Effects Of Intraperitoneal Bilirubin Administration On Infarct Area And Left Ventricular Function In A Rat Model Of Acute Coronary Occlusion

A Thesis

Presented in Partial Fulfillment of the Requirements for The Degree Master of Science in the Graduate School of The Ohio State University

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The Ohio State University
2011

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Abstract

Bilirubin was considered to be a toxin that accumulates after catabolism of heme by the enzyme heme oxygenase. However, a mounting body of evidence suggests that bilirubin, at physiological (non-toxic) doses, is a powerful antioxidant and anti-atherosclerotic agent. Recent clinical studies have shown that human beings with mild hyperbilirubinemia (Gilbert Syndrome) are protected against coronary heart disease. The purpose of this study was to investigate whether administration of exogenous bilirubin to normal rats would convey similar protective effects in an experimental model of coronary ischemia. Our hypothesis was that bilirubin administration (20uM/kg, IP, 1 hour before injury) would decrease infarct area and preserve left ventricular function when compared to non-treated rats. Coronary ischemia was induced by temporary (30 min) ligation of the left anterior descending coronary artery in control rats (n=5) and in bilirubin treated rats (n=5), followed by a 1 hour period of reperfusion. Left ventricular function was estimated non-invasively using echocardiographic measurements of fractional shortening and percent area shortening. Effects of anesthesia on cardiac function were controlled by using a sham group (n=5). There was a significant reduction of infarct size in the bilirubin treated group compared to the non-treated group (p<0.0067). Left ventricular systolic function decreased in both experimental groups after ischemia and reperfusion, although bilirubin seemingly provided a protective effect on fractional shortening during the period of ischemia (p=0.034). Based on these results, bilirubin supplementation appears to provide significant myocardial protection following
ischemia in this rodent model. However, protective effects on left ventricular function were noted only during the period of ischemia.
Dedication

Dedicated to my parents Gadi and Lia, and my beautiful wife Shir-Raz and my beautiful children Shai and Yonatan, who supported me throughout the years and during my residency
Acknowledgments

I wish to thank my advisor, Chris Adin, for keeping me focused and motivated during the process of completing this study. I thank my other committee members, John Bonagura and Robert Hamlin, for intellectual support and helping with this project.
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Heme oxygenase

Heme protein is degraded by the Heme oxygenase (HO), an enzyme that produces equimolar quantities of carbon monoxide (CO), biliverdin (BV) and iron (Fe$^{2+}$). Biliverdin is further degraded to Bilirubin (Br) by the enzymatic activity of bilirubin reductase. Currently, three forms of HO are recognized. HO-1 (known as heat shock protein 32), the inducible enzyme, is expressed in many cell types as a protective response to cell insult/injury. HO-2 is the constitutive expressed enzyme that regulates normal cell function and is present in high quantities in the testis and brain. The more recently identified HO-3, is similar to HO-2 but serves as a less efficient heme catalyst. The effects of HO-1 appear to be mediated in large part by the actions of its reaction byproducts, CO, iron and bilirubin. These metabolites have been previously shown to protect against oxidative stress, and to demonstrate effects that are anti-inflammatory, anti-apoptotic, and anti-atherogenic. Up regulating HO-1 has been shown to convey cytoprotection whereas deficiencies of HO cause harm. Numerous studies have supported the multifunctional roles of HO-1 in the vascular system, including regulation of vascular tone, suppression of smooth muscle proliferation, prevention of endothelial apoptosis, and stimulation of angiogenesis. Increasing tissue activity of HO-1, either by transgenic methods or by treatment with HO-1 inducers, has protected rodents from atherogenesis, thrombosis, renal, hepatic, or pulmonary injury, and ischemia-reperfusion injury; in many of these studies, concurrent administration of HO-1 inhibitors abolished this protection. Conversely, HO-
1 knockout mice (HO-1<sup>−/−</sup>) are more prone to atherogenesis and vein graft stenosis and calcification. Recently it was found that HO-1 overexpression protects the myocardium from ischemia and reperfusion injury. Masini et al showed that pretreatment of animals with hemin, an HO-I inducer, provides protection against ischemia-reperfusion damage. This was shown by the decrease both of the infarct area and of the increase of the markers of oxidative stress (MDA and tissue calcium). The effects were completely antagonized by ZnPP-IX (zinc protoporphyrin, an HO-1 inhibitor). Vulapalli et al tested whether HO-1 prevents cardiomyocyte apoptosis and cardiac dysfunction after ischemia-reperfusion (IR). In their study, the transgenic mice overexpressing HO-1 in the heart, showed an enhanced functional recovery during reperfusion after ischemia compared with nontransgenic controls. Other studies from several laboratories have demonstrated that HO-1 also exerts potent anti-atherogenic effects. Moreover, the inhibitory effects of CO and bilirubin on monocyte transmigration through endothelium, smooth muscle cell proliferation and inflammation appear to contribute to various degrees to the protective effect of HO-1 in atherosclerosis. Likewise, HO-1 overexpression in arterial walls reduces neointima formation subsequent to vascular injury through the anti-proliferative effects of CO and bilirubin and the anti-thrombotic and anti-inflammatory effects of CO. Carbon monoxide (CO), which was until recently regarded as a toxic gas, is now considered to have beneficial biological effects. Carbon monoxide acts as a powerful vasodilator, similar to NO. This molecule exerts its vasodilatory effects through activation of guanylate cyclase (GC), an enzyme that generates the second messenger cyclic guanosine monophosphate (cGMP) in target tissues. Carbon monoxide also exerts its vasodilatory effects by direct activation of the potassium channels in vascular smooth muscles. Similar to upregulation of HO-1, CO has been shown to inhibit platelet aggregation and proliferation of vascular smooth muscle cells, inhibit apoptosis, and stimulates angiogenesis. Carbon monoxide has an affinity for hemoglobin that is 90 times greater than that of oxygen. Elevated levels of carbon monoxide cause displacement of oxygen
from hemoglobin and development of tissue hypoxia that can result in death. Administration of
gaseous carbon monoxide would also pose a risk to health care providers that may experience
prolonged or repeated exposure. These factors, as well as the psychological difficulty associated
with administration of a known toxin that can be lethal at higher doses, have limited the clinical
application of carbon monoxide as a therapeutic agent in treatment of human cardiovascular
disease.

