLIC OPINION ON THE USE OF BDRS IN XENOTRANSPLANTATION

An alternate approach to the use of nonhuman primates in preclinical models of xenotransplantation could be the use of BDRS. We determine whether the public agrees with the use BDRS in xenotransplantation research. In order to do this, we presented participants with the following statement, “It is appropriate to use the bodies of brain dead subjects in experiments to help researchers develop alternative transplantation methods. This would involve transplanting animal organs into the human body.” We found that 59.7% of respondents thought that the use of brain dead subjects in medical research involving xenotransplantation was appropriate (Figure 5-6 and 5-7). Although a majority of the participants surveyed agreed with the use of brain dead subjects in xenotransplantation research, this type of study garnished the lowest amount of support among the research topics presented (Figure 5-6).
Figure 5-7 Public opinion of the use of brain dead research subjects in xenotransplantation research.

Fifty-nine point seven percent of respondents agreed that it is appropriate to use brain dead subjects in medical research involving xenotransplantation. p-Values for variables significant, or marginal significant, are shown in bold.
5.4.5 EDUCATION OF BRAIN DEATH AND MEDICAL RESEARCH

POSITIVELY INFLUENCES PUBLIC SUPPORT OF THE USE OF BDRS IN MEDICAL RESEARCH

Through conversation with subjects in this study, it became clear that members of the public were unaware of the current practices with respect to the utilization brain dead patients in medical fields, such as transplantation. We hypothesized that a lack of understanding of the definition of brain death and of current practices could have implications on the outcome of our study.

In order to determine the impact of educating the public on issues relating to brain death and xenotransplantation, we conducted a study wherein members of the public were invited to attend a symposium aimed at educating the participants on topics relating to brain death and xenotransplantation. During this study, participants were asked to complete the questionnaire (Appendix) before and after the symposium. We found that in every survey question asked, there was an increase in the support of the use of BDRS in medical research (Figure 5-8).
Figure 5-8 The effect of education on the use of brain dead subjects in medical research.
In order to determine the effect education on the public attitude towards the use of brain dead subjects in medical research, a symposium was conducted to inform the audience on the brain death and medical research. The questionnaire was administered before and after the symposium and responses were compared. In all cases, the support increased after education.

5.5 DISCUSSION

The use of BDRS in preclinical medical research could provide vast insight into the efficacy of newly developed therapies (117). However, the public opinion of the use of such preclinical models has not been determined. In this public survey study, we determined whether the public agreed with the current law defining brain death as death and whether the public approved of the use of BDRS in various types of medical research.
A potential cause of public concern regarding the use of BDRS in medical research could arise from a misunderstanding or disagreement with the law stating that brain death defines death. As is shown in figure 5.2, our study illustrated that 62% of study participants agreed with the law stating that brain death defines death. Next, we found that 75% of respondents agreed to the use of BDRS in medical research (Figure 5-3). We felt that the support of the use of BDRS in medical research was relatively high given the surprising low percentage of individuals who agree with the law defining brain death as death. Taken together, we feel that the favorability of using BDRS in medical research could increase if a higher percentage of the public agreed that brain death equated to death. It is worth noting that several study participants thought that brain death was the same as a coma, or a vegetative state and that brain death is, in some cases, reversible. Furthermore, these data illustrate that support for the use of BDRS was influenced by the type of research being conducted (Figure 5-6).

Finally, we showed that the support of the use of BDRS in medical research increases upon educating the public on brain death and medical research (Figure 5-8). It is possible that increased favorability of the use of BDRS in medical research increased as a result of a better understanding of brain death. We expect that respondents who thought that patients diagnosed with brain death could recover from this diagnosis would be less likely to support research which utilizes this type of model.

A concern with the use of BDRS in medical research is the public objection that could come from performing such potential ethically challenging studies. For this reason, various institutional review boards have been established specifically for the review of research proposals involving BDRS (118). Given the potential public pushback
regarding research involving the recently deceased, it is important to assess the public opinion before performing such studies.