**Bilirubin**

**Introduction:**

Bilirubin is a natural anti-oxidant that results from the catabolism of the heme. Not only it is a
major constituent of hemoglobin, but also serves as the backbone of numerous intracellular
enzymes (eg. cytochromes). It is important to note that, in the process of heme degradation, a
relatively non-toxic and water-soluble molecule (biliverdin) is converted into a toxic, non water-
soluble molecule, bilirubin, a process that seems counterproductive on initial consideration.
However, a mounting body of evidence suggests that bilirubin, at a physiological (non-toxic)
doses, serves many beneficial purposes. Bilirubin has been shown to have powerful antioxidant,
anti-inflammatory effects in various models of tissue injury. In particular, bilirubin appears to
have a major role in cardiovascular health, inhibiting vascular smooth muscle proliferation and
decreasing the risk of atherosclerosis in several animal models. Most importantly, serum bilirubin
levels have been shown to be inversely proportional to risk of cardiovascular disease in human
beings.\(^1,9,19,20,22,26,32-42\)

**Protective Effects:**

A study by Stocker et al first introduced the anti-oxidative properties of bilirubin.\(^22\) This study has
found that at micromolar concentrations in vitro, bilirubin efficiently scavenges peroxyl radicals.
Moreover, the antioxidant activity of bilirubin increases as the experimental concentration of oxygen is decreased from 20% (normal air) to 2% (physiological concentration). At low oxygen concentrations, bilirubin was found to suppress oxidation more than alpha-tocopherol, which was previously regarded as the most powerful antioxidant in the serum. Maines and colleagues have shown that reduction of oxidative stress following bilateral renal ischemia was proportional to the rate of bilirubin formation. Other studies have demonstrated similar anti-oxidant properties in injured neurons and cardiomyocytes. Accumulating evidence suggests a vital role for bilirubin in both cell growth and cell death, with a special role in the regulation of apoptosis. Recent experimental studies also provided vital information on the protective effect of bilirubin on vascular smooth muscle cell proliferation (VSMC), and association of coronary artery disease. VSMC have been used in models of vascular injury and serve as a target for therapy for atherosclerosis related diseases. Several authors suggested that abnormal proliferation of the VSMCs plays an important role in the very early stages of atherogenesis, leading to atherosclerosis and coronary heart disease, the number one cause of morbidity and mortality in the western world. A similar pathology appears to produce restenosis after vessel manipulation, stenting, bypass grafting, as well as in chronic allograft vasculopathy, where extensive proliferation of the VSMCs in the neointima causes narrowing of the vessels and leads to organ ischemia and subsequently to acute (e.g., stroke, myocardial infarction) or chronic (e.g., chronic allograft rejection) deterioration of organ function. In an experimental in vivo model it was shown that neointimal proliferation seen two weeks after angioplasty (balloon injury) in wild type rats was virtually absent in the hyperbilirubinemic rats. Furthermore, one-hour local pretreatment of the carotid artery with biliverdin (a precursor of bilirubin), significantly reduced neointima formation following balloon injury in rats.
Cardiovascular disease in humans:

According to the American Heart Association, coronary heart disease caused 425,425 deaths in 2006 and is the single leading cause of death in America today. This year an estimated 1.26 million Americans will have a new or recurrent coronary attack. In 2006, coronary heart disease death rates per 100,000 people were 176.3 for white males and 206.4 for black males; and 101.5 for white females and 130.0 for black females. The primary cause of coronary heart disease is atherosclerosis, an inflammatory disease process in which lipid deposition in arterial walls is central to the development of lesion formation. This process involves the uptake of oxidized low-density lipoprotein (oxLDL), a lipoprotein that is highly susceptible to oxidation by macrophages. As atherosclerotic lesions progress, migration and proliferation of smooth muscle cells and deposition of fibrous tissue lead to an advanced, complicated lesion.48,49 Furthermore, the development of coronary artery disease is also associated with the formation of oxygen and peroxyl radicals.50 Numerous studies have supported the multifunctional roles of HO-1 in the vascular system, including regulation of vascular tone, inhibition of smooth muscle proliferation, inhibition of endothelial apoptosis, and angiogenesis.25,51,52 The effects of HO-1 appear to be mediated in large part by the actions of its reaction byproducts, CO and bilirubin. Different forms of bilirubin (albumin-bound, conjugated, and free) were found to have protective effects against peroxyl radicals involved in ischemia-reperfusion injuries, and even protect against peroxidation of low-density lipoproteins.21,22,53 Mayer et al has found that atherosclerosis and coronary artery disease development in humans will decrease with mild elevations of bilirubin.20 In contrast to carbon monoxide (CO), which exerts its anti-proliferative effect on smooth muscle cells through a cGMP pathway, bilirubin appears to exert its anti-proliferative effects through its antioxidant properties.2,39 Ghem and colleagues compared 100 patients with coronary heart disease and a control group of 100 patients with normal coronaries. In their study they have found a reduced serum levels of bilirubin to be associated with a higher prevalence of coronary artery disease,
suggesting that bilirubin can serve as a new marker for risk of coronary artery disease.\textsuperscript{35} A report by Schwertner and colleagues also indicated that a 50\% decrease in total bilirubin was associated with a 47\% increase in the chance of having severe coronary artery disease. This study concluded that there is a significant inverse correlation between total bilirubin plasma concentration and the prevalence of coronary artery disease.\textsuperscript{19} Low serum bilirubin concentration was also proposed to be useful as a provisional risk factor of coronary artery calcification based on the finding that an increase of 1\(\mu\)mol/L in serum bilirubin concentration was associated with 14\% decrease in the coronary artery calcification scores.\textsuperscript{54} Another prospective study by Breimer et al. found that low concentration of serum bilirubin was associated with increased risk of ischemic heart disease in middle-aged British men.\textsuperscript{34} Another plausible mechanism of bilirubin action in the prevention of atherosclerosis is related to the immune reaction and inflammatory processes. Upregulation HO-1 leads to faster resolution of inflammation and bilirubin has been shown to inhibit compliment-dependent reactions and to suppress the release of tumor necrosis factor-\(\alpha\) in vitro.\textsuperscript{55,56}

Gilbert Syndrome is an innocuous recessive genetic variant in human beings that causes decreased expression of the enzyme UDP-glucuronosyltransferase type A1A(~30\% of normal). (UGT1A1), the enzyme almost solely responsible for bilirubin conjugation; the mutation is in the promoter region, leading to decreased expression of an enzyme with normal structure and specific activity. As a result, serum unconjugated bilirubin tends to be elevated by 2-3-fold; serum bilirubin is typically about 30\(\mu\)M in Gilbert subjects. It was found that in Gilbert Syndrome individuals the prevalence of ischemic heart disease is 2\% compared to 12\% of the general population.\textsuperscript{42,57} In the Framingham Offspring study involving 4276 men and women, higher serum bilirubin concentrations were found to be associated with a lower risk of myocardial infarction, coronary death, and any cardiovascular events in men.\textsuperscript{58}
**Chemistry:**

Consideration of bilirubin as a pharmacologic agent reveals many challenges that would need to be overcome prior to clinical trials. Bilirubin is a breakdown product of heme-containing proteins and mainly originates from hemoglobin in aging red blood cells. HO1 (Heme Oxygenase 1) converts heme to biliverdin, which is then reduced by biliverdin reductase to bilirubin. Bilirubin is non-water soluble, but is highly protein bound.\(^\text{42}\) In adults, 90% of bilirubin is bound to albumin, with normal plasma concentration of 5-15\(\mu\text{mol/L}\). The albumin-bilirubin complex not only improves its solubility but also reduce the potential toxic effects of this molecule when concentrations are greater then 50\(\mu\text{M}\).\(^\text{59}\) Once bound to albumin, it is then taken up across the basolateral membrane of hepatocytes, and conjugated in the hepatocyte by UGT1A1 (bilirubin UDP-glucuronosyl transferase 1 family, polypeptide Al; UGT1A1). Bilirubin conjugates are then actively secreted into the canaliculi.\(^\text{42}\) The bilirubin molecule exists in three isomers with XIII\(\alpha\) being the natural structure formed from heme catabolism. Its hydrophobic properties, as well as the molecule’s susceptibility to oxidation, extreme changes in pH (either low or high), and potential for photosensitization indicate a number of special considerations in using bilirubin as a therapeutic agent in the clinical arena. Bilirubin solubility at physiological pH (7.4) is low. With increase in pH values, bilirubin solubility increases from approximately 70\(\text{nM}\) (natural pH) to over 60\(\text{mM}\) (pH of >9.5).\(^\text{42,59}\) At low pH, bilirubin dissociates readily from albumin, reaching concentrations >50\(\mu\text{mol/L}\) and increasing the potential for cell toxicity effects including cell lysis or disruption of mitochondrial function.\(^\text{33,59}\) An experimental model of exogenous administration of bilirubin used a basic solution (pH 8) in order to overcome difficulties in dissolving bilirubin in aqueous solutions. This protocol was successful in reaching a target serum bilirubin level of 10-80\(\mu\text{mol/L}\). Addition of albumin or bovine alpha-fetoprotein can overcome bilirubin’s poor solubility in physiologic solutions through protein binding.\(^\text{61,62}\) Commercially available bilirubin
is typically derived from animal sources (swine) and not in a pure form that would be appropriate for marketing or administration in human beings. Another potential consideration when administering exogenous bilirubin is related to the method of delivery and the short half-life ($t_{1/2}$) of this molecule. In a rodent model, bolus administration caused a rapid increase in serum bilirubin levels, but bilirubin was eliminated completely within 3 hours, suggesting that a continuous intravenous infusion may be required to maintain concentrations in the therapeutic range. These observations enhance the challenges when dealing with exogenous administrations of bilirubin. In addition to the previously mentioned challenges, conditions such as hypoxia (which can change membrane integrity), hemolysis, or even drugs that compete with protein binding can modify the relative proportion of albumin-bound versus free diffusible bilirubin. Whether these conditions can affect the cardioprotective potential of bilirubin remains unknown.

**Models of hyperbilirubinemia:**

Genetic polymorphisms affecting expression or activity of UDPGT are described in both human beings and in rats. As previously described, Gilberts syndrome is a common genetic polymorphism that has a 6% prevalence in the human population. Gilberts syndrome involves an alteration in the promoter region for UGT1A1, decreasing expression of this gene by approximately 70%. The glucuronidating enzyme UDP-GT is fully functional in individuals with Gilberts syndrome, but partial expression of the enzyme results in mild increases in bilirubin concentrations (20-100 µmol/l) that protect against cardiovascular diseases, but have no described pathological effects. A more severe hyperbilirubinemia results from mutations within the actual gene for UDPGT - a disease termed Crigler-Najjar syndrome. Patients with Crigler Najjar Syndrome suffer from a variety of complications resulting from higher (toxic) concentrations of unconjugated bilirubin, including choleliths and pigment related renal injury, among others. There is no rat model for Gilbert’s syndrome and creation of a transgenic mouse
with alterations in the promoter region would be difficult to perform when compared to
generating knockout strains. The Gunn rat does provide an existing model of hyperbilirubinemia,
although the phenotype observed in this strain is more similar to Criggler Najjar syndrome due to
a single guanosine (G) base deletion within the UGT1A1 gene. The defect results in complete
absence of enzyme activity, and severe hyperbilirubinemia. Although rats do develop signs of
bilirubin toxicity as they age, younger Gunn rats have been used in several models of organ or
tissue stress to evaluate the protective effects of hyperbilirubinemia. Experiments using Gunn rats
either exposed to hypoxia, vascular balloon injury, or myocardial ischemia have found decrease
in serum lipid peroxidation and prevension of ischemic heart disease.\textsuperscript{57,70-72}