This study has provided evidence of the public’s support of the use of BDRS in medical research. The authors feel that this study should provide the medical community with the confidence to move forward with the use of BDRS in research studies that may not benefit from the use of classical pre-clinical models and are in need of a more clinically relevant model.
Chapter 6

Conclusions

6.1 SUMMARY

Currently, patients diagnosed with fulminant hepatic failure do not have a temporary means of support while awaiting liver transplantation. Extracorporeal liver perfusion with a porcine liver has the potential to serve as a temporary therapy capable of bridging patients in liver failure to either liver transplantation or recovery. Unfortunately, the use of a porcine liver in an extracorporeal liver perfusion device is limited by several forms of graft-vs-host responses. Rees et al. has previously illustrated that, when human blood is perfused through an ex vivo porcine liver, the porcine liver consumes nearly all of the human erythrocytes and after 72 hours of perfusion, the hematocrit falls to 2.5% of the original value (Figure 1-2) (62). Additionally, we have shown that this mechanism involves porcine Kupffer cells and is independent of complement activation or antibody opsonization (61, 63).

The goals of this thesis were two fold. First, we set out to better understand the role of pSn in mediating the recognition and subsequent destruction of human erythrocytes perfused through porcine livers. Second, we explored the feasibility of
nonhuman primates at serving as preclinical models of ECLP and subsequently explored the possibility of using brain dead research subjects as an alternative preclinical model in medical research. Chapter 1 discusses the history of liver perfusion by first introducing work done by Eiseman et al., Otto et al., and other pioneers in the field and concludes with more recent experiences using porcine livers to treat patients in FHF. Chapter 2 highlights the past barriers to the use of a porcine liver in the treatment of FHF patients using ECLP. Chapter 3 aimed at further elucidating the role of pSn in mediating the anti-human erythrocyte response seen when human blood is perfused through a porcine liver. This chapter utilized both in vitro and ex vivo experimentation to illustrate the role of pSn. The hypothesis driving the experimentation in Chapter 4 was borne out of the knowledge that porcine macrophages reject human erythrocytes through a mechanism dependant Neu5Ac expression on the surface of the erythrocyte and data published by Varki et al. suggesting that nonhuman primates lack Neu5Ac expression (108, 112, 125). Data collected in Chapter 4 explored the effects of the differences in the form of sialic acid expressed on the surface of human and nonhuman primate erythrocytes and whether or not these differences could affect the ability of nonhuman primates to serve as adequate preclinical models for studying ECLP. Finally, Chapter 5 explored the public opinion of the use of brain dead research subjects as alternative preclinical models in medical research, including, xenotransplantation research. Data collected in this chapter summarize public perception of brain death and whether or not the use of brain dead subjects in medical research, specifically research involving xenotransplantation, is an acceptable practice. The data presented in this thesis have led us to pursue the following research approaches:
• **Test a sialoadhesin knockout pig in the extracorporeal liver perfusion model.**

Previous studies performed in our laboratory have identified the role of pSn in mediating the destruction of human erythrocytes by porcine macrophages *in vitro* (75). Additionally, the *in vitro* and *ex vivo* data published by Waldman et al. (126), and presented in Chapter 3 of this thesis, help to further elucidate the role of pSn in mediating the anti-human erythrocyte response carried out by the porcine liver during an *ex vivo* liver perfusion. Though the addition of an anti-Sn monoclonal Ab to the circuit of a human blood to pig liver *ex vivo* perfusion significantly reduced the amount of erythrocyte destruction, the findings were not as definitive as what we had expected based on our *in vitro* data. The role of pSn in mediating the destruction of human erythrocytes may be more definitively elucidated by performing human blood-to-pig liver xenoperfusion with a liver derived from a pSn knockout pig. Recently, Prather et al. reported data highlighting progress towards the generation of a pSn− pig (127), collaborative efforts are underway that will allow our laboratory to perform perfusion studies using livers derived from these animals.

• **Determine alternative mechanisms of erythrocyte destruction.**

The effect of the addition of an anti-Sn monoclonal Ab on blocking erythrocyte destruction was very convincing in the *in vitro* model, however, the effect of blocking pSn with a monoclonal Ab in the *ex vivo* model was less convincing (126). It is possible that the inability to completely block erythrocyte consumption in the *ex vivo* model, was the result of incomplete blockade of all
pSn molecules expressed in the liver. The possibility remains that the lack of complete blockade of erythrocyte destruction resulted due to mechanisms other than those involving pSn.

There are several receptor-ligand pairs that have been proposed to be involved in mediating macrophage consumption of xenogeneic cells. The CD47- Signal inhibitory receptor protein (SIRP-α) pair have been suggested to be involved in mediating destruction of CD47 void autologous cells as well as xenogeneic cells and should be investigated to determine if it is involved in mediating the destruction of human erythrocytes in the human blood to pig liver xenoperfusion model (100, 101).

- **Assess the ability of a porcine liver to maintain liver function in a brain dead subject.**

Several groups have utilized NHP as preclinical models for studying orthotopic liver xenotransplantation as well and extracorporeal porcine liver xenoperfusion. Although these studies have served as valuable contributors towards understanding many of the rejection processes that limit the use of porcine livers in the clinic, these studies have not demonstrated the anti-erythrocyte response shown when human blood is perfused through an extracorporeal porcine liver. Data presented in Chapter 4 of this thesis suggest that studies utilizing NHP will fail to demonstrate the graft-vs-host anti-erythrocyte response seen when human blood is perfused through a porcine liver due to a difference in the form of sialic acid expressed on the surface of the NHP erythrocyte.
As a result of the differences between human and NHP sialic acid expression, it is important that we consider preclinical models that are capable of serving as a more accurate representation of what we would expect in the clinical setting. Chapter 5 of this thesis introduces the idea of utilizing brain dead subjects in medical research and assesses the public opinion of such practices. We propose that future studies utilize brain dead research subjects to more accurately assess the outcome of using porcine liver xenoperfusion to treat fulminant hepatic failure. Initial studies would test the safety of attaching the circulation of an intact human to a porcine liver and would also allow for the assessment of the immune mediated injury to the attached porcine liver. Subsequent studies utilizing brain dead research subjects could test the ability of a porcine liver to maintain liver function in a brain dead research subject with impaired liver function.

6.2 IMPLEMENTATIONS ON THE FIELD OF EXTRACORPOREAL PORCINE LIVER PERFUSION

The field of extracorporeal porcine liver xenoperfusion is currently limited by several forms of rejection, including: severe thrombocytopenia, and the loss of human erythrocytes. Work presented in this thesis is aimed at resolving the mechanisms of the graft-vs-host anti-human erythrocyte response elicited during ECLP with a porcine liver. Additionally, this work focuses on understanding the inadequacies of NHP in preclinical studies of porcine liver xenoperfusion and suggests an alternative preclinical model utilizing BDRS.
Fulminant hepatic failure is characterized by rapid deterioration of the patient from the onset of jaundice to the onset of hepatic encephalopathy (128). Exemplifying this data is the statistic from UNOS demonstrating that, between the years of 1995 and 2011, more than 38,000 patients were removed from the liver transplantation waiting list as a result of death or becoming too ill to be transplanted (129). Adding further evidence to the rapid progression of this disease is data published by Bernal et al. suggesting that patients who are treated within the first 36 hours of being diagnosed with FHF have increased survival rates (59). Given the data published by UNOS and the study performed by Bernal et al., along with the fact that the average wait time for a liver to become available is 6 days, it is important to pursue the development of a device capable of either bridging FHF patients to transplantation or allowing the patient’s own liver to recover. Currently, the length of time that ECLP with a porcine liver can be administered is limited by the loss of erythrocytes. This erythrocyte loss is mediated by the Kupffer cells in the liver. This study has contributed to the field of xenotransplantation and more specifically to the field of extracorporeal porcine liver xenoperfusion by offering insights into the mechanisms by which porcine macrophages destroy human erythrocytes.