\textbf{Other therapeutic strategies:}

Genetic manipulation (knock down) or pharmacological manipulation of UDPGTA1A expression
may provide one means of increasing serum bilirubin concentrations in animal models. Other
genes involved in bilirubin metabolism and red blood cell life span might also play an important
rule in gene therapy in the future. Uptake of bilirubin into the hepatocytes, is mediated largely by
SLCO1B1 (solute carrier organic anion transporter family, member 1B1), a major transport
protein localized on the basolateral membrane of hepatocytes. Many variants have been
identified, contributing to reduced uptake of bilirubin.\textsuperscript{73} Glucose-6-phosphate dehydrogenase
(G6PD), provides the NADPH essential for cells including erythrocytes. With its deficiency, the
life span of red blood cells may be shortened, and low-grade hemolysis occurs, leading to
increased bilirubin production. Neonatal hyperbilirubinemia is a result of G6PD deficiency,
which can result same condition in adults.\textsuperscript{74-77} Drugs that can inhibit UGT1A1 activity or
transport could be effective in increasing serum and tissue bilirubin concentrations. Among the
investigated drugs, probenecid, a UGT1A1 activity inhibitor and rifampicin, an SLCO1B1
transport activity inhibitor have been reported.\textsuperscript{66,78} However, inhibition of SLCO1B1 or UGT1A1
can negatively impact the conjugation and clearance of other drugs and toxins, which can either impractical or unsafe.

**Conclusion:**

The catabolism of heme, generating bilirubin, is mediated by heme oxygenase 1 (HO-1). This enzyme and its downstream products have been demonstrated to provide important antioxidant protection. Epidemiological studies have also demonstrated the relationship between serum bilirubin levels and atherogenic disease in humans, with serum bilirubin concentrations being inversely proportional to the rate of vascular diseases. Furthermore, upregulation of HO-1 was found to ameliorate post-ischemic myocardial dysfunction in isolated rat hearts.\(^1\) Despite this background, no investigators have studied the protective effects of direct supplementation of bilirubin in a model of cardiac ischemia reperfusion injury - a common cause of death in humans undergoing interventional procedures for coronary ischemia. Our goal in the following study was to investigate the protective effects of exogenous bilirubin in a rat model of acute myocardial infarction and reperfusion injury, simulating the clinical scenario of myocardial infarction and reperfusion that occurs in human beings. Once the mechanism of protection is identified, pharmacological methods and gene therapy can be exploited to induce a reversible hyperbilirubinemic state.
Introduction

Ischemic heart disease remains the most common cause of mortality in Western countries and is the predicted leading source of mortality worldwide by 2020. Blockage of coronary blood flow leads to myocardial ischemia. Persistent ischemia causes myocardial infarctions, resulting in profound myocyte death, irreversible myocardial damage and a permanent loss of contractile mass. Timely reperfusion of ischemic heart is the only way to preserve cardiac cell viability. However, reperfusion can trigger further damage to the myocardium termed ischemia/reperfusion (IRI). Reperfusion of ischemic tissues introduces oxygen to an environment containing free radicals, producing reactive oxygen species—that are extremely injurious to tissues. Several studies have shown that increased expression of myocardial stress proteins and/or antioxidant enzymes before an injury (a process called pre-conditioning) protects against postischemic injury. Heme oxygenase (HO)-1 appears to be one of the more important mediators involved in organ preconditioning. HO-1 is a stress response and cytoprotective protein, also known as hsp32, protects cells from death due to pathophysiological stress by degrading the pro-oxidant heme and generating the antioxidant bilirubin. Recent evidence has led to strong interest in manipulation of the HO-1 pathway as a means of cardioprotection. To test whether HO-1 protects against oxidative injury in the heart, a cardiac-specific transgenic mice overexpressing different levels of HO-1 was generated. Using a Langendorff preparation, hearts from transgenic mice were shown to have improved recovery of contractile performance during reperfusion after ischemia in an HO-1 dose-dependent manner. In vivo, myocardial ischemia and reperfusion experiments
showed that infarct size was only 14.7% of the area at risk in transgenic mice compared with 56.5% in wild-type mice. For the potential clinical application of HO-1 in the heart, Melo and colleagues studied gene transfer of human HO-1 with adeno-associated virus prior to ischemia/reperfusion injury. Gene transfer eight weeks prior to acute coronary artery ligation and release, resulted in sustained myocardial protection from ischemia/reperfusion injury in rats. Gene transfer also produced a marked reduction in infarct size and oxidative damage in the hearts. This study suggested a "pre-event" gene transfer approach to provide sustained tissue protection from future repeated episodes of injury, and a potential preventative therapy for patients at risk for developing coronary ischemic events. Although these data would suggest that HO-1 upregulation is strongly beneficial in the scenario of cardiac ischemia, the techniques required for delivery and regulation of cardiac gene therapy will likely delay the ability to utilize this therapy in human patients with clinical disease. A number of previously studies have demonstrated that heme end products (Br, and CO) are responsible for much of the biological activity of HO-1, including anti-inflammatory, antiapoptotic, antiproliferative and antioxidant effects. In particular, the production of bilirubin via the HO-1 system has been shown to be an important protective factor in cardiovascular disease. Bilirubin, at physiological levels, can provide cardioprotection by suppressing oxidation of lipid membranes and preventing endothelial cell death caused by hydrogen peroxide. Interestingly, data from the Framingham offspring study has solidified the relationship between serum bilirubin concentration and risk of coronary artery disease, vascular events and myocardial infarction, showing that serum bilirubin concentration is inversely proportional to cardiovascular risk. In addition, humans with Gilbert Syndrome, a genetic polymorphism that results in mild (30μM/L) hyperbilirubinemia, are significantly protected against coronary heart disease with the incidence of MI being reduced from 12% in the general population to 2% in individuals with Gilbert Syndrome. Although this clinical evidence supports the importance of bilirubin in preventing chronic vascular disease and
atherosclerosis, there have been no investigations of the protective effects of bilirubin in acute myocardial ischemia and reperfusion. Based on previous experience in models of acute organ ischemia, we suspected that administration of exogenous bilirubin therapy may provide a means of reducing tissue injury in the milieu of acute coronary IRI that occurs during clinical application of interventional cardiology (stenting and balloon angioplasty) or administration of thrombolytic therapies to humans with active myocardial infarction. We planned to perform initial investigations in a rat model of left anterior descending coronary artery occlusion (LAD) and reperfusion. Our central hypothesis was that bilirubin administration (20uM/kg, IP, 1 hour before injury) would decrease infarct area and preserve left ventricular function when compared to non-treated rats. Our eventual goal is to apply supplemental bilirubin therapy in human beings at risk for coronary heart disease.
CHAPTER 3: MATERIALS AND METHODS

Study Design

We proposed a single prospective, controlled, blinded experiment with a total of 3 experimental groups and one training group. The training group was used to practice the model, including baseline echocardiogram, occlusion, and repeated echocardiogram at the end of occlusion and reperfusion period, sample collection, staining and tissue storage.

Animals

Male Sprague Dawley rats, weighing 220-300 g, were purchased from Harlan Sprague Dawley (Indianapolis, IN). Animals were maintained in a temperature-controlled room and were fed with a standard diet and water ad libitum. All procedures were approved by The Ohio State University Institutional Animal Care and Use Committee and were performed in accordance with the institute for lab Animal Research Guide for the Care and use of Laboratory Animals.

Bilirubin treatment

BR solutions were prepared to a final concentration of 20µM/kg (0.02µmol/g). To make the final concentration, bilirubin was dissolved in 0.2N NaOH and adjusted to pH to 7.4 with HCl. Aliquots of 0.8ml per vial were stored at -80°C. The solutions were prepared before each experiment, and were protected from light at all times by a covering of aluminum foil. Treated animals received intraperitoneal injection of Bilirubin (Br) 1-hour prior to occluding the left anterior descending coronary artery.
**Surgical Procedure**

Each rat’s weight was recorded before the procedure. Tank induction was performed using 5% inhalant isoflurane in 100% oxygen (2-3 minutes). After induction, an injection of 0.4mL Ketamine (50mg/mL; 10mg/kg) was administered intra-peritoneally (IP). Ketamine was administered to maintain anesthesia during intubation and to decrease the subsequent requirement for isoflurane. Once induced, a facemask was then used to deliver 100% O₂ and isoflurane (1-2%) until endotracheal intubation was performed. A ventral midline cervical approach was made and a cut down to the trachea was performed to confirm and visualize appropriate insertion of the endotracheal tube (16 gauge intravenous catheter). Each catheter was secured to the skin using 3-0 silk suture, to prevent tube pullout during the surgical procedure. After intubation, anesthesia was maintained with positive pressure ventilation (Harvard Rodent Ventilator, Southnatick, Mass) using isoflurane (1-2.0%) in 100% oxygen (0.2L/min at a rate of 95 cycles/min and tidal volume of 2-2.5mL. Gas was scavenged through a scavenging system (f/air™, A.M. Bickford INC, Walles Center, NY). Each rat was then positioned in right lateral recumbency. A heat lamp was used to monitor and maintain body temperature at 37± 1˚, using a rectal thermometer (Physitemp™, Clifton, NJ). Following a left 4th intercostal thoracotomy, the heart was isolated and pericardiectomy was performed to facilitate anatomical location of the left anterior descending coronary artery. The left auricle was slightly retracted exposing the entire left main coronary artery system. Coronary ischemia was induced (group 1, 2) and maintained for 30 minutes by encircling the left anterior descending (LAD) branch of the left coronary artery, 1-3mm from tip of the normally positioned left auricle, with 6-0 silk suture with a tapered needle (Sofasilk™, Convidien, Norwalk, CT). The suture was tightened over a 2mm section of PE-50 tubing to prevent vascular injury and to permit the release of the ligature for later reperfusion. After release of the suture, reperfusion was allowed to occur for 60 minutes. Reperfusion of the previously occluded coronary artery was confirmed by visual inspection. Left ventricular function
was assessed with echocardiogram at three different time points (after isolating the heart but prior to ligation, at the end of ischemic period, and at the end of reperfusion period). Before termination of the experiment, the LAD was re-occluded and 1mL of 1% Evans blue solution (Sigma-Aldrich, St. Loius, MO) was perfused in a retrograde manner with a 22-gauge needle inserted into the ascending aorta (group 1, 2) to identify the area of risk. The heart was then removed for further analysis, which included infarct size measurement. The liver, kidney, lung, spleen were harvested for future analysis.

**Experimental groups**

Male Sprague Dawley rats were randomly assigned into one of three different treatment protocol groups. Group 1 was administered vehicle (PBS; n=5), Group 2 was administered Bilirubin (20µM/kg; IP; n=5), given 1 hour before occlusion. Effects of anesthesia on cardiac function were controlled for by using a sham group (n=5).