Data presented in chapter 3 of this thesis is focused on understanding the role of pSn in mediating the rejection of human erythrocytes by porcine macrophages. The expression of Sn is most often seen in resident macrophages, however, in diseases such as HIV and Systemic Sclerosis, Sn expression has been shown on circulating macrophages and monocytes, suggesting that it is involved in modulating immune responses to certain diseases (130, 131). The link between viral immunology and Sn in human pathology may not be surprising given the previously identified role of pSn in porcine macrophage
recognition of PRRSV (discussed in chapter 1). Data presented in chapter 3 of this thesis, as well as data published previously from our lab, suggests that pSn plays a role in porcine macrophage-mediated destruction of human erythrocytes (75).

This is not the first time that macrophages have been suggested to play a role in xenogeneic rejection. Wu et al. in 2000 and Goddard et al. in 2002 demonstrated that xenograft failure of whole organs was affiliated with macrophage infiltration into the graft (132, 133). Additionally, studies by Wu et al. showed that depletion of macrophages prior to xenografting increased graft survival, further suggesting the role of macrophages in xenograft rejection. Others have shown that porcine macrophages can facilitate the rejection of xenogeneic cells. Oldenborg et al. illustrated that porcine macrophages consume xenogeneic cells through a mechanism dependent on the CD47/SIRPα pathway (101). Data presented in chapter 3 of this thesis contributes to our understanding of the role macrophages play in mediating the rejection of xenogeneic cells and tissues. It is possible that pSn is involved in facilitating in the infiltration of macrophages into the host tissue during xenografting as Sn has previously been shown to act as an anchoring protein allowing macrophages to play a role in maintaining T and B-cell follicles in the lymph node as well as in T and B cell trafficking throughout the vasculature (92). Additionally, it would not be surprising if Sn was acting collaboratively with the CD47/SIRPα pathway to facilitate phagocytosis of xenogeneic cells. Based on the known functions of Sn described previously, it is possible that Sn is acting as the “anchor” allowing SIRPα to sample donor CD47 and to generate either an anti-phagocytic or permissive response.
The field of xenotransplantation and extracorporeal porcine liver xenoperfusion has benefited greatly from the use of NHP in preclinical studies. Studies utilizing NHP have shed light on the various rejection processes that have stood as barriers to the advancement of xenotransplantation and the use of porcine livers in ECLP in the clinic. The most deleterious forms of rejection which has been identified and continues to prevent successful outcomes in NHP models is thrombocytopenia (48). Initial studies of xenotransplantation utilizing NHP helped to identify the mechanisms of complement activation in xenotransplantation. These studies provided insight into the role of natural antibodies in triggering complement destruction, which cause severe vascular destruction in an acute setting and can lead to loss of the graft in only minutes when transplanting xenogeneic hearts and kidney, however livers do not appear to be as susceptible to complement mediated rejection. Furthermore, studies utilizing NHP have elucidated the mechanisms involved in the onset of severe thrombocytopenia, a form of rejection that occurs after only minutes of transplantation of the xenograft (48).

Experimentation to better understand the mechanism of xenogeneic erythrocyte rejection has primarily utilized ex vivo studies. To date, we are unaware of any study conducted with the aim of better understanding the loss of erythrocytes during ECLP with a porcine liver that utilized NHP. Based off of data discussed in Chapter 4 of this thesis, we would not expect NHP to serve as adequate models for studying the loss of human erythrocytes. As noted previously, the difference in SA expression between humans and NHP (Neu5Ac vs. Neu5Gc), as well as all other mammals, prohibit the ability of NHP to serve as adequate models for studying the loss of human erythrocytes.
Illustrating that NHP may fail to serve as an adequate model for studying erythrocyte loss during porcine ECLP has several potential implications on the field of xenotransplantation and medical research. First, we expect the data shown in this thesis illustrating the inadequacies of NHP will cause increased awareness of the potential pitfalls of using NHP in medical research as a final assessment before moving to clinical trial. This is not the first time that primates have been shown to be inadequate at predicting the future impact that a drug will have on patients. Several other examples where primates have failed to predict clinical outcomes in patients include: the development of the AIDS vaccine, AIDSVAX, and the development of Opren for arthritis, among others (134).