**Tissue sampling**

Once the heart was removed, it was placed in a rodent slicer matrix (Zivic instruments, Pittsburgh, PA) and cooled in a -80°C freezer for 10-15 minutes to facilitate slicing. Once the heart was frozen, 5-6 heart sections of 2 mm each were made. Sections of the ventricles from the level of the ligature to the apex were then incubated in 2% triphenyltetrazolium chloride (TTC; Sigma-Aldrich, St. Louis, MO; TTC) solution in a 37°C bath for 20 minutes to visualize the unstained infarcted region. After TTC staining, viable myocardium stains brick red and the infarct appears pale white (Figure 1). This staining technique relies on the ability of dehydrogenase enzymes in viable myocytes to convert the stain to brick red color. Heart slices were placed in buffered formalin and infarct size analysis of the 3rd most distal heart slice (from apex-to base) was performed after 24 hours. Other tissues (Liver, Spleen, Kidney, Lungs) were harvested for
Determination of Infarct Area

The apical side of each 3rd heart slice (from apex to base) was imaged. The area of infarction and left ventricle (LV) areas at risk were determined by computerized planimetry using an image analysis software program (Image/J, National Institutes of Health). After TTC staining, viable myocardium stained brick red (area at risk) and the infarct myocardium appeared pale white. Measurements were repeated 5 times for each heart and the average of these 5 measurements was used to calculate area at risk and infarct area. The infarct area was expressed in percentage as the ratio of infarct area divided by LV area of risk. The individual conducting the measurements (RBA) was blinded to the experimental group.

Echocardiogram analysis

The left hemithorax was shaved, and a left 4th intercostal thoracotomy was performed. The heart was isolated and a pericardectomy was performed. Before each echocardiographic measurement, saline was infused into the left hemithorax to minimize any potential air artifacts and the hemithorax was temporarily closed using 4-0 Nylon suture (Ethicon, Johnson and Johnson, New Brunswick, New Jersey). Heart function was assessed by echocardiography at three different time points (prior to ischemia, at the end of ischemia (30 minutes ligation), and at the end of reperfusion period (one hour post ligation) using echocardiographic system (Vivid7™, GE Medical Systems, Milwaukee, WI, USA) equipped with a 13-MHz linear phase arrayed transducer (GE i13L) and proprietary software for rodent imaging. Acquisition frame rates were 159/sec. The heart was first imaged with 2D echocardiography using the short axis-imaging plane at the level of the papillary muscles. The M-mode cursor was then placed perpendicular to the ventricular septum and LV posterior wall to acquire M-mode imaging of the LV. Images obtained
during the experimental portion of the study were stored in digital format on a magnetic optical
disk for subsequent review and analysis. Measurements of the LV internal diameter at end-
diastole (LVIDd) were taken at the time of maximal LV diastolic dimension, whereas
measurements of the LV internal diameter at end-systole (LVIDs) were taken at the time of the
apogee of the systolic excursion of the posterior wall (Figure 4). Five different measurements
were taken and the average number was used as a single data point for statistical analysis. Left
ventricular fraction shortening (FS) was calculated by the formula: FS (%) = (LVIDd – LVIDs / 
LVIDd) x 100. Fractional shortening area was calculated by tracing the endocardial border at the
maximal LV diastolic dimension, and minimal LV systolic dimension (Figure 5). Left Area
shortening (AS) was calculated by the formula AS(%) = (LVAd-LVAs/LVAd) x 100.

**Statistical analysis**

Statistical calculations were performed using a computer software program (Statview, SAS
Institute, Inc, Cary, NC). Data were tested for distribution and assumptions for analysis of
variance. Data for each time period and treatment group were summarized and are reported as
means ± SD. Comparisons of fractional shortening (FS %), area shortening (AS%) over the 3
different time periods were compared, as well as differences between treatment groups, using a
two-way analysis of variance for repeated measures with time as the within-groups and treatment
as the between-groups factors. Comparison of infarct size % was made using a student t-test.
A p<0.05 was considered statistically significant.
CHAPTER 4: RESULTS

Twenty-five rats were included in the experimental model (10 from Group 1 and 2 respectively); and 5 from Group 3). Rats were excluded from results analysis if infarct area measurements could not be performed due to problems with staining techniques and imaging processing, or if premature death occurred during the anesthetic event resulting in premature termination of the experiment. Overall, five rats from group 1, five rats from group 2 and four rats from the sham groups were used for statistical analysis.

**Assessment of area at risk and infarct size:**

Successful coronary artery occlusion and reperfusion were confirmed in several ways. First, during ligation, visual confirmation of ischemia was confirmed by identification of a visibly pale, non-contractile and slightly bulging area in the left ventricle. In contrast, reperfusion was evident as the tissue became red and often returned to a contractile state. Myocardial infarction was also identified by echocardiography as any new segmental wall motion abnormality such as hypokinesis, akinesis and dyskinesis. Finally vital staining was used to confirm the area of ischemia. Left anterior descending artery occlusion consistently created a large area at risk, as shown by delineation using Evans blue dye (non-ischemic zone). All sections of the ventricle above the site of the ligature were uniformly blue whereas sections of the ventricle from the level of the ligature to the apex were brick red and the infarct appeared pale-white. Staining with TCC delineated the infarct site in the transverse heart section (3rd section from apex), as shown in figure 1 and 2 with the area of infarction appearing pale, and the area at risk in red. The infarct
size reported as a percentage of the area at risk in the non-treated rats mean was 25.51% ±4.9 SD or 2.2 SEM whereas the mean infarct size in the treated bilirubin group was 13.34% ± 5.7 SD or 2.5 SEM. Infarct size was significantly smaller in the bilirubin treated group when compared to the non-treated group (p<0.0067, Figure 3). The Average of each 5 measurements is presented in Table 1.

<table>
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<th>Area at risk</th>
<th>Infarct area</th>
<th>% Infarct</th>
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Table 1: The Average of each 5 measurements. NT: No-treatment; T: Treatment. Infract % = Infarct area/Area at risk
**Figure 1.** Bilirubin treatment. TCC assessment of infarct size at section 3 (apex to base). Note the infarct area is pale-white, compared to the area at risk which appears as red and to non-affected area that appeared in blue. **Infarct size=9.5%** (infarct area/area at risk)
Figure 2. Control. TCC assessment of infarct size at section 3 (apex to base). Note the infarct area is pale-white, compared to the area at risk which appears as red and to non-affected area that appears blue. **Infarct size=33.58%** (infarct area/area at risk).
Figure 3: Infarct size (IS) is reported as a percentage of the area at risk in the treated and non-treated rats. Mean IS was 25.51% ±4.9 SD or 2.2 SEM whereas the mean IS in the treated bilirubin group was 13.34% ± 5.7 SD or 2.5 SEM. Infarct size was significantly smaller in the bilirubin treated group when compared to the non-treated group (p<0.0067)

Echocardiogram analysis:
Left ventricular function decreased in both experimental groups after ischemia and reperfusion. Although bilirubin provided a protective effect on FS% during the period of ischemia (25.8% ± 5.89 SD or 2.63 SEM vs 18.8% ± 2.16 SD or 0.97 SEM; P= 0.034), there was no significant difference in measurements of left ventricular function at the time of reperfusion. Left ventricular function remained constant throughout the anesthetic period in sham rats, validating the model
and echocardiographic technique (Figure 6). For AS%, left ventricular function decreased in both experimental groups after ischemia and reperfusion. Although bilirubin provided a protective effect on AS% during the period of ischemia (34% ±12.58 SD or 5.62 SEM vs 26%± 3.00 SD or 1.342 SEM) this was not statistically significant (P=0.548). Furthermore, there was no significant difference in measurements of left ventricular AS% at the time of reperfusion. Left ventricular function remained constant throughout the anesthetic period in sham rats, validating the model and echocardiographic technique (Figure 7). Table 2-4 represent the average measurement results at 3 different time points (Baseline; Ligation; Reperfusion) of each group.

Figure 4: M-mode imaging of the LV (at base line)
Figure 5: Measurements of the LV internal diameter at end-diastole (LVIDd) and LV internal diameter at end-systole (LVIDs).
### Table 2: Non-treatment group. Average measurement results at 3 different time points (Baseline; Ligation; Reperfusion)

<table>
<thead>
<tr>
<th>Rat #</th>
<th>Baseline FS%</th>
<th>Ligation FS%</th>
<th>Reperfusion FS%</th>
<th>Baseline Area%</th>
<th>Ligation Area%</th>
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### Table 3: Br-treatment group. Average measurement results at 3 different time points (Baseline; Ligation; Reperfusion)

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<th>Rat #</th>
<th>Baseline FS%</th>
<th>Ligation FS%</th>
<th>Reperfusion FS%</th>
<th>Baseline Area%</th>
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### Table 4: Sham group. Average measurement results at 3 different time points (Baseline; Ligation; Reperfusion)

<table>
<thead>
<tr>
<th>Rat #</th>
<th>Baseline FS%</th>
<th>Ligation FS%</th>
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<th>Baseline Area%</th>
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Figure 6: Fractional shortening measurements (FS%) of sham, Br and control groups at three different time points. Bilirubin provided a protective effect on FS% during the period of ischemia (25.8% ± 5.89 SD or 2.63 SEM vs 18.8% ± 2.16 SD or 0.97 SEM; P= 0.034), there was no significant difference in measurements of fractional shortening at the time of reperfusion. Left ventricular function remained constant throughout the anesthetic period in sham
**Figure 7:** Area fractional shortening measurements (AS%) of sham, Br and control groups at three different time points. AS%, decreased in both experimental groups after ischemia and reperfusion (34% ±12.58 SD or 5.62 SEM vs 26%± 3.00 SD or 1.342 SEM). Although AS% was higher in Br treated rats at the end of ischemia, this was not statistically significant (P=0.548). Furthermore, there was no significant difference in measurements of left ventricular AS% at the time of reperfusion. Left ventricular function remained constant throughout the anesthetic period in sham rats.
CHAPTER 5: DISCUSSION

Our results demonstrate that bilirubin pre-treatment significantly reduced infarct size in a rodent model of acute LAD occlusion and 1 hour reperfusion, with mean infarct size being nearly 50% less in the treated group when compared to untreated controls. These results correlate with the protective effect seen after pharmacologic or genetic upregulation of HO-1 prior to tissue injury\textsuperscript{15,18,83} and suggest that, in fact, bilirubin therapy may offer a simple means to substitute for the effects of HO-1 upregulation in a safe and readily achievable manner. Although high doses of bilirubin are potentially toxic in neonates\textsuperscript{89-91}, the doses used in our current study are barely above the physiologic range and would not be expected to incur any risk in a healthy adult. IP administration of bilirubin has been shown to increase plasma levels of bilirubin, reaching a peak at approximately 1 hour after administration.\textsuperscript{92} Bilirubin is then quickly eliminated from the circulation and redosing or chemical modification of the molecule would be necessary to create a sustained effect. Preconditioning therapies are typically most effective when initiated prior to the onset of tissue injury. Although bilirubin had significant protective effects in this model, treatment 1 hour before myocardial infarction may be impractical in clinical treatment of human myocardial infarction. Future studies will investigate the efficacy of bilirubin administration during ischemia, but prior to reperfusion- a dosing protocol that would be more clinically applicable. Alternatively, creation of targeted drugs that can produce more sustained increases in serum bilirubin concentration may facilitate prophylactic treatment of individuals that are at risk of myocardial infarction. We are currently working with individuals in the pharmaco-thearapeutics department to achieve this goal. Due to a significant difficulty in performing the
delicate surgical and staining procedures, a large number of animals were dedicated towards model development and a limited number of rats remained available for the data collection (n=5 per treatment group). Limitations in-group size restricted our ability to measure function and infarct size at multiple time points after injury in this initial study. Based on the positive effects seen in this initial study, future experiments involving multiple time points including recovery models are indicated.