Data presented in Chapter 4 serves as a reminder to the medical community that, although NHP serve as adequate models for studying the predicted clinical outcomes of many new drugs, the unforeseen differences between NHP and humans sometimes prohibits the usefulness of primates in certain types of studies. Perhaps Dr. Alan Kirk, M.D., Ph.D., Editor-in-Chief of the American Journal of Transplantaiton, stated it best when he wrote:

“As for interpreting failure of a therapy in an animal model or failure of a model to predict efficacy in humans, it is typically not a failure of the model but a failure to recognize critical differences between the animal and humans that were not appreciated when the study was performed”(135)

Making known the potential pitfalls of NHP at serving as preclinical models in the development of novel therapies, has the potential to prevent the loss of time and money caused by faulty data obtained from a faulty model. I expect that Chapter 4 of this thesis will have a similar effect on the field of ECLP with a porcine liver. Given the data
highlighting potential pitfalls in using NHP to study porcine liver ECLP, perhaps the FDA will consider alternative strategies that could lead to approval of a clinical trial.

Various other groups have demonstrated efficacy of BDRS in preclinical medical research. However, to our knowledge no one has assessed whether the public is in support of such research practices. Chapter 5 of this thesis presents data summarizing the public’s opinion regarding brain death and the use of BDRS in medical research. The findings showed more enthusiastic support than we had expected. First, we found that the majority of the general public agrees with the law defining brain death as death. Second, we observed that the majority of the general public supports the use of BDRS in medical research. Finally, we provide evidence that education on brain death and the issues involved in medical research have a positive effect on the support of using BDRS in medical research. Taken together, this data suggests that medical research using BDRS is supported by the general public and should be considered as an alternative research model when it is expected that NHP will fail to recapitulate clinical outcomes.

Data in Chapter 5 have the potential to impact the porcine liver xenoperfusion community in that it sheds light on an alternative preclinical model potentially capable of predicting clinical outcomes. This is important as it will help to better elucidate the unforeseen obstacles that could manifest themselves when ECLP with a porcine liver is introduced into the clinic. It would be inappropriate to proceed to clinic trials and experience failure because a “successful” preclinical NHP study failed to elicit the “anti-host” response seen towards human blood.

The implication of the data presented in Chapter 5 of this thesis have the potential of being more far reaching than porcine liver xenoperfusion alone. As noted previously,
prior medical research has been conducted utilizing BDRS (117). However, given the potential ethical dilemma of such practices, the development of practices utilizing BDRS in medical research has been stagnated. Having demonstrated evidence that the public supports this practice, the study in Chapter 5 may provide researchers and clinicians the confidence to consider utilizing BRDS as preclinical models when NHP and other preclinical models fail to predict outcomes seen in humans. The use of alternative preclinical models could ultimately decrease the time and costs associated with drug development.
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Appendix A
Medical Research Questionnaire

Thank you for volunteering to complete this survey. Your participation is optional and you may choose to withdraw at any time. All results will remain anonymous and cannot be linked directly to you.

This survey is intended to ask your opinion regarding the use of brain dead humans in medical research. Brain dead humans are legally defined as those who are irreversibly unconscious with a complete loss of brain function. However, if the patient or the patient’s family expresses interest in organ donation, the heart and other organs are kept alive with pumps and breathing machines. Only those brain dead patients who have given consent to organ donation have their organs kept alive by artificial life support until organ donation is possible. All other brain dead patients have life support removed shortly after the declaration of brain death.

Section 1

Unfortunately not all brain dead patients who have agreed to organ donation are healthy enough to donate their organs. A new idea is to have these brain dead patients serve as research subjects for experiments that may eventually lead to improved methods for treating living patients. The goal of this research is to assess public opinion of research using brain dead patients.

Please check the response you feel best reflects your opinion:

1. Do you agree with the law stating that a diagnosis of brain death accurately defines death?
   - Yes ☐
   - No ☐
   - No Opinion ☐

2. Is it appropriate to conduct medical research on these brain dead patients (who have previously consented to the use of their organs)?
   - Yes ☐
   - No ☐
   - No Opinion ☐

Please explain: ____________________________________________________________
3. In your opinion, how long is it appropriate to use the bodies of brain dead subjects for research purposes?

For a period of hours:    Yes ☐  No ☐  No Opinion ☐
For a period of days:    Yes ☐  No ☐  No Opinion ☐
For a period of weeks:   Yes ☐  No ☐  No Opinion ☐
For a period of months:  Yes ☐  No ☐  No Opinion ☐
For a period of years:   Yes ☐  No ☐  No Opinion ☐
For no period of time:   Yes ☐  No ☐  No Opinion ☐

Section 2
Please check the response you feel best reflects your opinion:

1. It is appropriate to use the bodies of brain dead subjects in experiments that determine the effects of substances we are commonly exposed to, such as the sun (UV rays), skin creams, and deodorants. This would not require entering the body or puncturing the skin

   Agree ☐    Disagree ☐    No opinion ☐

2. It is appropriate to use the bodies of brain dead subjects in experiments to help researchers develop better treatments. This would involve entering the body or puncturing the skin to inject drugs, bacteria, cancer cells, or viruses

   Agree ☐    Disagree ☐    No opinion ☐

3. It is appropriate to use the bodies of brain dead subjects in experiments to help researchers develop alternative transplant methods. This would involve transplanting animal organs into the human body

   Agree ☐    Disagree ☐    No opinion ☐
4. It is appropriate to use the bodies of brain dead subjects in experiments to help researchers learn about arm, leg, and face transplants. This would involve physical alteration of body parts

Agree ☐  Disagree ☐  No opinion ☐

Section 3
1. Age: (in years) _____________________________________________

2. Race/Ethnicity: (please check one)
   ☐ African American, Black ☐ Native Hawaiian/Pacific islander
   ☐ Asian ☐ Multiracial
   ☐ Caucasian/White ☐ Declined
   ☐ Hispanic/Latino ☐ Native American

3. Sex: ☐ Male ☐ Female

4. Occupation: ______________________

5. Education:
   ☐ No high school diploma ☐ College degree
   ☐ High school diploma ☐ Graduate school degree
   ☐ Some college ☐ Technical school certificate

6. Marital Status:
   ☐ Single (never married) ☐ Separated
   ☐ Married ☐ Widowed
   ☐ Divorced ☐ With long-term partner

7. Religion:
   ☐ Christian ☐ Judaism
   ☐ Islam ☐ Other (specify) ____________
   ☐ Hinduism ☐ No Religious Affiliation
8. Combined yearly **household income**:

- □ less than or equal to 20,000
- □ 20,001 – 40,000
- □ 40,001 – 60,000
- □ More than 60,000

9. **Number of children**:

- □ 0
- □ 1 or 2
- □ 3
- □ 4
- □ 5
- □ 6
- □ 7 or more

10. **Are you an organ donor?**

- Yes □
- No □

11. If yes, would you be willing to contribute to medical research upon brain death if your organs were unsuitable for transplantation?

- Yes □
- No □
- Not an organ donor □

Thank you for completing this survey. The results will be published and analyzed by ethicists, physicians, scientists, and other professionals before research using previously consented brain dead patients is further considered.