We elected to use a rodent model of myocardial infarction and reperfusion for this initial study. The rat model of myocardial infarction has been used for decades in many research laboratories to study left ventricular function, the impact of pharmacological or surgical interventions, and the effect of gene transfer and cell transplantation.\textsuperscript{1,12,16-18,32,61,83} Coronary artery ligation is often used to produce experimental myocardial infarction (MI). The relationship between infarct size and ventricular performance after left coronary artery occlusion was previously investigated using a rat model. In that study, the impairment of left ventricular function was directly related to the loss of myocardium.\textsuperscript{93} Although rodents are low on the phylogenetic tree, there are several advantages to the use of this rodent model including minimal expense, decreased inter-animal variation due to the availability of inbred strains, and the lack of coronary collateral circulation in the rat, leading to consistent and reproducible infarcts after LAD occlusion.

A variety of methods are available for measurement of infarct size, although vital staining techniques are considered the standard method. Triphenyltetrazolium chloride (TCC) is typically used to stain viable tissue where active dehydrogenases in myocardial cells convert the water-soluble compound into a brick-red, insoluble precipitate. The infarct region is identifiable as non-stained myocardium (lacking dehydrogenase activity), which often appears pale-white. Standard planimetry techniques can be used to quantify the perfused, non-ischemic (blue), viable area under risk (brick-red), and infarcted (pale) regions. Tetrazolium staining is a well-validated method that allows the early detection of myocardial infarction.\textsuperscript{94,95} Tissue lacking hydrogenase
enzyme activity is either dead or destined to die. It should be noted however, that tissue that stains positive is not necessarily healthy and may succumb hours or even days later. The longer the reperfusion period, the more reliable the staining method becomes for discriminating between dead and viable tissue. While some researchers state that reperfusion times of less than 3 hours (2 hours for perfused hearts) can make this technique less reliable, other report that the technique is standardly used at 1 hour of reperfusion without significant problems.

Several studies have evaluated the use of myocardial contrast echocardiography in the rat model of myocardial infarction. The authors concluded that myocardial contrast echocardiography could quantify infarct area in rats with myocardial ischemia. A single measurement at the mid-papillary muscle level was found to be a simple, efficient and reliable approach for in vivo infarct size assessment. Newer non-invasive quantification methods of infarct size include molecular level analysis, computer tomography, magnetic resonance imaging speckle tracking, and tissue doppler echocardiography. In the future, collaboration with other investigators will allow additional parameters to be evaluated and may offer the distinct advantage of studying the effects of bilirubin on infarct size over multiple time points in the same animal by using non-invasive techniques.

Our results showed minimal differences in echocardiographic function at the time of reperfusion despite a demonstration of decrease in infarct size in the bilirubin pretrtreated group. The marked decrease in cardiac function seen in both control and bilirubin treated hearts is likely due to a phenomenon termed myocardial stunning. Reversibly damaged myocytes do not contract as efficiently as they did in the control state. Myocardial stunning is a reversible reduction in cardiac function that is not accounted for by tissue damage; rather, ischemia results in conversion to anaerobic glycolysis and lactate accumulation with eventual cessation of contraction in the entire area at risk persists for hours or days. It has been shown that myocytes reperfused late in the
reversible phase of ischemia still exhibit stunning 48-72 hours after being reperfused.\textsuperscript{100,101} This reverse phenomenon would not have been encountered in our acute model. It is interesting to note that Bolli \textit{et al}, have shown that abundant products of oxygen-derived free radical reactions are present in coronary sinus blood beginning at the time tissue is reperfused. Moreover, there is evidence that part of the stunning phenomenon is due to oxygen-derived free radicals, because stunning can be ameliorated greatly if free radical scavengers are given immediately before reperfusion.\textsuperscript{102} A long-term recovery model would be required to eliminate this phenomenon.

We elected to use transthoracic echocardiography to estimate LV function. Although echocardiographic measurements of FS\% and AS\% are well established as methods for estimation of systolic function, the disadvantage of these techniques in a focal MI model is that they are intended for use with a symmetrically contracting ventricle. Since FS\% and AS\% assess ventricular function only at the level being interrogated, these measurements can result in a misleading estimate of global ventricular function. Furthermore, these modalities (M-mode); interrogate only a single line and are not a comprehensive anatomic screening method. Another plausible explanation for minimal differences in echocardiographic function relate to the phenomenon of tethering effect. Tethering is an impact that an abnormal segment has on a normal adjacent border segment, leading to either over-representation or under-representation of the true segmental wall motion abnormalities. Other non-invasive quantitative methods, such as speckle tracking and Doppler tissue imaging may be used in future experimental models to detect regional left ventricular function.

Due to the invasive nature of this study, all measurements were obtained with the rats under general anesthesia maintained with isoflurane. It is known from previous rodent studies that the dose and type of anesthetic may influence heart rate, left ventricular (LV) systolic and diastolic function, LV thickness and LV cavity dimensions. When comparing isoflurane (ISF) and ketamine to pentobarbital used as anesthetic agents, isoflurane and ketamine yielded
echocardiographic LV structural and functional data different to those obtained in conscious rats, with isoflurane anesthesia resulting in significantly lower FS% and AS% as compared with other anesthetic agents $^{103-106}$. ISF and ketamine have also been shown to affect echocardiographic estimation of LV mass. Although general anesthesia is likely to have affected cardiac functional parameters in our model, measurements of LV function (FS% and AS%) remained constant throughout the anesthetic period in sham-operated rats, validating the model and echocardiographic technique.
References


